AD_____

GRANT NUMBER: DAMD17-93-J-3004

TITLE: Custom Tailoring Therapy for Breast Cancer Using erbB-2

PRINCIPAL INVESTIGATOR: Soonmyoung Paik, M.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: 5 Jun 95

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	5 Jun 95	Final Report (15 Dec 92 - 14 Dec 94)

4. TITLE AND SUBTITLE
Custom Tailoring Therapy for Breast Cancer Using erbB-2

5. FUNDING NUMBERS
DAMD 17-93-J-3004

6. AUTHOR(S)
Soonmyoung Paik, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

In order to examine the role of erbB-2 as a therapeutic response variable in breast cancer, 937 cases from NSABP B-14 and 207 cases from NSABP B-11 were examined using immunohistochemical assay. Both studies failed to demonstrate the value of erbB-2 in predicting response to specific treatment regimens used in the studies (tamoxifen or adriamycin).
In order to clone additional therapeutic response variables that may predict response to tamoxifen, we have used gene trap approach. A novel estrogen suppressed gene was partially cloned. In addition, we were able to confirm estrogen regulation of bcl-2 gene, which could be a potential predictor of tamoxifen response.

14. SUBJECT TERMS
breast cancer, therapeutic response variable, erbB-2, bcl-2 gene trap, transcriptional repression, tamoxifen

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

X Where copyrighted material is quoted, permission has been obtained to use such material.

X Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

___ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

___ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Soon Pai 4/26/97

PI - Signature Date

TABLE OF CONTENTS

Final report:

Title: Custom tailoring chemotherapy with erbB-2

Summary:

Identification and characterization of markers that predict response to specific therapeutic modality has been the major focus of this laboratory. The project focused on testing whether erbB-2 overexpression fits into that category.

Recent CALGB study demonstrated the selective benefit from adriamycin dose intensification in erbB-2 positive patients. In addition, in vitro studies suggested that erbB-2 positive breast cancers may be more sensitive to adriamycin containing regimen over others. We tested this hypothesis with the cases from NSABP trial B-11, where node positive patients were randomized between PF (L-PAM and 5-FU) and PAF (PF plus adriamycin). Blocks from 207 cases were available for the study. 97 of them received PF and 110 received PAF. ErbB-2 overexpression was found in 45 case (46.3%) in PF arm and 51 cases (46.3%) of PAF arm. ErbB-2 was prognostic overall based on Cox models controlling for treatment, nodal status, and age (p=0.051 for Disease Free Survival). On the other hand, there was no statistical evidence that treatment effect is different between erbB-2 negative and erbB-2 positive patients (p=0.36 for Disease Free Survival, based on tests for treatment-by-erbB-2 interaction using Cox proportional Hazards models, controlling for treatment, nodal status, age, and erbB-2 expression).

The negative interaction of erbB-2 and estrogen receptor (ER) signalling has prompted us to focus on the latter marker initially. In order to examine the value of erbB-2 as a therapeutic response variable that predicts response to tamoxifen in breast cancer patients, we have examined 937 cases of primary breast cancer from National Surgical Adjuvant Breast Project (NSABP) protocol B-14. 18% of the cases showed overexpression of erbB-2. The analysis of B-14 showed no positive correlation between erbB-2 and survival outcome of the patients (p=0.3 for disease free survival, p=0.6 for overall survival). ErbB-2 did not predict response to tamoxifen. Multivariate analysis of with other factors indicate that %s-phase, tumor size, progesterone receptor, and nuclear grade are independent predictors of disease free survival. All but nuclear grade predict for survival. The benefit from tamoxifen in disease free survival was independent of those prognostic factors. Thus we have not identified a single factor that could predict response to tamoxifen for these node-negative estrogen receptor positive tumors.

