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PRINCIPAL INVESTIGATOR: H. Kim Lyerly, M.D.

CONTRACTING ORGANIZATION: Duke University

Durham, NC 27705

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H. Kim Lyerly, M.D.

E-Mail: kim.lyerly@dm.duke.edu

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14. ABSTRACT
We hypothesize that immune responses to HER3 will block HER3 mediated endocrine-resistance, provide an effective therapy for endocrine- resistant patients, and eliminate the emergence of resistant clones. The study objective is to generate a clinically applicable HER3 cancer vaccine using advances in vector design that will optimize vaccine performance in clinical settings. Our preliminary data suggests that a vaccine targeting HER3 can generate polyclonal HER3 specific immune responses that have antitumor activity. Our translational studies intend to generate a GMP version of our HER3 vaccine, test its safety and immunogenicity, and then first evaluate whether HER3 vaccination in combination with standard anti-hormonal therapy in an anti-hormonal resistant population leads to increased efficacy of the standard therapy. These studies will support the long term goal of vaccinating against HER3 to prevent resistance. We have generated 4 different Ad5(E2b-)HER3 vectors and confirmed induction of anti-HER3 cellular and humoralimmune response and antitumor effect by Ad-HER3 vaccine in mice as preclinical basis for selection of vector construct for clinical use. We have established multiple Tam-resistant ER+/HER3 expressing human breast cancer cell lines and developed HETR3 activated pathway signatures using heregulin (HRG) as an activator of the HER3 pathway. These models will be used to identify the protein architecture associated with HER3 pathway activation.

15. SUBJECT TERMS
HER3, adenoviral vector, immunotherapy, tamoxifen resistance, breast cancer

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1. Introduction

ER+ breast cancer accounts for 70% of all new cases and can be effectively treated with surgery, radiation therapy, and appropriate anti-estrogen therapies such as selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AI) in the adjuvant and metastatic setting. Nonetheless, approximately one-third of women treated with anti-estrogen therapy will have recurrent disease within 15 years. Resistance to anti-estrogen therapy appears to result from a complex compensatory network ("escape routes"), and inhibition of a single pro-survival pathway may ultimately lead to the utilization of other, related pathways to potentiate the survival of the breast cancer cells, therefore, overcoming these mechanisms will likely require multiple strategies. We propose a novel approach that has the potential to target multiple pathways for the lifetime of the patient, specifically **active immunotherapy directed toward tumor associated antigens evoked by the compensatory resistance mechanisms**. As proof of concept, we have chosen to focus on a single candidate antigen in a critical pathway, realizing that additional pathways may require inhibition to completely overcome therapeutic resistance. We will focus on the HER3 axis as a model since the HER3 growth factor heregulin (HRG) and HER3 have been implicated in the development of resistance to all classes of anti-estrogen therapies.

We hypothesize that an immune response to HER3 will block HRG and Akt activation-induced endocrine-resistance, provide an effective therapy for endocrine- resistant patients, and eliminate the emergence of resistant clones. Our objective is to generate a clinically applicable HER3 cancer vaccine using advances in vector design that will optimize vaccine performance in clinical settings.

Specific Aims: In the first phase of this proposal, (Years 1-2) we will complete advanced translational studies, obtain IRB and HRPO approvals, and obtain the appropriate FDA approvals:
Aim 1: Generation of GMP Ad(E2b-)HER3
Aim 2: Pre-clinical testing of activity and toxicity of the GMP Ad(E2b-)HER3
Aim 3: Regulatory pathway to first in human testing: obtain US FDA IND, and IRB and HRPO approvals of the phase I clinical trial.

In the second phase of this proposal (Years 3-5) we will initiate and execute two prospective clinical trials.
Aim 4: Perform a first in human clinical trial of Ad(E2b-)HER3
Aim 5: Perform a randomized phase II clinical trial of Ad(E2b-)HER3

Study Design: The pre-clinical studies in the first phase will support the submission of an IND. The second phase will support clinical studies will include the performance of a "first in human" clinical trial at Duke of Ad-HER3 as an open label single arm dose escalation study of vaccination with Ad(E2b-)HER3 to determine safety, tolerability, and estimate the induced HER3 specific immune response.

The phase II dose determined from the phase I study will be used in a multi-site open label, double arm, randomized phase II study as a "proof of concept" comparing best available care (currently Exemestene) to best available care plus Ad(E2b-) HER3 to determine progression free survival. It is expected that approximately 10 study sites will participate in the trial. Additional sites may be added, as appropriate. 130 patients will be randomized in a 1:1 ratio to

receive either Ad(E2b-)-HER3 + exemestane or exemestane alone. We anticipate that the median PFS in the exemestane alone arm will be about 4 months, and we hope to find statistical evidence that the exemestane plus Ad-HER3 vaccine arm has a median of at least 7 months. Secondary objectives: overall survival (OS) defined as the time from randomization to death due to any cause; objective response defined as a confirmed CR or PR according to RECIST criteria. Rate of HER3-specific T cell response by ELISPOT. Rate of HER3-specific antibody response by ELISA Exploratory objective: Correlation of a HER3 signaling pathway analysis with clinical benefit. The trial will use a two-stage statistical design that allows the trial to be closed early for futility while controlling the overall alpha level at a one-sided 0.10. Log-rank tests stratified by the two stratification factors listed above will be used to test for futility in stage 1 and superiority in stage 2. The superiority test will test the null hypothesis that the arm hazard ratio is ≤ 1.0 against the alternative hypothesize that the HR is ≥ 1.75 (7/4). The futility test will be calculated after 25 events are observed (theoretically, at about 7 months).

Impact: These studies will have significant impact as introduces an entirely new concept in the armamentarium to eliminate breast cancer, by vaccinating against the emergence of resistant tumors. The model antigen used for these studies, HER3, may also be targeted as a resistance mechanism for HER2 targeted therapy, as well as EGFR targeted therapy. In addition, our new vaccine technologies to be used in this proposal will provide insight into targeting other molecular pathways that lead to a preventive vaccine for breast cancer.

2. Keywords

SERM- selective estrogen receptor modulators

AI- aromatase inhibitors

ER- Estrogen Receptor

PR- Progesteron Receptor

HER3- Human Epidermal growth factor Receptor 3

HER2- Human Epidermal growth factor Receptor 2

HRG- heregulin,

NRG- neuregulin

EGFR- epidermal growth factor receptor

TKI- Tyrosine Kinase Inhibitor

siRNA- small interfering RNA

cMET- MNNG HOS Transforming gene, proto-oncogene that encodes a protein known as hepatocyte growth factor receptor (HGFR)

PI3K- Phosphoinositide (PI) 3-kinase

AKT- member of the non-specific serine/threonine-protein kinase

Ad- adenovirus

Ad5- adenovirus serotype 5

E1- E1 region encodes the E1A proteins, which share common domains, and two entirely distinct E1B proteins, namely the E1B 55 kDa protein (E1B 55K) and the E1B 19 kDa protein (E1B 19K)

E2b- E2b-encoded Adenovirus DNA polymerase

E3- adenovirus early transcription unit 3 (E3) encodes multiple immunosubversive functions

ICD- intracellular domain

ECD- extracellular domain

ELISPOT- enzyme linked immunosorbent spot

VIA- vaccine induced antibodies

ADCC- antibody-dependent cell-mediated cytotoxicity

CDC- complement dependent cytotoxicity

GFP- green fluorescent protein

LacZ- β-galactosidase

IHC- immunohistochemical

CR- complete response

TNBC- triple negative breast cancers

Keywords (cont'd.)

MTD- maximum tolerated dose

CROs- contract research organizations

GLP- good laboratory practice

IND- investigational new drug

3. Overall Progress Summary

In Year 1, we generated four different adenoviral vectors encoding human HER3 genes, (Ad-hHER3-FL, Ad-hHER3/ECD, Ad-hHER3/ECD-TM, Ad-hHER3/ECD-mC1C2), and tested their immunogenicities in immune-competent BALB/c mice. Anti-tumor effect by prevention vaccine with these Ad-vectors were also analyzed in BALB/c mice using HER3 transfected JC murine breast cancer cell line (JC-hHER3). We confirmed anti-tumor effect of the vaccines by the induction of anti-HER3 immunity in Balb/C mice.

Two major developments have influenced our progress in year 2. First, in response to the peer review critique, we were advised to complete our preclinical studies in a model tolerant of human HER3, specifically human HER3 transgenic animals. We requested, and have imported the human HER3 transgenic FVB mice generated by Dr. Stan Gerson at Case Western Reserve, and begin a breeding program to develop a colony of animals to be used for preclinical testing. We had to establish immunogenicity of our vaccine candidates, and a syngenic FVB implantable breast tumor model to test the anti-tumor activity of the vaccine candidates. We used a number of constructs, but eventually developed the JC-HER3 model implanted into cross breed mice to perform our down selection studies. We continue to search for a reliable murine in vivo model of endocrine resistance. Although models of endocrine resistance have been reported, we have been unable to confirm these in our laboratory.

Second, published reports regarding the state of the art research for endocrine resistance emerged in 2013. A number of reports were published found that somatic mutations in the estrogen receptor served as a significant factor in the failure of anti-estrogen therapy. These new findings were unknown and unappreciated at the start of this grant. To address this, we held an External Scientific Advisory Committee (ESAC) meeting July 8, 2014 at Duke, meeting with experts in the field of hormone therapy resistance. The ESAC included Drs. Matthew Ellis, Suzanne Fuqua, Rachel Schiff, Geoffrey Green, and William Muller. After review of research progress and discussion of opportunities, ESAC comments and recommendations were made.

In general, the ESAC felt that we had assembled an excellent team of researchers and clinicians who collectively bring effective and complementary expertise needed to develop and test a novel adenoviral HER3 delivery vector that takes advantage of an E2b deletion to evade natural immunity to Ad, a major problem with other Ad targeted vaccines. The goals of the 2-year Phase I portion of this project have been largely achieved, demonstrating that HER3 is an effective and fairly broad spectrum target for vaccination using the Ad5 vector since HER3 plays an important role in endocrine therapy resistance and is expressed and/or up regulated in many progressive breast cancers and also plays an important pro-survival role in HER2 signaling.

Regarding preclinical testing in mice, it is notable that the full length Ad-hHER3FL vaccine appears to be the most effective at inhibiting JC-HER3 tumor growth, as measured by tumor volume, compared to truncated versions that express only the ECD or ECDTM. Similarly, the survival rate in the same animal model was highest for Ad-hHER3FL. At this point, GMP Ad5 [E1-, E2b-]-HER3 is under way for upcoming preclinical toxicity studies, projected to start this fall. The projected start date of Q1, 2016 for a Phase 1 clinical trial seems reasonable. A summary of our progress is presented in the following sections.

Aim 1: Generation of GMP Ad5(E2b-)HER3

1A: Generate Ad5(E2b-)HER3 and Ad5(E2b-)HER3 C1C2 constructs

Four Ad[E1-,E2b-]HER3 vector constructs were generated, sequenced, tittered and cyropreserved for experiments as outlined in **Figures 1 and 2**, and detailed in our previous publications and pre-IND package to the FDA (Appended).

Figure 1. Schema for generation Ad-Human Her3

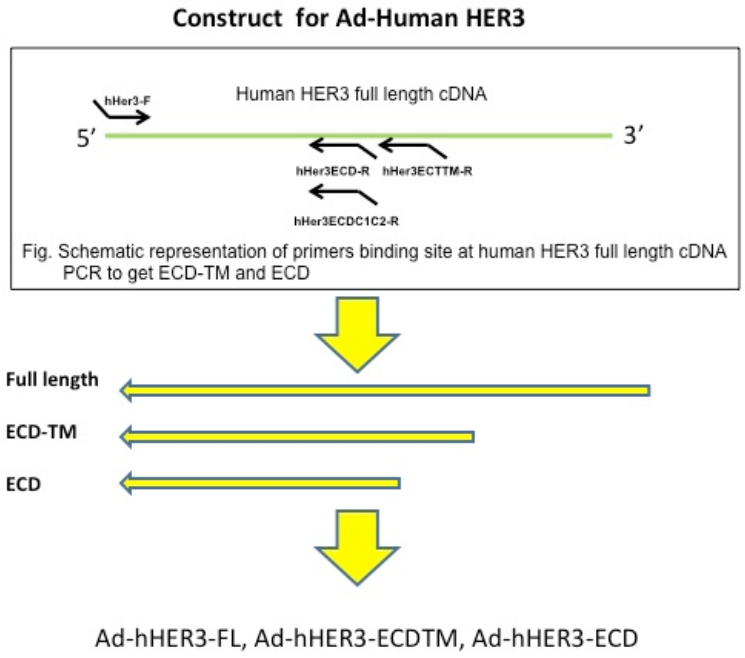
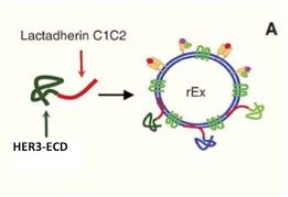
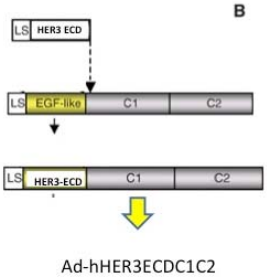


Figure 2. Schema for generation of C1C2 exosome trafficking vaccines





1. Exosomes are vesicular bodies and released in the extracellular milieu.

2. Exosomes transfer cellular antigens directly to APCs.

3. Targeting antigens to exosomes would increase their trafficking to APCs and therefore stimulate their immunogenicity.

4. Fusing protein to the Lactadherin C1C2 domain yields chimeric proteins that bind to exosomes.

Vaccine 29, 9361; 2011 ,
Cancer Res 71, 5235; 2011

1B: Preclinical immunogenicity testing of Ad5(E2b-)HER3 (84 mice required, but additional testing of vaccines in combination drugs likely to be used in the clinical studies, for example vaccination plus anti- estrogen therapy, anti- HER2 therapy, or mTOR inhibitors to confirm immunogenicity in those settings)

a) Crossbreeding of BALB/c and HER3 transgenic mice for vaccine experiment.

We started crossbreeding BALB/c to the HER3 Transgenic mice (FVB background) that we had obtained from Dr. Stan Gerson at Case Western Reserve. These mice had been cryopreserved, were first established as a breeding colony at Case Western Reserve, and then imported to Duke, where they we re-established as a breeding colony.

We confirmed homozygous HER3 transgenic mice in our breeding colony, and thus used them for crossbreeding. We have made more than 20 breeding cages for this crossbreeding, one parent from BALB/c strain, and the other parent from HER3 Transgenic (MMTV-neu/MMTV-hHER3). All neonates are expected to be HER3-positive, and confirmed to be so by PCR based genotyping as described in the previous quarterly technical report. Although the initial mating did not yield many neonates, the average litter size became stable and relatively large (10~12). By now (April 8th), we obtained more than 90 female F1 hybrid mice, and more pups are born in breeding cages.

To assess the vaccine efficacy of newly made Ad-HER3 vectors, we used HER3 transgenic mice model with implantation of human HER3-expressing murine breast cancer cells that derived from MMTV-neu mice (FVB background). We have established MNX5 murine breast cancer cell line from spontaneously occurring tumors in MMTV-neu female mice, and confirmed its tumorigenicity in MMTV-neu mice and HER3 Transgenic mice. Then, we generated MNX5-hHER3 cells using lentiviral vector encoding human HER3, with puromycin resistant gene as a selection marker. However, MNX5-hHER3 cells grew in HER3 Transgenic mice for about two weeks, but eventually rejected by immune system.

We hypothesized that puromycin-resistant gene, which is a foreign gene for mice, is inducing immune response in HER3 transgenic mice and leading to rejection of MNX5 HER3 transfected cells. Therefore, we constructed new HER3 transfectant cells without any selection markers. To select HER3-positive cells from the whole MNX5 cell population, FACS sorting for HER3 expressing cells was repeated 3 times, and more than 95% of cells were HER3 positive. Additional FACS sorting and expansion of HER3-positive cells was performed, and MNX5-hHER3 cells were implanted to HER3 transgenic mice to test tumorigenicity.

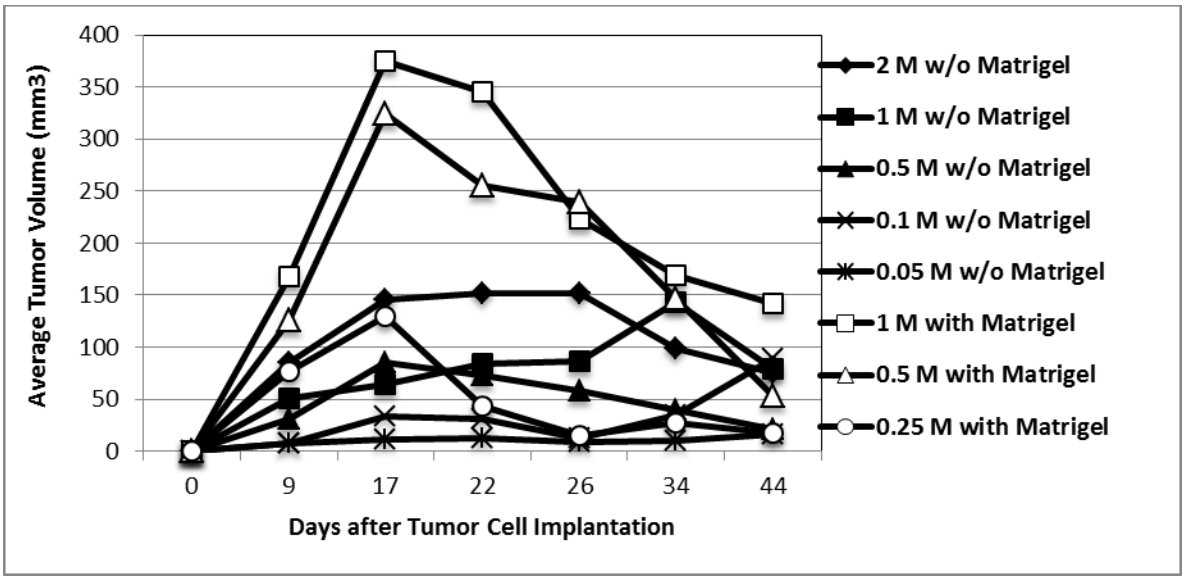


Figure 3. Tumorigenicity of MNX5-hHER3 cells in HER3 transgenic mice. Different number of MNX5-hHER3 tumor cells were resuspended in saline or in 50% Matrigel (100 µL/injection), and subcutaneously injected into the flank of HER3 Transgenic mice.

As shown in **Figure 3**, MNX5-HER3 tumor grew in first 17~22 days, however, many of them started shrinking until day 44 after tumor cell implantation. Thus, we decided the model of MNX5-hHER3 cells in HER3 transgenic mice was not a suitable model to determine the treatment efficacy of Ad-HER3 vaccine, and thus we decided to switch our animal model to use JC-hHER3 cells.

a) Crossbreeding of BALB/c and HER3 transgenic mice for vaccine experiment

We crossbred BALB/c and HER3 Transgenic mice (FVB background), and decided to use JC-hHER3 cells (BALB/c background). We confirmed homozygous HER3 transgenic mice in our breeding colony, and thus used them for crossbreeding. All neonates were HER3-positive. We have made more than 20 breeding cages for this crossbreeding, one parent from BALB/c strain, and the other parent from HER3 Transgenic. **Figure 4** shows the typical example of genotyping of human HER3 gene.

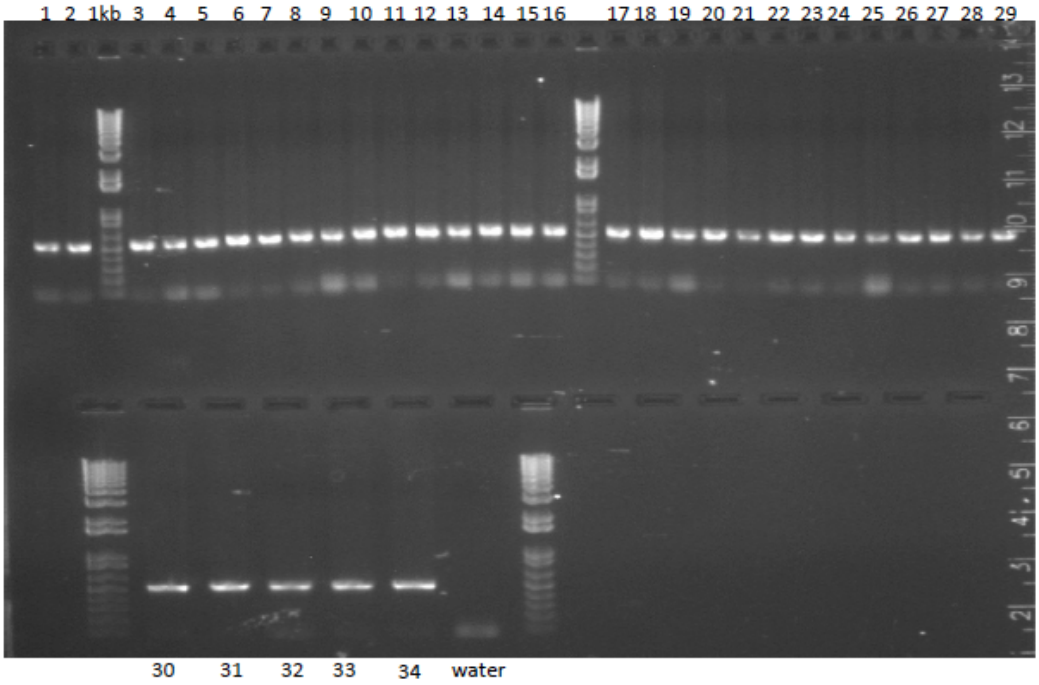


Figure 4: Genotyping of HER3 gene for crossbreed of BALB/c and HER3 transgenic mice
Homozygous HER3 Transgenic mice were used as one of parents, and bred with BALB/c mice. Genotyping was performed with the tail of pups to analyze HER3 gene expression.

We planned to confirm the tumorigenicity of JC-hHER3 cells in F1 mice, before conducting tumor-treatment experiments with Ad-HER3 vaccines.

b) Tumorigenicity test of JC-hHER3 cells in F1 hybrid mice

We confirmed tumorigenicity of JC-hHER3 cells (BALB/c background) in F1 Hybrid mice before conducting a tumor treatment experiment. Six weeks old female F1 Hybrid (BALB/c x MMTV-neu/MMTV-hHER3) mice were injected with 3, 1, 0.3 or 0.15 million JC-hHER3 cells in to the flank. As a control, BALB/c mice and MMTV-neu/MMTV-hHER3 Tg mice were also injected with JC-hHER3 cells (1 or 0.3 million cells/mouse). Tumor size was measured twice a week until some mice reached humane endpoint.

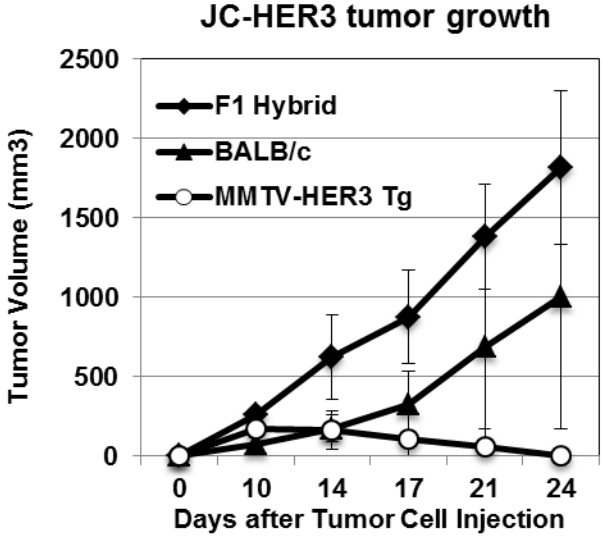


Figure 5. Tumor growth after JC-hHER3 cell injection in F1 hybrid mice. JC-hHER3 cells (1 x 10E6 cells/mouse) were resuspended in 50% Matrigel/50% saline and injected to the flank of F1 Hybrid mice, BALB/c mice or HER3 Transgenic mice (two or three mice for each strain). Mean tumor sizes for each mouse strain are shown. Error Bar: SD.

JC-hHER3 tumors grew in all F1 Hybrid mice injected with different number of cells. With 1x10E6 cell injection, two mice out of three reached humane endpoint by day 24 after cell implantation. With 0.15 x 10E6 cell injection, tumor volume was about 1,000 mm3. However, as expected, JC-hHER3 cells (derived from BALB/c mouse strain) were rejected by MMTV-hHER3 Transgenic mice (FVB background) by 24 days after cell implantation (**Figure 5**). JC-hHER3 tumors grew also in BALB/c mice, while the growth speed was a slightly slower than in F1 Hybrid mice, suggesting the possibility of immune response against human HER3 antigen and resultant delay in tumor growth.

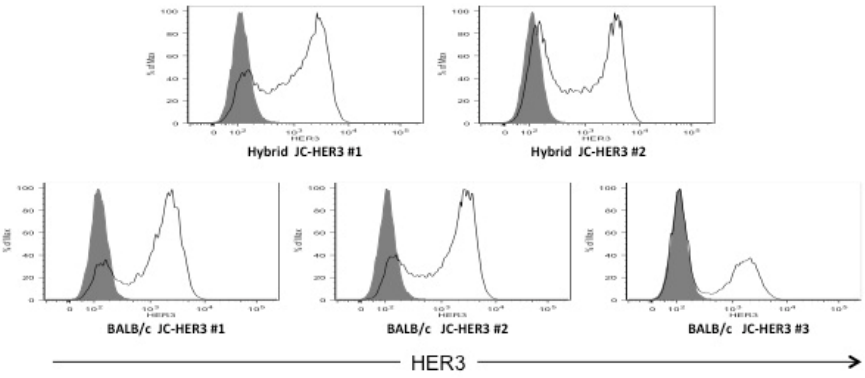


Figure 6. HER3 expression by JC-hHER3 tumors grown in F1 hybrid mice and BALB/c mice. JC-hHER3 tumors grown in BALB/c mice and F1 Hybrid mice were harvested, digested with triple enzyme buffer (collagenase type III, hyarulonidase, DNase) for 1 h, and then incubated for 4 days for recovery. Tumor cells were stained with PE-conjugated anti-hHER3 antibody (open histograms) or PE-conjugated isotype control (black histograms).

As shown in **Figure 6**, JC-hHER3 tumor cells grown in Hybrid mice maintained HER3 expression, but some tumors grown in BALB/c mice had decreased HER3 expression level. Based on these results, JC-hHER3 cells will maintain HER3 expression when implanted in F1 Hybrid mice. We confirmed that JC-hHER3 tumors in HER3 transgenic F1 Hybrid mice is an optimal model for the treatment experiment of HER3 vaccine.

c) Treatment experiment with Ad-HER3 vaccine: Preliminary

To test Ad-HER3 vaccine efficacy in the new model of JC-hHER3 tumors in F1 hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3), we conducted tumor treatment experiment in a smaller scale. We made vaccination with Ad[E1-E2b-]-hHER3 full length, Ad[E1-E2b-]-hHER3/ECD-TM, or Ad[E1-E2b-]-GFP (negative control), and assessed antitumor effect with the vaccine treatment. Each group consisted with 5 mice. As shown in table 1 below, 4 and 11 days after JC-hHER3 cells implantation (1 x 10^6 cells/mouse) to female F1 hybrid mice, mice were vaccinated with Adenovirus (2.6x10^{10} vp/mouse) via footpad injection. Tumor size was measured twice a week.

Table 1: Treatment and Assay Schedule

Group	**Mouse #**	**Day 0** Tumor Inj/ Sample Harvest	**Day 4, 11** Vaccine 1, 2	**Day 40** or humane endpoint Assessment
A	5	1) JC-HER3 cell inoculation (**1 x 10^6** cells /mouse)	Ad[E1-E2b-]-**HER3 full length**	Tumor volume measure until Day 40 or they reach 2000 mm^3 with harvested samples (tumor, spleen, serum) 1. Immunohistochemistry (hHER3 expression) 2. ELISPOT (HER3 ICD/ECD) 3. Flow assay (anti-HER3 Ab) 4. Cell-based ELISA
B	5		Ad[E1-E2b-]-**HER3/ECD-TM**	
C	5		Ad[E1-E2b-]-GFP	

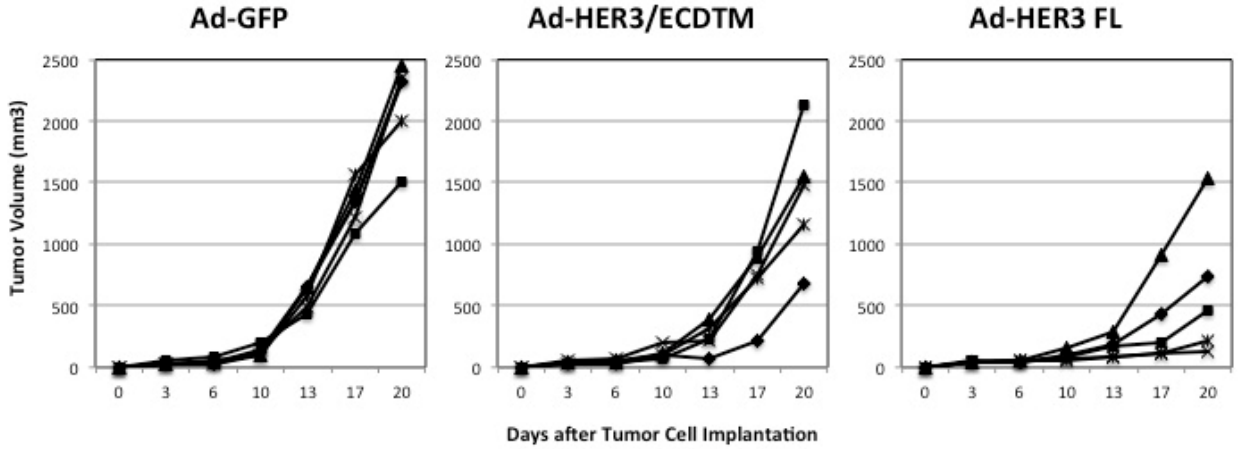


Figure 7A. JC-hHER3 tumor growth in F1 Hybrid mice treated with Ad-HER3 vaccine (individual). JC-hHER3 cells (1 x 10^6 cells/mouse) were injected to the flank of female F1 Hybrid mice on day 0, and were treated with Ad-HER3 full length, Ad-HER3/ECD-TM, or Ad-GFP vaccine (2.6 x 10^{10} vp/mouse) on days 4 and 11. Tumor size was measured twice a week until humane endpoint. Individual tumor volumes are shown in each graph.

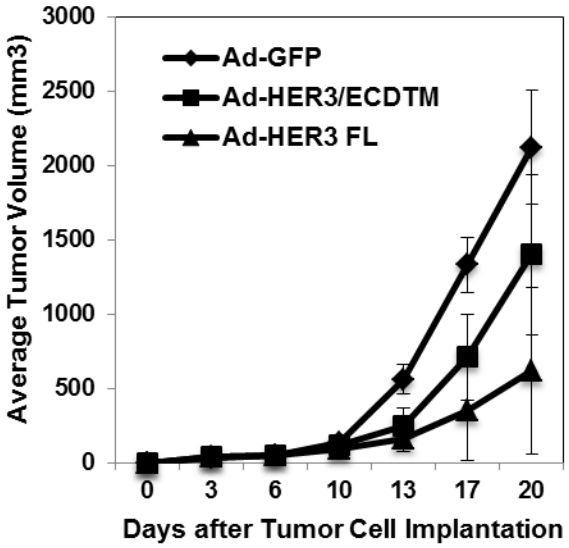


Figure 7B. JC-hHER3 tumor growth in F1 hybrid mice treated with Ad-HER3 vaccine (Average of Groups). Average tumor volumes of each group are shown. On day 20, some mice in Ad-GFP group reached humane endpoint, and thus the experiment was terminated. Error bar: SD.

As shown in **Figures 7A** and **7B**, there were statistical differences between Ad-GFP vs. Ad-HER3/ECD-TM (T-test; days 13 & 17: $p<0.005$, day 20: $p<0.05$) and between Ad-GFP vs. Ad-HER3 full length (T-test; day 13: $p<0.001$, day 17: $p<0.001$, day 20: $p<0.005$). There were no statistical difference between Ad-HER3/ECD-TM and Ad-HER3 full length.

Using splenocytes from the mice, IFN-gamma ELISPOT assay was performed and the result is shown in **Figure 8**. Because the assay was performed with splenocytes from mice implanted with HER3 expressing JC-hHER3 cells, even Ad-GFP vaccinated mice had weak anti-HER3 cellular immune response. Ad-HER3/ECD-TM vaccine induced a little stronger response against HER3 ECD peptides compared to Ad-HER3 full length vaccine. On the contrary, Ad-HER3 full length induced stronger response to HER3 ICD peptides in mice. When stimulated with HER3 ECD and ICD peptide mix in the assay, both Ad-HER3 vectors showed similar number of IFN-gamma+ spots. We could confirm that anti-HER3 cellular immune response could be induced even in the treatment model with HER3 transgenic mice (F1 hybrid).

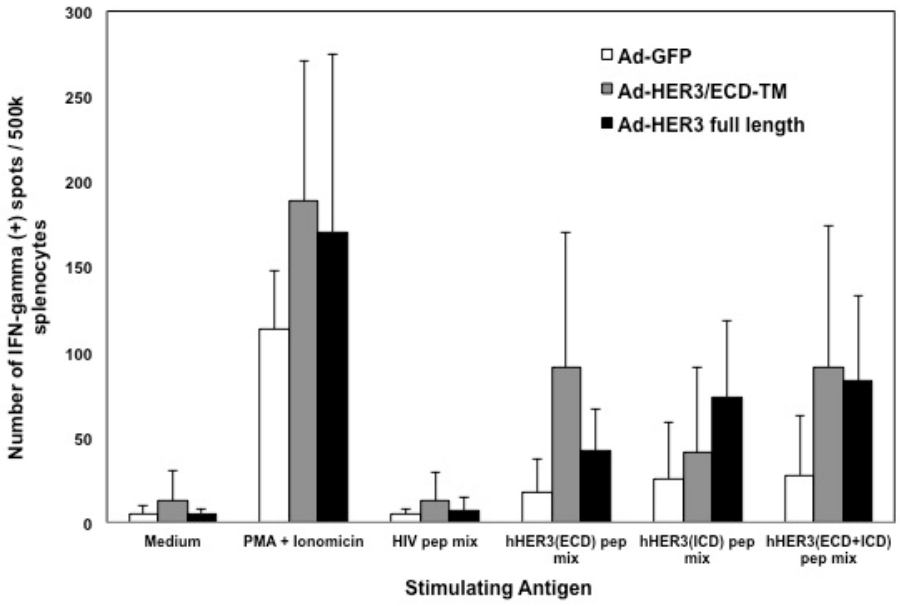


Figure 8. Anti-HER3 cellular immune response in F1 hybrid mice treated with Ad-HER3 vaccine. Splenocytes were stimulated with HER3 ECD peptide mix and/or ICD peptide mix. HIV peptide mix was used as a negative control and PMA+Ionomycin as a positive control. Five mice for each group were analyzed and the mean values are shown. Error bar: SD.

Anti-HER3 antibody production in mice treated with Ad-HER3 vaccine was analyzed by cell-based ELISA using 4T1 cells and 4T1-hHER3 cells for coating plates (**Figure 9**).

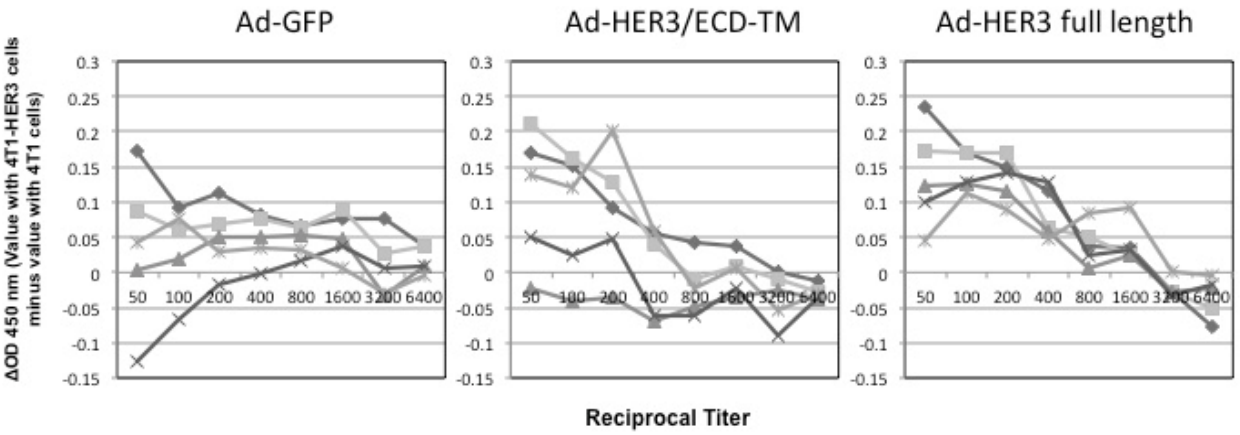


Figure 9. Anti-HER3 antibody levels in Ad-HER3 vaccinated F1 hybrid mice (Cell-based ELISA assay). Female F1 hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3) were implanted with JC-hHER3 cells (1 x 10^6 cells/mouse) on day 0, and then vaccinated twice on days 4 and 11 with Ad-HER3 full length, Ad-HER3/ECD-TM or Ad-GFP (2.6 x 10^{10} vp/mouse). On day 20, mice were sacrificed, and serum was collected from each mouse. Serum was used for cell-based ELISA (4T1-HER3 and 4T1 cells as

plating cells). Serum were titrated from 1:50 to 1:6400. HRP-conjugated goat anti-mouse IgG was used as secondary Ab, and color was developed with TMB substrate and reaction was stopped by H_2SO_4. Individual OD 450 nm values (OD value with 4T1-HER3 cells minus OD value with 4T1 cells) are shown.

As shown in **Figure 9**, both Ad-HER3/ECD-TM and Ad-HER3 full length vaccine induced anti-HER3 humoral response, but Ad-HER3 full length vaccine induced stronger antibody production. Some mice treated with control Ad-GFP vaccine showed anti-HER3 antibody in the serum, which might be induced because of HER3 expression by implanted JC-hHER3 cells.

We performed confirmatory study (Ad-HER3 vaccine treatment experiment) with current model (JC-hHER3:F1 Hybrid mice). We compared all 4 Ad[E1-E2b-]-HER3 vectors that were produced in the year 1, with 10 female F1 hybrid mice per group in this tumor treatment experiment. Details are shown in **Table 2**.

Table 2. Treatment and Assay Schedule

Group	Mouse #	Day 0 Tumor Injection	Day 3, 10,17 Vaccine 1, 2, 3	Day 40 or humane endpoint Assessment	Assessment
A	10	JC-hHER3 cell injection (**5 x 10^5** cells/mouse)	Ad[E1-E2b-]-HER3FL	Tumor volume measure until Day 40 or they reach 2000 mm^3	•ELISPOT (hHER3 pep mix) •Cell-based ELISA or Flow-assay •Immunohistochemistry & pathology (hHER3 expression) •Proliferation assay
B	10		Ad[E1-E2b-]-HER3/ECD		
C	10		Ad[E1-E2b-]-HER3 /ECD-TM		
D	10		Ad[E1-E2b-]-HER3 /ECD-C1C2		
E	10		Ad[E1-E2b-]-GFP		
F	10		Ad[E1-]HER3		
G	10		saline		

In the confirmatory study, we planned to determine the survivals of animals, and thus when mouse tumors reached humane endpoint (such as tumor ulceration or tumor volume equal to or over 2,000 mm^3), mice were sacrificed individually but not as a whole group. All the mice in the saline control group died by day 34, we terminated the experiment on day 34, and all surviving mice were euthanized.

Using splenocytes from the mice, IFN-gamma ELISPOT assay was performed and the result is shown in **Figure 10**. Similar with the result of our Pilot study, Ad-HER3/ECD-TM vaccine induced a little stronger response against HER3 ECD peptides compared to other Ad-HER3 vaccine including Ad-HER3 full length. Also in this confirmatory study, we could confirm that anti-HER3 cellular immune response could be induced even in the treatment model with HER3 transgenic mice (F1 hybrid). Among 4 Ad-HER3 vectors generated for this project, overall

strength of induced anti-HER3 cellular response was similar among full-length, ECD, and ECD-C1C2 vectors, but Ad-HER3/ECD-TM induced significantly stronger cellular response against HER3 antigen (t- test: $p<0.0001$).

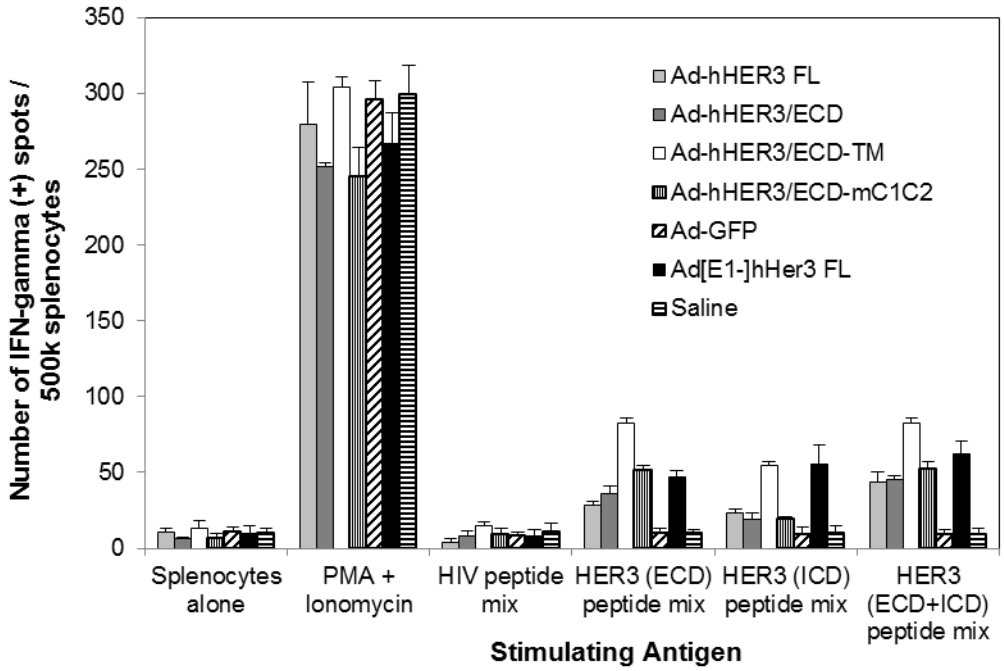


Figure 10. Anti-HER3 cellular response induced by Ad-HER3 vaccination. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECDTM, ECD-C1C2), Ad[E1-E2b-]GFP, Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. When tumor volume reached humane endpoint, mice were sacrificed. ELISPOT plates were coated with anti-IFNg mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. The mean of 4 mice from each group are shown.

Anti-HER3 antibody production in mice treated with Ad-HER3 vaccine was analyzed by cell-based ELISA using 4T1 cells and 4T1-hHER3 cells for coating plates (**Figure 11**). The assay was performed in exactly the same method as we performed in our pilot study. Serum titration was made from 1:50 to 1:6400. Four serum samples from each group were analyzed, and individual mouse serum data are shown as the difference in OD 450 nm (OD450 of 4T1-HER3 cells minus OD450 of 4T1 cells). As shown in the graph, all 4 Ad-HER3 vectors induced strong anti-HER3 antibody production in the serum, with slightly lower titer in Ad-HER3/ECD-mC1C2 vaccinated group (significantly different at 1:800: Ad-HER3 full-length vs. Ad-HER3/ECD-C1C2, $p<0.05$ by t-test). Negative control groups (saline, Ad-GFP) showed mostly negative for the Cell-based ELISA, that was reasonable because the mice were not receiving vaccine for HER3. Again as we've seen in the pilot study, the induction of anti-HER3 humoral response was confirmed in Ad-HER3 vaccinated HER3 transgenic mice, suggesting that Ad-HER3 vaccine with different constructs can overcome the tolerance in vivo.

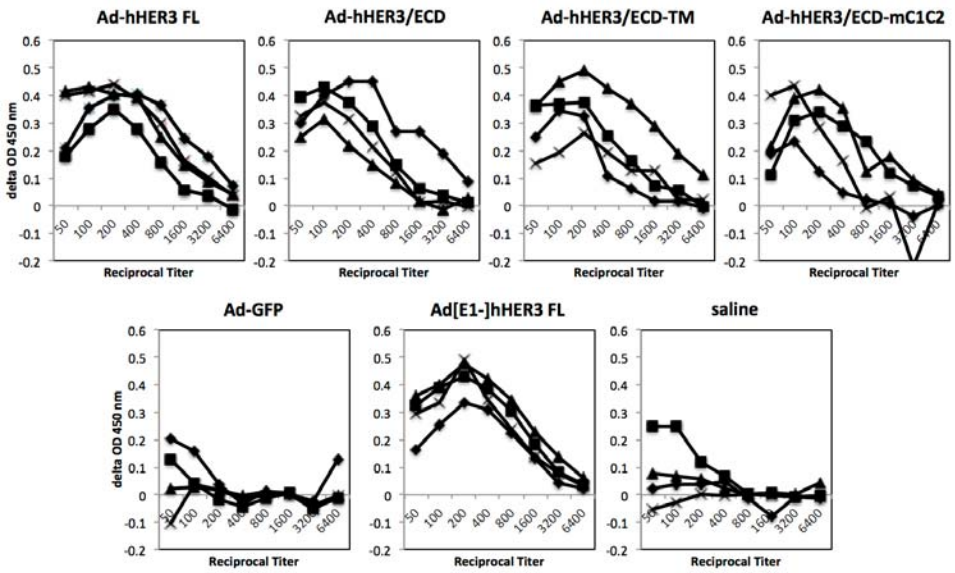


Figure 11. Anti-HER3 antibody levels in Ad-HER3 vaccinated F1 hybrid mice (Cell-based ELISA assay). Female F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3) were implanted with JC-hHER3 cells (5 x 10^5 cells/mouse) on day 0, and then vaccinated twice with Ad-HER3 (full length, ECD, ECD-TM, ECD-C1C2), Ad-GFP or Ad[E1-]-HER3 full length (2.6 x 10^{10} vp/mouse). Once the tumor volume reached humane endpoint, mice were sacrificed, and blood was collected from each mouse. Serum was used for cell-based ELISA (4T1-HER3 and 4T1 cells as plating cells). HRP-conjugated goat anti-mouse IgG was used as secondary Ab, and color was developed with TMB substrate and reaction was stopped by H_2SO_4. Individual OD 450 nm values (OD value with 4T1-HER3 cells minus OD value with 4T1 cells) are shown.

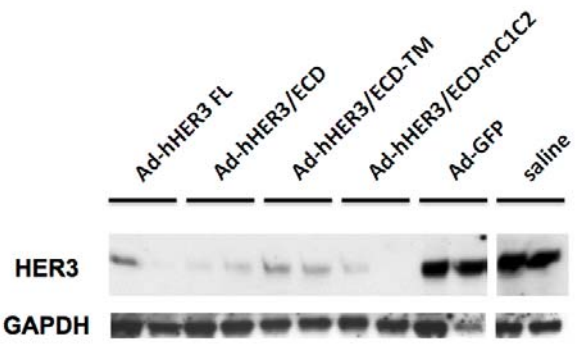


Figure 12. HER3 expression by JC-hHER3 tumors treated with Ad-HER3 vaccines. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECD-TM, ECD-mC1C2), Ad[E1-E2b-]GFP, Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline. When tumor volume reached humane endpoint, mice were sacrificed and tumor tissues were collected. Western blot was performed with anti-hHER3 antibody (Santa Cruz) or anti-GAPDH antibody as a control, followed by HRP-conjugated anti-mouse IgG (Cell Signaling) and chemiluminescent development kit.

To test the effect of Ad-HER3 vaccine treatment on HER3 expression status by in vivo tumors, we performed Western Blot analysis with the tumor lysates derived from collected tumors. Since the mice where sacrificed when the tumor reached the humane endpoint, the tumor tissues were frozen stocked at each timepoint, and two representative cases from each treatment group were used for lysate making and Western Blot. The same amount of protein was loaded to each lane (25 µg/lane). As shown in **Figure 12**, tumors treated with Ad-HER3 vaccination showed downregulation of HER3 expression, while saline and Ad-GFP control groups showed similar levels of strong HER3 expression by tumors. Therefore, Ad-HER3 vaccine has potentials to not only inhibit the growth of HER3 expressing tumors by targeting surface expressing HER3 molecules, but also can affect the

HER3 expression level of tumor cells and thus affect the downstream signaling pathway of HER3 molecule.

JC-hHER3 tumor sizes were measured twice a week after implantation. In **Figure 13**, mean tumor volume for each group is shown until day 21, when several tumors reached the humane endpoint and thus some mice were sacrificed. In **Figure 14**, tumor growths in individual mice are shown. Vaccination was done on days 3 and 10 as described above. Ad-HER3 full length suppressed the tumor growth most significantly (P=0.02 compared to saline group). However, for other groups receiving Ad-HER3 ECD, ECD-TM or ECD-C1C2 vaccines, the tumor growth suppression was less significant. At the end of experiment (on day 34), four mice in full-length, five mice in ECD, four mice in ECD-TM, two mice in ECD-C1C2, and 0 mouse in saline group were surviving, and eight all mice were dead by day 28 in Ad-HER3/ECD-C1C2 group while the first three mice died on day 28 in Ad-HER3 full-length group. These results suggest that Ad-HER3/ECD-C1C2 vaccine was less effective compared to other Ad-HER3 vaccines. In this experiment, Ad-GFP vaccine showed some effect on tumor growth for unknown reason, and thus making statistical analysis difficult. Therefore, we focused on Ad-HER3 full length and Ad-HER3/ECD-TM vectors and statistically analyzed antitumor response by combining data from the pilot and confirmatory studies (**Figure 15**).

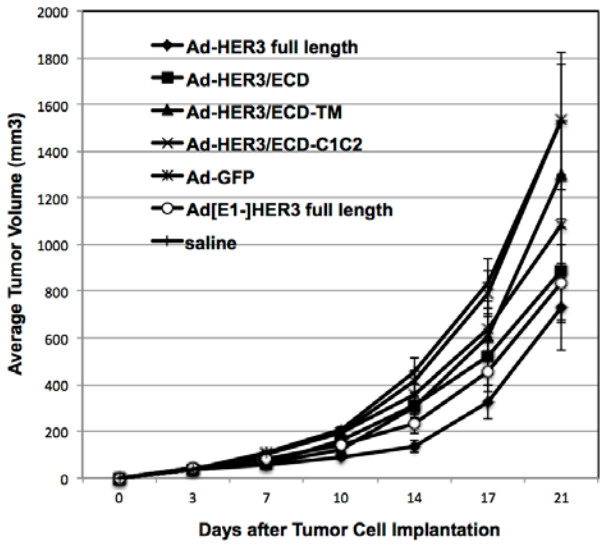


Figure 13. JC-HER3 tumor growth in HER3+ F1 hybrid mice treated with Ad-HER3 vaccines. F1 Hhybrid mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECDTM, ECD-C1C2), Ad[E1-E2b-]GFP, Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. Tumor size was measured twice a week. Mean tumor volume for each group is shown. Error Bar: SE.

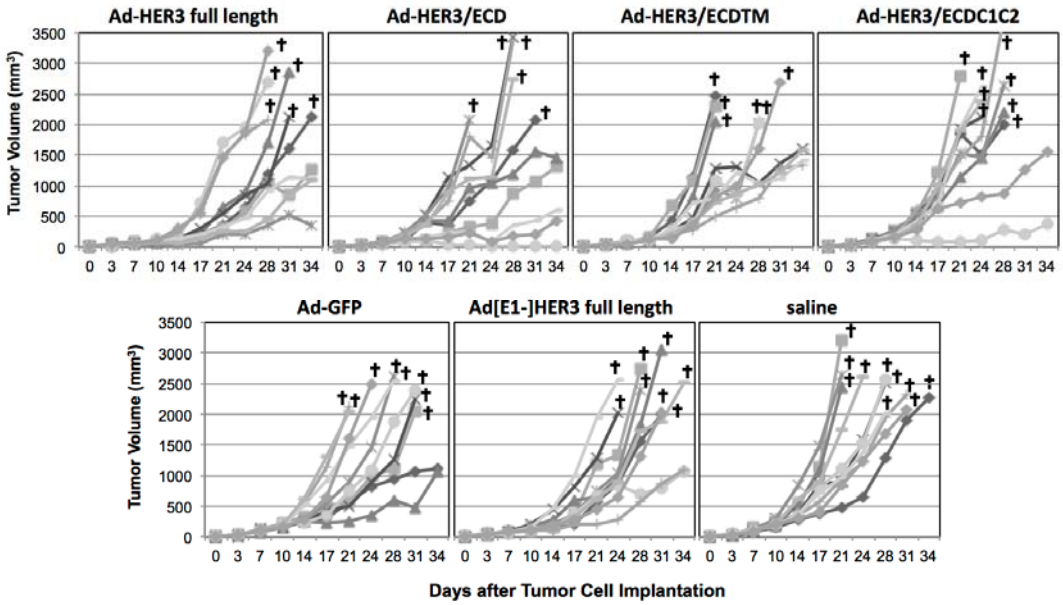


Figure 14. Individual JC-HER3 tumor growth in HER3+ F1 hybrid mice treated with Ad-HER3 vaccines. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECDTM, ECD-C1C2), Ad[E1-E2b-]GFP, Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. Tumor size was measured twice a week. Individual tumor growth for each group is shown.

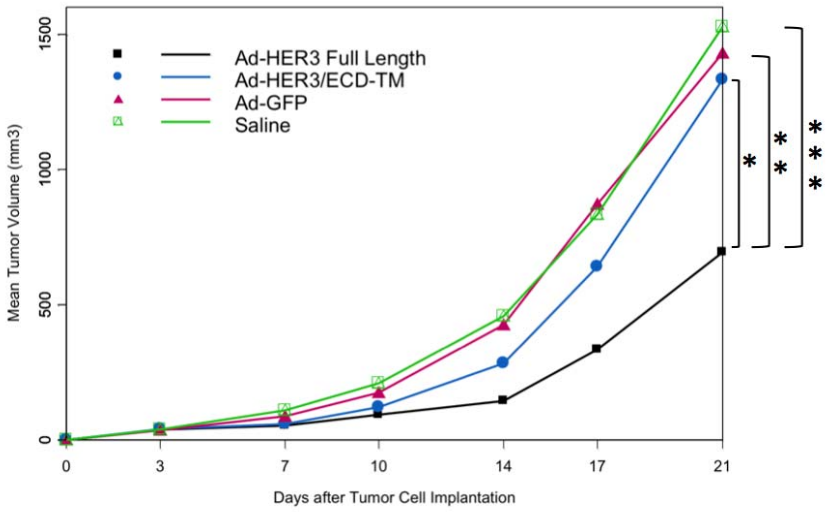


Figure 15. Mean tumor growth from combined pilot and confirmatory studies: JC-HER3 tumor growth in mice treated with Ad[E1-E2b-]HER3 vaccine. Tumor growth data from the pilot study and confirmatory studies (which had the same treatment schedule) were combined and statistically analyzed. The longitudinal mixed effects model with the maximum likelihood variance estimation method was used to model tumor volume over time. Ad-HER3 FL, Ad-HER3/ECD-TM, Ad-GFP: Fifteen mice for each group, saline: 10 mice. * p< 0.05, **p< 0.005, ***p< 0.01

As shown in **Figure 15**, Ad-HER3 full length vaccine suppressed the JC-hHER3 tumor growth significantly, compared to saline (p<0.01), Ad-GFP vaccine (p<0.005) and Ad-HER3/ECD-TM (p<0.05). Importantly, the combined data analysis demonstrated that Ad-HER3 full length was more potent in inhibiting HER3 expressing tumor growth than the other Ad vector, Ad-HER3/ECD-TM.

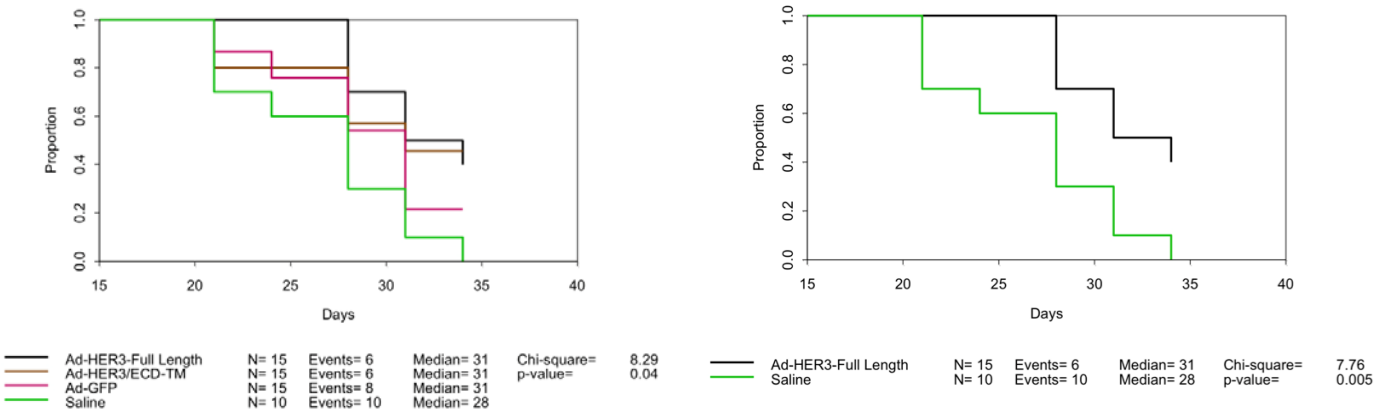


Figure 16. Survival of JC-HER3 tumor-bearing mice treated with Ad[E1-E2b-]HER3 vaccine. HER3 Transgenic F1 Hybrid female mice (BABL/c x MMTV-neu/MMTV-hHER3) were injected with JC-hHER3 cells (5 x 10^5 cells/mouse) on day 0, and treated twice with Ad[E1-E2b-]-HER3 (full length, ECD-TM), Ad[E1-E2b-]-GFP (2.6 x 10E10 vp/mouse) or saline. Tumor size was measured twice a week. Mice were sacrificed and when the tumor volume reached the humane endpoint. Survival curves for each group was estimated by combining the data of two independent experiments (a pilot and a confirmatory study) that had identical treatment schedules. The Kaplan-Meier method was used to estimate overall survival and groups were compared using a 3 d.f. score test from the proportional hazards model.

We also analyzed the survival of mice until day 34 (**Figure 16**). The log rank test was performed to test the null hypothesis of no survival differences among the four treatment groups. As shown in the right graph, Ad-HER3 full-length vaccine improved the overall survival of mice significantly compared to saline treatment (p=0.005).

Treatment experiment with Ad-HER3 vaccine: Confirmatory

In the previous period, to test Ad-HER3 vaccine efficacy in the new model of JC-hHER3 tumors in F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3), we conducted a pilot study of tumor treatment experiment in a smaller scale (Figure 3A and 3B in Year 2 Quarter 2 report). We vaccinated the mice with Ad[E1-E2b-]-hHER3 full length, Ad[E1-E2b-]-hHER3/ECD-TM, or Ad[E1-E2b-]-GFP (negative control); and assessed the antitumor effect of the vaccine treatment.

Statistical analysis was performed by Duke University Medical Center, Cancer Center Biostatistics (Drs. Gloria Broadwater, Bercedis Peterson). The longitudinal mixed effects model with the maximum likelihood variance estimation method was used to model tumor volume over time. To correct for skewness the log transformation of tumor growth was modeled. The model included quadratic terms for fixed effects and a time-continuous autoregressive model for the covariance of repeated measures. The full quadratic model contained the group effect, a continuous time effect, time squared effect, the group-by-time interaction, and group-by-time squared interaction. Pairwise treatment comparisons were tested using two degree of freedom

chi-square tests from the difference between the -2 log likelihoods of the reduced and full models. The two-sided alpha level for all pairwise comparisons was 0.05. There was statistically significant difference between Ad-HER3 full length vs. Ad-GFP ($p<0.001$) in the trajectory of tumor volume across time, but the difference was not significant between Ad-HER3/ECD-TM vs. Ad-HER3 full-length ($p=0.06$).

Based on the tumor growth data of the pilot study, and the data from immune assays, we could demonstrate that the tumor-mouse system (JC-hHER3 cells in F1 hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3)) works for the treatment model with Ad-HER3 vaccine. Thus, we conducted Ad-HER3 vaccine treatment experiment in full scale. We compared all 4 Ad[E1-E2b-]-HER3 vectors that were produced in the year 1. We made vaccination with Ad[E1-E2b-]-hHER3 full length, Ad[E1-E2b-]-hHER3/ECD-TM, , Ad[E1-E2b-]-hHER3/ECD, , Ad[E1-E2b-]-hHER3/ECD-mC1C2, Ad[E1-E2b-]-GFP (negative control), and Ad[E1-]-hHER3 full length (positive control) on days 3 and 10 after JC-hHER3 tumor cell implantation (5×10^5 cells/mouse) to female F1 hybrid mice. Vaccination was done via footpad injection of Adenovirus (2.6×10^{10} vp/mouse). Each group consisted with 10 mice. Tumor size was measured twice a week. Detailed schedule and grouping are shown below.

Figure 17. Confirmatory study schedule

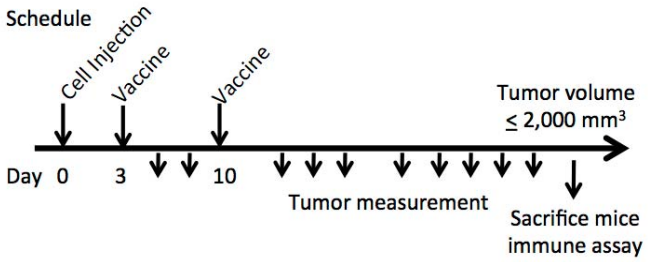


Table 3. Treatment and assay schedule: Confirmatory study

Group	**Mouse #**	**Day 0** Tumor Injection	**Day 3, 10** Vaccine 1, 2	**Day 40** or humane endpoint Assessment	**Assessment**
A	10	JC-hHER3 cell injection (**5 x 10^5** cells /mouse)	Ad[E1-E2b-]-HER3FL	Tumor volume measure until Day 40 or they reach 2000 mm^3	•ELISPOT (hHER3 pep mix) •Cell-based ELISA or Flow-assay •Immunohistochemistry & pathology (hHER3 expression) •Proliferation assay
B	10		Ad[E1-E2b-]-HER3/ECD		
C	10		Ad[E1-E2b-]-HER3 /ECD-TM		
D	10		Ad[E1-E2b-]-HER3 /ECD-C1C2		
E	10		Ad[E1-E2b-]-GFP		
F	10		Ad[E1-]HER3		
G	10		saline		

In the confirmatory study, we planned to determine the survivals of animals, and thus when mouse tumors reached humane endpoint (such as tumor ulceration or tumor volume equal to or over 2,000 mm^3), mice were sacrificed individually but not as a whole group. All the mice in the saline control group died by day 34, we terminated the experiment on day 34, and all surviving mice were euthanized.

Using splenocytes from the mice, IFN-gamma ELISPOT assay was performed and the result is shown in **Figure 18**. Similar with the result of our Pilot study, Ad-HER3/ECD-TM vaccine induced a little stronger response against HER3 ECD peptides compared to other Ad-HER3 vaccine including Ad-HER3 full length. Also in this confirmatory study, we could confirm that anti-HER3 cellular immune response could be induced even in the treatment model with HER3 transgenic mice (F1 hybrid). Among 4 Ad-HER3 vectors generated for this project, overall strength of induced anti-HER3 cellular response was similar among full-length, ECD, and ECD-C1C2 vectors, but Ad-HER3/ECD-TM induced significantly stronger cellular response against HER3 antigen (t-test: $p<0.0001$).

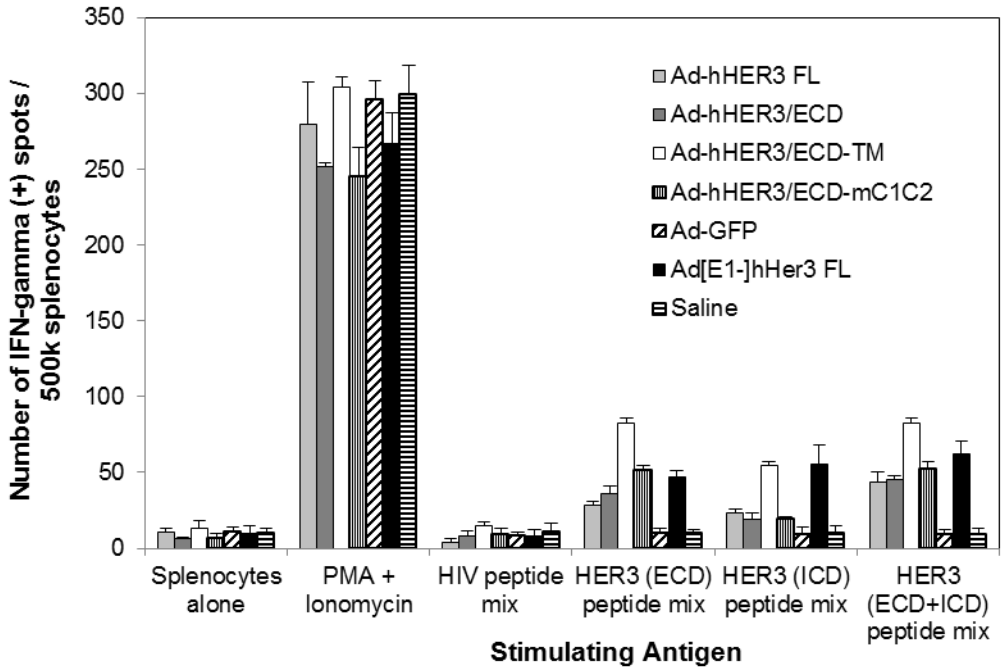


Figure 18. Anti-HER3 cellular response induced by Ad-HER3 vaccination. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECDTM, ECD-C1C2), Ad[E1-E2b-]GFP, Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. When tumor volume reached humane endpoint, mice were sacrificed. ELISPOT plates were coated with anti-IFNg mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. The mean of four mice from each group are shown.

Anti-HER3 antibody production in mice treated with Ad-HER3 vaccine was analyzed by cell-based ELISA using 4T1 cells and 4T1-hHER3 cells for coating plates (**Figure 19)**. The assay was performed in exactly the same method as we performed in our pilot study. Serum titration was made from 1:50 to 1:6400. Four serum samples from each group were analyzed, and individual mouse serum data are shown as the difference in OD 450 nm (OD450 of 4T1-HER3 cells minus OD450 of 4T1 cells). As shown in the graph, all 4 Ad-HER3 vectors induced strong anti-HER3 antibody production in the serum, with slightly lower titer in the Ad-HER3/ECD-mC1C2 vaccinated group (significantly different at 1:800: Ad-HER3 full-length vs. Ad-HER3/ECD-C1C2, $p<0.05$ by t-test). Negative control groups (saline, Ad-GFP) showed mostly negative for the Cell-based ELISA; this was expected because the mice were not receiving the vaccine for HER3. Again as we've seen in the pilot study, the induction of anti-HER3 humoral response was confirmed in Ad-HER3 vaccinated HER3 transgenic mice, suggesting that Ad-HER3 vaccine with different constructs can overcome the tolerance in vivo.

