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13. ABSTRACT (Maximum 200 Words)

The purpose of this research is to demonstrate that gene amplification of *HER-2/neu* is associated with loss of expression of the tetraspanin metastasis suppressor gene, *KAI1/CD82*, in breast cancer cell lines and patients' primary breast tumors.
The scope is limited to breast cancer and mechanisms of metastasis.
Results

- We identified three cell lines with gene amplification of *HER-2/neu* and negative *KAI1/CD82* expression to serve as model systems and corroborated these results in primary breast tumors.
- We found that *CO-029*, a tetraspanin metastasis promoter gene, was up-regulated in *HER-2/neu*+ lines.
- We ectopically expressed *HER-2/neu* in MCF-7 cells and demonstrated that over-expression of *HER-2/neu* resulted in loss of expression of *KAI1/CD82*, but increased expression of *CO-029*.
- Finally, we knocked down expression of *HER-2/neu* by RNAi and demonstrated that KAI1/CD82 levels increased four-fold.

Significance
HER-2/neu signaling regulates tetraspanin gene expression to promote metastatic potential. This novel mechanism may explain why women with gene amplification of *HER-2/neu* in their breast tumors are at higher risk for metastasis and death. This is the first demonstration of a receptor tyrosine kinase (RTK) that regulates tetraspanin metastasis genes, and this may be the first example of a broader paradigm linking RTK oncogenes to metastasis genes.

14. SUBJECT TERMS
KAI1, CD82, HER-2/neu, tetraspanin, breast cancer, metastasis, metastasis suppressor gene, CO-029, proto-oncogene, EGFR

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Table of Contents

Introduction

Subject The gene amplification of *HER-2/neu* (*HER2*) in breast tumors is associated with poor prognosis in women with breast cancer (Slamon 1987). Patients with breast cancer die of metastatic disease, primarily to the liver, lungs, bone, and brain. Tetraspanins are cell membrane proteins that are critical in cellular adhesion/fusion, invasion/penetration, and motility/metastasis (Hemler 2002). Dynamic regulation of cell adhesion, invasion, and motility are vital to cells that have the ability to metastasize. The molecular mechanisms linking $p185^{erbB2}$ signaling to increased metastatic potential remain largely elusive, and no one has linked *HER2* to tetraspanin expression. *KAI1/CD82* (*KAI1*) is a tetraspanin metastasis suppressor gene that is expressed in normal breast ductal epithelia (Lombardi, unpublished results). Its expression is down-regulated in the progression of breast cancer (Yang 2000). Transfection of *KAI1* into a lowly *KAI1* expressing breast cancer cell line results in decreased metastasis in a murine xenograft model (Yang 2001). Loss of *KAI1* expression in breast tumors has been correlated with inferior survival proportions in women with breast cancer (Huang 1998). Purpose I hypothesized that women with breast tumors that contain gene amplification of *HER2* are at increased risk of early relapse and death because in a subset of these patients, $p185^{erbB2}$ signaling results in the down-regulation of *KAI1*. Scope After I had stated the above hypothesis, my colleague, Joseph Geradts, M.D., then at the University of Oxford, used gene expression profiling to study regulated genes in *HER2* positive and *HER2* negative primary breast tumors. Among over 5,000 genes and expressed sequence tags (ESTs) analyzed, *KAI1* was the third most down-regulated gene in the *HER2*+ subset, and its expression was reduced by 15-fold (Wilson 2002). In Specific Aim 1 of this proposal, we first used Hamon Cancer Center (HCC) breast cancer cell lines as a model system to validate the microarray data. Second, we wanted to be sure that this inverse relationship existed in primary breast tumor specimens and was not the result of an *in vitro* artifact of cell culture. Therefore, we analyzed protein expression of the antigens by immunohistochemistry (IHC) in archival tumor blocks that corresponded to the cell lines. Third, we used transfection experiments to ask whether over-expression of *HER2* would result in the down-regulation of *KAI1*. We found, as seen below, that not only was this true, but over-expression of *HER2* also resulted in the increased expression of *CO-029*, a tetraspanin metastasis promoter gene. Therefore, at that point, the scope was broadened to include the characterization of *CO-029*, as a possible downstream mediator of *HER2*'s pro-metastatic properties. Fourth, we used RNA interference (RNAi) to study the effects of down-regulation of $p185^{erbB2}$ on tetraspanin expression. We reasoned that decreasing HER2 mRNA would decrease CO-029 and increase KAI1 protein levels. Fifth, in the near future (months 8-12 of Specific Aim 1), we will assay for $p185^{erbB2}$, KAI1, and CO-029 protein expression in a large, retrospective, CALGB archival tumor bank. We intend to ask whether high $p185^{erbB2}$ and CO-029 levels and low KAI1 levels confer a worse survival rate compared to *HER2*-positive tumors that retain high levels of KAI1 and low CO-029 levels. The long-term goal of this research, outside the scope of this Physician-Scientist Training Award, is to discover therapies that will alter tetraspanin metastasis gene expression to inhibit the formation of metastasis in women with breast cancer. For example, because CO-029 is a cell surface protein, we ultimately aim to inhibit its function by developing reagents (antibodies, peptides, etc.) that will inhibit CO-029's pro-metastatic function. The research

conducted in Specific Aim 1 led to the discovery of the *HER-2/neu – CO-029* link, and will form the basis for future grant submissions.

BODY

Statement of Work

The Metastasis Suppressor Gene, *KAI1/CD82*, and the Proto-oncogene, *HER-2/neu*, as Molecular Determinants of Metastasis in Women with Breast Cancer

Please note that this "Annual Summary" only reflects eight months of laboratory work on Specific Aim 1. I decided to leave the University of Texas Southwestern Medical School and accept a new position at Washington University School of Medicine. Once I did made decision, I was informed by my Research Associate in Dallas that she wanted to transfer to another laboratory for job security reasons. Therefore, this report reflects work performed from July 21st, 2003 until March 24th, 2004. I have just hired two people in my new laboratory in St. Louis and will be set up shortly to resume experiments.

