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Award Number: W81XWH-04-1-0627

TITLE: The Role of HER-2 in Breast Cancer Bone Metastasis

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REPORT DATE: July 2005

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-05	Final	07/01/04-06/30/05

4. TITLE AND SUBTITLE
The Role of HER-2 in Breast Cancer Bone Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0627

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas
M. D. Anderson Cancer Center
Houston, TX 77030

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. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: The major goal of thus Concept award is to define the role for the growth factor receptor, HER2, in breast cancer growth in the bone, thus involvement in bone metastasis. To achieve this, we proposed three tasks:

Analysis of orthotropic model of breast cancer growth in the bone
Immunohistochemical analysis of bone resorption and bone resorption markers
Identification of potential HER2 targets involved in bone resorption

We have found that over-expression of HER2 could enhance breast cancer bone metastases. In our pilot study, we found that when injected into the tibia of nude mice, breast cancer cells that over-express Her2 induced osteolytic lesions that were more aggressive than that of the parental cell line expressing low levels of HER2. Thus, HER2 may contribute to an increase in osteolytic activity of breast cancer bone metastases and further experiments may show that HER2 may serve as a therapeutic target for controlling breast cancer bo9ne metastases. During the one-year funding period of this grant we experienced setbacks in our model system that hindered that completion of the tasks outlined in this award. We have remedied these obstacles and are actively pursuing the proposed experiments. Therefore, we would like to **ask for a one-year non-funded extension of this grant** to facilitate the completion of the outlined tasks.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)* 301-619-7325

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Introduction

It has long been recognized that breast cancers have the ability to invade and grow as metastases in the bone (Mundy GR 1997). In patients, the development of bone metastases causes extreme morbidity. HER2 (a.k.a. **ErbB2**) is a 185 kDa transmembrane glycoprotein, which is a receptor tyrosine kinase that belongs to the epidermal growth factor receptor subfamily (Yamomoto et al 1986). Overexpression of HER2 initiates aberrant activation, causing deregulation of downstream target genes. HER2 overexpression is found in approximately ~30% of breast cancers (Slamon et al 1987), and many other cancer types. Overexpression of HER2 correlates with poor clinical outcome and increased metastastic potential (Tan et al 1997) along with cancer recurrence. It has also been shown that bone marrow micrometastases express more HER2 than their primary tumor (Putz et al 1999) suggesting a selection bias for HER2 overexpressing cells in the bone microenvironment. Here we sought to understand the role of HER2 in breast cancer bone metastasis, thus seeking clinical impact through prognostic tests and preventative therapy, potentially decreasing HER2 mediated bone destruction.

To determine the role of HER2 in breast cancer bone metastasis, we proposed three Tasks. First, we sought to understand the biology of the bone environment when occupied by HER2 low and high expressing breast cancer cells, using intra-tibia injection. Next, in Task 2 we wished to perform immunohistochemical analysis, observing bone resorption in a site-specific manner for microscopic early stage bone resorption. Finally, we sought to identify potential HER2 targets causing bone resorption by using an unbiased approach to compare the expression levels of certain key targets

between the HER2 transfectants and their parental cell lines. Identification of novel targets would allow elucidation of the mechanism behind HER2-mediated bone resorption.

Body

In Task 1, we proposed to study HER2 over-expression involvement in breast cancer bone resorption/metastasis. To accomplish this task we proposed to inject 3 different HER2 low expressing parental breast cancer cells (MDA-MB-435, MCF-7, and MDA-MB-231) and their isogenic HER2 high expressing stable breast cancer cell lines (435.eB, MCF-7.eB, and 231.eB) into the tibias of female nude mice, monitoring growth and osteolytic activity through radiograph analysis. Unfortunately, after very limited passage number, western blot analysis revealed that our 435.eB and 231.eB HER2 stable transfectants expressed only moderate to low levels of HER2 (Figure 1). To address this problem we created an HER2-expressing retrovirus (pLPCX-CMV-HER2) and infected the parental cell lines MDA-MB-435 to generate new 435.eB stable clones (Figure 2, clones 46, 71, and 115). To remedy the lack of expression of HER2 in the 231.eB cell line, we have collaborated with Dr. Patricia Steeg at NIH-NCI and obtained parental 231.EGFP and its HER2 transfectant, 231.ErbB2 1.1

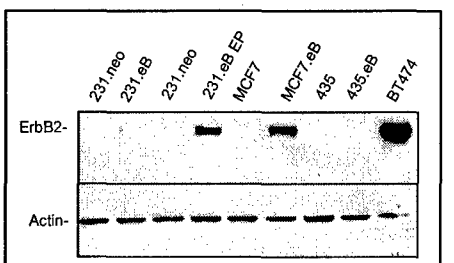

Figure 1. ErbB2 levels or breast cancer cell lines and their isogenic ErbB2 transfectants. Western Blot analysis shows loss of ErbB2 was observed after limited cell culture passage. BT474, an endogenous ErbB2 over-expressing breast cancer cell line serves as a positive control. (EP – Early Passage)