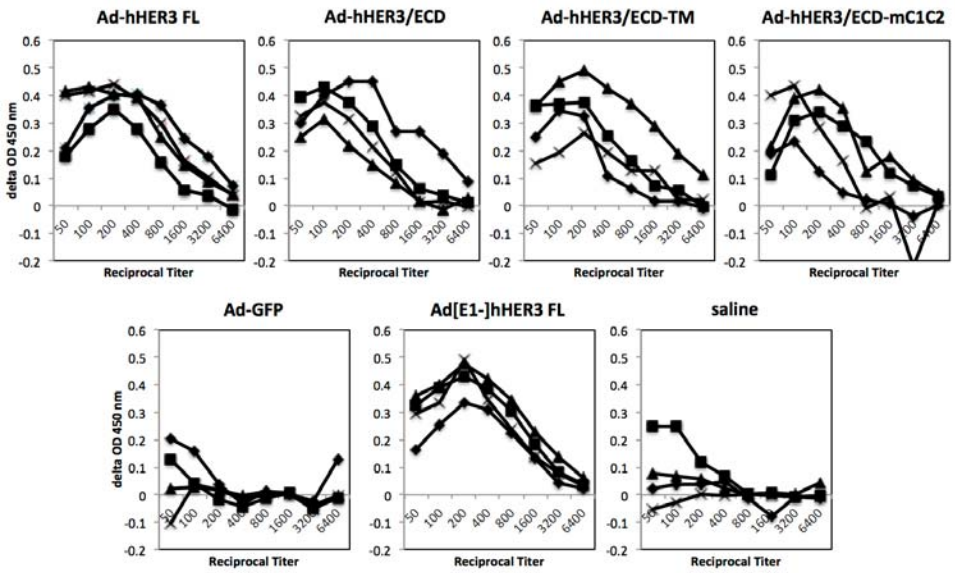


Figure 19. Anti-HER3 antibody levels in Ad-HER3 vaccinated F1 hybrid mice (cell-based ELISA assay). Female F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3) were implanted with JC-hHER3 cells (5 x 10^5 cells/mouse) on day 0, and then vaccinated twice with Ad-HER3 (full length, ECD, ECD-TM, ECD-C1C2), Ad-GFP or Ad[E1-]-HER3 full length (2.6 x 10^{10} vp/mouse). Once the tumor volume reached humane endpoint, mice were sacrificed, and blood was collected from each mouse. Serum was used for cell-based ELISA (4T1-HER3 and 4T1 cells as plating cells). HRP-conjugated goat anti-mouse IgG was used as secondary Ab, and color was developed with TMB substrate and reaction was stopped by H_2SO_4. Individual OD 450 nm values (OD value with 4T1-HER3 cells minus OD value with 4T1 cells) are shown.

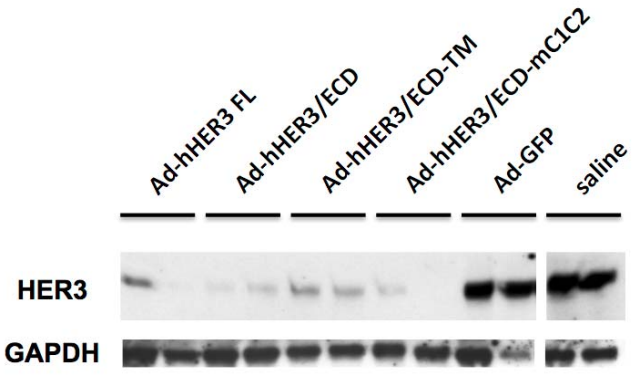
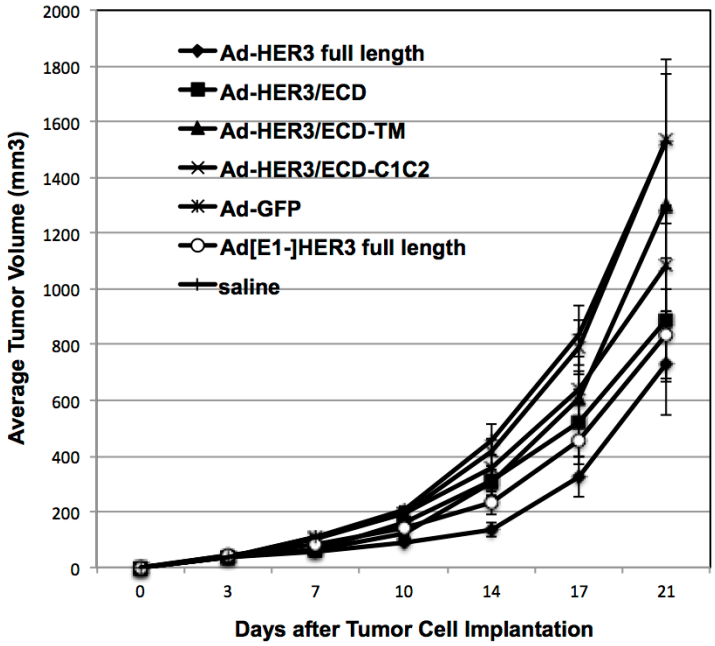


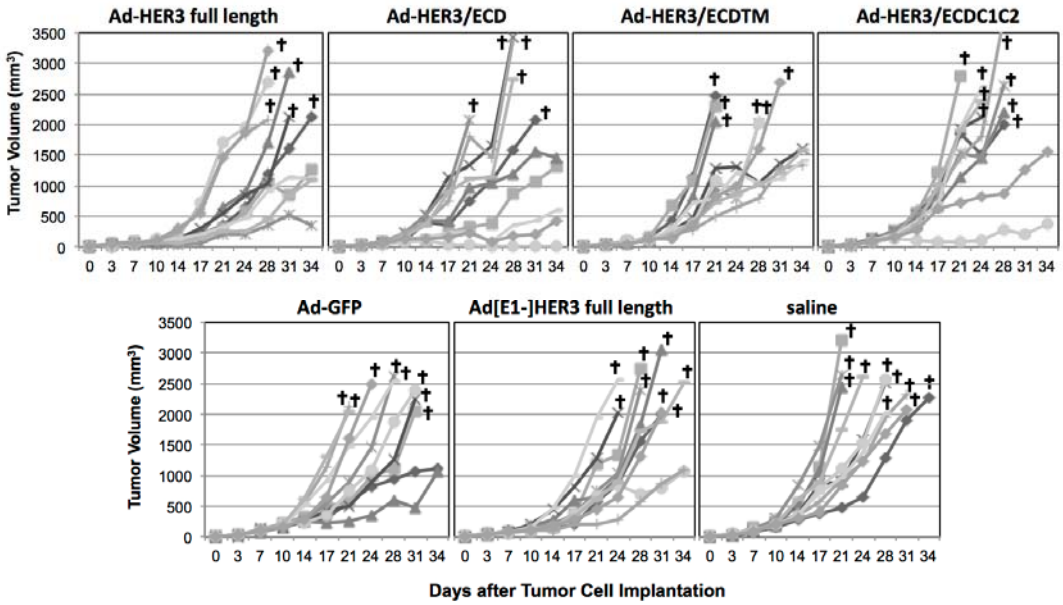
Figure 20. HER3 expression by JC-hHER3 tumors treated with Ad-HER3 vaccines. F1 hybrid mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECD-TM, ECD-mC1C2), Ad[E1-E2b-]GFP, Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline. When tumor volume reached the humane endpoint, mice were sacrificed and tumor tissues were collected. Western blot was performed with anti-hHER3 antibody (Santa Cruz) or anti-GAPDH antibody as a control, followed by HRP-conjugated anti-mouse IgG (Cell Signaling) and chemiluminescent development kit.

To test the effect of Ad-HER3 vaccine treatment on HER3 expression status by in vivo tumors, we performed Western Blot analysis with the tumor lysates derived from collected tumors. Since the mice where sacrificed when the tumor reached the humane endpoint, the tumor tissues were frozen stocked at each time point, and two representative cases from each treatment group were used for lysate making and Western Blot. The same amount of protein was loaded to each lane (25 µg/lane). As shown in **Figure 20**, tumors treated with Ad-HER3 vaccination showed downregulation of HER3 expression, while saline and Ad-GFP control groups showed similar levels of strong HER3 expression by tumors. Therefore, Ad-HER3 vaccine has potentials to not only inhibit the growth of

HER3 expressing tumors by targeting surface expressing HER3 molecules, but also can affect the HER3 expression level of tumor cells and thus affect the downstream signaling pathway of HER3 molecule.

JC-hHER3 tumor sizes were measured twice a week after implantation. In **Figure 21**, mean tumor volume for each group is shown until day 21, when some tumors reached humane endpoint and thus some mice were sacrificed. In **Figure 22**, tumor growths in individual mice are shown. Vaccination was done on days 3 and 10 as described above. Ad-HER3 full length suppressed the tumor growth most significantly (P=0.02 compared to saline group). However, for other groups receiving Ad-HER3 ECD, ECD-TM or ECD-C1C2 vaccines, the tumor growth suppression was less significant. At the end of experiment (on day 34), four mice in full-length, five mice in ECD, four mice in ECD-TM, two mice in ECD-C1C2, and 0 mouse in saline group were surviving, and all eight mice were dead by day 28 in Ad-HER3/ECD-C1C2 group while the first three mice died on day 28 in Ad-HER3 full-length group. These results suggest that Ad-HER3/ECD-C1C2 vaccine was less effective compared to other Ad-HER3 vaccines. In this experiment, Ad-GFP vaccine showed some effect on tumor growth for unknown reason, and was likely a false positive error. Therefore, we focused on Ad-HER3 full length and Ad-HER3/ECD-TM vectors and analyzed antitumor response by the statistical analysis of the combined data from the pilot and confirmatory studies (**Figure 23**).





Figures 21 and 22. JC-HER3 tumor growth in HER3+ F1 hybrid mice treated with Ad-HER3 vaccines. F1 hybrid mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECDTM, ECD-C1C2), Ad[E1-E2b-]GFP, Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. Tumor size was measured twice a week. Mean tumor volume (Figure 21) and individual tumor growth (Figure 22) for each group is shown. Error Bar: SE.

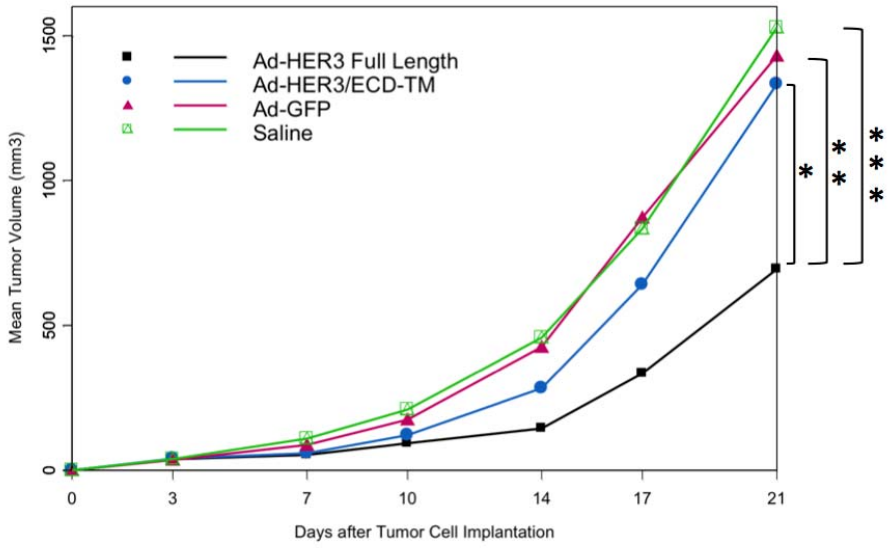


Figure 23. Combined tumor growth data from pilot and confirmatory studies: JC-HER3 tumor growth in mice treated with Ad[E1-E2b-]HER3 vaccine. Tumor growth data from pilot study and confirmatory study with the same treatment schedule were combined and analyzed.

The longitudinal mixed effects model with the maximum likelihood variance estimation method was used to model tumor volume over time. Ad-HER3 FL, Ad-HER3/ECD-TM, Ad-GFP: 15 mice for each group, saline: 10 mice. * p< 0.05, **p< 0.005, ***p< 0.01

As shown in **Figure 23**, Ad-HER3 full length vaccine suppressed the JC-hHER3 tumor growth significantly, compared to saline (p<0.01), Ad-GFP vaccine (p<0.005) and Ad-HER3/ECD-TM (p<0.05). Importantly, the combined data analysis demonstrated that Ad-HER3 full length was more potent in inhibiting HER3 expressing tumor growth than the other Ad vector, Ad-HER3/ECD-TM.

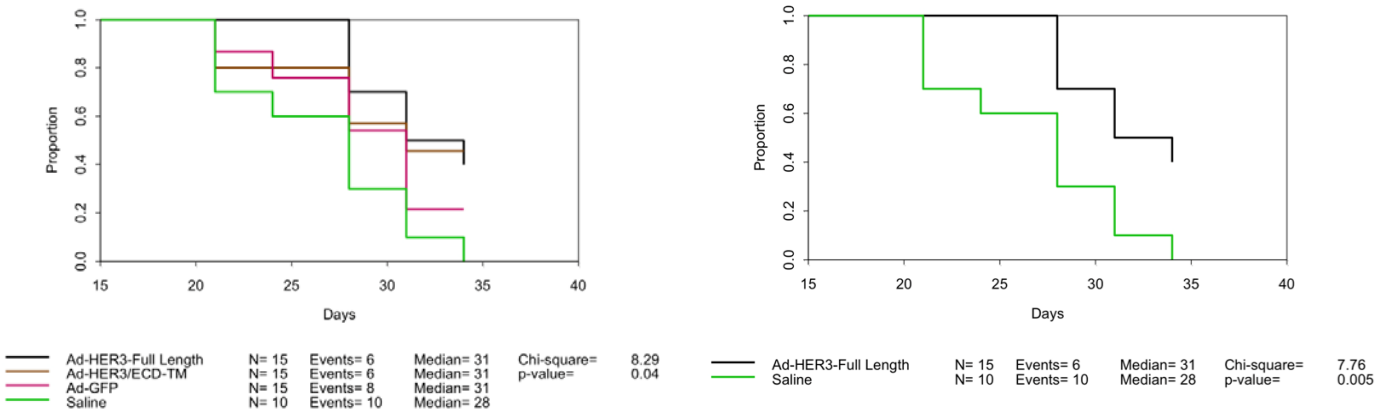


Figure 24. Survival of JC-HER3 tumor-bearing mice treated with Ad[E1-E2b-]HER3 vaccine. HER3 Transgenic F1 hybrid female mice (BABL/c x MMTV-neu/MMTV-hHER3) were injected with JC-hHER3 cells (5 x 10^5 cells/mouse) on day 0, and treated twice with Ad[E1-E2b-]-HER3 (full length, ECD-TM), Ad[E1-E2b-]-GFP (2.6 x 10E10 vp/mouse) or saline. Tumor size was measured twice a week. Mice were sacrificed if and when they reached the human endpoint. Survival curves for each group were estimated by combining the data from two independent experiments (a pilot and and a confirmatory study) that had identical treatment schedules. The Kaplan-Meier method was used to estimate the distribution of overall survival, and groups were compared using a 3 d.f. score test from the proportional hazards model.

We also analyzed the survival of mice until day 34 (**Figure 24**). The log rank test was used to test the null hypothesis of no survival differences among the four treatment groups. As shown in the right graph, Ad-HER3 full-length vaccine improved the overall survival of mice significantly compared to saline treatment (p=0.005).

Summary:

1. We confirmed the immunogenicity of Ad-HER3 vaccines we have generated.
2. We confirmed that Ad-HER3 vaccine has antitumor effect in JC-hHER3 tumor models in F1 hybrid HER3 Transgenic mice.
3. Based on tumor growth suppression and overall survival, Ad-HER3 full-length vector is the most potent vaccine.
4. HER3 downregulation of tumors by Ad-HER3 vaccine was confirmed.

1C: Generation of GMP Ad5[E1-E2b-)HER3

In order to identify manufacturing conditions for a large-scale GMP manufacture a small scale 5L Cell Bioreactor Process Development (PD) run was performed. In this study several parameters for the GMP manufacture were identified including cell growth culture conditions, the settings for the Cell Bioreactor and the overall set up for downstream processing were determined. The final quality of the product established criteria suitable for transfer to large scale GMP manufacturing of Ad5 [E1-, E2b-]-HER3 immunotherapeutic product.

1. 5L Cell Bioreactor Culture
Using the optimal conditions previously determined for production of Etubics recombinant Ad5 [E1-, E2b-]-based vectors, a 5L Cell Bioreactor run was performed under GLP conditions to produce the Ad5 [E1-, E2b-]-HER3 product. One vial of E.C7 cells obtained from the E.C7 Master Cell Bank (MCB) was thawed and transferred to a T-225 flask seeded at $4.0X10^4$ viable cells/cm^2 (vc/cm^2) and cultured in SGM (DMEM, 10% FBS, and 4mM L-glutamine). After three days of incubation at 37^O C in 5% CO_2, cells were transferred to a T-225 flask seeded at $3.0X10^4$ vc/cm^2. The cultures were split every 3-4 days and re-seeded between $2.0X10^4$ to $3.0X10^4$ vc/cm^2 in T-225 flasks and expanded into a 5-layer CellSTACK (CS-5) and 10-layer CellSTACKs (CS-10s). The cells were hard-transitioned from adherent to suspension culture using FreeStyle 293 serum-free medium at the time of seeding into the Cell Bioreactor with a 5L cell culture working volume. The cells were cultured for 24 hours at 37^O C in 5-10% CO_2. After culture, the cells were infected with Ad5 [E1-, E2b-]-HER3 virus particles (VP) at a multiplicity of infection (MOI) of 3 infectious viral particle unit (IU)/E.C7 cell. Post-infection, the cells were incubated for approximately 48 hours at 37^O C in 5-10% CO_2. After incubation, the cells were harvested and concentrated using a hollow fiber tangential flow filtration system (Spectrum) to a final retentate volume of 1041 mL. Tween-20 was added to the final retentate to a final concentration of 1% v/v, with intermittent shaking. This chemical lysis step to release the Ad5 [E1-, E2b-]-HER3 VP from the E.C7 cells was carried out for ~90 minutes at room temperature. The lysate was then transferred to a freezer and stored at $\leq$-65^O C prior to further processing.

Just prior to performing ion exchange chromatography to purify the Ad5 [E1-, E2b-]-HER3 product, the lysate was processed by thawing in a 37^OC water bath, followed by treatment with Benzonase (100 units per 1 mL of lysate) and a 75-minute (target range 60 to 90 min) incubation at room temperature. The Benzonase reaction was quenched by addition of 5M NaCl (50mL per every liter of lysate) and the lysate was then clarified by centrifugation and filtration through a 3.0 μm/0.8μm depth filter.

2. Purification of Ad5 [E1-, E2b-]-HER3 by Ion Exchange Chromatography.
A). <u>AEX Q Sepharose XL Purification (Step #1).</u>
A BPG 100 column was packed with Q Sepharose XL resin and sanitized with 0.5N NaOH. The column was washed with three column volumes (CV) of Buffer B (2.0M NaCl/50mM Tris/2mM $MgCl_2$; conductivity 130 - 160 mS/cm pH 7.8 - 8.3) and then equilibrated with three CV of Load Buffer (390mM NaCl/50mM Tris/2mM $MgCl_2$; conductivity: 37 - 41 mS/cm pH 7.8 - 8.3). The cell culture lysate containing Ad5 [E1-, E2b-]-HER3 was loaded at 200 mL/min using the AKTA Pilot Chromatography platform. Eight CV were used at the wash step and the product was eluted by step elution using elution buffer (540mM NaCl/50mM Tris/2mM $MgCl_2$; conductivity: 49 - 53 mS/cm pH 7.8 - 8.3). The main elution peak (410 mL) containing Ad5 [E1-, E2b-]-HER3 was carried forward to the next ion exchange purification step.

B). AEX Source 15Q Purification (Step #2).
A HiScale26 column was packed with Source 15Q resin and sanitized. The column was washed with three CV of Buffer B and then equilibrated with three CV of Buffer A (200mM NaCl/50mM Tris/2mM $MgCl_2$; conductivity 19.0 - 23.0 mS/cm pH 7.8 - 8.3). The eluate containing the Ad5 [E1-, E2b-]-HER3 from the first anion exchange chromatography purification was diluted 1:2 using dilution buffer (50mM Tris/2mM $MgCl_2$, pH 8.0). The diluted material was then loaded on the AKTA Explorer chromatography system (18 mL/min). Product was eluted starting at 100% Buffer A running through a gradient to 50% Buffer B. This gradient took place over a total of 30 CV. A total of 71.4 mL was collected as the Ad5 [E1-, E2b-]-HER3 product peak. The eluate was sampled and the Ad5 [E1-, E2b-]-HER3 VP concentration was measured by OD 260nm assay readings. The Ad5 [E1-, E2b-]-HER3 product was stored overnight at 2 to 8^{O} C.

3. *Downstream Processing and QC Testing of Purified Ad5 [E1-, E2b-]-HER3.*
A). Tangential Flow Filtration (TFF).
The following day, the eluate containing the purified Ad5 [E1-, E2b-]-HER2/*neu* product was processed through a TFF system with a 500K Nominal Molecular Weight Cut off membrane. The product was diluted to 88.3 mL with ARM Formulation Buffer (2.5% Glycerol/25mM NaCl/20mMTris, pH 8.0) to a targeted concentration of 1.2 X 10^{12} VP/mL base on OD 260 nm readings. The material was diafiltered by five additions (88 mL each) of ARM Formulation Buffer, with the material being brought back down to the original volume (~88 mL) after each addition. Following the last diafiltration round, the material was recovered and 83.2 mL of Ad5 [E1-, E2b-]-HER3 product was recovered post-sampling.

B). Final Filtration and Storage.
The purified Ad5 [E1-, E2b-]-HER3 product was sterile-filtered using a 0.2µm pre-wetted Sterivex-GV membrane filter. The recovered filtered product (81.3 mL and referred to as bulk drug substance; "BDS") was sampled to test for mycoplasma, bioburden, endotoxin, and infectious units titer (IU) using a Hexon staining assay. An HPLC chromatogram profile of the final product demonstrated that the purified Ad5 [E1-, E2b-]-HER3 exhibited a very high degree of purify (see **Figure 25** below).

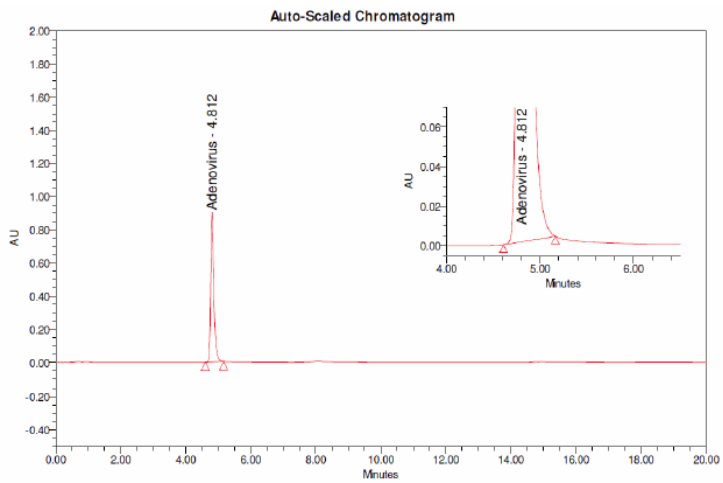


Figure 25. **Bulk Drug Substance HPLC trace.** Note the high degree of purity of the final Ad5 [E1-, E2b-]-HER3 product.

The remaining product was dispensed into one 78.6 mL aliquot and frozen at ≤-65^{O} C as BDS. As shown in **Table 4**, the 5L Cell Bioreactor Ad5 [E1-, E2b-]-HER3 product produced yielded product within an acceptable range of final product yield, infectious VP (IU) activity, and total VP to IU ratio.

Table 4 - Ad5 [E1-, E2b-]-HER3 5L Cell Bioreactor Run Product Yield

Parameter	Final Yield per mL	Final Total Product Yield
Final virus particle (VP) Yield (OD 260 nm reading)	1.05 X10^{12} VP/mL	8.2530 X10^{13} VP
Final infectious virus particle unit (IU) Yield	3.60 X10^{10} IU/mL	2.8296 X10^{12} IU
Final VP/IU ratio	29:1	29:1

C). Quality Control Test Results.

Quality Control tests performed on the Ad5 [E1-, E2b-]-HER3 product determined that it was mycoplasma free, had no bioburden, and exhibited endotoxin levels less than 1.0 Endotoxin Unit (EU) per mL. These test results are the same as those conducted on a 5L Cell Bioreactor Ad5 [E1-, E2b-]-HER2/*neu* immunotherapeutic product Etubics has produced and on an Ad5 [E1-, E2b-]-CEA(6D) immunotherapeutic product Etubics has produced also at the 5L volume in a Cell Bioreactor.

In summary, these parameters for cell growth, the settings for the Cell Bioreactor, the overall set up for downstream processing, and the final quality of the product establish criteria suitable for transfer to large scale GMP manufacturing of Ad5 [E1-, E2b-]-HER3 immunotherapeutic product.

We had anticipated that we could have completed the manufacturing of GMP Ad5[E1-E2b-]HER3 by the end of year 2, but we did not complete the down selection studies until we established a new colony of HER3 transgenic animals as requested by the DoD. Based on our pre-clinical data in animals and discussion with the FDA in a pre-IND teleconference, we propose using the full length human HER3 vaccine. Due to the time required to complete the downstream selection studies, the anticipated timing of the GMP run is presented in the Gantt chart below (**Figure 25)**.

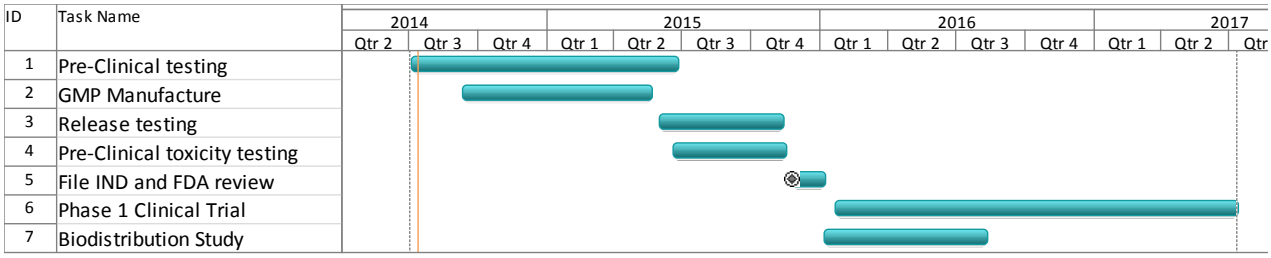


Figure 25. Gantt Chart

Etubics has contracted SAFC in Carlsbad, CA to generate cGMP material for clinical testing. The Company has chosen to use SAFC to manufacture the Ad5 [E1-, E2b-]-HER3 product because SAFC has manufactured Ad5 [E1-, E2b-] clinical grade material which has been used in human clinical trials and has extensive experience with the Etubics E.C7 manufacturing cell line. SAFC produced and stores the E.C7 Master Cell Bank. The use a different vendor would increase time and cost of the project in order to complete the necessary technology transfer and demonstration run(s) which have already been performed.

Etubics has now reserved a manufacturing suite at SAFC (Carlsbad, California) and it is estimated that production of clinical grade Ad5 [E1-, E2b-]-HER3 product will begin in the latter part of the fourth quarter of 2014. SAFC has begun generating the Batch Production Records which have been reviewed and approved by Etubics. All raw materials and equipment, including dedicated equipment, are being procured by SAFC in preparation for the manufacturing run. We do not anticipate any delays due to the preparation that has been accomplished.

The QC testing of the material generated during the GMP manufacture will take place at BioReliance, Rockville, MD. We utilize BioReliance as our preferred testing facility because SAFC has acquired BioReliance and now offer streamlined service and pricing discounts if material is produced at SAFC and is tested at BioReliance. This reduces the overall cost and time to completion of the manufacturing project.

1D: Development of a protein pathway signature of activated HER3 signaling

Introduction: Development of drug resistance to endocrine therapies used in the treatment of estrogen receptor positive (ER+) breast cancers remains a significant clinical dilemma, as it not only inevitably develops in the treatment of women with metastatic disease, but also in 25% of women with early stage cancer who eventually recur at metastatic sites. Activation of Human Epidermal Growth Receptor 3 (HER3) signaling has been implicated as playing a role in the development of therapeutic resistance to endocrine therapies used in the treatment of ER+ breast cancers. As the only HER family member with weak intrinsic autokinase activity (1) - it had long been considered a kinase dead receptor (2) - HER3 was considered an intractable target from the standpoint of the development of small molecule tyrosine kinase inhibitors. It can however be targeted through a vaccine-based approach. The goal of this CTRA is to develop a novel HER3 vaccine to prevent the development of resistance to endocrine therapies used in ER+ breast cancers. However, not all resistance is mediated by up-regulation and/or activation of HER3. *Therefore, our task is to develop a tumor signature of activated HER3 signaling with the objective to identify ER+ breast cancers that are more likely to respond to a HER3 vaccine*. Signatures can be identified using a number of “omic” strategies e.g. proteomic and genomic, which, in cell line studies have shown high discordance in many cell signaling pathways between genomic and proteomic (3). Since current therapeutic targets are primarily proteins rather than genes or RNA, we decided to initially focus on the identification of a protein/phosphoprotein-based profile(s) associated with resistance to endocrine therapies.

Research Accomplishments: The accomplishments over the past year can be summarized according to the following sub-tasks:

Sub-Task 1*: Development of tamoxifen resistant models.* Generating tamoxifen resistance is a challenge. In contrast to the development of resistance to other targeted therapies including tyrosine kinase inhibitors, resistance to tamoxifen is an exceedingly slow process. It has taken over 12 months to develop tamoxifen resistant ER+ MCF7, T47D, and MDA-MB-361, and more recently ZR-75-1, CAMA1, MDA-MB-134, and HCC 1428 (**Figure 26**). Considering most of the reported data on tamoxifen resistance involves one cell line (MCF7), we now have one of the larger collections of parental and isogenic matched tamoxifen resistant cell counterparts with which to establish a HER3 activation profile.

Using molecular knockdowns, we have shown that expression of HER3 alone is not sufficient to predict for dependence upon HER3 signaling. We have models where HER3 knockdown elicits an antitumor effect e.g. tam-resistant MCF7 and others where HER3 knockdown has no effect, e.g. tam-resistant T47D. As we characterize our panel of isogenic paired cell line into those that do or do not respond to HER3 knockdown, we will then characterize HER3 dependent and independent cell lines at the protein pathway activation and gene expression levels. We have developed the cell lines to be at the point where we can achieve our stated goal in Aim 1D.

Generate Tam-resistant ER+ cell lines

Established cell lines:

- MCF7
- T47D
- MDA-MB-361
- *MDA-MB-134
- *CAMA1
- *ZR-75-1

*recently established

Cell Line	EGFR	HER2	HER3	HER4
MCF7	Very low	Low	High	Low
T47D	Very low	Mod	High	Very high
MDA-MB-361	Mod	Mod/ High	High	Low
MDA-MB-134	Very low	Low	High	Very low
CAMA1	Very low	Low/ Mod	High	Very high
ZR-75-1	Low	Mod	Mod	Very High

Figure 26. ER+ breast cancer cell lines used to develop tamoxifen resistance. Each of these cell lines was cultured in the continuous presence of tamoxifen. After months, cell viability was maintained in the continuous presence of 1 μM tamoxifen.

Sub-Task 2*: Comparison of HER receptors and downstream signaling pathways in parental versus tam-resistant cells.*

It is important to point out that previously published data on tam-resistance is primarily based on MCF7 cells. However, breast cancer is a molecularly heterogeneous disease. Therefore, changes in HER receptor expression and downstream signaling pathways

associated with the development of tam-resistance will likely vary between different models of resistance. Accordingly, in Year 2 we have begun to interrogate the protein signaling pathways related to HER3 and other HER family members in models of tam-resistance in order to determine how they differ from not only each other, but also their isogenic matched tam-sensitive cell counterparts. We also sought to determine the role of HER3 in the regulation of the survival of tam-resistant cells. The following Western blots are representative of multiple experiments examining the impact of tam-resistance on HER receptor expression, and the activation status of downstream signaling pathways that mediate the growth and survival effects of HER3 and other HER receptors. The Western blots were performed using our previously published methods (4-6). As shown in **Figure 27**, ER expression remains essentially unchanged in resistant compared with tam-sensitive cell counterparts (compare lanes 1 and 3). For the most part, total HER3 expression is not increased with the development of resistance and may be decreased with tam-resistance. HER2 and EGFR appear to be slightly increased in tam-resistance particularly in MCF7 and MDA-MB-361 cells.

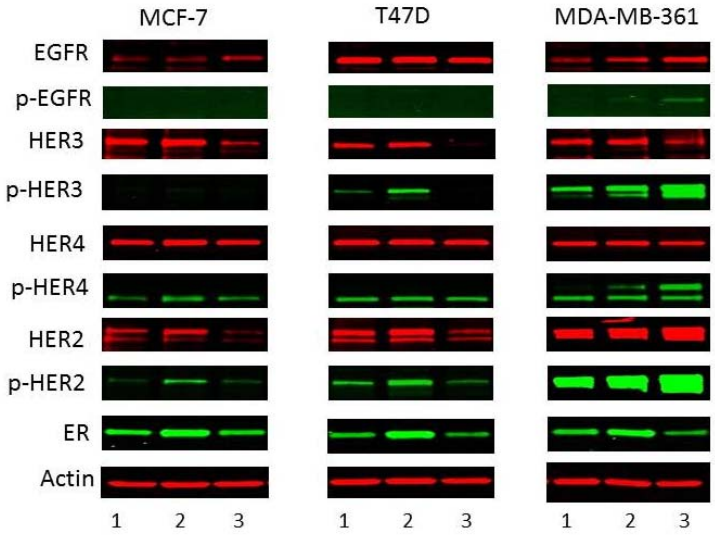


Figure 27. Changes in HER receptor expression with development of tam-resistance. Steady-state levels of the indicated proteins in control MCF7 and T47D cells treated with vehicle (0.01% DMSO alone (lane); parental cells treated with 1 μM tamoxifen for 96 hours (lane 2); and tamoxifen-resistant cells cultured in the continuous presence of 1 μM tamoxifen. Actin steady-state protein levels served as controls for equal loading of protein.

In other experiments we found that steady-state HER3 protein levels were slightly increased in tam-resistant MDA-MB-361 cells compared with tam-sensitive parental 361 cells (**Figure 28**). However, the activation state of downstream MAPK-Erk and PI3K-Akt signaling pathways were relatively unchanged.

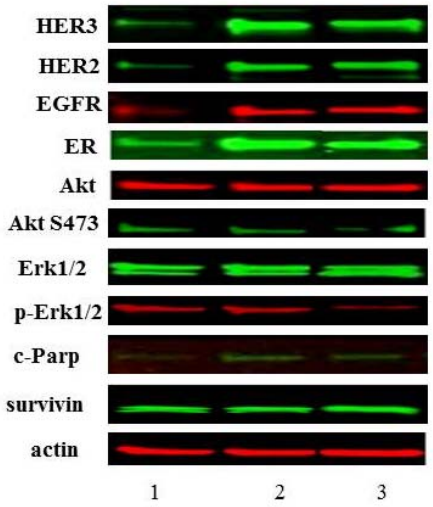


Figure 28. Increased HER3 in response to tam-treated MDA-MB-361 cells and tam-resistant 361 cells. Steady-state levels of the indicated proteins and phosphoproteins in control 361 cells treated with vehicle (0.01% DMSO) alone (lane 1); parental 361 cells treated with 1 μM tamoxifen for 72 hrs (lane 2); and tamoxifen-resistant 361 cells cultured in the continuous presence of 1μM tamoxifen. Actin steady-state protein levels served as controls for equal loading of protein

We also looked at redundant signaling pathways that have been linked to therapeutic resistance to other targeted therapies e.g. EGFR TKIs. For example, the non-membrane bound tyrosine receptor kinase c-Src has been linked to therapeutic resistance to the HER2/EGFR TKI lapatinib used in the treatment of HER2+ breast cancer. There are some reports that Src may also be involved in endocrine resistance in ER+ breast cancer. In our models of tam-resistance, we found that steady-state levels of total c-Src protein and that of Src phosphorylated on tyrosine 416, a site associated with the activated form of the kinase, were increased in tam-resistant MCF7 and CAMA-1 cells compared with their tam-sensitive matched counterparts, but not tam-resistant T47D and MDA-MB-361 cells (**Figure 29**), underscoring the heterogeneity of the cell signaling response to the development of tam-resistance.

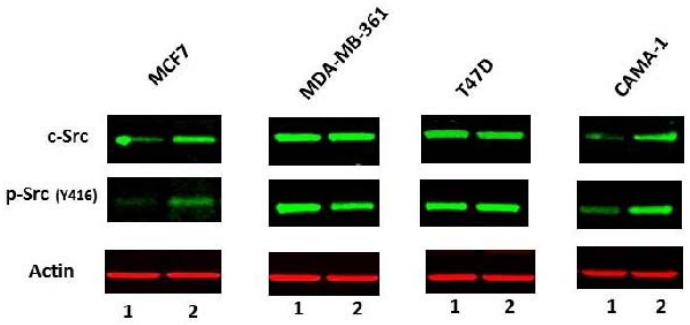
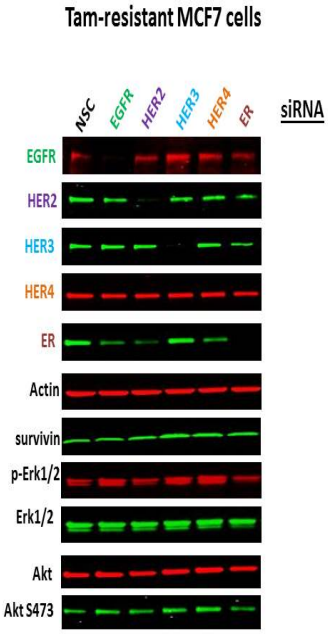
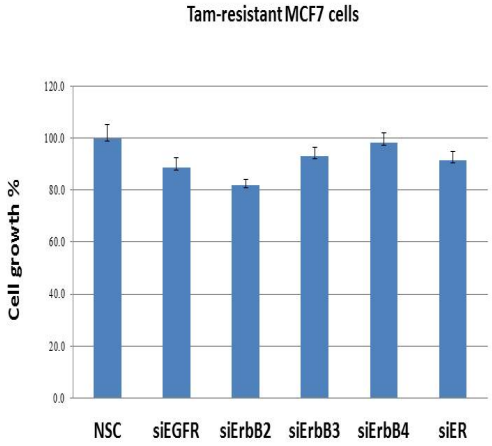


Figure 29. Src protein expression in tam-resistance. Steady-state levels of total and phosphor-Src (Y416) in parental (tam-sensitive) (lanes 1) and tam-resistant (lanes 2) for the indicated ER+ human breast cancer cell lines. Actin steady-state protein levels served as controls for equal loading of protein. The results are representative of independently repeated experiments.

***Sub-Task 3**: Evaluate the impact of HER receptors in regulating the survival of tam-resistant cells.* Not all tam-resistance will be mediated through HER3; therefore, it is important to identify those tam-resistant tumors that are more likely to respond to a HER3 vaccine when we start to develop the tumor profile of likely responders. Although HER3 will be used to demonstrate proof of concept for our vaccine approach, the efficacy of a HER3 vaccine may be enhanced in combination with other targeted strategies. We used a targeted molecular knockdown approach to ascertain the role of HER3 and other HER receptors, as well as ER in regulating survival in tam-resistant models. In Year 1, we showed that HER3 knockdown in tam-resistant MCF7 but not tam-resistant T47D had an effect on cell survival. We repeated the siRNA molecular knockdowns targeting each of HER receptor and ER in several models of tam-resistance (**Figure 30**). In addition to documenting effective knockdown of the targeted protein by Western blot analysis, we also determined the effects of each knockdown of tam-resistant cells (**Figure 30**).





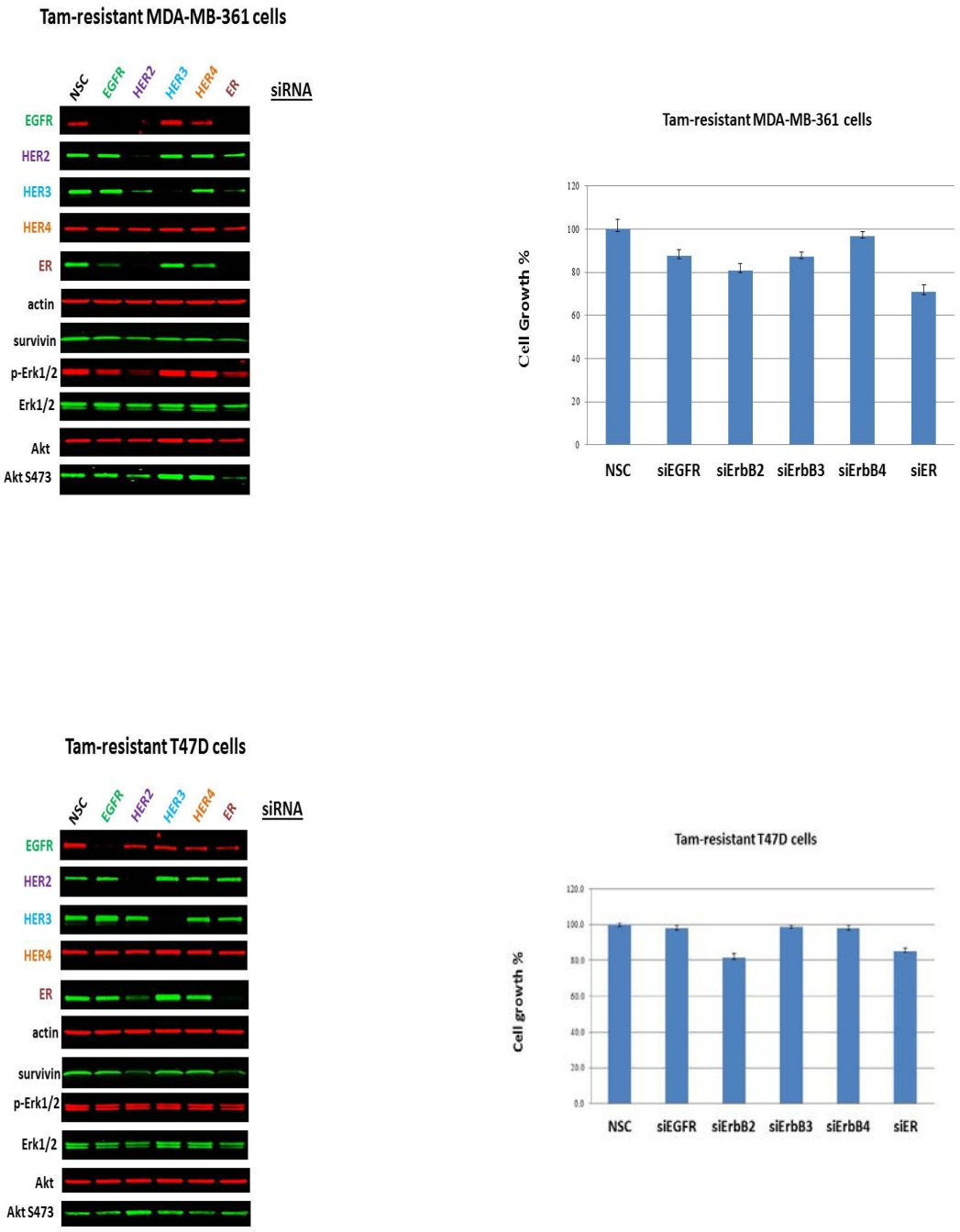


Figure 30. Molecular knockdowns of HER3 and other HER receptors. Western blot analysis (left side) of the indicated proteins in tamoxifen resistant MCF7, MDA-MB-361, and T47D at 72 hours following transfection with the indicated siRNA constructs. Scrambled siRNA (NSC) was used as a control. Steady-state actin protein levels were used to control for equal loading of protein in each lane. The effects of targeted molecular knockdowns on the growth and viability of the indicated tam-resistant cells were determined approximately 72 hours after siRNA transfections (right side). Results represent median +/- standard error of triplicate samples and are representative of three independent experiments.

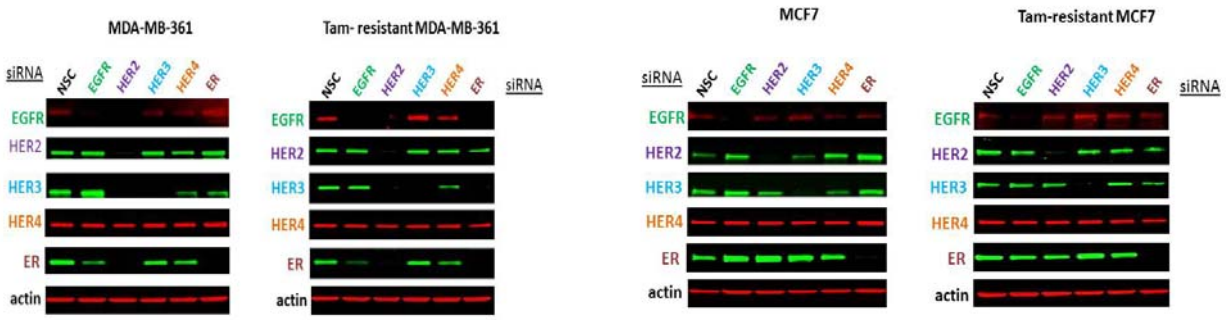


Figure 31. Effects of selectively knocking down HER3 and HER family members in parental versus tam-resistant cells. Parental and tam-resistant MDA-MB-361 and MCF7 cells were transfected with the indicated siRNA constructs. After 72 hours, cells were harvested Western blot analysis was performed on the indicated proteins. Steady-state actin protein levels were used to control for equal loading of protein in each lane.

We showed that ER knockdown in some tam-resistant cells lines resulted in marked reductions in the expression of selected HER receptors. We next sought to determine whether there were differences between in the effects of ER knockdown on HER receptor expression in parental versus tam-resistant cells. In tam-resistant MDA-MB-361 cells, ER knockdown lead to a concomitant reduction in HER3 and EGFR protein levels (**Figure 31**). In contrast, ER knockdown in parental MDA-MB-361 cells did not lead to reduced EGFR protein and some, but not complete inhibition of HER3 expression. Similar discrepancies were not observed in MCF7 parental and tam-resistant cell lines. These findings not only underscore the differences between tam-resistant models, but also the differences between the ER-HER receptor linkages in certain isogenic pairs of parental and tam-resistant cell lines. It suggests that targeting one receptor through a vaccine approach may also lead to the down-modulation of other HER receptors.

Summary

- Establishment of six Tam-resistant ER+/HER3 expressing human breast cancer cell lines. These models will be used to identify the protein architecture associated with HER3 pathway activation.
- We have demonstrated protein pathway heterogeneity among the tam-resistant cell lines, which could have important implications with regard to sensitivity to a HER3 vaccine strategy, although we recognize that results from HER3 molecular knockdown may not predict for response to a vaccine and its consequential immune effects e.g. CTL.
- Targeting multiple HER receptors and/or ER via vaccine approach or in the case of HER receptors, TKI, is an attractive next generation therapeutic strategy.
- Based on molecular knockdown studies, there appears to be a reciprocal relationship between the expression of ER and selected HER receptors. The nature of this relationship may be relevant to a vaccine strategy and is currently under investigation.

Reagents developed over the past year

- Three additional isogenic pairs of parental and Tam-resistant human ER+ breast cancer cell lines have been established.

The pathology core performed a series of immunohistochemical experiments using a new anti-HER3 antibody from Acris. The optimized staining protocol was applied to a series of cell lines and tissues.

HER3 IHC Protocol

- Primary antibody: rabbit monoclonal from Acris
- Antigen retrieval: citrate buffer for 35 min
- Primary incubation: 2 hours, 1:250 titer
- Detection reaction: Mach4 kit (BioCare)
- Chromogen DAB, counterstain hematoxylin
- Automated immunostainer (Intellipath)
- Positive controls: small/large bowel
- Staining pattern: cytoplasm, cell membrane

Mixed membrane and cytoplasmic staining was observed in cell line SKBR3. Cytoplasmic staining was seen in cell lines MDA-MB-468 and MCF10A. Unexpected nuclear staining was noted in cell lines BT474 and 4T1. No staining was present in cell lines MCF7, MDA-MB-231 and T47D **(Figure 32)**

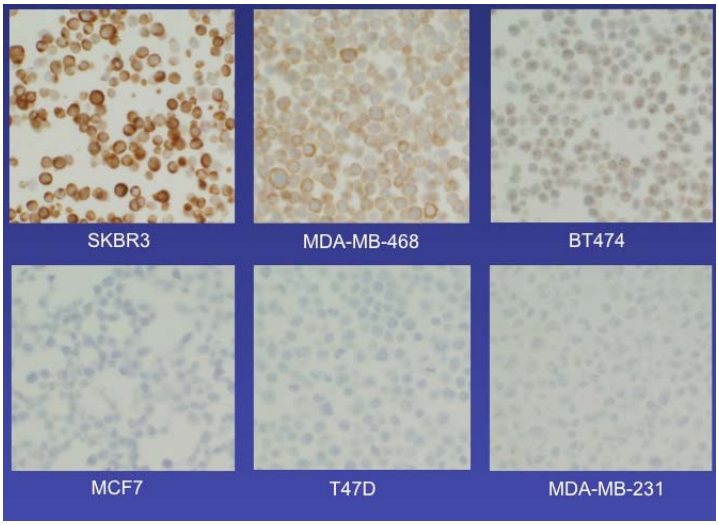


Figure 32. HER3 expression in cell lines

In archival tissue sections, we observed mixed membrane and cytoplasmic staining in epithelial cells of the colon and small bowel. Interestingly, it was also present in benign breast epithelium, usually in a heterogeneous distribution. In **Figure 33,** panel (a) shows a strongly reactive lobule, while other lobules in the same breast showed only weak or focal reactivity (b).

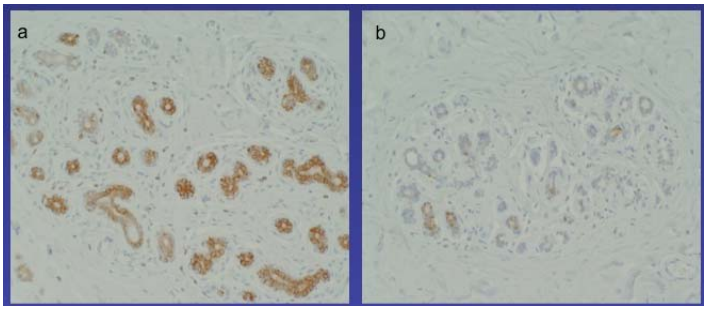


Figure 33. HER3 expression in benign breast epithelium

Positive membrane staining was also noted in one of six breast carcinomas (**Figure 34**).

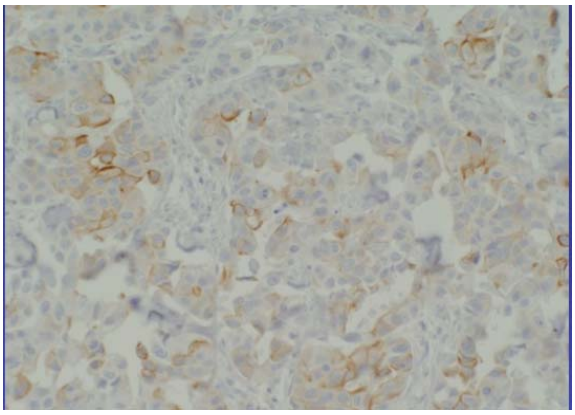


Figure 34: HER3 positive breast cancer

Two additional breast carcinomas showed weak cytoplasmic staining, while three tumors were negative.

Figure 35 depicts a HER3-negative invasive ductal carcinoma (panel b and black arrow in panel a). Interestingly, strong HER3 staining is observed not only in benign breast epithelium adjacent to the carcinoma (panel c and red arrow in panel a) but also in tumor infiltrating lymphocytes (TILs, panel c and green arrow in panel a).

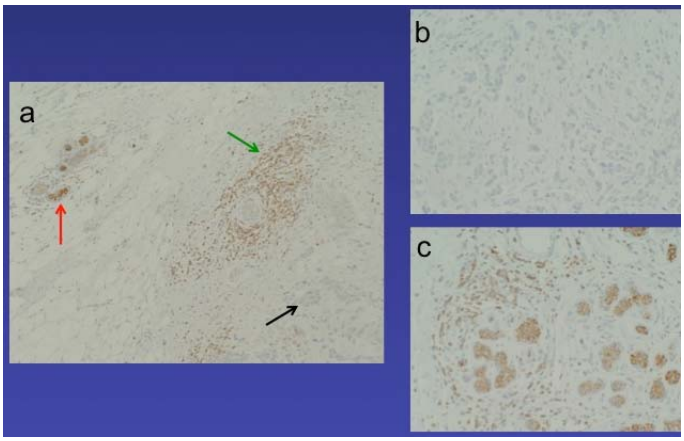


Figure 35: HER3 negative ductal carcinoma

Figure 36 shows the same reaction pattern at high power.

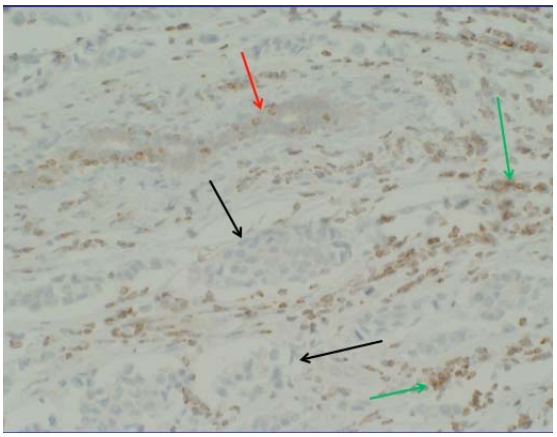


Figure 36: HER3 expression in tumor, benign epithelium and TILs

In addition, the Pathology core developed an immunohistochemical staining protocol for HER1 (EGFR) using a novel rabbit monoclonal antibody from Epitomics. Due to the high sensitivity of this antibody, we can use it at a very low titer (1:2000) and a short primary incubation time (30 min). Unlike other EGFR antibodies, the rabbit monoclonal has a good signal-to-noise ratio and yields good membrane staining with less nonspecific cytoplasmic reactivity.

EGFR IHC Protocol

- Primary antibody: rabbit monoclonal from Epitomics
- Antigen retrieval: EDTA buffer for 35 min
- Primary incubation: 30 min, 1:2000 titer
- Detection reaction: CellMarque HRP Universal Detection kit
- Chromogen DAB, counterstain hematoxylin
- Automated immunostainer (Intellipath)
- Positive controls: tonsil, MDA-MB-468 cell line
- Staining pattern: cell membrane

Inherent to the development of a protein or genomic signature of an activated HER3 signaling particularly in the context of resistance to endocrine therapy, is the establishment of ER+ breast cancer models of endocrine resistance. Isogenic paired parental and endocrine resistant cell counterparts can then be interrogated at a protein pathway or genomic level for evidence of an activated HER3 signaling pathway.

Generating in vitro models of endocrine therapy resistance

Demonstrating the effects of tamoxifen on the viability of ER+ breast cancer cells remains a challenge as changes in cell growth and viability are not typically seen until at least 96 hours after treatment, which is consistent with previous publications showing that it can up to 7 days before seeing significant cell death in response to hormonal therapies. As consequence, it has taken a considerably longer time to establish tamoxifen resistant ER+ human breast cancer cell lines then we had anticipated.

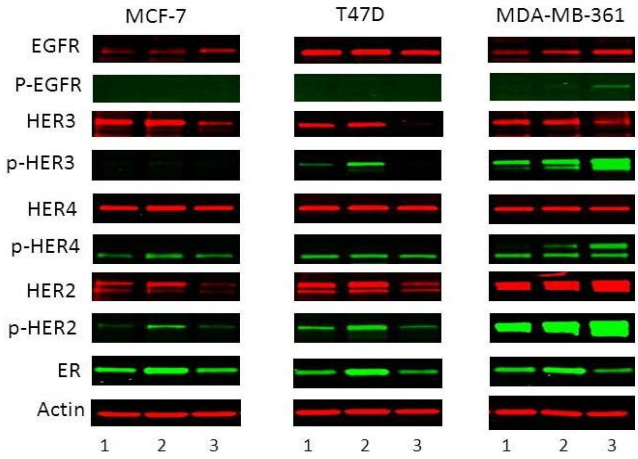


Figure 37. Expression of HER receptors in parental and tamoxifen resistant cells. Steady-state protein levels of the indicated proteins and their tyrosine phosphorylated (activated) forms were determined by Western blot analysis in MCF7, T47D, and MDA-MB-361 cells. Treatment conditions included: (lane 1) vehicle (0.01% DMSO) controls; (lane 2) parental treated with 1 μM tamoxifen x 96 hrs; (lane 3) established tamoxifen resistant cells growing in the continuous presence of 1 μM tamoxifen. Steady-state actin protein levels demonstrate equal loading of protein. These results are representative of three independent experiments.

As shown in **Figure 37**, there are baseline differences in the expression of EGFR, HER3, and HER2 and their activated, tyrosine phosphorylated forms in the three cell lines. Interestingly, in 361 cells, p-HER3 is markedly increased in tamoxifen-resistant cells. HER2 and p-HER2 is also markedly increased in 361 cells (parental and tam-resistant) compared with the other cell lines.

Recent reports have identified gain-of-function mutations in ER that are associated with resistance to endocrine therapy. These mutations render tumor cells largely independent of the effects of estradiol. We are currently sequencing our tamoxifen-resistant cell lines to determine whether they harbor these reported mutations. In the meantime, we sought to determine the effects of ER knockdown on the viability of our tam-resistant cells.

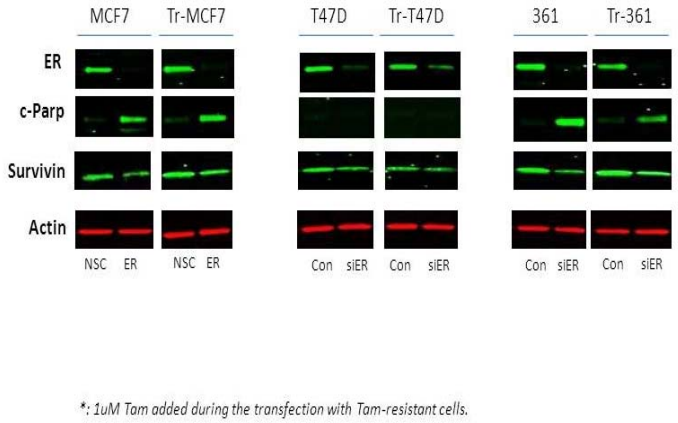
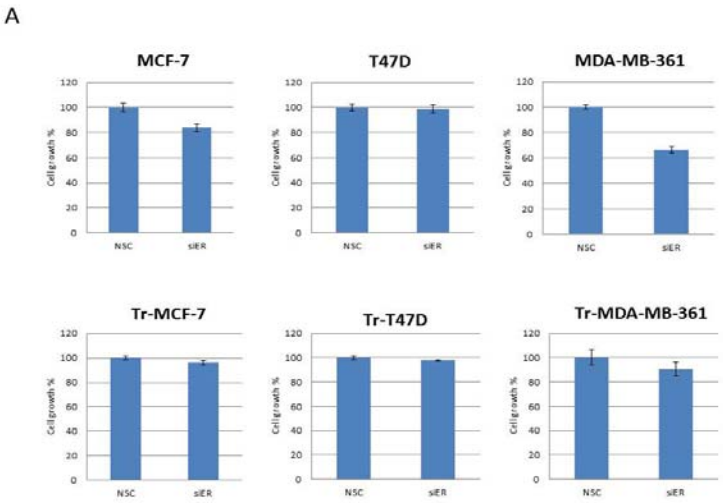


Figure 38. Effects of targeted molecular knockdown of ER in tam-resistant cells. Parental (MCF7; T47D; MDA-MB-361) and tam-resistant cell counterpart (e.g. Tr-MCF7) were transfected with control, scrambled siRNA (NSC) or siRNA targeting ER. Steady-state levels of the indicated proteins were analyzed by Western blot 72 hrs after transfection. Tam-resistant cells were maintained in 1 μM tamoxifen during siRNA transfection. Steady-state actin protein levels served to confirm equal loading of protein. Results are representative of three independent studies.

In addition to Western blot analysis, we also looked at cell viability at 72 (**Figure 39A**) and 96 hrs (**Figure 39B**) following transfection with either control, scrambled siRNA (NSC) or ER siRNA (as described in **Figure 38**).



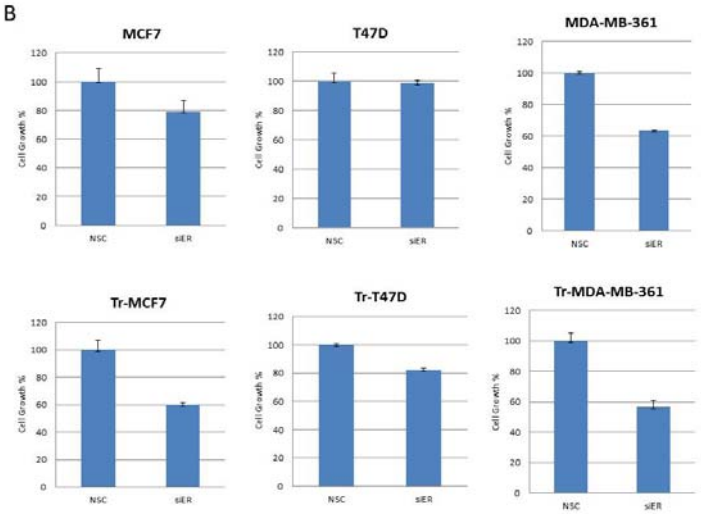
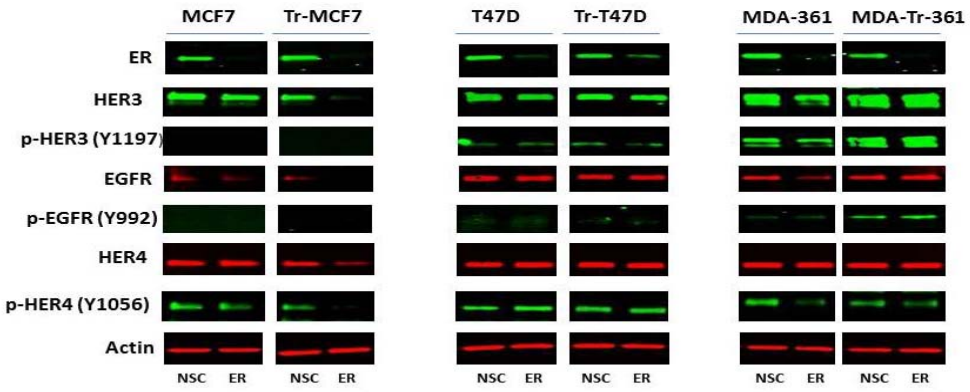


Figure 39. Effects of ER knockdown on cell viability in parental and tam-resistant cell counterparts. See Figure 36 for details of experimental design. Cell viability was evaluated at 72 hrs (A) and 96 hrs (B) after transfection with the indicated siRNA constructs. Values are the mean of triplicate samples with standard deviation error bars included. The data is representative of three independent experiments.

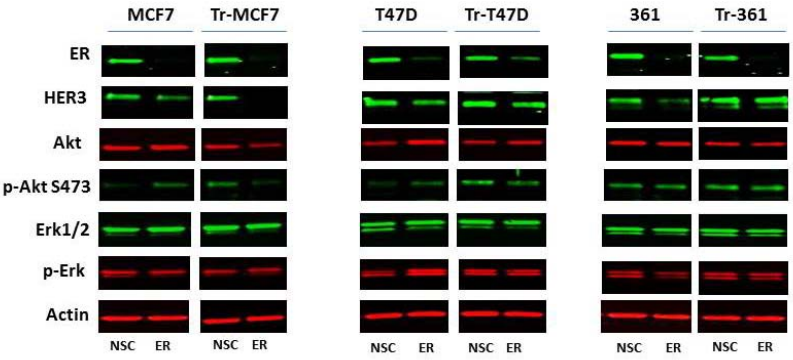
We also looked at the effects of ER knockdown on the expression of total and tyrosine phosphorylated forms of HER receptors, in addition to downstream PI3K-Akt and MAPK-Erk signaling pathways (**Figures 40A and 40B**).

A



B

**: 1uM Tam added during the transfection with Tam-resistant cells.*



**: 1uM Tam added during the transfection with Tam-resistant cells.*

Figure 40. Effects of ER knockdown on HER receptor signaling. Steady-state protein levels of the indicated proteins/phosphoproteins were determined by Western blot analysis. The experimental design is the same as that described in Figure 33. Cells were harvested 72 hrs after transfection of the indicated siRNA constructs, and equal amounts of protein loaded in each lane. Actin steady-state protein served as a control for equal loading of protein.

There is no consistent changes in HER receptor, phosphorylation, and downstream signaling that are associated with the increased sensitivity of MCF7 and MDA-MB-361 (parental and tam-resistant) cell lines to ER knockdown compared with T47D. Interestingly, in tam-resistant cell lines only, knockdown of ER also leads to reduced expression of HER3, EGFR, and to a lesser degree, HER4. These results underscore the heterogeneity of these parental and tam-resistant ER+ breast cancer cell lines and the likelihood that they will respond differently to therapies targeting HER receptors.

Summary

- Three stable tamoxifen resistant cell lines that appear to have different HER receptor profiles from their parental cell counterparts and from one another.
- As part of the characterization of our tam-resistant cell lines, we have assessed the continued role of ER in maintaining cell survival. It appears that two of the cell lines-tam-res MCF7 and MDA-MB-361 are still somewhat dependent upon ER for their survival as they undergo apoptosis in response to targeted molecular knockdown of ER.
    - We are in the process of sequencing ER in the tam-resistant cell lines to determine whether they express gain-of-function mutations in ER that have been associated with resistance to endocrine therapy. Cells lines that are still dependent upon ER for survival may not be amenable to a HER3 targeted therapeutic approach since these survival of these cells may not be dependent upon HER3.
- We have four additional ER+ cell lines that we are currently culturing in the continued presence of tamoxifen in order to generate additional tam-resistant cell lines for further characterization.

ER mutation analysis: Constitutively activating mutations in ER have been identified in approximately 15% of breast cancers. These mutations have been shown to contribute to endocrine resistance. The data presented in our last quarterly updated showed that certain tamoxifen resistant cell lines were still sensitive to targeted molecular knockdown of ER. Selection of cells containing activating ER mutations provides a potential explanation for persistent sensitivity of certain tamoxifen resistant cell lines to ER knockdown. It might also impact the sensitivity of cells to a HER3 targeted strategy. To address the ER status, we sequenced ER in our tamoxifen resistant cell lines. We generated PCR products that spanned known mutation hotspots and then sequenced those products. As shown below (**Figure 41**), we did not see evidence of ER mutations in any of our tamoxifen models.

ESR1 Mutations reported in Li (Ellis last author) Cell Reports 4:1116 '13								
		E380Q	V392I	S463P	P535H	L536R	Y537 C/N/S	D538G	R555C
MCF7- tam R		WT	WT	WT	WT	WT	WT	WT	WT
MDA-MB-361- TamR		WT	WT	WT	WT	WT	WT	WT	WT
T47D-TamR		WT	WT	WT	WT	WT	WT	WT	WT

Figure 41. ER receptor mutation status. We sequenced PCR products that spanned mutation hotspots to screen for ER mutations more reported in the literature to be more frequent in clinical samples.

Functional consequences of HER receptor knockdowns. Having evaluated the effects of ER knockdown in tamoxifen resistant models, we next sought to determine the consequences of selectively knocking down individual HER receptors. We used commercially available siRNA constructs from Origene and Santa Cruz Biotech. The Western blot analyses of the indicated proteins are shown in each cell line (**Figure 42**).

