

Level of HER-2/*neu* protein expression in breast cancer may affect the development of endogenous HER-2/*neu*-specific immunity

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Abstract

We questioned whether the incidence or magnitude of the humoral or cellular immune response to the self-tumor antigen HER-2/*neu* is influenced by the level of HER-2/*neu* protein overexpression as defined by immunohistochemical staining of tumors in breast cancer patients. We obtained peripheral blood from 104 women with stage II, III, and IV pathologically confirmed HER-2/*neu*-overexpressing breast cancer. Patients were categorized with +1 ($n = 14$), +2 ($n = 20$), or +3 ($n = 70$) HER-2/*neu* overexpression by institutional pathologic report. Circulating antibodies to HER-2/*neu* were evaluated using ELISA. T-cell responses to HER-2/*neu* were measured using an antigen-specific tritiated thymidine incorporation assay. Eighty-two percent of subjects with HER-2/*neu* antibodies were +3 overexpressors compared with 18% +2 overexpressors and 0% +1 overexpressors, a highly significant difference ($P < 0.001$), and there were significant differences in the magnitude of the HER-2/*neu*-specific antibodies between groups with varying HER-2/*neu* protein expression ($P = 0.022$). In addition, 65% of subjects with HER-2/*neu*-specific T cells were +3 overexpressors compared with 16% +2 overexpressors and 19% +1 overexpressors ($P = 0.001$). Data presented here indicate that endogenous HER-2/*neu*-specific humoral and T-cell immunity is greater in patients with +3 protein overexpression in their tumors than in patients with lower

levels of HER-2/*neu* expression. Overexpression of a self-tumor-associated protein is a potential mechanism by which immunogenicity is enhanced and may aid in the identification of biologically relevant proteins to target for immune-based molecular cancer therapies. [Mol Cancer Ther 2008;7(3):449–54]

Introduction

Many newly discovered tumor antigens are proteins that are overexpressed in tumor cells. Up-regulation of proteins involved in cell growth and cell cycle control play an essential role in malignant transformation, and it is known that cancer patients can have circulating antibodies specific for self-proteins. However, the relationship between protein overexpression and protein immunogenicity in human cancer is not well defined. Characterization of that relationship may help identify molecular immunologic targets particularly amenable to therapeutic intervention. HER-2/*neu* is a protein involved in the control of cell growth and is present at low concentration in normal cells. HER-2/*neu* protein expression is up-regulated in breast cancer cells, and patients with HER-2/*neu*-overexpressing tumors can have preexisting immunity to HER-2/*neu*. Thus, HER-2/*neu* is an ideal candidate for analysis, elucidating the connection between protein overexpression in tumor and endogenous immunity in patients.

We investigated the relationship between the level of endogenous HER-2/*neu*-specific antibody immunity in patients with HER-2/*neu*-overexpressing tumors and the level of HER-2/*neu* protein overexpression in their primary tumors. We found that increasing levels of HER-2/*neu* overexpression in primary tumors are associated with increasing levels of endogenous HER-2/*neu*-specific antibody immunity and that only those patients with moderate to high levels of overexpression had preexisting antibodies to HER-2/*neu*. To our knowledge, this work represents the first report of a quantifiable relationship between protein overexpression and endogenous immunity.

Materials and Methods

Study Population

Blood was collected from 104 patients with stage II ($n = 25$), III ($n = 32$), or IV ($n = 47$) breast cancer from 1996 to 2006. To be eligible, patients had stage II, III, or IV breast cancer and immunohistochemistry-documented HER-2/*neu* overexpression in either their primary tumor or metastasis.

Patients must have been off cytotoxic chemotherapy and/or treatment dose corticosteroids for at least 1 month before enrollment. Patients were not allowed to participate

Received 6/8/07; revised 10/10/07; accepted 1/10/08.

Grant support: National Cancer Institute grants K24 CA85218 and U54 CA090818, NIH grant MOI-RR-00037, and Athena Water and the Cancer Research Treatment Foundation.

