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13. ABSTRACT ***(Maximum 200 Words)***

In this study, we investigated the structural requirements of erbB2 for erbB2 mediated higher metastatic potential in human breast cancer cells. We found that the kinase domain of erbB2 receptor is required for erbB2 to enhance metastatic potential of human breast cancer cells. The C-terminal tyrosine of erbB2 may also play roles in erbB2 mediated higher metastatic potential of breast cancer cells. We also provided the evidence that HRG/egf can promote the metastatic potential of MDA-MB-435 cell erbB2 transfectants *in vivo*.

In addition, we investigated the signaling mechanism of heregulin enhanced MCF-7 and SKBR-3 cell aggregation. We found that HRG–β1-induced PI3-kinase activation is required in HRG enhanced MCF-7 and SKBR-3 breast cancer cell aggregation.

Our studies have generated important information about the molecular basis of breast cancer invasion and metastasis. Ultimately we may use the information to develop new prognostic indicators and novel therapies for breast cancer metastasis.

14. SUBJECT TERMS
Breast Cancer, erbB2/HER-2, Metastasis, Heregulin, Signal transduction

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Table of Contents

Cover......1

SF 298......2

Table of Contents......3

Introduction......4

Body......4

Key Research Accomplishments......9

Reportable Outcomes......10

Conclusions......11

References......11

Appendices......12

INTRODUCTION

Breast cancer is one of the most common malignancies among women in the United States, and metastasis from this cancer is the major cause of death for these patients. Therefore, it is extremely important to uncover the basis of breast cancer metastasis. Overexpression of the c-erbB2 (also known as HER-2, *neu*) gene has been shown to be correlated with poor prognosis and the number of lymph node metastases in breast cancer patients. Our recent work has demonstrated that stable transfection of the human c-erbB2 gene into the low c-erbB2-expressing MDA-MB-435 human breast cancer cells (named 435.eB transfectants) indeed enhanced the intrinsic metastatic potential of these cells (1). Because overexpression of the c-erbB2 gene has been found in ~ 30% of breast tumors, it is very important to examine the molecular mechanisms underlying the enhanced metastatic potential induced by c-erbB2 overexpression and then to design new strategies to treat this type of breast cancer metastasis. We hypothesize that the c-erbB2-encoded receptor tyrosine kinase (RTK) may enhance metastatic potential through the RTK-signaling molecules. The purpose of this proposed study is to test whether the increased c-erbB2 tyrosine kinase activity and tyrosine autophosphorylation on the carboxyl-terminal tail may be required for the downstream signaling involved in breast cancer metastasis. To address this question we will study: 1) The requirement of the tyrosine kinase domain and tyrosine autophosphorylation sites in the c-erbB2 receptor for mediating signals leading to metastasis. 2) The downstream signals of c-erbB2 that may contribute to increased metastatic potential.

BODY

Objective 1. To evaluate the requirement of the tyrosine kinase domain and tyrosine autophosphorylation sites in the erbB2 receptor for mediating signals leading to metastasis.

<u>Task 1</u>: Completed (See 1997-1998 annual report).

<u>Task 2</u>: Completed (See 1997-1998 annual report).

<u>Task 3</u>: Completed (See 1997-1998 annual report).

<u>Task 4</u>: Completed within this finding period. To evaluate the structural motifs of erbB2 that are required for erbB2 enhanced metastatic potential of human breast cancer cells, we need a panel of mutated erbB2 gene expressing cell lines with same genetic background. We transfected MDA-MB-435 human breast cancer cells with the four types of mutants. ErbB2-containing pCDNA3-erbB2 plasmids were composed of erbB2 cDNA sequences with mutations or deletion

as described above, and erbB2 gene was driven by pCMV promoter, which is same as the vector expressing wild-type erbB2. Western bolt analysis shows that these erbB2 mutants transfected cells express comparable levels of mutated erbB2 proteins (Fig. 1, Top).

To determine if the structural changes in erbB2 mutants may result in changes in tyrosine-phosphorylation of erbB2 proteins in erbB2 gene transfected MDA-MB-435 cells. Western blot analysis using anti-phosphotyrosine antibodies was performed (Fig. 1, bottom). As we expected, little tyrosine phosphorylation of erbB2 protein has been found in transfectants that expressing K753M mutant and C1025 mutant, because of kinase defective (K753M) and C-terminal phosphorylation sites deletion (C1025). We also detected a reduced tyrosine phosphorylation of erbB2 protein in transfectants expressing Y1248F compared to those transfectants expressing wild-type erbB2 (435.eB1). Further more, we detected higher tyrosine phosphorylation of erbB2 proteins in transfectants expressing the constitutive activated V659E mutant compared to 435.eB1, which indicates the higher intrinsic tyrosine kinase activities of this mutant.

