ErbB2 Immune Response in Breast Cancer Patients with Soluble Receptor Ectodomain

Vincenzo Visco,*[§] Roberto Bei,*[§] Enrica Moriconi,*
Walter Gianni,[†] Matthias H. Kraus,[‡] and
Raffaella Muraro[§]

From the Department of Experimental Medicine and Pathology*
and the Geriatric Oncology Unit,[†] University "La Sapienza",
Rome; the Department of Experimental Oncology,[‡] European
Institute of Oncology, Milan; and the Department of Oncology
and Neurosciences,[§] University "G. D'Annunzio", Chieti, Italy

Investigation of ErbB2 immunity in human breast cancer employing recombinant expression sources in immunoblot analysis revealed ErbB2-specific antibodies of the IgG isotype in sera of 14 of 71 cancer patients and 1 of 31 normal donors. Reactivity was confirmed on ErbB2-specific immunoprecipitates. Independent evidence of existing ErbB2 immunity was obtained after *in vitro* transformation of peripheral blood leukocytes from six positive patients. Furthermore, *in vitro* immortalization of B-lymphocytes unmasked existent ErbB2 immunity in 1 of 8 patients negative for ErbB2 serum antibodies. Determining shed ErbB2 extracellular domain as an indirect measure of tumor burden in ErbB2-positive malignancy, elevated serum levels were observed in 16 of 71 breast cancer and 1 of 31 normal donor sera. Strikingly, existing ErbB2 immunity correlated significantly with elevated shed ErbB2 ectodomain among the patients analyzed. Incidence of both ErbB2 immunity and elevated ErbB2 extracellular domain increased with a progressed disease stage and was significantly associated with metastatic breast cancer. These observations implicate soluble ErbB2 amounts *in vivo* in the development of ErbB2 immunity in breast cancer. They further project serum analysis of ErbB2 immunity and soluble ectodomain as potential markers of disease progression in ErbB2-positive malignancy. (Am J Pathol 2000, 156:1417–1424)

The ErbB2 (Neu/HER2) gene represents one of four structurally conserved members of the ErbB/EGFR tyrosine kinase subfamily^{1–4} that diverged during meta-zoan evolution. It is amplified and overexpressed at high transcript and protein levels in approximately 20 to 30% of primary human breast carcinomas.⁵ Although a specific ligand for the 185-kd transmembrane tyrosine kinase remains elusive, ErbB2 is known to critically determine ligand-dependent signals from all other ErbB receptors

by heterodimerization with EGFR or the neuregulin receptors ErbB3 and ErbB4.⁶ Recombinant cellular model systems as well as transgenic mouse studies provided a mechanistic basis for the concept of high constitutive ErbB2 activity associated with overexpression, to effect the neoplastic phenotype *in vitro* and *in vivo*. In clinical settings, ErbB2 gene amplification and overexpression correlate with a reduced disease-free and overall survival as well as resistance to adjuvant therapy.⁵ Moreover, recent clinical trials of metastatic breast cancer demonstrated that intravenous administration of a monoclonal ErbB2 antibody elicited an objective tumor inhibitory effect in some patients with advanced ErbB2-overexpressing malignancy.^{7,8}

ErbB2 alterations in breast carcinoma occur at early clinical stages and persist during disease progression.^{5,9,10} Circulating proteolytic cleavage fragments of the ErbB2 protein become detectable in serum as a possible consequence of tumor burden. Consistently, their appearance *in vivo* correlates with ErbB2 overexpression and disease progression or tumor size in human malignancy or animal models, respectively.^{11–18} Furthermore, it is well documented that some cancer patients harboring ErbB2 overexpressing tumors, may spontaneously develop an autologous anti-ErbB2 response in the course of their disease.^{19–22}