In order to identify novel markers that could be useful as a therapeutic response variable for tamoxifen treatment, we have focused on molecules involved in apoptotic process in breast cancer

cells.Breast cancer cells and normal ductal epithelial cells which express the estrogen receptor (ER) undergo apoptosis upon estrogen withdrawal. This suggest the importance of estrogen in preventing apoptosis of estrogen receptor positive mammary ductal epithelial cells. For estrogen to suppress apoptosis it has to either repress molecular inducers of apoptosis and/or induce the expression of suppressor of apoptosis. We have used the retroviral promoter trap U3lacZ to identify estrogen regulated genes from breast cancer cells since such genes could have an important role in the regulation of the apoptotic process in breast cancer cells. Clones in which the U3lacZ virus had integrated into genes which were either suppressed or induced by estrogen were identified. Beta-galatosidase reporter activity in 20 of 2000 clones mutated by U3lacZ were inducible by estrogen while 3 in 2000 were suppressed. Using this method, a novel estrogen repressed gene was cloned. The partial sequence of the gene is as follows:

1 agggctgtgg gcctgtatct tgtttgctcg tcatcctgtc agtcattttt ttctttccct

61 ttttttaaag acaaaatctg aacctagaaa caccgaagcc agagcaaaaa ctagatgcga

121 atccactatt tgtgcgaccc

On the other hand, 5'-flanking genomic DNA from one of the estrogen inducible clones showed 100% sequence identity with bcl-2 DNA. Estrogen induction of lacZ in this clone was completely blocked by the antiestrogen ICI-164,384. Thus we identify bcl-2 as a candidate therapeutic response variable that predicts response to tamoxifen.

The strategy we have developed could be useful in identifying genes regulated by hormones or growth factors. Clones in which genes inducible by estrogen are mutated by the U3lacZ retrovirus could be useful in screening anticancer drugs as the reporter gene lacZ faithfully follows the pattern of regulation of the endogenous genes.

Introduction:

Treatment of breast cancer has been revolutionized through three important stages. First was the development of surgical techniques to remove breast cancer tissue which improved the survival of patients dramatically. Second stage was the use of systemic adjuvant therapy (tamoxifen for estrogen receptor positive tumors and chemotherapy for estrogen receptor negative tumors). The final revolution was the early detection through screening program. Even after these improvements in the diagnosis and management, we still did not obtain 100% cure of breast cancer. Instead, about 30% of early invasive breast cancer will still recur within 10 years after surgery and radiation (1). Additional systemic therapy will reduce this recurrence rate to about 20%. Thus only small subset of patients actually benefit from systemic therapy. This modest benefit brings significant dilemma for clinical practice. Focus of our lab has been to identify and test markers that predict response to systemic therapy. We have tested whether erbB-2 is a predictor of response to adriamycin and/or tamoxifen. Our early attention to erbB-2 as a potential marker for tamoxifen response stems from in-vitro studies that demonstrated negative interaction between estrogen receptor signalling pathway and erbB-2 pathway (2). In the latter report, activation of erbB-2 receptor signalling prevented estrogen induction of progesterone receptor. However, using samples from NSABP B-11 and B-14, we failed to see any impact of erbB-2 in adriamycin or tamoxifen response (vide infra). This finding has forced us to redirect our attention to other potential markers that will be useful as a predictor of tamoxifen response.

One of the most important developments in clinical oncology in recent years has been the identification of apoptosis as a key process in cancer development and in chemotherapeutic action mechanism (3). We postulate that molecules involved in apoptotic pathway are potential candidates for therapeutic response variables. We have elected to use ER positive breast cancer cell line MCF-7 as a model system from which to identify and clone regulators and effectors of apoptotic process, since estrogen prevents their apoptosis (4). Estrogen deprivation of MCF-7 cells results in more than 50% of the cells undergoing apoptosis within 3 days in vitro and causes regression of xenograft in mice (5,6). Estrogen could achieve its protective function either through repression of cell death effectors or through induction of anti-apoptotic factors.