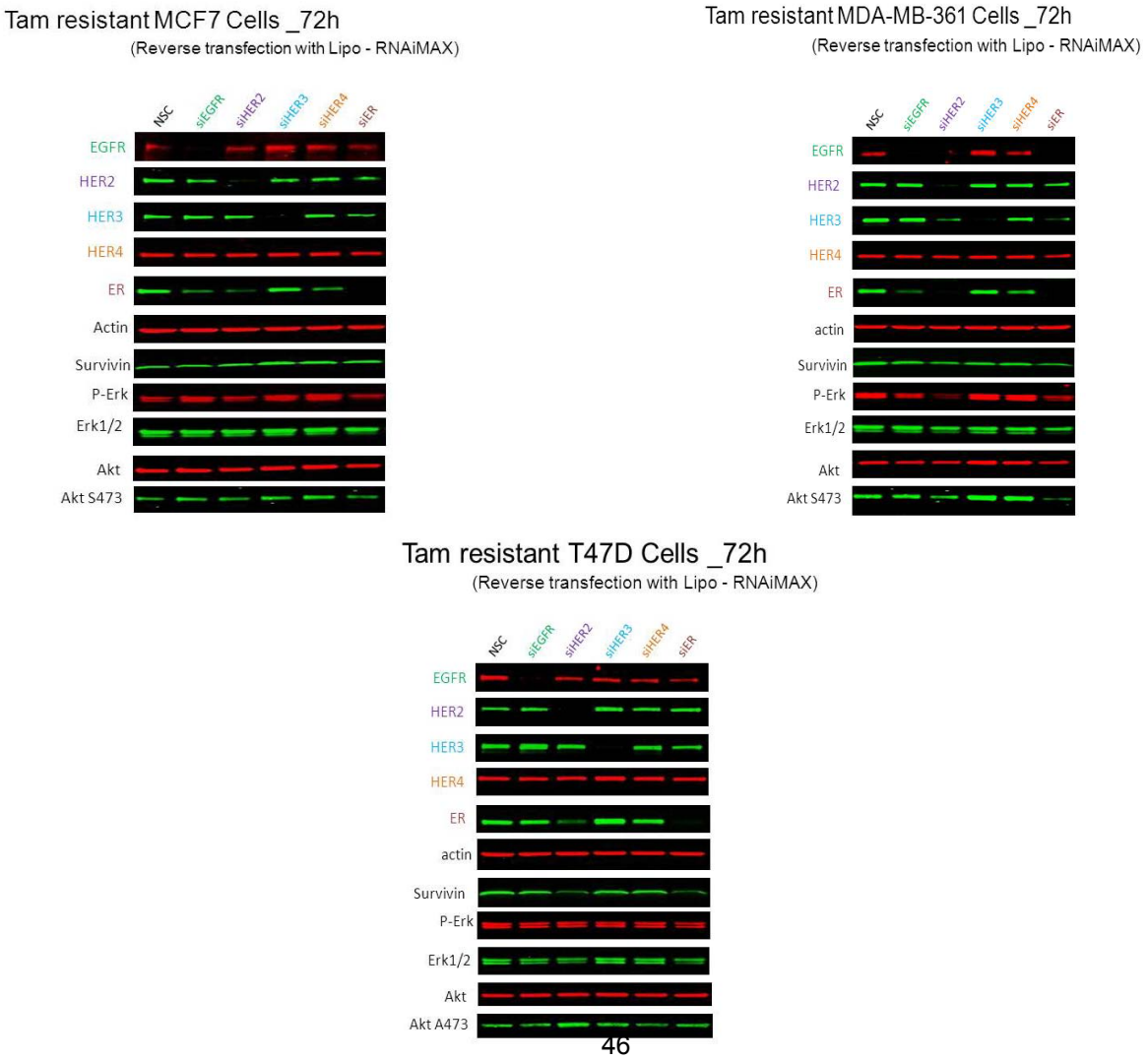


Figure 42. Molecular knockdowns of individual HER receptors. Western blot analysis of the indicated proteins in tamoxifen resistant MCF7, MDA-MB-361, and T47D 72 hours following transfection with the indicated siRNA constructs. Scrambled siRNA (NSC) was used as a control. Steady-state actin protein levels were used to control for equal loading of protein in each lane.

Now that we have the conditions to knockdown individual HER receptors, we will look at the functional consequences of those knockdowns.

Summary

- Sequencing ER mutation hotspots in tamoxifen resistant cell lines
    - No evidence of frequently reported activating ER mutations
- Targeted knockdown of individual HER receptors
- We have four additional ER+ cell lines that we are currently culturing in the continued presence of tamoxifen in order to generate additional tam-resistant cell lines for further characterization.

Aim 2: Pre-clinical testing of activity and toxicity of Ad5(E2b) HER3

2A: Pre- IND meeting with FDA

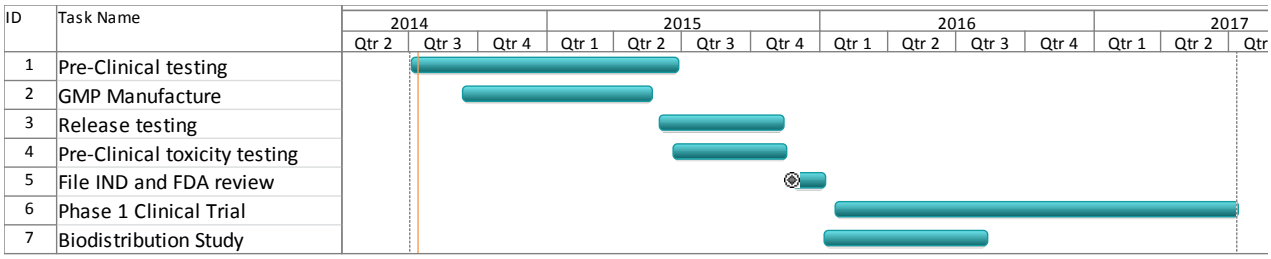
We began initial work on a pre-IND package in October 2013 which included assembly of preclinical data and generation of a complete protocol and consent forms. We then formally submitted a request for a pre-IND meeting (Type B meeting) to discuss the use of Ad[E2b-]huHER3 vaccine. The request was received by the FDA on April 14, 2014 and after nearly a 2 month delay by FDA, we received a response from FDA that the pre-IND teleconference will occur on July 2. In keeping with the requirement to submit meeting discussion materials 30 days prior to the meeting, we have submitted the pre-IND package to FDA as of May 30, 2014. Based on pre-clinical data using a HER3 transgenic mouse model, we have selected an Ad[E2b-] vector encoding full length HER3 as the best candidate to use in our proposed phase I clinical trial. Among the key questions to be discussed at the pre-IND meeting are whether they agree with the selection of the Ad-HER3 full length vaccine for clinical development and whether they agree with our toxicology testing strategy.

2B: Respond to Pre-IND review

In our pre-IND meeting, we reviewed proposed toxicology testing plans, our manufacturing plan, lot release testing, selection of the adenoviral vector encoding HER3, and our clinical trial design with the FDA. Based on feedback from the FDA, will begin the process of generating the clinical grade Ad-HER3 material.

2C: Pre- clinical activity and toxicity testing of GMP Ad5(E2b-)HER3

As noted above, our timeline for manufacture and testing of the GMP material is shown below.



Previously, a toxicology study on our Ad[E2b-]-CEA therapeutic vaccine was requested by the FDA in an application for an IND (IND- 14325. Our collaborator (Etubics) has proposed a similar toxicology strategy in our pre-IND meeting package. We will proceed with the FDA's requested plan.

Because our pre-clinical studies were performed in a different strain of mice than our previous toxicology work and since we will use a higher maximum dose in our proposed clinical study, it may be necessary to contract with a CRO to perform GLP toxicity studies to evaluate the Ad(E2b-)- HER3 therapeutic product in a similar manner as that performed for the Ad[E2b-]-CEA product. This approach has been acceptable to the FDA in support of the IND application. Typically, a total of 220 mice to be used (110 per treatment group, 55 of each sex).

2D: Begin prospective tissue collection of tumors resistant to anti- estrogen therapy and explore expression of HER3 and the HER3 signaling pathway

We have made substantial progress with regard to our proposed tissue procurement efforts. Surgery CRU approval was received on 4/27/2014, followed by Surgery Chair approval on 4/30. The Duke Cancer Protocol Committee gave its approval on 5/15. The Duke IRB approved the protocol on 6/9, followed by DOCR approval on 7/1. The protocol is now under review at the DOD. We have held a protocol start-up meeting with the groups critical in the prospective collection of the resistant tumors. Once DOD approval is granted, we are ready to start our tissue collection. This banking effort will partly utilize the existing tissue procurement infrastructure at Duke University Medical Center (specifically the Biospecimen Repository and Procurement Core, BRPC). The BRPC will collect a processing fee for each procured specimen. Informed consent will be obtained by appropriately trained study personnel. Tissue samples will be obtained both through image guided biopsies and from surgically excised specimens. They will be immediately frozen and accessioned by BRPC personnel. Frozen sections will be cut from each sample and will be evaluated by Dr. Geradts. The salient pathologic characteristics (diagnosis, tumor cellularity, extent of necrosis etc.) will be entered in an electronic database. The diagnoses of the research samples will then be compared to that of the paired pathologic specimens. In addition, a clinical database will be established to complement the procured sample characteristics and annotated pathology data.

In collaboration with Cedar-Sinai Medical Center we have created an offsite clinical annotation database. This platform for this database is REDCap. This database adheres to the Cedar-Sinai Enterprise Information Services (EIS) research database security standards. The database consists of eight Baseline forms and three follow-up forms. The clinical data elements collected

include: demographics, radiographic findings (mammography, U/S, MRI, CT, PET), pathologic diagnosis (histology, IHC, and FISH), surgical and radiation treatment received, systemic treatment received (dates and dosing regimes), and survival data.

Aim 3: Regulatory pathway to first in human testing: obtain US FDA IND, and IRB and HRPO approvals of the phase I clinical trial.

3A: Respond to Pre-IND meeting clinical review and design Phase I study

We will begin the process following our successful FDA teleconference July 2, 2014.

3B: RAC Submission and waiver of review

Based on guidance from the FDA pre-IND meeting, we will complete the package for submission to the NIH RAC.

3C: IND submission and approval

Following completion and analysis of the toxicology study, we will submit the IND.

3D: IRB and HRPO submission and approval

At the time of IND submission, we will submit the IRB application and following IRB approval, submit to HRPO.

Aims 4 and 5 were to be done in phase II of this proposal.

4. Key Research Accomplishments

- **Established human HER3 transgenic model of breast cancer in immunocompetent mice.**
- **Designed and synthesized human HER3 vaccine candidates in second generation recombinant adenovirus.**
- **Tested candidate HER3 vaccines in pre-clinical models and evaluated immunogenicity and anti-tumor effects.**
- **Held External Scientific Advisory Committee meeting at Duke.**
- **Prepared pre-IND package for FDA review.**
- **Held pre-IND teleconference with FDA.**
- **Developed plan for GMP manufacture of human HER3 vaccine.**
- **Established prospective tissue collection protocol for refractory breast tumors.**
- **Establish TCR deep sequencing for vaccine analysis.**
- **Developed alternative antigen targets and pre-clinical evaluation of alternative antigen targets.**

5. Conclusion

Generation and testing a HER3 vaccine

The required response to the critique to perform preclinical studies in human HER3 transgenic mice led to a delay in generating the preclinical models. The down selection process was then performed in the context of animals with tolerance to HER3, but this lead to a delay in selecting the HER3 candidate vaccine, and beginning the manufacturing process. Furthermore, based on feedback from the FDA during a pre-IND teleconference, and review of our preclinical data, we have selected the full length HER3 for production and clinical testing, based on the timeline below.

Plans for the upcoming year

Based on feedback from the FDA during a pre-IND teleconference, and review of our preclinical data, we have selected the full length HER3 for production and clinical testing, based on the timeline below.

Development of a pathway signature of HER3 signaling

HER3 has been implicated as playing a role in the development of resistance to endocrine therapies used in the treatment of ER+ breast cancer. In an attempt to identify patients who are more likely to respond to a HER3 vaccine, our objective is to develop a tumor signature(s) associated with an activated HER3 signaling pathway. Our working hypothesis is that tumors where tam-resistance is mediated by HER3 are more likely to be sensitive to the antitumor effects of a HER3 vaccine. To test this hypothesis, we have generated multiple tam-resistant models. At the completion of Year 2, we now have established six tam-resistant ER+ human breast cancer cell lines. We have begun to interrogate the protein signaling architecture of these models seeking to identify protein signatures that predict for an antitumor response to HER3 antibodies generated by our vaccine. The molecular knockdown studies suggest that overcoming or ideally preventing tam-resistance will likely require a multi-targeted strategy e.g. HER2/HER3; HER3/ER. Interestingly, total HER3 protein expression was not necessarily increased in tam-resistant cells compared with treatment naïve cell counterparts. In fact, HER3 protein appears to be decreased in two of the resistant cell lines compared to controls. In addition, HER3 and downstream MAPK-Erk and PI3K-Akt were not particularly activated in tam-resistant cells compared with parental controls. In contrast, we found that protein expression and activation (phosphorylation at Y416) of the non-membrane bound tyrosine kinase c-Src was increased in two of the four models of tam-resistance examined. Thus, targeting Src in these tam-resistant models may enhance the antitumor activity of a HER3 vaccine. Our molecular knockdown studies indicate that targeting a single molecule alone, whether HER3, HER2, EGFR, or ER may not be the optimal approach.

The unexpected nuclear staining pattern for HER3 in two breast cancer cell lines remains to be investigated.

One option to develop a signature of HER3 signaling in endocrine resistance is to generate cells resistant to therapy in 2D culture. Development of tamoxifen resistance in additional ER+ breast cancer cell lines remains a challenge due to the slow growth pattern of the parental cells and their delayed antitumor response to tamoxifen. We will continue to optimize the conditions e.g. dose escalation of tamoxifen for establishing additional tam-resistant cells lines.

Due to the challenges with the tam resistance models, we asked Dr. Suzanne Fuqua, an expert on endocrine resistance, to consult with o=us regarding models of Tam and AI resistance. She felt that 3D models, in vivo models of resistance were superior. She felt that patient samples should be the primary source of information about resistance. We hope she can provide her in vitro and in vivo models and expertise to our work.

Plans for the upcoming year

Having established six tam-resistant cell lines, we can now try to identify an activated HER3 signaling profile by gene expression analysis or reverse-phase protein/phosphoprotein microarrays (RPMA). As a control, we will use an ER+ breast cancer cell line that expresses activated HER3. Since HER3 has a low level autokinase activity, we will need to stimulate with the HER3 ligand, heregulin β1 (HRG). Before we send samples for gene expression and RPMA analysis, we will first make sure HRG activates HER3 and downstream HER3 regulated signaling pathways looking at targeted phosphoproteins e.g. p-HER3, AktS473, p-mTOR by Western blot analysis

Now that we have optimized immunohistochemical staining protocols, we can assess the expression of HER1, HER2 and HER3 in larger series of breast carcinomas.
We expect commencement of our prospective tissue procurement efforts within the next quarter.

We are in the process of developing four new tamoxifen resistance ER+ cell lines (CAMA-1; HCC1428; ZR-75-1; MDA-MB-231). Once these are established, we will profile their cell signaling pathways particular changes in the expression of HER family members. We will continue to determine the sensitivity of parental and tam-resistant cell counterparts using siRNA mediated HER3 molecular knockdowns.
Determine the functional consequences of knocking down individual HER receptors on the growth and viability of tamoxifen resistant cells.

We are also in the process of validating PCR primers to generate a fragment of ER where the gain-of-function mutations associated with endocrine therapy resistance have been identified. These primers will be used to generate PCR fragments from cDNA of the tam-resistant cell lines and then the fragment will be sequenced. If we find mutations in the tam-resistant cells, we can then go back and evaluate the parental cell lines.

Additional antigen targets for endocrine therapy resistance

The major change is the field has been reports that endocrine resistance is due to mutations in the estrogen receptor. We are actively generating reagents that will allow us to test the ER as a target to prevent resistance. We have also arranged a formal scientific advisory board meeting from experts in the field, including Drs. Matt Ellis, Geoffrey Greene, Rachel Schiff, Susanne

Fuqua, and William Muller, which met July 8, 2015 at Duke. The meeting allowed us to focus on progress, and consider state of the art information about potential targets for endocrine resistance based on recent reports of estrogen receptor mutations, and the role of HER2 and HER2 isoforms.

Regarding the possible targeting of other antigens, such as ESR1 mutations that have been observed in ~25% of metastatic tumors obtained from women who have experienced acquired endocrine therapy resistance, the major question is whether a selective immune response against these constitutively active ERs is feasible. In most cases, one or two point mutations are observed in the ERα LBD, such as Y537S or D538G, sometimes in conjunction with S463P. A proof of principle study would be needed to verify that antibodies (sic- immune responses) can be generated that will recognize corresponding conformational or sequence differences in mutant ERα that are presented on the surface of tumor cells. Unlike HER3, ERα is almost exclusively an intracellular protein, although a small percentage of ER may be expressed at the cell surface.

In addition the ESR1 mutations, the ESAC suggested that HER2 and isoforms of HER2 such as HER2del16 remain attractive candidates as vaccines targeting resistance to endocrine therapy.

Additionally, there was high interest in using human specimens from tumors progressing while on endocrine therapy, as a true source of information about antigens found on therapy resistant tumors. The ongoing collection of tumors from patients refractory to endocrine therapy, and establishing analysis and isolation of antigens was seen as the most direct to generate authentic evidence of antigens found in therapy resistant tumors.

Plans for the upcoming year

We expect commencement of our prospective tissue procurement efforts in the next reporting period. We anticipate collecting and annotating more than 20 resistant tumors per calendar year. We plan on expanding the collected tissues using the Rho kinase inhibitor co-culture methods and analyze these resistant tumors for antigen expression.

We will also prepare vaccine strategies targeting ESR1 mutations, HER2del16, and androgen receptors as candidate vaccines for additional resistance mechanisms. Based on our previous work with developing HER2 targeting vaccines, we first started work on a HER2del16 variant. We have found that:

- Mutant ESR1 genes seem to confer exogenous estrogen-independent ERE, PR, and RAR signaling
- In vitro assays to determine estrogen dependent growth have not demonstrated reliable phenotypes
- However, ESR1-Y537N can confer estrogen-independent growth to MCF-7 breast cancer(ER+) in vivo
- MCF-7 cells for all mutations have been constructed and will be tested in vivo
- Additionally, Ad vectors encoding ESR1-WT, Y537N, Y537S, and D538G have been constructed and can be tested for immunogenicity

The EMBO Journal Vol.18 No.8 pp.2149–2164, 1999

Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer

Peter M.Siegel[1,2], Eamonn D.Ryan[3], Robert D.Cardiff[4] and William J.Muller[1,2,3,5]

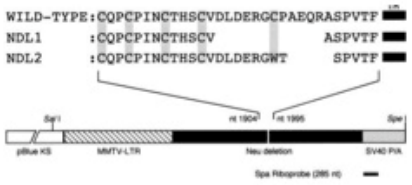


Figure 42

Mammary Tumor Formation and Metastasis Evoked by a HER2 Splice Variant

Abdullah Alajati, Nina Sausgruber, Nicola Aceto, et al.

Cancer Res 2013;73:5320-5327. Published OnlineFirst July 18, 2013.

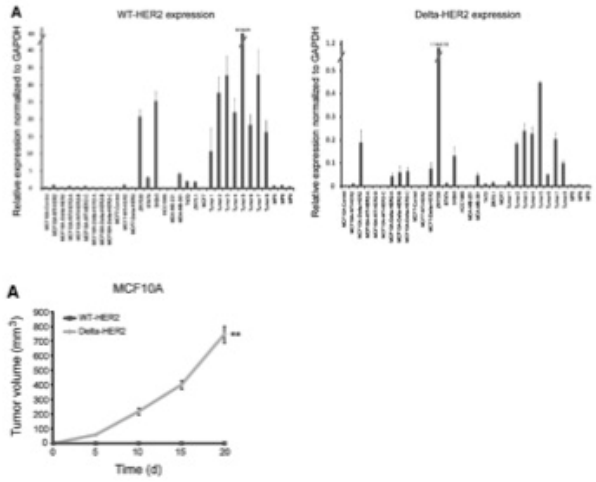


Figure 43. Gene block assembly of new genes or isoforms. Utilizing gene blocks and site-directed mutagenesis to create different mutants and gene specific isoforms.

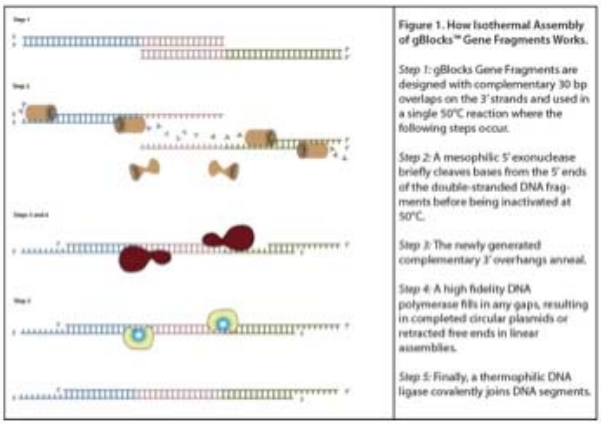


Figure 44. HER2 and HERd16 expression in non-malignant mouse mammary cells.

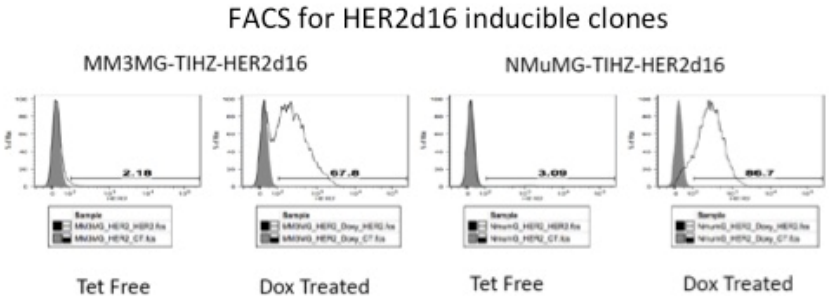


Figure 45. HER2d16 altered morphology

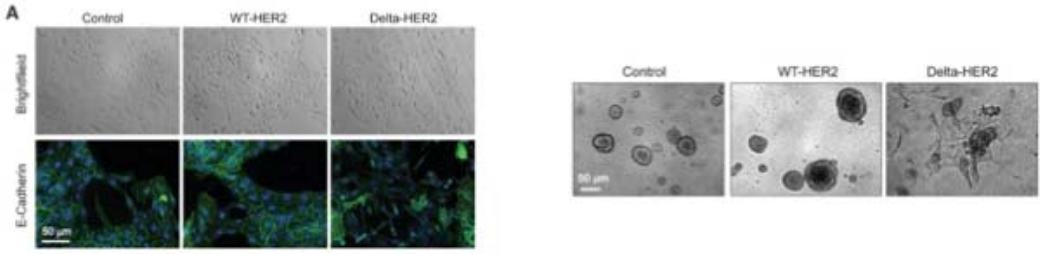


Figure 46. MCF-10a-HER2d16 cells display grossly altered morphology. Characterized by fibroblastic presentation, clustered growth,and cell detachment upon prolong culture. Other mammary cell types (HME1, MM3MG, and NMuMG) and other cells (293T) do not display this overt morphologic difference.

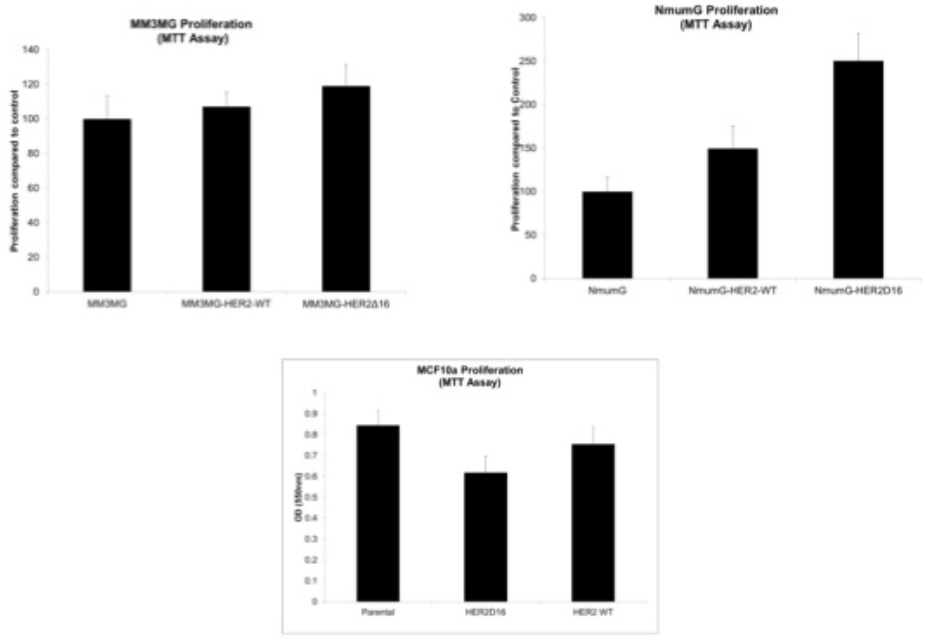
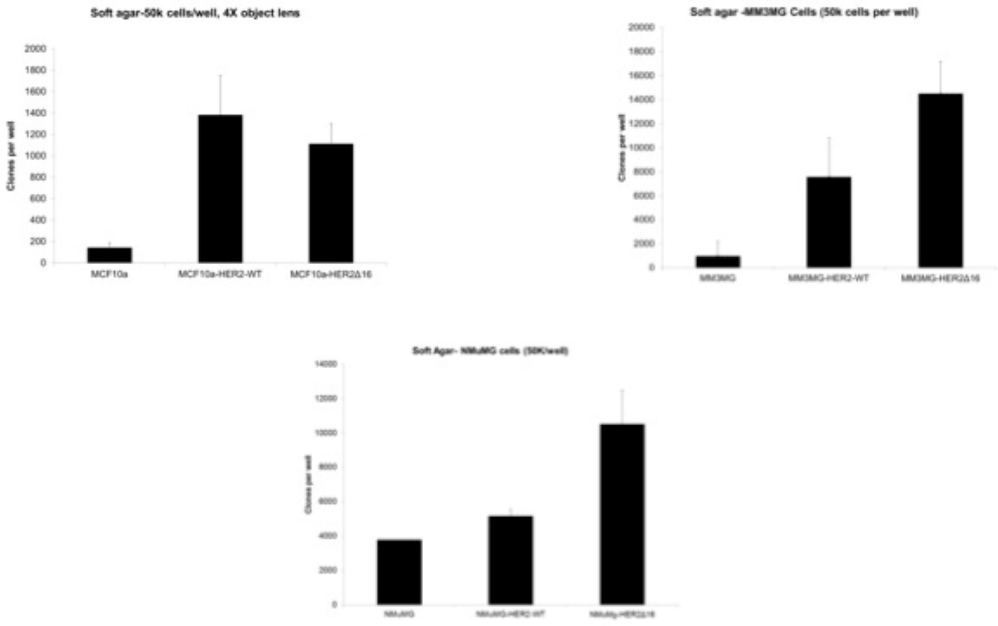


Figure 47. HER2D16 effect on proliferation is cell type dependent



*Colonies are small in MCF-10a cells in all conditions

Figure 48. HER2d16 elicits anchorage independent growth in all cell types. Colonies are small in MCF-10a cells in all conditions.

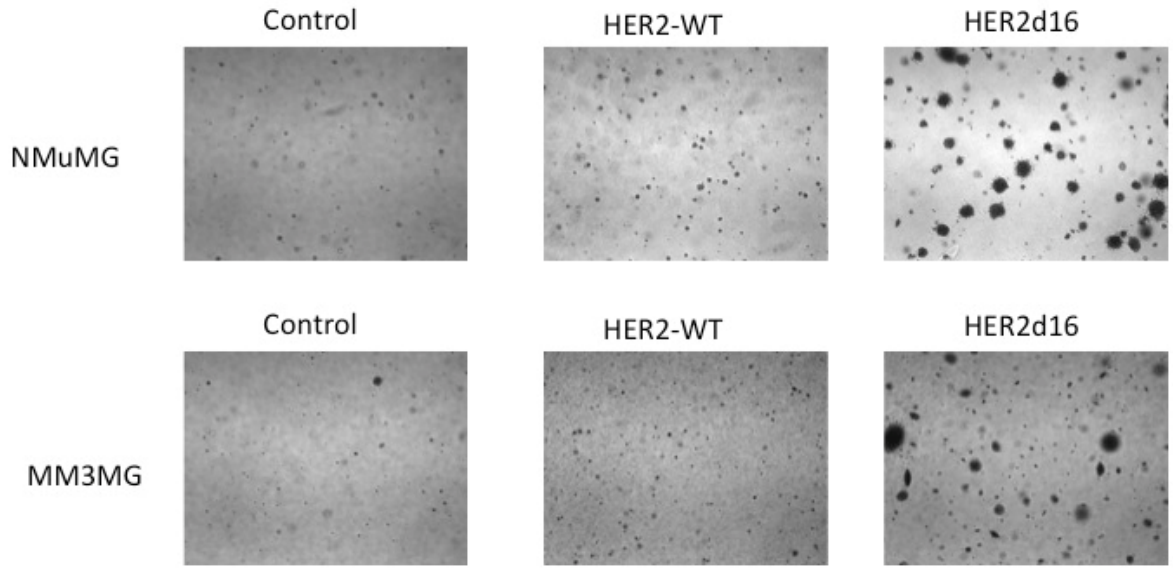


Figure 49. HER2d16 altered anchorage independent growth in murine breast epithelial cells

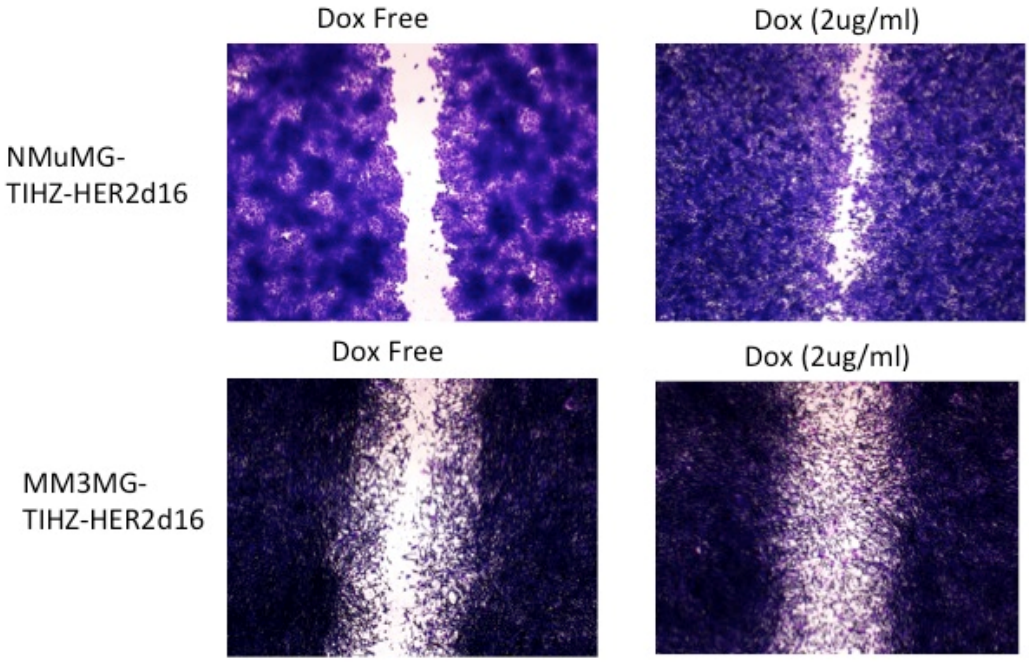


Figure 50. HER2d16 expression enhances cellular migratory capacity.

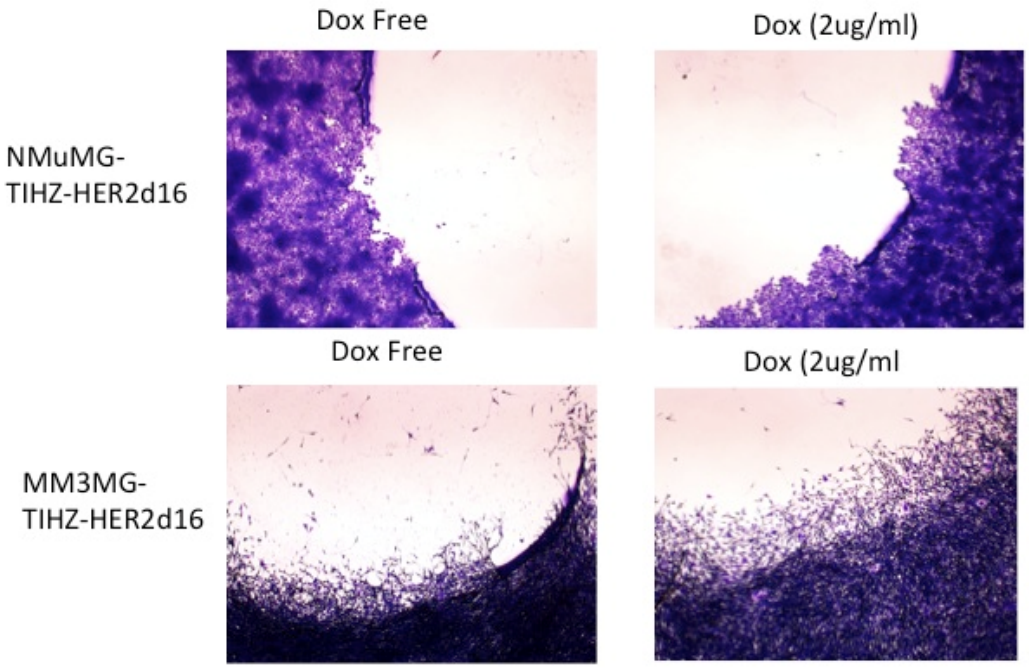


Figure 51. HER2d16 expression enhances cellular invasive capacity.

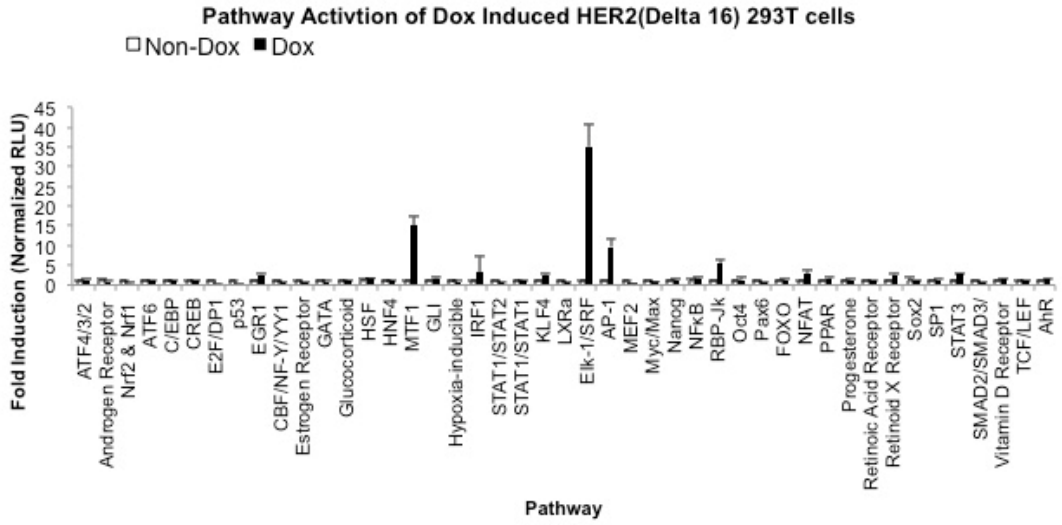


Figure 52. Analysis of HER2d16 signaling

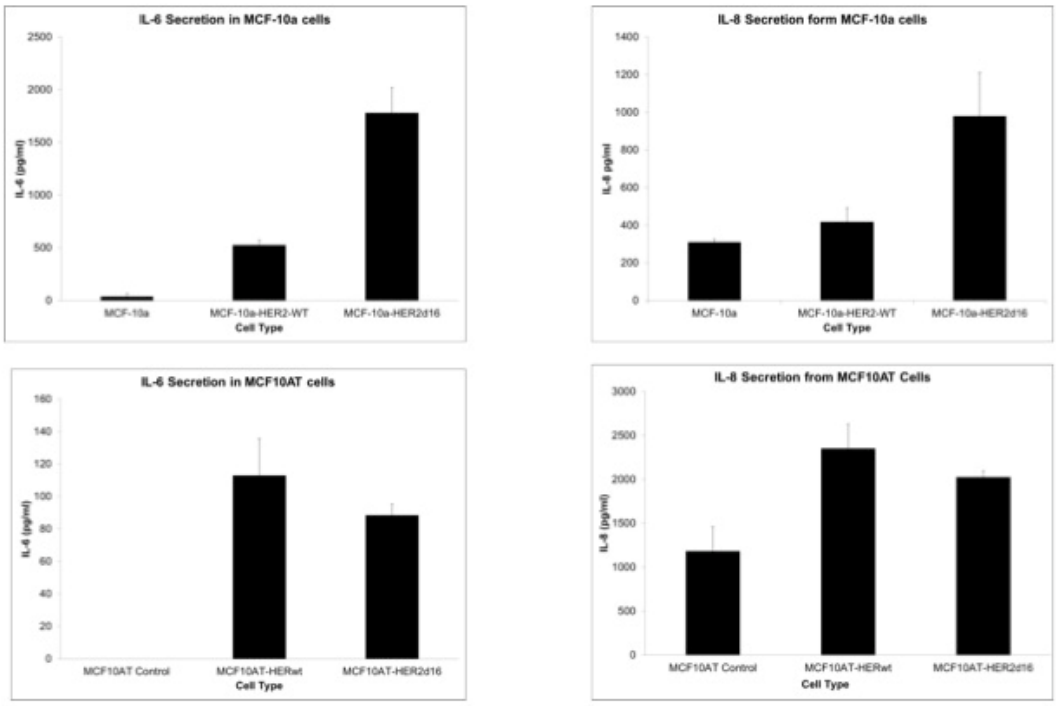


Figure 53. HER2 and HER2d16 induces expression of IL-6 and IL-8 in human premalignant mammary lines.

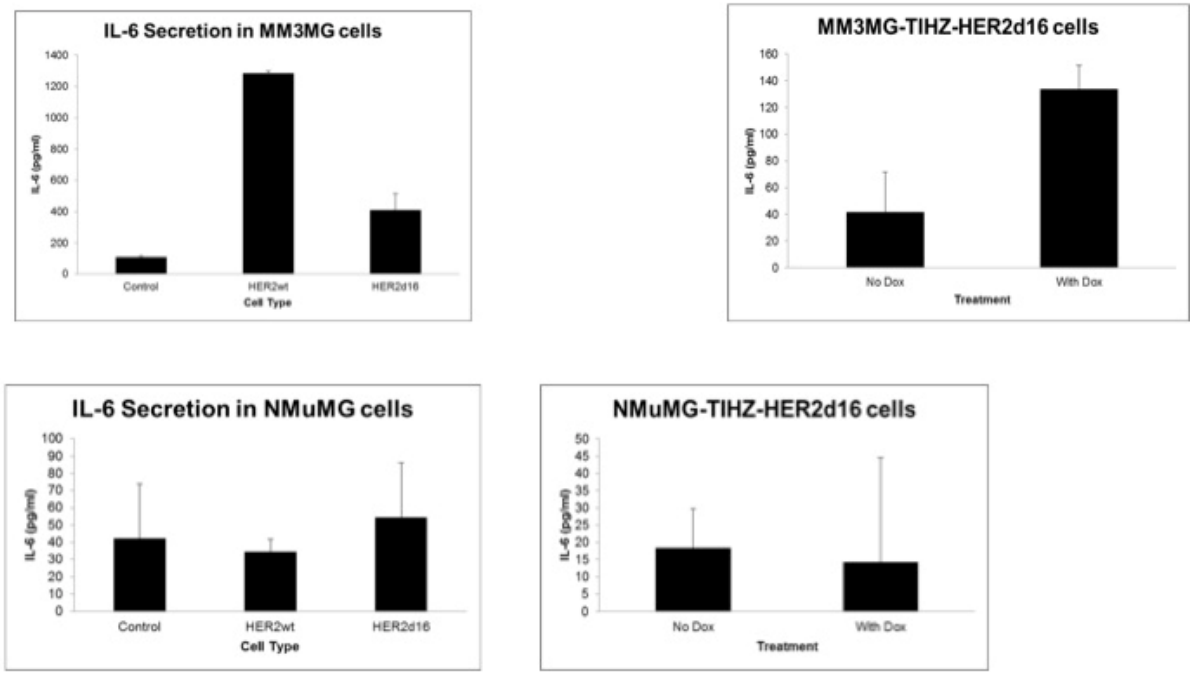


Figure 54. The relationship between HER2d16 is dependent upon cell line in murine models

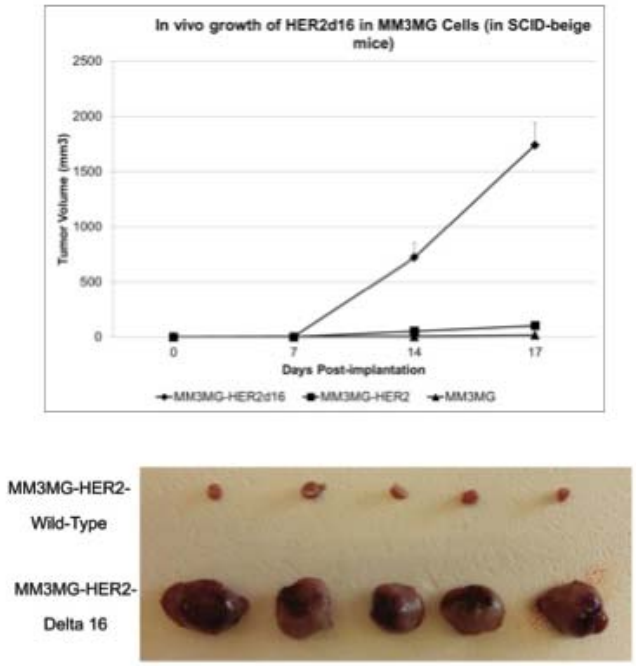


Figure 55. In vivo growth of HER2d16 tumors

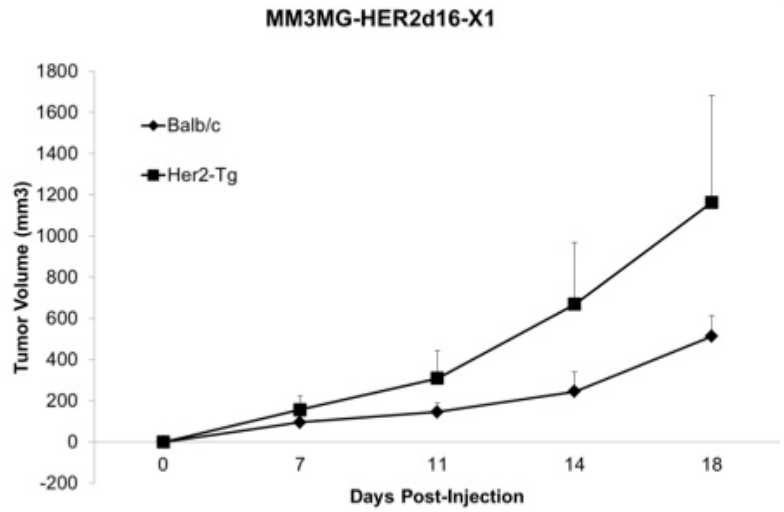


Figure 56. Expansion of MM3MG-HER2d16-X1 (from HER2-Tg F1 mouse)

We have constructed Ad-HER2d16-WT, Ad-HER2d16-kinase inactive, and Ad-HER2d16-TM vectors.

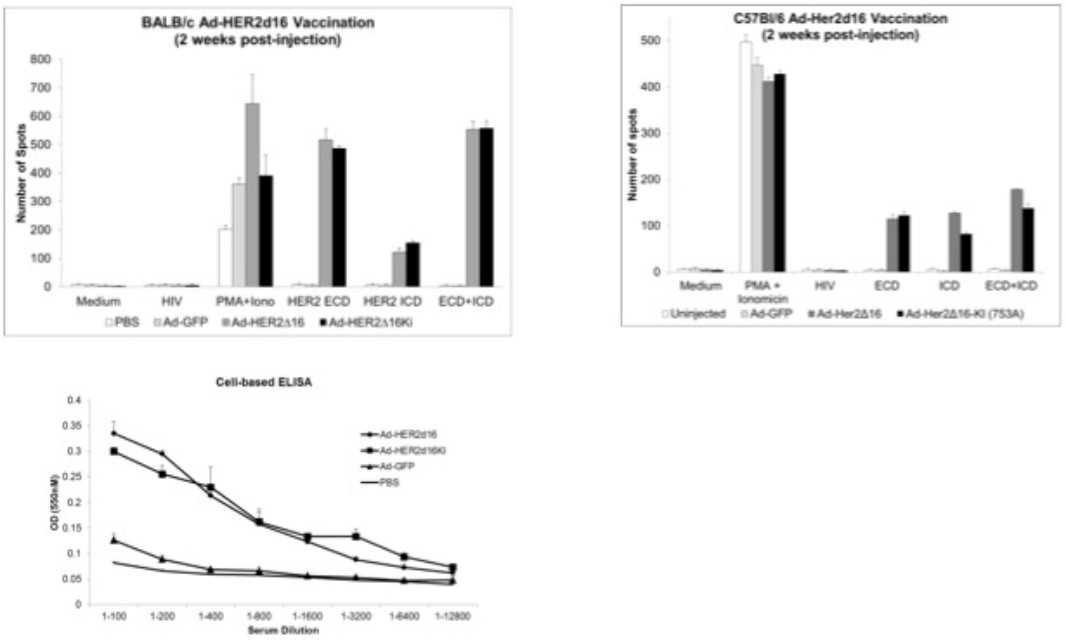


Figure 57. Immune response to Ad-HER2d16 viral vectors

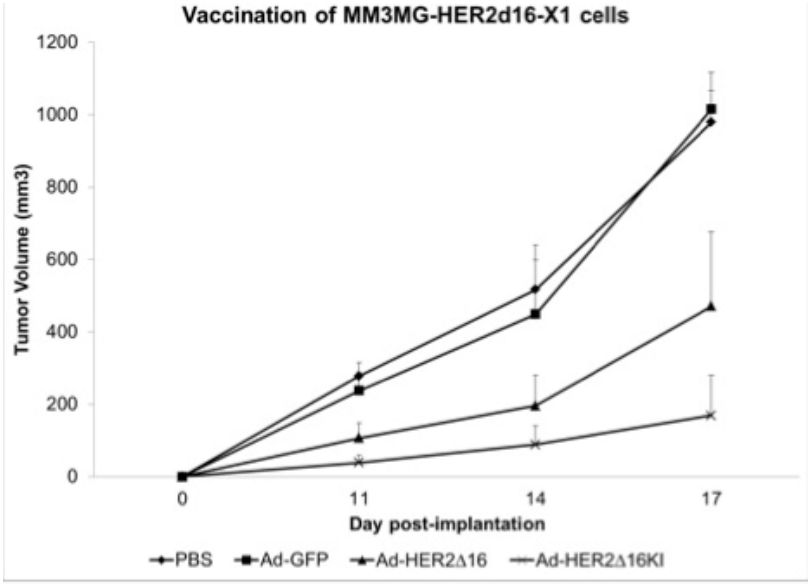


Figure 58. Ad-HER2d16 and Ad-HER216-kinase inactive can significantly suppress tumor growth in a treatment model.

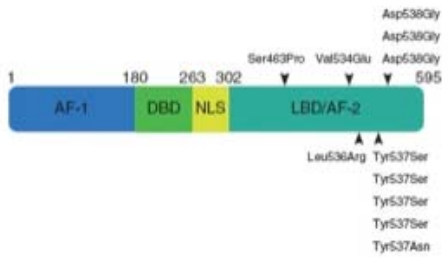


Figure 59. ESR1 mutations

Estrogen-independent ESR1 mutations were discovered in 1996. First found in ER+ breast cancer in 1997 by Fuqua. Recent papers have demonstrated that these DBD ESR1 mutations are found in approximately 12-50% of ER+ endocrine resistant breast cancers.

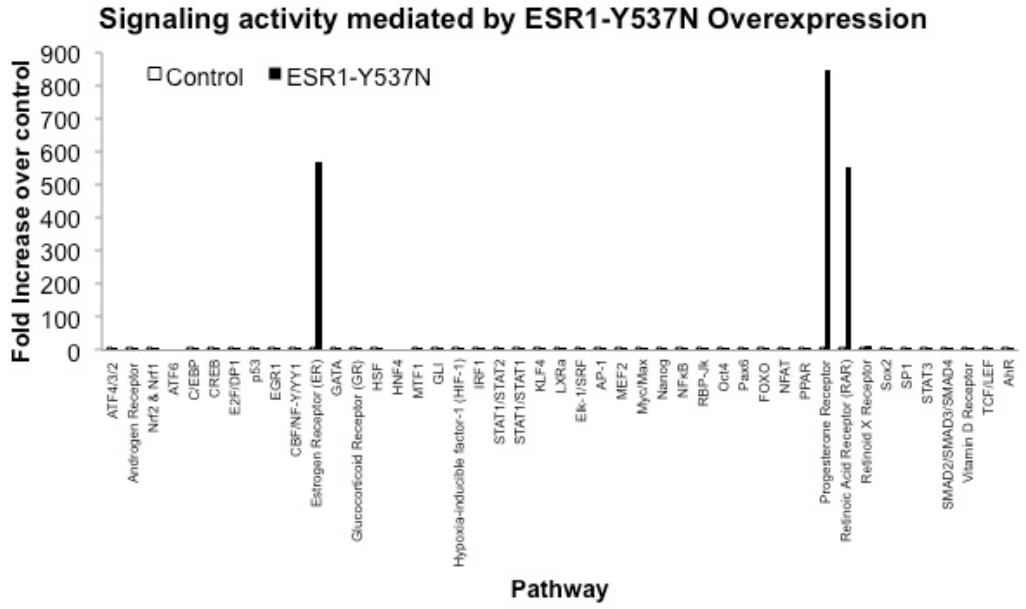


Figure 60. Analysis of ESR1-Y537N mutant signaling

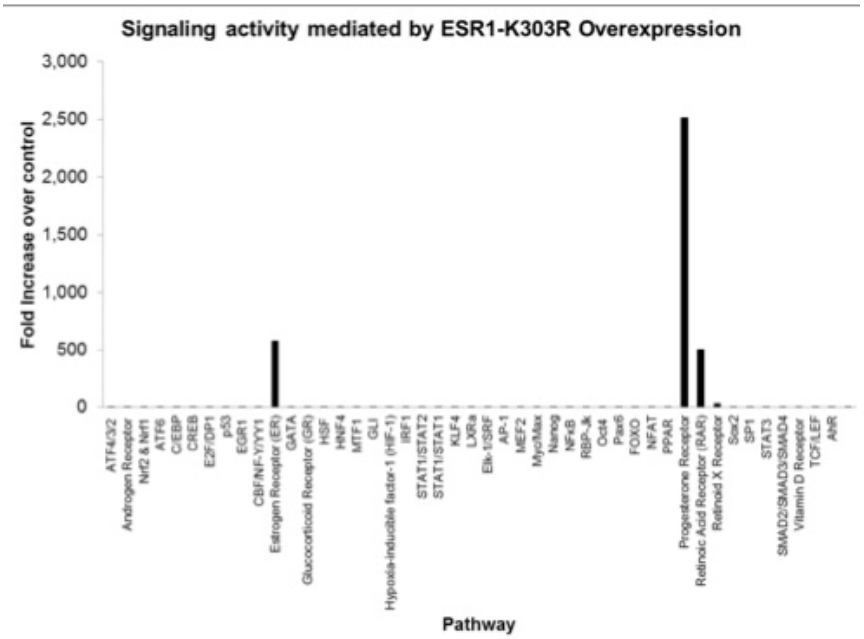


Figure 61. Analysis of ESR1-K303R mutant signaling

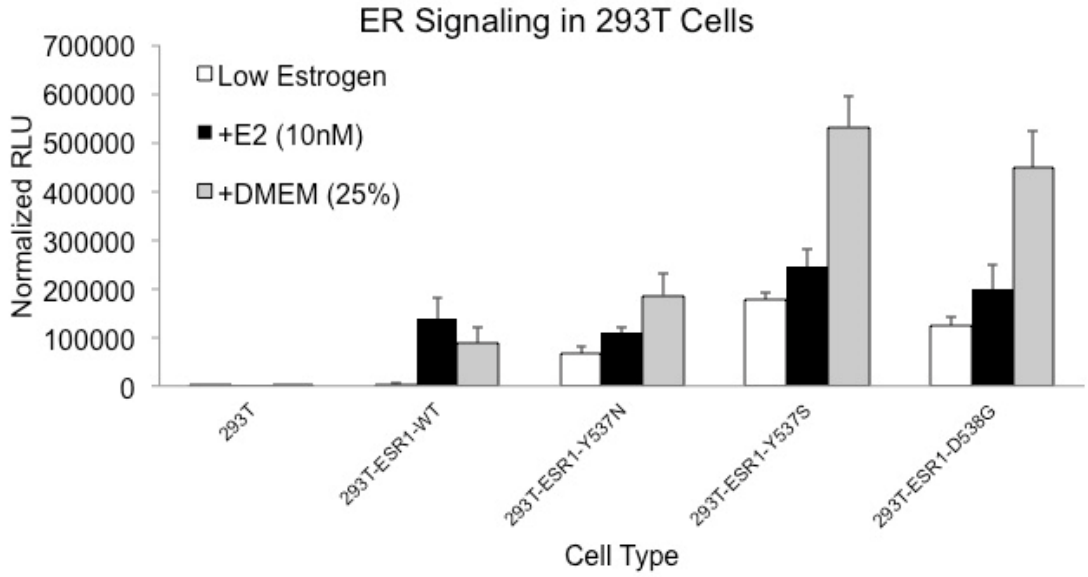


Figure 62. ESR1 ER Signaling with different mutants

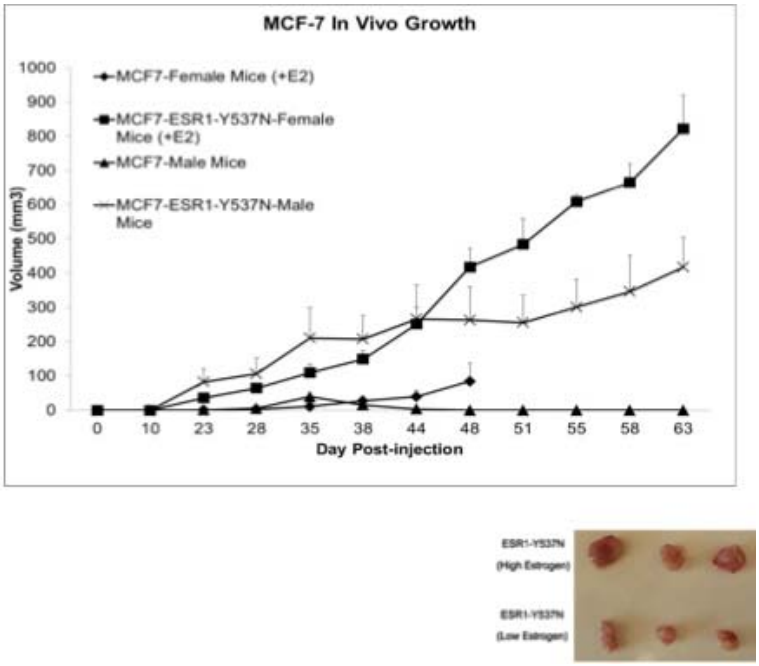


Figure 63. ESR1-Y537N in vivo growth

Defining ESR1 peptides presented by MHC molecules

Aim 2. Using proteomics to identify antigens in human DCIS using based on CNV and poor prognosis.

We hypothesized that peptide epitopes naturally presented by MHC class I molecules on the surface of cancer cells would be the most relevant immunologic targets. A novel strategy for identifying the truly relevant antigenic peptides is to analyze those actually presented by the MHC molecules on tumor cells, reducing the 118 potential antigens to number feasible to use in a vaccine. The analysis of the peptide repertoire associated with the MHC class I molecules of cancer cells can be identified, and those representative genes identified in Aim 1 will serves as a source for tumor antigens for development of a DCIS vaccine (8-10). We have utilized this approach to identify new antigens for cancer vaccines containing 10 antigens, which we tested in human clinical trials (9).

Preparation of cells for mass spectrometry analysis:

DCIS cell lines that express genes with CNA selected from Aim 1 will be expanded in appropriate media to obtain 5-10x10^8 total cells. Cells will be lysed by homogenization and freeze/thawed in buffer containing 1.0% NP40. The cell lysates will be cleared by centrifugation at 2000 rpm for 30 minutes to remove the cell debris and will be used for MHC/peptide complexes isolation.

Isolation and purification of MHC class I bound peptides

MHC/peptide complexes will be isolated by immunoaffinity chromatography using HLA-A2 antibody, (bb7.2 antibody) followed by pan class I antibody (W632) coated protein A/G beads (UltraLink Immobilized Protein A/G, Pierce, Rockford, IL). 400□l Protein A/G beads will be washed with low pH buffer followed by PBS rinses and incubated with 0.5mg of the antibody at room temperature for 2 hours. Labeled beads will be washed three times and incubated with the cell lysate for two-hours at room temperature. The beads will be separated from the lysate by centrifuging at 1000 rpm for 5 minutes. The bound MHC complexes will be eluted from the beads by the addition of 0.1% Trifluoroacetic acid, (TFA), pH 1.5 and heated at 85° C for 15 min to dissociate the bound peptides from the MHC molecules. The peptides will be purified from the antibody by centrifugation using Amicon Ultra-3 kDa molecular mass cutoff membrane filters (Millipore). The purified peptide mixture will be fractionated using C-18 reversed phase (RP) column (4.6mm diameter×150 mm length) using an offline ultimate 3000 HPLC (Dionex, Sunnyvale, CA). Mobile phase A will be 2% acetonitrile (ACN) and 0.1% formic acid (FA) in water, while mobile phase B will be 0.1% FA and 90% ACN in water. Peptides will be eluted from the column with an 80 min linear gradient from 5 to 80% buffer B at a flow rate of 200 µL/min. Fractions will be collected and dried to 6 µL under vacuum for LC/MS/MS analysis.

Mass spectrometry analysis

LC/MS/MS experiments will be carried out using an LTQ-Orbitrap velos (Thermo) instrument interfaced with nano ultimate HPLC (Dionex) instruments. RP-HPLC purified peptide fractions will be loaded onto a trap column of 100 um ID X 2 cm (L) packed with 5-µm Magic C18 AQ (200 A, 3 um, Michrom) and washed using 98% H2O, 2% ACN, 0.05% TFA buffer at a flow rate of 10 ul/min for 5 min. The peptides will be then separated by a self-packed 75um ID X 50 cm (L) fused cilia column packed with 3-µm Magic C18 AQ (200 A, 3 um, Michrom) using a linear gradient of Buffer B (0.1% formic acid, 80% acetonitrile) from 4% to 55% in 50 min at a flow rate

of 300 nl/min. The peptides will be analyzed in the Orbitrap operated at 60,000 resolution in full scan (300–2000m/z) followed by 10 Data-Dependent CIC MS/MS scans (100–2000m/z) with 7,500 resolution. Survey scans will be acquired in profile mode and MS/MS scans acquired in centroid mode. Maximum injection times for MS and MS/MS will be set to 500 and 1000ms, respectively. The precursor isolation width will be set at ±1.2 Da and monoisotopic precursor selection was enabled to exclude singly charged ions from MSMS. The minimum intensity threshold for MS/MS fragmentation in the Orbitrap analyzer will be 5000 counts and the dynamic exclusion set to 60 sec with repeat count as one. *The spectra data will be searched against 1) database containing the CNA genes; 2) genes associated and co-amplified with poor prognosis genes (~400 genes) and 3) Swissprot human non-redundant protein database using Proteome Discoverer (Thermo) to interpret the data and derive peptide sequences.* Epitopes derived from CNA and genes co-amplified with poor prognosis genes will be selected for immunogenicity characterization. Synthetic peptides will be made to validate the peptides selected. The synthetic peptides will be subjected to LC-MS/MS analysis under identical experimental conditions as described above and their sequences will be confirmed by comparison of their MS/MS spectra with that of their synthetic analogs.

Preliminary data supporting this aim:

In preparation for our work in identifying target antigens in cancer, we used LC/MS/MS analysis and identified MHC class I-presented peptides from ER+ cancer cells. Using the criteria of strong consensus for HLA-A2 binding we determined binding affinities. We then prioritized these peptides if the represented genes with amplified copy number. We found that tumor cells had processed and presented HLA A2 binding peptides representing ESRI, as described below.

MCF10

IQGNELEPL	A2	Estrogen receptor ESR1

Preparing for the vaccine trial. T cell receptor deep sequencing.

6. Publications, Abstracts and Presentations

None

7. Inventions, Patents and Licenses

Patent application for vaccination targeting HER3 to prevent therapeutic resistance.

Patent application for vaccination targeting other antigens to prevent therapeutic resistance.

8. Reportable Outcomes

None.

9. Other Achievements

None

10. References

1. Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. Proc Natl Acad Sci U S A. 2010;107:7692-7.

2. Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway KL: Insect cell-expressed p180ErbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci USA 1994; 91:8132-6.

3. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 2006;10:514-27.

4. Xia W, Gerard C, Lui L, Baudson N, Ory T, Spector NL. Lapatinib (GW572016), a small molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases synergizes with anti-ErbB2 antibodies to inhibit mediators of tumor cell survival and induce apoptosis in ErbB2 overexpressing breast cancer cells. Oncogene, 2005; 24: 6213-21.

5. Ren X-R, Wei J, Lei G, Wang J, Lu J, Xia W, et al. Polyclonal HER2-specific antibodies induced by vaccination mediate receptor internalization and degradation in tumor cells. Breast Cancer Res 2012;14: R89.

6. Xia W, Petricoin III EF, Zhao S, Liu L, Osada T, Cheng Q, et al. An heregulin EGFR-HER3 signaling axis can mediate acquired resistance in HER2+ breast cancer models. Breast Cancer Res 2013;15:R85.

11. Appendices

A. Meeting Request Granted for HER3 Vaccine

B. Pre-IND package

C. External Scientific Advisory Committee Roster

D. External Scientific Advisory Committee Agenda

FDA/CBER/OCTGT Teleconferencc Confirmation

Today's Date:	5/8/14
Meeting Request Received:	4/17/17
Requestor:	Duke University Medical Center
Contact:	H. KIM LYERLY, M.D.
Contact Phone:	919-684-0132
Meeting Type:	B / Teleconference
Product:	The Product Ad[e2b-]huHER3 is an E1 and E2b region deleted adenoviral vector, serotype 5, expressing HER3 cDNA encoding the full length HER3 receptor
Purpose:	to discuss Preclinical, product, and clinical issues for phase I and overall drug development
Indication:	Treatment of patients with locally advanced, recurrent, or metastatic cancer who have experienced progression of disease following standard therapy

Meeting Schedule:

Date:	Wednesday, July 2, 2014
Time:	12:00 – 13:00 EST

Tentative CBER Attendees:

AKSAMIT, ROBERT	BAILEY, ALEXANDER	BROSS, PETER
BRYAN, WILSON	CHAMRIN, RONALD	GEORGE, BINDU
GEORGE, BINDU	IRONY, ILAN	KAUL, SADHANA
LIU, KE	LU, JINHUA	SERABIAN, MERCEDES
TAKEFMAN, DANIEL		

Please note that pre-read materials need to be submitted at least **one month** prior to the scheduled meeting date. As part of the pre-read materials, please include a copy of your meeting request and the specific questions to be addressed by CBER. **Please submit 17 copies of the pre-reads.**

Due date for receipt of meeting pre-reads at OCTGT/RMS: Monday, June 2, 2014

A productive meeting depends on the timely receipt of adequate pre-read materials. Consequently CBER may cancel the meeting if the above conditions are not met. **Please be prepared to provide an update of your pediatric plan and timelines.**

Please contact Ron Chamrin at (301) 827-6536 to provide them with your arrangements and also supply call-in number info for this teleconference. CBER Meeting ID: 9417 / PTS # PS002425

Next Page | Export Data | Import Data | Reset Form

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

INVESTIGATIONAL NEW DRUG APPLICATION (IND)

(Title 21, Code of Federal Regulations (CFR) Part 312)

Form Approved: OMB No. 0910-0014
Expiration Date: April 30, 2015
See PRA Statement on page 3.

NOTE: No drug/biologic may be shipped or clinical investigation begun until an IND for that investigation is in effect (21 CFR 312.40)

1. Name of Sponsor
H. Kim Lyerly, MD

2. Date of Submission *(mm/dd/yyyy)*
05/30/2014

3. Sponsor Address

Address 1 *(Street address, P.O. box, company name c/o)*
Duke University Medical Center MSRB1 Research Drive, Room 433, Box 2714

Address 2 *(Apartment, suite, unit, building, floor, etc.)*

City: Durham
State/Province/Region: NC
Country: USA
ZIP or Postal Code: 27710

4. Telephone Number *(Include country code if applicable and area code)*
919-684-6408 Voice; 919-681-7970 Fax

5. Name(s) of Drug *(Include all available names: Trade, Generic, Chemical, or Code)*
Ad5 [E1-, E2b-]-huHER3

Continuation Page for #5

6. IND Number *(If previously assigned)*

7. (Proposed) Indication for Use
Cancer

Is this indication for a rare disease (prevalence <200,000 in U.S.)? ☐ Yes ☑ No

Does this product have an FDA Orphan Designation for this indication? ☐ Yes ☑ No

If yes, provide the Orphan Designation number for this indication:

Continuation Page for #7

8. Phase(s) of Clinical Investigation to be conducted ☑ Phase 1 ☐ Phase 2 ☐ Phase 3 ☐ Other *(Specify)*:

9. List numbers of all Investigational New Drug Applications (21 CFR Part 312), New Drug Applications (21 CFR Part 314) , Drug Master Files (21 CFR Part 314.420) , and Biologics License Applications (21 CFR Part 601) referred to in this application.

10. IND submission should be consecutively numbered. The initial IND should be numbered "Serial number: 0000." The next submission (e.g., amendment, report, or correspondence) should be numbered "Serial Number: 0001." Subsequent submissions should be numbered consecutively in the order in which they are submitted..