Mechanistically, at least a subset of B cells is amenable to positive selection, generation and maintenance of autoreactivity by self-antigens.^{23,24} Consistently, normal ErbB2 epitopes can elicit syngeneic immunity to self-ErbB2 protein,²⁵ providing a functional basis for the development of ErbB2 immunity in breast cancer. ErbB2 in the latter typically lacks mutations that activate the rat homologue in chemically induced neuroblastomas.²⁶ While pathophysiological roles of shed ErbB2 extracellular domain and native anti-ErbB2 immunity have not unequivocally been defined, their elucidation might hold important clues for disease monitoring and design of immunotherapeutic strategies. To this purpose, we evaluated in the present study occurrence of both ErbB2 specific immunity and soluble ErbB2 extracellular domain

Supported in part by grants from the Ministero dell'Università e della Ricerca Scientifica (MURST), the Consiglio Nazionale delle Ricerche (project CNR-ACRO), the Associazione Italiana per la Ricerca sul Cancro (AIRC), and the European Community (Program Biomed II).

Accepted for publication December 13, 1999.

Address reprint requests to Prof. Raffaella Muraro, Dipartimento di Oncologia e Neuroscienze, Università "G. D'Annunzio" Via dei Vestini, 66013 Chieti, Italy. E-mail: rmuraro@axrma.uniroma1.it.

in sera of breast cancer patients. An association of ErbB2 immune response with ErbB2 ectodomain serum levels was assessed and correlated with clinicopathological disease parameters.

Materials and Methods

Patient and Human Control Sera

Sera from 71 breast cancer patients and 31 healthy female donors were obtained with informed consent and stored in aliquots at -20°C . Tumor patient sera were collected before adjuvant therapy either the day of surgery or the day before. Tumor types included 62 infiltrating ductal, 8 infiltrating lobular, and 1 medullar carcinoma. The age ranges were 23 to 83 years for patients and 25 to 60 years for normal donors.

Cell Lines and Antibodies

NIH3T3 transfectants with human ErbB2²⁷ and NIH3T3 controls were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Polyclonal rabbit antiserum M6 raised against a cytoplasmic epitope of human ErbB2 and monoclonal antibody E2-1 specifically recognizing the ErbB2 extracellular domain have previously been described.²⁸ Purified mouse myeloma immunoglobulins (MOPC21; Cappel, Organon Teknika Corp., West Chester, PA) and normal rabbit serum served as negative controls.

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblot analysis for specific detection of human anti-ErbB2 antibodies were conducted essentially as described.²² For direct immunoblotting, 100 μg protein lysate of LTR-ErbB2 or control NIH3T3 transfectants were electrophoresed in denaturing 8% Tris-glycine polyacrylamide gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and proteins were transferred to a nitrocellulose membrane at 40 V for 1 hour. Membranes were reacted with patient sera (1:100), undiluted culture supernatants of immortalized PBLs, anti-ErbB2 specific (1:400), or preimmune rabbit serum (1:400). A dilution of 1:100 for patient sera was determined after titration to be the highest concentration lacking substantial background reactivity. After washing, the membranes were incubated with alkaline-phosphatase-conjugated goat anti-human IgG and IgM or goat anti-rabbit antiserum (Gibco BRL, Gaithersburg, MD). Bound antibody was visualized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro blue tetrazolium (NBT; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). For immunoprecipitation, 250 μg of LTR-ErbB2 transfectant lysate were precipitated in Staph A buffer using 3 μg of monoclonal antibody (mAb) E2-1 or the negative control immunoglobulin MOPC-21 and 20 μl of protein G sepharose. Following three washes in Staph A buffer, immunoprecipitates were processed for immunoblotting as described above.

Determination of Soluble ErbB2 in Patient and Healthy Sera

Quantitation of ErbB2 extracellular domain in human sera was performed by an enzymatic immunocapture assay using two anti-ErbB2 mAbs (Bender MedSystems, Boehringer Ingelheim Group, Vienna, Austria). According to manufacturer's recommendation, 96-well microtiter plates coated with anti-ErbB2, were incubated with human sera (diluted 1:20) along with ErbB2 extracellular domain protein standards. Bound ErbB2 protein was revealed by a second anti-ErbB2 mAb conjugated to horseradish peroxidase. Absorbance was measured at 450 nm, and ErbB2 serum concentrations were extrapolated from the standard curve. Serum levels above 6 ng/ml were considered positive, as indicated by the manufacturer.

