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13. ABSTRACT (Maximum 200) Overexpression of HER-2/neu (a member of the EGF receptor family) and inactivation of estrogen receptor (ER) or the tumor suppressor gene, Rb, are known to be involved in the development of human breast cancer. Expression of HER-2/neu can be regulated by ER or Rb. In addition, expression of other EGF receptor family genes (EGF receptor, HER-3 and HER-4) and Heregulin, a ligand for the HER-3 and HER-4 encoded receptor has also been found in some breast cancer cells and may contribute to malignant transformation of breast cancer. The current progress report focuses on the role of the EGF receptor family genes, Heregulin, ER, Rb, and their interrelationship in breast cancer. The technical objectives are: (1) Systematic studies on the expression of EGF receptor family genes, Heregulin, and ER in breast tumor specimens and correlation of the expression with tumor stages and patient survival; (2) Potential paracrine and autocrine loops for EGF receptor family molecules and Heregulin; (3) Effects of ER on malignant transformation phenotypes breast cancer cells; and (4) Effects of Rb on malignant transformation phenotypes breast cancer cells. Expression of EGF receptor family genes, Heregulin, and ER in the same breast tumor specimens were examined by immunohistochemical staining, and western blotting. The relationship between expression of these molecules, tumor grades, and patient's survival will be evaluated Using gene transfer technique, Heregulin, ER or Rb gene are being introduced into HER-2/neu-expressing breast cancer cells. The effect on their malignant transformations will be examined. This project may help to develop a more reliable molecular prognostic strategy and to understand how interactions among multiple genetic factors are involved in the development of breast cancer.					
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FOREWORD

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INTRODUCTION

A. Background

The data from the Surveillance, Epidemiology, and End Results (SEER) Program indicates breast cancer remains a major cause of death in this country (1). It was estimated that approximately 180,000 new cases of breast cancer will be diagnosed in the United States in 1995, and 46,000 women will die from this disease. Many oncogenes and tumor suppressor genes were shown to be involved in the development of breast cancer. The clinical value of each of these genes in prognostic and potential therapeutic applications has been studied to some extent (2). Yet, none of these molecular markers alone was found to be a better prognosis factor than the prognosis factors currently used for breast cancer patients (e.g., number of metastatic lymph nodes). **Identification of a set of related genes that are involved in breast cancer may be critical to develop a better molecular prognostic strategy. Studies on the role of a set of related genes and their interrelationship in breast cancer may also provide a more productive avenue to understand the basic biology of breast cancer cells.** Loss of estrogen receptor (ER) expression or function has been known for a long time to be associated with poor prognosis for breast cancer patients. The *HER-2/neu* oncogene encodes a growth factor receptor-like molecule and overexpression of *HER-2/neu* is also reported to correlate positively with poor survival for breast cancer patients. Some studies, including our own, demonstrate that estrogen-stimulated ER can repress *HER-2/neu* overexpression, suggesting a possible causal relationship between *HER-2/neu* overexpression and non-function of ER in breast cancer cells. The Rb gene is known to be inactivated in approximately 20% of breast tumors. It is not yet clear whether Rb might have prognosis value in breast cancer.

A-1. The *HER-2/neu* Proto-oncogene Encoding an Epidermal Growth Factor (EGF) Receptor-related Protein is a Potent Transforming Oncogene

The rat *neu* oncogene was a transforming gene originally isolated from rat neuroblastoma and later its cellular counterpart, the normal *neu* gene, was also isolated from rat and human libraries (3-6). Detailed structural and functional analysis of the transforming rat *neu* and the normal *neu* genes (*neu* proto-oncogene) indicates that a single point mutation in the transmembrane region is responsible for the conversion of the normal *neu* gene into a transforming *neu* oncogene (7). The human homologue (HER-2 or *c-erbB-2*) of rat *neu* oncogene was isolated based on its homology to chicken *v-erbB* gene (HER-2 represents Human EGF Receptor-2 and *c-erbB-2* means the second gene homologous to *v-erbB*; EGF receptor gene was the first). It is now known that *neu*, HER-2, and *c-erbB2* are the same gene. We will use *HER-2/neu* to represent this gene since *HER-2/neu* seems to be the most frequently used in the literature. Structural comparison between the *HER-2/neu*-encoded p185 protein and EGF receptor revealed significant sequence homology and identical gross structural organization including ligand-binding, transmembrane, and tyrosine-kinase domains between these two proteins (5-8). Both EGF receptor and p185 proteins can form either homodimers or heterodimers (9-11). The homodimer is believed to be an active receptor form for ligand binding. Two other EGF receptor-related genes, HER-3 and HER-4, have also recently been cloned. Recent experimental evidence indicates that these four EGF receptor-related proteins are able to form heterodimers among each other, suggesting different combination of heterodimers may interact with distinct ligands and induce signal transduction in specific cell type (12,13). A molecule (named Heregulin or NDF), thought to be a ligand for *HER-2/neu*, recently has been shown to be a ligand for HER-3 and HER-4 (14-19). Heregulin is able to bind HER-3 and HER-4 homodimers but cannot bind to *HER-2/neu* or EGF receptor homodimers. However, Heregulin is able to interact with *HER-2/neu* through heterodimerization of *HER-2/neu* with HER-3 or with HER-4 (12,13). Since Heregulin and the four EGF receptor-related proteins (EGF receptor, *HER-2/neu*, HER-3 and HER-4) can interact with each other, it is likely that they may all be involved in the development of breast cancer. In this Progress Report, we have begun to investigate their expression in breast tumor specimen by immunohistochemical staining.