Serial Number: 0001

11. This submission contains the following *(Select all that apply)*

☐ Initial Investigational New Drug Application (IND)
☐ Request For Reactivation Or Reinstatement
☐ Development Safety Update Report (DSUR)
☐ Response to Clinical Hold
☐ Annual Report
☑ Other *(Specify)*: CBER meeting ID: 9417/PTS#PS002425 preIND package
☐ Response To FDA Request For Information
☐ General Correspondence

Protocol Amendment(s)
☐ New Protocol
☐ Change in Protocol
☐ New Investigator
☐ PMR/PMC Protocol

Information Amendment(s)
☐ Chemistry/Microbiology
☐ Pharmacology/Toxicology
☐ Clinical ☐ Statistics
☐ Clinical Pharmacology

Request for
☐ Meeting
☐ Proprietary Name Review
☐ Special Protocol Assessment
☐ Formal Dispute Resolution

IND Safety Report(s)
☐ Initial Written Report
☐ Follow-up to a Written Report

12. Select the following only if applicable. *(Justification statement must be submitted with application for any items selected below. Refer to the cited CFR section for further information.)*

Expanded Access Use, 21 CFR 312.300

☐ Emergency Research Exception From Informed Consent Requirements, 21 CFR 312.23 (f)
☐ Charge Request, 21 CFR 312.8
☐ Individual Patient, Non-Emergency 21 CFR 312.310
☐ Individual Patient, Emergency 21 CFR 312.310(d)
☐ Intermediate Size Patient Population, 21 CFR 312.315
☐ Treatment IND or Protocol, 21 CFR 312.320

For FDA Use Only

CBER/DCC Receipt Stamp	DDR Receipt Stamp	Division Assignment
		IND Number Assigned

Previous Page | Next Page

13. Contents of Application – This application contains the following items *(Select all that apply)*

- [x] 1. Form FDA 1571 *(21 CFR 312.23(a)(1))*
- [x] 2. Table of Contents *(21 CFR 312.23(a)(2))*
- [x] 3. Introductory statement *(21 CFR 312.23(a)(3))*
- [x] 4. General Investigational plan *(21 CFR 312.23(a)(3))*
- [ ] 5. Investigator's brochure *(21 CFR 312.23(a)(5))*
- [x] 6. Protocol(s) *(21 CFR 312.23(a)(6))*
  - [x] a. Study protocol(s) *(21 CFR 312.23(a)(6))*
  - [ ] b. Investigator data *(21 CFR 312.23(a)(6)(iii)(b))* or completed Form(s) FDA 1572
  - [ ] c. Facilities data *(21 CFR 312.23(a)(6)(iii)(b))* or completed Form(s) FDA 1572
- 6. Protocol(s) *(Continued)*
  - [ ] d. Institutional Review Board data (21 CFR 312.23(a)(6)(iii)(b)) or completed Form(s) FDA 1572
- [x] 7. Chemistry, manufacturing, and control data *(21 CFR 312.23(a)(7))*
  - [ ] Environmental assessment or claim for exclusion *(21 CFR 312.23(a)(7)(iv)(e))*
- [ ] 8. Pharmacology and toxicology data (21 CFR 312.23(a)(8))
- [ ] 9. Previous human experience *(21 CFR 312.23(a)(9))*
- [x] 10. Additional information *(21 CFR 312.23(a)(10))*
- [ ] 11. Biosimilar User Fee Cover Sheet *(Form FDA 3792)*
- [ ] 12. Clinical Trials Certification of Compliance *(Form FDA 3674)*

14. Is any part of the clinical study to be conducted by a contract research organization? [x] Yes [ ] No

If Yes, will any sponsor obligations be transferred to the contract research organization? [x] Yes [ ] No

If Yes, provide a statement containing the name and address of the contract research organization, identification of the clinical study, and a listing of the obligations transferred *(use continuation page)*. Continuation Page for #14

15. Name and Title of the person responsible for monitoring the conduct and progress of the clinical investigations

Michael Morse, MD Associate Professor, Department of Medicine- Oncology, Duke University Medical Center

16. Name(s) and Title(s) of the person(s) responsible for review and evaluation of information relevant to the safety of the drug

Michael Morse, MD Associate Professor, Department of Medicine- Oncology, Duke University Medical Center;
H. Kim Lyerly, MD Professor, Department of Surgical Sciences, Duke University Medical Center

I agree not to begin clinical investigations until 30 days after FDA's receipt of the IND unless I receive earlier notification by FDA that the studies may begin. I also agree not to begin or continue clinical investigations covered by the IND if those studies are placed on clinical hold or financial hold. I agree that an Institutional Review Board (IRB) that complies with the requirements set forth in 21 CFR Part 56 will be responsible for initial and continuing review and approval of each of the studies in the proposed clinical investigation. I agree to conduct the investigation in accordance with all other applicable regulatory requirements.

17. Name of Sponsor or Sponsor's Authorized Representative

H. Kim Lyerly, MD/Amy Hobeika, PhD

18. Telephone Number *(Include country code if applicable and area code)*

919-684-6408/919-684-6112

19. Facsimile (FAX) Number *(Include country code if applicable and area code)*

919-681-7970

20. Address

Address 1 *(Street address, P.O. box, company name c/o)*
Duke University Medical Center, MSRB1 Rm 433 Research Dr, Box 2714

Address 2 *(Apartment, suite, unit, building, floor, etc.)*

City: Durham
State/Province/Region: NC
Country: USA
ZIP or Postal Code: 27710

21. Email Address

kim.lyerly@dm.duke.edu
amy.hobeika@duke.edu

22. Date of Sponsor's Signature *(mm/dd/yyyy)*

5/29/2014

23. Name of Countersigner

24. Address of Countersigner

Address 1 *(Street address, P.O. box, company name c/o)*

Address 2 *(Apartment, suite, unit, building, floor, etc.)*

City
State/Province/Region
Country: United States of America
ZIP or Postal Code

WARNING : A willfully false statement is a criminal offense (U.S.C. Title 18, Sec. 1001).

25. Signature of Sponsor or Sponsor's Authorized Representative

Sign

26. Signature of Countersigner

Sign

The information below applies only to requirements of the Paperwork Reduction Act of 1995.

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Department of Health and Human Services
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Office of Chief Information Officer
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DUKE UNIVERSITY MEDICAL CENTER

OFFICE OF H. KIM LYERLY, MD
DUMC, DURHAM, NORTH CAROLINA, USA
919-684-0132/681-7970 (FAX)

May 30, 2014

Food and Drug Administration
Center for Biologics Evaluation and Research
Document Control Center, HFM-99, Suite 200N
1401 Rockville Pike
Rockville, MD 20852-1448

Attn: Ronald Chamrin & Mehran Azari

Pre-IND Package Meeting CBER meeting ID: 9417/PTS#PS002425

Please find enclosed 17 desk copies of the Briefing Document for the 2 July 2014 Pre-IND meeting at 12:00 pm (CBER meeting ID: 9417/PTS#PS002425). This meeting has been requested to discuss the preclinical, product and clinical issues for a Phase I study of Ad5 [E1-, E2b-]-huHER3 in the treatment of patients with patients with advanced or metastatic solid tumor malignancies. The Principal Investigator for the clinical study will be Michael Morse, MD.

If there are any questions regarding this submission, please contact me, Amy Hobeika, PhD (amy.hobeika@duke.edu; 919-684-6112), or Michael Morse, MD (Michael.morse@dm.duke.edu; 919-684-5705). Drs. Hobeika and Morse can act on my behalf on any issue relating to this pre-IND package.

Below is the information regarding the call-in number for the teleconference.

US Toll-Free: 1-855-244-8681
Participant Passcode: 73715779
PIN: 6408

Sincerely,

H. Kim Lyerly, MD
Professor, Department of Surgery
Duke University Medical Center
MSRB1 Rm 433 Research Drive
Durham, NC 27710
Phone: 919-684-0132
Email: kim.lyerly@dm.duke.edu

PRE-IND MEETING BRIEFING DOCUMENT

A PHASE I STUDY OF ACTIVE IMMUNOTHERAPY WITH Ad [E1-, E2b-]-huHER3 VACCINE IN PATIENTS WITH ADVANCED OR METASTATIC MALIGNANCIES EXPRESSING HER3

PIND
CBER Meeting ID: 9417/PTS# PS002425

H. Kim Lyerly, MD
Professor, Department of Surgery
DUKE UNIVERSTIY MEDICAL CENTER

May 30, 2014

Table of Contents

1. OVERALL SUMMARY

1.1. Rationale

HER3 in malignancy

The human epidermal growth factor receptor (HER) family including: HER1 (also known as EGFR), HER2, HER3 and HER4 (also known as ErbB2, ErbB3, and ErbB4 respectively) is an important receptor family for the development of many malignancies. HER3 is overexpressed in breast, lung, gastric, head and neck, and ovarian cancer and melanoma and its overexpression is associated with poor prognosis (1-7). Because of the negligible tyrosine kinase function of HER3, it is typically present in heterodimers with HER1 or HER2, through which downstream signaling occurs involving extracellular-signal-regulated kinase (ERK) 1/2 and AKT (8). In breast cancer, HER3 is associated with resistance to anti-HER2 therapeutics. When HER2-driven breast cancer cell lines and xenografts were treated with anti-HER2 therapeuties, there was an increase in HER3 expression and signaling (9). HER3 is also one of several important causes of endocrine resistance in breast cancer (10,11). In patients with ER+ breast cancer who were treated with tamoxifen, HER3-overexpression was associated with a shorter progression-free survival (12). In vitro, HER3 expression was induced when ER-positive breast cancer cell lines MCF-7 and T47D were treated with fulvestrant (13). Overexpression of heregulin (HRG), the ligand for HER3, is also associated with resistance to antiestrogens in vitro and in vivo (14). These data demonstrate the key role of HER3 and its ligand to therapeutic resistance.

Figure 1 (adapted from 15, 16) illustrates a model of anti-estrogen therapy resistance in which ER blockade results in HRG overexpression and activation of the HER2/HER3 heterodimer.

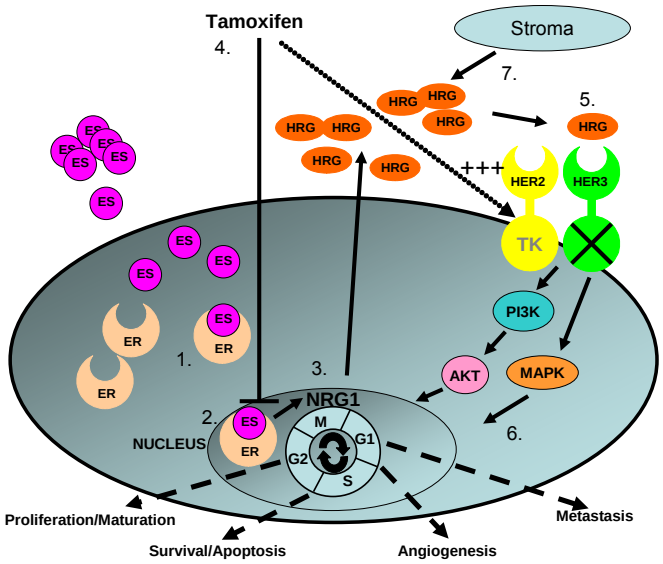

Figure 1. Our model of resistance: Estrogen-driven tumorigenesis followed by the onset of resistance to tamoxifen induced by increased hergulin (HRG) production leading to HER3 becoming the dominant driver of tumor growth. (1) Estrogen (ES) binds to the estrogen receptor (ER) on breast cells, triggering signaling via multiple pathways (2) leading to tumorigenesis and sustaining cancer growth. ER signaling also induces increased NRG1 expression (3) resulting in secretion of the HER3 ligand HRG. (4) Blockade of the ER receptor by tamoxifen can block ER signaling but also enhances HER2 signaling (+++). The increased production of HRG also leads to signaling through HER3 containing heterodimers (5) that bind HRG and lead to sustained cancer growth (6). HER3 thus becomes the dominant growth factor receptor driving tumor growth, leading to the failure of tamoxifen therapy. (7) Tumor stroma has also been implicated as an initial or contributing source of HRG. (Adapted from 15,16).

Based on these observations, it appears that targeting either HER2 or HER3 may be an effective strategy to overcome anti-endocrine therapies resistance.

Consistent with the notion, Liu et al showed that downregulation of ErbB3 by siRNA reversed HER2-driven tamoxifen resistance, and enhanced the ability of tamoxifen to inhibit growth and enhance apoptosis (17). Liu et al speculated that one possible strategy of overcoming tamoxifen resistance would be through the inhibition of ErbB3 driven activation of Akt. ErbB3-mediated resistance to tyrosine-kinase inhibitors targeting ErbB1 and ErbB2/HER2 also stems from the sustained activation of Akt, also linked to ErbB3 expression (18,19), suggesting that ErbB3 may be a broadly applicable resistance mechanism. Folgiero, et al identified an interaction between the ErbB3 receptor and the α6β4 integrin which assists in sustaining the PI3K/Akt survival pathway of breast cancer tumor cells (20). Further work demonstrates that in anti-estrogen resistant cell lines, continued signaling via Akt is important for continued cell growth (21)

Although HER3 protein alone is not oncogenic (22, 23) and expression itself has not been associated with poor outcome in breast cancer, contemporary approaches to identify the presence of an activated HER3 signaling cascade have recently suggested that the HER3 signaling was associated with a poor prognosis. For example, Spears and colleagues recently reported that detection of HER2:HER2 and HER2:HER3 dimers has prognostic significance in early breast cancer (24). In addition, we are aware that the presence of the HER3 ligand HRG could also be an indicator of an active HER3 signaling cascade. Therefore, we performed a gene expression analysis of heregulin/neuregulin HRG/NRG1 by compiling a collection of breast tumor gene expression data (n = 4010) derived from 23 data sets posted on the NCBI Gene Expression Omnibus (GEO). Our analysis of mRNA expression from these data sets revealed up-regulated mRNA expression of HRG/NRG1 was correlated with lower relapse free survival in ER+ HER2- breast cancer patients. Additionally, HRG/NRG1 mRNA was elevated in tumors from patients with both early recurrence (less than 5 years) or late recurrence (5-10 years) (25).

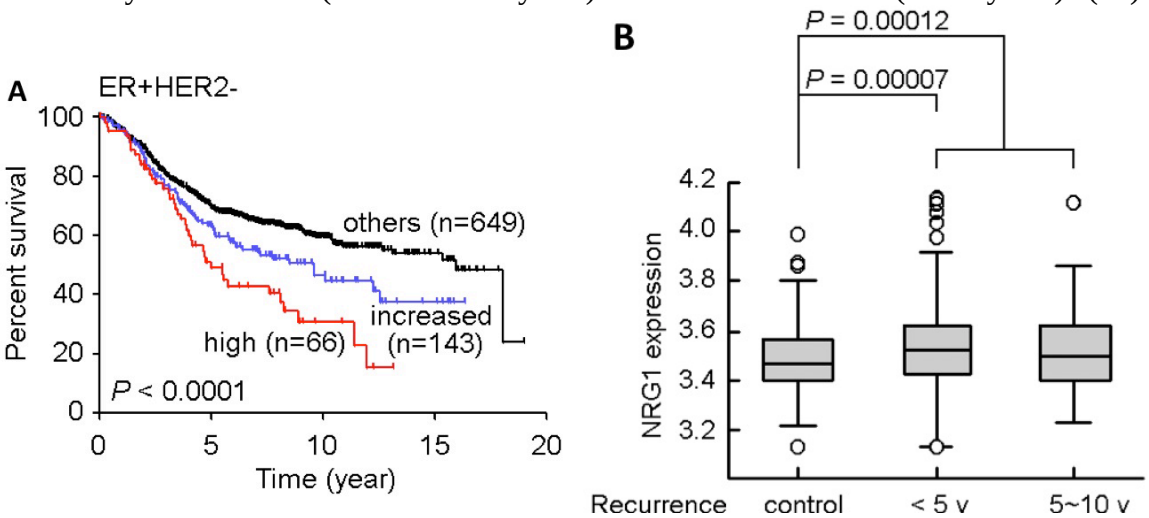

Fig. 2 A: Up-regulated mRNA expression of HRG/NRG1 was correlated with lower relapse free survival in ER+ HER2- breast cancer patients. B: HRG/NRG1 mRNA was elevated in tumors from patients with

both early recurrence (less than 5 years) or late recurrence (from 5-10 years) after diagnosis compared to non recurring tumors.

Therefore preclinical studies, clinical data, and our own analysis suggest that the HER3 signaling axis is associated with poor outcomes in ER+, HER2- breast cancer patients. Although HER2 signaling is implicated in resistance, and HER2 inhibition using small molecules or antibodies can be achieved clinically, our overarching hypothesis is that eliciting an anti-HER3 immune response with an adenoviral (Ad) vaccine targeting full length human HER3 will have greater anti-tumor efficacy.

Rational for vaccines targeting HER3

Cancer vaccines have recently demonstrated promising activity in clinical trials for malignancies, with improvements in overall survival being reported (26-28) and the first cancer vaccine recently received FDA-approval (Provenge). Cancer vaccines may have advantages over monoclonal antibody therapeutics, including their ability to elicit both cellular and humoral immunity, to target multiple epitopes, to perturb growth factor receptor signaling, to synergize with small molecule drugs (29), and to provide long lasting effects. They are generally well tolerated and do not have overlapping toxicities with conventional therapies, making them attractive in combination with existing drugs. Targeting HER3 is especially attractive as HER3, unlike the other HER family members that have intrinsic kinase activity, lacks a kinase domain and is not readily "drugable" using small molecule approaches.

While monoclonal antibody strategies to target HER3 are in commercial development (Amgen and Sanofi-Aventis/Merrimack), we believe that a cancer vaccine that induces polyclonal antibody and T cell responses should also be explored as this approach can provide long term anti-HER3 immune responses, which could provide the long term effects needed to prevent the emergence of resistant clones. In addition to the long term protection afforded by vaccination, polyclonal immune responses to a target protein may offer additional benefits. It has been established that the binding of multiple antibodies to different epitopes is more efficient than a single monoclonal antibody in mediating receptor internalization (30-35). For example, we have recently shown that polyclonal antibodies that mediate HER2 internalization and degradation both block HER2 signaling and have dramatic anti-tumor activity (29, 36). In addition, T cell responses induced by vaccination are also a potent mechanism of tumor rejection in numerous animal studies and the adoptive transfer of T cells in human clinical trials has shown clinical efficacy (37-41).

Although HER3 is expressed on a number of normal tissues, and is only rarely mutated in cancers, it remains an attractive immunotherapeutic target as it is not abundant on the cell surface in normal cells, tumor cells may have higher levels of membrane-bound HER3, and HER3 peptides are presented on the cell surface by MHC complexes (42) for presentation to T cells. Additionally, antibody targeting can occur as tumor cells upregulate HER3 at the cell surface after being exposed to therapeutic agents.

Finally, because HER3 is not typically expressed at high levels in normal tissues or in less heavily pretreated tumors, it is less likely that there would be self-tolerance to HER3, a major obstacle to the induction of clinically relevant levels of anti-tumor immunity. Vaccinating against antigens not normally present, but induced by resistance

mechanism, such as HER3, prior to the development of resistance, may circumvent these mechanisms. For example, as we have mentioned above, we believe that the HER3 receptor may represent a relevant target in endocrine therapy-resistant breast cancer, and therefore a HER3 cancer vaccine may prevent endocrine therapy-resistance by targeting HER3-mediated resistance. We believe that there would be a similar role of a HER3 vaccine for other malignancies in which HER3 is relevant for therapeutic resistance. Furthermore, the HER3 vaccine could be used in combination with other therapies such as endocrine therapy in breast cancer to prevent the onset of therapeutic resistance mediated by HER3 overexpression.

1.2. Ad5 Vaccines

Rationale for utilizing Adenovirus based vectors for targeting HER3:

Adenoviruses are a family of DNA viruses characterized by an icosohedral, non-enveloped capsid containing a linear double-stranded genome. Of the human Ads, none are associated with any neoplastic disease, and only cause relatively mild, self-limiting illness in immunocompetent individuals**.** The first genes expressed by the virus are the E1 genes, which act to initiate high-level gene expression from the other Ad5 gene promoters present in the wild type genome. Viral DNA replication and assembly of progeny virions occur within the nucleus of infected cells, and the entire life cycle takes about 36 hr with an output of approximately 10^4 virions per cell. The wild type Ad5 genome is approximately 36 kb, and encodes genes that are divided into early and late viral functions, depending on whether they are expressed before or after DNA replication. The early/late delineation is nearly absolute, since it has been demonstrated that super-infection of cells previously infected with an Ad5 results in lack of late gene expression from the super-infecting virus until after it has replicated its own genome. This is due to a replication dependent *cis*-activation of the Ad5 major late promoter (MLP), preventing late gene expression (primarily the Ad5 capsid proteins) until replicated genomes are present to be encapsulated.

Ad5 vectors

First generation, or E1-deleted adenovirus vectors ([E1-] Ad5) are constructed in a manner such that a transgene replaces only the E1 region of genes; thus, 90% of the wild-type Ad5 genome is retained in the vector. [E1-] Ad5 vectors have a decreased ability to replicate and cannot produce infectious virus after infection of cells not expressing the Ad5 E1 genes. The recombinant [E1-] Ad5 vectors are propagated in human cells (typically 293 cells) allowing for [E1-] Ad5 vector replication and packaging. [E1-] Ad5 vectors have a number of positive attributes; one of the most important is their relative ease for scale up and cGMP production. Currently, well over 220 human clinical trials utilize [E1-] Ad5 vectors, with more than two thousand subjects given the virus sc, im, or iv. Additionally, since Ad5 vectors do not integrate, (their genomes remain episomal) the risk for insertional mutagenesis and/or germ-line transmission is extremely low if at all. Conventional [E1-] Ad5 vectors have a large carrying capacity that approaches 7kb.

[E1-] Ad5 vectors used as a cancer vaccine

Arthur et.al. demonstrated that [E1-] Ad5 vectors encoding a variety of antigens could efficiently transduce 95% of *ex vivo* exposed DC's to high titers of the vector (41). Importantly, increasing levels of foreign gene expression were noted in the DC with increasing multiplicities of infection (MOI) with the vector, a finding repeated by others, as well as reproduced in our preliminary studies (44). It has been demonstrated that DC infected with [E1-] Ad5 vectors encoding a variety of antigens (including the tumor antigens MART-1, MAGE-A4, DF3/MUC1, p53, hugp100 melanoma antigen, polyoma virus middle –T antigen,) have the propensity to induce antigen specific CTL responses, have an enhanced antigen presentation capacity, and have an improved ability to initiate T-cell proliferation in mixed lymphocyte reactions (46-50). Immunization of animals with DC's previously transduced by Ad5 vectors encoding tumor specific antigens has been demonstrated to result in significant levels of protection for the animals when challenged with tumor cells expressing the respective antigen (51, 52). Interestingly, intra-tumoral injection of Ads encoding IL-7 was less effective than injection of DCs transduced with IL-7 encoding Ad5 vectors at inducing antitumor immunity, further heightening the interest in *ex vivo* transduction of DCs by Ad5 vectors (53). *Ex vivo* DC transduction strategies have also been used to attempt to induce tolerance in recipient hosts, for example, by Ad5 mediated delivery of the CTLA4Ig into DCs, blocking interactions of the DCs CD80 with the CD28 molecule present on T-cells (54).

Ad5 vector capsid interactions with DCs in and of themselves appear to trigger several beneficial responses, which may be enhancing the propensity of DCs to present antigens encoded by Ad5 vectors. For example, immature DCs, though specialized in antigen uptake, are relatively inefficient effectors of T-cell activation. DC maturation coincides with the enhanced ability of DCs to drive T-cell immunity. In some instances, Ad5 infection can result in direct induction of DC maturation (55, 56). Studies of immature bone marrow derived DCs from mice suggest that Ad vector infection of these cells resulted in upregulation of cell surface markers normally associated with DC maturation (MHC I and II, CD40, CD80, CD86, and ICAM-1) as well as down-regulation of CD11c, an integrin known to be down regulated upon myeloid DC maturation. In some instances, Ad vector infection triggers IL-12 production by DCs, a marker of DC maturation (56). These events may possibly be due to Ad5 triggered activation of NF-kB pathways (55-57). Similar studies in mature CD83+ human DC (derived from peripheral blood monocytes) demonstrated that mature DCs were efficiently transduced by Ad vectors, and did not lose their functional potential to stimulate the proliferation of naive T-cells at lower MOI. However, some studies also suggested that mature DCs were less infectable than immature ones (58, 59). Modification of capsid proteins have also been used as a strategy to optimize infection of DC by Ad vectors, as well as enhancing functional maturation, for example using the CD40L receptor as a viral vector receptor, rather than using the normal CAR receptor infection mechanisms (60).

Ad5 vectors offer a unique opportunity to allow for high level and efficient transduction of TAA. Unfortunately, one of the major problems facing Ad5 based vectors is the high propensity of pre-existing immunity to Ads in the human population, and how this may preclude the use of conventional, E1 deleted (first generation Ads) in most human populations, for any additional vaccine application.

The Use of Ad5 [E1-, E2b-] Vaccines to Overcome the problem of Pre-existing Anti-Ad5 Immunity

Studies in humans and animals have demonstrated that pre-existing immunity against Ad5 can be an inhibitory factor to commercial use of Ad-based vaccines (61, 62). The preponderance of humans have antibody against Ad5, the most widely used subtype for human vaccines, with two-thirds of humans studied having lympho-proliferative responses against Ad5 (63). This pre-existing immunity can inhibit immunization or re-immunization using typical Ad5 vaccines and may preclude the immunization of a vaccinee against a second antigen, using an Ad5 vector, at a later time. Overcoming the problem of pre-existing anti-vector immunity has been a subject of intense investigation. Investigations using alternative human (non-Ad5 based) Ad5 subtypes or even non-human forms of Ad5 have been examined. Even if these approaches succeed in an initial immunization, subsequent vaccinations may be problematic due to immune responses to the novel Ad5 subtype. To avoid the Ad5 immunization barrier, and improve upon the limited efficacy of [E1-] Ad5 vectors to induce optimal immune responses, we have constructed a next generation Ad vector based vaccine platform. The E2b-deleted Ad5 vectors (E1- and E2b-) have additional deletions in the E2b region, removing the DNA polymerase and the preterminal protein genes. They have an expanded cloning capacity that is sufficient to allow inclusion of many possible genes (64). E2b-deleted vectors have up to a 12 kb gene-carrying capacity as compared to the 7 kb capacity of [E1-] Ad5 vectors, providing space for multiple genes if needed. Deletion of the E2b region confers advantageous immune properties on our novel Ad vectors, eliciting potent immune responses to specific, non-viral antigens while minimizing the immune responses to Ad viral proteins.

Most importantly, [E1-, E2b-] Ad5 vectors induce a potent CMI, as well as antibodies against the vector expressed vaccine antigens even in the presence of Ad immunity (64). [E1-, E2b-] Ad5 vectors also have reduced adverse reactions as compared to [E1-] Ad vectors, in particular the appearance of hepatotoxicity and tissue damage (65-68). A key aspect of these Ad5 vectors is that expression of Ad late genes is greatly reduced (65, 69, 70). For example, production of the capsid fiber proteins could be detected *in vivo* for [E1-] Ad5 vectors, while fiber expression was ablated from [E1-, E2b-] Ad5 vector vaccines (67, 68, 71). The innate immune response to wild type Ad is complex and it appears that proteins deleted from [E1-] and E2b-deleted Ad5 vectors play an important role (72-75). Specifically, [E1-, E2b-] Ad5 vectors with deletions of preterminal protein or DNA polymerase display reduced inflammation during the first 24 to 72 hours following injection compared to [E1-] Ad5 vectors (74, 75). These data suggest that the lack of Ad5 gene expression renders infected cells invisible to anti-Ad activity and permits infected cells to produce the transgene for extended periods of time.

We hypothesize that the reduced inflammatory response against [E1-, E2b-] Ad5 vector viral proteins and the resulting evasion of pre-existing Ad immunity increases the capability for the [E1-, E2b-] Ad5 vectors to transduce dendritic cells, improving antigen specific immune responses in the vaccinee. [E1-, E2b-] Ad5 vectors not only are safer than, but appear to be superior to [E1-] Ad5 vectors in regard to induction of antigen specific immune responses, making them a platform to develop HER3 vaccines in a rapid and efficient manner.

Our goal is to further develop a therapeutic vaccine against HER3 by taking advantage of a new Ad5 vector system that overcomes barriers found with other Ad5 systems and permits the immunization of people who have previously been exposed to Ad5. The results of this study will establish the technical safety, and immunological merit of using this [E1-, E2b-] Ad5 vector HER3 vaccine.

1.3. DESCRIPTION OF STUDY

Study Objectives

Our primary objective for the proposed phase I study is to assess the safety and tolerability of Ad5 [E1-, E2b-]-huHER3, as a single agent in patients with advanced or metastatic solid tumor malignancies including breast cancer and combined with exemestane in post-menopausal women with ER+ and/or PR+ HER2- metastatic or locally advanced breast cancer.

Our secondary objectives are to evaluate HER3-specific antibody and T cell responses following vaccination and ER+/HER2- breast cancer refractory to anti-estrogen therapy for markers of HER3 signaling activation.

Rationale for schedule, dose, route of administration

The study design flows from pre-clinical studies in animals using the Ad5 [E1-, E2b-] vector platform. Etubics Corporation has performed a dose response evaluation using the Ad5 [E1-, E2b-] vector platform and have demonstrated that 10^{10} virus particles (VP) is a dose which results in a desired CMI response against a transgene product in a murine model. Furthermore, we have demonstrated in murine and non-human primate (NHP) models that three immunizations using 10^{10} VP separated by two to four weeks respectively results in the desired CMI. The route of immunization is chosen since a preponderance of DC reside in the dermis. Using this premise we have done multiple murine and NHP studies using a cutaneous injection protocol and found that a desired level of CMI was induced using the Ad5 [E1-, E2b-] platform employing CEA and other transgenes. We then performed a phase I/II study with cohorts of patients with advanced colorectal cancer who were immunized with escalating doses of Ad5 [E1-, E2b-]-CEA(6D) (See Previous human experience below; Morse, Cancer Immunol Immunother (2013) 62:1293–1301 (see appendix 2)). The Ad5 [E1-, E2b-]-CEA(6D) doses were delivered to patients as follows: cohort 1: dose of 1X10E9 VP in 0.5 ml subcutaneously (SQ) in the same thigh every 3 weeks for 3 treatments; cohort 2: dose of 1X10E10 VP in 0.5 ml SQ every 3 weeks for 3 treatments; cohort 3: dose of 1 x 10E11 in 0.5 ml SQ every 3 weeks for 3 treatments. Following the establishment of the dose of 1 x 1011 VP as safe, an additional 12 patients received Ad5 [E1-, E2b-]-CEA(6D) at this dose and schedule (phase II cohort). After completing the phase II cohort, an additional cohort (cohort 5) of six patients received a dose of 5 x 10E11 VP in 2.5 ml SQ every 3 weeks for 3 treatments to determine safety of the highest achievable dose. Importantly, there was minimal toxicity, and overall patient survival (48 % at 12 months) was similar regardless of preexisting Ad5 neutralizing antibody titers. CEA-specific CMI responses were

observed despite the presence of preexisting Ad5 immunity in a majority (61.3 %) of patients. The results demonstrate that, in cancer patients, the novel Ad5 [E1-, E2b-] gene delivery platform generates significant CMI responses to the tumor antigen CEA in the setting of both naturally acquired and immunization induced Ad5-specific immunity. Therefore, in this phase I clinical study we have chosen to use up to 3 dose levels of Ad5 [E1-, E2b-]-huHER3 (starting at 2×10^{10} viral particles (vp), then escalating to 1×10^{11} vp, and then escalating to 5×10^{11} vp given as three immunizations separated by three weeks via a needle subcutaneous delivery method).

Rationale for patient population

This protocol will enroll patients with advanced malignancies that are expected to express HER3 who have progressed after standard therapy known to lengthen survival. For these patients, clinical trials are considered an appropriate recommendation for management of their disease. HER3 is overexpressed in breast, colon, lung, prostate, ovarian, cervical, endometrial, gastric, pancreatic, bladder, head and neck, liver, and esophageal cancer (1-7).

Rationale for endpoints chosen

The intention of this phase I study is to identify a safe dose of the vaccine within a feasible range of dose levels. Because the expected mechanism of action for the vaccine is to induce T cell and antibody responses, we will also examine HER3-specific T cell and antibody responses from the peripheral blood. The standard assays for measuring this immune response are the ELISpot to enumerate the proportion of HER3-responsive T cells and cytokine flow cytometry which identifies the CD4+ and CD8+ T cell contributions to the immune response. HER3 specific antibody levels will be determined by ELISA.

1.4. PREVIOUS HUMAN EXPERIENCE

We have performed a Phase 1/2 clinical trial of ETBX-011 (Ad5 [E1-, E2b-]-CEA) (IND#14325), which has the same Ad5 vector backbone but expresses the tumor-associated antigen CEA. The findings of this study were submitted to the FDA under IND 14325, Serial No. 0014, August 23, 2012 in Attachment 2 entitled “Clinical Data Summary”. The results of the study have been published (see Appendix 2 for publication: Morse MA, Chaudhry A, Gabitzsch ES, Hobeika AC, Osada T, Clay TM, Amalfitano A, Burnett BK, Devi GR, Hsu DS, Xu Y, Balcaitis S, Dua R, Nguyen S, Balint Jr. JP, Jones FR, Lyerly HK. Novel adenoviral vector induces T-cell responses despite antiadenoviral neutralizing antibodies in colorectal cancer patients. Cancer Immunol Immunotherapy(2013)62:1293–1301).

The ETBX-011 Phase 1/2 study consisted of a dose-escalation study of four dosage levels (1×10^9, 1×10^{10}, 1×10^{11}, 5×10^{11} VP/dose) (Phase I component), and the maximally tolerated dose of ETBX-011 (Phase 2 component). ETBX-011 was administered by SQ

injection every three weeks for a total of three treatments. Thirty-two patients with metastatic colorectal cancer, median age 57.5 (range 38–77) who had failed a median of three prior chemotherapeutic regimens (range 2–5), had a performance status of 90 % (range 70–100 %), and had three sites of metastatic disease (range 1–4), were enrolled. The majority were able to receive all three immunizations. Four patients who stopped immunizations early did so due to significant disease progression.

A total of 94 immunization treatments were administered to all patients. There was no dose-limiting toxicity and no serious adverse events (SAE) that resulted in treatment discontinuation at any test agent dose level. The most common toxicity was a self-limited, injection site reaction. Other reactions occurred with less than a 10% incidence and included fever, flu-like symptoms, anorexia, chills, nausea, and headache. These symptoms were also self-limiting and did not require intervention other than symptomatic measures such as acetaminophen.

Biological effects of ETBX-011 injections were monitored by recording blood hematology, chemistry, and anti-nuclear antibody (ANA) values of individual patients in case record forms (CRFs) and were also reported in the FDA reported Clinical Data Summary (Appendix 3 of IND#14325). Of 34 total patients entered into the trial, 28 received all three treatments with ETBX-011 (Ad5 [E1-, E2b-]-CEA). For the 28 patients which received all three treatments, the blood hematology, chemistry, and ANA values at week 0 (prior to first treatment) were compared with those obtained at week 9 (three weeks after the third treatment). There were no significant changes in chemistry or ANA values after treatments with ETBX-011. There was only one significant change in the blood hematology values. The basophil count was significantly (P=0.0403) lower at week 9 after treatments. However, this value remained in the normal range for basophil counts and, overall, there appeared to be no significant biological effects.

2. INTRODUCTION

2.1. Product name

Ad[E1-, E2b-]huHER3

2.2. Chemical name and Structure

The product Ad[E1-, E2b-]huHER3 is an E1 and E2b region deleted adenoviral vector, serotype 5, expressing full length human HER3 cDNA.

2.3. Proposed Indication

The proposed indication is patients with locally advanced, recurrent, or metastatic cancer who have experienced progression of disease following standard therapy.

2.4. Purpose of Meeting

This meeting is to discuss preclinical, product, and clinical issues for Phase I and overall drug development program for the Ad[E1-, E2b-]huHER3 cancer vaccine. Questions relating to preclinical, product, and clinical protocol are listed in Section 2.5.

2.5. List of Specific Objectives

Agreement from FDA is sought on the following questions:

Nonclinical

a) Does the proposed toxicology testing plan for Ad[E1-, E2b-]huHER3 full length meet with FDA requirements? Specifically, the dose chosen for toxicology is (2 x 10^8 viral particles per each 25 µL injection) based on our starting dose in the clinical trial of 2 x 10E10 viral particles. Is this acceptable?

Product

a) Does the manufacturing plan from Etubics for the clinical grade Ad[E1-, E2b-]huHER3 full length product meet with FDA approval?
b) Does the Lot release testing plan for Ad[E1-, E2b-]huHER3 full length from Etubics meet with all FDA requirements such that we will be able to release the product for testing in patients?
c) Based on our preclinical data comparing different HER3 transgene constructs (see section 3.2.), we have selected the wildtype sequence that encodes the full length form of the HER3 receptor (Ad[E1-, E2b-]huHER3) for the proposed Phase I clinical trial. We request FDA feedback on the perceived safety of the human HER3 transgene sequence for the proposed clinical vector.
d) We will sequence the whole genome and transgene of the adenovirus vector. We request clarification on the issue of whether material from production pilot lots (same process as clinical) can be sequenced or if material from the final vialed clinical product must be sequenced.

Clinical

Our clinical trial design for Phase 1 safety testing of the Ad[E1-, E2b-]huHER3 vector, is a standard 3+3 dose escalation design, which will enroll 3 patients at 3 dose levels (2×10^{10} vp, 1×10^{11} vp, and 5×10^{11} vp) (See section 6.0 of the clinical study protocol). Additional patients will be enrolled at the MTD until a total of 12 patients have been treated at that dose level. During dose escalation through the first three patients of cohort 3, there will be a minimum of 1 week between enrolling successive patients. **DLT** (based on CTCAE4.0 criteria) is defined as any Grade 2, 3 or 4 immediate hypersensitivity reactions, Grade 3 or 4 fever that may possibly be associated with the immunization, Grade ≥2 autoimmune events except for vitilgo or fever for less than 2 days and less than <101.5 ºF, Grade ≥2 allergic reactions (grade 2 is defined as generalized urticaria as defined by version 4 CTC guide), or Grade ≥3 non-hematologic toxicity. Assessment of DLT for dose escalation will be made after all patients in this cohort have had a study visit at least 3 weeks after receiving their first dose of vaccine. If there are no DLT (as defined below), then patients may begin enrolling into the next cohort.

a) Is this an acceptable trial design?

b) We propose to perform the study at 2 or more sites. Is this acceptable?

2.6. Proposed Agenda

Event	Person responsible	Time allotted
Introductions	Morse (Duke) and FDA	5 min
Discussion of questions submitted	Morse (Duke)	20 min
Discussions of issues identified by the Agency	FDA	30 min
Summary of conclusions reached at the meeting	Morse (Duke)/FDA	5 min

2.7. List of Sponsor Attendees

Michael Morse, MD, Professor, Department of Medicine, Duke University
Amy Hobeika, PhD, Assistant Research Professor, Department of Surgery, Duke University
H. Kim Lyerly, MD, Professor, Department of Surgery, Duke University
William Gwin, MD, Fellow, Department of Medicine, Duke University
Bruce Burnett, PhD, Director of Regulatory Affairs, Duke University
Frank Jones, CEO, Etubics Corporation
Beth Gabitzsch, Vice President of Research, Etubics Corporation

2.8. List of Requested FDA Attendees

Raj K. Puri, MD, Ph.D., Director, Division of Cellular and Gene Therapies
Preclinical reviewer

Product reviewer
Medical reviewer

3. Synopsis of nonclinical studies

3.1. Human epidermal growth factor receptor-3 (ErbB3/HER3)

The human HER3 gene is a member of the Epidermal Growth Factor Receptor subfamily of receptor tyrosine kinases that includes the Epidermal Growth Factor Receptor (EGFR; *erbB gene;* HER1), HER2 (*erbB-2*), HER3 (*erbB-3*), and HER4 (*erbB-4*). HER3 is membrane bound protein encoded by the ERBB3 gene. Unlike EGFR or HER2, HER3 is considered kinase inactive but forms active heterodimers with other members of the ERBB family, including HER2. HER3 dimerization with other members of the EGFR family is thought to be one mechanism HER3 expression allows for escape tyrosine kinase inhibition of HER2.

Design of the HER3 transgene sequence

The HER3 transgene sequence used to produce the Ad[E1-, E2b-]huHER3 used in our preclinical studies and proposed for our clinical trial spans the full length of the wild type HER3 receptor.

Figure 3.1.-1. Schematic map of the shuttle plasmid used to produce Ad[E1-, E2b-]huHER3

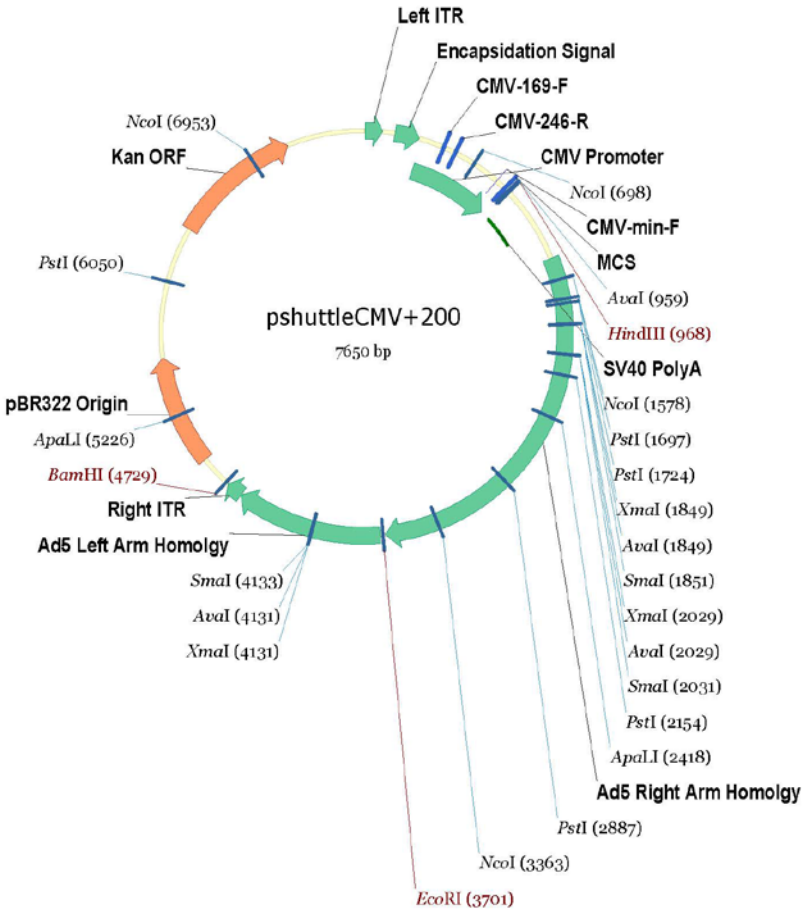

Figure 3.1.-2. Schematic map of the plasmid containing the human HER3 gene

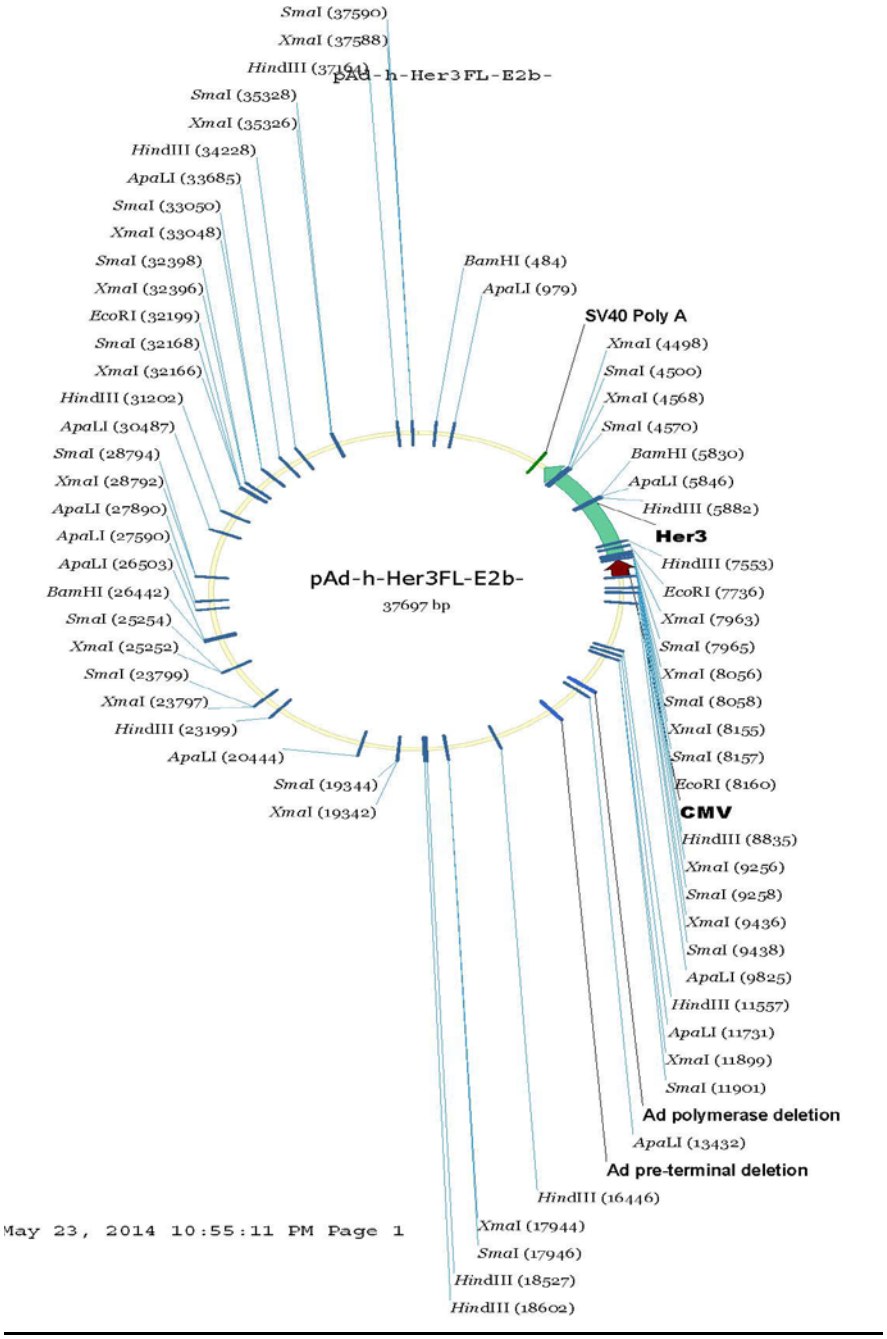

Figure 3.1.-3. HER3 full length sequence for the vector production

DNA Sequence for Ad-Human Her3 –full length construct

atgagggcgaacgacgctctgcaggtgctgggcttgcttttcagcctggcccggggctcc
gaggtgggcaactctcaggcagtgtgtcctgggactctgaatggcctgagtgtgaccggc
gatgctgagaaccaataccagacactgtacaagctctacgagaggtgtgaggtggtgatg
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cagtatggaggagtttgtgtagccagctgtccccataactttgtggtggatcaaacatcc
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agaacgtaa

3.2. Preclinical models

Our original preclinical studies involved proof of principle studies to show our Ad-HER3 vectors were capable of inducing in vivo anti-HER3 immune responses and anti-tumor responses. We therefore initially developed a recombinant first generation adenoviral (Ad5[E1-]) vaccine which targets full length human HER3 and induces T cell and antibody responses to multiple T cell and antibody epitopes.

We developed an Ad vector expressing full length human HER3, termed Ad-HER3 or Ad5 [E1-] huHER3 full length (FL), to generate our preliminary data. We first demonstrated that Ad-HER3 was immunogenic in wild type BALB/c mice and elicited T cell and antibody responses (**Figure 3.2.-1**).

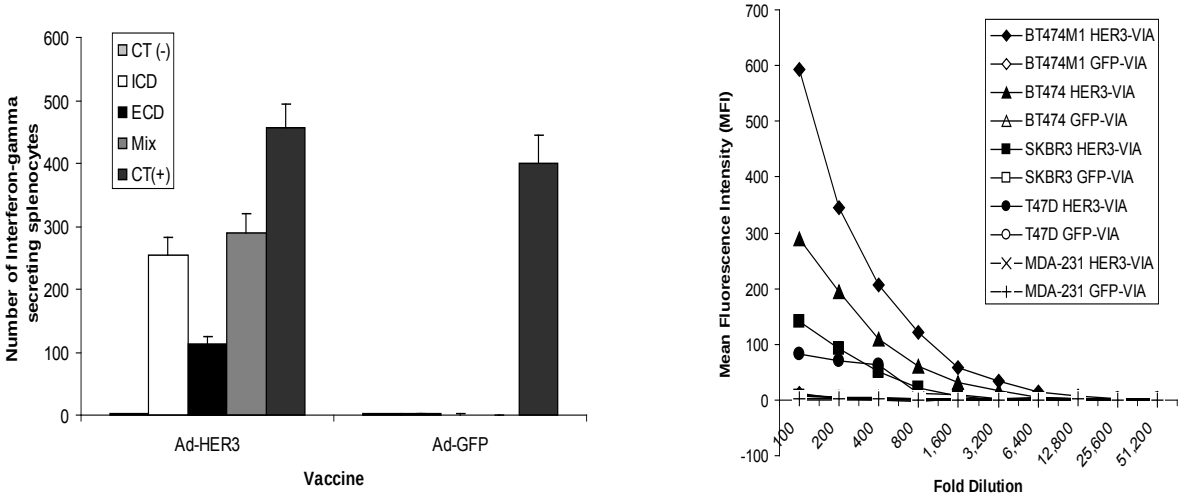


Figure 3.2.-1. Immunogenicity of Ad-HER3 vaccine. BALB/c mice (N=5) received two bi-weekly vaccinations with either Ad-HER3 or Ad-GFP as a control. Mice were euthanized two weeks after the second vaccinations and splenocytes and peripheral blood serum were isolated. **Left:** IFN-g ELISPOT analysis showing response of splenocytes from Ad-HER3 vaccinated or Ad-GFP vaccinated mice against saline (CT-), HER2-intracellular domain (ICD) peptide mix, HER2-extracellular domain (ECD) peptide mix, a mixture of ICD and ECD peptide mixes, and SEB (CT+) respectively. Error bars = standard deviation. **Right:** Flow cytometric assessment of purified serum antibody binding to HER3+ (BT474M1, BT474, SKBR3, T47D) and HER3- (MDA-231) human breast tumor cell lines at various serum dilutions (x-axis) from Ad-HER3 (HER3-VIA) or Ad-GFP (GFP-VIA) vaccinated mice (methodology described on our papers: 27,42). VIA = vaccine induced antibodies.

Epitope mapping studies using overlapping peptide arrays of human HER3 have identified 18 epitopes recognized by the HER3-VIA, demonstrating that the HER3-VIA is polyclonal (**Figure 3.2.-2, left**). These likely represent only a fraction of the antibody epitopes present because the overlapping peptide library we screened on the peptide array blots consisted of 15 amino acid peptides and secondary structure was likely not optimal. Our experience with vaccine induced antibodies to HER2 causing dramatic receptor internalization (29, 36) led us to examine if these HER3-VIA could also mediate receptor internalization. There is marked internalization of the HER3 receptor induced by HER3-VIA (**Figure 3.2.-2, right**).

HER3-VIA Antibody epitopes	
Epitope(s) position	Protein region
101-111	ECD
153-167	ECD
185-191	ECD
209-223	ECD
369-375	ECD
501-511	ECD
589-599	ECD
649-663	TM
681-691	ICD
865-875	ICD
881-895	ICD
901-915	ICD
981-988	ICD
1037-1051	ICD
1105-1119	ICD
1153-1163	ICD
1249-1255	ICD
1268-1279	ICD

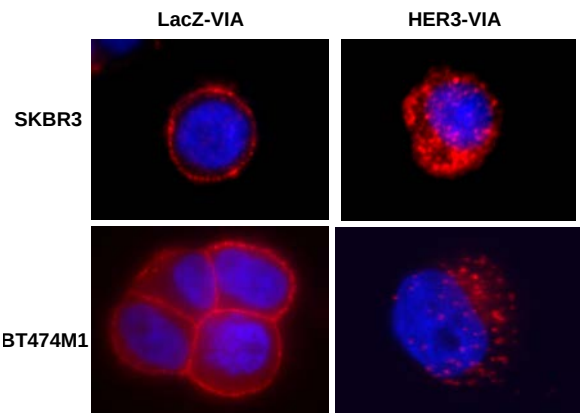


Figure 3.2.-2. Ad-HER3 vaccination elicits polyclonal vaccine induced antibodies (HER3-VIA) that mediate HER3 receptor internalization. Left: Epitope mapping results. Right: Human HER3+ breast cancer cells (SKBR3 or BT474M1) were stained with DAPI (nuclear stain, blue) and an anti-HER3 MAb (red). Cells were then incubated with either LacZ-VIA from Ad-LacZ vaccinated mice or HER3-VIA from Ad-HER3 vaccinated mice, and then visualized 2 hrs later by fluorescence microscopy. The HER3-VIA causes rapid receptor internalization.

In contrast to receptor internalization mediated by the natural ligand for HER3, polyclonal antibodies mediate receptor internalization and degradation as seen in **Figure 3.2.-3.** A high content assay for HER3 receptor internalization was developed using a mutant HER3 receptor which lacked a nuclear localization signal (**Fluorescent HER3 Construct YFP-HER3ΔNLS2)**. This results in constitutive HER3 membrane expression. Cells were then exposed to HER3 vaccine induced antibodies (HER3-VIA), or the HER3 ligand hergulin/neregulinB1 (NRGB1), or LacZ vaccine induced antibodies (Lacz-VIA)

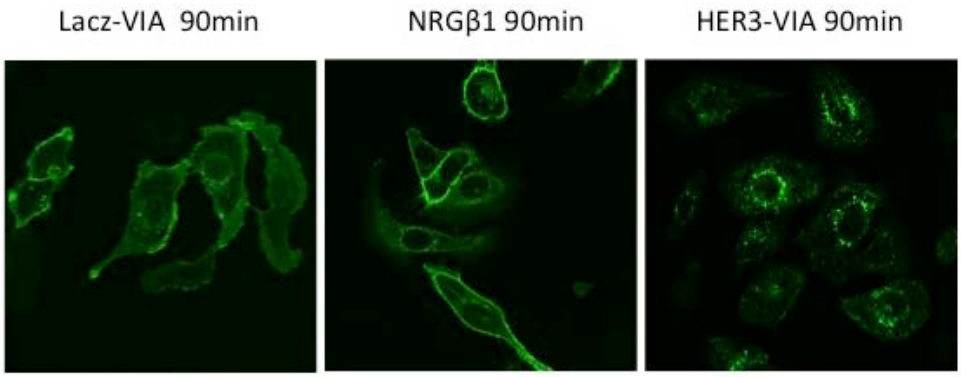


Figure 3.2.-3. HER3 vaccine induced antibodies (HER3 VIA) cause receptor internalization and degradation (Right panel). The HER3 ligand hergulin/nereulin (NRGB1) causes minimal receptor internalization (Middle panel). **Construction of Fluorescent HER3 Construct YFP-**

HER3ΔNLS2: HER3-YFP was constructed using a LTR-2/erbB-3(HER3) construct (provided by Dr. L. E. Samelson, NCI, Bethesda, MD, USA) as a PCR template and pcDNA3.1-mYFP construct as a vector (gift from Roger Y Tsien, University of California at San Diego). HER3 was PCR amplified by using the primers 5'-GGGGTACCGGAGTCATGAGGGCGAACGACGCTC -3' and 5'-ATAAGAATGCGGCCGCGTTCTCTGGGCATTAGCCTTGGG -3', and inserted into the vector by KpnI and NotI restriction sites. In order to delete "RRRR" NLS2 sequence, HER3-YFP as a PCR template was PCR amplified by using the primers 5'-GAGTATGAATACATGAACCACAGTCCACCTCATCCC -3' and 5'-GGGATGAGGTGGACTGTGGTTCATGTATTCATACTC -3'. HER3 cDNA was verified by sequencing.

We believe that internalization of the receptor is an important potential mechanism of action for cancer vaccines targeting growth factor receptors because it offers the possibility for receptor degradation to inhibit receptor signaling (29, 36). We assessed this and found that the HER3-VIA could inhibit proliferation of HER3+ human breast tumors *in vitro* and also mediated complement dependent cytotoxicity (**Figure 3.2.-4**).

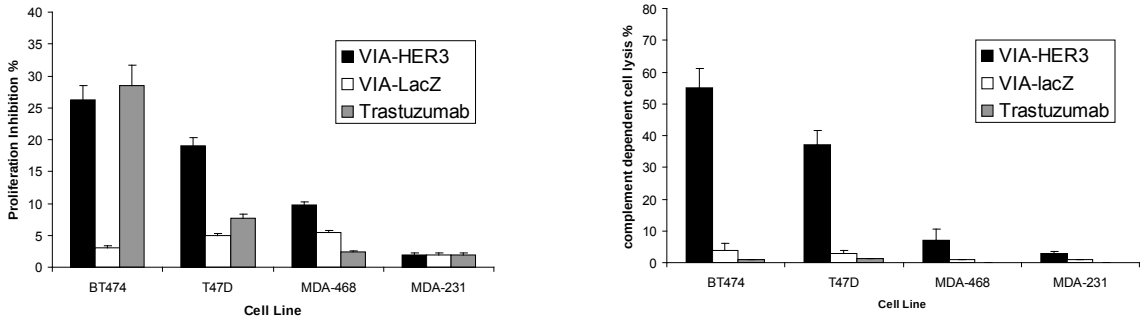
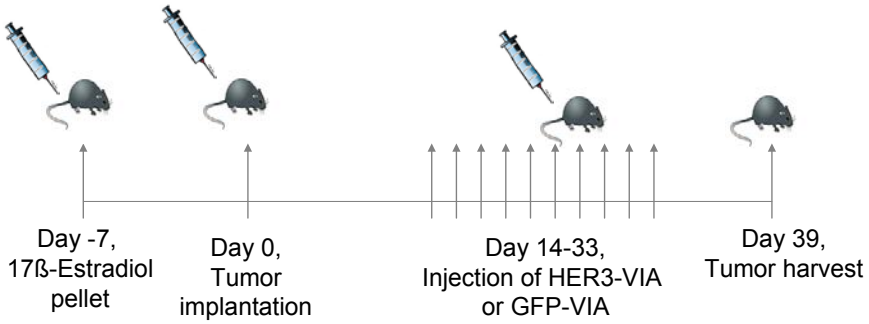


Figure 3.2-4. HER3-vaccine induced antibodies from the serum of mice vaccinated with Ad-HER3, mediate anti-proliferative effects (**left**) and complement dependent cytotoxicity (**right**) on HER3 expressing human breast cancer cell lines (BT474, T47D, MDA-468) but not HER3-non-expressing cells (MDA-231). Error bars represent standard deviation. Methodology as described (*27*).

As initial proof of antitumor activity of the HER3 vaccine, we took purified HER3-VIA mouse serum antibodies from mice vaccinated with the Ad-HER3 vaccine, or a control Ad-GFP vaccine, and passively transferred them to SCID mice bearing established BT474M1 tumors (HER2+, HER3+ human breast tumor cell line). The experiment is summarized in **Figure 3.2.-5**.



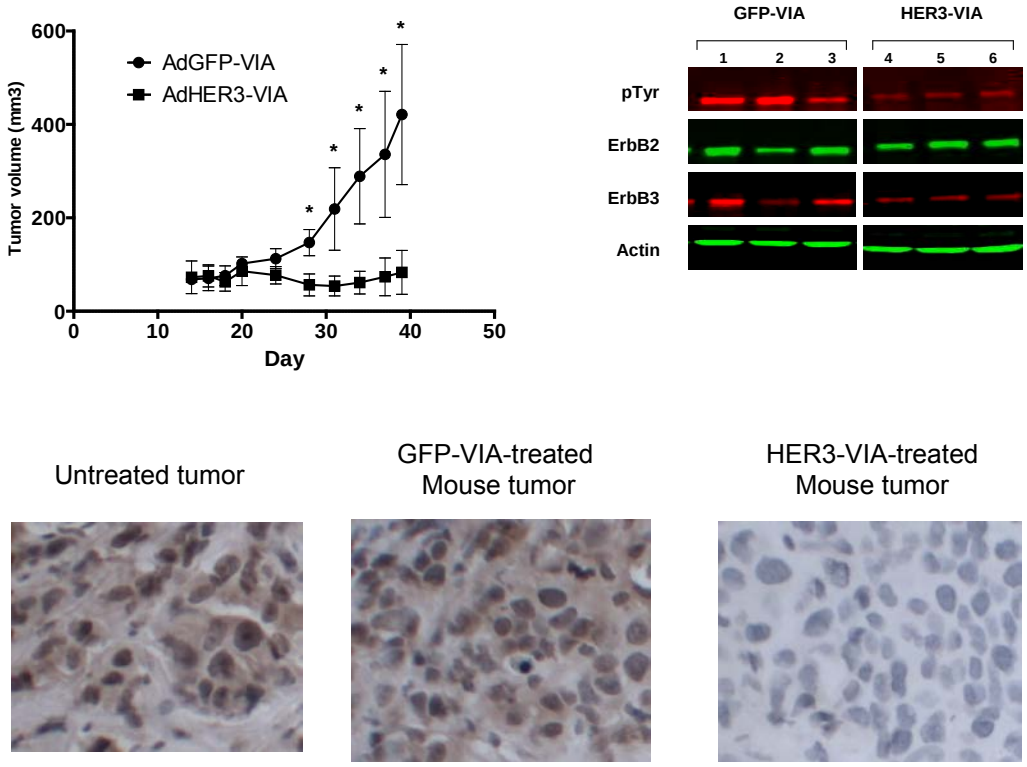


Figure 3.2.-5. HER3-vaccine induced antibodies (HER3-VIA) have antitumor activity *in vivo*. Top: Experimental schema for SCID xenograft studies of HER3 vaccine induced antibody (VIA) treatment of established HER3+ human breast tumors *in vivo.* Female SCID-B6.129S7-Rag1(tm1Mom) mice were implanted with 10 million BT474M1 cells on day zero. On day 8 tumors were measured and mice were randomized to receive either GFP-VIA (circle symbol) or HER3-VIA (square symbol), given every two days for 10 injections. Tumors volume was measured at two day intervals and tumors were harvested for further analysis at day 39. **Middle left:** Mean tumor volume is plotted. Error bars = Standard deviation. * represents $p < 0.0098$. **Middle right:** Western immunoblot of tumors excised from SCID xenograft mice treated with either HER3-vaccine induced antibodies or control GFP-VIA. Three representative tumors per treatment are shown. HER3-VIA results in marked decreases in pTyr (pHER2) and ErbB3 (HER3) protein levels compared to GFP-VIA treated mice. **Bottom panels:** Immunohistochemistry analysis of HER3 protein expression in excised tumors, showing a control tumor from a mouse that had received no treatment, a tumor from a GFP-VIA-treated mouse, and the loss of HER3 protein expression (brown) in the HER3-VIA treated mouse. Collectively, these data demonstrate that an Ad-HER3 vaccine is immunogenic and can mediate multiple mechanisms of action that have anti-tumor activity *in vitro* and *in vivo*.

Schedule, dose, route of immunization safety data

The clinical study design flows from pre-clinical studies in animals using the Ad5 [E1-, E2b-] vector platform. Initial studies were performed to evaluate and confirm that an Ad5 [E1-, E2b-] vector platform could express the antigen proteins on transfected cells. A-549 cells were transfected with vaccine platforms and analyzed by Western Blot Analysis. We observed that antigen proteins such as HIV-gag, HIV-pol, or HIV-nef were expressed on cells once they were transfected with the Ad5 [E1-, E2b-] vector platforms and a representative Western Blot is presented in Figure 3-6. Etubics Corporation has

performed a dose response evaluation using the Ad5 E1-, E2b-] vector platform and demonstrated that 10^{10} virus particles (VP) is a dose that results in a desired CMI response against a transgene product in a murine model.

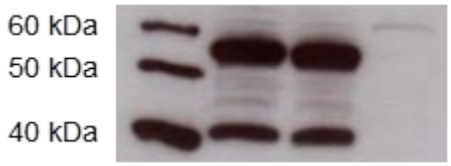


Figure 3.2.-6. Gag production by A-549 cells infected with Ad5 [E1-, E2b-]-gag. A representative immunoblot analysis of A-549 whole cell lysate infected at a MOI of 200 of Ad5 [E1-, E2b-]-gag or Ad5-null for 44 h. The blot was stained with a mouse monoclonal antibody against Gag. Lane 1, Magic Mark XP Western Standard (Invitrogen, CA), Lanes 2 and 3, Ad5 [E1-, E2b-]-gag, Lane 4, Ad5-null (empty). The upper band (55kDa) comprises the gag precursor and the lower band (41kDa) comprises the p17/p24 gag complex.

CMI responses were assessed by utilizing an ELISpot assay to detect interferon-gamma (IFN-γ) and IL-2 secreting cells (splenocytes) from spleens of mice. Furthermore, we have demonstrated in murine and non-human primate (NHP) models that three immunizations using 10^{10} VP separated by two weeks to four weeks, respectively, results in the desired CMI responses. In mice, we observed a greater degree of CMI responses after multiple immunizations as compared with one immunization only (Figure 3.2.-7).

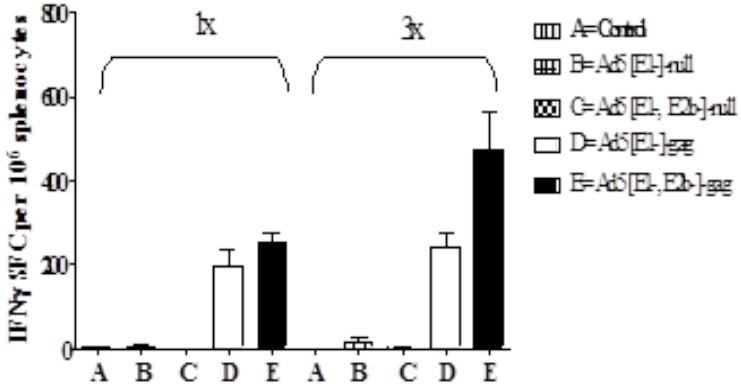


Figure 3.2.-7. Multiple immunizations induce a greater CMI (IFN-g ELISpot) response. Naïve BALB/C mice (n=5/group) were immunized once or three times at fourteen day intervals with 1010 VP of Ad5 [E1-]-null, Ad5 [E1-, E2b-]-null, Ad5 [E1-, E2b-]-gag, Ad5 [E1-]-gag or injection buffer alone (control). Fourteen days after the final immunization splenocytes were assessed for IFN-g secreting splenocytes by ELISpot analysis. For positive controls, splenocytes were exposed to Concanavalin A (Con A) (data not shown). The error bars depict the SEM.

In a NHP model, we first rendered the animals Ad5 immune by injection with wild type Ad5 virus. After detection of the presence of Ad5 neutralizing antibody (that confirmed the animals were immune to Ad5), the animals were vaccinated with an Ad5 [E1-, E2b-] vector platform three times at monthly intervals. As shown in Figure 3.2.-8, after immunizations, we detected the presence of robust CMI responses when the animals' PBMCs were assessed for IFN-γ and IL-2 secreting cells.

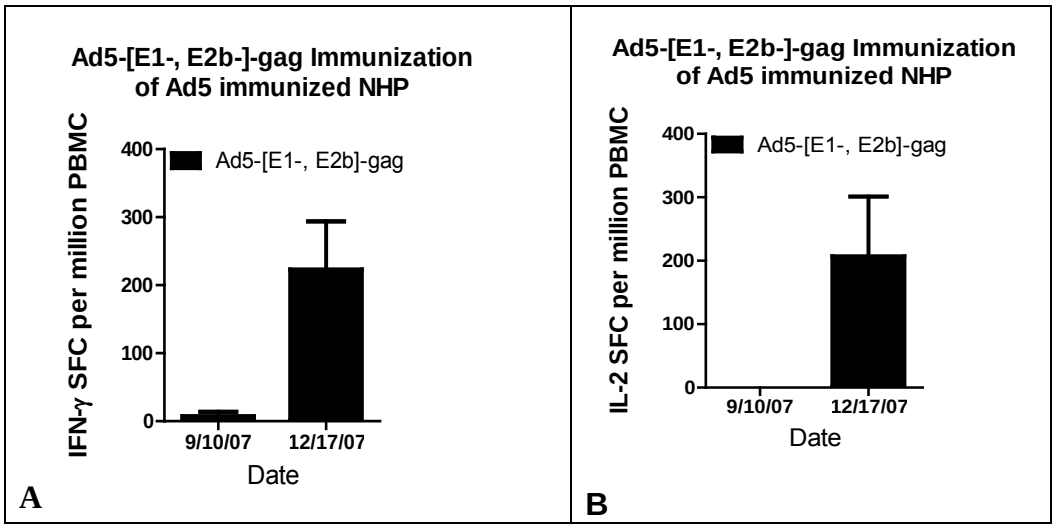


Figure 3.2.-8. ELISpot IFN-g (A) and IL-2 (B) analysis of PBMC from Cynomolgus Macaques (N=3) pre-immunized against Wild Type Ad5. When Ad5 neutralizing antibody titers reached 1:50 or greater they were immunized intradermally three times at 30 day intervals with Ad5-[E1-, E2b-]-gag at a dose of 1010 VP. The first immunization (Wild Type Ad5) was on 8/15/2007 and on 12/17/2007 (32 days after last vaccination) the NAb titers were equal to or greater than 1:1000. Note the presence of significantly elevated values ($P<0.05$) in the 12/17/2007 samples. For positive controls, splenocytes were exposed to Concanavalin A (Con A) (data not shown). Values represent mean +/- SEM.