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doi:10.1158/1535-7163.MCT-07-0386

if they had donated blood within the last 72 h, were concurrently receiving trastuzumab or other immune modulators, had symptoms of an infection including a "cold," or were ages <18 years. Clinical data were available for 84 of the 104 samples and were limited to age, stage of disease, response to treatment, estrogen receptor and progesterone receptor status, and site of disease and metastases. Thus, the multivariate analysis described was done on only those samples with clinical data. Patient demographics are listed in Table 1. After institutional review board approval and written consent were obtained, blood was drawn before vaccination to determine endogenous immunity to HER-2/*neu* and tetanus toxoid. Tumor expression of HER-2/*neu* protein was determined by immunohistochemical analysis before enrollment. Patients were categorized as having low (+1), moderate (+2), and high (+3) overexpressing primary tumors based on institutional pathology reports. Patients were categorized as estrogen receptor and progesterone receptor positive or negative based on institutional pathology reports. Response to treatment was defined as complete remission, stable disease, or progressive disease. Site of disease or metastases was categorized as bone, lung, subclavicular nodes, liver, lymph nodes, skin, local disease only, or not applicable when there was no evidence of disease after treatment. Peripheral blood mononuclear cells from two subjects were not available for T-cell analysis; thus, the total number of samples available for T-cell analysis was 102. All other patient serum and matched peripheral blood mononuclear cells were assessed for HER-2/*neu* immunity.

The reference population sera was derived from female volunteer donors ($n = 200$), contributing blood products

at the Puget Sound Blood Center. Individuals contributing the sera samples met all the health requirements associated with blood donation. Age range of the serologic control group was 18 to 72 years.

Evaluation of Endogenous HER-2/*neu* Antibody Response

Capture ELISA for HER-2/*neu*-specific and tetanus toxoid-specific antibodies was done as reported previously (1, 2). Briefly, 96-well microtiter plates (Dynex Technologies) were coated with the HER-2/*neu* monoclonal antibody 520C9 and serially diluted, purified human IgG (Sigma) provided a standard curve. After overnight incubation at 4°C, all wells were then blocked with 1% bovine serum albumin (Sigma). Plates were washed four times with 10% PBS-0.5% Tween (Amersham Biosciences) before addition to alternating columns of antigen derived from the HER-2/*neu*-overexpressing cell line SKBR3. Serum samples were added after an overnight incubation and washes. After serum incubation, plates were washed and goat anti-human IgG-horseradish peroxidase conjugate (Zymed Laboratories) was added at a dilution of 1:50,000 (50 μ L/well). After a final wash, developing reagent was added (75 μ L/well) and color reaction was assessed at an absorbance of 640 nm until the well containing the standard at a concentration of 0.16 μ g/mL was evaluated at 0.3 nm absorbance. Reaction was then stopped with 75 μ L/well 1 N HCl and read at an absorbance of 450 nm. A positive sample was defined as an antibody concentration above the nonparametric 95th percentile of 200 control samples evaluated for HER-2/*neu* antibodies. A sample was categorized as positive if the assay result was a HER-2/*neu*-specific IgG response of ≥ 1.13 μ g/mL (1).

Table 1. Patient characteristics

Characteristic	Ab+ ($n = 22$)	Immunohistochemistry		
		+1	+2	+3
Age (y), median (range)		51.5 (41-70)	49 (37-76)	49.5 (26-85)
Stage, %				
II	32	14	25	30
III	41	36	45	26
IV	27	50	30	44
Response, %				
Complete remission	75	36	60	64
Stable	13	64	40	21
Progressive	13	0	0	15
Hormone receptor status, %				
Estrogen receptor positive	50	67	77	51
Progesterone receptor positive	60	58	70	40
Site/metastasis, %				
NA	50	43	53	44
Bone	25	29	20	7
Lung	13	7	0	9
Supraclavicular lymph node	0	7	0	15
Local	13	0	20	17
Other*	0	14	7	7

*Skin, liver, or lymph nodes.