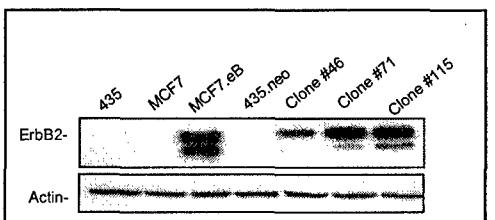

Figure 2. ErbB2 levels of stable transfectants. MDA-MB-435 cells were infected pLPCX-CMV-ErbB2 with retrovirus (10,000CFU). After clonal selection in puromycin, Western Blot analysis revealed 2 clones with high levels of ErbB2 expression and one with moderate levels.

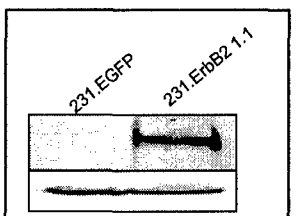

Figure 3. ErbB2 level of 231.ErbB2 1.1. Western Blot analysis shows ErbB2 levels of 231.ErbB2 1.1 as compared to Parental 231.EGFP control cells.

(Figure 3). In addition, these two cell lines express green fluorescence protein (GFP), which will also allow for in vivo imaging of occult metastasis and growth within the bone for future experimentation. Both cell lines have been characterized and are ready for experimentation. At the time of the original submission of this proposal, we proposed to use the breast cancer cell line MCF-7 and its stable HER2 transfectant MCF-7.eB. We have since learned that the MCF-7 breast cancer cell line is not a useful model for monitoring breast cancer cell growth within the bone. MCF-7 cells are ER positive and the mice require the supplementation of estrogen for proper MCF-7 cell growth. The effects of estrogen on bone growth, turnover and development is well established and add additional factors that create an unsuitable and complicated scenario for our studies. After the generation of our new 435.eB transfectants, we conducted a pilot in vivo study. As proposed, we first investigated and monitored tumor growth in the microenvironment of the bone, comparing, HER2 high expressing breast cancer cells, 435.eB to their parental MDA-MB-435, HER2 low expressing counterparts. We injected, each breast cancer cell line (5 X 10^5 cells /injection) intratibually into NCRNU-M female nude mice (Taconic Farms Inc., Germantown, NY), 5 mice per cell line, 10 mice total. After an initial growth period of one week, we took X-rays of each mouse once a week using Faxitron Specimen Radiography System (Figure 4, Appendix Figure 1,2).

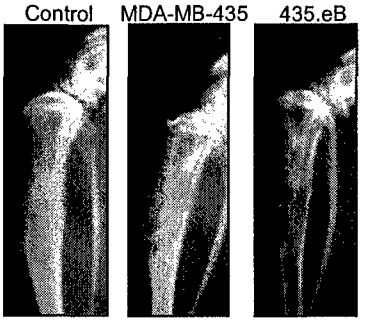

Figure 4: 435.eB cells show increased osteolysis *in vivo*. MDA-MB-435 and 435.eB ($5x10^5$) cells were injected intratibually into female nude mice. Weekly x-rays were taken for 8 weeks, figure depicts final image at 8 weeks.

Radiographic analysis indicated that both MDA-MB-435 and 435.eB were capable of growing in the bone microenvironment however the 435.eB breast cancer cells had an

increase in bone destruction and osteolytic activity. In addition, both cell lines produced distant metastases observed during necropsy, including lung and kidneys, suggesting that HER2 over-expressing 435.eB has an advantage in growing in the unique environment of the bone when compared to MDA-MB-435. Data collected from this pilot experiment is quite exciting and with our properly established system, we are currently continuing the original tasks outlined in the proposal. Therefore, we would like to **ask for a one-year non-funded extension of this grant** to facilitate the completion of this proposal.

Key Research Accomplishments

- New stable cell lines overexpressing HER2, 435.eB and 231.eB were created facilitating a proper model system for our proposed tasks
- Injection of MDA-MB-435 and 435.eB cells into the tibias of nude mice produced osteolytic lesions. Animals injected with 435.eB cells had an increase in osteolysis and bone degradation.