To determine the impact on metastatic potential from the structural changes in the erbB2 receptor, we tested the metastatic survival rate of these mice by injecting wild-type or mutant erbB2 expressing cell lines alone or with EGF domain of heregulin (HRG/egf) into the lateral tail vein of immunodificient ICR-SCID mice. Heregulin (HRG) is a family of polypeptide growth factors derived from alternatively spliced genes. HRG can bind to receptor tyrosine kinases erbB3 and erbB4, thereby inducing erbB3 and erbB4 heterodimerization with erbB2, leading to receptor tyrosine phosphorylation, and activating downstream signal transduction. The EGF-like domain is an important functional domain of heregulin. As the full-length extracellular domain of heregulin, HRG/egf was reported as an erbB receptor stimulator that can increase the phosphorylation of erbB receptors (1, 2). We injected mice the wild-type and mutated erbB2 expressing cells with HRG/egf to see whether HRG/egf could promote the metastatic potential of erbB2 overexpressing breast cancer cells.

Single-cell suspensions of each cell line were injected into the lateral tail veins of 8-week-old female ICR-SCID mice. 1 X 10^6 cells/mouse was injected without or with 5 μg/kg HRG/egf. The group injected with HRG/egf was injected HRG/egf 5 μg/kg three days after the first injection, the dose of HRG/egf then changed to 7.5 μg/kg once a week for another 7 weeks, totally 9 HRG/egf injections were administered. To test the impact of erbB2 structural

alterations on the metastatic potential of these cell lines and the impact on mice survival, after injection, all the mice were maintained under identical conditions and monitored regularly. Once a mouse was moribund or dead, euthanasia and/or necropsy was performed to determine the cause resulted in the death or moribund of the mouse. We found that the reason for death for all the dead mice that were injected with erbB2 mutant transfectants, except one was tumor metastasis to lungs or other organs. The reason for death of the mouse did not die of tumor metastasis is still not clear, but we did not find any tumors in this mouse when necropsy was performed. The mice metastatic survival curves were shown in Fig. 2. In mice injected with wild-type or mutant erbB2 expressing cells, 435.eB1 and V659E injected mice have significant lower metastatic survival rates comparing with erbB2 low expressing cell lines MDA-MB-435 and 435.neo (Fig. 2A). ($P=0.0420 < 0.05$, comparing 435.eB1 with 435.neo; $P=0.0252 < 0.05$, comparing V659E with 435.neo). However, although the erbB2 protein exprression levels are much higher in kinase-defective K753M cells than that in MDA-MB-435 and 435.neo cells, the metastatic survival rates of mice injected with K753M cell line were similar to those injected with parental and control cell lines MDA-MB-435 and 435.neo ($P=0.4641 >0.05$, comparing with 435.neo) (Fig. 2A). These data have no statistical significance, which may due to small sample size. However, we can see from the survival curves that the metastatic survival rates of Y1248F cell injected mice are lower than those of injected with MDA-MB-435 and 435.neo ($P=0.2346 > 0.05$, comparing with 435.neo), but are higher than those injected with 435.eB1 and V659E (Fig. 2A). These results indicate that an intact kinase domain is required for erbB2 mediated enhancement of metastatic potential of breast cancer cells, which indicates kinase activity of erbB2 is important in erbB2 mediated higher metastatic potential of human breast cancer. In addition, C-terminal tyrosine 1248 phosphorylation of erbB2 might also play a role in erbB2 enhanced metastasis. To our surprise, although a large portion of the carboxyl terminal tail of erbB2 was deleted in the mutant C1025, the metastatic survival rates of the mice injected with C1025 are much lower than the mice injected with the erbB2-low-expressing MDA-MB-435 and 435.neo cell lines ($P=0.0102 < 0.05$, comparing with 435.neo), indicating that this mutant retains high metastatic potential. It was noteworthy that this mutant does have an intact kinase domain and part of carboxyl terminal sequence. Therefore, this mutant may retain its tyrosine kinase activity and the remaining carboxyl terminal sequence may still functional in erbB2 mediated higher cancer metastatic potential. Further investigation on this mutant is

needed to clearly explain its behavior. In mice injected tumor cells with HRG/egf, similar results were obtained as mice injected with tumor cells alone, except the overall survival rates were lower in HRG/egf injected mice comparing to mice no HRG/egf injection, which further confirmed the above results (Fig. 2B).