In addition to the preliminary immunology studies performed in the initial vaccine trial in 3 NHP shown above, toxicity studies were also performed on the same NHP vaccinated with Ad5 [E1-, E2b-]-HIV gag. Animal temperatures and weights were assessed during the study period. The animals gained weight as they grew during the study period. No temperature differences were observed during the study period. Hematology studies were also performed on the vaccinated NHP. There appeared to be a small increase in the white blood cell count 2 weeks after the second vaccination that normalized thereafter. Other than fluctuation in values, there appeared to be no other differences in hematology values during the course of the study. Chemistry values were also determined in the NHP during the course of the study. Alkaline phosphatase levels declined slightly during the course of the study but remained in the normal range. Albumin levels declined slightly during the course of the study but remained in the normal. There were no other differences observed in the blood chemistries during the course of the study. The route of

immunization in this clinical study is chosen since the preponderance of DC reside in the dermis. Using this premise we have found that a desired level of CMI response was induced using the Ad5 [E1-, E2b-] platform employing CEA and other transgenes. Using an Ad5 [E1-, E2b-]-CEA vector platform, both non-Ad5 immune and Ad5 pre-immunized mice were injected three times with the vaccine. After immunizations, the splenocytes from mice were assessed by ELISpot for IFN-γ secreting cells. As shown in Figure 3.2.-9, elevated CMI responses were observed after immunizations and the levels of CMI responses were similar in both non-Ad5 immune and Ad5 pre-immunized mice. These results indicate that robust CMI responses can be induced despite the presence of pre-existing Ad5 immunity. In our proposed phase I clinical study, we have chosen to use three immunizations separated by three weeks via a needle subcutaneous delivery method.

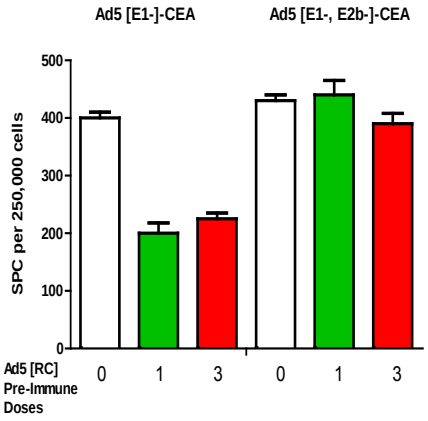


Figure 3.2.-9. ELISpot interferon-g secreting SPC from mice vaccinated with recombinant Ad5 CEA expression vectors. Note the lack of reduction in SPC from mice vaccinated with Ad5 [[E1-, E2b-]-CEA as compared with the reductions in SPC from Ad5 [E1-]-CEA vaccinated mice.

Generation of Ad5(E2b-)HER3 and Ad5(E2b-)HER3 C1C2 constructs

Following our proof of concept studies for targeting HER3 with an adenoviral vector, we modified the adenovirus construction methods to facilitate the production of the next generation Ad5 vectors with deletion of multiple early genes (E1, E2b, E3). Previous studies demonstrated that Ad5[E1-, E2b-] vectors are more potent immunogens compared 1st generation Ad (Ad5[E1-]) even in the presence of pre-existing anti-Ad5 immunity.

The human HER3 full length cDNA was obtained from OriGene (Rockville, MD). The truncated HER3 extracellular domain (ECD) and HER3 ECD plus transmembrane (TM) sequence were created using HER3 full length as templates in a PCR reaction using primers (see table 3.2.-1 and figure 3.2.-10 below).

Table 3.2.-1: Primers used in construction of truncated Ad5-human HER3

Primer	Sequence
hHER3-F	5’-cagggcggccgcaccatgagggcgaacgacgctct-3’
hHER3-ECDTM-R	5’-acaagcggccgcagttaaaaagtgccgcccagcatca-3’
hHER3-ECD-R	5’-acaagcggccgcatttatgtcagatgggttttgccgatc-3’
hHER3-ECDC1C2-R	5’-acaagcggccgcattgtcagatgggttttgccg-3’

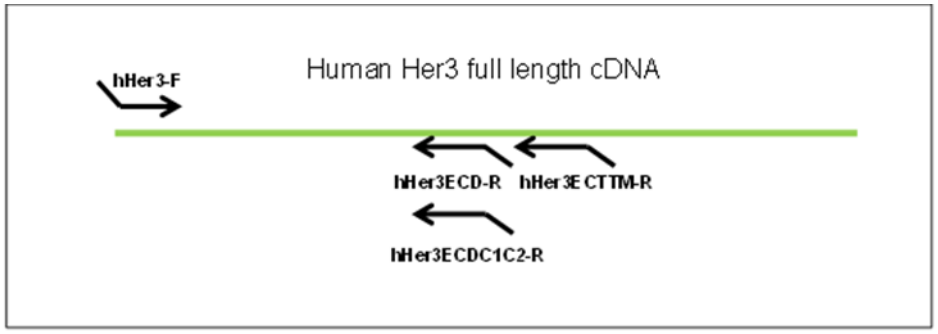


Figure 3.2.-10. Schematic representation of primers binding site at human HER3 full length cDNA

Briefly, full length HER3 cDNA and the PCR product are cut by restriction enzyme Not I and subcloned into Not I digested pShuttle-CMV or pShuttleCMV-C1C2 plasmid. Confirmation of correct insert of the full length and truncated DNA within pShuttle-CMV or pShuttle-CMV-C1C2 was confirmed by DNA sequence.

The pShuttle-CMV-HER3-FL, pShuttle-HER3ECD, pShuttle-HER3ECDTM and pShuttle-HER3ECDC1C2 were then linearized using digestion with Pme I, recombined into linearized (E1-,E2b-,E3-) serotype 5 pAd construct in BJ 5183 bacterial recombination-based system (Stratagene), and propagated in XL10-Gold Ultracompetent cells (Stratagene). Complementing C7 cell (which express E1 and E2b) were used to produce high titers of these replication-deficient Ad5 vectors, and cesium chloride density gradient was done to purify the Ad5-vectors. All Ad vector stocks were tested for replication-competent adenovirus via PCR-based replication-competent adenovirus assay.

We generated next generation human HER3 (E1-, E2b-, E3-) Adenovirus vectors as follows:

1. Ad5 [E1-, E2b-]HER3 FL; express human HER3 full length.
2. Ad5 [E1-, E2b-]HER3ECDTM; express human HER3 ECD and trans-membrane domain
3. Ad5 [E1-, E2b-]HER3ECD; express human HER3 ECD
4. Ad5 [E1-, E2b-]HER3ECDC1C2; express human HER3 ECD and C1C2 domain

Preclinical immunogenicity testing of Ad5[E1-]huHER3 full length

Prior to preclinical testing with the Ad5[E1-, E2b-] HER3 vectors, we tested our Ad5 [E1-]huHER3 vector by establishing a HER3 tumor prevention model using JC-HER3 mouse mammary tumor cells in BALB/c mice. JC murine breast cancer cell line

(BALB/c strain) was transfected with human HER3 using lentiviral vector. Immunogenicity and vaccine efficacy of Ad vectors were determined in BALB/c mice by assessing preventive effect of HER3 vaccination (Figures 3.2.-11).

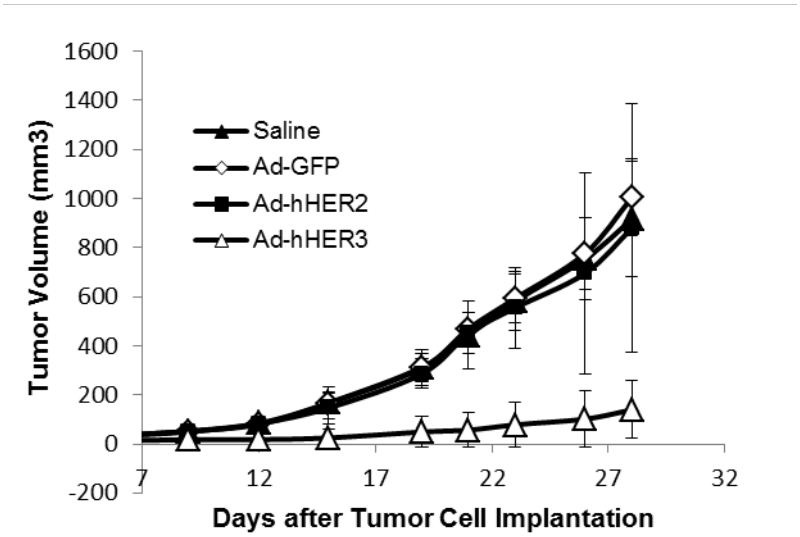


Figure 3.2.-11. Ad5[E1-]huHER3 vaccine inhibits JC-HER3 tumor growth. BALB/c mice were vaccinated twice (day-18, day-4) via footpad injection with Ad[E1-]GFP, Ad[E1-]huHER2 or Ad[E1-]huHER3 vectors (2.6×10^{10} particles/ mouse). Four day after boosting, at day 0, each mouse was implanted with 1,000,000 JC-HER3 mouse mammary tumor cells expressing human HER3. Tumor volume was measured every 3 days.

Only vaccination with the HER3 full length encoding vector prevented growth of HER3 expressing tumors.

To confirm the induction of HER3 specific immune response in Ad-HER3 vaccinated mice, we performed ELISPOT assay with splenocytes from vaccine treated mice as shown in Figure 3.2.-12.

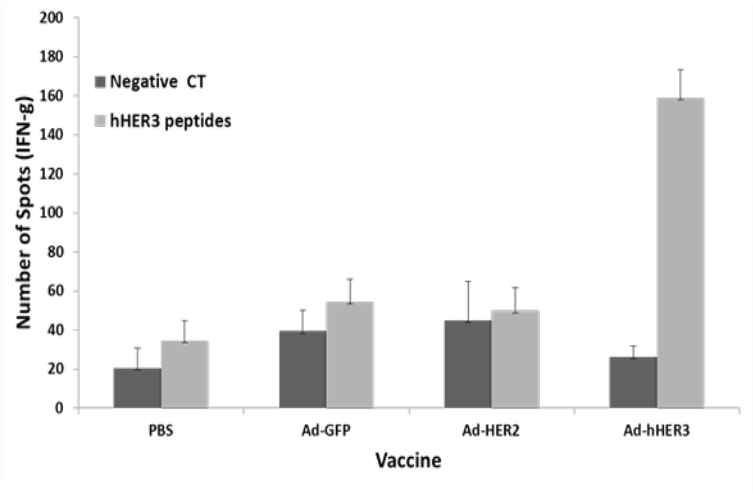


Figure 3.2.-12. Ad5[E1-]huHER3 vaccine induced HER3 specific T cell response. Splenocytes (500,000 cells/well) from Ad vaccinated BALB/c mice were collected on day 28 and stimulated with HER3 peptide mix (huHER3 peptides) (1µg/mL was used; JPT, Acton, MA) or HIV peptide mix (BD Bioscience) as a negative control (Negative CT) and analyzed in a interferon-gamma ELISpot assay

We confirmed the establishment of anti-HER3 cellular immune response in mice vaccinated with Ad-HER3. We hypothesized that Ad-huHER3 induced anti-HER3 immune response will affect the HER3 expression by tumors grown in mice. Therefore, we tested the HER3 expression in tumor tissue by Western Blot assay, as shown in Figure 3.2.-13.

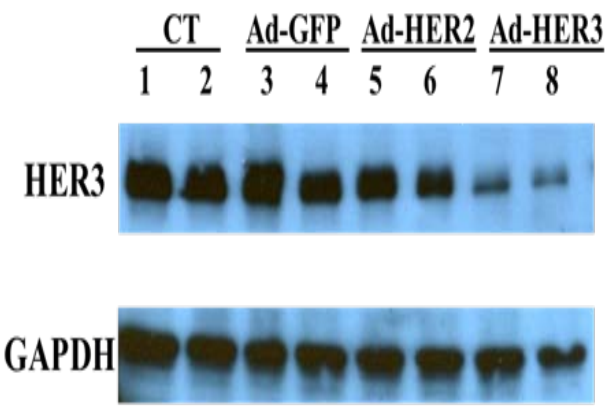


Figure 3.2.-13. Ad5[E1-]huHER3 vaccination causes degradation of HER3 on JC-hHER3 tumor. Tumors were isolated from vaccinated and control BALB/c mice (as indicated on figure) and immediately flash frozen. Tissue extracts were prepared by homogenization in RIPA buffer. Equal amounts of protein from each sample were used to visualize the indicated molecules by immunoblotting.

Immunization with Ad[E1-]-huHER3 led to a reduction of HER3 expression in the tumors while immunization with Ad[E1-]GFP or Ad[E1-]-huHER2 did not change HER3 expression by JC-HER3 tumors.

We also sought to test for the cell surface HER3 expression by tumors that grew in the HER3 vaccinated mice. Excised tumors were digested with collagenase /hyarulonidase /DNase, and collected tumor cells were stained with PE conjugated anti-HER3 mAb, and analyzed by flow cytometry as shown in Figure 3.2.-14.

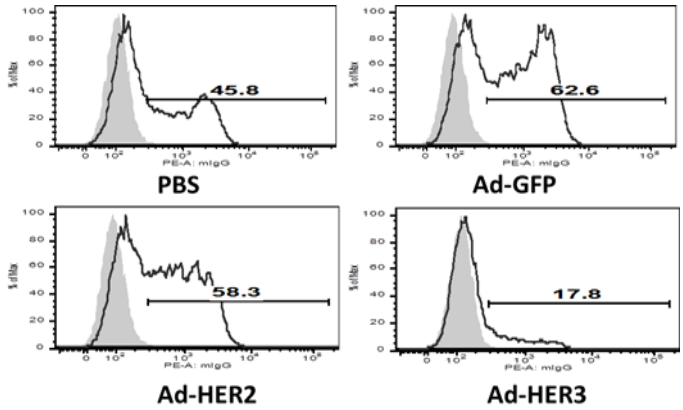


Figure 3.2.-14. Ad5[E1-]huHER3 vaccination decreases HER3 expression on JC-hHER3 tumor cells. JC-HER3 tumors were collected from vaccinated and control Balb/c mice (as indicated on figure) at day 28 and pooled by group. The tissues were minced and digested with an enzymatic cocktail (Hyaluronalse, DNAse, and Collagenase) overnight. After 3 days culture, the cells were harvested and HER3 expression determined by flow cytometry using PE-anti-hHER3 antibody. Solid line: anti-HER3 mAb. Grey histogram: PE-conjugated IgG.

As demonstrated in Figure 3.2.-14, the surface expression of HER3 was dramatically reduced in the tumors that did grow in the HER3 vaccinated mice, suggesting the

elimination of HER3-positive tumor cells or downregulation of HER3 expression by vaccine induced anti-HER3 immune response.

Preclinical immunogenicity testing of Ad5(E1-, E2b-)HER3 in BALB/c Mice

To compare the immunogenicity of the 4 different adenoviral vectors encoding human HER3 genes (Ad5[E1-, E2b-]HER3 FL, Ad5[E1-, E2b-]HER3ECD, Ad5[E1-, E2b-]HER3ECDTM, and Ad5[E1-, E2b-]HER3ECDC1C2) in BALB/c mice, female mice (10 mice/group) were vaccinated twice with 2 weeks interval, and human HER3 expressing murine breast cancer cell line (JC-HER3, 1 M cells/mouse) was injected to the flank of mice 4 days later. From each group, 3 mice were sacrificed before tumor cell implantation to collect blood and spleen for immune monitoring. Tumor volume was monitored for the rest of the mice until human endpoint is reached.

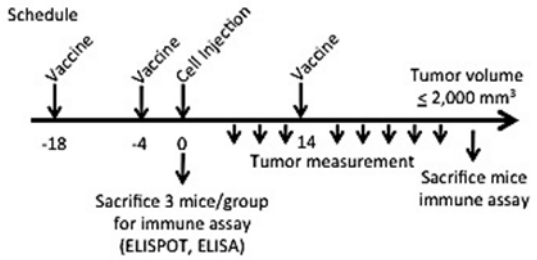


Figure 3.2.-15. **Scheme of Immunogenicity Testing and Antitumor Efficacy Testing.** On days -18 and -4, mice were vaccinated with Ad-vectors (2.6 x 10E10 vp/mouse), and 3 mice from each group were sacrificed for immune assays on day 0. Spleen was harvested for ELISPOT assay, and blood for the test of antibody production. For other 7 mice in each group, JC-HER3 cells were subcutaneously injected to the flank of BALB/c mice. Tumor size was measured until tumor volumes reach 2,000 mm3.

Humoral Immune Response

We analyzed established humoral immune responses against HER3 in these mice by flow-based assay.

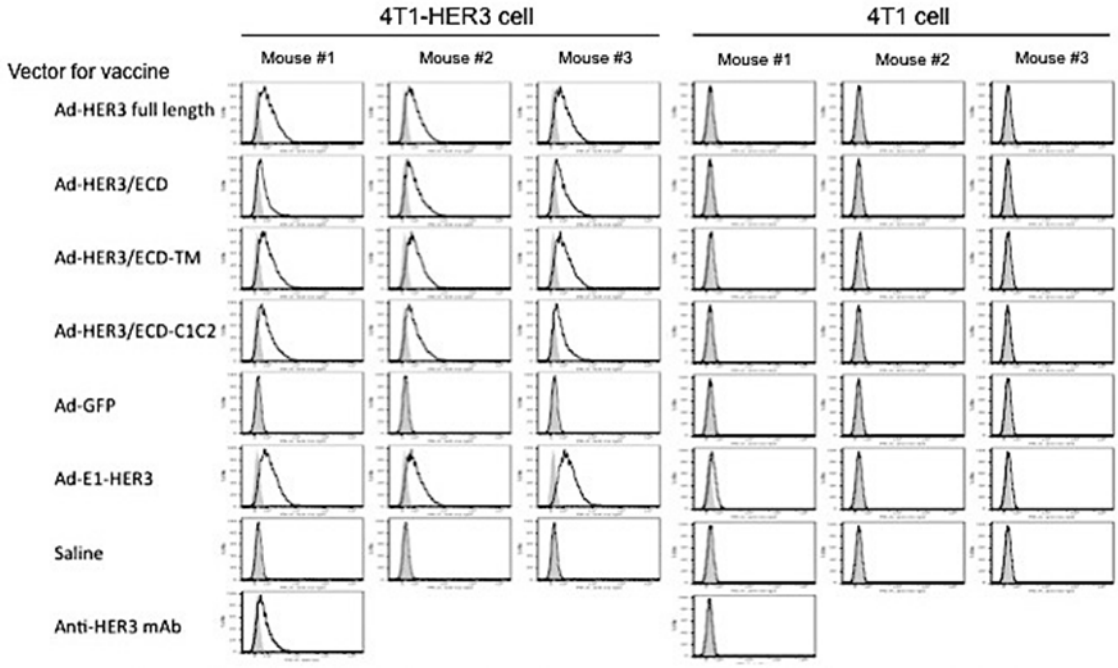


Figure 3.2.-16. Anti-HER3 antibody levels in the serum of Ad-HER3 vaccinated mice. Three mice from each group were sacrificed, and serum was collected. 4T1 (HER3-negative) and 4T1-HER3 (transfectant) were labeled with serum (1:100 dilution) and then with PE-conjugated anti-mouse IgG Ab. Open histograms (black line) show staining with mouse serum, and grey histograms show staining without serum (2ndary Ab only).

4T1 (HER3 negative) or 4T1-HER3 (HER3 transfectant) cells were incubated with mouse sera, which were diluted with saline (1:100 dilution), then with PE-conjugated secondary antibody (anti-mouse IgG). Sera from Ad5[E1-, E2b-]-GFP vaccinated mice were used as negative control, commercially available anti-HER3 mAb as positive control, and mouse serum from Ad5[E1-]huHER3 vaccinated mice were used for comparison purpose (Figures 3.2.-16 & 3-17). Mean Fluorescence Intensity for each serum is shown in the graph below (Figure 3.2.-17).

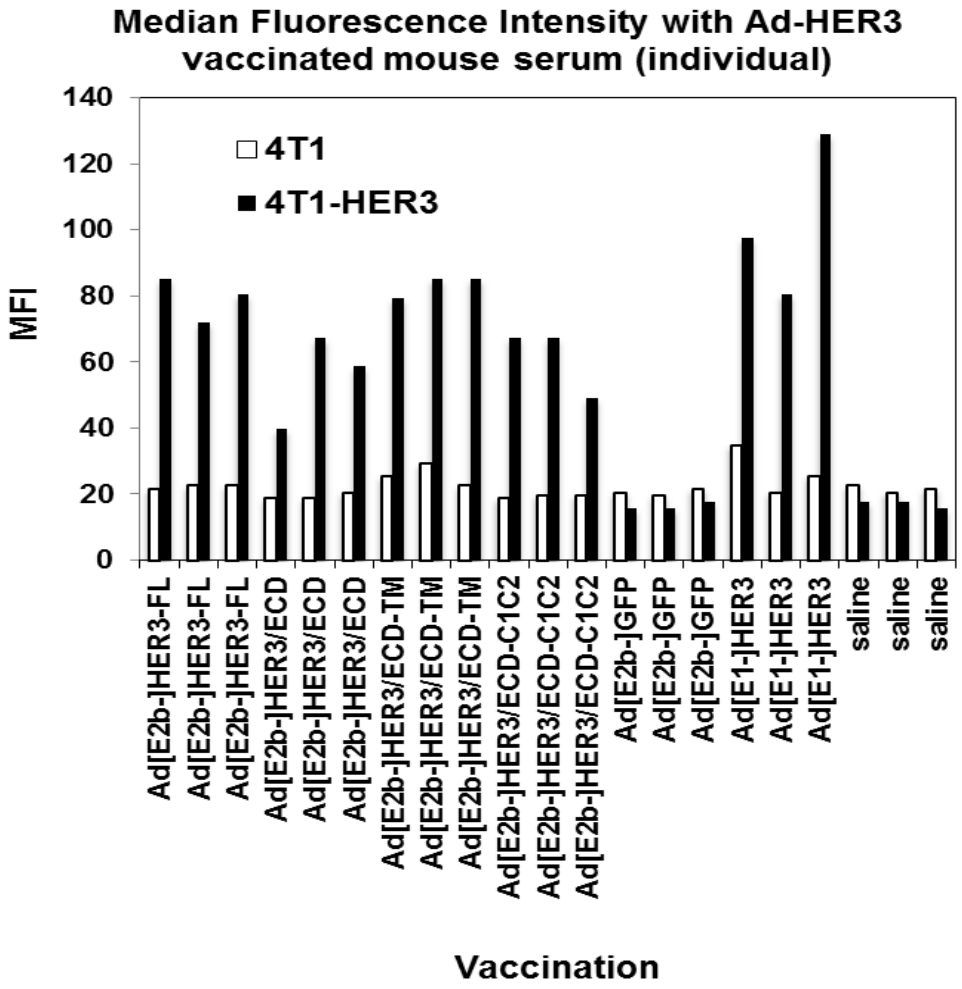


Figure 3.2.-17. Median Fluorescence Intensities for the staining of 4T1 and 4T1-HER3 cells with individual mouse serum.

As shown in the figure 3.2.-17, Ad5[E1-, E2b-]huHER3/ECD-TM and Ad5[E1-, E2b-]huHER3 FL induced slightly stronger anti-HER3 antibody production, and [E1-, E2b-]huHER3/ECD was the weakest among 4 newly generated Ad-HER3 vectors.

We further analyzed anti-HER3 antibody level in each serum by cell-based ELISA. 4T1 murine breast cancer cell line (HER3 negative) and human HER3 transfectant (4T1-HER3) were used in this assay. Sera from individual mice were titrated from 1:50 to 1:6400 (Figure 3.2.-18).

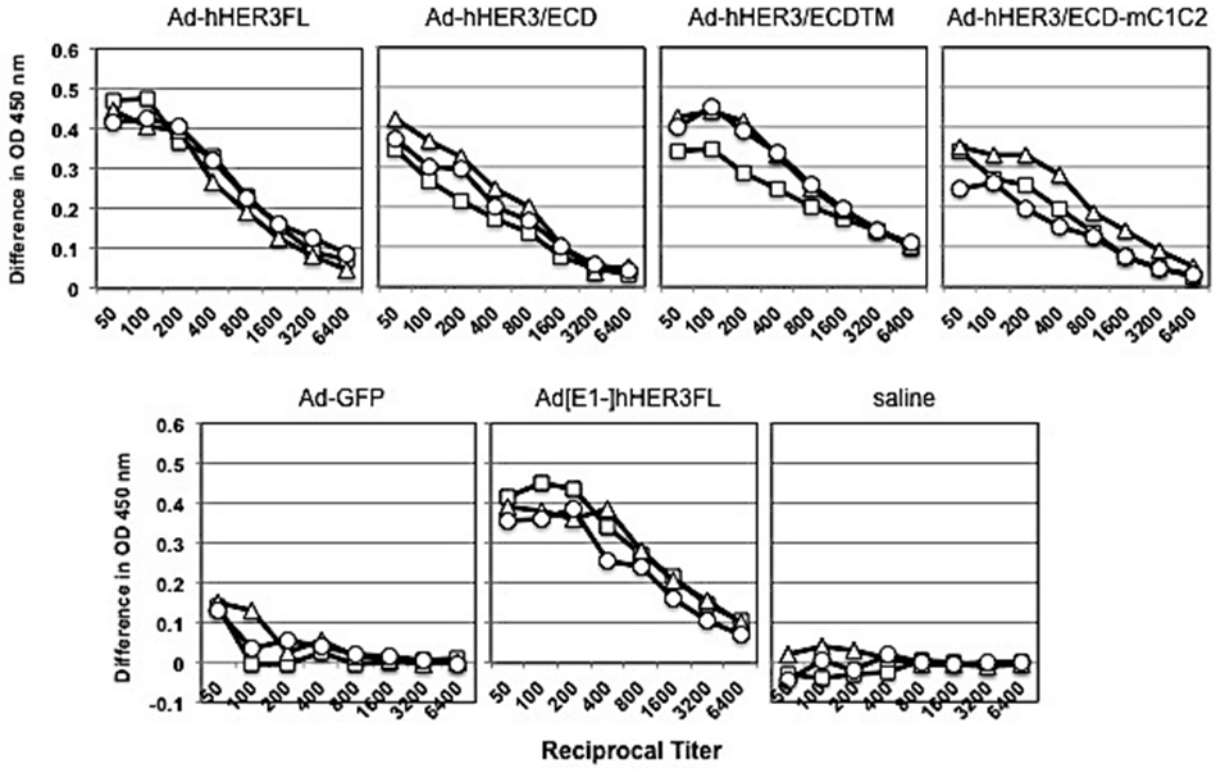


Figure 3.2.-18: Cell-based ELISA with mouse serum. 4T1 and 4T1-HER3 cells were seeded into 96 well plates. After overnight incubation, cells were washed with buffer, and mouse serum with serial dilutions were added (1:50 to 1:6400) and incubated for 1 h on ice. Then, cells were fixed with 4% formaldehyde, and HRP-labeled Goat anti-mouse IgG (1:1000) was added. After 1 h incubation, washed with PBS 3 times, and TMB was added for 5 min. Color development was stopped by adding H2SO4. Differences of OD450 values (=[value for 4T1-HER3] – [value for 4T1]) are shown.

Based on Cell-based ELISA, anti-HER3 antibody production in Ad-HER3 vaccinated mice were confirmed in all mice. These 4 newly made adenoviral vectors showed comparable efficacy in induction of humeral immunity, but Ad[E1-, E2b-]huHER3 FL and Ad[E1-, E2b-]huHER3/ECDTM induced slightly higher levels of anti-HER3 antibody and Ad[E1-, E2b-]huHER3/ECD induced slightly lower level.

Antigen-specific Cellular Response

We also analyzed antigen-specific cellular immune response by IFN-gamma ELISPOT assay with mouse splenocytes (Figure 3.2.-19). Splenocytes from each mouse were incubated with HER3 peptide pool (Extracellular domain (ECD), or Intracellular Domain (ICD)), and HIV peptide mix as a negative control, PMA+ Ionomycin as a positive control. As expected, only Adenoviral vectors encoding full length HER3 (Ad5[E1-,E2b-]huHER3 FL and Ad5[E1-]huHER3 FL) induced T cell response for the intracellular domain of HER3. T cell responses against the peptide mix of HER3 extracellular domain were variable. Ad5[E1-, E2b-]huHER3/ECD-TM induced the strongest cellular response

against extracellular domain of HER3. Ad5[E1-, E2b-]huHER3 virus encoding full length, however, induced only weak cellular response against extracellular domain.

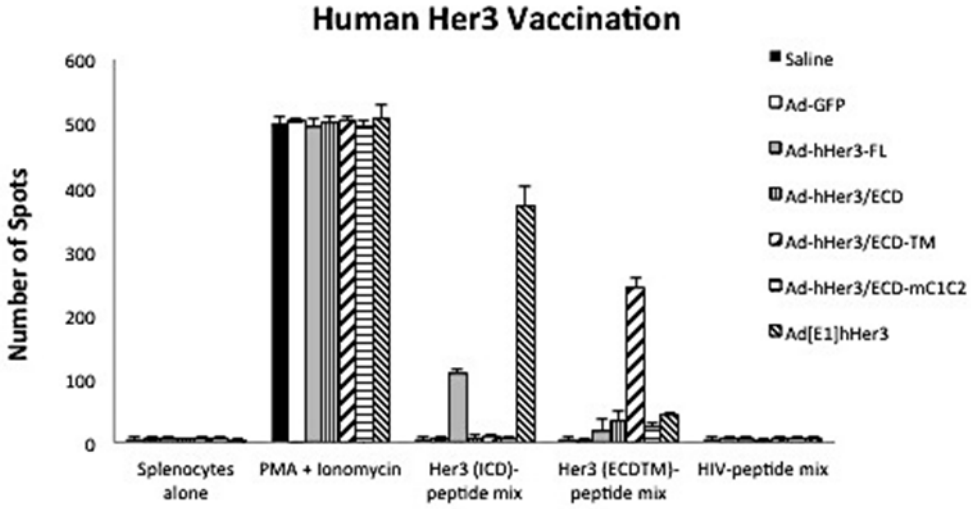


Figure 3.2.-19. Anti-HER3 cellular response induced by Ad-HER3 vaccination. Mice were vaccinated with Ad5[E1-, E2b-]-huHER3-full length(FL), Ad5[E1-, E2b-]huHER3/ECD, Ad5[E1-, E2b-]huHER3/ECD-TM, Ad5[E1-, E2b-]huHER3/ECD-mC1C2, or control Ad-GFP, Ad[E1-]HER3 (2.6 x 10^{10} vp /vaccination). Two weeks later, vaccination was repeated with the same Ad vectors, and 4 days later, spleen was collected to assess anti-HER3 cellular response. ELISPOT plates were coated with anti-IFNg mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. Average of 3 mice from each group is shown.

Antitumor Response

Tumor growth was measured twice a week until 34 days after tumor cell implantation. Once the tumor volume reached 2,000 mm3 or tumor had ulceration, mice were euthanized. Until day 20, all mice survived and the average tumor volume was calculated for each group and shown in the Figure 11. We are currently making statistical analysis to determine which group showed the strongest antitumor immune response. Our preliminary results for the statistical analysis are shown below.

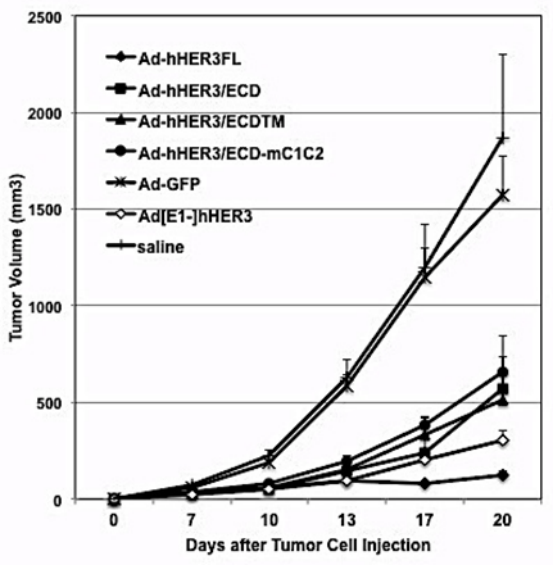


Figure 3.2.-20. Ad-HER3 vaccine effect on JC-HER3 tumor growth in BALB/c mice. BALB/c mice were vaccinated twice (day-18, day-4) before and once (day 14) after tumor cell implantation, with Ad[E1-E2b-]huHER3FL, Ad[E1-E2b-]huHER3/ECD, Ad[E1-E2b-]huHER3/ECDTM, Ad[E1-E2b-]huHER3/ECD-mC1C2, Ad-GFP, Ad[E1-]huHER3FL (2.6 x 10^{10} particles/ mouse) or saline via footpad injection. On day 0, each mouse was implanted

with JC-HER3 mouse mammary tumor cells expressing human HER3 (1 x 10^6 cells/mouse). Tumor volume was measured every 3 days. Error Bar: SE

A mixed model was used to analyze the data. Square root transformation was used for tumor volume to make the relation volume vs time linear and normalize the data. The model results clearly show that the tumor volume increases with time (Days) for the Saline group. The growth rate of tumor volume for the vaccine Ad5[E1-, E2b-]huHER3 FL, Ad5[E1-, E2b-]huHER3 /ECD, Ad5[E1-, E2b-]huHER3 /ECD-TM, Ad5[E1-, E2b-]huHER3 /ECD-mC1C2 are significantly slower than that in Saline, while the difference in the tumor growth in Saline and Ad-GFP is not significant.

Fixed effects: sqrt(vol) ~ groupname * days

	Value	Std.Error	DF	t-value	p-value
(Intercept)	0.4969292	1.9609008	238	0.253419	0.8002
groupnameAd[E2b-]hHER3FL	-0.4177898	2.7731325	42	-0.150656	0.8810
groupnameAd[E2b-]hHER3/ECD	-0.5374032	2.7731325	42	-0.193789	0.8473
groupnameAd[E2b-]hHER3/ECDTM	-0.4758268	2.7731325	42	-0.171585	0.8646
groupnameAd[E2b-]hHER3/ECD-mC1C2	-0.4648125	2.7731325	42	-0.167613	0.8677
groupnameAd[E2b-]GFP	0.0383965	2.7731325	42	0.013846	0.9890
groupnameAd[E1-]hHER3	-0.5313263	2.7731325	42	-0.191598	0.8490
days	1.9160718	0.1107739	238	17.297149	0.0000
groupnameAd[E2b-]hHER3FL:days	-1.3331182	0.1566579	238	-8.509742	0.0000
groupnameAd[E2b-]hHER3/ECD:days	-0.9370645	0.1566579	238	-5.981598	0.0000
groupnameAd[E2b-]hHER3/ECDTM:days	-0.9100369	0.1566579	238	-5.809072	0.0000
groupnameAd[E2b-]hHER3/ECD-mC1C2:days	-0.7315133	0.1566579	238	-4.669496	0.0000
groupnameAd[E2b-]GFP:days	-0.0986146	0.1566579	238	-0.629490	0.5296
groupnameAd[E1-]hHER3:days	-1.0963448	0.1566579	238	-6.998338	0.0000

All newly made Ad-HER3 vectors showed antitumor activity, inhibiting the JC-HER3 tumor growth in BALB/c mice compared to control groups (saline injection). Especially, Ad5[E1-, E2b-]huHER3 FL demonstrated the strongest inhibitory effect for tumor growth after day 17.

We also plotted individual tumor growth in each group (Figure 3.2.-21).

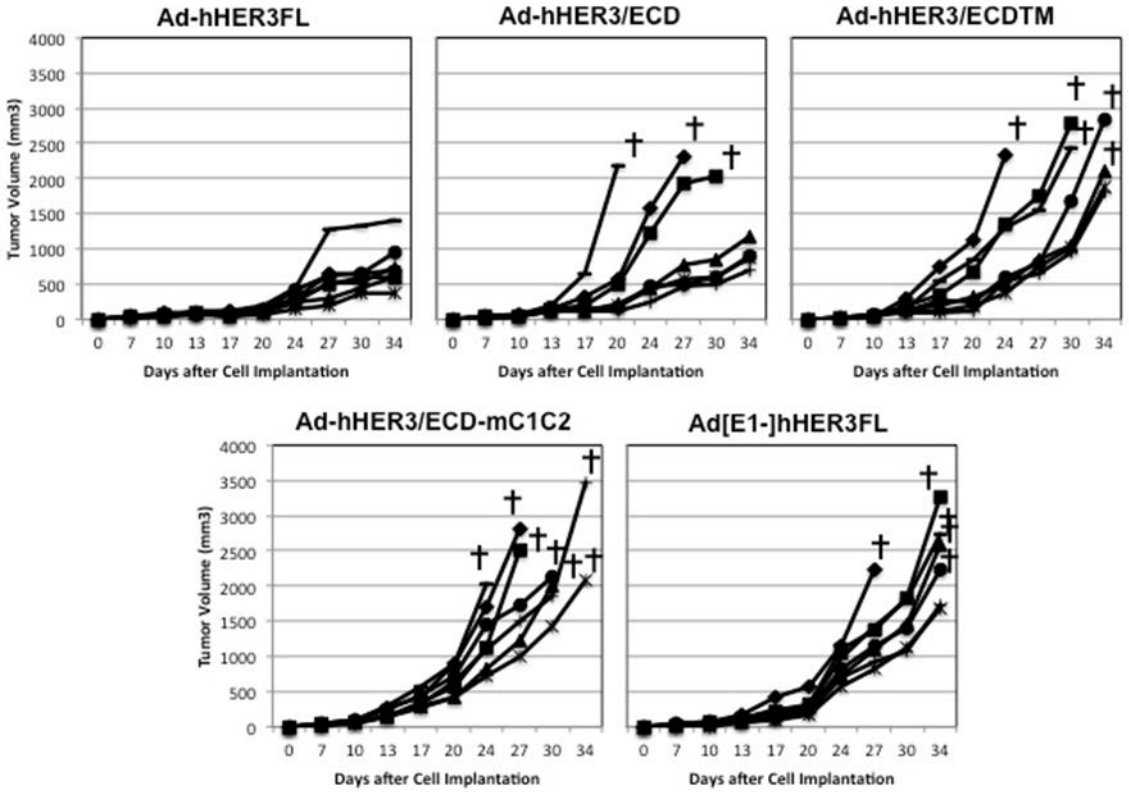


Figure 3.2.-21. Ad-HER3 vaccine effect on JC-HER3 tumor growth in BALB/c mice. Individual tumor growth. Mice were euthanized when tumor volume reached 2000 mm3 or had ulceration on the tumor.

By day 34 after tumor cell injection, 3 mice in Ad5[E1-, E2b-]huHER3/ECD, 5 mice in Ad5[E1-, E2b-]huHER3 ECD-TM, all 7 mice in Ad5[E1-, E2b-]huHER3/ECD-mC1C2 group died, but no mice died in Ad-hHER3-FL vaccine group. First generation Ad-viral vector, Ad5[E1-]-huHER3-FL showed strong tumor growth inhibition at earlier days, but after day 24, tumors started growing faster, and by day 34, 5 out of 7 mice died. These results may suggest the superiority of 2nd generation Ad5[E1-, E2b-]huHER3-FL viral vector above other Ad-HER3 vectors for vaccine use.

Immunogenicity Test in HER3 Transgenic Mice:

To confirm the findings of immunogenicity test of our new Ad-HER3 vectors performed in normal BALB/c mice, we conducted the immunogenicity test of these vectors in HER3 Transgenic mice.

IFN-gamma ELISPOT assay, Cell-based ELISA, flow-based assay were performed. Vaccinations were repeated with 2 weeks interval, and mice were sacrificed for immune assays a week after the boost vaccination.

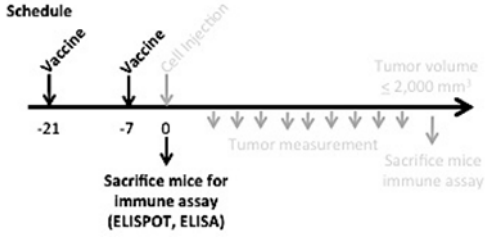


Figure 3.2.-22. Scheme of Immunogenicity Testing in HER3 Transgenic Mice. On days -18 and -4, mice were vaccinated with Ad-vectors (2.6 x 10E10 vp/mouse), and 4 mice from each group were

sacrificed for immune assays on day 0. Spleen was harvested for ELISPOT assay, and blood for test of antibody production.

Humoral Immune Response

We found established humoral immune responses in HER3 transgenic mice by flow-based assay as shown below in Figure 3.2.-23. 4T1 (HER3 negative) or 4T1-HER3 (HER3 transfectant) cells were incubated with mouse sera, which were diluted with saline (1:100 dilution), then with PE-conjugated secondary antibody (anti-mouse IgG). Sera from Ad-GFP vaccinated mice were used as negative control, commercially available anti-HER3 mAb as positive control, and mouse serum from Ad5[E1-]huHER3 vaccinated mice were used for comparison purpose.

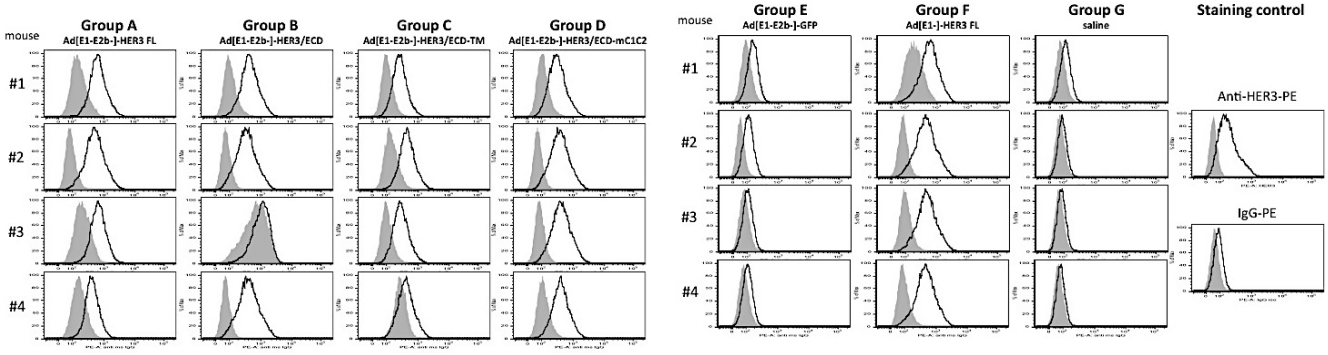


Figure 3-23. Anti-HER3 antibody levels in the serum of Ad-HER3 vaccinated HER3 transgenic mice. Mice were vaccinated with Ad-HER3-full length(FL), Ad-HER3/ECD, Ad-HER3/ECD-TM, Ad-HER3/ECD-C1C2, or control Ad-GFP, Ad[E1-]HER3 (2.6 x 1010 vp/vaccination). Two weeks later, vaccination was repeated with the same Ad vectors, and 7 days later, blood was collected to analyze the induction of anti-HER3 antibody. 4T1 cells (HER3-negative) and 4T1-HER3 cells (HER3 transfectant) were labeled with mouse sera (1:100 dilution) for 30 min, washed and then incubated with PE-conjugated 2ndary antibody (anti-mouse IgG) for 30 min. Open Histogram: 4T1-HER3 cells, Grey Histogram: 4T1 cells.

We further analyzed anti-HER3 antibody level in each serum by cell-based ELISA. 4T1 murine breast cancer cell line (HER3 negative) and human HER3 transfectant (4T1-HER3) were used in this assay. Sera from each mouse were titrated from 1:50 to 1:6400 (Figure 3.2.-24).

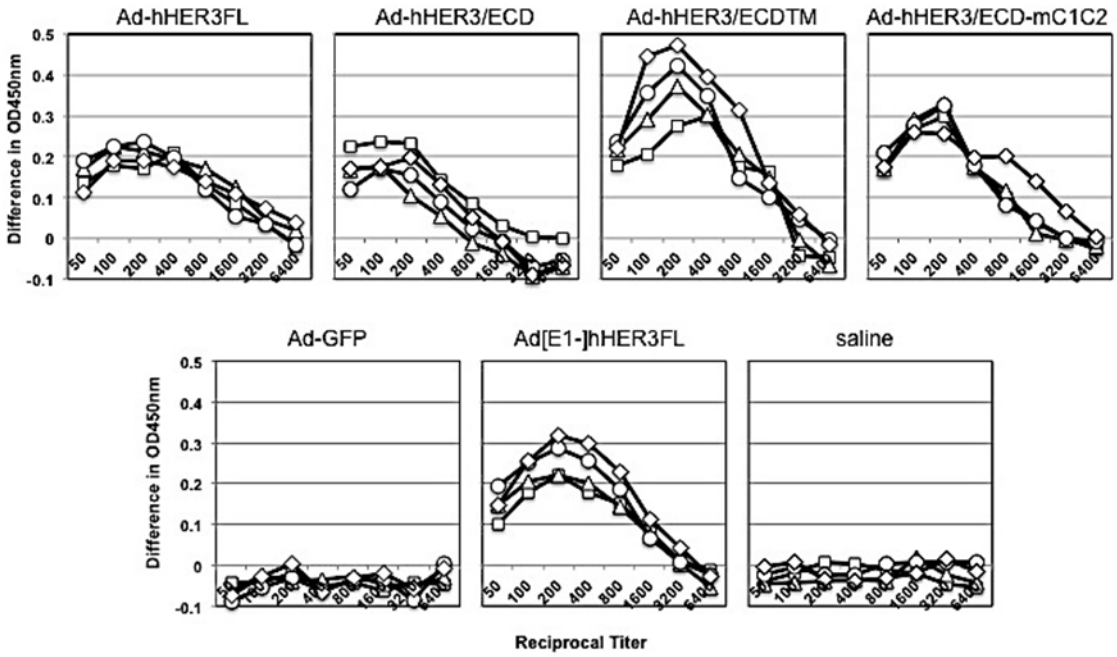


Figure 3.2.-24. Cell-based ELISA with mouse serum from HER3 Transgenic Mice. HER3 Transgenic mice were vaccinated twice with Ad5[E1-, E2b-]huHER3 vectors, Ad-GFP control or saline. Seven days after the last vaccine, mice were euthanized and serum was collected. 4T1 and 4T1-HER3 cells were seeded into 96 well plates. After overnight incubation, cells were washed with buffer, and mouse serum with serial dilutions were added (1:50 to 1:6400) and incubated for 1 h on ice. Then, cells were fixed, and HRP-labeled Goat anti-mouse IgG (1:2000) was added. After 1 h incubation, washed with PBS 3 times, and TMB was added for 5 min. Color development was stopped by adding H2SO4. Differences of OD450 values (value for 4T1-HER3) – (value for 4T1) are shown for individual mice.

Among the four Ad5[E1-, E2b-]huHER3 vectors, Ad5[E1-, E2b-]huHER3/ECD-TM induced the strongest humoral immune response against HER3, followed by Ad5[E1-, E2b-]huHER3/ECD-C1C2, Ad5[E1-, E2b-]huHER3-FL, and Ad5[E1-, E2b-]huHER3/ECD.

Antigen-specific Cellular Response

We also analysed antigen-specific cellular immune response in HER3 Transgenic mice by IFN-gamma ELISPOT assay (Figure 13). As expected, only Adenoviral vectors encoding full length HER3 (Ad5[E1-, E2b-]huHER3 FL and Ad[E1-]huHER3 FL) induced T cell response for the intracellular domain of HER3 antigen. T cell responses against the peptide mix of HER3 extracellular domain were variable. Ad5[E1-, E2b-]huHER3-FL, Ad5[E1-, E2b-]huHER3/ECD-TM, and Ad5[E1-, E2b-]huHER3/ECD-TM induced similar levels of strong cellular response against extracellular domain of HER3. Ad5[E1-]huHER3 virus encoding full length, however, induced only weak cellular response against extracellular domain, probably because of neutralization by anti-Ad antibody induced by the priming vaccine.

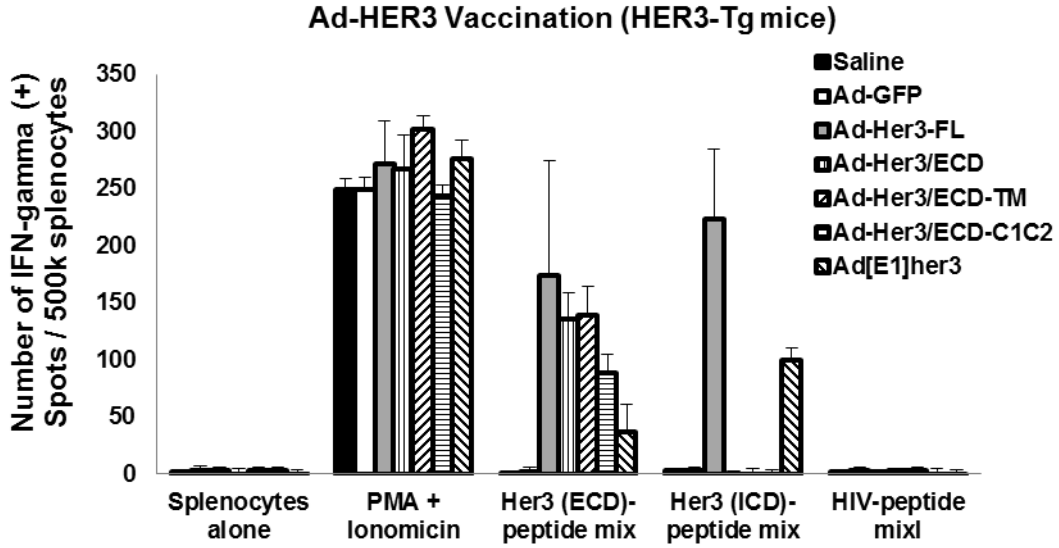


Figure 3.2.-25. Anti-HER3 cellular response induced by Ad-HER3 vaccination in HER3 Transgenic Mice. HER3 Transgenic mice were vaccinated with Ad5[E1-, E2b-]huHER3-full length(FL), Ad5[E1-, E2b-]huHER3/ECD, Ad5[E1-, E2b-]huHER3/ECD-TM, Ad5[E1-, E2b-]huHER3/ECD-C1C2, or control Ad-GFP, Ad5[E1-]HER3 (2.6 x 1010 vp/vaccination). Two weeks later, vaccination was repeated with the same Ad vectors, and 7 days later, spleen was collected to assess anti-HER3 cellular response. ELISPOT plates were coated with anti-IFNg mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. Average of 4 mice from each group are shown.

Antitumor Response

To assess the vaccine efficacy of the 4 Ad5[E1-, E2b-]huHER3 vectors, we used HER3 transgenic mice crossed with Balb/c mice (F1 generation) for tumorigenicity testing of the JC-hHER3 cell line. We initially confirmed tumorigenicity of JC-hHER3 cells (BALB/c background) in F1 Hybrid mice before conducting a tumor treatment experiment. Six weeks old female F1 Hybrid (BALB/c x MMTV-neu/MMTV-hHER3) mice were injected with 3, 1, 0.3 or 0.15 million JC-hHER3 cells in to the flank. As a control, BALB/c mice and MMTV-neu/MMTV-hHER3 Tg mice were also injected with JC-hHER3 cells (1 or 0.3 million cells/mouse). Tumor size was measured twice a week until some mice reached humane endpoint.

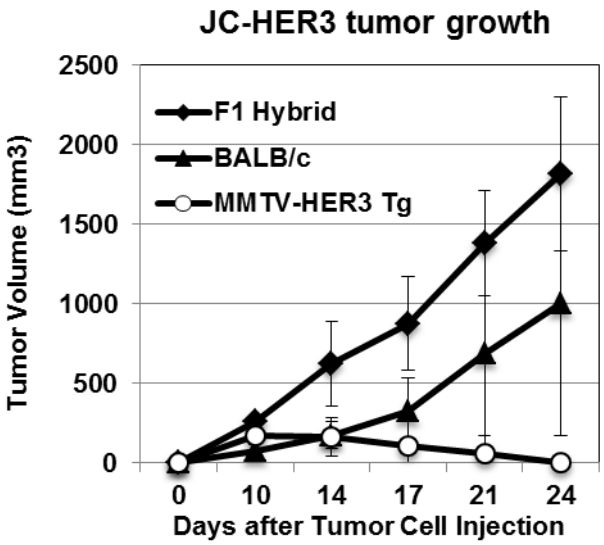


Figure 3.2.-26. Tumor growth after JC-hHER3 cell injection in F1 Hybrid mice. JC-hHER3 cells (1x10E6 cells/mouse) were resuspended in 50% Matrigel/50% saline and injected to the flank of F1 Hybrid mice, BALB/c mice or HER3 Transgenic mice (2 or 3 mice for each strain). Average tumor sizes for each mouse strain are shown. Error Bar: SD.

JC-hHER3 tumors grew in all F1 Hybrid mice injected with different number of cells. With 1x10E6 cell injection, 2 mice out of 3 reached humane endpoint by day 24 after cell implantation. With 0.15x10E6 cell injection, tumor volume was about 1,000 mm3. However, as expected, JC-hHER3 cells (derived from BALB/c mouse strain) were rejected by MMTV-hHER3 Transgenic mice (FVB background) by 24 days after cell implantation (Figure 3.2.-26). JC-hHER3 tumors grew also in BALB/c mice, while the growth speed was a slightly slower than in F1 Hybrid mice, suggesting the possibility of immune response against human HER3 antigen and resultant delay in tumor growth.

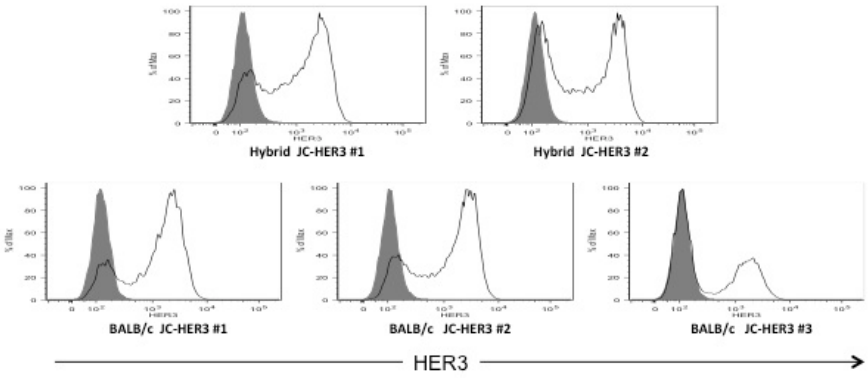


Figure 3.2.-27. HER3 expression by JC-hHER3 tumors grown in F1 Hybrid mice and BALB/c mice. JC-hHER3 tumors grown in BALB/c mice and F1 Hybrid mice were harvested, digested with triple enzyme buffer (collagenase type III, hyarulonidase, DNase) for 1 h, and then incubated for 4 days for recovery. Tumor cells were stained with PE-conjugated anti-hHER3 antibody (open histograms) or PE-conjugated isotype control (black histograms).

As shown in Figure 3.2.-27, JC-hHER3 tumor cells grown in Hybrid mice maintained HER3 expression, but some tumors grown in BALB/c mice had decreased HER3 expression level. Based on these results, JC-hHER3 cells will maintain HER3 expression when implanted in F1 Hybrid mice, suggesting that they will work as good target tumors in the treatment experiment of HER3 vaccine.

To test Ad-HER3 vaccine efficacy in the new model of JC-hHER3 tumors in F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3), we conducted tumor treatment experiment in a smaller scale. We made vaccination with Ad[E1-E2b-]-hHER3 full length, Ad[E1-E2b-]-hHER3/ECD-TM, or Ad[E1-E2b-]-GFP (negative control), and assessed antitumor effect with the vaccine treatment. Each group consisted with 5 mice. As shown in table 3-2 below, 4 and 11 days after JC-hHER3 cells implantation (1 x 10^6 cells/mouse) to female F1 Hybrid mice, mice were vaccinated with Adenovirus (2.6x10^{10} vp/mouse) via footpad injection. Tumor size was measured twice a week.

Table 3-2: Treatment and Assay Schedule

Group	**Mouse #**	**Day 0** Tumor Inj/ Sample Harvest	**Day 4, 11** Vaccine 1, 2	**Day 40** or humane endpoint Assessment
A	5	1) JC-HER3 cell inoculation (**1 x 10^6** cells /mouse)	Ad[E1-E2b-]-**HER3 full length**	Tumor volume measure until Day 40 or they reach 2000 mm³ with harvested samples (tumor, spleen, serum) 1. Immunohistochemistry (hHER3 expression) 2. ELISPOT (HER3 ICD/ECD) 3. Flow assay (anti-HER3 Ab) 4. Cell-based ELISA
B	5		Ad[E1-E2b-]-**HER3/ECD-TM**	
C	5		Ad[E1-E2b-]-GFP	

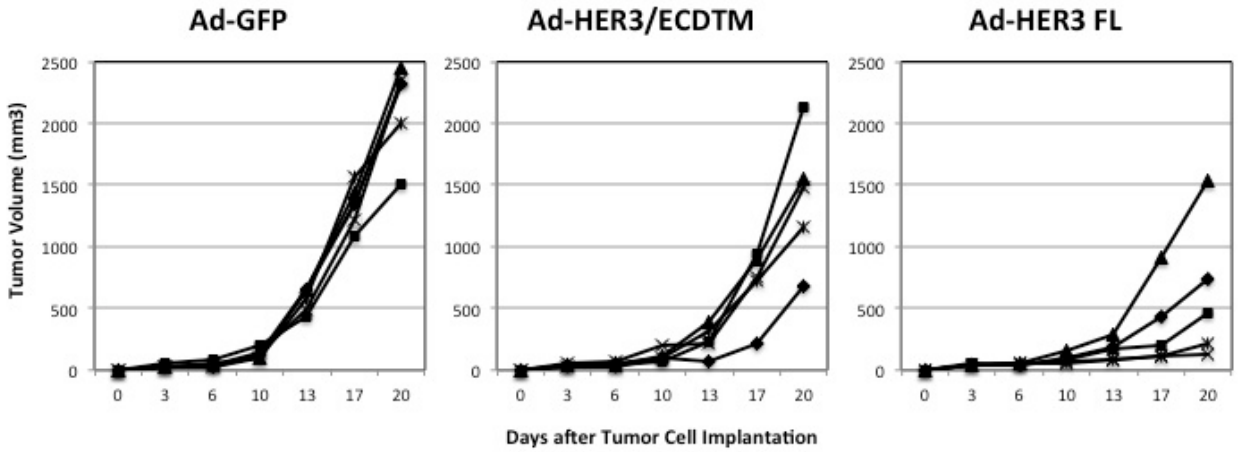


Figure 3.2.-28. JC-hHER3 tumor growth in F1 Hybrid mice treated with Ad-HER3 vaccine (individual). JC-hHER3 cells (1 x 10^6 cells/mouse) were injected to the flank of female F1 Hybrid mice on day 0, and were treated with Ad5[E1-, E2b-]huHER3 full length, Ad5[E1-, E2b-]huHER3/ECD-TM, or Ad-GFP vaccine (2.6 x 10^{10} vp/mouse) on days 4 and 11. Tumor size was

measured twice a week until humane endpoint. Individual tumor volumes are shown in each graph.

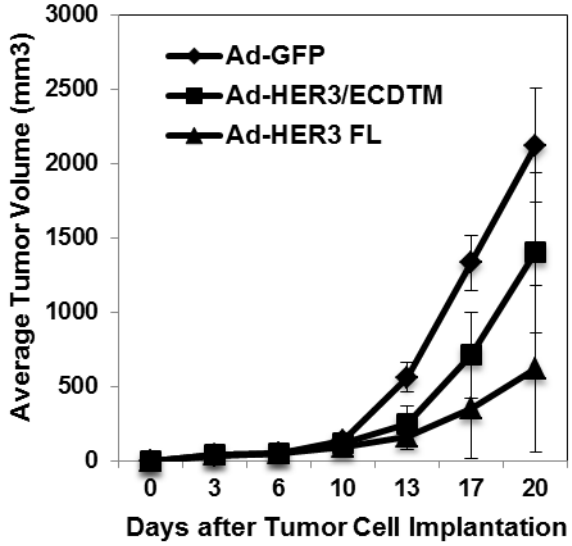


Figure 3.2.-29. JC-hHER3 tumor growth in F1 Hybrid mice treated with Ad-HER3 vaccine (Average of Groups). Average tumor volumes of each group are shown. On day 20, some mice in Ad-GFP group reached humane endpoint, and thus the experiment was terminated. Error bar: SD.

As shown in Figure 3.2.-28 and 3.2.-29, there were statistical differences between Ad-GFP vs. Ad5[E1-, E2b-]huHER3/ECD-TM (T-test; days 13 & 17: $p<0.005$, day 20: $p<0.05$) and between Ad-GFP vs. Ad-HER3 full length (T-test; day 13: $p<0.0005$, day 17: $p<0.001$, day 20: $p<0.005$). There were no statistical difference between Ad5[E1-, E2b-]huHER3/ECD-TM and Ad5[E1-, E2b-]huHER3 full length.

Using splenocytes from the mice, IFN-gamma ELISPOT assay was performed and the result is shown in Figure 3.2.-30. Because the assay was performed with splenocytes from mice implanted with HER3 expressing JC-hHER3 cells, even Ad-GFP vaccinated mice had weak anti-HER3 cellular immune response. Ad5[E1-, E2b-]huHER3/ECD-TM vaccine induced a little stronger response against HER3 ECD peptides compared to Ad-HER3 full length vaccine. On the contrary, Ad-HER3 full length induced stronger response to HER3 ICD peptides in mice. When stimulated with HER3 ECD and ICD peptide mix in the assay, both Ad-HER3 vectors showed similar number of IFN-gamma+ spots. We could confirm that anti-HER3 cellular immune response could be induced even in the treatment model with HER3 transgenic mice (F1 hybrid).

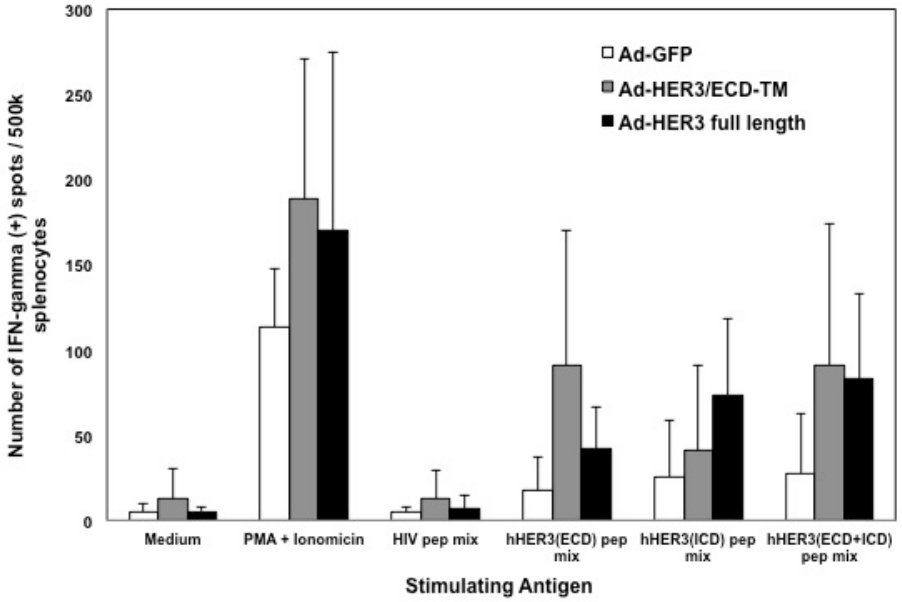


Figure 3.2.-30. Anti-HER3 cellular immune response in F1 hybrid mice treated with Ad-HER3 vaccine. Splenocyes were stimulated with HER3 ECD peptide mix and/or ICD peptide mix. HIV peptide mix was used as a negative control and PMA+Ionomycin as a positive control. Five mice for each group were analyzed and the average values are shown. Error bar: SD.

Anti-HER3 antibody production in mice treated with Ad-HER3 vaccine was analyzed by cell-based ELISA using 4T1 cells and 4T1-hHER3 cells for coating plates (Figure 3.2.-31).

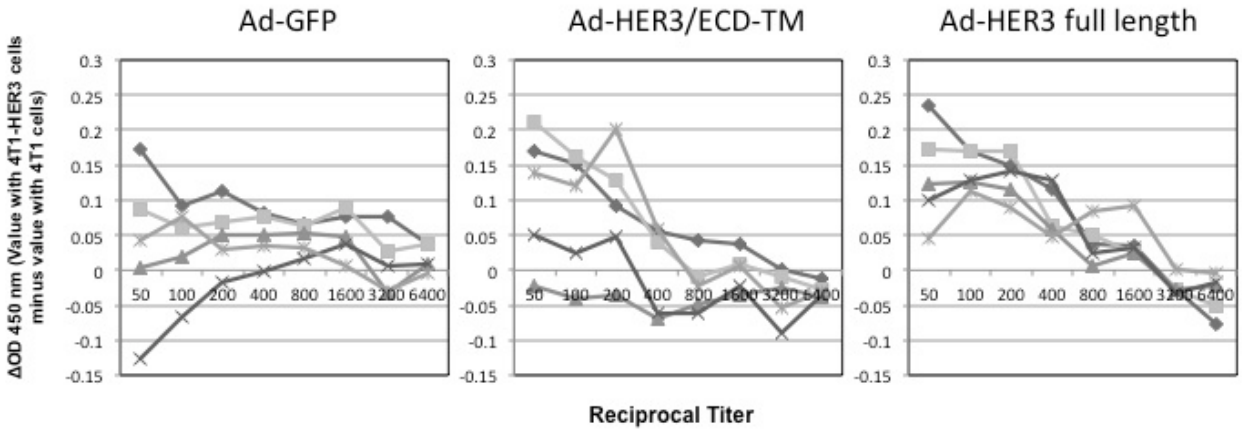


Figure 3.2.-31. Anti-HER3 antibody levels in Ad-HER3 vaccinated F1 Hybrid mice (Cell-based ELISA assay). Female F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3) were implanted with JC-hHER3 cells (1 x 10^6 cells/mouse) on day 0, and then vaccinated twice on days 4 and 11 with Ad5[E1-, E2b-]huHER3 full length, Ad5[E1-, E2b-]huHER3/ECD-TM or Ad-GFP (2.6 x 10^{10} vp/mouse). On day 20, mice were sacrificed, and serum was collected from each mouse. Serum was used for cell-based ELISA (4T1-HER3 and 4T1 cells as plating cells). Serum were titrated from 1:50 to 1:6400. HRP-conjugated goat anti-mouse IgG was used as secondary

Ab, and color was developed with TMB substrate and reaction was stopped by H_2SO_4. Individual OD 450 nm values (OD value with 4T1-HER3 cells minus OD value with 4T1 cells) are shown.

As shown in Figure 3.2.-31, both Ad5[E1-, E2b-]huHER3/ECD-TM and Ad5[E1-, E2b-]huHER3 full length vaccine induced anti-HER3 humoral response, but Ad5[E1-, E2b-]huHER3 full length vaccine induced stronger antibody production. Some mice treated with control Ad-GFP vaccine showed anti-HER3 antibody in the serum, which might be induced because of HER3 expression by implanted JC-hHER3 cells.