A-2. Amplification/Overexpression of the Human *HER-2/neu* Gene is Frequently Found in Human Cancers including Breast Cancer.

Unlike the rat *neu* oncogene that is activated by a single point mutation, the human *HER-2/neu* gene is activated by overexpression in human cancers. Amplification/overexpression of the *HER-2/neu* gene was first found in approximately 30% of human breast cancers (20-25) and later in many other human cancers

(31-36). In the case of breast, ovarian, lung and gastric cancers, several reports further indicated that *HER-2/neu* overexpression correlates with a poor survival rate (25-31), suggesting that *HER-2/neu* overexpression may be used as a prognosis factor (25-27, 20, 22). However, some studies disagree that *HER-2/neu* overexpression can be a poor prognostic factor in breast cancer (32-34). Although the discrepancy could be caused by reasons such as patient population and treatment differences, the methods and reagents used for detection of *HER-2/neu* overexpression and the way tumor specimens were collected, other more significant reasons may also contribute to this discrepancy. Considering the fact that the development of breast cancer requires multi-step activations, it is possible that *HER-2/neu* overexpression alone may not be an ideal prognosis factor for breast cancer patients. Combined data from the expression of multiple oncogenes and tumor suppressor genes involved in breast cancer may provide a more accurate prognosis.

A-3. Non-function of ER May Contribute to *HER-2/neu* Overexpression in Some Breast Cancer

Steroid hormones play an essential part in regulating the growth of both normal and neoplastic breast cells. Specifically, estrogen has a marked effect on the proliferation of breast cells *in vivo* and *in vitro*. Although the mechanisms by which estradiol (E2) induces proliferation in estrogen receptor (ER) positive breast cells are incompletely defined, modulation in expression of certain growth related cellular proto-oncogenes by estradiol stimulated estrogen receptor (E2/ER) has been well-described using cell lines established from human breast tumors. The function of ER in breast cancer is unclear; however, the significant correlation between loss of functional estrogen receptor and poor patient prognosis is very well described (35,36). Evidence that ER may play a role in the regulation of *HER-2/neu* expression comes from several diverse observations. Several studies of human breast tumor tissue specimens have shown an inverse relationship between ER and *HER-2/neu* expression (37-40). Furthermore, during development of rat mammary glands, expression of *HER-2/neu* is inversely related to ER status (41). Those breast cancer cell lines with the highest levels of *HER-2/neu* overexpression are generally ER negative or have very low levels of estrogen receptor. We and others have previously shown that ER through estrogen stimulation can negatively regulate the expression of *HER-2/neu* in ER positive but not ER negative breast cancer cell lines (42-44). We further demonstrated that the ER-mediated *HER-2/neu* repression can occur at the transcriptional level (44). The result suggests that *HER-2/neu* overexpression may be caused by inactivation of ER in some breast tumors. It also raises an interesting possibility that expression of ER in the ER negative and *HER-2/neu*-overexpressing breast cancer cells may suppress malignant transformation induced by *HER-2/neu* overexpression. If this indeed is correct, it may provide an interpretation for a well-known clinical phenomenon, namely, some ER positive breast cancer patients do not respond to hormone treatment such as Tamoxifen (TAM), an estrogen antagonist.

A-4. The Tumor Suppressor Gene, Rb, May Suppress Tumorigenicity of Human Breast Cancer Cells

The retinoblastoma susceptibility gene (Rb) is a well-characterized tumor suppressor gene (45). The existence of this gene was initially predicted based upon genetic predispositions to certain pediatric malignancies (46). Tumor formation or transformation occurs when these genes are inactivated, suggesting that their normal function is to limit cellular proliferation. Inactivation or deletion of Rb has been found in a variety of human cancers including breast cancer (45). Using retroviral-mediated gene transfer, it has been shown that the Rb gene can suppress tumor formation of retinoblastoma, osteosarcoma, and breast carcinoma in which the endogenous Rb gene is inactivated (47-49).

The Rb gene encodes a 105kDa protein (RB) and is known to form a protein complex with adenovirus E1A protein as well as large T (LT) antigen of SV40 virus and E7 protein of papilloma virus (50-52). It is believed that the DNA virus-associated proteins such as E1A, LT and E7 may inactivate the RB function through RB-E1A (or LT, E7) complex. Biochemically, RB can function as a transcriptional factor that can regulate transcription of cellular genes including *c-myc*, *TGF-b*, *c-fos* and *HER-2/neu* (53-56). More recently, RB has been shown to form a protein complex with a DNA-binding protein E2F and may, therefore, act as a transcriptional factor by complexing with other factors (57,58). In this progress report, we will focus on the effect of RB-associated E2F-1 on breast cancer cells.

B. THE PURPOSES

The major purposes are:

B-1. Systematic studies on the expression of EGF receptor family, Heregulin, ER in breast tumor specimens and correlation of the expression with tumor stages and patient survival.

Our hypothesis is that the combination of EGF receptor family, Heregulin, ER may be a better prognosis factor than each of these molecules individually. Therefore, expression of EGF receptor family, Heregulin, ER, in the same breast tumor specimens will be examined by immuno-histochemical staining. The relationship between expression of these molecules, tumor grades and patients' survival will be evaluated.

B-2. Potential paracrine and autocrine interactions between EGF receptor family and Heregulin in breast cancer cells.

Potential paracrine and autocrine loops between EGF receptor family and Heregulin ligand will be tested by using expression vectors and model cell lines. Effects of Heregulin on transformation phenotypes of breast cancer cells will be examined by growth properties, soft agar colonization assay, subcutaneous (s.c.) tumorigenicity and intraperitoneal (i.p.) survival assays. The Heregulin-mediated signal pathway will be studied, especially emphasize on involvement of NF-kB.