To test the effect of HRG/egf on erbB2 induced higher metastatic potential, we compared the mice injected tumor cells without and with HRG/egf for their metastatic survival rates. We found that mice injected with HRG/egf has significant lower metastatic survival rates compare to mice without HRG/egf injection ($P=0.0387 < 0.05$). The average surviving days for all the mice without HRG/egf injection is 102 days and mice with HRG/egf injection is 92 days. The metastatic survival curves for each cell line with or without HRG/egf injection are shown in Fig. 3, A to F. These results indicate that HRG/egf can promote metastatic potential of human breast cancer cell MDA-MB-435 erbB2 transfectants. It is noticeable that in erbB2 overexpressing cell lines, 435.eB1, V659E, Y1248F, K753M and C1025, the effect of HRG/egf is more obvious than those erbB2 low expressing cell lines MDA-MB-435 and 435.neo cells (Fig.3), which is consistent to previous reported role of erbB2 in potentiating heregulin induced signaling.

Task 5:Completed within this finding period. To examine the whether the invasion process may be different between wild-type and mutant erbB2 transfected MDA-MB-435 cells. Invasion assay has been performed by using matrigel-coated transwell (1). As shown in Fig. 4, 435.eB1, VE.ss and C1025 cells have higher invasion abilities. Y1248F mutant cells have lower invasion ability and Kinase defective mutant K753M cells has the lowest invasion ability among these mutants. These results are correlated with the in vivo metastatic survival rates of mice injected with these cell lines, which suggest that a intact kinase domain is also required for tumor cell invasion through constitutive basement membrane Matrigel.

Task 6: Completed within this funding period. To determine if the structural changes in erbB2 mutants may result in changes in tyrosine kinase activity or erbB2 tyrosine-phosphorylation in vivo. Western blotting analysis using anti-phosphotyrosine antibodies was performed (Fig.1 bottom). As we expected, little tyrosine phosphorylation of erbB2 protein has been found in transfectants that expressing the kinase-defective K753M mutant and c-terminal-deletion C1025 mutant. This result indicate that K753M and C1025 mutant proteins are kinase defective in

MDA-MB-435 cells. We also detected a reduction in tyrosine phosphorylation of erbB2 protein in MDA-MB-435 transfectants expressing either the Y1248F mutant protein compared to those expressing the wild-type erbB2 protein, which indicate relatively lower intrinsic tyrosine kinase activity of Y1248F. Further more, we detected a higher tyrosine phosphorylation of erbB2 proteins in MDA-MB-435 transfectants expressing the constitutive activated V659E mutant proteins compared to those expressing the wild-type erbB2 protein, which indicate higher intrinsic tyrosine kinase activities of this mutant.

Objective 2. To investigate the immediate downstream signals of erbB2 that may contribute to increased metastatic potential.

Task 7: Completed within this funding period. In addition to Ras-Raf--ERK pathway, we also studied the involvement of PI3-kinase pathway. Since Ras-Raf-ERK pathway and PI3-kinase pathway are two important signaling pathways, we tested whether expression of these mutants altered protein expression of Shc, ERK, p85 subunit of PI3-kinase, and PI3-kinase downstream signaling molecule Akt. The results showed that the erbB2 mutant protein expression has no effect on the expression of these signaling molecules (Appendices, Fig 1of Report 1999). We also tested the activation of Ras-Raf-ERK and PI3-kinase pathways in erbB2 wild-type and mutant cell lines. We used phosphorylated ERK and phosphorylated Akt specific antibodies (from New England BioLab) to test the activation of these two pathways. However, we did not see significant difference among these cells for ERK and Akt phosphorylation levels (Appendices, Fig 2 of Report 1999,). Since we did not see significant difference in ERK, Akt activation, we used HRG to stimulate these cells before our assays. We used phosphorylated ERK and phosphorylated Akt specific antibodies to test the activation of ERK and Akt by western analysis (Appendices, Fig 3 of Report 1999). After HRG treatment, the phosphorylation levels of ERK in wild-type, V659E, Y1248F cell lines increased dramatically. However, in the C1025, very weak phosphorylation of ERK was observed and in K753M mutant only moderately phosphorylation of ERK. The phosphorylation levels of Akt were increased dramatically in Wild-type, V659E, Y1248F cells. Slight phosphorylation was observed in C1025 cells, but not in

K753M cells. These results indicate that C-terminal of erbB2 protein is required for HRG induced Ras-ERK pathway signaling and erbB2 kinase activity is required for HRG induced PI3-kinase pathway activation. These result also indicated that erbB2 receptor is required for HRG induced Ras-Raf-ERK pathway and PI3-kinase pathway signaling.