TASK 1 (SPECIFIC AIM 1)

To demonstrate that gene amplification and over-expression of *HER-2/neu* (*HER2*) is associated with loss of expression of *KAI1/CD82* (*KAI1*) in breast cancer cell lines and patients' primary breast tumor samples (months 1-24)

- Analysis of expression of *KAI1* and *HER2* in Hamon Cancer Center (HCC) breast cancer cell lines using immunoblot and Northern blot techniques, and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (QR-T RT-PCR) (months 1-12)

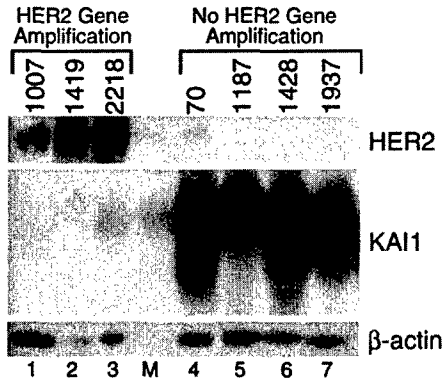

Figure 1. Gene amplification of *HER2* is associated with loss of *KAI1* expression in breast cancer cells. HCC1007, 1419, and 2218 have gene amplification of HER2 (HER2+). HCC70, 1187, 1428, and 1937 have one copy of HER2 gene per chromosome 17q (HER2 negative). 20 μg protein loaded per lane. Other experiments with adequate protein loading of HCC1419 showed identical KAI1 expression (data not shown).

Immunoblot techniques

In three *HER-2/neu* gene amplified HCC breast cell lines, (HCC1007, 1419, and 2218), there was little or no KAI1/CD82 expression detected by immunoblot. These results validate the earlier findings in the gene expression profiling experiment, and lend support to Specific Aim 1. The combination of gene amplification of *HER2* and high $p185^{erbB2}$ expression resulted in highly efficient axillary lymph node metastasis rate (59+ LNs/60 LNs excised=98%).

Two cell lines, HCC1569 and HCC1954, retain KAI1/CD82 expression despite having gene amplification of *HER-2/neu* (data not shown). We welcome results that counter our hypothesis because in the clinical arena, results are never (or nearly never) 100%. For example, while gene amplification of HER2 is associated with increased risk of metastasis, not all women will relapse with HER2+ tumors. Perhaps in these tumors, the signaling pathway from HER2 to KAI1 is abrogated, or, alternatively, the cell compensates successfully to counteract the pro-invasive, pro-metastatic program that HER2 turns on. Also, we suspect that the degree of gene amplification (gene dosage), and the numbers of cell surface HER2 receptors may influence the downstream pathways that lead to increasing metastatic potential in the primary tumor.

Northern blot analysis

We have analyzed approximately half of the HER2 negative HCC breast cancer cell lines, and have only found one transcript detectable in those lines that express KAI1. We find no evidence of alternative splice variant that has been reported in the literature (Lee 2003). We will complete the remainder of the Northern blot analysis within the first four months of transfer of the Award. However, we will need to analyze the HCC lines for the alternative spliced variant of KAI1 because this form of KAI1 may be pro-invasive and may increase metastatic potential. This spliced-KAI1 variant is important because the protein is present and may not look different on an immunoblot, but it does not function as the wild-type protein does. That is, exon 7 is deleted in the splice variant, and this corresponds to a 28 amino acid region that is located in the distal part of the extracellular domain 2 to the proximal part of the fourth transmembrane domain. Therefore, lateral protein interactions with integrins may be attenuated (Lee 2003). We are planning to look for alternative splice variants of KAI1 in the remainder of the HCC breast cancer cell lines, particularly in the two that retain some level of KAI1 expression (HCC1569 and HCC1954).

QR-T RT-PCR We are currently collaborating with the Matthew Ellis laboratory to design primers that will allow us to measure mRNA levels in the most quantitative manner possible. We plan to measure mRNA and protein levels side-by-side to see if mRNA levels correlate with protein levels. This issue is very important with tetraspanins because they regulate cellular adhesion, invasion, and motility by protein-protein interactions. In the future, the choice between using cDNA or proteomics as tools to assay for tetraspanin expression may depend on whether mRNA levels are reflective of protein levels. We developed primer pairs with the ABI Primer Express software, but will now use BioRad software because the Ellis laboratory has a BioRad QR-T RT-PCR machine.

- Analysis of HER2 gene amplification in HCC breast cancer cell lines by fluorescent in-situ hybridization (FISH) (months 1-12)

HCC Breast Cancer Cells	IHC Her-2/neu Score	FISH Score
HCC 1599	Negative	1.6
HCC 1937	Negative	1.12
HCC 1806	Negative	0.94
HCC 1954	3+	4.31
HCC 1143	Negative	1.22
HCC 38	Negative	1.07
HCC 202	3+	3.6
HCC 2218	3+	8.27
HCC 2185	2+	Needs more digestion
HCC 2157	Negative	1.03
HCC 1954	3+	4.01
HCC 1187	1+	1.04
HCC 1419	3+	7.41
HCC 712	Negative	0.86
HCC 1569	3+	4.06
HCC 1500	Negative	0.57
HCC 1007	3+	3.7
HCC 1739	Negative	0.81
HCC 1428	Negative to weak 1+	1.07
HCC 70	Negative	1.06

Table 1. FISH of HER2 in breast tumor blocks, with corresponding staining for HER2 protein by IHC. IHC scored as negative, 1+, 2+, or 3+. FISH score = # of copies of HER2 gene/17q chromosome. Scores ≥ 2.0 = gene amplification.

To be sure that we were testing the gene amplification of- and not merely the overexpression of- *HER-2/neu* in the HCC breast cancer cell lines, we performed FISH on 21 HCC tumors and cell lines. We have quantified the data as # of gene copies of *HER-2/neu* per # of copies of chromosome 17q. In this way, we correct for chromosomal instability and aneuploidy in the cell lines. In the HCC database, we have proven that seven of the 21 breast cancer cell lines have gene amplification of *HER-2/neu* and that 13 do not. HCC2185 expresses 2+ $p185^{erbB2}$, but we need to repeat the FISH to ascertain whether the expression results from amplification of the *HER2* gene or not. The latter 13 tend not to express levels of $p185^{erbB2}$ that can be detected by immunoblot techniques.