B-3. Effects of ER on malignant transformation phenotypes of *HER-2/neu*-overexpressing breast cancer cells.

Since we have found that estrogen-stimulated ER can repress *HER-2/neu* gene expression, ER expression vectors will be used to modulate *HER-2/neu* expression in *HER-2/neu* overexpressing breast cancer cells. The effect of ER on transformation phenotypes of *HER-2/neu* overexpressing breast cancer cells has been examined. The effect of receptor tyrosine kinase on ER will also be examined.

B-4. Effects of Rb on malignant transformation phenotypes of breast cancer cells.

The effects of Rb on *HER-2/neu* expression and transformation phenotypes have been analyzed. The effect of RB-associated E2F-1 on breast cancer cells will be explored.

BODY

1. Systematic studies on the expression of EGF receptor family, Heregulin, ER and Rb.

As mentioned in the last progress report, Heregulin is not a ligand for HER-2/*neu* (12-14), instead Heregulin is a ligand for HER-3 and HER-4 (also known as *c-erbB3* and *c-erbB4*) that are two recently identified EGF receptor family genes. Since HER-3 and HER-4 can form heterodimers with HER-2/*neu* and interactions between Heregulin and HER-2/*neu* is most likely through HER-3:HER-2/*neu* dimer or HER-4:HER-2/*neu* dimer (12-13), it is possible that HER-3 and HER-4 may also contribute to breast cancer. In addition, overexpression of EGF receptor is known to be involved in human breast cancer. In this Progress Report, we have expanded our expression study in breast tumor tissues for all 4 EGF receptor family (EGF receptor, HER-2/*neu*, HER-3 and HER-4).

We have used specific antibodies for EGF receptor, HER-2/*neu*, HER-3, HER-4, and Heregulin to stain the archival paraffin-embedded sections. Up to now, 250 tumor sections have been completed for all EGF receptor family including EGF receptor, HER-2/*neu*, HER-3 and HER-4. One set of representative data was shown in Fig. 1. In these cases, we can identify tumors with high, intermediate, low and negative expression. Quantitation of antibody staining is justified by relative intensity of positive staining by professional pathologist. It is obvious that differential expression of all four EGF receptor family can be found in the tumor tissues, suggesting their possible role in the development of breast tumors. We are currently collecting clinical status including tumor grades, patient survival, and ER status from the archival medical record. We will also continue to screen more tumor sections (we hope to have data more than 300 patients to make statistically significant conclusion). We expect that immunohistochemical staining will be completed within half year (300 tumor sections with multiple genes as proposed). At that point, we will analyze the relationship between clinical status and expression of each individual gene and a set of genes. Multiple variant analysis will be conducted.

As mentioned in the last progress report, immunohistochemical staining for Rb protein will not be a very informative strategy to detect inactivation of Rb in tumor tissues (This was also discussed in the original grant proposal.), we would, therefore, like to focus on EGF receptor family and Heregulin in the expression studies.

2. Potential Paracrine and Autocrine Interactions between HER-2/*neu* and Heregulin in Breast Cancer Cells.

In addition to our original approaches to establish stable transfectants of Heregulin in HER-2/*neu* and HER-3 positive breast cancer cell lines and anti-sense Heregulin in Heregulin-expressing cells (such as MDA-MB-231), we have also used another approach by adding Heregulin to the HER-2 and HER-3 positive cells. We used breast cancer cells, MCF7 (HER-3 positive) and its HER-2/*neu* transfectants, MCF-7/HER-2-11 and MCF-7/HER2-18 (HER-3 positive and HER-2/*neu* overexpression) and found that upon Heregulin stimulation, NF- κ B is activated in the transfectants (HER-3, HER-2 positive), but not in the parental MCF7 cells (HER-3 positive and HER-2/*neu* basal level) (Fig. 2), and this observation was correlated with increased cell survival (Fig. 3). Recently, activation of NF- κ B has been shown to have anti-apoptotic effect, the results shown in Figs 2 and 3 suggest that Heregulin may stimulate NF- κ B and this activation requires HER-2/*neu* overexpression (mostly through HER-3/HER-2 heterodimer). The Heregulin-mediated NF- κ B activation may produce anti-apoptotic activity and contribute to Heregulin-stimulated cell growth. This may provide a molecular mechanism for paracrine and/or autocrine interactions between HER-2/*neu* and Heregulin in breast cancer cells. A systematic study on this issue will be continued using MTT assay, thymidine incorporation assay and apoptotic assays including DNA ladders, TUNEL assay etc in the MCF7 and its HER-2/*neu* transfectants. In addition, we will also use the Heregulin stable transfectants mentioned earlier to address this issue

3. Effects of ER on Malignant Transformation Phenotypes of HER-2/*neu*-overexpressing Breast Cancer Cells.

In the last report, we have shown that our hypothesis is correct that estrogen suppresses transformation phenotype of ER⁺, HER-2/*neu* -overexpressing breast cancer cells. A reprint of the published paper (Oncology Report) was attached in the last report. It is known that HER-2/*neu* and its family receptors are tyrosine kinase receptors. In this report, we have extended our study on the involvement of tyrosine kinase pathway in ER signal pathway. An abstract describing these works was just submitted to AACR annual meeting. The abstract was described as following and several key data were shown as Fig 4-9. These results have provided a linkage between ER and tyrosine kinase signal pathways.