Epstein-Barr Virus (EBV) Transformation of Patient Peripheral Blood Leukocytes (PBL)

Heparinized blood was collected before adjuvant therapy from breast cancer patients with informed consent and diluted with equal volume of RPMI 1640 medium (Gibco BRL). Human PBL were isolated by density gradient separation through Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) and washed four times in PBS. Approximately 3×10^6 cells were immediately processed for transformation; the rest was frozen and stored in liquid nitrogen as previously described.²⁹ For transformation, PBL were pelleted and resuspended in 1 ml of RPMI 1640 medium. Two hundred microliters of concentrated EBV suspension derived from marmoset leukocyte supernatant were added and incubated for 2 hours at 37°C . Cells were washed twice in RPMI 1640 medium, and the pellet was resuspended at 5×10^4 cells/ml in Yssel's modified Iscove's medium supplemented with 15% heat-inactivated FCS, 2 mmol/L L-glutamine, and 50 $\mu\text{g}/\text{ml}$ gentamicin. EBV-transformed PBL were mixed with irradiated (7000 rad) Ltk-L transfectants ($10^5/\text{ml}$) recombinantly expressing the human ligand for CD40 (CD40 L).^{29,30} One hundred microliters of cell mixture were then seeded and cultured in growth medium. At days 12 and 19, culture supernatants were tested for the presence of ErbB2 antibodies by enzyme-linked immunosorbent assay (ELISA). Following expansion and subcloning at ~ 10 cells/well, supernatants were characterized by ELISA and Western blotting.

Detection of ErbB2-Reactive Immunoglobulins by ELISA

LTR-ErbB2 and NIH3T3 cells were seeded at 5×10^5 cells/well on 96-well plates and allowed to adhere overnight for detection of anti-ErbB2 immunoglobulins by ELISA. Nonspecific binding was blocked by incubation with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hour. Subsequently, cells were incubated with undiluted tissue culture supernatant for 1 hour at 37°C . After washing, horseradish peroxidase-

conjugated goat anti-human IgG+IgM (Gibco BRL) was added, and bound antibody detected with o-phenyl-diamine dihydrochloride (OPD; Sigma Chemical Co., St. Louis, MO). The reaction was stopped by addition of 25 μ l of 4N H₂SO₄, and the absorbance was measured at 492 nm with an automatic ELISA reader.

Statistical Analysis

Statistical significance of associations between anti-ErbB2 humoral response, shed receptor, and clinicopathological parameters was evaluated using Fisher's exact test. *P* values ≤ 0.05 were considered statistically significant.

Results

ErbB2 Immune Response in Sera of Breast Cancer Patients

Employing a recombinant expression model, sera of 71 breast cancer patients and 31 healthy donors were screened by immunoblotting for specific immunoreactivity to human ErbB2 essentially as reported.²² Analysis included 13 breast cancer and 21 control donor sera, which had previously been analyzed.²² Native anti-ErbB2 receptor antibodies as detected by immunoblot analysis were observed in 14 of 71 (20%) breast cancer patients and 1 of 31 controls, the latter observation confirming the known positivity of a single normal donor serum. Statistical analysis demonstrated significant association of an anti-ErbB2 serum response with breast cancer patients as compared to normal donor sera (*P* = 0.0342).