As estrogen regulated proteins in MCF-7 cells may play important roles in cell survival and growth, we have attempted to clone genes that are regulated by estrogen. In addition to cloning such genes, we desired to tag them with a reporter in order to study their transcriptional regulation. Resulting cell clones would be useful in screening pharmacological modulators of cell growth and apoptosis. For these purposes, we have used the retroviral gene trap, U3lacZ, described by Reddy et al (7). In this retrovirus, a promoter-less lacZ gene has been inserted into the U3 region of the

long terminal repeat. The promoter-less lacZ reporter is randomly inserted into the genome with the help of retroviral transduction. Expression of lacZ reporter in the resulting mutated cell clones is strictly dependent on the transcriptional activity of the genomic insertion site. Once the cell clones of interests are identified, flanking genomic DNA can be cloned using Polymerase Chain Reaction.

Body:

I. ErbB-2 overexpression does not predict added benefit from adding adriamycin to conventional regimen in node positive breast cancer.

Expression of erbB-2 protein in 207 cases of node-positive and ER negative breast cancer has been examined using immunohistochemical staining as described (8). These patients were subset of those enrolled into NSABP trial B-11 in which patients were randomized to receive PF or PAF after surgery. We hypothesized that only erbB-2 positive tumors will get additional benefit of adding adriamycin to the PF regimen. This hypothesis was based on CALGB data which demonstrated the selective benefit from adriamycin dose intensification in erbB-2 positive patients. Blocks from 207 cases were available for the study. 97 of them received PF and 110 received PAF. ErbB-2 overexpression was found in 45 case (46.3%) in PF arm and 51 cases (46.3%) of PAF arm. ErbB-2 was an independent prognostic factor based on cox models controlling for treatment, nodal status, and age ($p=0.051$ for Disease Free Survival). On the other hand, there was no statistical evidence that treatment effect is different between erbB-2 negative and erbB-2 positive patients ($p=0.36$ for Disease Free Survival, based on tests for treatment-by-erbB-2 interaction using Cox proportional Hazards models, controlling for treatment, nodal status, age, and erbB-2 expression). Thus we failed to demonstrate the selective advantage of adding adriamycin in treatment of erbB-2 positive tumors. The study was not designed to test whether erbB-2 is a marker of drug resistance in general.

II. erbB-2 does not predict response to tamoxifen for ER positive node-negative tumors.

Expression of erbB-2 protein in 937 cases of node-negative and ER positive breast cancer has been examined using immunohistochemical staining as described (8). This group of 937 patients represented 35% of the entire study population. Detailed statistical analysis showed that this 937 cases are representative of the entire 2661 patients in the trial. Thus there was no bias in patient selection that could influence the analysis of the result. Although the 937 cases represent only 35% of the initial study population, this is the largest study ever performed for node-negative ER positive group of patients with unified treatment. In addition to erbB-2 staining, following data were available for analysis; histopathological parameters including tumor size and nuclear grading, progesterone receptor status, DNA ploidy and S-phase measurement by flow cytometry, and survival outcome. Flow cytometric data was available from 759 (29%) of the cases. Univariate analysis showed that there were no significant differences in either disease free survival ($p=0.3$) nor survival ($p=0.6$) according to erbB-2 status. Patients with high S-phase had worse survival

(p=0.001). Results from multivariate analysis (Cox model) indicate that % S-phase, tumor size, progesterone receptor, and nuclear grade are independent predictors of disease free survival. All but nuclear grade predict for survival. However, benefit from tamoxifen therapy was evidenced in any subgroup examined including erbB-2, tumor size, and % S-phase.

Thus we failed to identify any subgroup of ER positive patients that may not benefit from tamoxifen treatment. This data suggest that all ER positive node-negative tumors should receive tamoxifen, since there is no predictive marker for response. Furthermore, the need for predictive marker for tamoxifen response is realized from this study.

III. Gene trap identifies multiple cells clones in which potentially important estrogen regulated genes are trapped.

In order to identify and clone genes that could be involved in the regulation of apoptotic process and could be used as therapeutic response variables, we have used U3lacZ gene trap. As shown in figure 1, after infection of 10 million MCF-7 cells with U3lacZ at a multiplicity of infection of less than one and G418 selection for the clones that contain proviral inserts, lacZ positive cells were enriched 10 folds by FDG-FACS (9), and approximately 2000 individual clones were randomly selected. At the time of cloning, replicas of individual clones were plated in 96 wells in quadruplicate and expression of lacZ was examined by beta-D-galactopyranoside (X-gal) staining after treating with estradiol, partial antagonist tamoxifen, pure antiestrogen ICI-164384, or vehicle (11,12,13). LacZ regulation in positive clones was verified using the ortho-nitrophenyl-beta-galactopyranoside (ONPG) assay (14).