- Analysis of cyclin D1 expression using immunoblot, Northern blot, and qR-T RT-PCR; signaling molecules in the HER-2 pathway (months 6-12)

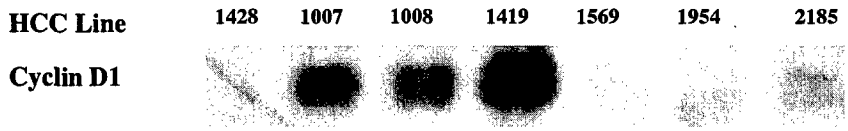

Figure 2. Immunoblot of HCC breast cancer cell lines with mAb to cyclin D1. 20 µg protein /lane. HCC1428 and 2185 are *HER2*-negative. HCC1007, 1008, 1419, 1569, and 1954 are *HER2*-positive (gene amplified).

HCC lines 1007, 1008, and 1419 express cyclin D1 and do not express KAI1. HCC lines 1569 and 1954 do not express cyclin D1, but retain expression of KAI1. This expression pattern may be significant and will be explored in the HER2 negative lines. HCC1428 and 2185 are used as controls (HER2 negative). HCC1419 may be useful in animal models for HER2+ breast cancer; it expresses high levels of VEGF and could support its own blood supply.

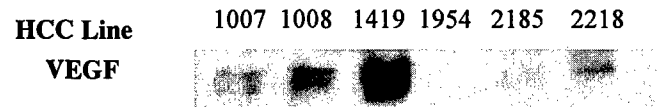

Figure 3. Immunoblot of VEGF (A) expression in HCC breast cancer cell lines. HCC1007, 1008, 1419, 1954, and 2218 contain gene amplification of HER2. HCC2185 FISH not finalized.

- Analysis of KAI1 and HER2 protein expression in patients' primary breast tumor specimens in HCC Breast Tumor Repository using immunohistochemistry (IHC) techniques (months 6-12)

Surgery Accession #	KAI1 protein	$p185^{erbB2}$	Corresponding HCC Breast Cell Line
U92-8061	0.9	0.6	HCC 202
92-9115 B7	1.6	0	HCC70
93-20685 C	2.1	0.9	HCC712
94-10691 O	0.2	2.3	HCC1007
94-13862 C	3.2	1.5	HCC1143
94-14675 B	3.5	1.4	HCC1187
94-20149 B	0	0.4	HCC1395
94-20901 A	0	5.0	HCC 1419
95-10118 F	2.8	0.6	HCC1739
95-12700 C	2.4	0	HCC1806
95-2428 U	0	1.4	HCC1500
96-3684 G	2.3	1.6	HCC2157
95-4110 U	3.5	5.2	HCC 1569
96-4303 I	3.5	0	HCC 2185
U96-672 D	1.4	4.8	HCC 2218

Table 2. Protein expression of KAI1 and $p185^{erbB2}$ in breast tumor samples that correspond to the HCC breast cancer cell lines. $p185^{erbB2}$ protein levels were measured using standard techniques (Wang 2002) that are used clinically in surgical specimens. Results of ≥ 2.0 reflects gene amplification and over-expression of the protein. The range is between 0.0 and 5.2. The same software was used to quantify KAI1 expression; KAI1 is also a surface membrane protein.

In Table 2, we report the IHC scores of KAI1 and $p185^{erbB2}$ protein expression. As a group, these data in the tumor blocks correlate well with those from the HCC cell lines. Formal statistical analysis will be done once all of the data are available. HCC1419 has amplified HER2, over-expression of $p185^{erbB2}$, and negative expression of KAI1. This cell line will be a candidate line for development of a xenograft model in future studies. HCC1569, despite its

gene amplification and over-expression of HER2, retains ample KAI1 expression and will be a useful line to study signal transduction pathways that do not lead to down-regulation of KAI1. Similar findings were observed in HCC1954 (data not shown). Clinical and pathological data are available from these patients and are presented in Table 3.

Cell Line	FISH	$p185^{erbB2}$ IHC	KAI1 IHC	Axillary Lymph Node
HER-2/neu amplified	Score	Score	Score	# mets/# LN Excised
HCC1007	3.7	2.3	0.2	12/12
HCC1419	7.4	5.0	0.0	5/5
HCC1569	4.1	5.2	3.5	4/18
HCC1954	4.3	5.6	3.3	0/27
HCC2218	8.3	4.8	1.4	42/43

The three HCC cell lines with gene amplification of HER-2/neu and low/negative expression of KAI1, HCC1007, 1419, and 2218, have IHC scores from primary breast cancer tumor blocks that approximate the protein expression observed *in vitro*. Moreover, the corresponding patients developed axillary lymph node metastasis rates of 98% (a total of 59 positive nodes/60 nodes excised). For the two lines that retain robust KAI1 expression in vitro and in vivo, the efficiency of axillary lymph node metastases is markedly less (9%; 4+ mets/45 LN excised). We do not think that it is a coincidence that CO-029 protein expression is virtually the opposite expression of KAI1 in this model system.

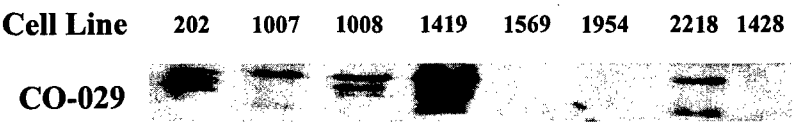

Figure 4. Immunoblot of CO-029 protein expression in HCC breast cancer cell lines. 20 μg protein/lane. Actin controls show equal loading of protein in each lane (data not shown).

The two lines that have significant amounts of KAI1 protein expressed, HCC1569 and 1954, have no expression of CO-029. These data are supportive of other data in the literature where KAI1 has metastasis suppressor properties, while CO-029 has metastasis promoter properties. The lines with the lowest KAI1 expression, HCC1007, 1008, 1419, and 2218, all express CO-029. HCC1007 is derived from a primary breast tumor, while the HCC1008 was derived from an axillary lymph metastasis from the same patient. We note that CO-029 expression is higher in the metastasis compared with that from the primary tumor. We also note an extra band, which we will need to further characterize (glycosylation variant vs. alternative spliced variant vs. post-translational processing of protein, etc.).