Detection specificity of ErbB2 by direct immunoblotting was further substantiated on ErbB2 immunoprecipitates for 9 positive and 2 negative human sera. Figure 1 representatively illustrates specific detection of an anti-ErbB2 response in human serum by direct immunoblotting (Figure 1A) or following immunoprecipitation (Figure 1B). Identical results were obtained by both procedures in the 11 cases simultaneously subjected to immunoprecipitation, confirming adequate specificity and sensitivity of detection. Development of separate immunoblots using IgM- and IgG-specific secondary antibodies revealed IgM-specific anti-ErbB2 immunoglobulins in 3 patients, while all positive sera contained ErbB2-specific antibodies of the IgG isotype. The latter observation implicated T helper cell activity in the development of an anti-ErbB2 response.

ErbB2 Immunity of Patients' B Lymphocytes Immortalized in Vitro

In direct serum analysis, an existent anti-ErbB2 response could have escaped detection due to a low serum titer *in vivo*. In search of independent confirmation for existent ErbB2 immunity, we screened patients' B lymphocytes for evidence of ErbB2-specific priming. To this purpose, peripheral blood leukocytes (PBL) were isolated from

Figure 1. Detection of ErbB2 antibodies in human sera. Immunoblot analysis using rabbit α -ErbB2 and pre-immune serum, patient serum, and normal human donor serum was conducted on 100 μ g lysate from ErbB2 transfectants and control NIH3T3 (A) and after immunoprecipitation of LTR-ErbB2 using ErbB2-specific mAb E2-1 or nonimmune MOPC-21 (B).

heparinized blood, activated by CD40 ligand and immortalized by EBV *in vitro*. PBL of 15 patients, including 7 positive and 8 negative for anti-ErbB2 serum antibodies, were selected for analysis. Tissue culture supernatants of at least 3×10^6 transformed PBL from each patient were tested for ErbB2-specific immunoreactivity by ELISA on LTR-ErbB2 and NIH3T3 negative controls. Supernatants from B lymphocytes with the relatively highest ErbB2-specific ELISA values (Table 1) were then subjected to immunoblotting using the recombinant expression model. Figure 2 depicts immunoblot analysis of two positive and two negative cases. Supernatants of transformed PBL from patients 1 and 2 recognized a single ~ 185 -kd protein species in LTR-ErbB2, but not control NIH3T3, comigrating with ErbB2 protein visualized by a receptor-specific antiserum. By contrast, PBL supernatants of patients 10 through 15 were negative in ErbB2 detection by ELISA and immunoblot analysis indicating that an ErbB2 response was not a general consequence of *in vitro* manipulation, but rather reflected ErbB2 immunological properties inherent to PBL *in vivo* (Table 1).

Table 1 lists for each patient the maximal ErbB2-specific ELISA value determined from supernatants of immortalized B cells and reactivity of these supernatants in immunoblot analysis in comparison with native ErbB2 antibody serum response *in vivo*. ErbB2-specific immunoreactivity by Western blot analysis was observed in supernatants of B cells derived from 7 patients, whereas

Table 1. Detection of Anti-ErbB2 Response *in Vivo* and *in Vitro*

Patient no.	Native serum reactivity	<i>In vitro</i> reactivity of B cells	
		ELISA*	Western blot
1	pos	1.737	pos
2	pos	0.894	pos
3	pos	0.714	pos
4	pos	1.115	pos
5	pos	0.934	pos
6	neg	0.739	pos
7	pos	1.024	pos
8	neg	0.035	neg
9	pos	0.200	neg
10	neg	<0.001	neg
11	neg	<0.001	neg
12	neg	<0.001	neg
13	neg	0.013	neg
14	neg	<0.001	neg
15	neg	<0.001	neg
pos. ctrl. [†]	N/A	1.716	N/A
neg. ctrl. [‡]	N/A	<0.001	N/A

*ELISA values reported as ΔOD_{492} (LTR-ErbB2-NIH3T3).

[†]Murine anti-ErbB2 IgG.

[‡]Murine nonimmune IgG MOPC21.

N/A, not applicable.