Figure 1

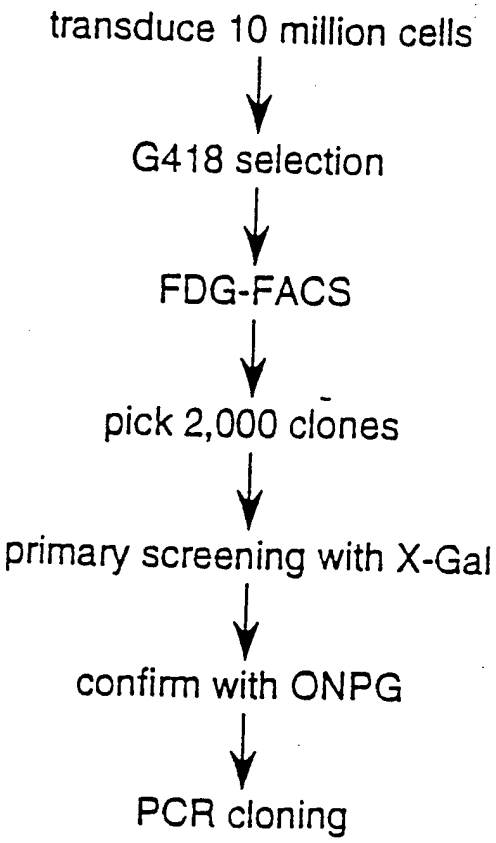


FIG. 1. Generation of gene trap clones regulated by estrogen

METHODS. 10 million MCF-7 cells were plated in estrogen depleted medium (IMEM (Biofluid) with 5% charcoal stripped calf serum (CCS)) containing 10nM ICI-164,384. 1 day after cells were infected with U3lacZ as described by Reddy (7). G418 selection was started 48 hours after infection at a concentration of 400 ug/ml. Two weeks later, all surviving colonies were FDG-FACS sorted as described by Nolan (9). There was approximately 10 fold enrichment of positive cells as determined by X-gal staining of the colonies before and after sorting. The sorted cells were plated at cloning density in the presence of G418. 2,000 colonies were randomly selected with cloning cylinder. At the time of cloning, cells were replica plated in 96 wells in quadruplicate and treated with vehicle alone, 10^{-9}M estradiol, 10^{-7}M 4-hydroxytamoxifen, or 10^{-7}M ICI-164,384 for 3 days. Plates were fixed with 2%glutaraldehyde/0.2% formaldehyde for 15 minutes, washed twice with PBS and stained with X-gal. Clones with estrogen regulated pattern of staining were further expanded. Cells were then plated into three 100mm dishes in estrogen depleted medium, treated with vehicle alone, 10^{-9}M estradiol, or 10^{-7}M ICI-164,384, and lacZ enzyme activity assayed with ONPG assay using beta-galactosidase assay kit (Promega)(14). 5' DNA flanking the viral insertion site was obtained using the PCR In Vitro Cloning Kit (Takara) with the following nested primers for proviral sequence; 5'-cggaaaccaggcaaagc-3' and 5'-atcatcgcgagccatggtggcctc-3'. Sequence homology searching was done using NCBI WWW Blast Server (15).

Of the 2,000 individual G-418 resistant clones tested with X-gal staining assay, lacZ activity was induced by estrogen in 20, and inhibited by estrogen in 3 clones.

IV. Bcl-2 is an estrogen regulated gene in breast cancer cells

In clone 134, lacZ activity is induced three to four fold by estradiol in a dose and time dependent manner (figure 2). This induction was completely blocked by co-incubation with antiestrogen (figure 2a). Thus, we concluded that the trapped gene in clone 134 is an estrogen inducible gene.