Based on the tumor growth data, and the data from immune assays, we could demonstrate that the system (JC-hHER3 cells in F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3)) works for the treatment experiments with Ad-HER3 vaccine. Although we could demonstrate statistically significant tumor growth suppression by Ad-HER3/ECD-TM vaccine and Ad-HER3 full length vaccine when compared to Ad-GFP vaccine, we couldn't show the significant difference between these 2 Ad-HER3 vectors. Therefore, we repeated Ad-HER3 vaccine treatment experiment with current model (JC-hHER3:F1 Hybrid mice). We compared all 4 Ad[E1-E2b-]-HER3 vectors (Ad5(E2b-)HER3 FL, Ad5(E2b-)HER3ECD, Ad5(E2b-)HER3ECDTM, and Ad5(E2b-)HER3ECDC1C2). Details are shown in Table 3.2.-3.

Table 3.2.-3. Treatment and Assay Schedule

Group	**Mouse #**	**Day 0** Tumor Injection	**Day 3, 10,17** Vaccine 1, 2, 3	**Day 40** or humane endpoint Assessment	Assessment
A	10	JC-hHER3 cell injection (**5 x 10^5** cells/mouse)	Ad[E1-E2b-]-HER3FL	Tumor volume measure until Day 40 or they reach 2000 mm^3	• ELISPOT (hHER3 pep mix) • Cell-based ELISA or Flow-assay • hHER3 expression
B	10		Ad[E1-E2b-]-HER3/ECD		
C	10		Ad[E1-E2b-]-HER3 /ECD-TM		
D	10		Ad[E1-E2b-]-HER3 /ECD-C1C2		
E	10		Ad[E1-]HER3		
F	10		saline		

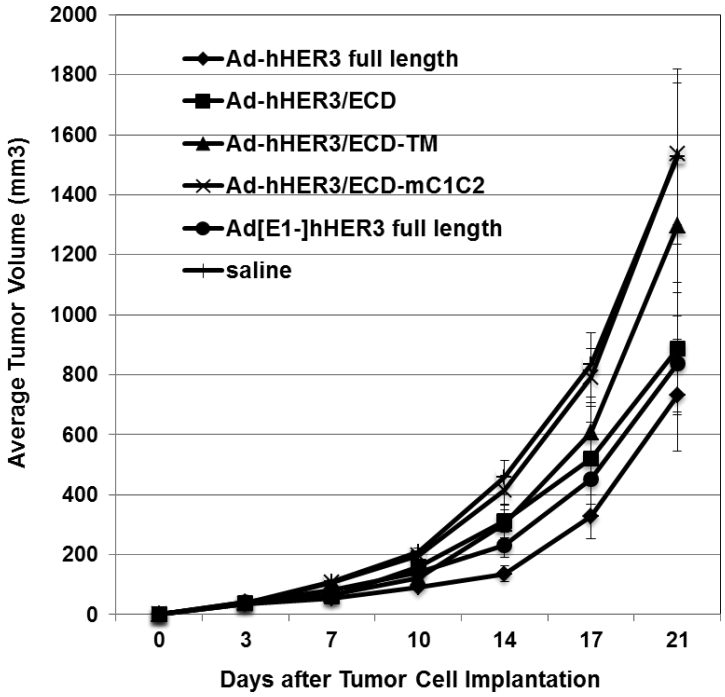


Figure 3.2.-32: JC-HER3 Tumor Growth in HER3+ F1 Hybrid mice treated with Ad-HER3 vaccines. . F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECDTM, ECD-C1C2), Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. Tumor size was measured twice a week. Individual tumor growth is shown. Error Bar: SE.

As shown in Figure 3.2.-32, Ad5[E1-E2b-]huHER3full length resulted in the best overall reduction in tumor growth in this treatment model, consistent with our previous experiment.

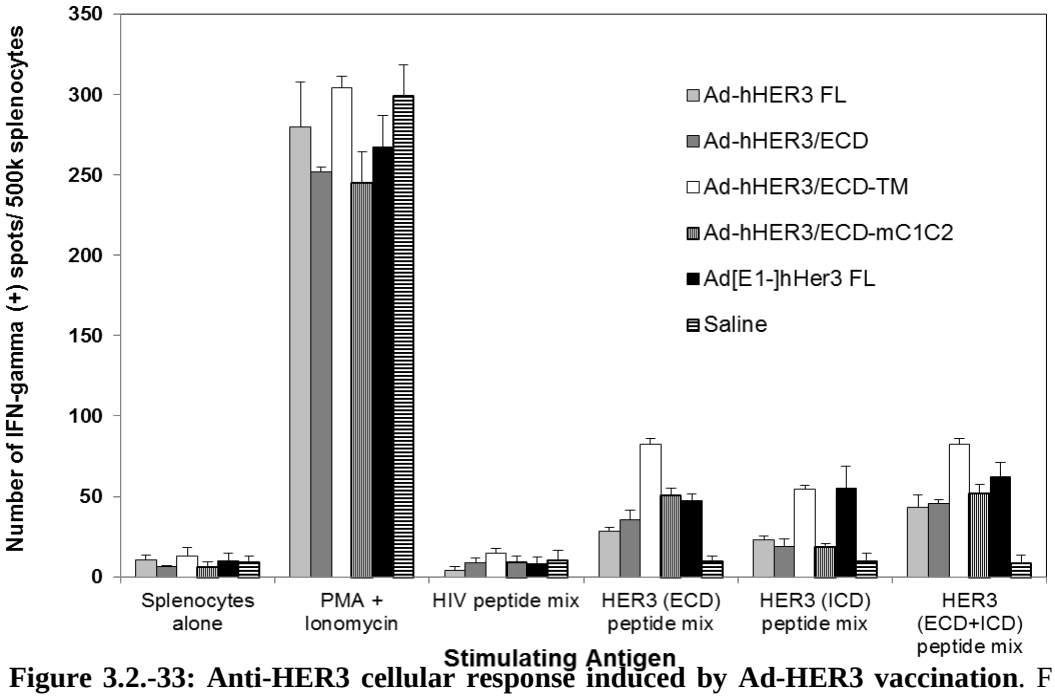


Figure 3.2.-33: Anti-HER3 cellular response induced by Ad-HER3 vaccination. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 105 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]huHER3 (full lengh, ECD, ECDTM, ECD-C1C2), Ad[E1-]huHER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. When tumor volume reached humane endpoint, mice were sacrificed. ELISPOT plates were coated with anti-IFNg mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. Average of 4 mice from each group are shown.

As shown in Figures 3.2.-33 and 3.2.-34, all 4 of the tested Ad5[E1-, E2b-]huHER3 resulted in T cell responses by IFNg ELISpot and antibody responses by HER3 cell based ELISA. The cell based ELISA indicates the Ad-HER3 induced antibodies can recognize and bind HER3 conformation expressed on the cell surface.

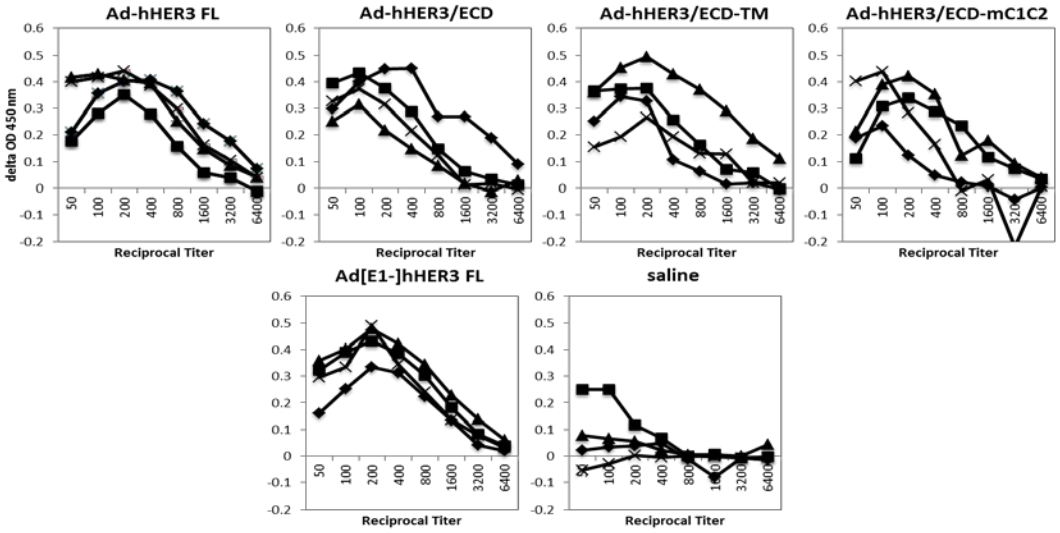


Figure 3.2.-34: Anti-HER3 antibody levels in Ad-HER3 vaccinated F1 Hybrid mice (Cell-based ELISA assay). Female F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3) were implanted with JC-hHER3 cells (5 x 10^5 cells/mouse) on day 0, and then vaccinated twice on days 3 and 10 with Ad5[E1-, E2b-]huHER3 (full length, ECD, ECD-TM, ECD-C1C2) or Ad5[E1-]huHER3 full length (2.6 x 10^{10} vp/mouse). Once the tumor volume reached humane endpoint, mice were sacrificed, and blood was collected from each mouse. Serum was used for cell-based ELISA (4T1-HER3 and 4T1 cells as plating cells). HRP-conjugated goat anti-mouse IgG was used as secondary Ab, and color was developed with TMB substrate and reaction was stopped by H_2SO_4. Individual OD 450 nm values (OD value with 4T1-HER3 cells minus OD value with 4T1 cells) are shown.

We additionally looked at HER3 expression on tumors in the mice following Ad-huHER3 vaccination (Figure 3.2.-35). In mice vaccinated with Ad-huHER3, HER3 expression was decrease in tumors compared to saline control showing the anti-HER3 response induced by the Ad-huHER3 vectors not only reduces tumor growth but also reduces expression of HER3 on the tumors.

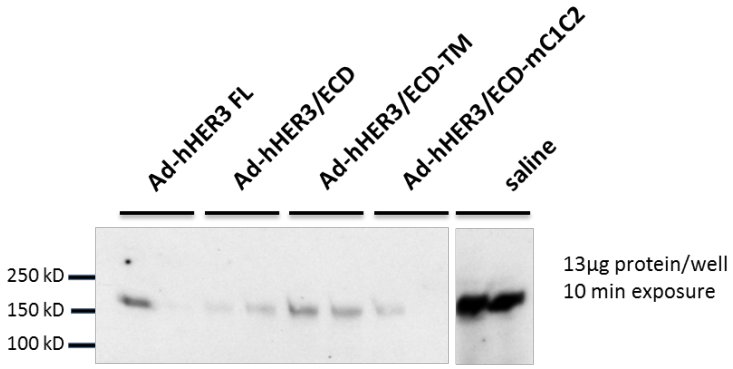


Figure 3.2.-35: HER3 Expression by JC-HER3 Tumors treated with Ad-HER3 Vaccines. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad5[E1-E2b-]huHER3 (full length, ECD, ECD-TM, ECD-mC1C2), Ad5[E1-]huHER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. When tumor volume reached humane endpoint, mice were sacrificed. Western blot was performed with anti-hHER3 antibody, followed by biotin-conjugated anti-mouse IgG and streptavidin-HRP.

Figure 3.2.-36 shows the survival curves from the 2 combined JC-HER3 treatment experiments comparing Ad-huHER3 Full length and saline control. We find a significant increase in survival time with Ad5[E1-E2b-]huHER3 full length.

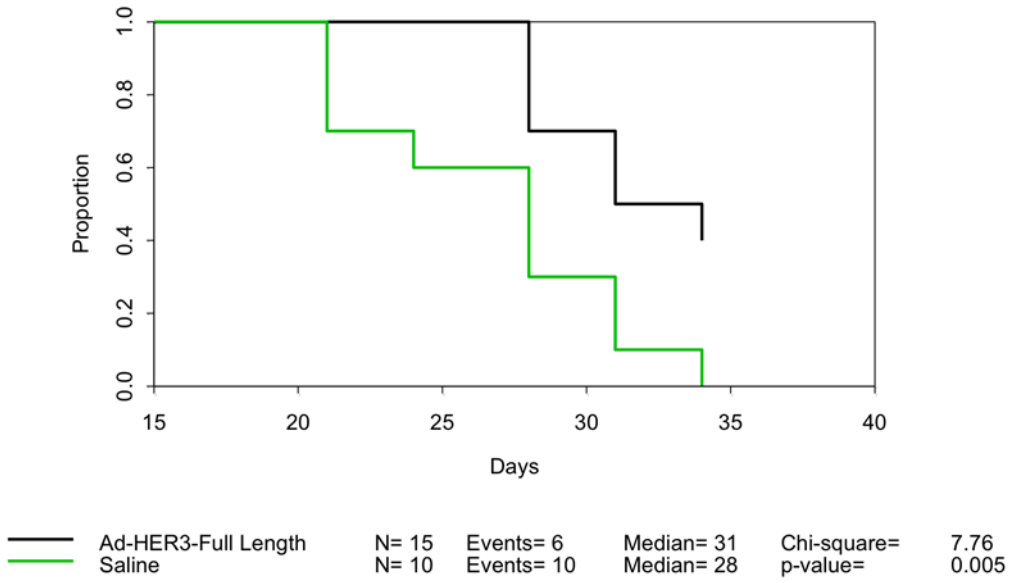


Figure 3.2.-36: Survival of JC-HER3 Tumor-bearing F1 Hybrid Mice treated with Ad-HER3 Vaccine. HER3 Transgenic F1 Hybrid female mice (BALB/c x MMTV-neu/MMTV-hHER3) were injected with JC-hHER3 cells (5 x 10^5 cells/mouse) on day 0, and treated with Ad5[E1-E2b-]huHER3 (full length, 2.6x10E10 vp/injection), or saline on days 3 and 10. Tumor size was measured twice a week. Mice were considered dead at the time the tumor volume reached humane endpoint. Survival curve for each group was made from survival data of two independent experiments with identical treatment schedule. The Kaplan-Meier method was used to estimate overall survival and groups were compared using a two-sided log-rank test.

Summary Rationale for choice of Ad [E1-, E2b-]-huHER3 full length vector

1. Second generation Ad5 [E1-, E2b-]—vectors induce more potent immune responses despite neutralizing antibodies than first generation Ad[E1-] vectors
2. Ad5 [E1-, E2b-]-huHER3 full length resulted in longer survival and greater tumor growth control than truncated versions such as Ad5 [E1-, E2b-]-huHER3 ECDTM or Ad5 [E1-, E2b-]-huHER3 ECD

3. Full length HER3 alone is not oncogenic (22, 23).

Based on this preclinical data demonstrating the greatest antitumor activity in a HER3 transgenic mouse model was achieved with the Ad5[E1-E2b-]huHER3 full length, we selected Ad5[E1-E2b-]huHER3 expressing the full length HER3 transgene for the vaccine to use in our proposed Phase I clinical trial “A Phase I Study of Active Immunotherapy with Ad [E1-, E2b-]huHER3 Vaccine in Patients with Advanced or Metastatic Malignancies Expressing HER3”.

4. SYNOPSIS OF PROPOSED CLINICAL STUDIES

See Appendix 1 for clinical protocol and draft informed consent.

5. CHEMISTRY, MANUFACTURING AND CONTROL INFORMATION

The manufacturing process used to produce the Ad5 [E1-, E2b-]huHER3 drug product will be similar to the process we have used to produce the ETBX-011 (Ad5 [E1-, E2b-]-CEA) drug product that has been evaluated in a Phase 1/2 clinical trials under IND#14325. One change will be a tangential flow diafiltration (TFF) process to concentrate the manufacturing E.C7 cells prior to viral harvest which replaces centrifugation isolation to allow for scale-up. In the manufacturing process the product peak from the second anion exchange column is diafiltered using a Hollow Fiber Filter Cartridge to perform a buffer exchange and to concentrate the formulated bulk viral particles. An additional TFF process to further concentrate the Ad5 [E1-, E2b-]huHER3 product may be needed to achieve a higher bulk particle concentration. We have employed this isolation SOP in an additional manufacture of ETBX-011 and found it to be superior with respect to product recovery. The product release assays for Ad5 [E1-, E2b-]huHER3 will be the same as those performed during ETBX-011 manufacture.

6. PHARMACOLOGY AND TOXICOLOGY INFORMATION

The purpose of the toxicology study will be to assess the toxicity of Ad5 [E1-, E2b-]-huHER3 following subcutaneous injections on Days 1, 22 and 43 in mice. This study will consist of four control groups and four test article-treated groups of 10 animals each per sex. One control group and one test article treatment group each will be sacrificed at the following time points to assess toxicity: Day 3, Day 50, Day 64 and Day 85. The dose level (2×10^8 viral particles per each 25 µL injection) selected for the test article-treated animals was based on the proposed clinical dose. The vehicle control mice will be dosed with 25 µL per injection of 0.9% sterile saline.

Table 1. Dosing and Termination schedule for the toxicity study of the Ad5 [E1-, E2b-]-HER3

Group	Males	Females	Study Day (SD) of Dose Administration	Study Day Termination
Group 1: Saline Control	10	10	SD 1	SD 3
Group 2: Saline Control	10	10	SD 1, 22, 43	SD 50
Group 3: Saline Control	10	10	SD 1, 22, 43	SD 64
Group 4: Saline Control	10	10	SD 1, 22, 43	SD 85
Group 5: Ad5 [E1-, E2b-]-HER3	10	10	SD 1	SD 3
Group 6: Ad5 [E1-, E2b-]-HER3	10	10	SD 1, 22, 43	SD 50
Group 7: Ad5 [E1-, E2b-]-HER3	10	10	SD 1, 22, 43	SD 64
Group 8: Ad5 [E1-, E2b-]-HER3	10	10	SD 1, 22, 43	SD 85
Total Animals	**80**	**80**		

Animal body temperatures will be recorded on Day 1, and on each day of dosing as well as on the day of sacrifice (Day 3, 50, 64, and 85). Body weight and food consumption data for individual animals will be recorded on Day 1 and weekly thereafter as well as on dosing days and on the day of sacrifice (including Day 3 (Groups 1 and 5), Day 50 (Groups 2 and 6), Day 64 (Groups 3 and 7) and Day 85 (Groups 4 and 8). All animals will be observed twice daily for moribundity and mortality and also on dosing days (within 2 hours after completion of dosing the last animal from each group) for clinical signs of toxicity (cage side observations). In addition, a more thorough detailed hands-on examination will be performed at the time they were weighed (Day 1 and weekly thereafter, including on the day of sacrifice). Animals will be bled from the retro-orbital sinus for clinical pathology evaluation, coagulation measurements and antibody analysis and were weighed to the nearest 0.1 gram prior to sacrifice by CO_2 overdose (on Day 3, 50, 64 or 85) and will be necropsied. A comprehensive necropsy will be performed on each animal with an accompanying complete gross pathology assessment. Tissues from the brain, heart, spleen, kidneys, adrenal glands, liver, lymph nodes (mesenteric and mandibular), lungs and bronchi, bone marrow (femur and sternum), gross lesions, skin

from non-injection site and injection site will be assessed for gross and micro-pathological effects of the test article. Blood collected from experimental and control mice will be assessed for blood chemistries and hematology. The chemistry panel will include sodium, potassium, chloride, calcium, phosphorus, bicarbonate, creatine phosphokinase, lactate dehydrogenase, aspartate transaminase, alkaline transaminase, alkaline phosphatase, gamma glutamyl transferase, glucose, blood urea nitrogen, creatinine, cholesterol, triglycerides, total bilirubin, total protein, albumin, globulin and albumin/globulin ratio determination. For all unscheduled deaths, a comprehensive histopathological evaluation will be performed in an attempt to determine the cause of death. A PCR necropsy will be performed on half of the mice and these tissues will be archived for bio distribution analysis at a later date.

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Appendix 1: Study Protocol and Draft Informed Consent

Appendix 2: Key References

A PHASE I STUDY OF ACTIVE IMMUNOTHERAPY WITH Ad5 [E1-, E2b-]-huHER3 VACCINE IN PATIENTS WITH ADVANCED OR METASTATIC MALIGNANCIES EXPRESSING HER3

Principal Investigator:	Michael Morse, M.D. Duke University Medical Center
Research Monitor:	
Study Location:	Duke University Medical Center, Durham, NC FWA00009025
Agents Used in This Study:	Ad5 [E1-, E2b-]huHER3 (also referred to as Ad5 [E1-, E2b-]huHER3 full length)

IND Holder: H.Kim Lyerly, M.D.

IND#:

Names and addresses of key study personnel:

Study Chairman and Principal Investigator **Name: Michael Morse, MD** **Address:** Duke University Medical Center Box 3233, Durham, NC 27710 **Telephone:** (919) 681-3480 **Fax:** (919) 681-7970 **Email:** michael.morse@duke.edu	
Other Applicable Staff **Title:** Regulatory Consultant **Name:** Amy Hobeika, PhD **Address:** MSRB1 Research Dr Box 2606 Duke University Medical Center Durham, NC 27710-2926 **Telephone:** (919) 684-6112 **Fax:** (919) 681-7970 **Email:** amy.hobeika@duke.edu	**Biostatistician:** **Name:** Bercedis Peterson, PhD **Address:** Duke Box 3958 Hock Plaza 2424 Erwin Rd., Room 8021 Durham, NC 27705 **Telephone:** 919 416 0942 **Fax:** 919 681 8028 **E-mail:** bercedis.peterson@dm.duke.edu
Other Applicable Staff **Title:** Acting Medical Director **Name:** H, Kim Lyerly, MD **Address**: Duke Comprehensive Cancer Center**,** Duke University Medical Center, Durham, NC 27710 **Telephone:** (919) 684-5613 **Fax:** (919) 684-5653 **Email**: lyerl001@mc.duke.edu	**Pharmacist or Person Responsible for Drug Accountability** **Name:** Cheryl Morgan **Address:** Morris Pharmacy, Duke University Medical Center, Trent Drive, Durham, NC 27710 **Telephone:** (919) 668-0657 **Fax:** (919) 668-3895 **Email:** cheryl.morgan@duke.edu
Other Applicable Staff: **Title:** CEO and CSO**,** Etubics Corporation **Name:** Frank R. Jones, Ph.D. **Address**: Etubics Corporation 410 West Harrison Street Seattle, WA 981119 **Telephone:** 206-838-5110 Ext 101 **Fax:** 206-838-2978 **Email:** frj@etubics.com	**Other Consultants:** William Gwin, MD

LIST OF ABBREVIATIONS

β–HCG	β–Human chorionic gonadotropin
AE	Adverse event
AJCC	American Joint Committee on Cancer
ALT	Alanine transaminase
AST	Aspartate transaminase
BSA	Body surface area
BUN	Blood urea nitrogen
CBC	Complete blood count
CFR	Code of Federal Regulations
Cl	Chloride
CNS	Central nervous system
CO_2	Carbon dioxide
CR	Complete response
CT	Computed tomography
DLT	Dose-limiting toxicity
DMSO	Dimethylsulfoxide
DOD	Department of Defense
ECD	Extracellular domain
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal Growth Factor Receptor
FDA	Food and Drug Administration
HER2	Human Epidermal Growth Factor Receptor 2
HIPAA	Health Insurance Portability and Accountability Act
IHC	Immunohistochemistry
IRB	Institutional Review Board
IV	Intravenous
K	Potassium
LD	Longest diameter
MRI	Magnetic resonance imaging
MUGA	Multiple gated acquisition (scan)
Na	Sodium
NCI	National Cancer Institute

NCI CTCAE V3.0	National Cancer Institute Common Terminology Criteria for Adverse Events Version 3.0
ORR	Objective response rate
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PD	Progressive disease
PFS	Progression-free survival
PR	Partial response
RECIST	Response Evaluation Criteria in Solid Tumors
SAE	Serious adverse event
SD	Stable disease
TTP	Time to progression
TTR	Time to response
USA	United States of America
WBC	White blood cell

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1. PROTOCOL SUMMARY

Title	**A PHASE I/II STUDY OF ACTIVE IMMUNOTHERAPY WITH AD5 [E1-, E2b-]-HUHER3 VACCINE IN PATIENTS WITH ADVANCED OR METASTATIC MALIGNANCIES EXPRESSING HER3**
Objectives	**Objectives:** Primary: To assess the safety and tolerability of Ad5 [E1-, E2b-]-huHER3, as a single agent in patients with advanced or metastatic solid tumor malignancies including breast cancer. Secondary: 1) Evaluate HER3-specific antibody and T cell responses following vaccination; 2) In patients with ER+ and/or PR+/HER2- breast cancer refractory to anti-estrogen therapy, evaluate for markers of HER3 signaling activation.
Major Inclusion/ Exclusion Criteria	Subjects ≥18 yrs with histologically confirmed, advanced solid tumors which have progressed after standard therapy known to lengthen survival, ECOG PS 0-1, at least 3 weeks since prior chemotherapy, with normal hematologic, renal, and hepatic function. Subjects will not be treated until 3 or more weeks after any prior chemotherapy or radiation therapy, but may be receiving non-cytotoxic targeted therapy (bevacizumab, cetuximab, trastuzumab, erlotinib, or gefitinib) or anti-hormonal therapy. They must not have a history of autoimmune disease, serious intercurrent chronic or acute illness, active hepatitis, or known HIV, or be receiving steroid or immunosuppressive therapy. Pregnant women and nursing mothers are excluded.
Study Design	A standard "3+3" phase I design will be employed where dose limiting toxicity (DLT) is defined as any Grade 2, 3 or 4 immediate hypersensitivity reactions or neurological toxicity, and other Grade 3 or 4 allergic or major organ toxicity within 1 month of the first dose of vaccine. There will be up to 3 dose levels of Ad5 [E1-, E2b-]-huHER3 (starting at 2×10^{10} viral particles (vp), then escalating to 1×10^{11} vp, and then escalating to 5×10^{11} vp). The Ad5 [E1-, E2b-]-huHER3 will be administered subcutaneously in the same limb (preferably the thigh), every 3 weeks for 3 doses). Up to three patients will be enrolled initially into a dose level cohort. If 0 of 3 experience DLT, then escalation will occur to the next cohort. If 1 of 3 experience DLT, then another 3 will be enrolled at that dose level. If no additional DLT occur, then escalation will proceed to the next dose level. If 1 or more additional DLT occurs, then dose de-escalation to the next lowest level will occur. If the 5×10^{11}

	vp dose is achieved with 0/3 or 1/6 DLT, then it will be declared the MTD. Otherwise the next lowest level at which there is 0/3 or 1/6 DLT will be declared the MTD. Once the MTD is achieved, more patients will be enrolled until a total of 12 have been treated at that dose level. In this expanded cohort, if 33% or more of the treated patients have DLT, we will drop back to the next lowest dose cohort (which would be declared the new MTD) and expand to 12 total patients. In patients with accessible tumor, we will request a core or punch biopsy of tumor tissue before the immunizations and after the final immunization to be cryopreserved for analysis of HER3 signaling.
Risks/toxicities	Potential risks associated with the vaccine include anaphylaxis, fever, skin reaction, autoimmunity (colitis), and hepatic insufficiency.
Number of Patients	Planned: 18 evaluable patients (plus up to 12 replacements); may require 30 patients if DLT occur and replacements are also required.
Duration of Study	6 months after the last patient is enrolled; approximately 2 years total
Criteria for Evaluation	Toxicity will be assessed using CTC toxicity criteria. HER3-specific immune response will be measured by ELISpot and ELISA. Time to recurrence will be determined by RECIST criteria.
Statistical Analysis	We will evaluate safety in each cohort during and at least 3 weeks after the last patient in the previous cohort has received their first injection. A dosing scheme will be considered safe if <33% of patients treated at a dosage level experience DLT (e.g., 0 of 3, ≤1 of 6, or ≤3 of 12). A patient will be considered evaluable for safety if treated with at least one immunization. At the end of the trial, the proportion of patients experiencing a DLT will be calculated within dose cohort. All toxicities will be tabulated by type and grade within dose cohort.

2. STUDY OBJECTIVES

Primary: To assess the safety and tolerability of Ad5 [E1-, E2b-]huHER3 in patients with advanced or metastatic solid tumor malignancies including breast cancer.

Secondary:

1) Evaluate HER3-specific antibody and T cell responses following vaccination;

2) In patients with ER+ and/or PR+/HER2- breast cancer, refractory to anti-estrogen therapy, evaluate for markers of HER3 signaling activation.

3. BACKGROUND AND SIGNIFICANCE

3.1. HER3 in malignancy

The human epidermal growth factor receptor (HER) family including: HER1 (also known as EGFR), HER2, HER3 and HER4 (also known as ErbB2, ErbB3, and ErbB4 respectively) is an important receptor family for the development of many malignancies. HER3 is overexpressed in breast, lung, gastric, head and neck, and ovarian cancer and melanoma and its overexpression is associated with poor prognosis (1-7). Because of the negligible tyrosine kinase function of HER3, it is typically present in heterodimers with HER1 or HER2, through which downstream signaling occurs involving extracellular-signal-regulated kinase (ERK) 1/2 and AKT (8). In breast cancer, HER3 is associated with resistance to anti-HER2 therapeutics. When HER2-driven breast cancer cell lines and xenografts were treated with anti-HER2 therapeuties, there was an increase in HER3 expression and signaling (9). HER3 is also one of several important causes of endocrine resistance in breast cancer (10,11). In patients with ER+ breast cancer who were treated with tamoxifen, HER3-overexpression was associated with a shorter progression-free survival (12). In vitro, HER3 expression was induced when ER-positive breast cancer cell lines MCF-7 and T47D were treated with fulvestrant (13). Overexpression of heregulin (HRG), the ligand for HER3, is also associated with resistance to antiestrogens in vitro and in vivo (14). These data demonstrate the key role of HER3 and its ligand to therapeutic resistance.

Figure 1 (adapted from 15, 16) illustrates a model of anti-estrogen therapy resistance in which ER blockade results in HRG overexpression and activation of the HER2/HER3 heterodimer.

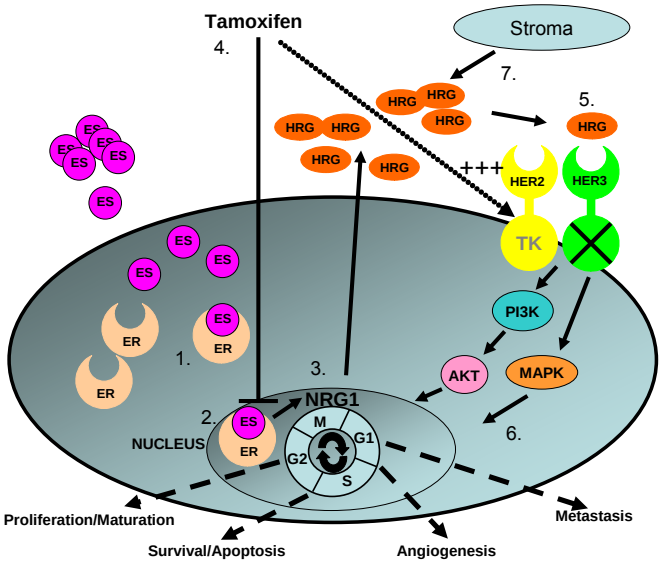


Figure 1. Our model of resistance: Estrogen-driven tumorigenesis followed by the onset of resistance to tamoxifen induced by increased hergulin (HRG) production leading to HER3 becoming the dominant driver of tumor growth. (1) Estrogen (ES) binds to the estrogen receptor (ER) on breast cells, triggering signaling via multiple pathways (2) leading to tumorigenesis and sustaining cancer growth. ER signaling also induces increased NRG1 expression (3) resulting in secretion of the HER3 ligand HRG. (4) Blockade of the ER receptor by tamoxifen can block ER signaling but also enhances HER2 signaling (+++). The increased production of HRG also leads to signaling through HER3 containing heterodimers (5) that bind HRG and lead to sustained cancer growth (6). HER3 thus becomes the dominant growth factor receptor driving tumor growth, leading to the failure of tamoxifen therapy. (7) Tumor stroma has also been implicated as an initial or contributing source of HRG. (Adapted from 15,16).

Based on these observations, it appears that targeting either HER2 or HER3 may be an effective strategy to overcome anti-endocrine therapies resistance.

Consistent with the notion, Liu et al showed that downregulation of ErbB3 by siRNA reversed HER2-driven tamoxifen resistance, and enhanced the ability of tamoxifen to inhibit growth and enhance apoptosis (17). Liu et al speculated that one possible strategy of overcoming tamoxifen resistance would be through the inhibition of ErbB3 driven activation of Akt. ErbB3-mediated resistance to tyrosine-kinase inhibitors targeting ErbB1 and ErbB2/HER2 also stems from the sustained activation of Akt, also linked to ErbB3 expression (18,19), suggesting that ErbB3 may be a broadly applicable resistance mechanism. Folgiero, et al identified an interaction between the ErbB3 receptor and the α6β4 integrin which assists in sustaining the PI3K/Akt survival pathway of breast cancer tumor cells (20). Further work demonstrates that in anti-estrogen resistant cell lines, continued signaling via Akt is important for continued cell growth (21)

Although HER3 protein alone is not oncogenic (22, 23) and expression itself has not been associated with poor outcome in breast cancer, contemporary approaches to identify the presence of an activated HER3 signaling cascade have recently suggested that the HER3 signaling was associated with a poor prognosis. For example, Spears and colleagues recently reported that detection of HER2:HER2 and HER2:HER3 dimers has prognostic significance in early breast cancer (24). In addition, we are aware that the presence of the HER3 ligand HRG could also be an indicator of an active HER3 signaling cascade. Therefore, we performed a gene expression analysis of heregulin/neuregulin HRG/NRG1 by compiling a collection of breast tumor gene expression data (n = 4010) derived from 23 data sets posted on the NCBI Gene Expression Omnibus (GEO). Our analysis of mRNA expression from these data sets revealed up-regulated mRNA expression of HRG/NRG1 was correlated with lower relapse free survival in ER+ HER2- breast cancer patients. Additionally, HRG/NRG1 mRNA was elevated in tumors from patients with both early recurrence (less than 5 years) or late recurrence (5-10 years) (25).

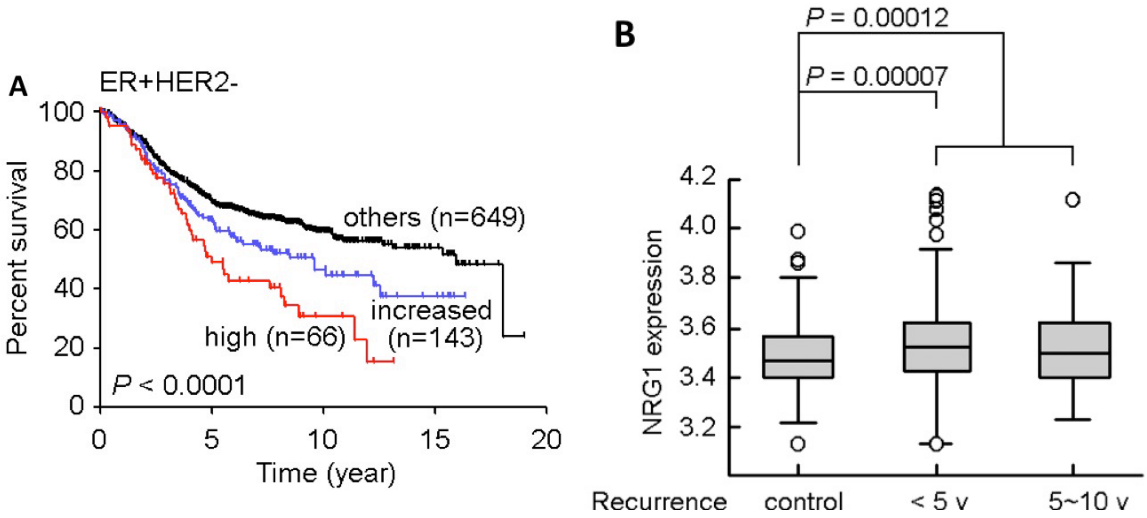


Fig. 2: Up-regulated mRNA expression of HRG/NRG1 was correlated with lower relapse free survival in ER+ HER2- breast cancer patients. B: HRG/NRG1 mRNA was elevated in tumors from patients with both early recurrence (less than 5 years) or late recurrence (from 5-10 years) after diagnosis compared to non recurring tumors.

Therefore preclinical studies, clinical data, and our own analysis suggest that the HER3 signaling axis is associated with poor outcomes in ER+, HER2- breast cancer patients. Although HER2 signaling is implicated in resistance, and HER2 inhibition using small molecules or antibodies can be achieved clinically, our overarching hypothesis is that eliciting an anti-HER3 immune response with an adenoviral (Ad) vaccine targeting full length human HER3 will have greater anti-tumor efficacy.

Rational for vaccines targeting HER3

Cancer vaccines have recently demonstrated promising activity in clinical trials for malignancies, with improvements in overall survival being reported (26-28) and the first cancer vaccine recently received FDA-approval (Provenge). Cancer vaccines may have advantages over monoclonal antibody therapeutics, including their ability to elicit both cellular and humoral immunity, to target multiple epitopes, to perturb growth factor receptor signaling, to synergize with small molecule drugs (29), and to provide long lasting effects. They are generally well tolerated and do not have overlapping toxicities with conventional therapies, making them attractive in combination with existing drugs. Targeting HER3 is especially attractive as HER3, unlike the other HER family members that have intrinsic kinase activity, lacks a kinase domain and is not readily "drugable" using small molecule approaches.

While monoclonal antibody strategies to target HER3 are in commercial development (Amgen and Sanofi-Aventis/Merrimack), we believe that a cancer vaccine that induces polyclonal antibody and T cell responses should also be explored as this approach can provide long term anti-HER3 immune responses, which could provide the long term effects needed to prevent the emergence of resistant clones. In addition to the long term protection afforded by vaccination, polyclonal immune responses to a target protein may offer additional benefits. It has been established that the binding of multiple antibodies to different epitopes is more efficient than a single monoclonal antibody in mediating receptor internalization (30-35). For example, we have recently shown that polyclonal antibodies that mediate HER2 internalization and degradation both block HER2 signaling and have dramatic anti-tumor activity (29, 36). In

addition, T cell responses induced by vaccination are also a potent mechanism of tumor rejection in numerous animal studies and the adoptive transfer of T cells in human clinical trials has shown clinical efficacy (37-41).

Although HER3 is expressed on a number of normal tissues, and is only rarely mutated in cancers, it remains an attractive immunotherapeutic target as it is not abundant on the cell surface in normal cells, tumor cells may have higher levels of membrane-bound HER3, and HER3 peptides are presented on the cell surface by MHC complexes (42) for presentation to T cells. Additionally, antibody targeting can occur as tumor cells upregulate HER3 at the cell surface after being exposed to therapeutic agents.

Finally, because HER3 is not typically expressed at high levels in normal tissues or in less heavily pretreated tumors, it is less likely that there would be self-tolerance to HER3, a major obstacle to the induction of clinically relevant levels of anti-tumor immunity. Vaccinating against antigens not normally present, but induced by resistance mechanism, such as HER3, prior to the development of resistance, may circumvent these mechanisms. For example, as we have mentioned above, we believe that the HER3 receptor may represent a relevant target in endocrine therapy-resistant breast cancer, and therefore a HER3 cancer vaccine may prevent endocrine therapy-resistance by targeting HER3-mediated resistance. We believe that there would be a similar role of a HER3 vaccine for other malignancies in which HER3 is relevant for therapeutic resistance. Furthermore, the HER3 vaccine could be used in combination with other therapies such as endocrine therapy in breast cancer to prevent the onset of therapeutic resistance mediated by HER3 overexpression.

3.2. Ad5 Vaccines

3.2.1 Rationale for utilizing Adenovirus based vectors for targeting HER3:

Adenoviruses are a family of DNA viruses characterized by an icosohedral, non-enveloped capsid containing a linear double-stranded genome. Of the human Ads, none are associated with any neoplastic disease, and only cause relatively mild, self-limiting illness in immunocompetent individuals**.** The first genes expressed by the virus are the E1 genes, which act to initiate high-level gene expression from the other Ad5 gene promoters present in the wild type genome. Viral DNA replication and assembly of progeny virions occur within the nucleus of infected cells, and the entire life cycle takes about 36 hr with an output of approximately 10^4 virions per cell. The wild type Ad5 genome is approximately 36 kb, and encodes genes that are divided into early and late viral functions, depending on whether they are expressed before or after DNA replication. The early/late delineation is nearly absolute, since it has been demonstrated that super-infection of cells previously infected with an Ad5 results in lack of late gene expression from the super-infecting virus until after it has replicated its own genome. This is due to a replication dependent *cis*-activation of the Ad5 major late promoter (MLP), preventing late gene expression (primarily the Ad5 capsid proteins) until replicated genomes are present to be encapsulated.

3.2.2 Ad5 vectors: First generation, or E1-deleted adenovirus vectors ([E1-] Ad5) are constructed in a manner such that a transgene replaces only the E1 region of genes; thus, 90% of the wild-type Ad5 genome is retained in the vector. [E1-] Ad5 vectors have a decreased ability to

replicate and cannot produce infectious virus after infection of cells not expressing the Ad5 E1 genes. The recombinant [E1-] Ad5 vectors are propagated in human cells (typically 293 cells) allowing for [E1-] Ad5 vector replication and packaging. [E1-] Ad5 vectors have a number of positive attributes; one of the most important is their relative ease for scale up and cGMP production. Currently, well over 220 human clinical trials utilize [E1-] Ad5 vectors, with more than two thousand subjects given the virus sc, im, or iv. Additionally, since Ad5 vectors do not integrate, (their genomes remain episomal) the risk for insertional mutagenesis and/or germ-line transmission is extremely low if at all. Conventional [E1-] Ad5 vectors have a large carrying capacity that approaches 7kb.

3.2.3 Ad5 [E1-] vectors used as a cancer vaccine: Arthur et.al. demonstrated that [E1-] Ad5 vectors encoding a variety of antigens could efficiently transduce 95% of *ex vivo* exposed DC's to high titers of the vector (41). Importantly, increasing levels of foreign gene expression were noted in the DC with increasing multiplicities of infection (MOI) with the vector, a finding repeated by others, as well as reproduced in our preliminary studies (44). It has been demonstrated that DC infected with [E1-] Ad5 vectors encoding a variety of antigens (including the tumor antigens MART-1, MAGE-A4, DF3/MUC1, p53, hugp100 melanoma antigen, polyoma virus middle –T antigen,) have the propensity to induce antigen specific CTL responses, have an enhanced antigen presentation capacity, and have an improved ability to initiate T-cell proliferation in mixed lymphocyte reactions (46-50). Immunization of animals with DC's previously transduced by Ad5 vectors encoding tumor specific antigens has been demonstrated to result in significant levels of protection for the animals when challenged with tumor cells expressing the respective antigen (51, 52). Interestingly, intra-tumoral injection of Ads encoding IL-7 was less effective than injection of DCs transduced with IL-7 encoding Ad5 vectors at inducing antitumor immunity, further heightening the interest in *ex vivo* transduction of DCs by Ad5 vectors (53). *Ex vivo* DC transduction strategies have also been used to attempt to induce tolerance in recipient hosts, for example, by Ad5 mediated delivery of the CTLA4Ig into DCs, blocking interactions of the DCs CD80 with the CD28 molecule present on T-cells (54).

Ad5 vector capsid interactions with DCs in and of themselves appear to trigger several beneficial responses, which may be enhancing the propensity of DCs to present antigens encoded by Ad5 vectors. For example, immature DCs, though specialized in antigen uptake, are relatively inefficient effectors of T-cell activation. DC maturation coincides with the enhanced ability of DCs to drive T-cell immunity. In some instances, Ad5 infection can result in direct induction of DC maturation (55, 56). Studies of immature bone marrow derived DCs from mice suggest that Ad vector infection of these cells resulted in upregulation of cell surface markers normally associated with DC maturation (MHC I and II, CD40, CD80, CD86, and ICAM-1) as well as down-regulation of CD11c, an integrin known to be down regulated upon myeloid DC maturation. In some instances, Ad vector infection triggers IL-12 production by DCs, a marker of DC maturation (56). These events may possibly be due to Ad5 triggered activation of NF-kB pathways (55-57). Similar studies in mature CD83+ human DC (derived from peripheral blood monocytes) demonstrated that mature DCs were efficiently transduced by Ad vectors, and did not lose their functional potential to stimulate the proliferation of naive T-cells at lower MOI. However, some studies also suggested that mature DCs were less infectable than immature ones (58, 59). Modification of capsid proteins have also been used as a strategy to optimize infection of DC by Ad vectors, as well as enhancing functional maturation, for example using the CD40L

receptor as a viral vector receptor, rather than using the normal CAR receptor infection mechanisms (60).

Ad5 vectors offer a unique opportunity to allow for high level and efficient transduction of TAA. Unfortunately, one of the major problems facing Ad5 based vectors is the high propensity of pre-existing immunity to Ads in the human population, and how this may preclude the use of conventional, E1 deleted (first generation Ads) in most human populations, for any additional vaccine application.

3.3. Ad5 [E1-, E2B-]-huHER3 Vaccine

3.3.1 The Use of Ad5 [E1-, E2b-] Vaccines to Overcome the problem of Pre-existing Anti-Ad5 Immunity:

Studies in humans and animals have demonstrated that pre-existing immunity against Ad5 can be an inhibitory factor to commercial use of Ad-based vaccines (61, 62). The preponderance of humans have antibody against Ad5, the most widely used subtype for human vaccines, with two-thirds of humans studied having lympho-proliferative responses against Ad5 (63). This pre-existing immunity can inhibit immunization or re-immunization using typical Ad5 vaccines and may preclude the immunization of a vaccinee against a second antigen, using an Ad5 vector, at a later time. Overcoming the problem of pre-existing anti-vector immunity has been a subject of intense investigation. Investigations using alternative human (non-Ad5 based) Ad5 subtypes or even non-human forms of Ad5 have been examined. Even if these approaches succeed in an initial immunization, subsequent vaccinations may be problematic due to immune responses to the novel Ad5 subtype. To avoid the Ad5 immunization barrier, and improve upon the limited efficacy of [E1-] Ad5 vectors to induce optimal immune responses, we have constructed a next generation Ad vector based vaccine platform. The E2b-deleted Ad5 vectors (E1- and E2b-) have additional deletions in the E2b region, removing the DNA polymerase and the preterminal protein genes. They have an expanded cloning capacity that is sufficient to allow inclusion of many possible genes (64). E2b-deleted vectors have up to a 12 kb gene-carrying capacity as compared to the 7 kb capacity of [E1-] Ad5 vectors, providing space for multiple genes if needed. Deletion of the E2b region confers advantageous immune properties on our novel Ad vectors, eliciting potent immune responses to specific, non-viral antigens while minimizing the immune responses to Ad viral proteins.

Most importantly, [E1-, E2b-] Ad5 vectors induce a potent CMI, as well as antibodies against the vector expressed vaccine antigens even in the presence of Ad immunity (64). [E1-, E2b-] Ad5 vectors also have reduced adverse reactions as compared to [E1-] Ad vectors, in particular the appearance of hepatotoxicity and tissue damage (65-68). A key aspect of these Ad5 vectors is that expression of Ad late genes is greatly reduced (65, 69, 70). For example, production of the capsid fiber proteins could be detected *in vivo* for [E1-] Ad5 vectors, while fiber expression was ablated from [E1-, E2b-] Ad5 vector vaccines (67, 68, 71). The innate immune response to wild type Ad is complex and it appears that proteins deleted from [E1-] and E2b-deleted Ad5 vectors play an important role (72-75). Specifically, [E1-, E2b-] Ad5 vectors with deletions of preterminal protein or DNA polymerase display reduced inflammation during the first 24 to 72 hours following injection compared to [E1-] Ad5 vectors (74, 75). These data

suggest that the lack of Ad5 gene expression renders infected cells invisible to anti-Ad activity and permits infected cells to produce the transgene for extended periods of time.

We hypothesize that the reduced inflammatory response against [E1-, E2b-] Ad5 vector viral proteins and the resulting evasion of pre-existing Ad immunity increases the capability for the [E1-, E2b-] Ad5 vectors to transduce dendritic cells, improving antigen specific immune responses in the vaccinee. [E1-, E2b-] Ad5 vectors not only are safer than, but appear to be superior to [E1-] Ad5 vectors in regard to induction of antigen specific immune responses, making them a platform to develop HER3 vaccines in a rapid and efficient manner.

3.4. Ad5[E1-, E2b-]-huHER3 Preliminary data

We initially tested a recombinant first generation adenoviral (Ad) vaccine encoding full length human HER3 (termed Ad-HER3 below but also referred to as Ad5 [E1-] huHER3 full length elsewhere). We first demonstrated that Ad-HER3 was immunogenic in wild type BALB/c mice and elicited T cell and antibody responses (**Figure 3**).

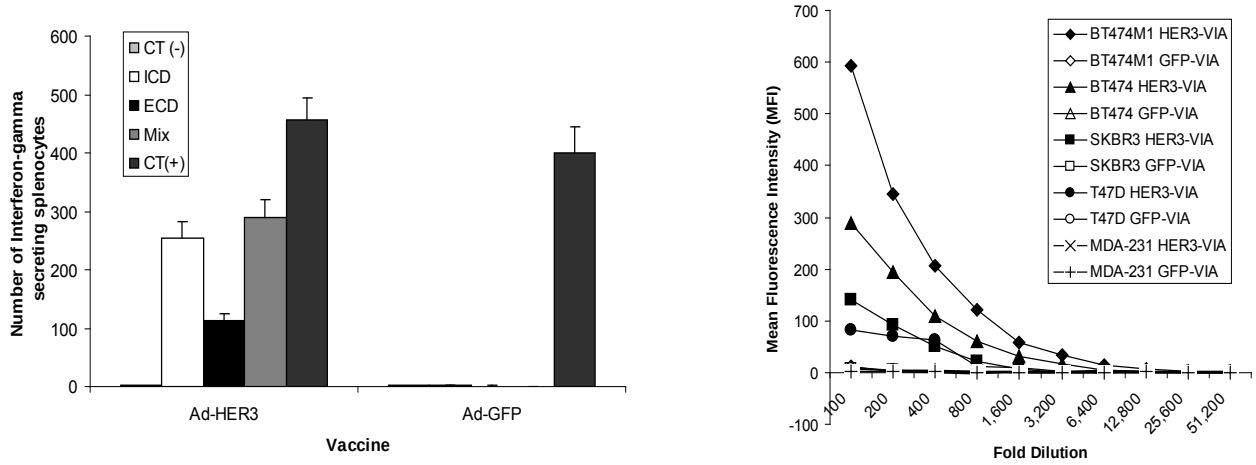


Figure 3. Immunogenicity of Ad-HER3 vaccine. BALB/c mice (N=5) received two bi-weekly vaccinations with either Ad-HER3 or Ad-GFP as a control. Mice were euthanized two weeks after the second vaccinations and splenocytes and peripheral blood serum were isolated. **Left:** IFN-g ELISPOT analysis showing response of splenocytes from Ad-HER3 vaccinated or Ad-GFP vaccinated mice against saline (CT-), HER2-intracellular domain (ICD) peptide mix, HER2-extracellular domain (ECD) peptide mix, a mixture of ICD and ECD peptide mixes, and SEB (CT+) respectively. Error bars = standard deviation. **Right:** Flow cytometric assessment of purified serum antibody binding to HER3+ (BT474M1, BT474, SKBR3, T47D) and HER3- (MDA-231) human breast tumor cell lines at various serum dilutions (x-axis) from Ad-HER3 (HER3-VIA) or Ad-GFP (GFP-VIA) vaccinated mice (methodology described on our papers: 27,42). VIA = vaccine induced antibodies.

Epitope mapping studies using overlapping peptide arrays of human HER3 have identified 18 epitopes recognized by the HER3-VIA (vaccine induced antibodies), demonstrating that the HER3-VIA is polyclonal (**Figure 4, left**). These likely represent only a fraction of the antibody epitopes present because the overlapping peptide library we screened on the peptide array blots consisted of 15 amino acid peptides and secondary structure was likely not optimal. Our experience with vaccine induced antibodies to HER2 resulting in dramatic receptor internalization (29, 36) led us to examine if these HER3-VIA could also mediate receptor

internalization. There is marked internalization of the HER3 receptor induced by HER3-VIA (**Figure 4, right**).

HER3-VIA Antibody epitopes	
Epitope(s) position	Protein region
101-111	ECD
153-167	ECD
185-191	ECD
209-223	ECD
369-375	ECD
501-511	ECD
589-599	ECD
649-663	TM
681-691	ICD
865-875	ICD
881-895	ICD
901-915	ICD
981-988	ICD
1037-1051	ICD
1105-1119	ICD
1153-1163	ICD
1249-1255	ICD
1268-1279	ICD

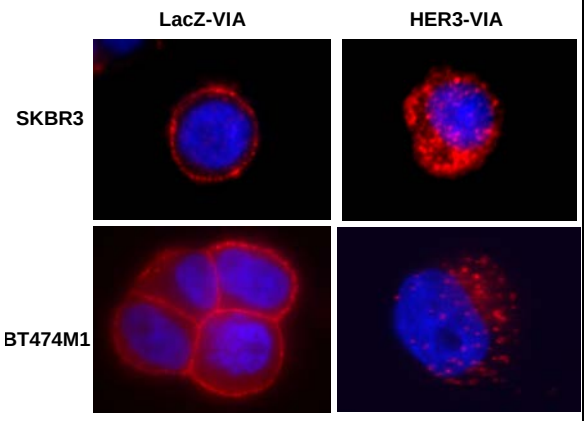


Figure 4. Ad-HER3 vaccination elicits polyclonal vaccine induced antibodies (HER3-VIA) that mediate HER3 receptor internalization. Left: Epitope mapping results. **Right:** Human HER3+ breast cancer cells (SKBR3 or BT474M1) were stained with DAPI (nuclear stain, blue) and an anti-HER3 MAb (red). Cells were then incubated with either LacZ-VIA from Ad-LacZ vaccinated mice or HER3-VIA from Ad-HER3 vaccinated mice, and then visualized 2 hrs later by fluorescence microscopy. The HER3-VIA causes rapid receptor internalization.

We believe that internalization of the receptor is an important potential mechanism of action for cancer vaccines targeting growth factor receptors as it offers the possibility for receptor degradation and thus inhibition of receptor signaling (29, 36). We assessed this and found that the HER3-VIA could inhibit proliferation of HER3+ human breast tumors *in vitro* and mediated complement dependent cytotoxicity (**Figure 5**).

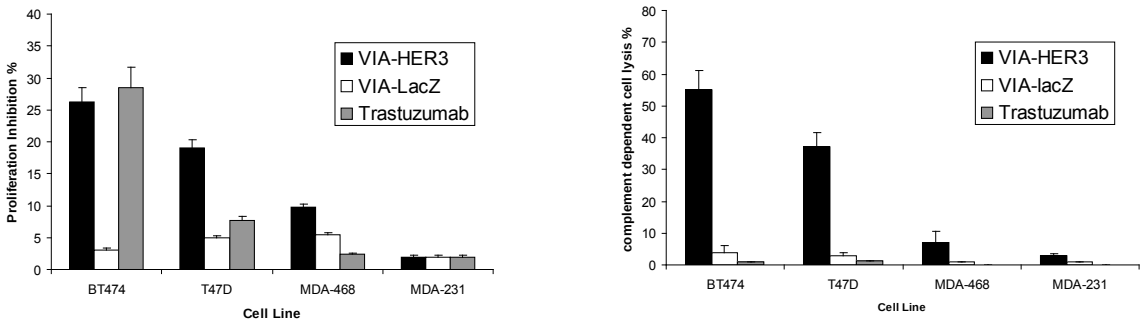


Figure 5. HER3-vaccine induced antibodies from the serum of mice vaccinated with Ad-HER3, mediate anti-proliferative effects (**left**) and complement dependent cytotoxicity (**right**) on HER3 expressing human breast cancer cell lines (BT474, T47D, MDA-468) but not HER3-non-expressing cells (MDA-231). Error bars represent standard deviation. Methodology as described (*28*).

Generation of Ad5[E1-, E2b-]-)huHER3 and Ad5[E1-, E2b-]huHER3 C1C2 constructs

Following our proof of concept studies for targeting HER3 with an adenoviral vector, we modified the adenovirus construction methods to facilitate the production of the next generation Ad5 vectors with deletion of multiple early genes (E1, E2b, E3). Previous studies demonstrated

that Ad5[E1-, E2b-] vectors are more potent immunogens compared 1st generation Ad (Ad5[E1-]) even in the presence of pre-existing anti-Ad5 immunity.

We generated next generation human HER3 (E1-, E2b-, E3-) Adenovirus vectors as follows:
1. Ad5 [E1-, E2b-]HER3 FL; express human HER3 full length.
2. Ad5 [E1-, E2b-]HER3ECDTM; express human HER3 ECD and trans-membrane domain
3. Ad5 [E1-, E2b-]HER3ECD; express human HER3 ECD
4. Ad5 [E1-, E2b-]HER3ECDC1C2; express human HER3 ECD and C1C2 domain

We established a HER3 tumor growth prevention model using JC-HER3 mouse mammary tumor cells in BALB/c mice. JC murine breast cancer cell line (BALB/c strain) was transfected with human HER3. Immunogenicity and vaccine efficacy of Ad vectors were determined in BALB/c mice by assessing preventive effect of HER3 vaccination (Figure 6).

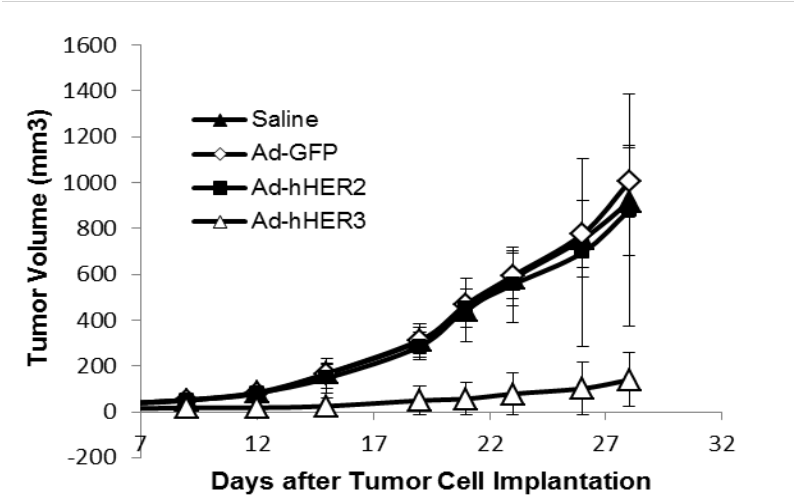


Figure 6. Ad-huHER3 vaccine inhibits JC-HER3 tumor growth. BALB/c mice were vaccinated twice (day-18, day-4) via footpad injection with Ad[E1-]GFP, Ad[E1-]huHER2 or Ad[E1-]huHER3 vectors (2.6×10^{10} particles/ mouse). Four day after boosting, at day 0, each mouse was implanted with 1,000,000 JC-HER3 mouse mammary tumor cells expressing human HER3. Tumor volume was measured every 3 days.

Only vaccination with the HER3 encoding vector prevented growth of HER3 expressing tumors.

To confirm the induction of HER3 specific immune response in Ad-HER3 vaccinated mice, we performed ELISPOT assay with splenocytes from vaccine treated mice as shown in Figure 7.

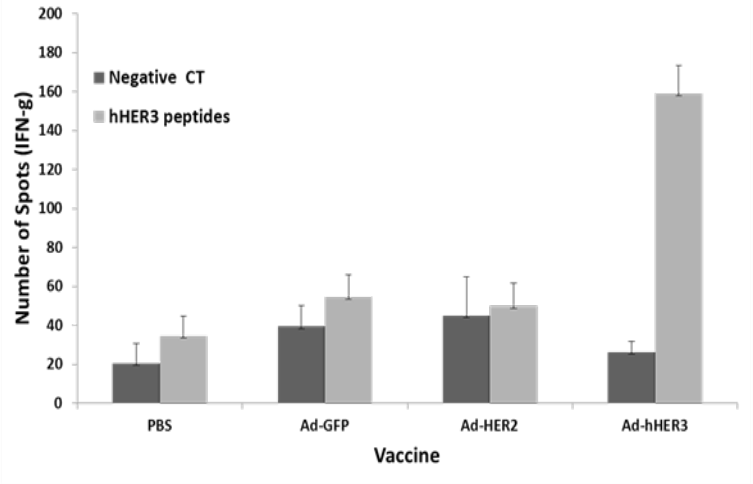


Figure 7. Ad-huHER3 vaccine induced HER3 specific T cell response. Splenocytes (500,000 cells/well) from Ad vaccinated BALB/c mice were collected on day 28 and stimulated with HER3 peptide mix (huHER3 peptides) (1µg/mL was used; JPT, Acton, MA) or HIV peptide mix (BD Bioscience) as a negative control (Negative CT) and analyzed in a interferon-gamma ELISpot assay.

We hypothesized that Ad-huHER3 induced anti-HER3 immune response will affect the HER3 expression by tumors grown in mice. Therefore, we tested the HER3 expression in tumor tissue by Western Blot assay, as shown in Figure 8.

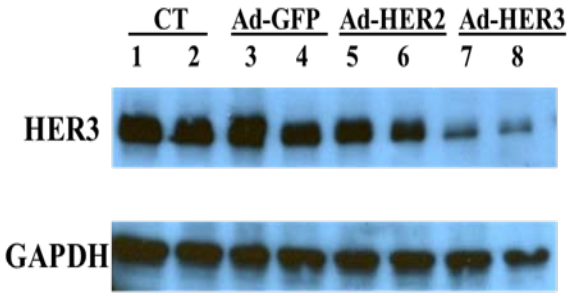


Figure 8. Ad[E1-]huHER3 vaccination causes degradation of HER3 on JC-hHER3 tumor. Tumors were isolated from vaccinated and control BALB/c mice (as indicated on figure) and immediately flash frozen. Tissue extracts were prepared by homogenization in RIPA buffer. Equal amounts of protein from each sample were used to visualize the indicated molecules by immunoblotting.

Immunization with Ad[E1-]-huHER3 led to a reduction of HER3 expression in the tumors while immunization with Ad[E1-]GFP or Ad[E1-]-huHER2 did not change HER3 expression by JC-HER3 tumors suggesting the downregulation of HER3 expression by vaccine induced anti-HER3 immune response.

Preclinical immunogenicity testing of Ad5(E1-, E2b-)huHER3 in BALB/c Mice

To compare the immunogenicity of the 4 different adenoviral vectors encoding human HER3 genes (Ad5[E1-, E2b-]huHER3 FL, Ad5[E1-, E2b-]huHER3ECD, Ad5[E1-, E2b-]huHER3ECDTM, and Ad5[E1-, E2b-]huHER3ECDC1C2) in BALB/c mice, female mice (10 mice/group) were vaccinated twice with 2 weeks interval, and human HER3 expressing murine breast cancer cell line (JC-HER3, 1 M cells/mouse) was injected to the flank of mice 4 days later.

Humoral Immune Response

We analyzed anti-HER3 antibody level in each serum by cell-based ELISA. 4T1 murine breast cancer cell line (HER3 negative) and human HER3 transfectant (4T1-HER3) were used in this assay. Sera from individual mice were titrated from 1:50 to 1:6400 (Figure 9).

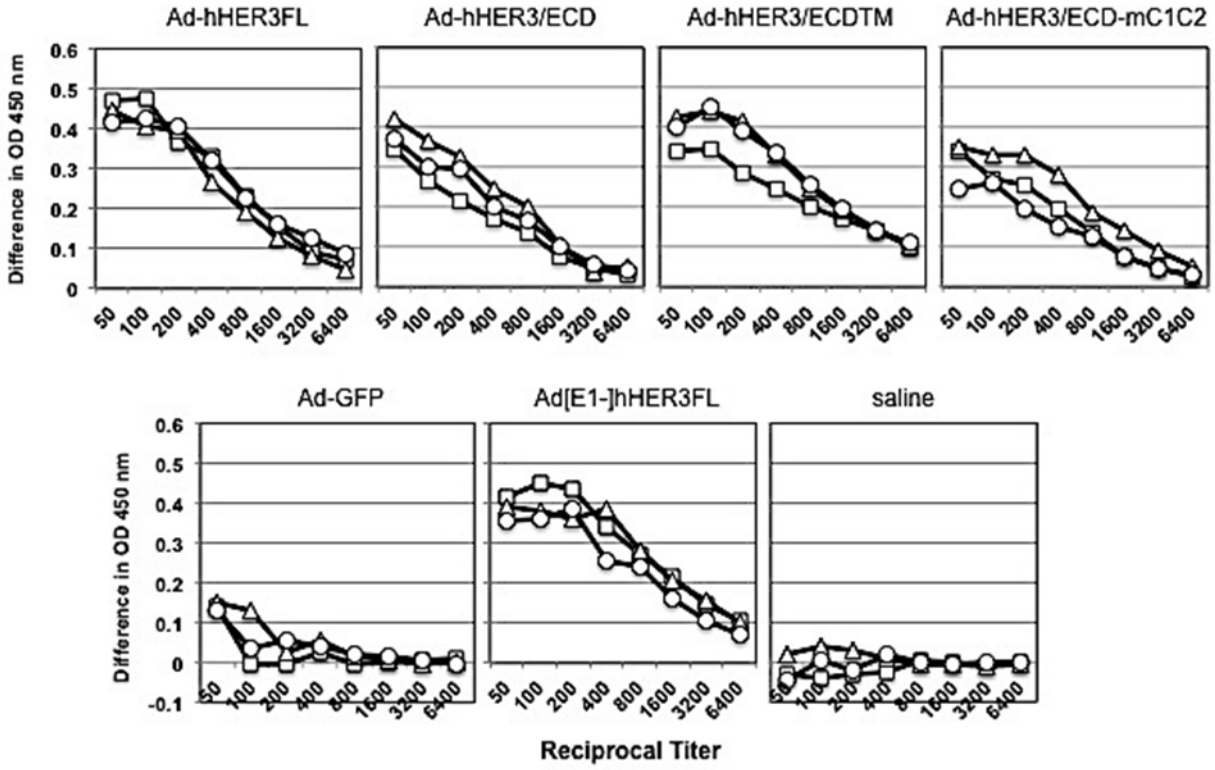


Figure 9: Cell-based ELISA with mouse serum. 4T1 and 4T1-HER3 cells were seeded into 96 well plates. After overnight incubation, cells were washed with buffer, and mouse serum with serial dilutions were added (1:50 to 1:6400) and incubated for 1 h on ice. Then, cells were fixed with 4% formaldehyde, and HRP-labeled Goat anti-mouse IgG (1:1000) was added. After 1 h incubation, washed with PBS 3 times, and TMB was added for 5 min. Color development was stopped by adding H2SO4. Differences of OD450 values (=[value for 4T1-HER3] – [value for 4T1]) are shown.

Based on cell-based ELISA, anti-HER3 antibody production in Ad-HER3 vaccinated mice were confirmed in all mice. These 4 newly made adenoviral vectors showed comparable efficacy in induction of humoral immunity, but Ad5[E1-, E2b-]huHER3FL and Ad5[E1-, E2b-]huHER3/ECDTM induced slightly higher levels of anti-HER3 antibody and Ad5[E1-, E2b-]huHER3/ECD induced slightly lower level.

Antigen-specific Cellular Response

We also analyzed antigen-specific cellular immune response by IFN-gamma ELISPOT assay with mouse splenocytes (Figure 10). Splenocytes from each mouse were incubated with HER3 peptide pool (Extracellular domain (ECD), or Intracellular Domain (ICD)), and HIV peptide mix as a negative control, PMA+ Ionomycin as a positive control. As expected, only Adenoviral vectors encoding full length HER3 (Ad5[E1-, E2b-]huHER3 FL and Ad5[E1-]huHER3 FL) induced T cell response for the intracellular domain of HER3. T cell responses against the peptide mix of HER3 extracellular domain were variable. Ad5[E1-, E2b-]-huHER3/ECD-TM induced the strongest cellular response against extracellular domain of HER3. Ad5[E1-, E2b-]huHER3 virus encoding full length, however, induced only weak cellular response against extracellular domain.