Task 8: Since we have demonstrated that Ras-raf-ERK pathway activation is correlated with the activation of erbB2 receptor by HRG. We modified our original proposal by deleted the studies on the binding ability of Grb2 to erbB2 receptor.

Task 9: See the modification of study described above.

Task 10: See the modification of study described above.

Task 11: Completed. A thesis on the role of erbB2 in human breast cancer metastasis has been written by P.I. based on this research.

KEY RESEARCH ACCOMPLISHMENTS

Key research accomplishments of this research are as follows,

- Established a panel of wild-type and mutant erbB2 gene transfectants, which can be used as the experimental system to study the mechanism of erbB2 gene enhanced human breast cancer metastasis.
- Investigated the structural requirements of erbB2 receptor for erbB2 mediated higher metastatic potential in human breast cancer cells. We found that the kinase domain of erbB2 receptor is required for erbB2 to enhance metastatic potential of human breast cancer cells. The C-terminal tyrosine of erbB2 may also play roles in erbB2 mediated higher metastatic potential of breast cancer cells. We also provided the evidence that HRG/egf can promote the metastatic potential of MDA-MB-435 cell erbB2 transfectants *in vivo*.
- Investigated the signaling mechanism of heregulin enhanced MCF-7 and SKBR-3 cell aggregation. We found that HRG–β1-induced PI3-kinase activation is required in HRG

enhanced MCF-7 and SKBR-3 breast cancer cell aggregation.

REPORTABLE OUTCOMES:

Publications in which USAMRC was acknowledged

1. **Tan M.**, Grijalva R., Yu D. Heregulin β1-activated phosphatidylinositol 3-kinase enhances aggregation of MCF-7 breast cancer cells independent of extracellular signal-regulated kinase. *Cancer Research*, 59:1620-1625, 1999.
2. Yu, D., Jing, T., Liu, B., Yao, J., **Tan, M.**, McDonnell, T.J., Hung, M.C. Overexpression of ErbB2 blocks Taxol induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. *Molecular Cell*, 2:581-91, 1998.
3. Yao, J., Pollock, R., Lang, A., **Tan, M.**, Pisters P.W., Goodrich, D., El-Naggar, A., Yu, D. Infrequent mutation of the p16/MTS1 gene and overexpression of cyclin-dependent kinase 4 in human primary soft-tissue sarcoma. *Clinical Cancer Research*, 4:1065-70, 1998
4. Jing T., Lee S., **Tan M.**, Yao J. Liu J., Arlinghaus R. B., Hung M. C., Yu D. Direct phosphorylation on tyrosine-15 of p34Cdc2 by erbB2 receptor tyrosine kinase is involved in inhibition of p34Cdc2 activation and resistance to Taxol-induced apoptosis. Submitted, 2000.

-Patents and licenses applied for and/or issued:

None.

-Degrees obtained that are supported by this award:

The principal investigator of this grant, Ming Tan, obtained his Ph D. degree from the University of Texas M.D. Anderson Cancer Center in May, 2000.

-Development of cell lines, tissue or serum repositories:

Four erbB2 mutant transfectants were developed while performing the proposed research.

-Funding applied for based on work supported by this award:

None.

-Employment or research opportunities applied for and/ or received on experiences/training supported by this award:

P.I. of this study obtained a postdoctoral position in the University of Texas M.D.

Anderson Cancer Center.

CONCLUSIONS:

In summary, our studies on erbB2 gene-transfected MDA-MB-435 breast cancer cells have provided explanations for the reported clinical correlation between erbB2 overexpression and breast cancer metastasis. We have demonstrated in this study that overexpression of the erbB2 gene can enhance the intrinsic metastatic potential of human breast cancer cells.