Figure 2A

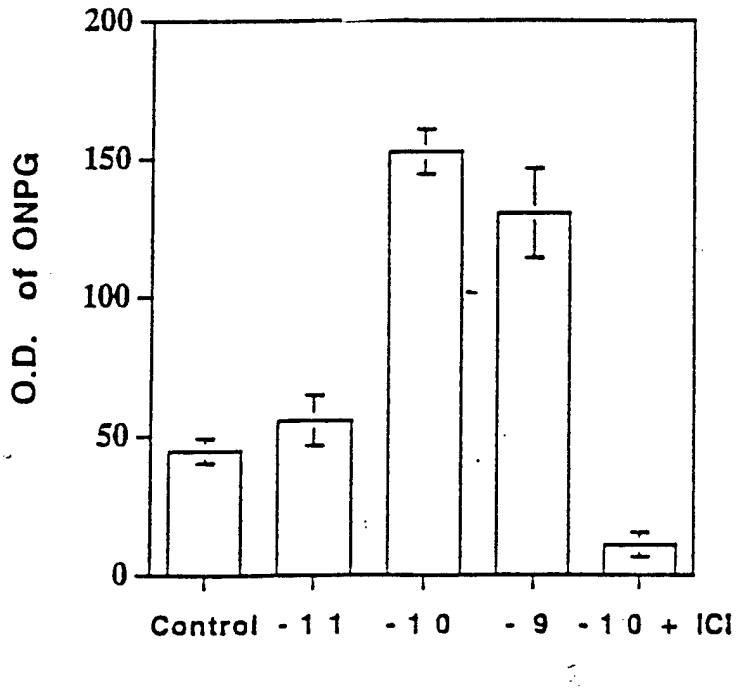


Figure 2B

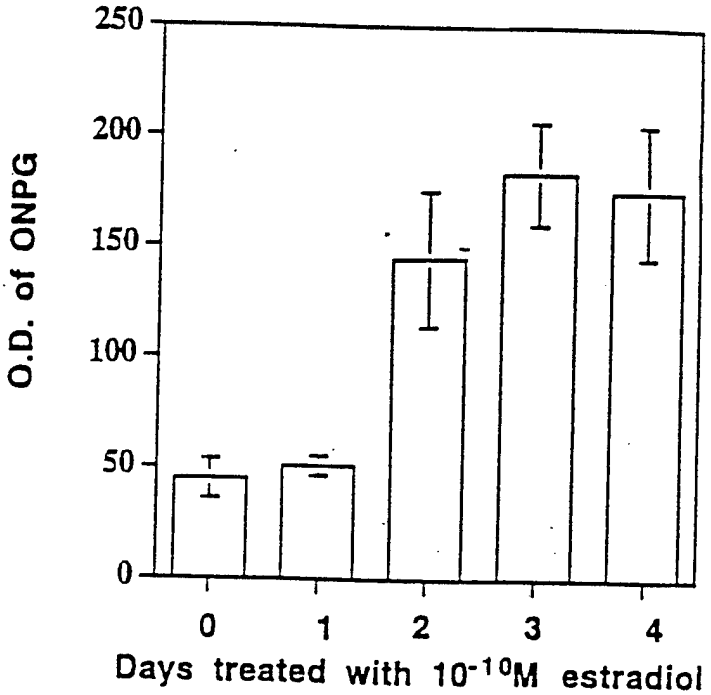


FIG. 2. Regulation of lacZ reporter activity in clone 134.

a) The antiestrogen ICI-164,384 blocks estrogen induction of lacZ activity in clone 134. The effect of estradiol with or without antagonist ICI-164,384 on lacZ reporter activity in clone 134 was measured using the ONPG assay. Log molar concentration of estradiol is shown in the legend. ICI is 10^{-7}M ICI-164,384. Error bars indicate standard errors from triplicate values.

b) Time course of lacZ reporter activity by estradiol treatment in clone 134. After plating cells in 100 mm dishes on day 0 in estrogen deprived medium, cells were treated with 10^{-10}M estradiol at varying time points.