In an attempt to study the involvement of tyrosine kinase pathway in estrogen receptor signaling pathway, we used emodin, a tyrosine kinase inhibitor, to treat MCF-7 breast cancer cell line which express high level of estrogen receptor protein. Here we reported that emodin inhibited estrogen-induced expression of bcl-2 protein in MCF-7 breast cancer cells. Unexpectedly, treatment of the cells with emodin rapidly depletes cellular levels of estrogen receptor protein in a dose- and time-dependent manner. The pulse chase experiment showed that the decrease was resulted from enhanced degradation of estrogen receptor protein, not the rate of synthesis. To examine the mechanism involved in the emodin-enhanced degradation of estrogen receptor, inhibitors of the lysosomal (chloroquine), proteasome (PSI and MG115), and calpains pathways were used. We found that only PSI and MG115, which specifically inhibit the chymotrypsin-like activity of proteasome, blocked emodin induced depletion of estrogen receptor protein levels. The results suggest that the proteasome proteolytic pathway may be involved in the emodin-induced decreases in estrogen receptor protein levels. We then examined the effect of emodin on the hsp90-estrogen receptor heteromeric complex formation. We found that there was a marked increase in the complex formation. The data demonstrate that emodin may inhibit the dissociation of hsp90 from estrogen receptor, resulting in the degradation of estrogen receptor. Overall these findings indicate that proteasome-mediated protein degradation can modulate estrogen receptor protein level, hsp90 can mediate the degradation of the estrogen receptor, and the possible use of emodin in the therapeutic manipulation of this process.

4. Effects of Rb on Malignant Transformation Phenotypes of Breast Cancer Cells

Using adenoviral vector expressing Rb, we have shown in the last report that Wt Rb can suppress transformation phenotype of Rb-defective breast cancer cells such as MDA-MB-468 and BT-549. Since RB is known to bind to E2F-1, we extend our study to examine effect of E2F-1 overexpression on breast cancer cells. We made an adenoviral vector that express E2F-1 and found that overexpressing of E2F-1 induces apoptosis in human breast and ovarian cancer cells. A manuscript describing these works have just appeared in Cancer Research (Hunt et al., Cancer Res. 57:4722-4726, 1997). A reprint is attached in lieu of detailed description.

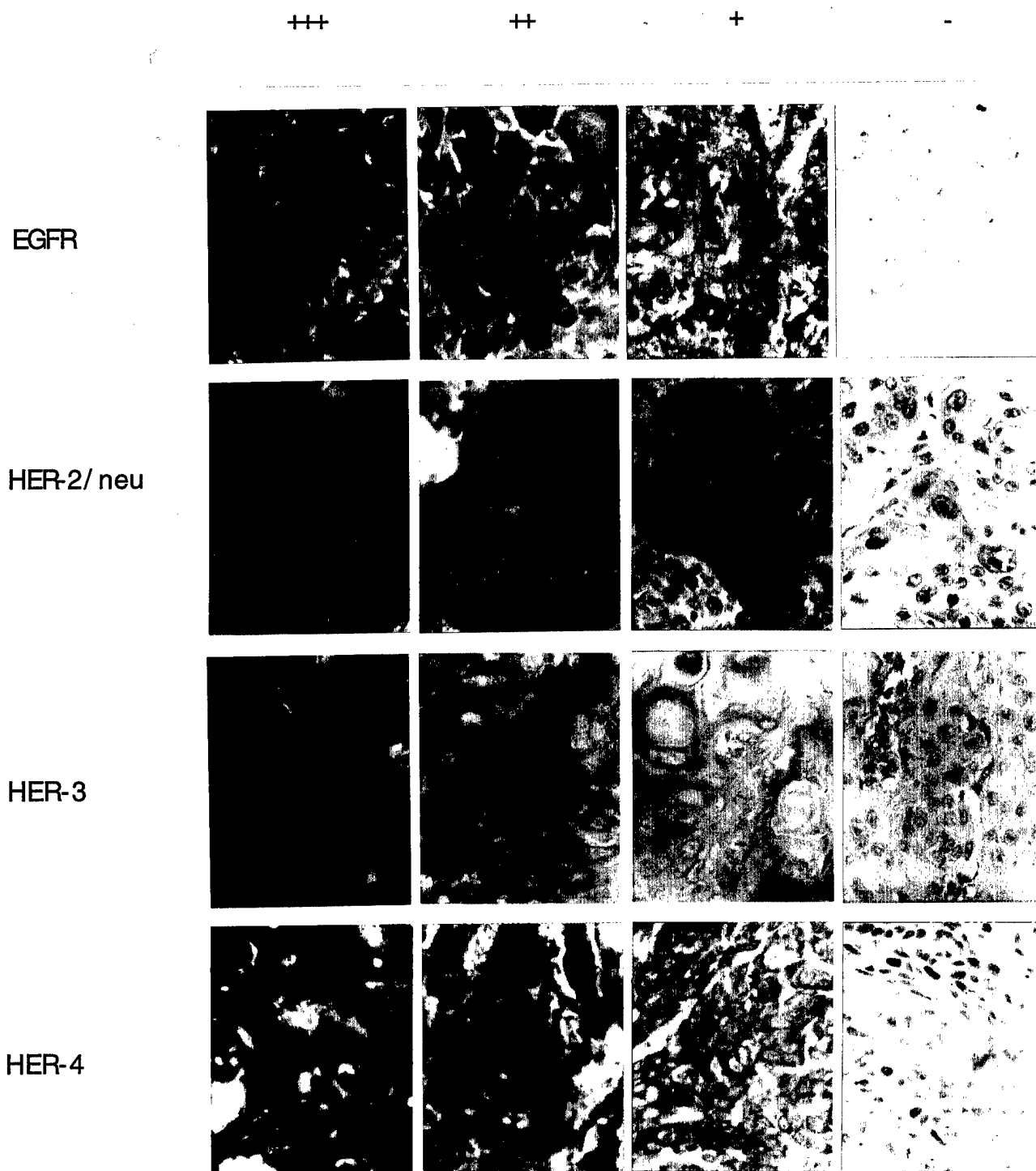


Fig.1. Immunohistostaining of tumor sections for EGF receptor family.
The relative intensities were marked by +++, ++, +, and - .