For future studies, we will optimize staining for CO-029 in these primary breast tumors that have been formalin-fixed and paraffin-embedded.

Thus far, we have presented data that support a direct correlation between HER-2 and CO-029 expression and an inverse correlation between HER2 and KAI1 expression. To prove a causal relationship, we turned to transfection experiments.

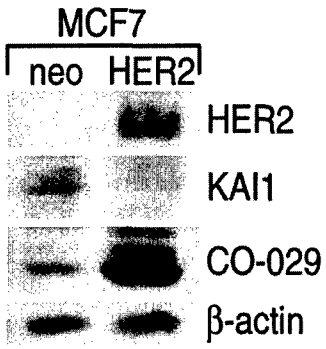

Figure 5. Ectopic Expression of *HER-2/neu* in MCF-7 Cells Increases CO-029 Expression, but Decreases KAI1 Expression. Immunoblot as described above. Neo depicts transfection with the cassette only. Beta-actin is used as protein loading control.

In Figure 5, we show that the transfection and over-expression of HER2 into MCF-7 breast cancer cells results in the 4.4-fold up-regulation of CO-029 and a 2.0 fold reduction in KAI1 expression. This line is not optimal for analyzing KAI1 expression because it expresses very low KAI1 in wild-type cells. This experiment will be repeated in a HER2-negative HCC line that expresses enough KAI1 to be seen on a Western blot.

If the relationships between HER2 and the two tetraspanins are true and consistent, we should be able to obtain opposite results by knocking down HER-2/neu expression.

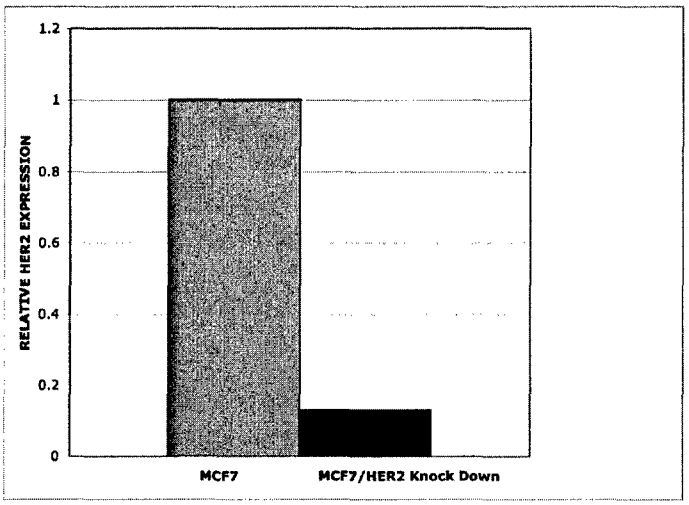

Figure 6. Knock down of $p185^{erbB2}$ protein expression using RNAi in MCF-7/HER-2/neu breast cancer cells.

In Figure 6, we show excellent (~85%) knock down of $p185^{erbB2}$ protein expression with an RNA interference oligonucleotide directed against HER2 mRNA.

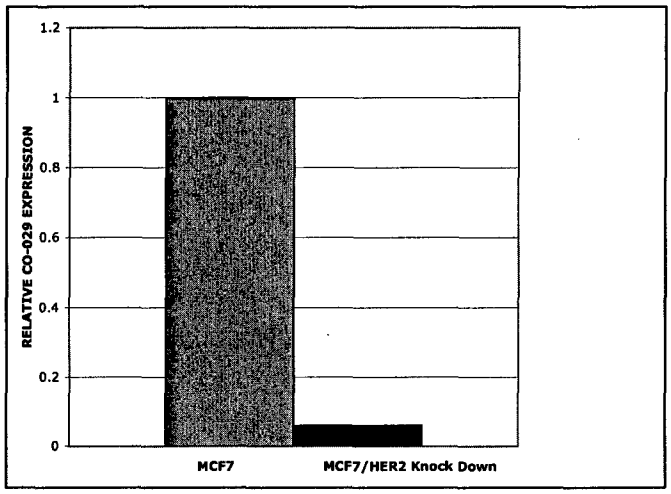

Figure 7. Knockdown of *HER-2/neu* expression results in down-regulation of *CO-029* expression in MCF-7/*HER-2/neu* cells.

As predicted, the knock down of $p185^{erbB2}$ protein expression by RNAi resulted in the robust down-regulation of CO-029, thus demonstrating a direct correlation between the expression levels of the two proteins. We proceeded to test whether KAI1 expression would be up-regulated using the same technique in another cell line.

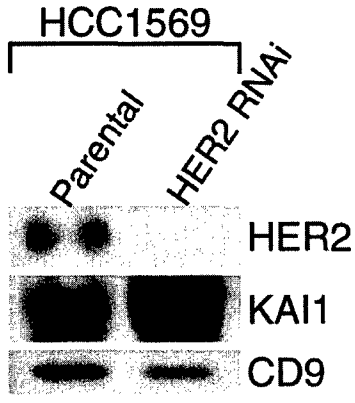

Figure 8. Loss of *HER-2/neu* Expression by RNAi Results in Up-regulation of *KAI1* Expression in a *HER2+* Cell Line. CD9 levels mirror those of beta-actin (not shown). Data subjected to densitometry analysis.

In Figure 8, we show that KAI1 expression is up-regulated four-fold in HCC1569 breast cancer cells that have had $p185^{erbB2}$ expression knocked down. Another tetraspanin metastasis suppressor protein, CD9/MRP-1 is not affected, suggesting specificity to the inverse relationship between HER2 and KAI1.

- Analysis of the activated, tyrosine-phosphorylated (p-Tyr1248) form of HER2 in HCC cell lines and their corresponding primary tumor blocks (months 6-12)

The phosphotyrosine antibody (Thor 2000) did not work in our hands. We will use an alternative assay, using a co-immunoprecipitation with a Santa Cruz mAb, followed by immunoblotting with a phospho-tyrosine antibody (Agus 2002).