In addition, we have investigated the structural requirements of erbB2 for erbB2 mediated higher metastatic potential in human breast cancer cells. We found that the kinase domain of erbB2 receptor is required for erbB2 to enhance metastatic potential of human breast cancer cells. The C-terminal tyrosine of erbB2 may also play roles in erbB2 mediated higher metastatic potential of breast cancer cells. We also provided the evidence that HRG/egf can promote the metastatic potential of MDA-MB-435 cell erbB2 transfectants *in vivo*.

Finally, we investigated the signaling mechanism of heregulin enhanced MCF-7 and SKBR-3 cell aggregation. We found that HRG–β1-induced PI3-kinase activation is required in HRG enhanced MCF-7 and SKBR-3 breast cancer cell aggregation.

Our studies have generated important information about the molecular basis of breast cancer invasion and metastasis. Ultimately we may use the information to develop new prognostic indicators and novel therapies for breast cancer metastasis.

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1. Tan M., Yao J., and Yu D. C-erbB-2 overexpression enhanced intrinsic metastatic potential in human breast cancer cells. Cancer Res. 57: 1199-1205, 1997.
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3. Landgraf, R., Pegram, M., Slamon, D. J., and Eisenberg, D. (1998). Cytotoxicity and specificity of directed toxins composed of diphtheria toxin and the EGF-like domain of heregulin beta1. Biochemistry 37, 3220-8.

APPENDICES:

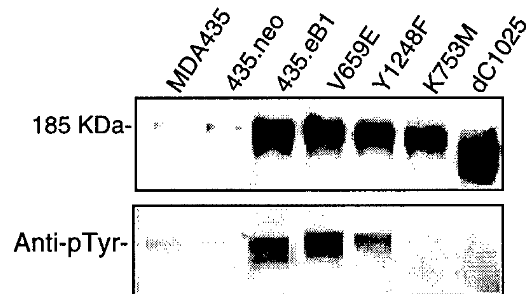
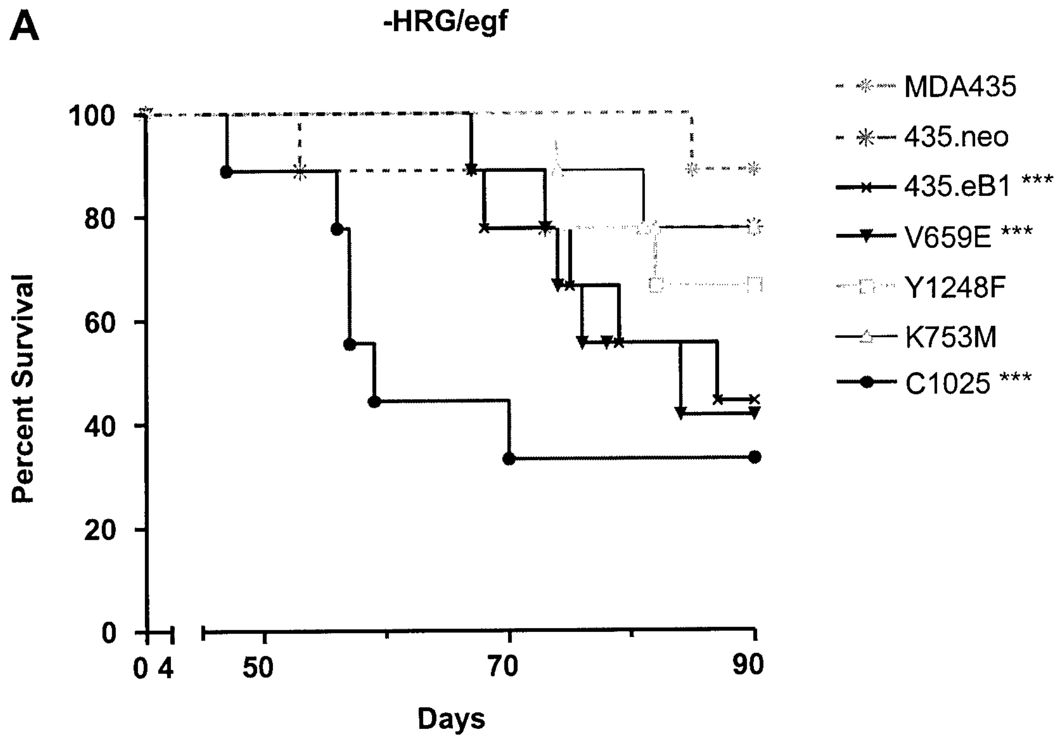


Fig. 1. erbB2 expression level and tyrosine phosphorylation of wild-type and mutated erbB2 expressing cells. 100 μg of protein from each sample was electrophoresed on 6% SDS-PAGE and transferred to nitrocellulose. The filters were incubated with the primary antibody against erbB2 extracellular domain. Position of the p185 is indicated on the left (Top). The membrane was stripped, and rehybridized with antibodies against phosphotyrosine (bottom). The tyrosine phosphorylated p185 was indicated on the left.