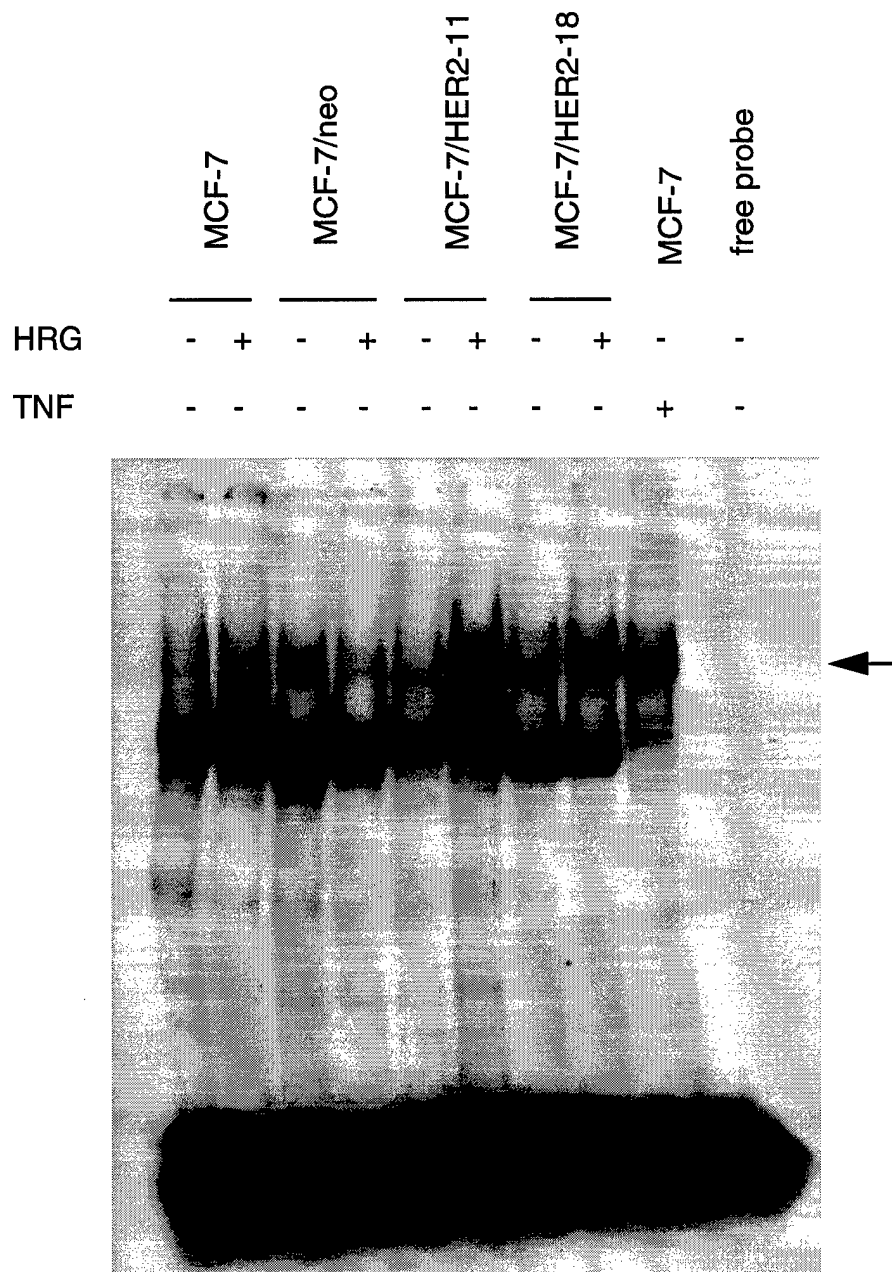


Fig. 2 Heregulin activates NF- κ B in MCF-7 cells overexpressing HER-2/neu. Cells were treated in presence or absence of heregulin (5ng/ml) for 1 hr in PBS/ 0.1% BSA. Equal amounts of nuclear extracts were subjected to electrophoretic mobility shift analysis to detect NF- κ B DNA binding activity. MCF-7/neo, MCF-7 transfected with vector; MCF-7/HER2-11 and MCF-7/HER2-18, two transfectant cell lines overexpressing HER-2/neu. Arrow indicates p50/RelA complex bound to oligonucleotides containing κ B site.