Reportable Outcomes

None

Conclusions

After the development and characterization of our new model system, we now have breast cancer cell lines with appropriate levels of HER2 to carry out the proposed tasks. In the pilot study, we were able to demonstrate that both MDA-MB-435 and 435.eB grow in the microenviroment of the bone. In addition we saw an increase of bone degredation from the 435.eB cells suggesting that over-expression of HER2 in breast

cancer cells causes an increase of osteolytic activity. Further experimentation and completion of the proposed tasks will determine the role of HER2 in breast cancer bone metastasis.

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Appendix

Figure 1: MDA-MB-435 breast cancer cell line (5 X 10^5 cells /injection) were injected intratibually into 5 NCRNU-M female nude mice. After an initial growth period of one week, X-rays of each mouse were taken once a week using Faxitron Specimen Radiography System.

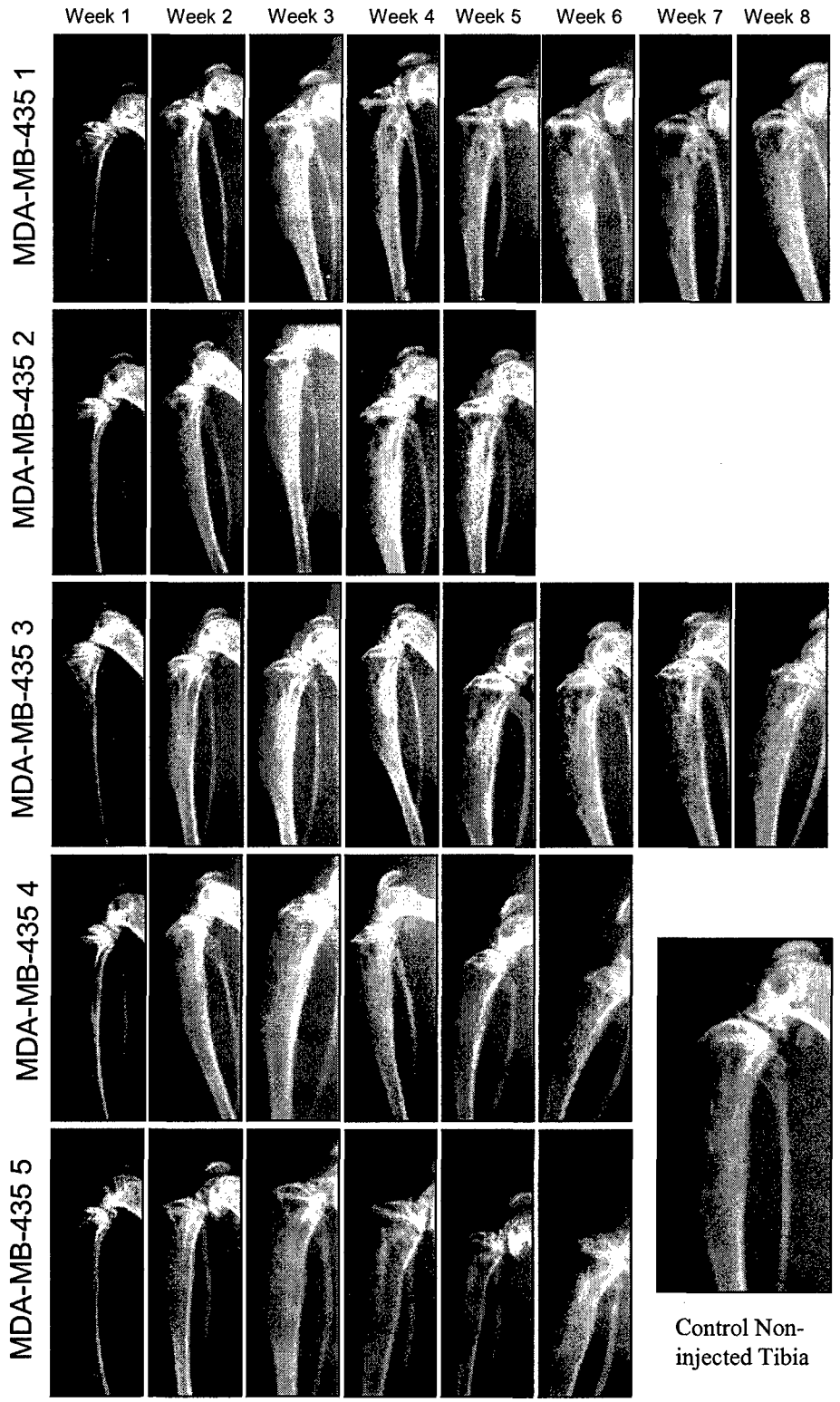

Figure 2: 435.eB breast cancer cell line (5 X 10^5 cells /injection) were injected intratibually into 5 NCRNU-M female nude mice. After an initial growth period of one week, X-rays of each mouse were taken once a week using Faxitron Specimen Radiography System.