ErbB2 will be immunoprecipitated using a monoclonal antibody (Ab-3, Oncogene Sciences, Boston, MA) covalently coupled to an affinity gel (Affi-Prep 10, Bio-Rad, Hercules, CA). 10 μL of gel slurry containing ~ 8.5 μg of immobilized Ab-3 will be added to each lysate and the samples will be mixed at room temperature for 2 hr. The gels will be collected by centrifugation, washed three times with lysis buffer, mixed with SDS sample buffer, and heated for 2 min in a boiling water bath. Supernatants will be collected and run on the appropriate SDS-PAG and electroblotted onto appropriate membranes for analysis.

- an analysis of protein expression of KAI1 and HER2 using immunohistochemistry techniques in a large retrospective CALGB database to correlate their expression with clinical endpoints such as time to progression, time to distant metastasis, disease-free survival, and overall survival (months 12-24)

Dr. Matthew Ellis and I are discussing which CALGB database we would like to use for this experiment. We want to study a sufficient number of tumor samples to ask whether HER2 and CO-029 are directly related, and HER2 and KAI1 are inversely related, in primary breast tumors. I will discuss the project with our statistical consultants to make sure that we have the power to detect meaningful differences. Washington University is a member of the CALGB.

KEY RESEARCH ACCOMPLISHMENTS

- Validation of my initial hypothesis that *HER-2/neu* gene amplification in breast cancer cells results in the down-regulation of the tetraspanin metastasis suppressor gene, *KAI1/CD82*. We found an inverse association of HER2 and KAI1 protein expression in both HCC breast cancer cell lines and tissue specimens.

- Discovery of *CO-029* as a tetraspanin that is directly correlated with *HER2* in these same breast cancer cell lines.

- Demonstration that *HER2* over-expression results in the up-regulation of *CO-029* and the down-regulation of KAI1 in MCF-7 cells transfected with HER-2/neu.

- Showing that the knock-down of *HER2* by RNAi resulted in the down-regulation of CO-029 protein expression and the up-regulation of KAI1 expression in HCC1569 cells.

REPORTABLE OUTCOMES

- Abstracts

  AACR-sponsored meeting: Advances in Breast Cancer Research:
  Genetics, Biology, and Clinical Implications
  Huntington Beach, California
  October 8-12, 2003
  Title: Gene Amplification of *HER-2/neu* in Breast Cancer Cells Results in Altered Tetraspanin Gene Expression to Promote Metastatic Potential
  See Appendix for abstract content

- Presentations

  "Molecular Mechanisms of Metastasis in Women with Breast Cancer"
  Advances in Oncology Conference, September 30, 2003
  Washington University School of Medicine
  St. Louis, Missouri

- Funding applied for based on work supported by this award

  American Cancer Society Institutional Research Grant, 2004
  Barnes-Jewish Foundation Grant Program, 2004

- Employment or Research Opportunities Applied for and/or received based on Experience/Training supported by this award:

  Offered and accepted a faculty position as:
  Assistant Professor of Medicine
  Division of Oncology, Section of Medical Oncology
  Washington University School of Medicine
  May 17th, 2004

  Member, Siteman Cancer Center
  Member, Breast Cancer Research Program, Matthew Ellis, M.D., Ph.D., Director

- Development of cell lines

  The work performed thus far adds to the biochemical classification of the HCC breast cancer cell lines. These lines have been deposited into the ATCC repository and are available to the scientific community. For our own future work, the characterization of these lines is a prelude to our development of xenograft models for these cell lines.

- Informatics such as databases and animal models

  Based on the work performed thus far, we will soon attempt to grow the HCC breast cancer cell lines in murine mammary glands to make new and improved xenograft mouse models for human breast cancer. We will apply for funding within the

National Cancer Institute (NCI) Alliance for Nanotechnology in Cancer. Washington University will make an application for a "Nanotechnology Center of Excellence" on March 1st, 2005. The principal investigator, Samuel A. Wickline, M.D., is excited about our model system and has invited me to submit a proposal for the overall grant. His laboratory, in collaboration with Greg Lanza, M.D., Ph.D., works on detection of angiogenesis using nanotechnology and molecular imaging tools. They are very interested in collaborating with me on the establishment of xenograft models of breast cancer. Using their expertise in nanotechnology and molecular imaging, we would explore: interactions between tumor cells and blood vessels in vivo; early detection of metastasis in vivo; interactions of integrin alpha v beta 3 on tumor microvasculature and tetraspanin expression in tumor cells and on tumor microvasculature (tetraspanins bind to and modulate integrin function; Yanez-Mo 2000; Berditchevski 2001;); imaging platelets *in vivo* and their binding to tumor cells: effects of metastatic cell survival in the circulation.

The ER-negative, PR-negative, HER-2/neu-negative breast cancer cell phenotype

At Washington University, the Breast Cancer Research Group is going to apply for an AVON-NCI grant in March 2005. One project will address the urgent need to understand the ER- PR- HER-2/neu – phenotype of breast tumors that behave very aggressively in women with breast cancer. Nine of our HCC cell lines have this "triple negative phenotype" and should provide good models for understanding DNA repair (with Simon Powell, new Chairman of the Department of Radiation Therapy), chemoresistance, metastatic potential, etc.

Circulating Tumor Cells

The second focus of the grant will be on circulating tumor cells. We want to set up an animal model system for metastatic breast cancer where we procure breast cells from normal breast ductal epithelia, primary breast tumor, circulating tumor cells, axillary lymph node metastases, and distant metastases. The central question in metastasis research is whether the metastatic potential of a breast tumor is hard-wired into the majority of the primary breast tumor cells or whether only a few of these cells have acquired the ability to spread in the evolution of the primary tumor. We are currently comparing different methodologies for the detection of circulating tumor cells in animal models and in patients as well. One company, Miltenyi Biotec (from Germany) is helping us evaluate magnetic cell sorting technologies and linking them to a flow cytometer that they are unveiling in December 2004.

CONCLUSIONS

In summary, the work completed for this Award has identified several HCC breast cancer cell lines that can serve as model systems to study the signal transduction pathways that lead from amplified and activated $p185^{erbB2}$ receptors to the regulation of tetraspanin gene expression. We have not only verified the findings from a previous microarray experiment (Wilson 2002), but we have discovered that the tetraspanin gene, *CO-029* is up-regulated in *HER-2/neu* gene amplified cells. CO-029 is the only tetraspanin that is positively correlated with metastasis. *In vitro*, CO-029 decreases cellular adhesion and increases invasion and motility. *In vivo*, CO-029 increases metastases and is associated with a consumptive coagulopathy in a rat pancreatic carcinoma model. We found that *CO-029* expression is higher in the matched axillary lymph node metastasis line, HCC1008 compared with that of the primary breast carcinoma cell line, HCC1007.