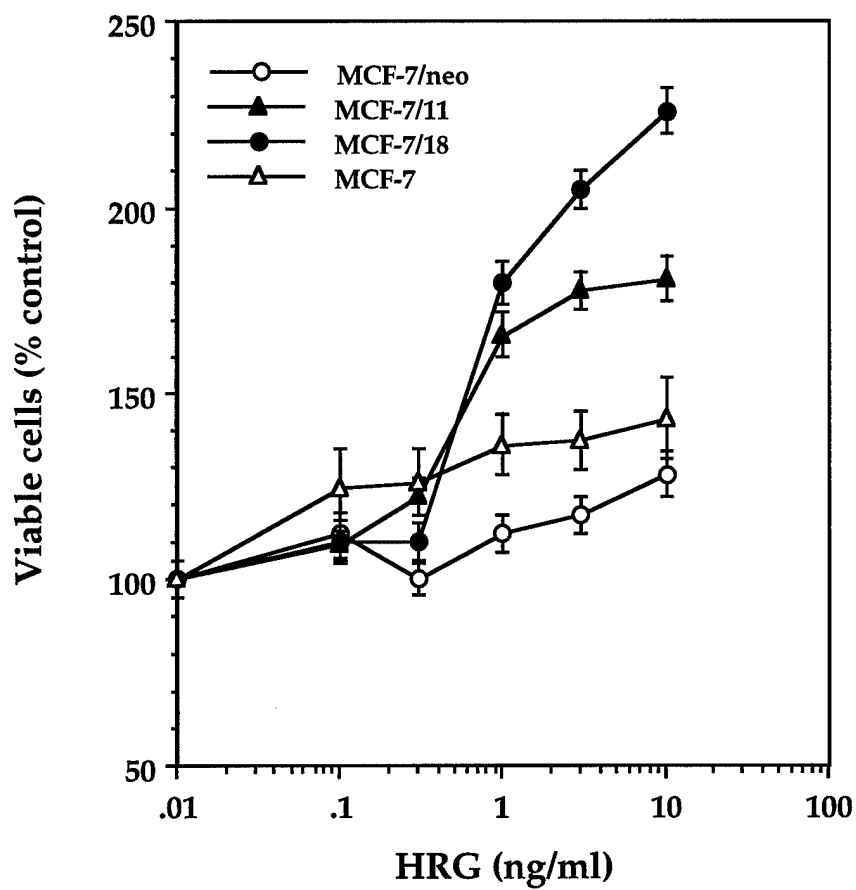


Fig. 3 Heregulin stimulates cell growth/survival in MCF-7 transfectants overexpressing HER-2/neu. Cells were seeded at 2×10^4 cells/ml in a 96-well microtiter plate overnight in 10% serum medium. Subsequently cells were incubated for additional 72 hrs in a medium containing 0.1% serum and varying amounts of heregulin. Cell viability was measured by MTT assay.

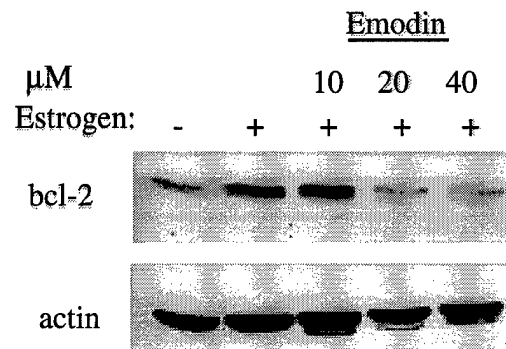


Fig. 4. Emodin inhibits estrogen-induced bcl-2 protein expression in MCF-7 cells. MCF-7 cells were grown in the estrogen depleted medium for one day before the initiation of the experiment. The cells were then stimulated with estrogen (10 nM). Different concentrations of emodin were added simultaneously. After 24 hours, total cell lysates were prepared and immunoblotting analysis for bcl-2 protein were determined. The membrane was stripped and reprobed with anti-actin antibody to show the protein loading.