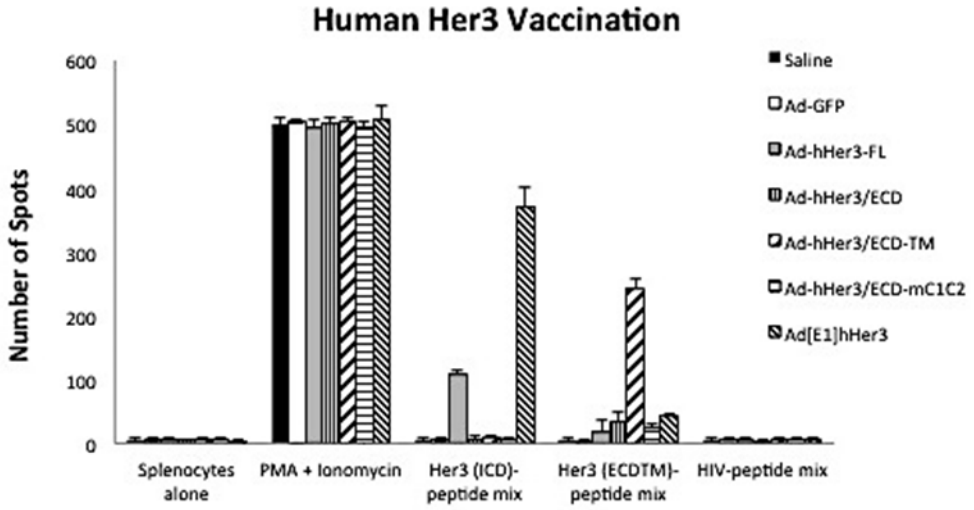


Figure 10. Anti-HER3 cellular response induced by Ad5[E1-, E2b-]huHER3 vaccination. Mice were vaccinated with Ad5[E1-, E2b-]-huHER3-full length(FL), Ad5[E1-, E2b-]huHER3/ECD, Ad5[E1-, E2b-]huHER3/ECD-TM, Ad5[E1-, E2b-]huHER3/ECD-mC1C2, or control Ad-GFP, Ad[E1-]HER3 (2.6×10^{10} vp /vaccination). Two weeks later, vaccination was repeated with the same Ad vectors, and 4 days later, spleen was collected to assess anti-HER3 cellular response. ELISPOT plates were coated with anti-IFNg mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. Average of 3 mice from each group is shown.

Antitumor Response

Tumor growth was measured twice a week until 34 days after tumor cell implantation. Once the tumor volume reached 2,000 mm3 or tumor had ulceration, mice were euthanized. Until day 20, all mice survived and the average tumor volume was calculated for each group and shown in the Figure 11.

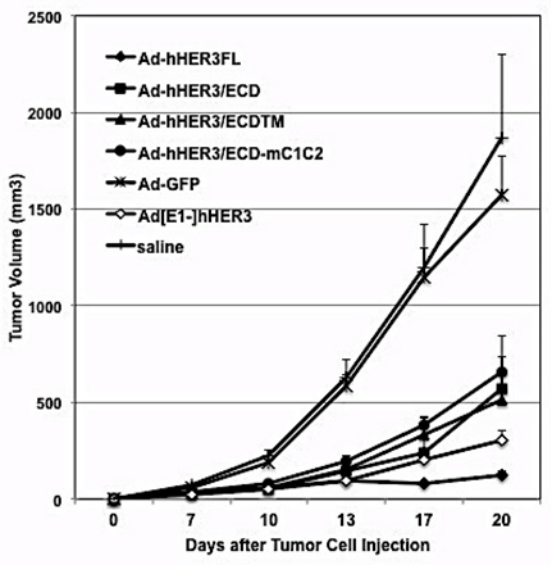


Figure 11. Ad5[E1-, E2b-]huHER3 vaccine effect on JC-HER3 tumor growth in BALB/c mice. BALB/c mice were vaccinated twice (day-18, day-4) before and once (day 14) after tumor cell implantation, with Ad[E1-E2b-]huHER3FL, Ad[E1-E2b-]huHER3/ECD, Ad[E1-E2b-]huHER3/ECDTM, Ad[E1-E2b-]huHER3/ECD-mC1C2, Ad-GFP, Ad[E1-]huHER3FL (2.6×10^{10} particles/ mouse) or saline via footpad injection. On day 0, each mouse was implanted with JC-HER3 mouse mammary tumor cells expressing human HER3 (1×10^{6} cells/mouse). Tumor volume was measured every 3 days. Error Bar: SE

All 4 Ad5[E1-, E2b-]huHER3 vectors showed antitumor activity, inhibiting the JC-HER3 tumor growth in BALB/c mice compared to control groups (saline injection). Ad5[E1-, E2b-]huHER3-FL demonstrated the strongest inhibitory effect for tumor growth after day 17.

Immunogenicity Test in HER3 Transgenic Mice:
To confirm the findings of immunogenicity test of our new Ad-HER3 vectors performed in normal BALB/c mice, we conducted the immunogenicity test of these vectors in HER3 Transgenic mice. Vaccinations were repeated with 2 weeks interval, and mice were sacrificed for immune assays a week after the boost vaccination.

Humoral Immune Response
We found established humoral immune responses in HER3 transgenic mice by cell-based ELISA. 4T1 murine breast cancer cell line (HER3 negative) and human HER3 transfectant (4T1-HER3) were used in this assay. Sera from each mouse were titrated from 1:50 to 1:6400 (Figure 12).

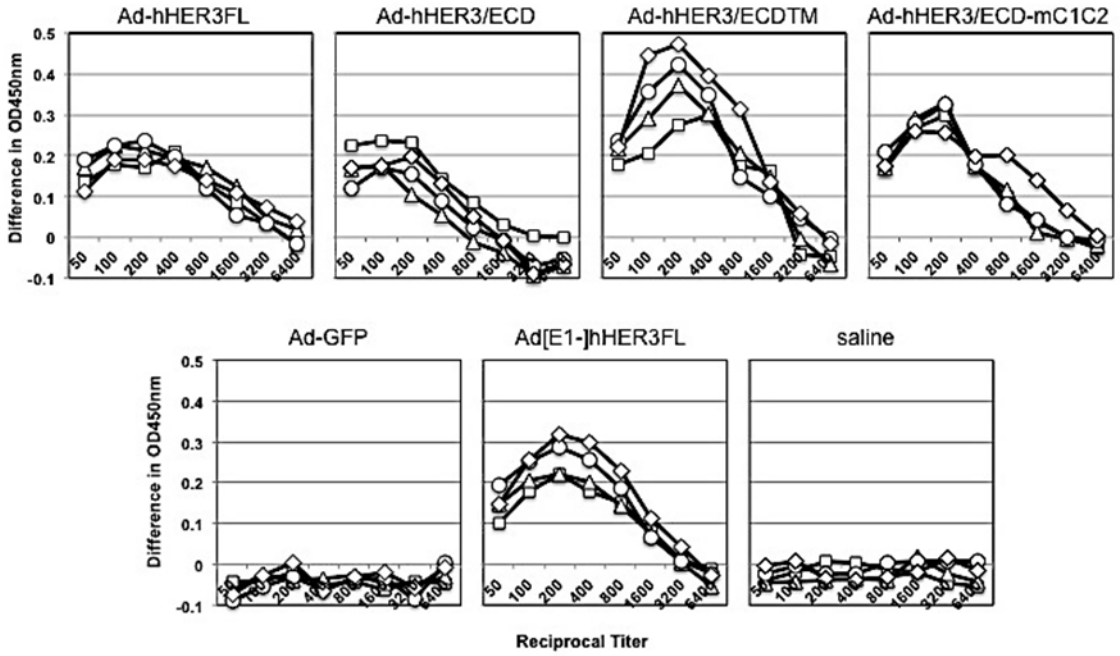


Figure 12. Cell-based ELISA with mouse serum from HER3 Transgenic Mice. HER3 Transgenic mice were vaccinated twice with Ad5[E1-, E2b-]huHER3 vectors, Ad-GFP control or saline. Seven days after the last vaccine, mice were euthanized and serum was collected. 4T1 and 4T1-HER3 cells were seeded into 96 well plates. After overnight incubation, cells were washed with buffer, and mouse serum with serial dilutions were added (1:50 to 1:6400) and incubated for 1 h on ice. Then, cells were fixed, and HRP-labeled Goat anti-mouse IgG (1:2000) was added. After 1 h incubation, washed with PBS 3 times, and TMB was added for 5 min. Color development was stopped by adding H2SO4. Differences of OD450 values (value for 4T1-HER3) – (value for 4T1) are shown for individual mice.

Among the four Ad5[E1-, E2b-]huHER3 vectors, Ad5[E1-, E2b-]huHER3/ECD-TM induced the strongest humoral immune response against HER3, followed by Ad5[E1-, E2b-]huHER3/ECD-C1C2, Ad5[E1-, E2b-]huHER3-FL, and Ad5[E1-, E2b-]huHER3/ECD.

Antigen-specific Cellular Response

We also analysed antigen-specific cellular immune response in HER3 Transgenic mice by IFN-gamma ELISPOT assay (Figure 13). As expected, only Adenoviral vectors encoding full length HER3 (Ad5[E1-, E2b-]huHER3 FL and Ad[E1-]huHER3 FL) induced T cell response for the intracellular domain of HER3 antigen. T cell responses against the peptide mix of HER3 extracellular domain were variable. Ad5[E1-, E2b-]huHER3-FL, Ad5[E1-, E2b-]huHER3/ECD-TM, and Ad5[E1-, E2b-]huHER3/ECD-TM induced similar levels of strong cellular response against extracellular domain of HER3. Ad5[E1-]huHER3 virus encoding full length, however, induced only weak cellular response against extracellular domain, probably because of neutralization by anti-Ad antibody induced by the priming vaccine.

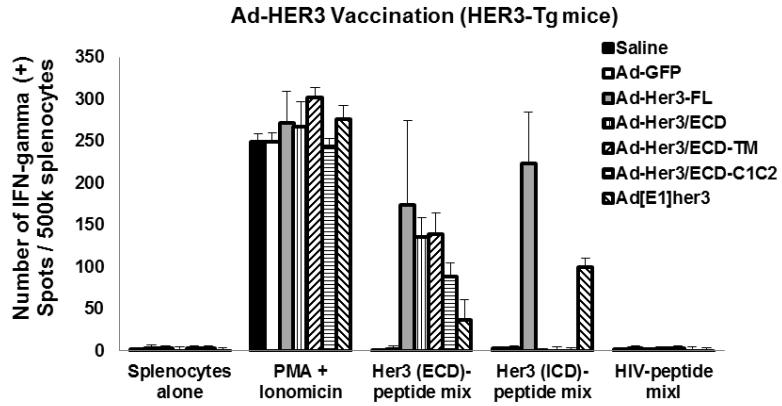


Figure 13. Anti-HER3 cellular response induced by Ad-HER3 vaccination in HER3 Transgenic Mice. HER3 Transgenic mice were vaccinated with Ad5[E1-, E2b-]huHER3-full length(FL), Ad5[E1-, E2b-]huHER3/ECD, Ad5[E1-, E2b-]huHER3/ECD-TM, Ad5[E1-, E2b-]huHER3/ECD-C1C2, or control Ad-GFP, Ad5[E1-]HER3 (2.6 x 1010 vp/vaccination). Two weeks later, vaccination was repeated with the same Ad vectors, and 7 days later, spleen was collected to assess anti-HER3 cellular response. ELISPOT plates were coated with anti-IFNg mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. Average of 4 mice from each group are shown.

Antitumor Response

To assess the vaccine efficacy of the 4 Ad5[E1-, E2b-]huHER3 vectors, we used HER3 transgenic mice crossed with Balb/c mice (F1 generation) for tumorigenicity testing of the JC-hHER3 cell line. We initially confirmed tumorigenicity of JC-hHER3 cells (BALB/c background) in F1 Hybrid mice before conducting a tumor treatment experiment.

To test Ad-HER3 vaccine efficacy in the new model of JC-hHER3 tumors in F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3), we compared the 4 Ad-HER3 vectors (Ad[E1-,E2b-]-HER3 vectors (Ad5[E1-,E2b-]huHER3 FL, Ad5[E1-, E2b-]huHER3ECD, Ad5[E1-, E2b-]huHER3ECDTM, and Ad5[E1-,E2b-]huHER3ECDC1C2) with the JC-hHER3:F1 Hybrid mice model.

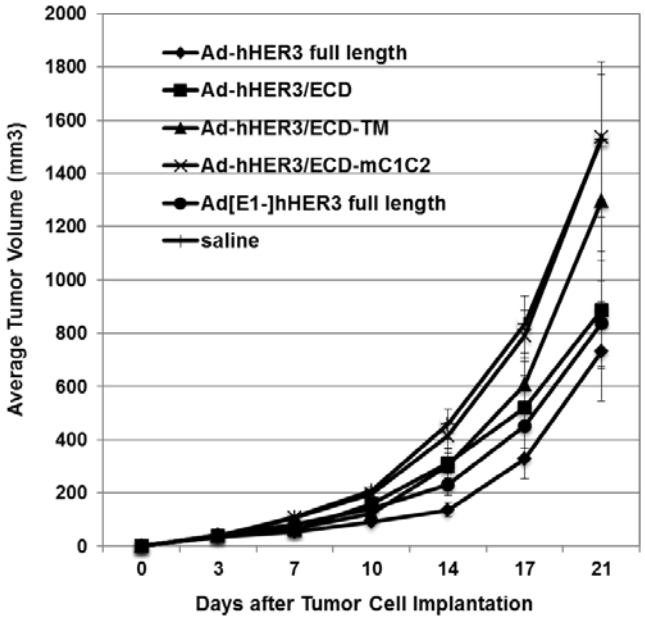


Figure 14: JC-HER3 Tumor Growth in HER3+ F1 Hybrid mice treated with Ad-HER3 vaccines. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]HER3 (full length, ECD, ECDTM, ECD-C1C2), Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. Tumor size was measured twice a week. Individual tumor growth is shown. Error Bar: SE.

As shown in Figure 14, Ad5[E1-E2b-]huHER3full length resulted in the best overall reduction in tumor growth in this treatment model, consistent with our previous experiment.

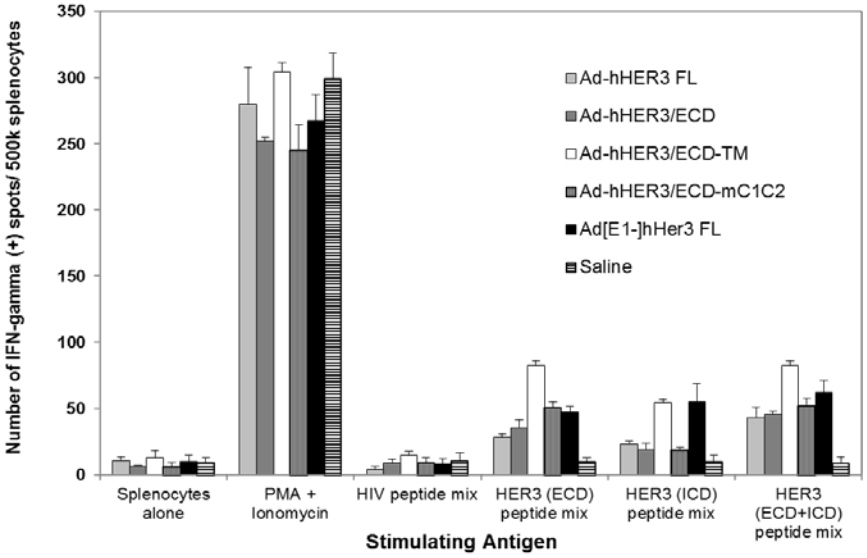


Figure 15: Anti-HER3 cellular response induced by Ad-HER3 vaccination. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 105 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad[E1-E2b-]huHER3 (full lengh, ECD, ECDTM, ECD-C1C2),

Ad5[E1-]huHER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. When tumor volume reached humane endpoint, mice were sacrificed. ELISPOT plates were coated with anti-IFNg mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. Average of 4 mice from each group are shown.

As shown in Figures 16, all 4 of the tested Ad5[E1-, E2b-]huHER3 resulted in T cell responses by IFNg ELISpot and antibody responses by HER3 cell based ELISA. The cell based ELISA indicates the Ad-HER3 induced antibodies can recognize and bind HER3 conformation expressed on the cell surface.

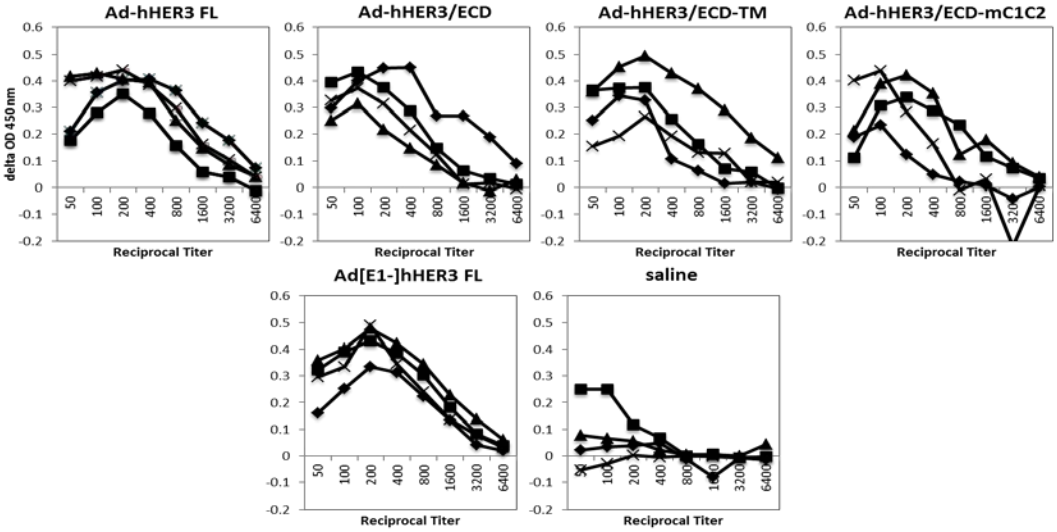


Figure 16: Anti-HER3 antibody levels in Ad-HER3 vaccinated F1 Hybrid mice (Cell-based ELISA assay). Female F1 Hybrid mice (BALB/c x MMTV-neu/MMTV-hHER3) were implanted with JC-hHER3 cells (5 x 10^5 cells/mouse) on day 0, and then vaccinated twice on days 3 and 10 with Ad5[E1-, E2b-]huHER3 (full length, ECD, ECD-TM, ECD-C1C2) or Ad[E1-]-HER3 full length (2.6 x 10^{10} vp/mouse). Once the tumor volume reached humane endpoint, mice were sacrificed, and blood was collected from each mouse. Serum was used for cell-based ELISA (4T1-HER3 and 4T1 cells as plating cells). HRP-conjugated goat anti-mouse IgG was used as secondary Ab, and color was developed with TMB substrate and reaction was stopped by H_2SO_4. Individual OD 450 nm values (OD value with 4T1-HER3 cells minus OD value with 4T1 cells) are shown.

We additionally looked at HER3 expression on tumors in the mice following Ad5[E1-, E2b-]huHER3 vaccination (Figure 17). In mice vaccinated with Ad-huHER3, HER3 expression was decrease in tumors compared to saline control showing the anti-HER3 response induced by the Ad5[E1-, E2b-]huHER3 vectors not only reduces tumor growth but also reduces expression of HER3 on the tumors.

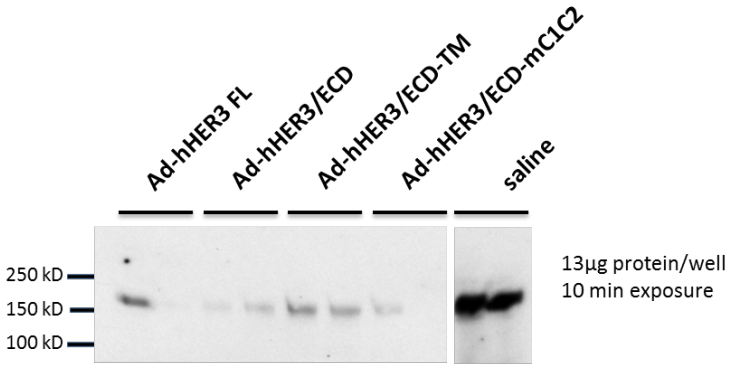


Figure 17: HER3 Expression by JC-HER3 Tumors treated with Ad-HER3 Vaccines. F1 Hybrid Mice (BALB/c x MMTV-neu/MMTV-HER3) received JC-HER3 tumor cell injections (5 x 10^5 cells/mouse, in 50% Matrigel) on day 0, and treated with Ad5[E1-E2b-]HER3 (full lengh, ECD, ECD-TM, ECD-mC1C2), Ad[E1-]HER3 (2.6 x 10E10 vp/injection) or saline on days 3 and 10. When tumor volume reached humane endpoint, mice were sacrificed. Western blot was performed with anti-hHER3 antibody, followed by biotin-conjugated anti-mouse IgG and streptavidin-HRP.

Figure 18 shows the survival curves JC-HER3 treatment experiments comparing Ad5[E1-, E2b-]huHER3 Full length and saline control. We find a significant increase in survival time with Ad5[E1-E2b-]HER3 full length.

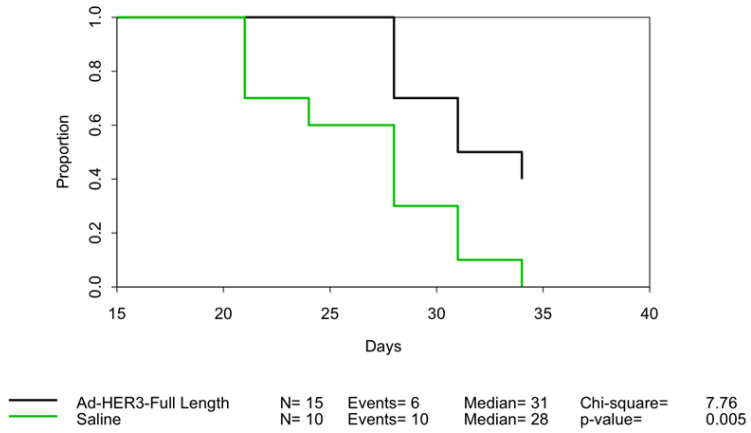


Figure 18: Survival of JC-HER3 Tumor-bearing F1 Hybrid Mice treated with Ad-HER3 Vaccine. HER3 Transgenic F1 Hybrid female mice (BALB/c x MMTV-neu/MMTV-hHER3) were injected with JC-hHER3 cells (5 x 10^5 cells/mouse) on day 0, and treated with Ad5[E1-E2b-]huHER3 (full length, 2.6x10E10 vp/injection), or saline on days 3 and 10. Tumor size was measured twice a week. Mice were considered dead at the time the tumor volume reached humane endpoint. Survival curve for each group was made from survival data of two independent experiments with identical treatment schedule. The Kaplan-Meier method was used to estimate overall survival and groups were compared using a two-sided log-rank test.

Summary Rationale for choice of Ad5 [E1-, E2b-]-huHER3 full length vector

1. Second generation Ad5 [E1-, E2b-]—vectors induce more potent immune responses despite neutralizing antibodies than first generation Ad[E1-] vectors
2. Ad5 [E1-, E2b-]huHER3 full length resulted in longer survival and greater tumor growth control than truncated versions such as Ad5 [E1-, E2b-]huHER3 ECDTM or Ad5 [E1-, E2b-]huHER3 ECD
3. Full length HER3 alone is not oncogenic.

Based on this preclinical data demonstrating the greatest antitumor activity in a HER3 transgenic mouse model was achieved with the Ad[E1-E2b-]huHER3 full length, we selected Ad[E1-E2b-]huHER3 expressing the full length HER3 transgene (subsequently referred to as Ad5 [E1-, E2b-]-huHER3) for the vaccine to use in our phase I clinical trial.

Rationale for patient population

This protocol will enroll patients with advanced malignancies that are expected to express HER3 who have progressed after standard therapy known to lengthen survival. For these patients, clinical trials are considered an appropriate recommendation for management of their disease. HER3 is overexpressed in breast, colon, lung, prostate, ovarian, cervical, endometrial, gastric, pancreatic, bladder, head and neck, liver, and esophageal cancer (1-7).

Rationale for endpoints chosen

In the phase I portion of the study, the intention is to identify a safe dose of the vaccine within a feasible range of dose levels. Because the expected mechanism of action for the vaccine is to induce T cell and antibody responses, we will also examine HER3-specific T cell and antibody responses from the peripheral blood. The standard assays for measuring this immune response are the ELISpot to enumerate the proportion of HER3-responsive T cells and cytokine flow cytometry which identifies the CD4+ and CD8+ T cell contributions to the immune response. HER3 specific antibody levels will be determined by ELISA.

We developed a vaccine against HER3 by taking advantage of a new Ad5 vector system that overcomes barriers found with other Ad5 systems and permits the immunization of people who have previously been exposed to Ad5. The results of this study will establish the technical safety and immunological merit of using this [E1-, E2b-] Ad5 vector HER3 vaccine.

4. PATIENT SELECTION

4.1. Criteria for Patient Eligibility

4.1.1. Histologically confirmed advanced solid tumor where HER3 expression is expected (this includes breast, colon, lung, prostate, ovarian, cervical, endometrial, gastric, pancreatic, bladder, head and neck, liver, and esophageal cancer, but other tumors will be considered based on emerging HER3 expression

data). Note: Demonstration of HER3 expression is not required for enrollment. Because this is a safety and immunogenicity study, patients are NOT required to have measurable or evaluable disease by Response Evaluation Criteria in Solid Tumors (RECIST).

4.1.2. Patients must have received at least 1 line of treatment with standard therapy known to have a possible overall survival benefit and at least 3 weeks since prior chemotherapy or radiotherapy.

For the following common cancers, the following eligibility criteria apply:

- Colorectal cancer: Must have received and progressed through at least one line of palliative chemotherapy consisting of one of the following regimens:
  - Palliative chemotherapy for metastatic colorectal cancer with 5-fluorouracil (or capecitabine) and oxaliplatin.
  - Palliative chemotherapy for metastatic colorectal cancer with 5-fluorouracil (or capecitabine) and irinotecan.
  - Palliative chemotherapy regimen for metastatic colorectal cancer that includes bevacizumab.
  - Colorectal cancer patients currently receiving palliative single-agent bevacizumab or cetuximab will be eligible for this trial and may continue these therapies concomitant with study treatment (if they have been on these single agent therapies for at least 3 months).
- Breast cancer: Must have received and progressed through at least one line of therapy for metastatic breast cancer consisting of one of the following regimens:
  - Palliative anthracycline, capecitabine, or taxane-based chemotherapy
  - Patients with tumors that over express HER2 (IHC 3+ or FISH+) must have received and progressed through at least one line of palliative therapy with trastuzumab, pertuzumab, TD-M1 or lapatinib with or without chemotherapy. Breast cancer patients currently receiving palliative HER2 targeted therapy (trastuzumab, pertuzumab, TD-M1, lapatinib) are eligible and may continue these therapies concomitant with study treatment (if they have been on these therapies for at least 3 months).
  - Palliative endocrine therapy (including aromatase inhibitors, tamoxifen, Fulvestrant, GnRH agonists). Breast cancer patients currently receiving palliative endocrine therapy may continue these therapies concomitant with study treatment (if they have been on these therapies for at least 3 months).
- Lung cancer: Must have received and progressed through at least 1 line of therapy consisting of one of the following regimens:

- Palliative platinum-based (cisplatin or carboplatin) chemotherapy if the patient has not received chemotherapy previously.
- Palliative taxane-based (docetaxel or paclitaxel) or vinorelbine chemotherapy if the patient has received chemotherapy previously.
- Lung cancer patients currently receiving palliative single-agent erlotinib,gefitinib, or crizotinib will be eligible for this trial and may continue these therapies concomitant with study treatment (if they have been on these single agent therapies for at least 3 months).

- Pancreatic cancer: Must have received and progressed through at least 1 line of therapy with the following:
  - Gemcitabine alone or with other drugs
  - Fluorouracil with oxaliplatin and/or irinotecan
  - Pancreatic cancer patients currently receiving palliative single-agent erlotinib will be eligible for this trial and may continue this therapy concomitant with study treatment (if they have been on this single agent therapy for at least 3 months).
- For other malignancies, if a first line therapy with survival or palliative benefit exists, it should have been administered and there should have been progressive disease.
- Patients who have received and progressed through first-line palliative chemotherapy must be advised regarding second-line therapy before being enrolled on this investigational study.

4.1.3. ECOG 0 or 1

4.1.4. Estimated life expectancy > 3 months

4.1.5. Age ≥ 18 years

4.1.6. Adequate hematologic function, with WBC ≥ 3000/microliter, hemoglobin ≥ 9 g/dL (it is acceptable to have had prior transfusion), platelets ≥ 75,000/microliter; PT-INR <1.5, PTT <1.5X ULN

4.1.7. Adequate renal and hepatic function, with serum creatinine < 1.5 mg/dL, bilirubin < 1.5 mg/dL (except for Gilbert's syndrome which will allow bilirubin ≤ 2.0 mg/dL), ALT and AST ≤ 2.5 x upper limit of normal or if liver metastases are present < 5 x upper limit of normal

4.1.9. Patients who have received prior immunotherapy are eligible for this trial, if this treatment was discontinued at least 3 months prior to enrollment.

4.1.10. Female patients must be of non child-bearing potential or use effective contraception, e.g., use of oral contraceptives with an additional barrier method (since the study drug may impair the effectiveness of oral contraceptives), double barrier methods (diaphragm with spermicidal gel or condoms with contraceptive foam), Depo-Provera, partner vasectomy, total abstinence, and willing to continue the effective contraception method for 30 days after the last dose of study drug;

4.1.11. Patients who are taking medications that do not have a known history of immunosuppression are eligible for this trial.

4.1.13. Written informed consent and HIPAA authorization (applies to covered entities in the USA only) obtained from the patient prior to performing any study-related procedures, including screening visits. However, CT scans, bone scans, MUGA, Echocardiogram, EKG,and labs performed as standard of care prior to signing consent can be used to fulfill eligibility requirements if they were performed within 8 weeks of the first dose of study drug (for the MUGA or echocardiogram) and within 4 weeks of the first dose of study drug for the remainder of the studies

4.1.14. Ability to understand and provide signed informed consent that fulfills Institutional Review Board's guidelines.

4.1.15. Ability to return to Duke University Medical Center for adequate follow-up, as required by this protocol.

4.2. Criteria for Patient Exclusion

4.2.1 Patients with concurrent cytotoxic chemotherapy or radiation therapy should be excluded. There are no exclusions based on the number of prior chemotherapy, biologic, hormonal, or experimental regimens. There must be 3 weeks between any other prior cytotoxic chemotherapy and/or radiotherapy and study treatment. Patients must have recovered to grade 1 acute toxicities from prior treatment.

4.2.2. Patients may have received prior radiation including for brain metastases.

4.2.3. Patients with CNS progression are ineligible until this CNS progression is treated either with whole brain radiation or SRS and have an MRI of the affected CNS lesion(s) 3 months after radiation therapy (per NCCN guidelines) demonstrating stable (or improved) disease prior to proceeding with enrolment on the AVX901 study. Patients also must be off all steroids prior to initiating the AVX901 protocol, as outlined below in 4.2.h.

4.2.4. History of auto-immune disease such as, but not restricted to, inflammatory bowel disease, systemic lupus erythematosus, ankylosing spondylitis, scleroderma, or multiple sclerosis. Prior history of autoimmune thyroiditis or vitiligo is permitted.

4.2.5. Serious chronic or acute illness considered by the P.I. to constitute an unwarranted high risk for investigational drug treatment.

4.2.6. Medical or psychological impediment to probable compliance with the protocol.

4.2.7. Concurrent or prior second malignancy (within the past 5 years) other than non-melanoma skin cancer, controlled superficial bladder cancer or controlled cervical cancer.

4.2.8. Presence of active infection or systemic use of antimicrobials within 72 hours prior to the first injection

4.2.9. Patients on continuous steroid therapy for at least 72hrs (or other continuous immunosuppressives such as azathioprine or cyclosporine A) are excluded on the basis of potential immune suppression. Patients must have had 6 weeks of discontinuation of any continuous steroid therapy (taken for at least 72 hrs

duration) prior to enrollment (except steroids used for allergic reactions or as anti-emetics for systemic chemotherapy which are permitted).

4.2.10. Presence of a known active acute or chronic infection including HIV or viral hepatitis (Hepatitis B and C)). 4.2.11. Pregnant or nursing women

4.3. Accrual

Patients will be accrued into the following sequential cohorts (if there are no DLTs):

1. Ad5 [E1-, E2b-]huHER3 at 2 x 10^{10} viral particles (vp) (3 subjects)
2. Ad5 [E1-, E2b-]huHER3 at 1 x 10^{11} vp (3 subjects)
3. Ad5 [E1-, E2b-]huHER3 at 5 x 10^{11} vp (12 subjects)

We expect to accrue a minimum of 18 evaluable patients (plus up to 12 replacements for screen failures and patients removed from the study prior to completion of the assigned vaccine schedule for any reason other than toxicity). We will consent up to 40 patients total.

Assignment of study number: Patients will be assigned study numbers in order of their screening using the following: AdHER3-01- 001, 002, 003 etc. including screened patients.

Assignment of study day: Subjects will receive study drug every 3 weeks for a total of 3 injections. Day 0/week 0 is the day of the first immunization. The second immunization is at Day 21/week 3 and the third immunization is Day 42/week 6.

Inclusion of Women and Minorities
This trial is open to both genders and all racial and ethnic groups (See appendix).

Inclusion of Children: Only individuals 18 and older will be enrolled. As this is a phase I study with an agent that has never been tested in humans previously, the potential harms to children are entirely unknown. We do not believe that it is appropriate to enroll children, until data regarding safety are available, so that a better calculation of the risks can be made.

Subject Recruitment & Informed Consent Process

The patient's oncologist will contact the research nurse assigned to the study to explain the study to the patient in person. The research nurse will explain the study procedures, review the consent with them and answer any questions they may have. In addition, Drs. Lyerly, Gwin and/or Morse will be available to discuss the study. The consent is likely to occur in the clinics of Duke University Medical Center and will take place at the time the patient is seeing their oncologist. The consent process is expected to take 60-120 minutes and the patient can take the consent home to discuss with their family if desired. Specifically, the chronological order of events for the patient include: patient's physician identifies they may be a candidate for the study; patient's physician contacts research nurse; the research nurse discusses study and obtains

consent. For patients that are non-English speaking, the informed consent may be translated and approved by the Duke IRB in order to obtain consent.

5. PRE-TREATMENT EVALUATION

(See also Schema in Appendix 1.) The following pre-treatment evaluations will be completed within 1 month (+/- 2 weeks) before starting study treatment:

- **History and physical exam,** to include ECOG Performance Score ß-HCG for women with childbearing potential
- **Hematological, biochemical and immunological tests:**

  CBC with differential

  PT INR and PTT

  Na, K, Cl, CO_2, BUN, creatinine, Ca, total protein, albumin, total bilirubin, alkaline phosphatase, AST, ALT and glucose

  Urinalysis
- **Peripheral blood for immune monitoring**

  Subjects will have a peripheral blood draw (90 ml total will be requested) prior to initiating the immunizations. Serum and PBMCs will be collected and stored for immune analyses of T cell and antibody responses.

  In addition to 90mL, 20 mL of blood may be drawn during the first clinic visit, when the patient history and physical exam are conducted, at the discretion of the immune monitoring laboratory for analysis of immune responses.
- **Imaging studies:**

  Imaging studies (available CT of Chest, abdomen, and pelvis and bone scans) will be reviewed prior to initiating the injections. It is preferred to have these imaging studies will be performed within 1 month before starting study treatment to document the presence and size of any measurable metastatic disease that might present.

6. TREATMENT PLAN

1) **Cohort 1:** Three patients will receive Ad5 [E1-, E2b-]huHER3 at a dose of 2×10^{10} viral particles in 0.5 mL subcutaneously (SQ) in the same thigh every 3 weeks for 3 immunizations. Immunizations should be separated by 5 cm. Diary card to record any adverse reactions for 2 days after treatment (24 hours and 48 hours post injection) will be given to each patient at the first treatment along with a ruler. Patient can fax (# 919-684-2311) the completed diary form prior to next appointment date. The diary card will be placed in the patient file and a copy given to the Principal Investigator for his records. Assessment of DLT for dose escalation will be made after all patients in this cohort have had a study visit at least 3 weeks after receiving their first dose of vaccine. If there are no DLT (as defined below), then patients may begin enrolling into cohort 2. If there is 1 DLT then an additional 3 patients will be enrolled at this dosage level. Assessment of DLT for dose escalation will be made after the 3 additional patients have had a study visit at least 3 weeks after receiving their first dose of vaccine. If none of these latter 3 patients have DLT, then patients may begin enrolling into cohort 2. If 2 patients have DLT at this lowest dosage level, dosing will be de-escalated to $1x10^8$ particles and a new cohort instituted.

2) **Cohort 2:** Three patients will receive Ad5 [E1-, E2b-]huHER3 at a dose of 5×10^{11} particles in 0.5 mL SQ in the same thigh every 3 weeks for 3 immunizations. Immunizations site should be separated by 5 cm. Assessment of DLT for dose escalation will be made after all patients in this cohort have had a study visit at least 3 weeks after receiving their first dose of vaccine. If there are no DLT, then patients may begin enrolling into cohort 3. If there is 1 DLT then an additional 3 patients will be enrolled at this dosage level. Assessment of DLT for dose escalation will be made after the 3 additional patients have had a study visit at least 3 weeks after receiving their first dose of vaccine. If none of these latter 3 patients have DLT, then patients may begin enrolling into cohort 3. If 2 patients have DLT at this dosage level, the dosage level in cohort 1 will be considered the MTD. If only 3 patients were enrolled in cohort 1, an additional 3 patients will be enrolled at that dosage before proceeding to phase II.

3) **Cohort 3:** Three patients will receive Ad5 [E1-, E2b-]huHER3 at a dose of 5×10^{11} particles in 0.5 mL SQ in the same thigh every 3 weeks for 3 immunizations. Immunization sites should be separated by 5 cm. Assessment of DLT for proceeding to phase II enrollment will be made after all patients in this cohort have had a study visit at least 3 weeks after receiving their first dose of vaccine. If there are no DLT, then up to 9 more patients will be enrolled (total 12 in this cohort) to assess immune response. If there is 1 DLT then an additional 3 patients will be enrolled at this dosage level. Assessment of DLT for dose escalation will be made after the 3 additional patients have had a study visit at least 3 weeks after receiving their first dose of vaccine. If none of these latter 3 patients have DLT, then up to 6 more patients will be enrolled (up to a total of 12 in the cohort). If 2 patients have DLT at this dosage level, the dosage level in cohort 2 will be considered the MTD. If only 3 patients were enrolled in cohort 2, an additional 9 patients will be enrolled at that dosage to obtain immunogenicity data.

DLT (based on CTCAE4.0 criteria) is defined as any Grade 2, 3 or 4 immediate hypersensitivity reactions, Grade 3 or 4 fever that may possibly be associated with the immunization, Grade ≥2 autoimmune events except for vitilgo or fever for less than 2 days and less than <101.5 ºF, Grade ≥2 allergic reactions (grade 2 is defined as generalized urticaria as defined by version 4 CTC guide), or Grade ≥3 non-hematologic toxicity.

During dose escalation through the first three patients of cohort 3, there will be a minimum of 1 week between enrolling successive patients. The first patient will be called to check on their condition prior to enrolling the second patient since patients can be enrolled after 1 week of initiation of cohort 3. If no DLT have been observed at this point, then further enrollment can occur in cohort 3 and phase II component without the 1-week waiting period. Between dosage levels, assessment of DLT for dose escalation will be made after all patients in a cohort have had a study visit at least 3 weeks after receiving their first dose of vaccine and all the available safety data and laboratory results have been reviewed by the Principle Investigator. If DLT occurs in <33% of patients in a given dosage level cohort, progression to the next dosage level will proceed. If DLT occurs in ≥33% of patients in a given cohort, the next lower dosage level will be defined as the maximum tolerated dose (MTD). If DLT occurs in <33% of patients in the highest dosage level tested, that dosage level will be defined as the MTD. In phase II, 12 additional patients will be enrolled at the MTD. In phase II, if at any time the rate of DLT in patients enrolled at the MTD (for the phase I and phase II cohorts combined) is ≥33%, the MTD will be re-defined as the next lower dosage level, and phase II will proceed with enrollment of additional patients at this lower dosage level. Additional details of this dose escalation and de-escalation plan are provided below and in Figures 3A, 3B and Table 3.

Study Visits

1. Vaccine administration at each visit (weeks 0, 3, 6)
   No premedication will be given.

   Ad-HER3: each dose is injected subcutaneously in thigh. The same thigh will be used for each injection at the discretion of the study PI.

   Patients will remain in the clinic for 1 hour after immunization with vital signs checked at 15 minutes, 30 minutes, and 1 hour.
2. Patients will have up to 90 mL peripheral blood drawn prior to each immunization and approximately 3 weeks after the third immunization (Week 9) to determine whether there is an effect on the immune response at specific time points during the study and/or after a specific number of immunizations.
3. Time to progression will be measured using CT scans performed at approximately 3 month intervals (based on clinical standard of care).
4. For all patients, if scheduling conflicts arise, the scheduled 3-week interval between immunizations may be modified so that the interval between immunizations is between 20 and 28 days (3 weeks -1 day to 3 weeks +7 days). If the second and/or third

immunization is delayed, the subsequent immunizations should occur no earlier than 20 days after the previous immunization.

5. The following safety events will trigger a temporary suspension of study vaccinations:
   a) If one or more patients develop a Grade 4 allergic reaction without a clear attributable cause, other than study vaccine
   b) Death not attributed to disease.

Assessment of these halting rules is a review of cumulative events for all study participants, and should not be confused with reasons for delaying or terminating the immunization schedule of any individual patient.

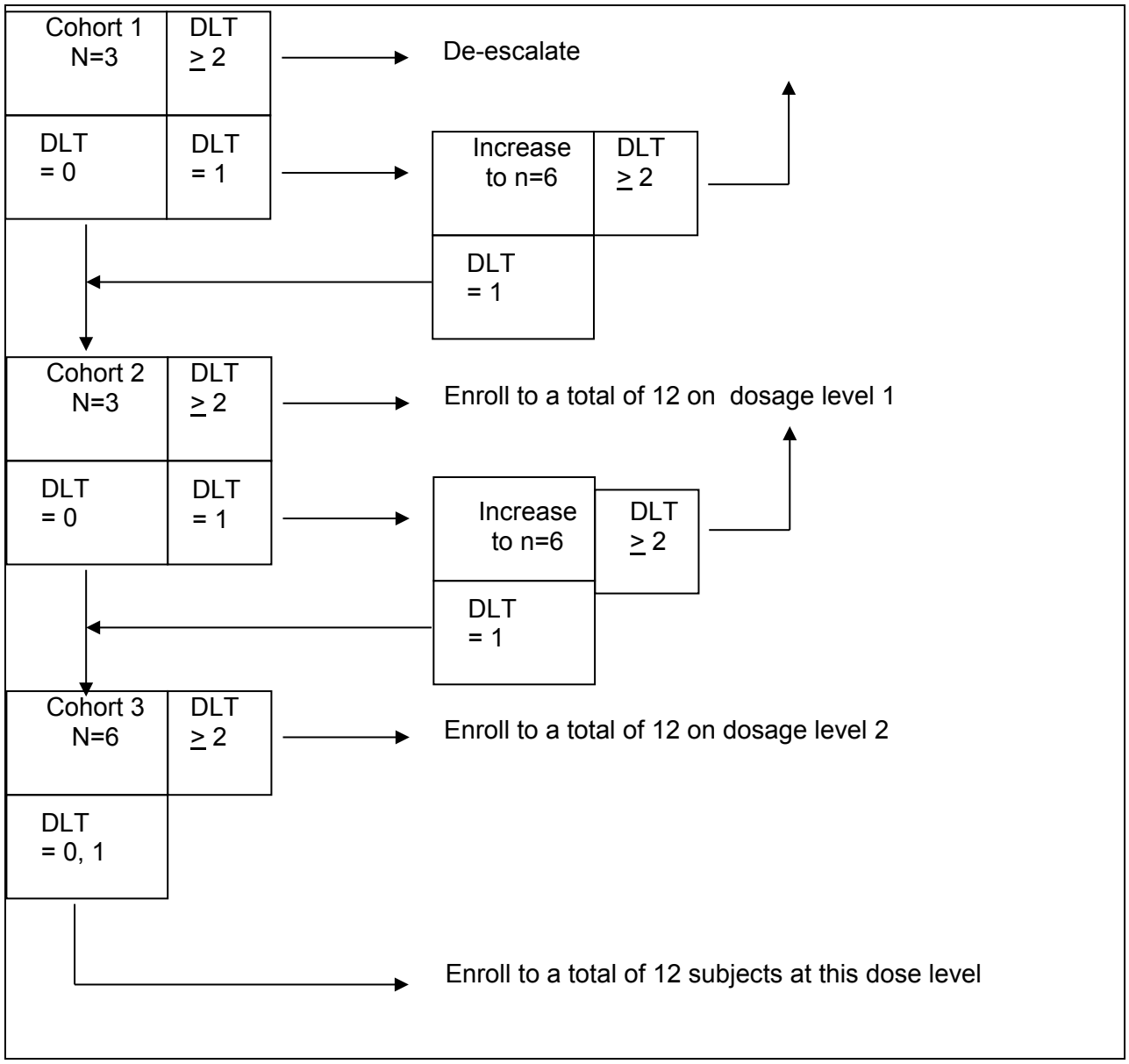
The Scientific Monitoring Subcommittee of the Cancer Protocol Committee (CPC) will fully review all available safety data, consult with the principal investigator, medical monitor and the FDA as needed, before determining if resuming vaccinations is appropriate. If it is determined that study vaccinations can resume, the halting rules will apply to each subsequent event that meets the criteria described above.

Vaccinations may also be suspended for safety concerns other than those described above if, in the judgment of the principal investigator or sponsor, participant safety is threatened.

6.1. Study Stopping Rules

- Death possibly related to the study agent.
- Two patients having a Grade 4 toxicity event that is possibly/probably related to the study agent.

Figure 3A


Cohort 1
N=3
DLT
≥ 2
De-escalate
DLT
= 0
DLT
= 1
Increase
to n=6
DLT
≥ 2
DLT
= 1
Cohort 2
N=3
DLT
≥ 2
Enroll to a total of 12 on dosage level 1
DLT
= 0
DLT
= 1
Increase
to n=6
DLT
≥ 2
DLT
= 1
Cohort 3
N=6
DLT
≥ 2
Enroll to a total of 12 on dosage level 2
DLT
= 0, 1
Enroll to a total of 12 subjects at this dose level

Pharmaceutical Information

6.1.1 Dosage and Administration

Patients will receive Ad5 [E1-, E2b-]huHER3 at a dose of 2 x 10^{10}, 1 x 10^{11}, or 5 x 10^{11} viral particles subcutaneously (SQ) in 0.5 mL of a buffered saline solution every 3 weeks for a total of 3 immunizations.

6.1.2 How Supplied

Ad5 [E1-, E2b-]huHER3 will be provided in a frozen state in a 2 ml vial with a fill volume of 0.5 ml of extractable vaccine which contains 1X10^{11} total virus particles. The volume of injection for 1x10^{11} virus particles is 0.5 mL. The lower doses will be produced by dilution in 0.9% saline using the following directions. The product should be stored at </= –20°C until used.

Instructions for dose preparation:
A detailed description of dose preparation is described in the study IPHP and clinic SOP.

1. To administer 2x10e10 virus particles by subcutaneous injection:
Perform 2 serial dilutions of vialed vaccine as follows:
Draw 4.5 mL of sterile saline into a syringe. Using a second syringe, withdraw 0.5 mL of previously thawed Ad5 [E1-, E2b-]huHER3 from the supplied vial. Remove the needle from syringe containing the 4.5 mL saline, and inject the 0.5 mL of Ad5 [E1-, E2b-]huHER3 from the second syringe into the syringe containing saline. Mix. This new solution has a concentration **2 x 10^{10} vp/ml.**
Use a new sterile syringe with a needle and repeat above procedure. Withdraw 4.5 mL of sterile saline. In the second syringe withdraw 0.5 mL from the syringe containing **2 x 10^{10} vp/ml**. Remove the needle from the syringe containing the 4.5 mL saline, and inject the 0.5 mL of 2 x 10^{10}vp/mL from the second syringe.
Place a new needle on the 10-mL syringe (**Syringe C from Step 9 above**) and mix the two solutions. This solution now has a concentration **2 x 10^{9} vp/mL (**1 x 10^{9} vp per 0.5 mL)**.**
Label a new 1-mL sterile syringe ETBX-011, 1 x 10^{9} vp and withdraw 0.5 mL from the syringe containing **2 x 10^{9} vp/mL**. This prepared vaccine (Ad5 [E1-, E2b-]huHER3**, 1 x 10^{9} vp)** can be kept at room temperature for four hours prior to administering to the patient.

2. To administer 1x10e10 virus particles by subcutaneous injection:
Draw 4.5 mL of sterile saline into a syringe. Using a second syringe, withdraw 0.5 mL of previously thawed Ad5 [E1-, E2b-]huHER3 from the supplied vial. Remove the needle from syringe containing the 4.5 mL saline, and inject the 0.5 mL of Ad5 [E1-, E2b-]huHER3 from the second syringe into the syringe containing saline. Mix. This new solution has a concentration **2 x 10^{10} vp/ml.**

Label a new 1-mL sterile syringe, 1 x 10^{10} vp and withdraw 0.5 mL from the syringe containing **2 x 10^{10} vp/mL**. This prepared vaccine (Ad5 [E1-, E2b-]-huHER3**, 1 x 10^{10} vp)** can be kept at room temperature for four hours prior to administering to the patient.

3. **To administer 5x10e11 virus particles by subcutaneous injection:**
Withdraw 0.5mL of contents (Ad5 [E1-, E2b-]-huHER3) from vial and administer each subject without any further manipulation.

6.1.3 Disposal of Unused Vaccine

Unless other arrangements are agreed in writing, all unused vaccine should be delivered to Dr. H. Kim Lyerly at or before the completion of the clinical study.

7. TREATMENT EVALUATION

7.1. Short-Term Evaluation During and After Active Immunotherapy

On vaccine administration days, blood will be drawn before administration. Evaluations will also be conducted for patients who discontinue from the study if they have received any treatment. The investigator will determine the degree of evaluation based on the patient's condition and/or reason for discontinuation from the study.

7.1.1 General Evaluations (each visit for immunization)

General evaluations include medical history, Karnofsky performance status, and complete physical examination with weight. Any other treatments, medications, biologics, or blood products that the patient is receiving or has received since the last visit will be recorded. Patients will remain in the clinic for approximately 30 minutes following receipt of vaccine to monitor for any adverse reactions. Local and systemic reactogenicity after each dose of vaccine will be assessed daily for 3 days (on the day of immunization and 2 days thereafter) using diary cards to report symptoms and a ruler to measure local reactogenicity.

7.1.2 Hematological and Biochemical Assessment

Blood chemistry and hematology, including CBC with differential, Na, K, Cl, CO_2, BUN, creatinine, Ca, total protein, albumin, total bilirubin, alkaline phosphatase, AST, ALT and glucose will be drawn at Week 0,3,6,9, at discontinuation of treatment (if treatment is discontinued early), and as clinically indicated.

7.1.3 Biological Markers

Serum from research blood draws to measure antibodies to HER3 and the Ad5 vector will be obtained at Week 0, approximately 3 weeks after the last immunization (approximately Week 9), and every 3 months thereafter at discretion of the PI.

7.1.4 Immunological Assessment

Peripheral blood (approximately 90mL total; yellow tops collected for PBMC, red tops for serum) will be drawn prior to each immunization and approximately 3 weeks after the last immunization to determine whether there is an effect on the immune response at specific time points during the study and/or after a specific number of immunizations. Peripheral blood mononuclear cells (PBMC) will be assayed for T cell responses to HER3 using ELISPOT, plus, if possible and at the discretion of the investigator, additional immune assays such as proliferation assays, multi-parameter flow cytometric analysis, TCR sequencing, and cytoxicity assays. Serum will be archived from the initial and Week 9 blood draws for evaluating antibody levels and other serum markers.

7.2. Long-Term Follow-Up

Patients will be requested to continue long-term follow-up at Duke University Medical Center every 3 months for 1 year, while on the study (i.e., have not progressed or been removed from the study for other reasons). At each visit, a medical history and physical exam and labs (Blood chemistry and hematology, including CBC with differential, Na, K, Cl, CO_2, BUN, creatinine, Ca, total protein, albumin, total bilirubin, alkaline phosphatase, AST, ALT and glucose) will be drawn.

At each visit, 40-90 mL of peripheral blood for immune analysis may be drawn, if there was previous evidence of an immune response or at the discretion of the investigator.

7.3. Management of Intercurrent Events

7.3.1 Concomitant Medications

Patients will be removed from the protocol treatment if they initiate concomitant chemotherapeutic agents, corticosteroids, or other immunosuppressive agents, or other forms of immunotherapy. After meeting the inclusion criteria, all other medications deemed appropriate for the patient, by the investigator, may be administered to the patient. All medications and changes in medication during treatment will be recorded. Hormonal therapy and bisphosphonates may be continued in breast cancer patients if they have been stable on the agents for at least 1 months prior to enrollment.

7.3.2 Adverse Events

7.3.2.1 Toxicity will be graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 (which can be downloaded from the CTEP web site (http://ctep.cancer.gov).) Dose-limiting toxicity (DLT) is defined in section 6.

7.3.2.2 Possible side effects from immunization may include local effects (pain, tenderness, rednesss or swelling), systemic effects (malaise, fatigue, myalgia, arthralgia, headache, nausea, vomiting, chills or fever), and allergic reactions

such as hives, rash or anaphylactic reactions. Induction of auto-immunity, manifest as arthritis, serositis, nephritis, thyroiditis, colitis, neutropenia, etc., is theoretically possible, but has not been observed in our prior CEA vaccine studies. Also, liver function test abnormalities and liver failure are theoretically possible.

7.3.3 Treatment of Toxicity: Bronchospasm, stridor, wheezing, respiratory depression (RR < 8) cardiac arrhythmia, generalized urticaria, systolic BP ≤ 80mm Hg, angioedema, shock, or loss of consciousness

1. Stop infusion and remain at patient bedside
2. Have another nurse notify MD
3. NS at KVO, if hypotensive then give NS 500ml bolus
4. Start oxygen for dyspnea, stridor, wheezing or respiratory depression at 2 liters/nasal cannula, initiate continuous pulse oximetry and call RT
5. Diphenhydramine 50 mg IVP** x 1
6. Give methylprednisolone 125mg IV** x 1
7. Give epinephrine 0.3mg (auto-injector) x 1 IM x **OR** epinephrine 0.3mg (1:1000) SQ x 1 (may repeat x 1 in 5 minutes)
8. If no response to above interventions within 5 minutes or patient condition worsens, call a code 911 or x115 (Duke), x222 (Durham Regional), 3111 (Duke Raleigh)
9. Vital signs with pulse oximetry Q2 minutes until patient is stable, then Q5 minutes x 30 minutes and Q15 minutes for 1 hr or as the patient's condition requires

**If no IV access or IV access lost, may give these agents IM as appropriate

7.3.4 Active Immunotherapy will be Discontinued for:

7.3.4.1 Life-threatening anaphylactic reactions related to active immunotherapy

7.3.4.2 Dose-limiting toxicity related to active immunotherapy

7.3.4.3 Disease progression (by RECIST criteria). Patients will be offered referral to a medical oncologist at Duke University Medical Center for discussion of other treatment options, and for continued medical care.

7.3.4.3.1 Disease progression prior to completing the 3 study immunizations: In the event that a patient undergoes reimaging studies prior to the completion of their 3 study immunizations and is found to have disease progression, they will be permitted to continue on the study as long as the progression has been 50%.

If a patient is removed from the study prior to completion of the assigned vaccine schedule for any reason other than toxicity, that patient will be replaced, in order to obtain data to help determine the toxicity of the immunizations. We will allow up to 3 replacements per cohort (Phase I, Dose levels 1 and 2; Phase II, MTD).

8. STATISTICAL CONSIDERATIONS

8.1. Safety:

We will evaluate safety continuously in a cohort. We will make our overall assessment of whether to escalate to the next dose level at least 3 weeks after the last patient in the previous cohort has received their first injection. This decision will be made by the medical monitor and the Principle Investigator. A note will be generated following the assessment decision and filed in study binder. A dosage level will be considered safe if <33% patients treated at a dose level experience dose-limiting toxicity (i.e., 0 of 3, ≤1 of 6, ≤3 of 12). Dose-limiting toxicity is defined in section 6. Safety will be evaluated in 3 or 6 patients at each dosage level in phase I. A patient will be considered evaluable for safety if treated with at least one immunization. DLTs will be observed through 9 weeks to accommodate safety evaluation of all 3 product doses.

8.2. Rate of Immune Response:

Immune responses against HER3 and other antigens will be evaluated from the peripheral blood of patients from among the following studies at the discretion of the Principle Investigator (ELISpot, cytokine flow cytometry, and antibody responses). We will determine the percentage of patients with a positive immune response. We define a positive immune response by ELISpot as described at the 2002 Society of Biologic Therapy Workshop on "Immunologic Monitoring of Cancer Vaccine Therapy", i.e. a T cell response is considered positive if the mean number of spots adjusted for background in six wells with antigen exceeds the number of spots in six control wells by 10 and the difference between single values of the six wells containing antigen and the six control wells is statistically significant at a level of $p \leq 0.05$ using the Student's t test. Immunogenicity assays will occur prior to each immunization and at week 12. Immune response will be assessed among the 12 patients treated at the MTD. The therapy will be considered of further interest if 12 of 12 patients treated at the MTD dose exhibit an immune response as defined above. Meeting this criterion establishes that the immune response rate is at least 33% with approximately 90% confidence. At significance level 0.1 there is 82% power to test the null hypothesis that the immune response rate is ≤ 0.33 versus the alternative that the immune response rate is ≥ 0.58. The magnitude of response will also be described. A patient will be considered evaluable for immune response if they receive at least 3 immunizations.

8.3. Determination of Clinical Response

Among patients with measurable/evaluable disease, response determination will be made according to the RECIST criteria:

Complete Response (CR):	Disappearance of target lesion, confirmed at 4 weeks
Partial Response (PR):	30% decrease in longest dimension of target lesion, confirmed at 4 weeks
Stable Disease (SD):	Neither PR nor PD
Progressive Disease (PD):	20% increase in longest dimension of target lesion; no CR, PR, or SD documented before increased disease.

The exact binomial confidence interval for the proportion of subjects with a clinical response of CR or PR will be calculated.

9. PATIENT WITHDRAWAL

Patients may be removed from the study for the following reasons:

- Dose-limiting toxicity, as defined in section 6.
- Patient voluntarily decides to withdraw.
- Patient non-compliance with the study protocol.
- Intercurrent disease which in the opinion of the patient's treating physician would affect the ability of the patient to continue on the clinical study.

In the event of withdrawal due to toxicity, a patient will be requested to have safety evaluations performed as per the protocol for a one year duration post treatment. This may include having up to 90 mL of blood drawn for immunologic testing..

10. STUDY CONDUCT AND ETHICAL AND REGULATORY CONSIDERATIONS

10.1. Departure from the Protocol

There should be no departure from the protocol if at all possible. If an emergency occurs that requires departure from this protocol, the investigator or other physician in attendance in such an emergency will, if circumstances and time permit, contact the principal investigator (Dr. Michael Morse) or in his absence, Dr. H. Kim Lyerly, immediately by telephone (Page operator 919 684-8111). Such contacts with the principal investigator will be made to permit a decision as to whether or not the patient will be continued on the study. Such departures need to be clearly documented and reported to the IRB and the sponsor (Etubics) by the principal investigator.

10.2. Informed Consent

In accordance with guidelines in the Federal Register, Vol. 48, No. 17, 1982, pp. 8951-2, all patients are required to sign a statement of informed consent. This phase I/II study involves research that presents risk, but holds the prospect of direct benefit to the individual patient (46.405-45 Code of the Federal Regulations part 46). The investigator will report to the IRB and the sponsor (Etubics) will report to FDA changes in the research protocol and all unanticipated problems involving risks to human patients and others, and no changes will be made in the research activity without IRB approval.

10.3. Institutional Review

This study must be approved by the Institutional Review Board (IRB). IRB approval of the protocol and the informed consent form for this study must be given in writing. The IRB must also approve any significant changes to the protocol as well as a change of principal investigator. Records of all study review and approval documents must be kept on file by the investigator and are subject to FDA inspection during or after completion of the study. Adverse events must be reported to the IRB. The IRB will receive notification of the completion of the study and final report within three months of study completion or termination. The investigator

must maintain an accurate and complete record of all submissions made to the IRB, including a list of all reports and documents submitted.

10.4. Documentation and Monitoring

Data will be collected for all patients. Accurate completion of the computer data forms for all patients is the responsibility of the investigator.

10.4.1 Case Report Forms

Case Report Forms (CRFs) are used to record study data and are an integral part of the study and subsequent reports. Therefore, all reports must be legible and complete. All forms should be filled out using a black ballpoint pen. Errors should be lined out but not obliterated and the correction inserted, initialed, and dated by the principal investigator, co-investigators, study coordinator, or data manager. A Case Report Form must be completed and signed by the principal investigator for each patient enrolled, including those removed from the study for any reason. The reason for removal must be noted on the Final Report Form by the investigator for each patient. Case Report Forms must be kept current to reflect patient status at each phase during the course of the study. Patients are not to be identified on case report forms by name; appropriate coded identification and patient initials must be used. The investigator must keep a separate log of patient names and addresses. This log is subject to FDA inspection. Because of the potential for errors, inaccuracies, and illegibility in transcribing data onto case report forms, originals of laboratory and other test results must be kept on file with patient's case report form or clinical chart. Case report forms and copies of test results must be available at all times for inspection by the FDA.

10.4.2 Maintenance of Study Documentation

The following will be maintained:

a. Case Report Forms - which must be kept legible, accurate, and up-to-date.
b. Patient Files/Signed Informed Consent - which substantiates the data entered on the case report forms for all required test and evaluation procedures and verifies that the patient has signed an informed consent to enter the study.
c. Patient Exclusion Record - which should reflect the reason any patient was screened and found ineligible for the study.
d. Monitoring Log - listing dates of monitor visits.
e. Regulatory Documents - including protocol, investigator brochure, FDA Form 1572, CVs, IRB correspondence, IRB approval/renewals and IRB approved consent form.
f. Adverse Experience Report Form - which should explain any serious or unexpected adverse experiences.

All study documentation pertaining to the conduct of the study must be kept on file by the investigator for a period of 2 years following the date a marketing application is approved for the drug for the indication for which it is being investigated; or, if no application is to be filed or if the application is not approved for such indication, until 2 years after the investigation is

discontinued and FDA is notified. The sponsor will notify the investigator if a marketing application is approved or if the investigation is discontinued and the FDA notified.

10.5. Monitoring of the Protocol

Protocol data and safety will be monitored by the Scientific Monitoring Subcommittee of the Cancer Protocol Committee (CPC) with the following plan:

1. Purpose: This is a phase I/II clinical study with more than minimal risk and as such will be monitored for the occurrence of a greater frequency of AEs.
2. Monitoring: Up to 24 patients will be enrolled. The principal investigator will continuously monitor the study. The principal investigator will review the data and safety of the study after the enrollment of three patients in cohort 1, after enrollment of three patients in cohort 2, and after enrollment of six patients in cohort 3. Formal, independent monitoring by CPC will occur after enrollment of the three patients in cohort 1 and again after enrollment of three patients in cohort 2; then a scientific progress review will occur yearly, assuming a result of "satisfactory" on the initial review. The exceptions are as follows: If more than one patient experiences a Grade 4 or greater allergic reaction, the principal investigator will request a monitoring review by CPC. If at any time, more than 50% of patients experience a Grade 3 or 4 major organ toxicity, we will request a monitoring review by CPC.
3. Description of Monitoring: Adverse event reports will be reviewed with tabulation of all Grade 2, 3 or 4 toxicity.
4. Toxicity: see section 7.3.2 of the protocol.
5. Reporting Adverse Events: An adverse experience is any adverse change from the study patient's baseline (pre-treatment) condition, including any clinical or laboratory test abnormality that occurs during the course of the proposed clinical study after treatment has started. All adverse experiences that are classified as serious as described in section 10.7 of the protocol should be reported to the sponsor (Etubics) by telephone or fax within 24 hours, and reported in writing to the sponsor (etubics) within 72 hours. All study-related deaths should be reported to the IRB within 24 hours; all other serious adverse experiences should be reported to the IRB within 5 business days. All deaths, whether considered study-related or not, must also be reported immediately to the principal investigator, with a copy of the autopsy report and the death certificate. All adverse experiences will be recorded on the Adverse Experience Case Report Form. This report form should include severity, duration, outcome, and the investigator's judgment as to the relationship of the adverse experience to treatment.
6. Reporting of Pregnancy: If a participant becomes pregnant during the study, treatment will be discontinued (*i.e.*, no additional dose of study vaccine will be given) and the participant will be encouraged to continue to have regularly scheduled follow-up visits and evaluations. The occurrence of pregnancy, and the outcome of any pregnancy, in a subject treated with study vaccine, must be reported to the sponsor (Etubics), and to the IRB.

10.6. CTCAE Term (AE description) and Grade:

The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 4.0. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site (http://ctep.cancer.gov). The CTCAE displays Grades 1 through 5 with unique clinical descriptions of severity for each AE based on the following guideline:

Grade 1	Mild AE
Grade 2	Moderate AE
Grade 3	Severe AE
Grade 4	Life-threatening or disabling AE
Grade 5	Death related to AE

10.7. Serious Adverse Event Reporting

Events are classified as SERIOUS if they meet any of the following criteria [per the US Code of Federal Regulations (CFR) 21 CFR 312.32 and the recommendations of the International Conference on Harmonization (ICH)]:

An SAE is any sign, symptom or medical condition that emerges during the study or during a post-study follow-up period that 1) was not present at the start of the study and is not a chronic condition that is part of the patient's medical history, OR 2) was present at the start of the study or as part of the patient's medical history but worsened in severity and/or frequency during study participation, AND that meets any of the following regulatory serious criteria:

- any death
- any life-threatening event, i.e., an event that places the patient, in the view of the investigator, at immediate risk of death from the event as it occurred (does not include an event that, had it occurred in a more severe form, might have caused death)
- any event that requires or prolongs in-patient hospitalization
- any event that results in persistent or significant disability/incapacity
- any congenital anomaly/birth defect diagnosed in a child of a patient who participated in this study and received investigational drug
- other medically important events that in the opinion of the investigator may jeopardize the patient or may require intervention to prevent one of the other outcomes listed in the definition above (e.g. allergic bronchospasm requiring intensive treatment in an emergency room, convulsions occurring at home that do not require in-patient hospitalization, any blood dyscrasias, or the development of drug dependency or drug abuse).