The implication is that the proto-oncogene and receptor tyrosine kinase, *HER-2/neu*, and its signaling lead to a pro-metastatic phenotype in a subset of breast tumors that have gene amplification of *HER2*. Increased $p185^{erbB2}$ signaling led to the increased expression of the tetraspanin metastasis promoter, *CO-029*, and the down-regulation of the tetraspanin metastasis suppressor, *KAI1/CD82*. Future work on this project will now include an emphasis on *CO-029* in parallel with *KAI1*.

Since CO-029 is expressed more highly in metastatic tumors than in primary tumors, we may be able to target the protein to try to inhibit the formation or further spreading of metastatic disease. It is easier to knock down function of a positive regulator than it is to develop strategies that will up-regulate the expression of a suppressor (such as KAI1). We have an antibody to CO-029 and will test it to see if has inhibitory properties. In the future, we will collaborate with our Antibody/Hybridoma Core Facility to make new antibodies or peptides that inhibit CO-029 associations.

<u>"So What"</u>

The identification of CO-029 as a tetraspanin metastasis gene may lead to therapeutics that would attempt to down-modulate the protein's function as a metastasis promoter. Monoclonal antibodies or small peptide molecules could be made to inhibit the:

-spreading of established metastases
-metastatic spread of breast cancer following definitive local therapy
-metastatic rate in HER2+ breast cancer.

In patients with breast cancer, if the combination of HER2 gene amplification, high CO-029 expression, and low KAI1 expression is associated with an even worse prognosis than that for all HER2+ tumors, then novel treatment strategies could be designed to rationally tailor more aggressive therapy.

At a more basic science level, these findings could lead to:

- the identification of promoter sequences that KAI1 and CO-029 have in common that are targets of transcription factors that regulate tetraspanin gene expression.

- the identification of protein-protein interactions, such as tetraspanin-tetraspanin, tetraspanin-integrin, tetraspanin-erbB family members that further our understanding of cellular adhesion, invasion, and motility. These three properties are altered in the epithelial to mesenchymal transition that accompanies the acquisition of metastatic potential.

References

Agus DB, et al. Targeting ligand-activated erbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell 2002; 2: 127-37.

Berditchevski F. Complexes of tetraspanins with integrins: more than meets the eye. Journal of Cell Science 114: 4143-51.

Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. Annual Review of Cell and Developmental Biology 2003; 19: 397-422.

Huang C, et al. Correlation of reduction in MRP-1/CD9 and KAI1/CD82 expression with recurrences in breast cancer patients. American Journal of Pathology 1998; 153(3): 973-83.

Lee JH, et al. Expression of a splice variant of KAI1, a tumor metastasis suppressor gene, influences tumor invasion and progression. Cancer Research 2003; 63(21): 7247-55.

Slamon DJ, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987; 235(4785): 177-82.

Thor AD, et al. Activation (tyrosine phosphorylation) of erbB2 (HER-2/*neu*): a study of incidence and correlation with outcome in breast cancer. Journal of Clinical Oncology 2000; 18(18): 3230-9.

Wang S, et al. Aneusomy 17 in breast cancer: its role in HER-2/neu protein expression and implication for clinical assessment of HER-2/neu status. Modern Pathology 2002; 15(2): 137-145.

Wilson KS, et al. Differential gene expression patterns in HER2/neu-positive and –negative breast cancer cell lines and tissues. American Journal of Pathology 2002; 161(4): 1171-85.

Yanez-Mo M, et al. Tetraspanins in intercellular adhesion of polarized epithelial cells: spatial and functional relationship to integrins and cadherins. Journal of Cell Science 114: 577-87.

Yang X, et al. KAI1 protein is down-regulated during the progression of human breast cancer. Clinical Cancer Research 2000; 6: 3424-9.

Yang X, et al. Overexpression of KAI1 suppresses in vitro invasiveness and in vivo metastasis in breast cancer cells. Cancer Research 2001; 61(13): 5284-8.

APPENDICES

Appendix 1.

American Association for Cancer Research (AACR) Special Conference
"Advances in Breast Cancer Research: Genetics, Biology, and Clinical Implications"
October 8-12, 2003
Hyatt Regency Huntington Beach Resort and Spa, Huntington Beach, California

ABSTRACT

Introduction The purpose of this study was to test the hypothesis that the tetraspanin metastasis genes, *KAI1/CD82* and *CO-029*, are downstream effectors of the receptor tyrosine kinase, HER2, in breast cancer cells that have gene amplification of *HER-2/neu*. Women with breast tumors that have gene amplification of *HER-2/neu* suffer from early metastatic relapse and shortened survival times. The mechanisms by which HER2 overexpression confer increased metastatic potential are unclear. The tetraspanin family consists of 30 genes, some of which are central to co-stimulation, signal transduction, and cell fusion (between egg and sperm, myotubules, syncytium formation, etc.). Three tetraspanin members, *CD9*, *CD63*, and *CD82/KAI1*, are metastasis suppressor genes, as proven by their ability to inhibit metastasis in animal models. Only one tetraspanin, *CO-029*, seems to promote metastasis *in vivo*. The products of these genes are cell surface proteins that modulate cellular adhesion, invasion, and motility, three core characteristics of metastatic cells. Our aim was to define a molecular link between *HER-2/neu* and metastasis in breast cancer patients.

Experimental Procedures 1. Gene expression profiling was used to study regulated genes in HER2 positive vs. HER2 negative primary breast tumors. 2. Hamon Cancer Center (HCC) breast cancer cell lines were used as a model system to validate the microarray data. We studied protein expression of HER2, KAI1, and CO-029 by immunoblot techniques using monoclonal antibodies. 3. We analyzed protein expression of the three antigens by immunohistochemistry (IHC) in archival tumor blocks that corresponded to the cell lines. 4. We used transfection experiments to ask whether HER2 could alter tetraspanin gene expression. 5. Finally, we used RNA interference (RNAi) to study the effects of downregulation of HER2 on tetraspanin expression.