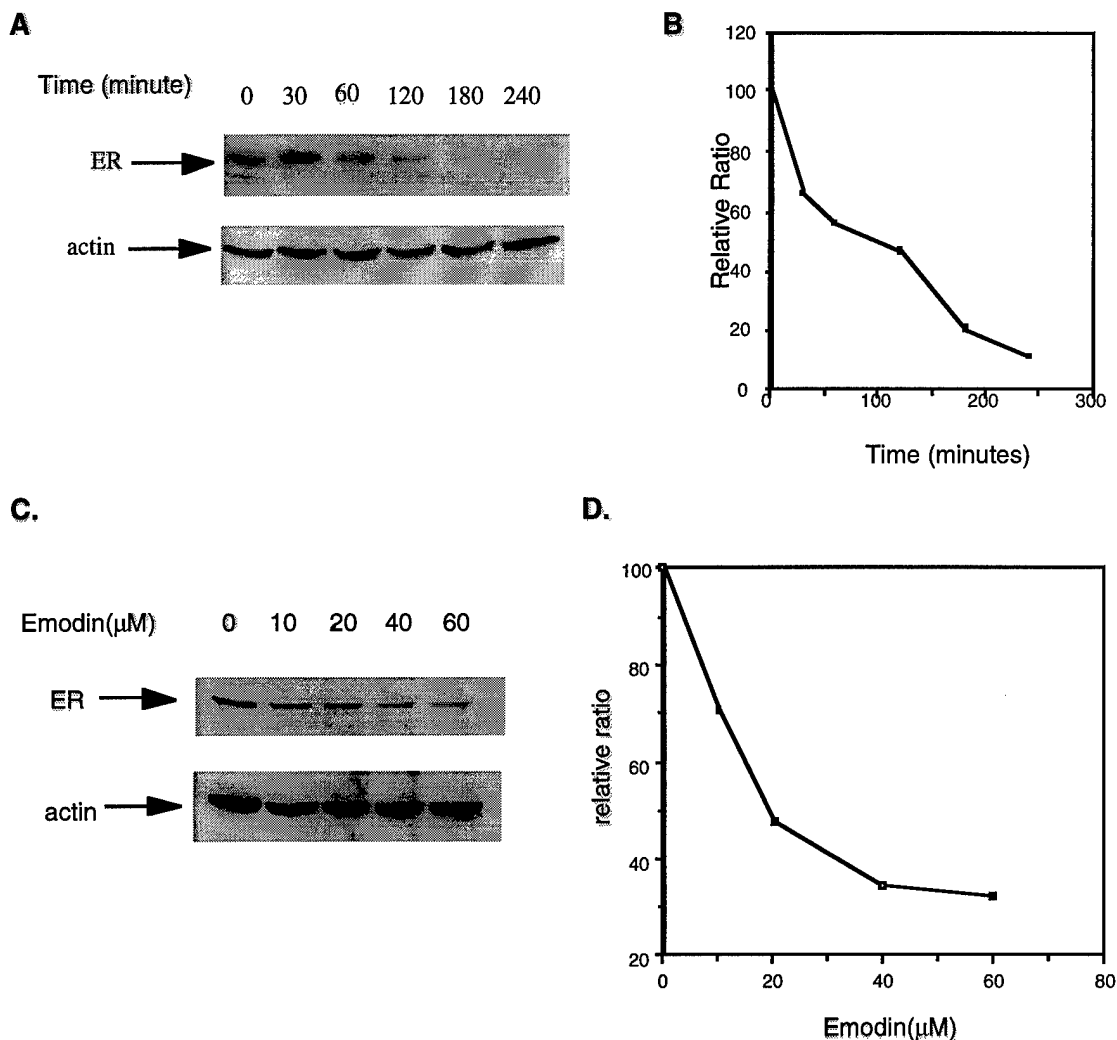


Fig. 5. Depletion of estrogen receptor protein in estrogen receptor positive cells by emodin. A, MCF-7 cells were exposed to 40 μ M emodin for various time intervals and extracted as described in. Estrogen receptor protein levels in MCF-7 cells as measured by immunoblotting with monoclonal antibody D75. The same membrane was stripped and reprobed with anti- β -actin antibody to show the protein loading. B, the proteins were quantitated by NIH Image software and plotted as the % control (without emodin) and normalized with actin. C, MCF-7 cells were incubated with different concentrations of emodin for 4 hours. The protein levels were then examined by immunoblotting as described above. The protein levels were quantitated as described above and plotted as showed in panel D.

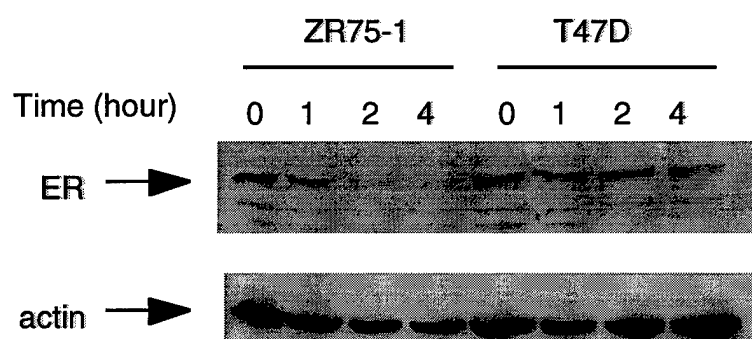


Fig.6. Effect of emodin on estrogen receptor protein levels was examined in two other estrogen receptor positive cell lines, ZR75-1 and T47D. The cells were treated with 40 μ M emodin for different time intervals. Immunoblotting analysis for estrogen receptor protein was performed. The membrane was stripped and reprobbed with anti-actin antibody to show the protein loading.