Fig. 2. See next page for legend



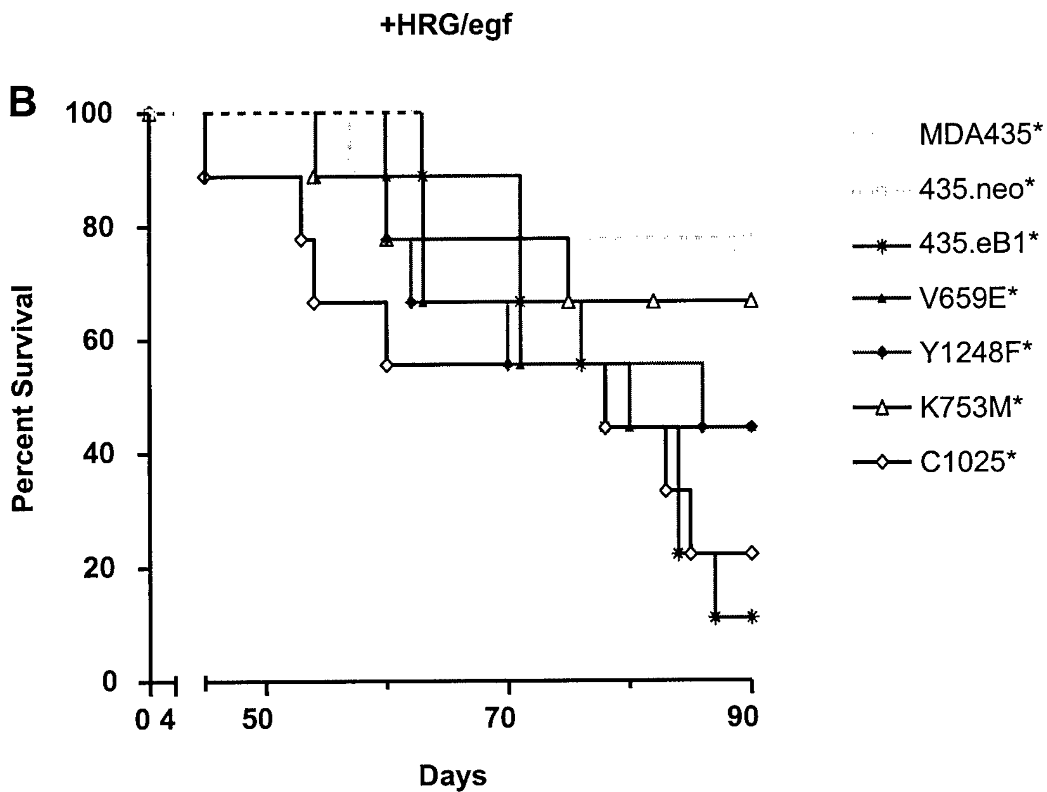


Fig. 2. The metastatic survival curves for mice injected erbB2 transfectants without (A) or with (B) HRG/egf. Single-cell suspensions of each cell line were injected into the lateral tail veins of 8-week-old female ICR-SCID mice (Harlan Sprague-Dawley, Inc., Madison, WI). 1 X 10^6 cells/mouse was injected without or with 5 μg/kg HRG/egf. The groups injected with HRG/egf were injected HRG/egf 5 μg/kg three days after the first injection, the dose then changed to 7.5 μg/kg once a week for another 7 weeks, totally 9 injections were administered. There are 9 mice in each control or experimental group. The injected mice were maintained under identical conditions and were monitored regularly. Once a mouse was moribund or dead, euthanasia and/or necropsy was performed to determine the cause resulted in the death of the mouse. The survival curves were drawn and analyzed by using the PrismSoftware, GraphPad Software, Inc. San Diego, CA. The metastatic survival curves were compared by Mantel-Haenszel logrank test. *, injected tumor cells with HRG/egf.