Results 1. In a microarray experiment, *KAI1* was the third most downregulated gene among 5,500 genes and ESTs in HER2 positive vs. HER2 negative tumors. *KAI1* expression was 15-fold lower in HER2 positive vs. HER2 negative breast cancers ($p<0.01$). 2. Gene amplification of *HER-2/neu* was associated with relatively low KAI1 expression in three HER2 positive vs. four HER2 negative cell lines ($p<0.05$). 3. For each cell line, we retrieved the correspondent tumor block and used IHC to show that HER2 expression was inversely correlated with KAI1 expression ($p<0.04$). Therefore, the relationship between HER2 and KAI1 expression was not an *in vitro* artifact. 4. In transfection studies, we ectopically expressed HER-2/neu in MCF-7 breast cancer cells and demonstrated that overexpression of HER2 resulted in loss of expression of KAI1, but increased expression of CO-029 (4.4-fold; $p=0.03$). 5. Finally, we knocked down expression of HER-2/neu by RNAi and demonstrated that KAI1 levels increased four-fold ($p=0.05$).

Conclusions Gene amplification of *HER-2/neu* results in downregulation of the metastasis suppressor, *KAI1*, and the up-regulation of the metastasis promoter, *CO-029*. This novel mechanism may explain, in part, why women with gene amplification of *HER-2/neu* in their breast tumors are at high risk for metastasis and early death. Furthermore, this is the first demonstration of a receptor tyrosine kinase (RTK) that regulates tetraspanin metastasis genes, which may signify a new paradigm that links RTK oncogenes to metastasis genes.

Appendix 2.

CURRICULUM VITAE

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Birth Date May 30, 1960

Birthplace Boston, Massachusetts

Citizenship United States of America

Education

1982-1986	M.D.	University of Rochester School of Medicine Rochester, New York
1978-1982	A.B.	Bowdoin College Brunswick, Maine *Magna cum laude* High Honors in Biochemistry Senior Thesis

Education (continued)

(Independent Laboratory Research Study)

1974-1978	Wilmington High School Wilmington, Massachusetts Valedictorian

Board Certification

2003	Medical Oncology, Re-certification
1991	Medical Oncology
1989	American Board of Internal Medicine

Medical Licensure

2004-present	Missouri
1999-present	Texas
1996-2000	North Carolina
1995	Tennessee, Virginia, Montana, and Oregon
1991	Washington, D. C.

Employment/Fellowships/Residency/Internship/Research

2004-present	Washington University School of Medicine Division of Oncology Section of Medical Oncology Department of Medicine Siteman Cancer Center
1999-2004	University of Texas Southwestern Medical Center Assistant Professor of Internal Medicine Division of Hematology-Oncology Department of Internal Medicine Member, Simmons Cancer Center Principal Investigator Hamon Center for Therapeutic Oncology Research Attending Physician Parkland Memorial Hospital Zale-Lipshy University Hospital St. Paul University Hospital
1998-1999	The University of North Carolina at Chapel Hill Research Assistant Professor

Employment/Training (continued)

1996-1999	Duke University Medical Center National Institute of Environmental Health Sciences
1996-1999	The University of North Carolina at Chapel Hill Training Program for Physicians in Environmental Clinical Sciences
1996-1999	National Institute of Environmental Health Sciences Postdoctoral fellowship in Molecular Biology in the Laboratory of J. Carl Barrett, Ph.D., Scientific Director, NIEHS Currently, Director, Center for Cancer Research National Cancer Institute
1996-1999	UNC Hospitals Clinical Instructor Division of Hematology/Oncology
1995-1996	Locum Tenens in Medical Oncology Cancer Carepoint, Inc. Medical Doctor Associates, Inc. in Tennessee, Virginia, Montana, Oregon
1994-1996	Sibley Memorial Hospital Washington, D.C. Hospitalist (Internal Medicine)
1992-1994	National Cancer Institute Biotechnology Fellow in Molecular Genetics Laboratory of Ilan R. Kirsch, M.D., currently Head, Genetics Branch Center for Cancer Research National Cancer Institute
1992-1994	NCI/Navy Medical Oncology Branch Senior Clinical Fellow/ Assistant Attending Physician
1990-1992	National Cancer Institute (NCI) Postdoctoral Fellow in Molecular Genetics Laboratory of Ilan R. Kirsch, M.D.
1989 - 1992	National Institutes of Health National Cancer Institute

Training (continued)

Medicine Branch
Medical Staff Fellow in Medical Oncology

Chairmen:
Charles E. Myers, M.D., formerly
Director
University of Virginia Cancer Center

Robert E. Wittes, M.D., currently,
Physician-in-Chief
Memorial Hospital
Memorial Sloan-Kettering Cancer Center

Carmen J. Allegra, M.D., formerly,
Chairman, Medicine Branch
National Cancer Institute

Trained Under:
Bruce A. Chabner, M.D.
Director, Division of Cancer Treatment, NCI
Currently, Clinical Director and
Chief, Division of Hematology and Oncology
Massachusetts General Hospital Cancer Center

1986 - 1989 University of Michigan Hospital
Department of Internal Medicine
Residency
Internship

Department of Internal Medicine
Chairman: William N. Kelley, M.D., formerly
CEO and Dean University of Pennsylvania School of Medicine

1980-1984 Harvard Medical School
Department of Microbiology and
Molecular Genetics
Summer Research Student in Laboratory of
Harold Amos, Ph.D.,

Professional Societies

American Association for Cancer Research
American Cancer Society
American Society of Clinical Oncology
Society for Molecular Imaging

Honors and Awards

2002	Physician-Scientist Training Award Department of Defense U.S. Army Medical Research and Materiel Command Breast Cancer Research Program
2000	Developmental Project/Career Development Award University of Texas SPORE in Lung Cancer
1998	Clinical Associate Physician (CAP) Award A Career Development Award National Institutes of Health University of North Carolina at Chapel Hill
1989	House Officer Research Award University of Michigan Medical School Department of Internal Medicine First place among 40 3rd-year House Officers "Anaerobic bacteremia: incidence, patient characteristics, and clinical significance"
1987	Golden Beeper Award Award for Excellence in the Teaching of Medical Students Internship Year University of Michigan Medical School
1985	Lange Medical Publications Student Award Award for development of new curriculum University of Rochester School of Medicine
1982	Colonel William Henry Owen Premius Award for leadership in the Chapel Program Bowdoin College
1980 Summer	Alvan T. and Viola D. Fuller Summer Student Undergraduate Research Fellow