Procedures for adverse event reporting

1. PI notified by medical staff of a SAE
   PI: Michael Morse, MD
   Address: Duke University Medical Center
   Seeley Mudd Building

Box 3233, Durham, NC 27710
Telephone: 1-919-681-3480
Fax: 1-919-681-7970
Pager 1-919-970-5626
Email: michael.morse@duke.edu

2. PI calls sponsor to report an unexpected SAE associated with the use of the drug

Sponsor: Carol Jones, Vice President of Administration
Address: 410 West Harrison Street, Suite 100, Seattle, WA 981119
Telephone: 206-838-5110 ext. 102
Cell: 1-206-818-2985
Fax: 1-206-838-2978
Email: cj@etubics.com

3. Carol Jones reports the SAE to one of the following in this order:

A. Chief Scientific Officer: Frank Jones, PhD
Address: 410 West Harrison Street, Suite 100, Seattle, WA 98119
Telephone: 206-838-5110 ext. 101
Cell: 206-818-2857
Fax: 206-838-2978
Email: frj@etubics.com

B. Laboratory Manager: Joseph Balint, PhD
Address: 410 West Harrison Street, Suite 100, Seattle, WA 98119
Telephone: 206-838-5110 ext. 107
Fax: 206-838-2978
Email: joe@etubics.com

C. Vice President Research: Elizabeth S. Gabitzsch
Address: 410 West Harrison Street, Suite 100, Seattle, WA 98119
Telephone: 206-838-5110 ext. 103
Cell: 970-402-2598
Fax: 206-838-2978
Email: beth@etubics.com

4. Contacted person in three (3) above notifies the FDA on MedWatch 3200A form.
   a. If the SAE results in death or is life-threatening, report the SAE to the FDA within 7 days
   b. All other SAEs must be reported to FDA within 15 days

5. If the SAE requires input from a physician then the Acting Medical Director is consulted:

H,. Kim Lyerly, MD
Telephone: (919) 684-5613
Fax: (919) 684-5653
Email: lyerl001@mc.duke.edu

All SAE reports will be recorded on the Duke IRB Adverse Event Reporting form and will be reviewed and signed by the Principal Investigator. Only adverse events that are deemed to be serious, unexpected and related or possibly related to the research must be reported to the IRB (this is in accordance with Duke's IRB reporting policy). All reportable events will be forwarded to the IRB via campus mail or fax:

IRB: Duke Medical Center Institutional Review Board:
Hock Plaza, 4th floor
2424 Erwin Road
Box 2991
Durham, NC 27705
Fax: 919-668-5125

In accordance with FDA regulations and ICH guidelines, investigators will be notified of the occurrence of new, serious, unexpected adverse events associated with the use of the study medication (i.e. there is a reasonable possibility that the adverse event may have been caused by the drug) within 15 calendar days via a written report. It is the responsibility of the investigator to promptly inform the relevant Institutional Review Board (IRB) of these new adverse events/risks to patients, in accordance with 21 CFR 312.66. It is also the responsibility of the investigator and the sponsor (Etubics) in conducting gene transfer research to promptly inform the NIH Office of Biotechnology Activities (OBA) and relevant Scientific Review Board of these new adverse events/risks to patients, in accordance with NIH Guidelines for Research Involving Recombinant DNA Molecules, in particular Appendix M. It is the responsibility of the sponsor (Etubics) to report these serious adverse events to the FDA. The SAE report will be forwarded to the FDA after recording the event data via the FDA MedWatch form 3500A.

MedWatch 3500A Reporting Guidelines:

In addition to completing appropriate patient demographic and suspect medication information, the report should include the following information within the Event Description (section 5) of the MedWatch 3500A form:

Treatment regimen (dosing frequency, combination therapy)

Protocol description (and number, if assigned)

Description of event, severity, treatment, and outcome, if known

Supportive laboratory results and diagnostics

Investigator's assessment of the relationship of the adverse event to each investigational product and suspect medication

Follow-up information:

Additional information may be added to a previously submitted report by any of the following methods:

- Adding to the original MedWatch 3500A report and submitting it as follow-up
- Adding supplemental summary information and submitting it as follow-up with the original MedWatch 3500A form

- Summarizing new information and faxing it with a cover letter including patient identifiers (i.e. D.O.B., initials, patient number), protocol description and number, if assigned, suspect drug, brief adverse event description, and notation that additional or follow-up information is being submitted (The patient identifiers are important so that the new information is added to the correct initial report.)

10.8. Assessing Causality:

Investigators are required to assess whether there is a reasonable possibility that study medications caused or contributed to an adverse event. The following general guidance may be used.

Yes: if the temporal relationship of the clinical event to study drug administration makes a causal relationship possible, and other drugs, therapeutic interventions or underlying conditions do not provide a sufficient explanation for the observed event.

No: if the temporal relationship of the clinical event to study drug administration makes a causal relationship unlikely, or other drugs, therapeutic interventions or underlying conditions provide a sufficient explanation for the observed event.

10.9. Safety Reporting Requirements for IND Holders

In accordance with 21 CFR 212.32, sponsor-investigators of studies conducted under an IND must comply with following safety reporting requirements:

a. Expedited IND Safety Reports:

***7 Calendar-Day Telephone or Fax Report*:**

The sponsor is required to notify the FDA of any fatal or life-threatening adverse event that is unexpected and assessed by the investigator to be possibly related to the use of the investigational product. An unexpected adverse event is one that is not already described in the Investigator's Brochure. Such reports are to be telephoned or faxed to the FDA within 7 calendar days of first learning of the event.

***15 Calendar-Day Written Report*:**

The sponsor is also required to notify the FDA and all participating investigators, in a written IND Safety Report, of any serious, unexpected AE that is considered possibly related to the investigational product. An unexpected adverse event is one that is not already described in the Investigator Brochure.

Written IND Safety Reports should include an Analysis of Similar Events in accordance with regulation 21 CFR § 312.32. All safety reports previously filed with the IND concerning similar events should be analyzed. The new report should contain comments on the significance of the new event in light of the previous, similar reports.

Written IND safety reports with Analysis of Similar Events are to be submitted to the FDA and all participating investigators within 15 calendar days of first learning of the event. The FDA prefers these reports on a MedWatch 3500A Form but alternative formats are acceptable (e.g. summary letter).

b. IND Annual Reports

In accordance with the regulation 21 CFR § 312.32, the sponsor shall within 60 days of the anniversary date that the IND went into effect submit a brief report of the progress of the investigation. Please refer to Code of Federal Regulations, 21 CFR § 312.32 for a list of the elements required for the annual report.

c. Data and Safety Monitoring

Data will be collected by: the principal investigator, co-investigators, and the protocol coordinator. The protocol coordinator under the supervision of the principal investigator will report the AEs. The data will be audited by the CPC and Etubics' CRA.

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12. APPENDIX 1 – SCHEMA

Procedure / Test	Pre-treatment [a]	Week 0 [a]	Week 3 [b]	Week 6 [b]	Week 9	Months 6, 9, etc. [c]	Off Treatment
H & P	X	X	X	X	X	X	X
Karnofsky Status	X	X	X	X	X	X	X
β-HCG	X						
CBC & diff	X	X	X	X	X	X	X
PT/PTT	X						
Chemistries/LFTS	X	X	X	X	X	X	X
Urinalysis	X						
Immune Monitoring	X	X	X	X	X	X [d]	X
MRI/CT Scan	X				X [e]	X	X
Immunization		X	X	X			

Notes: H & P = history & physical examination, Karnofsky = performance score of 70% or higher , β-HCG = human chorionic gonadotrophin pregnancy test, CBC & diff = complete blood count and white blood cell differential, MRI/CT = magnetic resonance imaging/computed tomography.

[a] CEA and or other biological markers and with tumor that are universally CEA positive testing for biomarkers will be performed at the discretion of PI

[b] Immunizations may be performed -1 to +7 days after the specified week. Subsequent immunizations should be 3 weeks afterwards and keep to the every 3 week interval.

[c] Follow-up evaluations to be performed every 3 months after the Week 9 visit.

[d] Immune monitoring if there was evidence of an immune response or at the discretion of the immune monitoring laboratory.

[e] MRI/CT scan to be requested 1-4 weeks after the third immunization.

DUKE UNIVERSITY HEALTH SYSTEM

Subject
Name:_____

Duke
History Number:_____

INTRODUCTION

You are being asked to take part in this research study because you have an advanced cancer that may have HER3 protein on it, but have had the cancer grow or come back despite prior treatment. HER3 is a protein expressed by some cancer cells including (but not limited to) those found in breast, colon, lung, prostate, ovarian, cervical, endometrial, gastric (stomach), pancreatic, bladder, head and neck, liver, and esophageal cancers. Research studies include only people who choose to take part. Please read this consent form carefully and take your time making your decision. As your study doctor or study staff discusses this consent form with you, please ask him/her to explain any words or information that you do not clearly understand. We encourage you to talk with your family and friends before you decide to take part in this research study. The nature of the study, risks, inconveniences, discomforts, and other important information about the study are listed below.

Please tell the study doctor or study staff if you are taking part in another research study.

This study is being conducted by Dr. Michael Morse it is funded by a grant from the U.S. Department of Defense (DOD). Portions of Dr. Morse's and his staff's salaries will be paid by these grants.

WHO WILL BE MY DOCTOR ON THIS STUDY?

If you decide to participate, Dr. Morse or your regular Duke cancer doctor will be your doctor for the study and will be in contact with your regular health care provider throughout the time that you are in the study and afterwards, if needed.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to find out what effects (good and bad) that a cancer vaccine has on you and your cancer. The cancer vaccine is called Ad5 [E1-, E2b-]-huHER3. HER3 is a protein. Proteins are made from a gene or gene product. Proteins are the building blocks of your body, cells, and organs. A gene contains information that determines in part the traits, such as eye color, height, or disease risk, that are passed on from parent to child. The vaccine in this study is made from a virus that contains a fragment of the HER3 gene. Once this virus is given to you as a vaccine, it can enter certain cells in your body such as some types of immune cells. This vaccine is based on a virus called an adenovirus but it has been changed to express the protein HER3 that is found on some cancer cells. The virus has been made to

transfer a copy of the gene fragment that makes the HER3 protein to your cells. This transfer of a gene fragment should cause extra copies of the HER3 protein to be made in your cells. These cells will break up the HER3 protein and show pieces of it to other immune cells to tell them to attack cancer cells expressing HER3.

Ad5 [E1-, E2b-]-huHER3 is an investigational drug. The word "investigational" means Ad5 [E1-, E2b-]-huHER3 is still being tested in research studies and has not been approved by the U.S. Food and Drug Administration (FDA).

This is the first time Ad5 [E1-, E2b-]-huHER3 has been used in humans. The goal of the study is to determine if Ad5 [E1-, E2b-]-huHER3 is safe and to see if there are any side effects that cause problems for the study participants. The study is also being done to see if AVX901 can cause an immune response against your cancer.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

Up to 40 people will take part in this study.

HOW DO THESE DRUGS WORK?

Ad5 [E1-, E2b-]-huHER3 is an investigational vaccine that causes immune cells to attack cancer cells expressing the HER3 protein.

WHAT IS INVOLVED IN THE STUDY?

Once you understand what is involved with participating in this study and all your questions have been answered, you will be asked to sign and date this consent form to show that you want to take part in this research study.

For your safety, you will be monitored to be sure any side effects are lessened. This will require regular clinic visits, blood and urine tests, and radiology tests (such as CT scans, which are a computerized series of x-rays) that are part of routine care. Routine care means that they are part of care that you would receive as part of treatment for your cancer even if you were not participating in this study. If you have side effects or have other medical problems, you may need more monitoring or other tests.

In addition, you will have some tests that are only for research purposes. These tests are not part of your regular care. All of the research tests are being done to help find out which

people are most likely to benefit and/or have side effects from this study drug combination. These additional tests are described below.

Research Tests for this Study:

Blood Tests for immunogenicity:

Immunogenicity tests show whether a drug can cause an immune response by the body. One way we can tell if the body is producing an immune response is by analyzing blood samples. This information will not change how you are treated, but it is hoped that this will provide information that may benefit future people with cancer. Six (6) tablespoons of blood will be taken before each dose of Ad5 [E1-, E2b-]-huHER3 and approximately 3 weeks after your final dose. Additional blood samples may be drawn during follow up investigations at the discretion of the investigator.

Study Drugs

The study vaccine will be injected under the skin on your thigh every 3 weeks for three injections total. Each time you will be requested to remain at the hospital or clinic for 30 minutes afterwards.

Your participation in this study is voluntary. If you do not sign this consent form, you will continue to receive care, but not as a part of this study.

Study Calendar

If you take part in this study, you will have the following tests and procedures which are listed in the following table and then explained below the table.

Procedure / Test	Pre-treatment	Week 0	Week 3	Week 6	Week 9	Months 6, 9, etc.	Off Treatment
H&P	X	X	X	X	X	X	X
KPS	X	X	X	X	X	X	X
Pregnancy Test	X						
CBC & diff	X	X	X	X	X	X	X
PT/PTT	X						
Chemistries	X	X	X	X	X	X	X
Urinalysis	X						
ANA	X				X		X
HIV	X						
Hepatitis B and C	X						
Immune Tests	X	X	X	X	X	X	X
Biological Markers	X	X			X	X	X
MRI/CT Scan	X				X	X	X
Injections of Vaccine		X	X	X			

DUKE UNIVERSITY HEALTH SYSTEM

Subject
Name:_____

Duke
History Number:_____

The following are tests that are part of regular cancer care and may be done even if you do not join the study. These tests and procedures may also be repeated throughout the study.

- H&P means a review of your medical history and physical exam
- KPS is the Karnofsky performance score – an evaluation of your general well-being
- CBC & diff and chemistries mean routine blood tests (about1 tablespoon of blood)
- PT/PTT is a test that measures how well your blood clots
- Biological markers: a blood test that has been done to look for evidence of your cancer (CEA expression, about 1 teaspoon of blood).
- MRI/CT Scan: A review of X-rays and scans of your disease that have been done

The following tests will be done evaluate your eligibility for the study and they will be part of your medical record.

- Pregnancy test (if you are a woman of childbearing potential) (about a teaspoon of blood will be taken from a vein in your arm)
- Test for hepatitis B and hepatitis C and for HIV (human immunodeficiency virus, which is the virus that causes the acquired immunodeficiency syndrome [AIDS]) (about a teaspoon of blood). You will be notified of the results of the testing, and counseled as to the meaning of the results, whether they are positive or negative. If the test indicates that you are infected with hepatitis B, hepatitis C or HIV, you will receive additional counseling about the significance of your care and possible risks to other people. We are required to report all positive results to the North Carolina Division of Public Health. The test results will be kept confidential to the extent permissible under the law. If you do not want to be tested for hepatitis B or hepatic C or HIV, then you should not agree to participate in this study. If you have hepatitis B or hepatitis C or HIV you cannot participate in this study because you may have immunosuppression that may render you unable to respond to the vaccine.
- ANA: A blood test that detects whether your immune system might be activated against your bodies normal cells
- Immune tests on your blood cells (up to 6 tablespoons of blood before each injection and after all the injections)

  If you are eligible to participate in the study, at each visit of the study, a medical history and a complete physical examination will be performed. You will have up to 100 mL of blood (9 tablespoons) drawn from a vein by needle stick, for routine tests such as blood count and chemistries, as well as for testing for research purposes only (tests to determine the response

of your immune system). The total amount of blood you will have drawn over the first 6 weeks of the study is about 30 tablespoons or a pint of blood.

HOW LONG WILL YOU BE IN THE STUDY?

You will be on the study until 1 month after the last injection. You may also choose to return for a follow up phase for this study. The follow up phase is optional. Long-term follow-up for research involving a viral vector containing a gene or gene fragment allows for the collection of important information on safety and side effects. We will also ask you to participate in the follow-up phase if you leave the study early.

The follow up phase consists of visits every 3 months for one year. During these visits, you will not receive study drug. At each of the visits, we will ask about any medical problems you have had and we may draw up to 6 tablespoons of blood to test your immune function.

Thereafter, we may contact you by phone, email or in person (if you come for a clinic visit) at least once a year for up to 15 years in order to obtain information about the long term safety of the study vaccine.

You can choose to stop participating in the study or in the long term follow-up at any time without penalty or loss of any benefits to which you are entitled. However, if you decide to stop participating in the study, we encourage you to talk to your doctor first. Please initial beside your choice below:

_____I agree to participate in the follow up phase of the study.

_____ I do not agree to participate in the follow up phase of the study.

Federal guidelines related to this type of cancer vaccine study require us to notify you that if you should die at any point after having received the study vaccine, we will request permission from your family for an autopsy to be performed to find out if there have been any unexpected effects of the study vaccine on your body.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study. Your doctor may decide to take you off this study if your condition gets worse, if you have serious side effects, or if your study doctor determines that it is no longer in your best interest to continue. The sponsor or regulatory agencies may stop this study at anytime without your consent. If this occurs, you will be notified and your study doctor will discuss other options with you.

WHAT ARE THE RISKS OF THE STUDY?

While on this study, you are at risk for side effects. You should discuss these with your doctor. In addition to the side effects listed below, there may be other side effects that the researchers cannot predict. Other drugs may be given to make side effects less serious and uncomfortable. Many side effects go away shortly after the medications are stopped but, in some cases, side effects can be serious, long-lasting and permanent or may result in death.

The study vaccine, which transfers a gene into your cells, is designed so that it should not be able to survive and grow in your body. It is not designed to make any long-lasting changes to your cells or your DNA, and should only be in your cells for a short time. Although a viral vector containing a gene can in rare cases cause a disease or a new cancer, the chance of this is very small. The Ad5 [E1-, E2b-]-huHER3 virus has been designed to minimize this risk.

The known side effects and possible risks of this research study are:

Study Vaccine Injections

Minor side effects could include:

- fever
- chills
- headache

Major side effects could include:

- allergic reactions with swelling in such places as your throat or lungs. This could lead to shortness of breath, breathing failure or death. Medications that might be needed to treat these side effects include antihistamines such as diphenhydramine (Benadryl), epinephrine and corticosteroids.
- your immune system could be stimulated to attack your own body (called autoimmunity) leading to a low white blood cell count which could lead to developing an infection, skin rash, joint swelling, intestinal inflammation (chronic colitis), or fluid around the heart and lungs.
- Liver toxicity

Medications may be given to make any side effects you experience less serious and uncomfortable.

There may also be risks, discomforts, drug interactions or side effects that are not yet known. Some side effects may occur that do not need medical attention and may go away while you are receiving the study drug as your body adjusts. Others may be severe and need immediate attention or lead to hospitalization. Seek emergency care and contact your doctor immediately if severe side effects occur.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study. Your doctor may decide to take you off this study if your condition gets worse, if you have serious side effects, or if your study doctor determines that it is no longer in your best interest to continue. The sponsor or regulatory agencies may stop this study at any time without your consent. If this occurs, you will be notified and your study doctor will discuss other options with you.

For Those of Reproductive Potential
Female
Being a part of this study while pregnant may expose the unborn child to significant risks, some of which may be unforeseeable. Therefore, pregnant women will be excluded from the study. If you are a woman of childbearing potential, a blood pregnancy test will be done (using 1 teaspoon of blood drawn from a vein by needle-stick), and it must be negative before you can continue in this study. If sexually active, you must agree to use appropriate contraceptive measures for the duration of the study and for 1 month after your last dose of the vaccine. Medically acceptable contraceptives include: (1) surgical sterilization (such as a tubal ligation or hysterectomy), (2) approved hormonal contraceptives (such as birth control pills, patches, implants or injections), (3) barrier methods (such as a condom or diaphragm) used with a spermicide, or (4) an intrauterine device (IUD). Contraceptive measures such as Plan B(TM), sold for emergency use after unprotected sex, are not acceptable methods for routine use. If you do become pregnant during this study or if you have unprotected sex, you must inform your study physician immediately. You also must agree not to breastfeed your child for the duration of the study and for 1 month after the last dose of the vaccine.

Male
The study drug used in this study could affect your sperm and could potentially harm a child that you may father while on this study. Such harm may be currently unforeseeable. You must agree to avoid fathering a child during the course of this study and for a period of 1 month after the study vaccinations are completed due to unknown risks of the study vaccine to a mother and/or developing fetus. If you are sexually active, you must agree to use at least one medically acceptable form of birth control during and for a period of 1 month after the last vaccine. Medically acceptable contraceptives include: (1) surgical sterilization, or an (2) impermeable condom used with a spermicide. You should inform your partner of the potential

for harm to an unborn child. She should know that if pregnancy occurs, you will need to report it to the study doctor, and she should promptly notify her doctor.

Additional Risks Related to Study Procedures
Financial Risk
There may be financial risk in participating in this study. It is your responsibility to contact your insurance provider to discuss your coverage prior to making a decision to participate in this study (not all insurance providers cover clinical trials). Please discuss this thoroughly with your family, doctor and insurance provider(s) to make sure all your questions have been answered.

Blood draws: Taking blood from a vein in your arm by needle stick. Risks associated with drawing blood from your arm include momentary discomfort and/or bruising. Infection, excess bleeding, clotting, or fainting are also possible, although unlikely.

For all study participants:
You should not donate blood while you are in this study and for possibly longer. If you are male, you should also not donate any sperm while you are in this study. Please discuss with your study doctor how long you should wait before donating any blood or, if applicable, sperm.

CAN I CONTINUE TO TAKE MY CURRENT MEDICATIONS?

To participate in this study, you may need to stop or change some of your current medications because they may affect how well the study drugs work. For your safety, you must tell the study doctor or nurse about all the prescribed medical foods and drugs, herbal products, over-the-counter (OTC) drugs, vitamins, natural remedies, and alcohol that you are taking before you start the study and before starting to take any of these products while you are on the study. You will be notified if any of your current medications need to be stopped or changed to allow you to participate in this study. If there are any changes, your doctor will let you know of any risks that may be associated with changes in your current medications. If you participate in this study, there may also be limitations to the other medications and supplements or foods that you can take while you are taking the study drug. Changing or limiting the medications you take for other conditions may be associated with additional inconvenience, costs, and/or side effects.

There may be risks, discomforts, drug interactions or side effects that are not yet known.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

There is no guarantee that being in this study may help you. Your cancer might not get any better while you are in this study. If you do not personally benefit, the knowledge learned from your participation may help doctors and researchers learn more about the use of these study drugs to help other people with cancer.

WHAT OTHER OPTIONS ARE THERE?

You do not have to be in this study to get care for your cancer. Instead of being in this study, you could take standard chemotherapy chosen by your physician or participate in another clinical research study if one were available to you or choose to take no further cancer therapy. Please talk to your doctor about these and other options.

WHAT ABOUT CONFIDENTIALITY?

Study records that identify you will be kept confidential as required by law. Federal Privacy Regulations provide safeguards for privacy, security, and authorized access. Except when required by law, or as outlined in this consent, you will not be identified by full name, social security number, address, or telephone number in study records disclosed outside of Duke University Health System (DUHS). However, your initials, dates of service, date of birth, or other identifiers may be disclosed. For records disclosed outside of DUHS, you will be assigned a unique code number. The key to the code will be kept in a locked file in Dr. Morse's office. This is to protect your study data by making it anonymous for most study purposes.

Because this study involves a viral vector containing a human gene, safety information must be reported to the Recombinant DNA advisory Committee (RAC) of the National Institutes of Health. This information is available to the public. However, no information by which you can be identified will be reported with the safety information.

As part of the study, Dr. Morse and his study team will report the results of tests and procedures related to your participation in this study. Some of these studies would have been done as part of your regular care. These test results will be recorded in your medical record. Your doctor will use them both to guide your treatment and to complete this research. The results of these tests will be reported to the data office at Duke and may be reported to DOD. Results of medical tests and studies done solely for this research study and not as part of your regular care will also be included in your medical record. The results of the immune

monitoring tests will be performed only for research and will be recorded in your research record but not in your medical record.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: Food and Drug Administration, Duke University Health System Institutional Review Board, Duke Cancer Institute, Recombinant DNA advisory Committee (RAC) of the National Institutes of Health, and the U.S. DOD. If your research record is reviewed by any of these groups, they may also need to review your entire medical record. If this information is disclosed to outside reviewers for audit purposes, it may be further disclosed by them and may not be covered by the federal privacy regulations.

The study results will be retained in your research record for at least 15 years or until after the study is completed, whichever is longer. At that time either the research information not already in your medical record will be destroyed or information identifying you will be removed from the study results at DUHS. Any research information in your medical record will be kept indefinitely. Data that has been sent outside of DUHS may be further disclosed. If it is further disclosed, the information is no longer covered by the federal privacy regulations.

While the information and data resulting from this study may be presented at scientific meetings or published in a scientific journal, your identity will not be revealed.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

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ARE THERE COSTS TO ME FOR PARTICIPATING IN THIS RESEARCH?

The Ad5 [E1-, E2b-]-huHER3 is provided free of charge for use in this study. There will be no additional costs to you as a result of being in this study. However, routine medical care for your condition (care you would have received whether or not you were in this study) will be charged to you or your insurance company. You may wish to contact your insurance representative to discuss this further before making your decision about participating in the study. In order to make sure that tests and studies done solely for research purposes are charged correctly, your Duke Hospital and Clinic charges will be closely monitored as long as you are participating in this study.

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DUKE UNIVERSITY HEALTH SYSTEM

Subject
Name:_____

Duke
History Number:_____

WHAT ABOUT COMPENSATION?

You will not be paid for your participation in this study.

WHAT ABOUT RESEARCH RELATED INJURIES?

Immediate and necessary medical care is available at Duke University Medical Center in the event that you are injured as a result of your participation in this research study. However, there is no commitment by Duke University, Duke University Health System, Inc., your Duke physician, the study funding sources U.S. DOD, to provide monetary compensation or free medical care to you in the event of a study-related injury. For questions about the study or a research related injury, contact Michael Morse, M.D. at 919-684-5705 during regular business hours and at 919-970-5626 after hours and on weekends and holidays.

FUTURE RESEARCH

There may be blood samples left over from this study. If you are willing to allow these samples to be used for research purposes that are not specifically related to this study, you will be asked to sign a separate consent form. Participation in this future research is optional and no matter what you choose, it will not affect your participation in the rest of this study.

WHAT IF I WANT TO LEAVE THE STUDY?

Participation in this study is voluntary. You are free to withdraw your consent and to discontinue participation in the study at any time.

If you withdraw from the study, no new data about you will be collected for study purposes unless the data is concerning an adverse event (a bad effect) related to the study. If such an adverse event occurs, we may need to review your entire medical record. All data that have already been collected for study purposes, and any new information about an adverse event related to the study, will be sent to the study sponsor.

Your decision not to participate or to withdraw from the study will not involve any penalty or loss of benefits to which you are entitled, and will not affect your access to health care at Duke. If you do decide to withdraw, we ask that you contact Dr. Morse in writing and let him know that you are withdrawing from the study.

His mailing address is:

c/o Protocol Office
Box 3233
DUMC
Durham, NC 27710.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Your doctor may decide to take you off this study if your condition gets worse, if you have serious side effects, or if your study doctor determines that it is no longer in your best interest to continue. If it is discovered that you did not give an accurate medical history or did not follow the instructions for the study given by your Study Doctor and/or study nurse you may be taken off the study at any time. If you are taken off the study, you will no longer receive the study drug. The sponsor or regulatory agencies may stop this study at any time without your consent. If this occurs, you will be notified and your study doctor will discuss other options with you.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, or to discuss problems, concerns or suggestions related to the research, or to obtain information or offer input about the research, contact Dr. Morse at (919) 668-1861 during regular business hours. After hours and on weekends and holidays page Dr. Morse at (919) 970-5626 or call (919)-684-8111 (the Duke paging operator), and ask the operator to page Dr. Michael Morse.

For questions about your rights as a research participant or to discuss problems, concerns or suggestions related to the research, or to obtain information or offer input about the research, contact the Duke University Health System Institutional Review Board (IRB) Office at (919) 668-5111.

DUKE UNIVERSITY HEALTH SYSTEM

Subject
Name:_____

Duke
History Number:_____

SIGNATURE

"The purpose of this study, procedures to be followed, risks and benefits have been explained to me. I have been allowed to ask questions, and my questions have been answered to my satisfaction. I have been told whom to contact if I have questions, to discuss problems, concerns, or suggestions related to the research, or to obtain information or offer input about the research. I have read this consent form and agree to be in this study, with the understanding that I may withdraw at any time. I have been told that I will be given a signed and dated copy of this consent form."

_____
Printed Name of Subject

_____ _____ _____
Signature of Subject Date Time

"I have explained the research to the subject and answered all of his/her questions. I believe that he/she understands the information described in this document and freely consents to participate."

_____
Printed Name of Person Obtaining Consent

_____ _____ _____
Signature of Person Obtaining Consent Date Time

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ORIGINAL ARTICLE

Novel adenoviral vector induces T-cell responses despite anti-adenoviral neutralizing antibodies in colorectal cancer patients

Michael A. Morse · Arvind Chaudhry · Elizabeth S. Gabitzsch · Amy C. Hobeika · Takuya Osada · Timothy M. Clay · Andrea Amalfitano · Bruce K. Burnett · Gayathri R. Devi · David S. Hsu · Younong Xu · Stephanie Balcaitis · Rajesh Dua · Susan Nguyen · Joseph P. Balint Jr. · Frank R. Jones · H. Kim Lyerly

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Abstract First-generation, E1-deleted adenovirus subtype 5 (Ad5)-based vectors, although promising platforms for use as cancer vaccines, are impeded in activity by naturally occurring or induced Ad-specific neutralizing antibodies. Ad5-based vectors with deletions of the E1 and the E2b regions (Ad5 [E1-, E2b-]), the latter encoding the DNA polymerase and the pre-terminal protein, by virtue of diminished late phase viral protein expression, were hypothesized to avoid immunological clearance and induce more potent immune responses against the encoded tumor antigen transgene in Ad-immune hosts. Indeed, multiple homologous immunizations with Ad5 [E1-, E2b-]-CEA(6D), encoding the tumor antigen carcinoembryonic antigen (CEA), induced CEA-specific cell-mediated immune (CMI) responses with antitumor activity in mice despite the presence of preexisting or induced Ad5-neutralizing antibody. In the present phase I/II study, cohorts of patients with advanced colorectal cancer were immunized with escalating doses of Ad5 [E1-, E2b-]-CEA(6D). CEA-specific CMI responses were observed despite the presence of preexisting Ad5 immunity in a majority (61.3 %) of patients. Importantly, there was minimal toxicity, and overall patient survival (48 % at 12 months) was similar regardless of preexisting Ad5 neutralizing antibody titers. The results demonstrate that, in cancer patients, the novel Ad5 [E1-, E2b-] gene delivery platform generates significant CMI responses to the tumor antigen CEA in the setting of both naturally acquired and immunization-induced Ad5-specific immunity.

Keywords Immunotherapy · Ad5 vector · CEA · Cell-mediated immunity

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M. A. Morse (✉) · D. S. Hsu
Department of Medicine, Duke University Medical Center, Rm 403 MSRB 1, Box 3233, Durham, NC 27710, USA
e-mail: morse004@mc.duke.edu

M. A. Morse · B. K. Burnett · D. S. Hsu · H. K. Lyerly
Duke Cancer Institute, Durham, NC 27710, USA

A. Chaudhry
Medical Oncology Associates, Spokane, WA 99208, USA

E. S. Gabitzsch · Y. Xu · S. Balcaitis · R. Dua · S. Nguyen · J. P. Balint Jr. · F. R. Jones
Etubics Corporation, Seattle, WA 981119, USA

A. C. Hobeika · T. Osada · G. R. Devi · H. K. Lyerly
Department of Surgery, Duke University Medical Center, Durham, NC 27710, USA

T. M. Clay
R&D Immunotherapeutics, GlaxoSmithKline Biologicals, Rue de l'Institut 89, 1300 Rixensart, Belgium

A. Amalfitano
Michigan State University, East Lansing, MI 48824, USA

Introduction

Cancer immunotherapy achieved by delivering tumor-associated antigens (TAA) has recently demonstrated survival benefits [1, 2]; however, limitations to these strategies exist and more immunologically potent vaccines are needed. To address the low immunogenicity of self-tumor antigens, a variety of advanced, multi-component vaccination strategies including co-administration of adjuvants and immune-stimulating cytokines have been employed [3, 4]. Alternatives include the use of recombinant viral vectors that inherently provide innate pro-inflammatory signals while simultaneously engineered to express the antigen of interest. Of particular interest are adenovirus serotype-5 (Ad5)-based immunotherapeutics that have been repeatedly used in humans to induce robust T-cell-mediated immune (CMI) responses all while maintaining an extensive safety profile [5–7]. In addition, Ad5 vectors can be reliably manufactured in large quantities and are stable for storage and delivery for outpatient administration [6–8]. Nonetheless, a major obstacle to the use of first-generation (E1-deleted) Ad5-based vectors is the high frequency of preexisting anti-adenovirus type 5 neutralizing antibodies. These antibodies can be present in a potential vacinee due to either prior wild-type adenovirus infection [8, 9] or induction of adenovirus neutralizing antibodies by repeated injections with Ad5-based vaccines, each resulting in inadequate immune stimulation against the target TAA [10].

Attempts to overcome anti-Ad immunity have included use of alternative Ad serotypes and/or alternations in the Ad5 viral capsid protein, each with limited success and the potential for significantly altering biodistribution of the resultant vaccines. Therefore, a completely novel approach was attempted by further reducing the expression of viral proteins from the E1-deleted Ad5 vectors, proteins known to be targets of preexisting Ad immunity. Specifically, a novel recombinant Ad5 platform has been described with deletions in the early 1 (E1) gene region and additional deletions in the early 2b (E2b) gene region (Ad5 [E1-, E2b-]) [11]. Deletion of the E2b region (that encodes DNA polymerase and the pre-terminal protein) results in decreased viral DNA replication and late phase viral protein expression. This vector platform has been previously reported to successfully induce CMI responses in animal models of cancer and infectious disease [10, 12–18], and more importantly, this recombinant Ad5 gene delivery platform overcomes the barrier of Ad5 immunity and can be used in the setting of preexisting and/or vector-induced Ad immunity [10, 12–19], thus enabling multiple homologous administrations of the vaccine. We have constructed and tested an Ad5 [E1-, E2b-] platform containing a gene insert for the tumor antigen carcinoembryonic antigen (CEA) with a modification that enhances T-cell responses (Ad5 [E1-, E2b-]-CEA(6D) [12, 16, 19, 20]. Multiple immunizations with this Ad5 platform induced CEA-specific CMI responses with antitumor activity despite the presence of existing Ad5 immunity in mice [12, 16]. We now present results of a first-in-man, phase I/II clinical trial to determine the safety and immunogenicity of dose escalation of the Ad5 [E1-, E2b-]-CEA(6D) vector in advanced stage colorectal cancer patients to determine whether CMI could be induced and whether there was an effect on clinical outcome relative to the existence of preexisting Ad5 immunity.

Methods

Construction and production of Ad5 [E1-, E2b-]-CEA(6D)

The cDNA sequence containing the modified CEA with the CAP1(6D) mutation was produced at Duke University [21]. Clinical grade Ad5 [E1-, E2b-]-CEA(6D) was constructed as previously described [12] and manufactured using the E.C7 cell line [12] under GMP at SAFC, Carlsbad, California, and provided by Etubics Corporation.

Protocol schema and patient treatment

The clinical study was performed under an FDA-approved Investigational New Drug Exemption (IND14325) and registered at ClinicalTrials.gov (NCT01147965). Participants were recruited from medical oncology clinics at Duke University Medical Center, Durham, NC, and Medical Oncology Associates, Spokane, WA. Patients provided informed consent approved by the respective Institutional Review Boards (IRB). Eligibility requirements included metastatic cancer expressing CEA and adequate hematologic, renal, and hepatic function. Trial participants were required to have received treatment with standard therapy known to have a possible overall survival benefit or refused such therapy. Exclusion criteria included chemotherapy or radiation within the prior 4 weeks, history of autoimmune disease, viral hepatitis, HIV, or use of immunosuppressives. Patients who had been receiving bevacizumab or cetuximab for at least 3 months prior to enrollment were permitted to continue receiving these antibodies. Prior CEA immunotherapy was permitted. The study employed a standard 3 + 3 dose escalation strategy with dose-limiting toxicities (DLT) defined as grade 3 or 4 major organ toxicity. The Ad5 [E1-, E2b-]-CEA(6D) doses were delivered to patients as follows: cohort 1: dose of $1X10^9$ VP in 0.5 ml subcutaneously (SQ) in the same thigh every 3 weeks for 3 treatments; cohort 2: dose of $1X10^{10}$ VP in 0.5 ml SQ every 3 weeks for 3 treatments; cohort 3: dose of 1×10^{11} in 0.5 ml SQ every 3 weeks for 3 treatments.

Following the establishment of the dose of 1×10^{11} VP as safe, an additional 12 patients received Ad5 [E1-, E2b-]-CEA(6D) at this dose and schedule (phase II cohort). After completing the phase II cohort, an additional cohort (cohort 5) of six patients received a dose of 5×10^{11} VP in 2.5 ml SQ every 3 weeks for 3 treatments to determine safety of the highest achievable dose. PBMCs were collected from patients just prior to the immunizations at weeks 0, 3, 6, and three weeks following the last treatment. The PBMCs were frozen in liquid nitrogen until ELISPOT assays were performed. In cohort 5, fresh PBMCs were analyzed in preliminary flow cytometry assays for polyfunctional CD8+ T lymphocytes.

Assessment of clinical activity

Clinical activity was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST 1.0 criteria [22]) using computed tomography (CT) or magnetic resonance imaging (MRI) scans obtained at baseline and after treatments were completed. Toxicity was assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 [23]. Peripheral blood CEA levels, hematology, serum chemistries, and anti-nuclear antibody titers were compared at baseline and 3 weeks following the final treatment. Survival was measured from the day of the first immunization until death from any cause.

Analysis of CMI responses by ELISPOT assay

An ELISPOT assay for IFN-γ-secreting lymphocytes was adapted from our previous animal studies and performed as described [12]. Briefly, isolated PBMCs (2×10^5 cells/well) from individual patient samples were incubated 36–40 h with a CEA peptide pool (15mers with 11aa overlap covering full-length CEA with the 6D modification; 0.1 μg/well) to stimulate IFN-γ-producing T cells. CMI responses to Ad5 were determined after exposure of patient PBMC to Ad5 null (empty vector). Cells stimulated with concanavalin A (Con A) at a concentration of 0.25 μg/well served as positive controls. Colored spot-forming cells (SFC) were counted using an Immunospot ELISPOT plate reader (Cellular Technology, Shaker Heights, OH), and responses were considered to be positive if 50 SFC were detected/10^6 cells after subtraction of the negative control and SFC were ≥twofold higher than those in the negative control wells.

Determination of Ad5 neutralizing antibody (NAb) titers

Endpoint Ad5 NAb titers were determined as previously described [12–14]. Briefly, dilutions of heat-inactivated test sera in 100 μL of DMEM containing 10 % fetal calf serum were mixed with 4×10^7 VP of Ad5 [E1-]-null and incubated for 60 min at room temperature. The samples were added to microwells containing HEK293 cells cultured in DMEM containing 10 % heat-inactivated calf serum at 2×10^3 cells/well for 24 h at 37 °C in 5 % CO_2. The mixture was incubated for an additional 72 h at 37 °C in 5 % CO_2. An MTS tetrazolium bioreduction assay (Promega Corp. Madison, WI) [24] was used to measure cell killing and endpoint Ad5 NAb titers. Endpoint titers with a value less than 1:25 were assigned a value of 0.

Statistics

Statistical analyses comparing immune responses were performed employing the Mann–Whitney test (PRISM, GraphPad). Survival comparisons were made employing Kaplan–Meier plots (PRISM, GraphPad). Ad5 NAb titer and CEA-specific CMI were analyzed as continuous variables. The association of Ad5 NAb titer with change in CEA-specific CMI was tested with the Spearman correlation coefficient. The association of Ad5 NAb titer with survival was tested with the Wald test of the proportional hazards model. All tests used a two-sided alpha of 0.05.

Results

Patient demographics and safety and tolerability

Thirty-two patients with metastatic colorectal cancer, median age 57.5 (range 38–77) who had failed a median of three prior chemotherapeutic regimens (range 2–5), had a performance status of 90 % (range 70–100 %), and had three sites of metastatic disease (range 1–4), were enrolled (Table 1). The majority were able to receive all three immunizations. All four patients who stopped immunizations early did so due to significant disease progression. There was no dose-limiting toxicity and no serious adverse events (SAE) that resulted in treatment discontinuation at any vaccine dose level. The most common toxicity (see Supplemental Table 1) was a self-limited, injection site reaction. Other reactions occurred with less than a 10 % incidence and included fever, flu-like symptoms, anorexia, chills, nausea, and headache. These symptoms were also self-limiting and did not require intervention other than symptomatic measures such as acetaminophen. Routine hematology and chemistry studies showed no significant biologic changes during the immunization period (Supplemental Table 2). In particular, the total lymphocyte count remained stable (pre and post). Overall, comparisons of ANA titers at baseline and 3 weeks after the last immunization revealed no significant difference in values across all patient groups (Supplemental Table 2).

Table 1 Patient demographics

Patient ID/cohort	Dose (VP)	Dx	Age	Sex	KPS	# prior CTx	Mets (# of sites)	# of doses	++Disease Status after tx	Survival (Months)
002/1	10^9	C	67	M	70	>3	4	3	PD	3 (−)
003/1	10^9	R	63	M	100	5	2	3	PD	9 (−)
004/1	10^9	C	53	F	100	2	3	3	PD	11 (−)
005/2^	10^{10}	C	60	M	100	3	3	3	SD	12 (+)
007/2	10^{10}	C	52	M	80	2	5	1	PD	1 (−)
008/2	10^{10}	C	42	F	100	3	3	3	PD	12 (+)
010/2	10^{10}	C	58	M	90	3	3	3	PD	12 (−)
011/3	10^{11}	R	50	M	100	5	1	3	PD	12 (+)
012/3	10^{11}	C	48	M	100	1	2	3	PD	12 (+)
013/3	10^{11}	R	62	M	100	3	2	3	PD	4 (−)
500/3	10^{11}	C	55	M	80	4	3	3	PD	12 (+)
015/3	10^{11}	C	58	F	80	3	4	3	PD	10 (−)
016/3@	10^{11}	C	53	F	100	3	4	3	PD	6 (−)
017/3*	10^{11}	R	52	F	90	3	2	3	PD	3 (−)
501/II	10^{11}	R	54	M	90	1	1	3	PD	12 (+)
502/II	10^{11}	C	66	F	80	1	2	2	PD	3 (−)
019/II	10^{11}	C	69	M	90	1	3	3	PD	12 (+)
020/II^	10^{11}	C	59	M	100	5	4	3	SD	12 (+)
021/II^	10^{11}	C	51	F	100	4	3	3	PD	12 (+)
506/II	10^{11}	C	77	F	80	2	2	3	PD	3 (−)
023/II	10^{11}	C	51	F	100	3	4	3	PD	4 (−)
504/II	10^{11}	C	57	M	90	3	3	3	PD	12 (+)
507/II	10^{11}	R	58	M	90	2	2	3	PD	12 (+)
024/II	10^{11}	C	67	M	90	2	3	3	PD	12 (+)
025/II	10^{11}	C	62	F	100	2	4	3	PD	7 (−)
026/II	10^{11}	C	53	M	100	3	2	2	PD	4 (−)
030/5	5×10^{11}	C	38	M	90	4	3	3	PD	10 (+)
031/5	5×10^{11}	R	72	F	90	4	2	3	SD	9 (+)
032/5@	5×10^{11}	R	53	M	90	4	3	3	PD	6 (−)
033/5	5×10^{11}	R	48	F	90	>3	2	3	PD	5 (−)
034/5	5×10^{11}	C	62	M	100	5	4	3	PD	7 (+)
035/5	5×10^{11}	C	60	F	90	3	5	2	PD	2 (−)

Dx diagnosis, *C* colon, *R* rectal cancer, *KPS* Karnofsky performance status, *PD* progressive disease, *SD* stable disease

* concurrent cetuximab; ^concurrent bevacizumab; @ concurrent panitumumab

++Represents disease status at 9 weeks post-initiation of immunizations

(+) Alive; (−) Dead at last follow-up

Determination of Induced CMI Responses to CEA

ELISPOT analysis was performed on cryopreserved PBMC samples drawn before each immunization and after the completion of the final immunization to assess CEA-specific CMI responses. We observed a dose–response effect with the highest magnitude CEA-specific CMI responses occurring in patients who received the highest dose of Ad5 [E1-, E2b-]-CEA(6D) (Fig. 1). Of the doses received, 0/3 (0 %) patients in cohort 1 exhibited positive CEA-directed CMI responses, 1/4 (25 %) patient in cohort 2 exhibited positive CEA-directed CMI responses, 10/19 (53 %) patients in cohort 3/phase II exhibited positive CEA-directed CMI responses, and 4/6 (67 %) patients in cohort 5 exhibited positive CEA-directed CMI responses. The time course of induction of CEA-specific CMI (Supplemental Fig. 1) demonstrated that there may be plateau in the magnitude of CEA CMI prior to the last dose although small numbers could affect this finding. In the largest group of patients who received the same dose (cohort 3 plus phase II), we observed a significant increase over baseline in the average CEA-directed CMI responses at the week 6

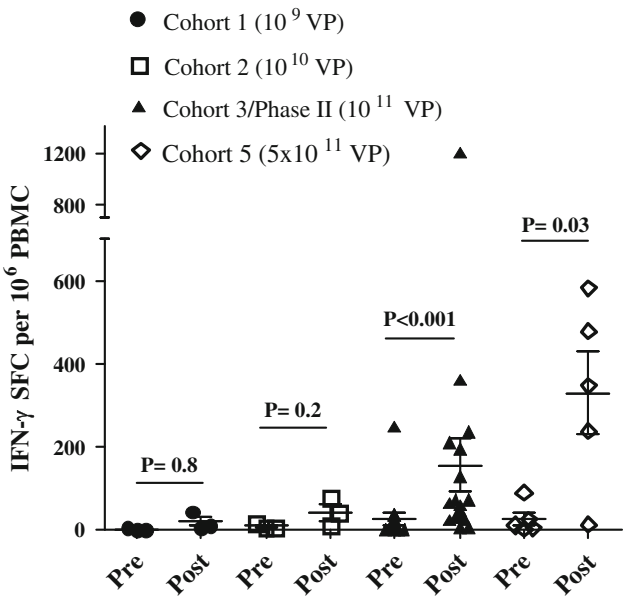

Fig. 1 CEA-directed CMI responses in treated patients. CMI (IFN-γ secretion) was assessed at baseline (pre) and after administrations of Ad5 [E1-, E2b-]-CEA(6D) (post). The highest CMI responses (regardless of time point) observed in the patients after treatment revealed a dose response. The highest CMI levels occurred in patients that received the highest dose of 5×10^{11} VP (Cohort 5). The CMI responses for cohort 3/phase II and cohort 5 were significantly elevated (*Mann–Whitney test*) as compared to their baseline (pre) values. Specificity of the responses was demonstrated by the lack of reactivity with the irrelevant antigens β-galactosidase and HIV-gag (data not shown). For positive controls, PBMCs were exposed to concanavalin A (data not shown). *Horizontal line* and *error bar* indicate the mean ± SEM for each cohort

evaluation ($P < 0.05$, Mann–Whitney test), averaging 94 SFC/10^6 PBMC, which increased further by the week 9 evaluation (Supplementary Fig. 1). One patient (patient ID 13) had a highly elevated baseline CEA-specific immune response (1100 SFC) and had elevated CMI at week six (2305 SFC) but did not return for week 9 evaluation and therefore was not included in CEA CMI data analysis.

We also measured Ad5 NAb and CMI against Ad5 and correlated it with CEA-specific CMI. Each patient had their serum and PBMC sample tested at baseline (prior to treatment) and at 9 weeks after completion of 3 treatments. Nineteen of 31 patients (61.3 %) tested in this study had Ad5 neutralizing activity in serum samples prior to the onset of treatment with the CEA(6D)-expressing Ad vaccine. The mean pre-treatment Ad5 NAb titer value obtained among all patients was 1:189 ± 1:71 SEM (geometric mean 1:21), and the mean pre-treatment Ad5 NAb titer among seropositive patients was 1:308 ± 1:108 (geometric mean 1:146). Analysis of serum samples from patients who received 3 immunizations revealed Ad5 NAb titers that were significantly increased ($P < 0.0001$, *Mann–Whitney test*) by week 9 (mean 1:4767 ± 1:1225 SEM) (geometric mean 1:1541) when compared with their respective baseline values (Fig. 2a). Analysis of PBMC for CMI responses to Ad5 also revealed a significant increase ($P < 0.01$, Mann–Whitney test) in Ad5-directed CMI responses after immunizations with Ad5 [E1-, E2b-]-CEA(6D) (Fig. 2b). Only ELISPOT assays were performed for CMI, and we did not assess the relative contribution of CD4+ and CD8+ T cells; thus, it is unclear whether both cell types are responding or whether responses are associated preferentially from one group.

Comparison of week 9 CEA-directed CMI responses from patients with low baseline preexisting Ad5 immunity (Ad5 NAb ≥200) versus those with high baseline Ad5 immunity (Ad5 NAb >200) revealed no significant difference in responses ($P > 0.4$, Mann–Whitney test). Further, when the highest CEA-specific CMI responses were compared with preexisting or vector-induced Ad5 NAb activity, there was no correlation between levels of CEA CMI and Ad5 NAb activity (Fig. 3). These data indicate that immunizations with Ad5 [E1-, E2b-]-CEA(6D) were able to induce CEA-specific immune responses in colorectal cancer patients despite the presence of existing and/or immunization-induced Ad5 immunity.

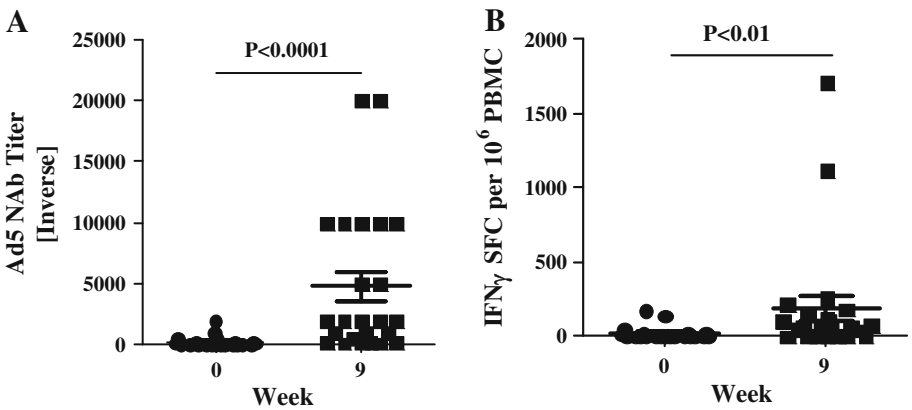

Fig. 2 Ad5 immune responses. Ad5 NAb titers **a** and CMI responses **b** to Ad5 were determined in patients at baseline (week 0) and 3 weeks (week 9) after the third immunization. The number of IFN-γ-secreting PBMCs from patients that were specific for Ad5 was determined by ELISPOT. Both the Ad5 NAb titers and Ad5 CMI responses were significantly elevated at week 9 (*Mann–Whitney test*). *Horizontal line* and *error bar* indicate the mean ± SEM

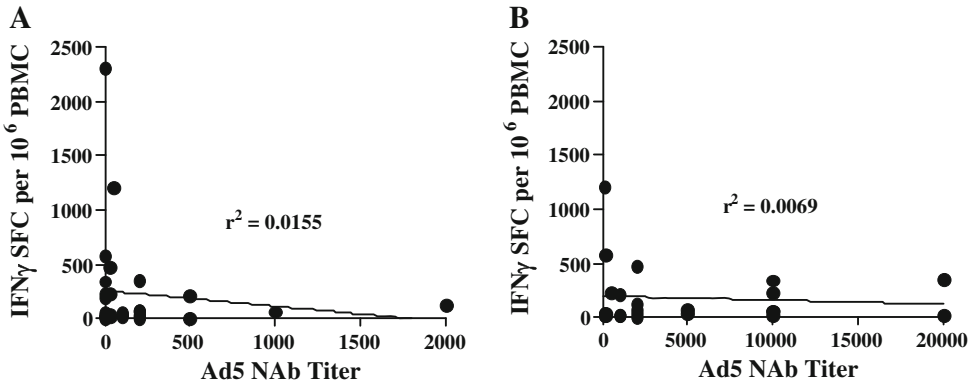

Fig. 3 CEA-specific immunity in patients and comparisons with Ad5 immunity. Correlation between preexisting Ad5 NAb activity and highest levels of induced CEA CMI responses **a**. Correlation between vector-induced Ad5 NAb activity and CEA CMI responses **b**. The r^2 values revealed no correlation between preexisting or vector-induced Ad5 NAb activity and CEA CMI ELISPOT responses

Clinical outcomes

Carcinoembryonic antigen levels in serum at baseline and week 9 were assessed in patients. Among those with CEA levels available at baseline and follow-up, three had no increase in CEA levels at the end of the immunization period while the remaining patients showed increased CEA levels. There were three patients with stable disease who remained so during the 9-week study period. All other patients experienced some level of progressive disease (Table 1). Patients in cohorts 1, 2, 3, and phase II who received at least 2 treatments ($n = 25$) were followed for survival and Kaplan–Meier plots and survival probabilities performed. Patients in cohort 5 ($n = 6$) have not completed the 12-month follow-up period and, therefore, were not evaluated for survival by Kaplan–Meier plots. Six patients in cohorts 1 and 2 experienced a 12-month survival probability of 33.3 % (Fig. 4). Nineteen patients in the combined group of cohort 3 and phase II experienced a 12-month survival probability of 52.6 % (Fig. 4). With a median follow-up of 12 months, all 25 patients as a group (cohorts 1, 2, 3, and phase II) experienced a 12-month survival probability of 48 % (Fig. 4). There was no association between Ad5 NAb and survival using Ad5 NAb both as a continuous variable and as a variable dichotomized between <200 and ≥200 (P values 0.48 and 0.44, respectively). These data indicate that preexisting Ad5 NAb did not significantly impact survival outcomes following immunization with the Ad5 [E1-, E2b-]-CEA(6D) vaccine.

Discussion

Adenoviral vectors have significant potential for use as cancer therapeutic vaccines because of their propensity to induce robust adaptive immune responses specifically against transgene products in general; however, recombinant first-generation Ad5 [E1-] vectors used in homologous prime/boost regimens have been greatly limited in their potential efficacy due to the presence of preexisting Ad5 immunity as well as vector-induced immunity [7–10]. Specifically, Ad5-directed immunity mitigates immune responses to TAA that have been incorporated into earlier

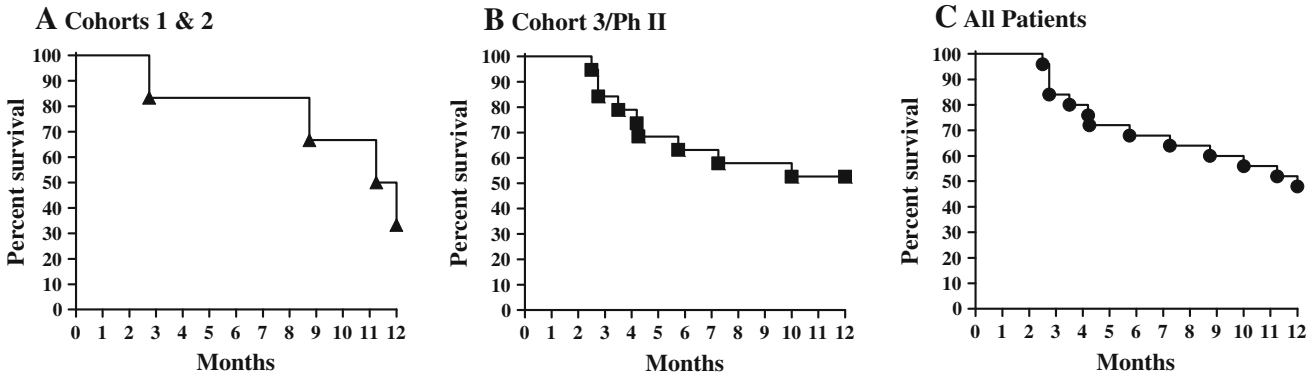

Fig. 4 Kaplan–Meier survival plots of patients treated with Ad5 [E1-, E2b-]-CEA(6D). Patients treated at least two times with Ad5 [E1-, E2b-]-CEA(6D) were followed for survival. *Panel* **a** represents 6 patients in cohorts 1 and 2 that were followed for survival. There were 4 events in this group. *Panel* **b** represents 19 patients in cohort 3 and phase II that were followed for survival. There were 9 events in this group. *Panel* **c** represents all 25 patients (cohorts 1,2, 3, and phase II) that were followed for survival. There were 13 events in this group

generation Ad5 [E1-]-based platforms [10]. The Ad5 [E1-, E2b-] platform utilized in the present study was intended to accommodate a homologous prime–boost regimen, by avoiding presentation of antigens that are the targets of preexisting Ad5 immunity [2, 8, 25–28]. Since CEA has been identified as one of the priority cancer antigens by the National Cancer Institute [29], we investigated this TAA as a transgene to be incorporated into the new Ad5 [E1-, E2b-] vector platform for use as a cancer therapeutic vaccine. CEA expression in adults is normally limited to low levels in the gastrointestinal epithelium, whereas CEA is over-expressed in adenocarcinomas of the colon and rectum and in many breast, lung, and pancreas cancers [30, 31]. We chose the HLA A2-restricted CAP1(6D) modification of CEA because, compared with the wild-type CAP1 epitope, CAP1(6D) has been shown to enhance the sensitization of CTLs [19, 20] and has been included in our recent CEA-based vaccine constructs [32, 33]. Although we did not test for HLA type because we used full-length CEA that is not HLA-restricted, A*0201 is the allele observed most frequently in Caucasians (allele frequency 0.2717) and is common in other populations [34]. However, in expanded trials, we plan to test patients for HLA type and assess whether or not there may be a relationship between HLA type and clinical and/or CMI responses.

Previously, we tested multiple subcutaneous immunizations employing three administrations of a single dose level (1×10^{10} VP) of this class of Ad5 vaccine expressing the TAA CEA, (Ad5 [E1-, E2b-]-CEA(6D)) in a preclinical murine model of CEA-expressing cancer. In mice with preexisting Ad5 immunity, we demonstrated the induction of potent CEA-directed CMI responses that resulted in anti-tumor activity and noted that these CMI and anti-tumor responses were significantly greater than those responses induced by a current generation Ad5 [E1-]-based vector vaccine [12, 16]. We have also demonstrated in additional animal models (both cancer and infectious disease targeted) [10, 12–18] that multiple subcutaneous immunizations with vaccines based on the new Ad5 [E1-, E2b-] platform induce CMI responses that were superior to those of current generation Ad5 [E1-]-based vaccines, can overcome the barrier of Ad5 immunity, and can be utilized in multiple immunization regimens requiring a generation of robust CMI responses. In our present report, the greatest magnitude of CEA-directed CMI responses occurred in patients receiving the highest dose of the vector. We observed that a CEA-directed CMI response was induced in a dose–responsive manner despite the presence of pre-existing and/or vector-induced Ad5 immunity. We did not assess CAP1(6D)-specific CMI responses in this phase I/II clinical study and plan to assess CAP1(6D) and other CEA epitope-directed CMI responses in our expanded clinical trials. No CEA-directed antibody responses were observed either pre- or post-vaccination employing an ELISA technique [21]. In a preliminary analysis (data not shown), we also observed a population of polyfunctional CD8+ T cells (those that secrete more than one cytokine when activated) after immunizations, a sign of greater functionality of T cells induced by the vaccine. These data support the use of the Ad5 [E1-, E2b-]-CEA(6D) vector in homologous prime–boost regimens designed to induce and increase CEA-directed CMI responses in patients with advanced colorectal adenocarcinoma, as well as any number of other vaccine amenable diseases or applications.

Although the precise mechanism(s) of how the Ad5 [E1-, E2b-] vector platform accomplishes tumor antigen-specific immune induction in the setting of existing or induced Ad5 immunity is not fully understood at present, we believe there are factors that contribute to the favorable activity of this new platform. As compared to earlier generation Ad5 [E1-] vectors containing deletion in the early 1 (E1) gene region, the Ad5 [E1-, E2b-] vector platform with additional deletions in the early 2b (E2b) gene region exhibits significantly reduced inflammatory responses directed at the vector [11, 35, 36]. This can result in longer transgene expression and a reduction in elimination of transgene expressing cells (e.g., antigen-presenting cells) that would otherwise occur due to induced inflammatory responses [35, 37]. Since Ad5 late gene antigen expression is significantly reduced as compared to earlier generation Ad5 platforms [8, 11], this could enable the Ad5 [E1-, E2b-] platform to evade Ad5 immune-mediated neutralizing activity for significantly longer periods of time resulting in greater longevity and amplification of TAA expression. In addition, the E2b gene product, polymerase, is a known target of human cellular memory immune responses to Ad5 infection and its elimination from the vaccine could be furthering its capability in the setting of preexisting Ad5 immunity [38]. The extended and/or greater expression of TAA by the vector in this milieu could result in a more effective immune response against the target antigen. However, it is also possible that this vector configuration produces better transgene expression, different biodistribution, or different innate/adaptive immune effects that impact the effectiveness of this vector, rather than escape from preexisting immunity.

Our patient demographics, albeit limited in size, compares favorably with previously published studies of patients with chemotherapy-refractory colorectal cancer [39–41]. Of interest is the observation that treated patients in our study exhibited favorable survival probability. Overall, all 25 patients treated at least two times with Ad5 [E1-, E2b-]-CEA(6D) exhibited a 12-month survival probability of 48 % and this was achieved despite the presence of significant levels of preexisting Ad5 neutralizing antibody titers. However, the true impact of this new

immunotherapy on overall survival will only be determined in a statistically controlled and randomized trial with larger numbers of patients.

In other clinical trials, immunotherapeutic agents have been found to increase overall survival without having a direct impact on time to objective disease progression, a trend noted in our study as well [1, 42–44]. By engaging the patient's immune system, active immunotherapeutics, such as Ad5 [E1-, E2b-]-CEA(6D), could induce continuous immunologic anti-tumor responses over a long period of time that could result in a "deceleration" or alteration in specific aspects of the rapid growth rate or spread of the tumor not measured by standard response assessments [39, 45]. Indeed, we have observed slower tumor progression in Ad5 immune mice harboring established CEA-expressing tumors following treatment with Ad5 [E1-, E2b-]-CEA(6D) [12]. Moreover, it has been noted that overall survival might be the only true parameter for the determination of clinical efficacy of any potential cancer (immune) therapy [46].

As with any new treatment modality, safety is an important factor. In this phase I/II trial, we demonstrate that the Ad5 [E1-, E2b-]-CEA(6D) could be manufactured to scale, as well be easily and repeatedly administered by conventional subcutaneous injection techniques. The most common adverse effects were site of injection reactions and flu-like symptoms consisting of fever, chills, headache, and nausea. There was no impact on blood hematology or serum chemistries, and overall, the treatments were well tolerated. Specifically, no SAE were noted, and no treatments were stopped due to adverse events, indicating that a dose limitation to use of Ad5 [E1-, E2b-]-CEA(6D) in this clinical application had not been met.

These data suggest that patients with advanced colorectal cancer which are treated with Ad5 [E1-, E2b-]-CEA(6D) do not have serious adverse effects and may experience extension of life even if they have preexisting immunity to Ad5; however, this study had a small number of patients in a trial that was not randomized against a control population. The results of this trial are encouraging enough to advance to a large, randomized, single-agent trial. The observation that some of the patients experienced an increase in CMI which is dose dependent could be an indication that this may play a role in their clinical outcome. We plan to initiate a large multicenter trial which should give us the opportunity to evaluate in greater detail the influence of Ad5 [E1-, E2b-]-CEA(6D) treatment on safety, overall survival, time to progression following treatment, the levels of induction of CMI, and the relationship of induced CMI responses with clinical outcome.

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Conflict of interest The following authors declare financial conflict of interest: Elizabeth S. Gabitzsch, Younong Xu, Stephanie Balcaitis, Rajesh Dua, Susan Nguyen, Joseph P. Balint, Jr., Frank R. Jones are employees of Etubics Corporation. All other authors do not have any conflict of interest.

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CTRA DoD ESAC Members

Suzanne A.W. Fuqua, Ph.D.
Baylor College of Medicine
Lester and Sue Smith Breast Center
One Baylor Plaza
MS 600
Houston, TX 77030
Ph: 713-798-1671
Email: sfuqua@bcm.edu

Matthew Ellis, M.B., B.Chir., Ph.D.
Baylor College of Medicine
Director, Lester and Sue Smith Breast Center
One Baylor Plaza
Houston, TX 77030
Email: mellis@bcm.edu

Geoffrey Greene, Ph.D.
Professor and Chair
The Ben May Department for Cancer Research
The University of Chicago
GCIS W421
929 East 57th Street
Chicago, IL 60637
Ph: 773-834-3410
Email: ggreene@uchicago.edu

Debra Madden
National Coalition for Cancer Survivorship
Phone: 203-748-2551, x371
Cell: 203-733-0879
Email: draemadden@gmail.com

Marc E. Lippman, M.D.
Professor and Chair
Department of Medicine
University of Miami School of Medicine
P.O. Box 016760, R 760
Miami, FL 33101
Email: mlippman@med.miami.edu

William Muller, Ph.D.
Professor, Department of Biochemistry
The Cancer Research Building
McGill University
1160 Pine Avenue
Room 516
Montreal, Quebec H3A 1A3
Canada
Ph: 514-398-5847
Email: william.muller@mcgill.ca

Rachel Schiff, Ph.D.
Baylor College of Medicine
Lester and Sue Smith Breast Center
One Baylor Plaza
Houston, TX 77030
Ph: 713-798-1676
Email: rschiff@bcm.edu

External Scientific Advisory Committee Meeting

Department of Defense Clinical Translation Research Award

Project title: Developing a HER3 Vaccine to Prevent Resistance to Endocrine Therapy

PI: H. Kim Lyerly, M.D.

July 8, 2014

R. David Thomas Executive Conference Center · Duke University · Bellsouth Room

Agenda

Time	Title	Speaker(s)
July 7: 6:45 p.m.	Dinner in the Fairmont Restaurant, Washington Duke Inn and Golf Club	
July 8: 7:30 a.m.	Transportation departs Washington Duke Inn to Thomas Center	
7:40 – 8:00 a.m.	Continental Breakfast	
8:00 – 8:10 a.m.	Introductions & Overview of Grant Objectives	H. Kim Lyerly, M.D.
8:10 – 8:20 a.m.	Patient Perspectives	Patty Spears, Mary Jackson
8:20 – 8:50 a.m.	Down selection of HER3 vaccine candidate	Takuya Osada, Ph.D.
8:50 – 9:05 a.m.	A Phase I clinical Trial of Ad5-[E1-E2b-]-huHER3 vaccine	Kim Blackwell, M.D. and Michael Morse, M.D.
9:05 – 9:20 a.m.	Discussion	
9:20 – 9:35 a.m.	Identification of a HER3 Driven Tumor	Neil Spector, M.D. and David Alcorta, Ph.D.
9:35 – 9:50 a.m.	Discussion	
9:50 – 10:05 a.m.	DoD CTRA Tissue Collection, Analysis, and Annotation	Joseph Geradts, M.D. and William Gwin, M.D.
10:05 – 10:20 a.m.	Discussion	
10:20 - 10:35 a.m.	Break/Refreshments	
10:35 – 10:45 a.m.	Generation and Production of GMP Ad5 [E1-, E2b-]-HER3 Immunotherapeutic	Frank Jones, Ph.D.
10:45 – 11:00 a.m.	Discussion	
11:00 – 11:30 a.m.	Questions & Discussion	All
11:30 – 12:00 p.m.	Summary Comments & Action Items	H. Kim Lyerly, M.D.
12:00 – 1:00 p.m.	Lunch/ESAC Executive Session	
1:00 – 1:30 p.m.	State of the Science: Mechanisms of Hormone Resistance in Breast Cancer	Suzanne Fuqua, Ph.D.
1:30 – 1:45 p.m.	State of the Science: Targeted Resistance in Breast Cancer	Rachel Schiff, Ph.D
1:45 – 2:00 p.m.	State of the Science: Understanding and Targeting Acquired ESR1 Mutations in Hormone Resistant Breast Cancers	Geoffrey Greene, Ph.D.
2:00 – 2:15 p.m.	State of the Science: The Role of c-Src /EZH2 Signaling Axis in Tumourigenesis and Drug Resistance	William Muller, Ph.D.
2:15 – 3:00 p.m.	Discussion/Potential Collaborations to Advance	All
3:00 p.m.	Adjourn – Transportation departs Thomas Center for RDU Airport	