Fig. 3. See next page for legend

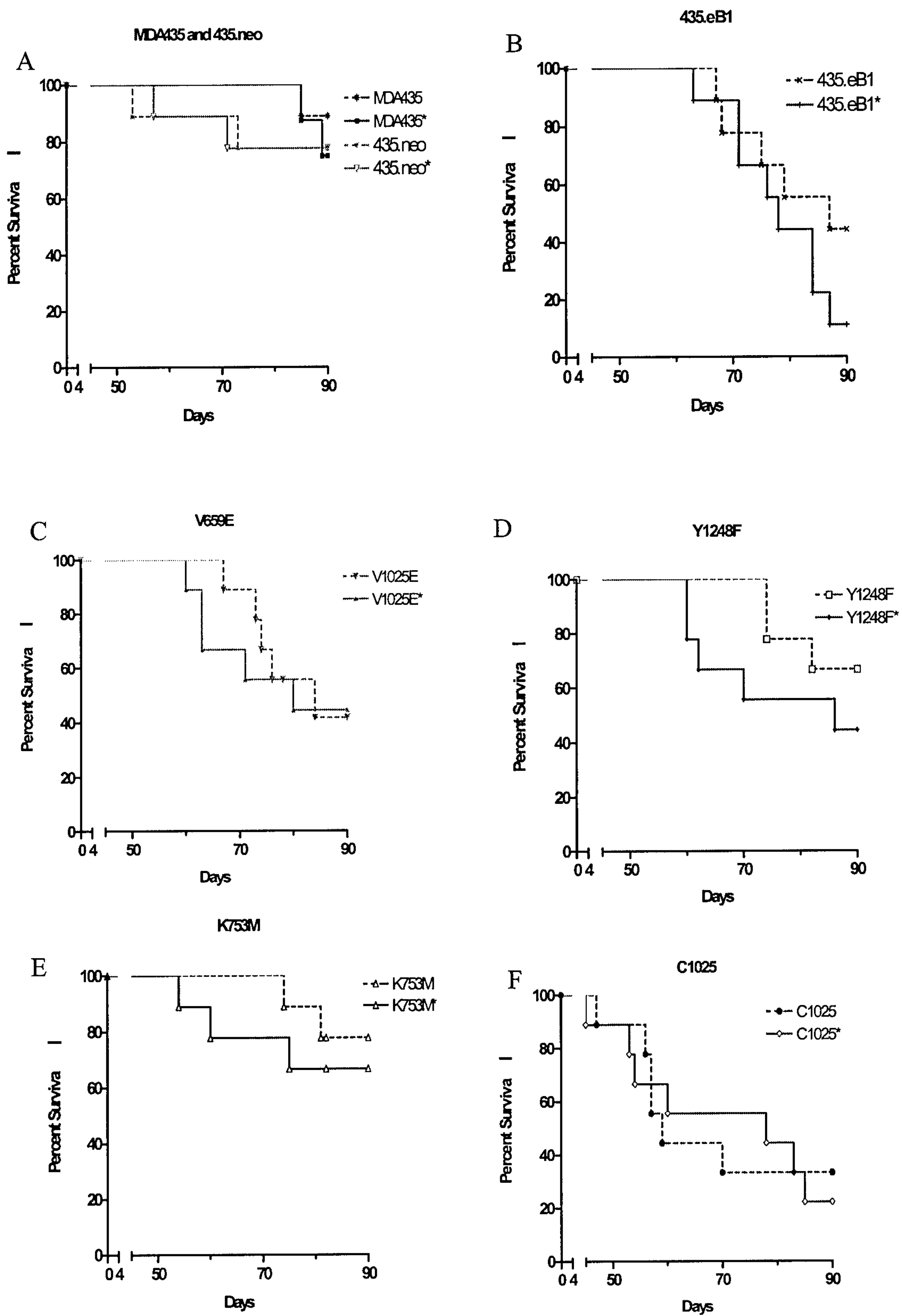


Fig. 3. The metastatic survival curves for the mice injected without or with HRG/egf of each cell lines. The name of each cell line was labeled on the upper right corner of each panel. Doted lines represent tumor cells injected without HRG/egf; solid lines represent tumor cells injected with HRG/egf. The survival curves were drawn and analyzed by using the Prism Software, GraphPad Software, Inc. San Diego, CA. The one-tailed student t-test was used to analyze the significance of difference between HRG/egf treatment and no treatment groups. *, injected tumor cells with HRG/egf.