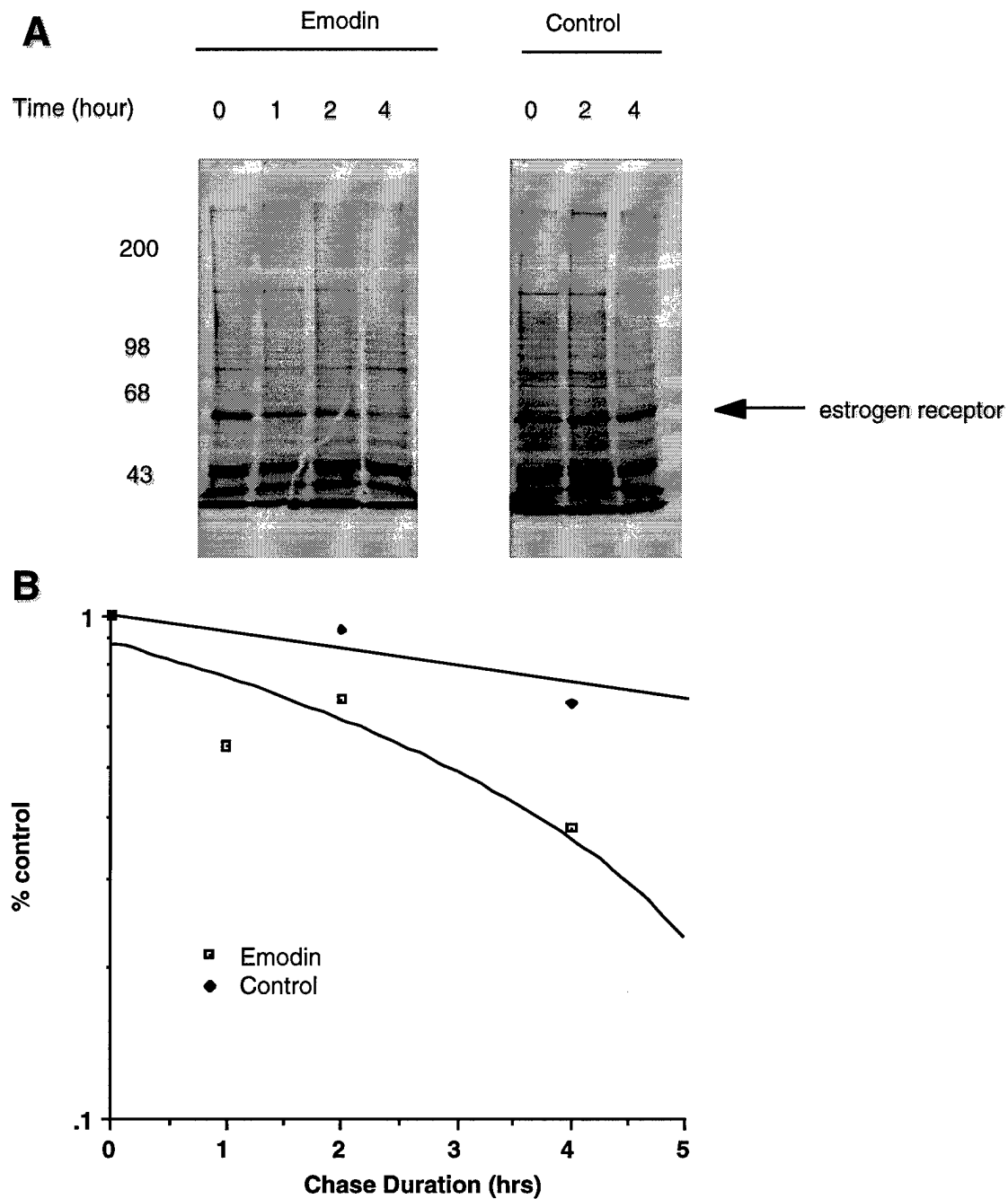


Fig.7. Emodin-enhanced estrogen receptor protein degradation. Pulse chase experiment was performed to determine the stability of the estrogen receptor proteins after treatment with emodin . A, MCF-7 cells were treated with emodin at different time intervals. B, MCF-7 cells were treated with DMSO at various time intervals. C, proteins were quantitated by NIH Image software and plotted as the % of the value at the beginning of the chase.

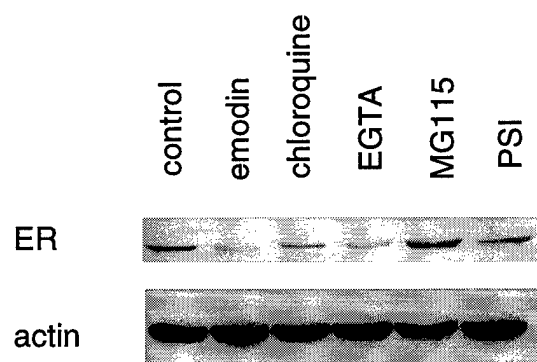


Fig. 8. Effect of different protease inhibitors on emodin-induced estrogen receptor protein degradation. Chloroquine (100 μ M), EGTA (5mM), MG115 (25 μ M), and PSI (25 μ M) were added to MCF-7 cells simultaneously with 40 μ M emodin. PBS and DMSO were added to the control. The cells were then harvested and the expression level of estrogen receptor protein were measured by western blotting analysis.

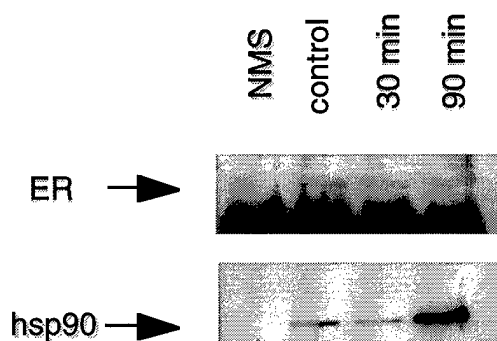


Fig. 9. Enhanced association of hsp90 and estrogen receptor protein in MCF-7 cells after incubation with emodin. Estrogen receptor immunoprecipitates (by anti-estrogen receptor antibody, SRA1010) from MCF-7 cells were analyzed by SDS/PAGE and immunoblotting with anti-hsp90 (AC88). Normal mouse serum (NMS) was used instead of anti-estrogen receptor antibody as a control. The membrane was then stripped and reprobed with anti-estrogen receptor (D75) antibodies.

CONCLUSION:

Task 1: Screening of 250 tumor sections for EGF receptor, HER-2/*neu*, HER-3, and HER-4 has been completed. Due to the complexity caused by the newly identified HER-3 and HER-4 (12, 13), we will emphasize more on the staining of EGF receptor, HER-2/*neu*, HER-3, HER-4 and Heregulin. We also have begun to collect data and analyze between gene expression and clinical status from medical record.

Task 2: Construction of Heregulin-expression vectors and anti-sense Heregulin plasmids have been completed. Stable transfectants of both Heregulin and anti-sense Heregulin are completed. Heregulin was found to activate NF- κ B activity in the HER-3 positive and HER-2/*neu*-overexpressing cells but not in the HER-3 positive and HER-2/*neu* low-expressing cells.

Task 3: Characterization of E2 on ER⁺, HER-2/*neu*-overexpressing breast cancer cells was completed. A manuscript describing these works was published last year. A tyrosine kinase inhibitor was found to inactivate ER through depletion of ER via proteosome proteolytic pathway.

Task 4: Expression of Rb is able to suppress transformation phenotype of Rb-defective breast cancer cells. The Rb-associated E2F-1 protein was found to induce apoptosis in breast cancer cells through a p53-independent pathway.

In addition to the those described in the BODY Section, several studies relating to HER-2/*neu* oncogene in breast cancer have been completed. The funding support from the current project has been appropriately acknowledged in the resulting publications. These include:

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