Evaluation of Endogenous HER-2/*neu* T-Cell Response

Tritiated thymidine incorporation measuring antigen-specific proliferation was done as reported previously (2, 3). Briefly, 2×10^5 peripheral blood mononuclear cells/well were plated into 96-well plates in 24-well replicates in medium consisting of equal parts EHAA 120 (Biofluids) and RPMI 1640 (Life Technologies) with L-glutamine, penicillin/streptomycin, 2-ME, and 10% AB serum (ICN Flow) in the presence or absence of 25 $\mu\text{g}/\text{mL}$ HER-2/*neu* ICD protein (Corixa) or 0.5 units/mL tetanus toxoid (Lederle). After 5 days, wells were pulsed with 1 μCi [^3H]thymidine for 8 to 10 h and counted. Stimulation index (SI) is defined as the mean counts/min of the response of the antigen-stimulated cells divided by the mean of the response of cells cultured without antigen. Responses are quantified as the number of responding wells of 24-well replicates. Data are expressed as a standard SI that is defined as the mean of all of the 24 experimental wells divided by the mean of the control wells (no antigen). A positive sample was defined as a SI of ≥ 2 based on the mean plus 3 SDs of a reference population (3).

Statistical Analysis

Differences in HER-2/*neu* overexpression levels among positive responders were analyzed by χ^2 test. Magnitude of HER-2/*neu* antibody and T-cell responses were compared with immunohistochemical status or stage of disease by Kruskal-Wallis analysis. Differences in overexpression levels among positive responders were analyzed by χ^2 test, and the association between HER-2/*neu*-specific responses and stage or tetanus toxoid-specific responses was analyzed by Spearman's rank correlation. Multivariate analysis of patients with clinical data was done using linear regression. HER-2/*neu*-specific antibodies, age, and HER-2/*neu*-specific T-cell proliferation were included in the model as continuous variables, stage, response to treatment, and site of disease/metastases as categorical variables and hormone receptor status as a dichotomous variable. All analyses were done using SPSS 13.0.

Results

HER-2/*neu*-Specific Antibodies Are Found Significantly More Often in Patients with +3 Overexpression of HER-2/*neu* Protein in Their Primary Tumor Than in Patients with Lower Levels of Overexpression

Antibody responses were dichotomized by categorizing all responses of $\geq 1.13 \mu\text{g}/\text{mL}$ as positive. A total of 22 patients were positive for circulating antibodies to HER-2/*neu*. None of the +1 subjects' sera ($n = 14$) showed evidence of HER-2/*neu*-specific antibodies. Eighteen percent of subjects positive for HER-2/*neu* antibodies were +2 overexpressors, and 82% of subjects with endogenous HER-2/*neu* antibodies were +3 overexpressors (Fig. 1). Thus, subjects with endogenous HER-2/*neu* humoral immunity were significantly more likely to have +3 HER-2/*neu* protein-overexpressing tumors ($P < 0.001$).

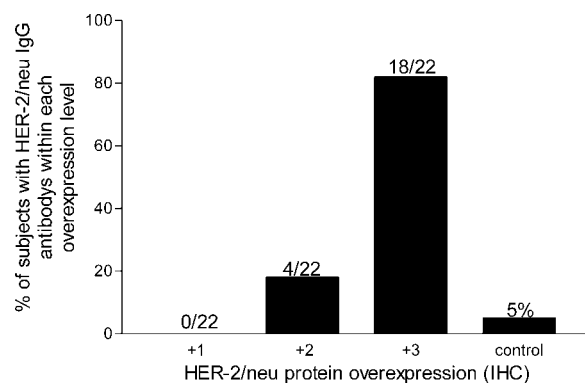


Figure 1. Patients with HER-2/*neu*-specific antibodies are more likely to have a high level of HER-2/*neu* protein overexpression in their primary tumor. Proportion of the 22 patients with circulating antibodies to HER-2/*neu* who had low (+1), moderate (+2), or high (+3) HER-2/*neu* protein overexpression in their primary tumor as assessed by immunohistochemistry. Control data are included for comparison.