Fig 4. See next page for legend

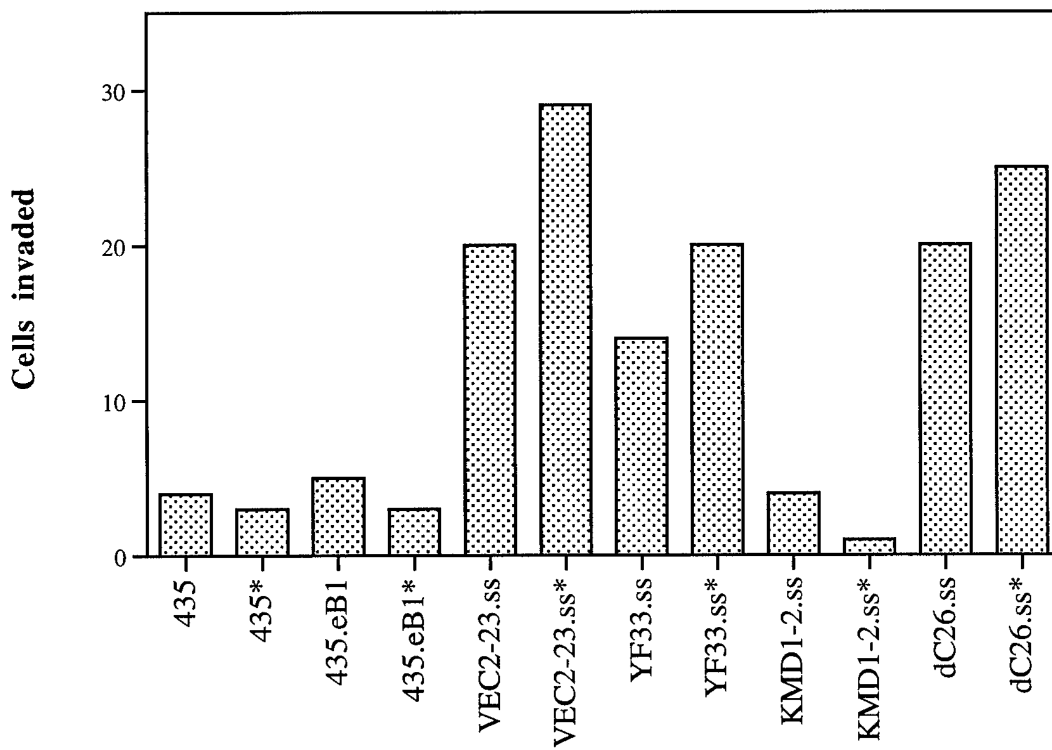


Fig 4. Invasion of Matrigel by the erbB2 transfectants. Cells were treated with or without HRG as previously described. Chemoinvasion was measured by using 24-well Biocoat Matrigel Invasion Chambers (Becton Dickinson Labware, MA 01730) with an 8-μm pore size polycarbonate filter coated with Matrigel. The lower compartment contained 0.6 ml of laminin at different concentration in DMEM/F12 as chemoattractants. The cells (1 X 10^5 cells/0.1 ml DMEM/F12 containing 0.1% bovine serum albumin) were placed in the upper compartment and incubated for 72 h at 37°C in a humidified 5% CO_2 atmosphere. After the incubation, the filters were fixed with 3% glutaraldehyde in phosphate-buffered saline and stained with Giemsa. We determined chemoinvasion activity by counting the number of cells per High Power Field (HPF, X200) that had migrated to the lower side of the filter. The Matrigel-invaded cells were counted at least three HPFs per filter.

BIBLIOGRAPHY

Publications

See above reportable outcomes.

Meeting Abstracts

4. MingTan, Rebecca Grijalva and Dihua Yu. HRG activated PI3-kinase enhances aggregation of MCF-7 breast cancer cells independent of ERK. Miami Nature Biotechnology Winter Symposia. February 6-10, 1999.
5. Ming Tan, Kristine Klos, Rebecca Grijalva and Dihua Yu. Heregulin-activated erbB receptor signaling and human breast cancer cell aggregation, invasion and metastasis. Joint regulation of signaling pathways by integrins and growth factors, Keystone Symposia. March 25-31, 2000.

PERSONNEL RECEIVING PAY

Ming Tan, the principal investigator of this research, received his stipend provided by this award.