Honors and Awards (continued)

American Cancer Society
Harvard Medical School

1978-1982 — James Bowdoin Scholar
Equivalent to Dean's List
Bowdoin College

1978 — Student Leader of the Year
at Wilmington High School
The Golden Scroll for Scholastic Achievement
American Academy of Achievement, California

Reviewer

Ad-hoc
For Cancer Research, Oncogene, Clinical Cancer Research
Endocrine-Related Cancers

Committees

2002-2004 — Physician Liaison, Subcommittee on Quality Cancer
Care, UT Southwestern Medical Center

Chairman, Subcommittee on Preparation for
American College of Surgeons Survey
Parkland Memorial Hospital Cancer Program
Result: Certification Approved for a full 3-year accreditation
#1 rating

Member, Zale-Parkland Cancer Committees
UT Southwestern Medical Center

1999-2004 — American Cancer Society
Texas Division/Prostate Cancer Committee
Chairman, Research Subcommittee

Grant Support

1. Current

Source — Department of Defense/U.S. Army Medical Research
Type — Physician-Scientist Training Award

Grant Support (continued)

Title	The Metastasis Suppressor Gene, *KAI1/CD82*, and the Proto-oncogene, *HER-2/neu*, as Molecular Determinants of Metastasis in Breast Cancer Patients
Dates	07/21/03 - 07/20/08
% Of time allocated	70%
Award	$371,916

2. Past

Source	National Institutes of Health
Type	Developmental Project/Career Development Award University of Texas SPORE in Lung Cancer
Title	The Metastasis Suppressor Genes *KAI1* and *MRP-1* in Lung Cancer
Grant number	P50 CA70907
Dates	02/01/00 – 08/01/00
% Of time allocated	5%
Award	$25,000, direct costs

3. Past

Source	National Institutes of Health
Type	Clinical Associate Physician (CAP) Award
Grant number	3 MO1 RR00046-3852
Dates	07/01/98 - 06/30/99
% Of time allocated	80%
Award	$95, 153

BIBLIOGRAPHY

Mandel, Kenneth G., Lively, Mary K., Lombardi, Donald, and Amos, Harold. Reactivation of NAD (H) biosynthetic pathway by exogenous NAD+ in Nil cells severely depleted of NAD(H). Journal of Cellular Physiology *114*: 235-244, 1983.

Aplan, Peter D., Lombardi, Donald P., Ginsberg, Ann M., Cossman, Jeffrey, Bertness, Virginia L. , and Kirsch, Ilan R. Disruption of the human *SCL* locus by "Illegitimate" V-(D)-J recombinase activity. Science *250*: 1426-9, 1990.

Aplan, Peter D., Lombardi, Donald P., Kirsch, Ilan R. Structural characterization of *SIL*, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. Molecular and Cellular Biology *11*(11): 5462-9, 1991.

Foss, RD, Laskin, WB, Lombardi, DP, Morton, AL, Snyder, J. Anaplastic large cell lymphomas. Head & Neck *13*: 545-48, 1991.

BIBLIOGRAPHY (continued)

Lombardi, Donald, Engleberg, N. Cary. Anaerobic bacteremia: incidence, patient characteristics, and clinical significance. American Journal of Medicine *92*: 53-60, 1992.

Aplan, PD, Lombardi, DP, Reaman, GH, Sather, H, Hammond, D, Kirsch, IR. Involvement of the putative hematopoietic transcription factor *SCL* in T-cell acute lymphoblastic leukemia. Blood *79*(5): 1327-33, 1992.

Kirsch, IR, Abdallah, JM, Bertness, VL, Hale, M, Lipkowitz, S, Lista, F, Lombardi, DP. Lymphocyte-specific genetic instability and cancer. Cold Spring Harbor Symposia on Quantitative Biology, *Volume LIX*, The Molecular Genetics of Cancer, The Cold Spring Harbor Laboratory Press, pp. 287-95, 1994.

Abdallah, JM, Lombardi, DP, Kirsch, IR. Genetic instability in patients with Hodgkin's Disease undergoing chemotherapy. Journal of Clinical Investigation *96*: 2744-7, 1995.

Geradts, Joseph, Maynard, Robert, Birrer, Michael J., Hendricks, Denver, Abbondanzo, Susan L., Fong, Kwun M., Barrett, J. Carl, Lombardi, Donald P. Frequent loss of *KAI1* expression in squamous and lymphoid neoplasms: an immunohistochemical study of archival tissues.
American Journal of Pathology, *154*(6): 1665-71, 1999.

Lombardi, Donald P., Geradts, Joseph, Foley, Julie F., Chiao, Chia, Lamb, Pattie W., Barrett, J. Carl. Loss of *KAI1* expression in the progression of colorectal cancer. Cancer Research, *59*(22): 5724-31, 1999.

Garry, Vincent F., Tarone, Robert E., Kirsch, Ilan R., Abdallah, Jorge M., Lombardi, Donald P., Long, Leslie K., Burroughs, Barbara L., Barr, Dana B., and Kesner, James S. Biomarker correlations of urinary 2,4-D levels in foresters: genomic instability and endocrine disruption. Environmental Health Perspectives, *109*(5): 495-500, 2001.

Li, Yingming, Okegawa, Takasugu, Lombardi, Donald P., Frenkel, Eugene P., and Hsieh, Jer-Tsong. Enhanced transgene expression in androgen-independent prostate cancer gene therapy by taxane chemotherapeutic agents. Journal of Urology *167* (1): 339-346, 2002.

Invited Preview

Crowder, Robert J, Lombardi, Donald P., and Ellis, Matthew J. Successful targeting of ErbB2 receptors—is PTEN the key? Preview; Cancer Cell *6*(2): 103-4, 2004.