Significant Increases in the Magnitude of the HER-2/*neu* Antibody Response Occur with Increasing HER-2/*neu* Protein Overexpression in Tumor

Samples from patients with +1 ($n = 14$), +2 ($n = 20$), and +3 ($n = 70$) tumor HER-2/*neu* protein overexpression were assessed for HER-2/*neu*-specific IgG antibodies. The mean level of antibody response among 104 breast cancer patients with HER-2/*neu*-overexpressing tumors increased from 0.00 $\mu\text{g}/\text{mL}$ among the +1 immunohistochemical group (no positive responses) to 1.13 $\mu\text{g}/\text{mL}$ among the +2 immunohistochemical group (range 0-5.41 $\mu\text{g}/\text{mL}$) to 1.22 $\mu\text{g}/\text{mL}$ in the +3 immunohistochemical group (range, 0-8.96 $\mu\text{g}/\text{mL}$; Fig. 2). The differences in magnitude of antibody responses between overexpression levels were significant ($P = 0.022$). Multivariate analysis of clinical data showed that only level of HER-2/*neu* protein overexpression was a significant predictor of HER-2/*neu*-specific antibody response ($P = 0.016$). In this study population, clinical data were limited to age, stage, HER-2/*neu*-specific T-cell proliferation, response to treatment, site of disease/metastases, and hormone receptor (estrogen receptor and progesterone receptor) status. These were not significant predictors for level of HER-2/*neu* protein overexpression ($P = 0.546, 0.097, 0.486, 0.058, 0.970, 0.741, \text{ and } 0.897$, respectively).

To investigate the possibility that the level of HER-2/*neu*-specific antibody immunity is simply a reflection of immune status, we evaluated antibody immunity to tetanus toxoid as a measure of immunocompetence and compared levels of HER-2/*neu*-specific IgG with levels of tetanus toxoid-specific IgG in a subset of 90 patients with all stages of disease (Fig. 3A). We found no correlation between HER-2/*neu* antibody responses and tetanus toxoid antibody responses ($P = 0.848$). We have found previously an association between presence of endogenous antibodies to tumor antigen and stage of disease in ovarian cancer (4). To further investigate the possibility of a relationship between stage of disease and level of

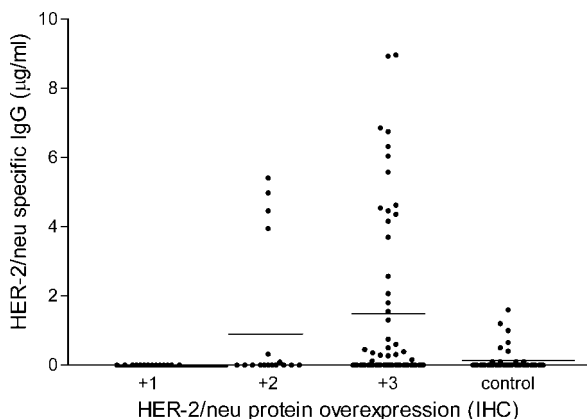


Figure 2. Magnitude of the HER-2/neu-specific antibody response increases as the level of HER-2/neu protein overexpression increases. Circles, HER-2/neu-specific antibody responses for patients with low (+ 1), moderate (+ 2), and high (+ 3) HER-2/neu protein overexpression in their primary tumors. Control data are included for comparison.

preexisting antigen-specific immunity, we compared the level of HER-2/neu-specific antibody response with stage of disease (Fig. 3B). We found no correlation between antibody response and stage of disease. Responses ranged between 0.00 and 8.96 µg/mL with a mean response of