METHODS. Clone 134 was kept in IMEM (Biofluid) with 10% fetal bovine serum (FBS) (Biofluid) until the time of experiments and plated on 100mm dishes in estrogen depleted medium. 24 hours after plating, cells were treated with vehicle alone (ethanol), or varying concentrations of estradiol. After 3 days treatment, cell lysates were obtained and the ONPG assay was performed as instructed by the supplier (Promega). The optical density (O.D.) at 420nm was measured with Dynatek ELISA plate reader and normalized for the amount of total protein in the cell extracts. For the time course experiment, cells were incubated in estrogen depleted medium for 5 days. At day 0, 10^{-10}M estradiol was added and cells harvested at each time point and assayed as above.

Sequencing of the 5'-flanking DNA sequence from clone 134 obtained by polymerase chain reaction revealed a following sequence, 5'-AAT CAG CTA TAA CTG GAG AGT GCT GAA GAT TGA TGG GAT CGT TGC CTT ATG CAT TTG TTT TGG TTT TAC AAA AAG GAA ACT TGA CAG AGG ATC ATG CTG TAC TTA AAA AAT ACA A-3', that was 100% identical to published bcl-2 cDNA sequence, suggesting that the provirus inserted into the 5' untranslated region of bcl-2 gene at position +954 (16,17). This provides direct evidence that bcl-2 is an estrogen regulated gene.

One mechanism by which estrogen may inhibit apoptosis is by increasing expression of Bcl-2. Peak of apoptosis in the mammary gland and endometrium during menstrual cycle coincides with low levels of bcl-2 and estrogen (23.24). Breast cancers that express ER tend to also express high levels of Bcl-2 (18-22). We postulate that estrogen induces the generation of more terminal ductules during the reproductive years by not only promoting growth but also by suppressing apoptosis. We further postulate that withdrawal of estrogen at menopause decreases the expression of bcl-2 and thus induces apoptosis. The isolation of Bcl-2 gene by the gene trap

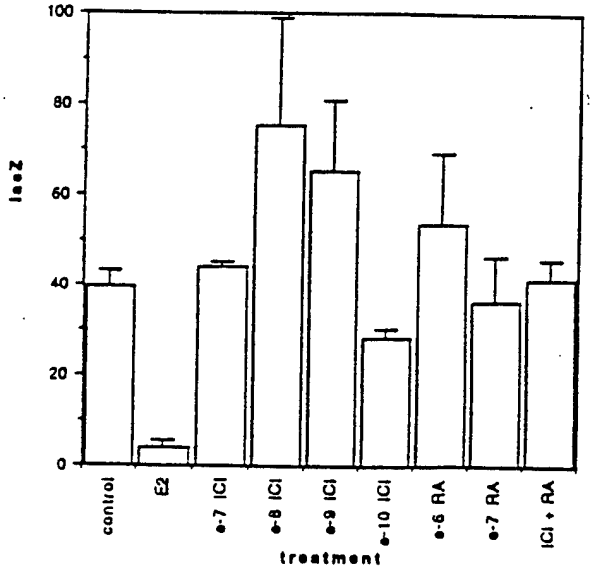
U3lacZ provides direct evidence that estrogen stimulates Bcl-2 expression in ER positive cells.

ER expression in breast cancer is a predictor of response to antiestrogen therapy (25). Unfortunately, many ER positive breast cancer do not respond to antiestrogen perhaps due to the escape from normal estrogen regulation (25). Since bcl-2 is a marker of ER function, bcl-2 could be used as a predictor of response to antiestrogen therapy. This concept is supported by clinical data by Gasparini et al. (26). Thus, bcl-2 seems to be a therapeutic response variable for tamoxifen treatment as well as a indicator for the presence of functional estrogen receptor in the tumor cells. Its role in chemotherapy response awaits further studies. We have verified the potential of our gene trap method in identifying and cloning genes that are involved in apoptosis by identifying a clone in which bcl-2, an anti-apoptotic regulator, is trapped by U3lacZ.