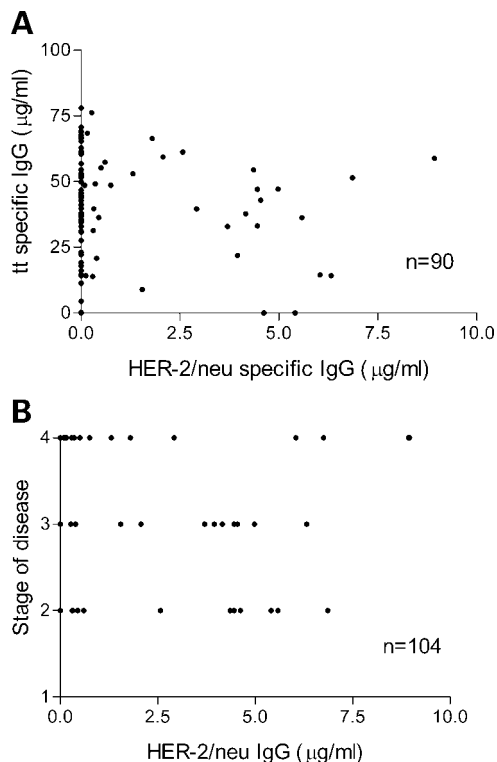


Figure 3. Presence of a HER-2/neu-specific antibody response is not associated with the presence of a tetanus-specific antibody response or with the stage of disease. **A**, X axis, HER-2/neu antibody responses; Y axis, tetanus toxoid responses. **B**, X axis, HER-2/neu antibody responses; Y axis, stage of disease.

1.27 µg/mL for patients with stage II disease, 1.10 µg/mL for patients with stage III disease, and 0.83 µg/mL for patients with stage IV disease ($P = 0.308$). Only 12 of the patients included in the study had ever received Herceptin, but to address the possibility of an association between HER-2/neu antibody response and prior Herceptin use, we did the above analyses excluding these patients, with no resulting change in significance. We also did not find a correlation between level of overexpression and antibody response ($P = 0.755$).

HER-2/neu-Specific T Cells Are Found Significantly More Often in Patients with +3 Overexpression of HER-2/neu Protein in Their Primary Tumor Than in Patients with Lower Levels of Overexpression

Peripheral blood mononuclear cell samples were assessed for HER-2/neu-specific T-cell response by tritiated thymidine incorporation. T-cell responses were dichotomized by categorizing all responses above a SI of 2 as positive. A total of 31 patients showed HER-2/neu protein-specific T-cell immunity. Nineteen percent of the subjects with HER-2/neu protein-specific T cells were +1 overexpressors and 16% were +2 overexpressors, whereas 65% of patients with endogenous HER-2/neu-specific T cells were +3 overexpressors (Fig. 4). Thus, subjects with endogenous HER-2/neu-specific cellular immunity were significantly more likely to have +3 HER-2/neu protein-overexpressing tumors ($P = 0.001$).

Mean HER-2/neu-specific SI among 102 breast cancer patients with HER-2/neu-overexpressing tumors increased from a SI of 2.64 (range, 0.6-11.1) among the +1 immunohistochemical group to a SI of 2.67 among the +2 immunohistochemical group (range, 0.8-18.5) to a SI of 2.70 among the +3 immunohistochemical group (range, 0.2-24.0; data not shown). The difference in magnitude of T-cell responses between overexpression levels was not significant ($P = 0.753$).

We found no correlation between HER-2/neu T-cell responses and tetanus toxoid T-cell responses ($P = 0.076$).

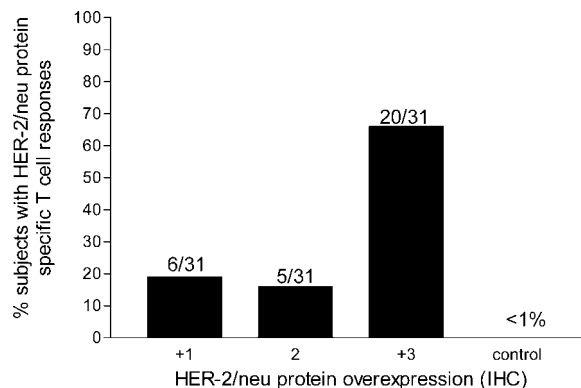


Figure 4. Patients with HER-2/neu protein-specific T cells are more likely to have a high level of HER-2/neu protein overexpression in their primary tumor. Proportion of the 30 patients with HER-2/neu-specific T cells who had low (+ 1), moderate (+ 2), or high (+ 3) HER-2/neu protein overexpression in their primary tumor as assessed by immunohistochemistry. Control data are included for comparison.

on comparison of HER-2/*neu* protein-specific SI levels to tetanus toxoid-specific SI levels in a subset of 90 patients with all stages of disease. We also found no correlation between HER-2/*neu* protein-specific T-cell response and stage of disease ($P = 0.162$).

Discussion

Human tumor-associated proteins are immunogenic in patients with breast cancer. The mechanisms by which self-proteins become immunogenic in the malignant state are largely unknown. For example, proteins found in normal cells may, in tumor cells, be altered due to gene mutation or aberrant post-translational modification, potentially increasing immunogenicity. Recent studies of microsatellite instability in colorectal cancers show that many of the abnormal peptides produced through mismatch repair deficiency induce proinflammatory cytokines and peptide-specific T-cell responses (5). Normal proteins that have been inappropriately modified post-translationally have also proven immunogenic. Improperly glycosylated carbohydrates are immunogenic in some tumors and serve as a marker of malignancy in breast cancer (6). Many overexpressed proteins have also been reported as tumor antigens in patients with cancer. Data presented here suggest that overexpression of self-proteins within cancer cells increases protein immunogenicity.

Within the last decade, it has become generally recognized that self-antigens can be immunogenic in cancer patients when a protein that is normally silent or expressed at low levels is aberrantly overexpressed or abundant in tumor cells. For example, it has been shown that patients with various cancers produce antibodies to p53. Endogenous p53 antibodies respond to both wild-type and mutated epitopes in the protein (7). What are the mechanisms by which abundance of a self-protein within a cancer cell enhances immunogenicity? One possibility is that cryptic epitopes, which have the potential for immune recognition, are presented in the presence of costimulatory molecules evoked by inflammatory conditions surrounding tumor growth, thus eliciting an immune response (8). It has also been postulated that the increased presence of tumor-associated protein stimulates an increase in number of peptides available for complexing to MHC class I molecules and subsequent recognition by the immune system. Such a mechanism using the HER-2/*neu* model was illustrated when investigators showed that increases in receptor degradation induced by trastuzumab led to enhanced HER-2/*neu*-specific cytotoxic T-cell function (9).

Growth-related proteins involved in initiating and maintaining the malignant phenotype are often found in abundance in cancer cells, and identification of proteins that are both demonstrably immunogenic and drivers of malignant transformation is important to the discovery of appropriate immunotherapeutic targets. The cyclin family of proteins regulates cell cycle. Members of this family have been implicated in tumorigenesis and have also proven immunogenic in cancer patients. Cyclins are normally

expressed intermittently at low levels during specific stages of the cell cycle but may be found constitutively expressed at higher levels in tumor cells. Patients with various types of cancer have naturally occurring cyclin B1-, cyclin D1-, and cyclin E1-specific antibodies and/or T cells (10). Patients with breast cancer can have preexisting antibody immunity to proteins associated with the apoptotic pathway, which are also expressed abundantly in tumor cells. Survivin is an inhibitor of cellular apoptosis, which is virtually undetectable in normal cells but abundantly present in tumor cells. Endogenous antibody immunity to survivin has been found in breast cancer patients with all stages of disease (11, 12). Humoral immunity to these antigens has been proposed as a diagnostic marker for malignancy (10, 13) and has allowed for identification of targets that have proven vulnerable to therapeutic intervention (14).

The patient population studied included 12 women who had, at some point during their course of treatment, received Herceptin (trastuzumab). Theoretically, individuals treated with Herceptin may be more likely to develop endogenous antibodies to HER-2/*neu* via antibody-mediated cell-dependent cytotoxicity mediated by Herceptin (15). However, our analysis did not suggest that Herceptin treatment significantly induced endogenous antibodies.

This study shows that a naturally occurring protein normally present at low levels in nonmalignant cells can, when present in higher levels in tumor cells, stimulate endogenous immunity. We found endogenous antibodies and T cells to HER-2/*neu* in 21% of patients before vaccination. A minority response was expected based on literature regarding HER-2/*neu* autoantibodies in breast cancer (16, 17). However, to our knowledge, no other group has reported information on a relationship between those patients who do have endogenous HER-2/*neu* immunity and the level of HER-2/*neu* protein overexpression in their tumors. Our finding of a "dose-dependent" response may indicate that portions of the protein not readily available for immune recognition when the protein is expressed at basal levels become immunogenic when the protein is increasingly abundant in the cancer cell. Recognition of the relationship between level of protein overexpression and presence of preexisting, tumor-specific immunity may aid in identification of biologically relevant immune targets for therapeutic development.

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