III. Cloning of a novel antiestrogen repressed gene.

As shown in figure 3, lacZ activity in a gene trap cell clone is repressed by estrogen and induced with ICI-164,384.

Figure 3



Anchor mediated PCR cloning of 5'-DNA flanking retroviral insert resulted in a 350 bp product. Sequencing of this PCR product revealed hereto undescribed gene with sequence as follows;

1 agggctgtgg gcctgtatct tgtttgctcg tcatcctgtc agtcattttt ttctttccct

61 ttttttaaag acaaaatctg aacctagaaa caccgaagcc agagcaaaaa ctagatgcga

121 atccactatt tgtgcgaccc

Full length cDNA cloning and examination of its role in apoptotic process and in endocrine and/or chemotherapy response is the current focus of this lab.

Conclusion:

I. Role of erbB-2 as a therapeutic response variable that predicts selective benefit from adriamycin could not be demonstrated using cases from NSABP B-11.

II. Role of erbB-2 as a therapeutic response variable for tamoxifen therapy could not be demonstrated using cases from NSABP B-14.

III. Gene trap U3lacZ was used to demonstrate that Bcl-2, an anti-apoptosis regulator, is an estrogen regulated gene in breast cancer cells, which explains its role as a therapeutic response variable for tamoxifen treatment.

IV. Gene trap U3lacZ was used to identify and clone a novel antiestrogen repressed gene. The mechanism of repression and the role of this gene in breast cancer need further study.

References

1) Rove, R.R., *J. Clin. Oncol.* **7**,803-815 (1989)

2) Wright C, et al.British J Cancer 65(1):118-121

3) Steller, H. *Science* **267**,1445-1449 (1995)

4) Aitken, S.C. & Lippman, M.E., *Cancer Res.* **45**, 1611-1620 (1985)

5) Wari, AM *et al. J. Natl. Cancer Inst.* **85**, 1412-1418 (1993)

6) Kyprianou, N. *et al. Cancer Res.* **51**, 162-166 (1991)

7) Reddy, S. *et al. J. Virol.* **65**, 1507-1515 (1991)

8) Paik S; Simpson S; King CR; Lippman ME; *Methods in Enzymology*, **198**:290-300 (1991)

9) Nolan, G.P. *et al. Proc. Natl. Acad. Sci. USA* **85**,2603-2607 (1988)

11) Wakeling, A.E. & Bowler, J., *J. Endocrinolo.* **112**, R7-R10 (1987)

12) Sanes, J.R., Rubenstein, J.L.R., Nicolas, J.F., *EMBO J.* **5**, 3133-3142 (1986)

13) Jordan, V.C., et al. J. Endocrin. 73, 305-316 (1977)

14) Rosenthal, N., *Meth. Enzymolo.* **152**, 704-720 (1987)

15) Altschul, S.F., *et al. J. Mol. Biol.* **215**,403-410 (1990)

16) Tsujimoto, Y. & Croce, C.M. *Proc. Natl. Acad. Sci. USA* **83**, 5214-5218 (1986)

17) Isegawa, Y., *et al. Molec. Cell. Probes* **6**, 467-475 (1992)

18) Bhargava, V., *et al. Am. J. Patholo.* **145**, 535-540 (1994)

19) Nathan, B., *et al. Ann. Oncol.* **5**, 409-414 (1994)

20) Doglioni, C., *et al. Virchows Arch.* **424**, 47-51 (1994)

21) Leek, R.D., *et al. Br. J. Canc.* **69**, 135-139 (1994)

22) Silvestrini, R., *et al. J. Natl. Canc. Inst.* **86**, 499-504 (1994)

23) Sabourin, J.C., *et al. Int. J. Cancer* **59**,1-6 (1994)

24) Gompel, A. *et al. Am. J. Patholo.* **144**, 1195-1202 (1994)

25) Paik, S. *et al. Br. Canc. Res. Treat.* **31**, 301-307 (1994)

26) Gasparini, G., *et al. Clinical Canc. Res.* **1**,189-198 (1995)