8 patients scored negative (Table 1). Thirteen of 15 patients, including 6 positive and 7 negative for ErbB2 serum antibodies, scored similarly in direct serum analysis and following PBL activation *in vitro*. Furthermore, activated PBL of patient 6 displayed ErbB2 reactivity in the absence of detectable ErbB2 serum antibodies implying that antibody titer *in vivo* was below the threshold of detection. On the other hand, PBL supernatant of patient 9 exhibiting ErbB2 serum antibodies *in vivo*, failed to reveal ErbB2 reactivity by immunoblotting (Figure 2). Given the relatively low albeit specific reactivity in ELISA (Table 1), it is conceivable that antibody titer of this PBL supernatant was below detection in immunoblot analysis. All of these observations suggested rather sensitive detection of ErbB2 serum antibodies *in vivo*, as merely 1 of 8 serum-negative patients revealed ErbB2 immunity following *in vitro* activation of her lymphocytes. Moreover, *in*

Table 2. Association between Anti-ErbB2 Immune Response and Disease Parameters in Breast Cancer Patients

Clinicopathological parameters	ErbB2 immunoreactivity (positive/total)
Tumor histology	
infiltrating ductal Ca.	14/62
infiltrating lobular Ca.	1/8
medullar Ca.	0/1
Grade of differentiation	
G1	0/3
G2	7/38
G3	8/30
Lymph node status	
negative	3/45
positive	12/26
pos. vs. neg.	($P = 0.0002$)
Stage of disease	
stage I	1/28
stages II–IV	14/43
I vs. II–IV	($P = 0.0030$)
I–II vs. III–IV	($P < 0.0001$)

Fisher's exact test was used to compute P values.

vitro activation of PBL independently confirmed a natural ErbB2-specific immune response in the majority of these patients.

Comparison of ErbB2 Immune Response and Clinical Disease Parameters

To explore whether an ErbB2 immune response *in vivo* was associated with a particular disease condition, correlation with clinical disease characteristics were evaluated including tumor histology, grade, stage, lymph node, steroid receptor and menopausal status. ErbB2 immunoreactivity was preferentially detected in ductal breast carcinoma representing the majority of cases ($n = 62$) under analysis. No significant association was observed with grade of differentiation (G1–G3) taking into account that only few cases of G1 ($n = 3$) were analyzed. Furthermore, there was no significant correlation between ErbB2 immune response and steroid receptor or menopausal status. While detected at all stages of breast carcinoma, ErbB2 immunity correlated with disease progression as reflected by lymph node status and disease stage (Table 2). Three of 45 lymph node-negative patients and 12 of 26 lymph node-positive patients revealed anti-ErbB2 serum antibodies indicating a significantly higher prevalence following lymph node metastasis ($P = 0.0002$). Similarly, 1 positive of 28 stage I tumor patients as compared to 14 of 43 stage II–IV patients demonstrated significant association of ErbB2 serum antibodies with an advanced disease stage ($P = 0.0030$). Statistical significance increased to $P < 0.0001$ when comparing frequency of ErbB2 immune responses in stages I and II ($n = 58$) versus stages III and IV ($n = 13$) tumor patients, thus suggesting an association with overall disease progression rather than lymph node status itself (Table 2).

Figure 2. Reactivity of transformed PBL with human ErbB2. Immunoblot analysis of LTR-ErbB2 transfectants and NIH3T3 controls employed ErbB2-specific rabbit antiserum or supernatants of transformed PBL isolated from breast cancer patients, as indicated.

Association of Anti-ErbB2 Response and Shed ErbB2 Ectodomain in Breast Cancer Patients

Experimental animal models established that serum concentration of soluble ErbB2 extracellular domain correlates with tumor burden of ErbB2-overexpressing malignancies.¹¹ We therefore quantified soluble ErbB2 extracellular domain in serum of all patients and normal donors using a commercial double-sandwich ELISA. Considering a cutoff threshold of 6 ng/ml as recommended by the manufacturer, 16 of 71 breast cancer patients revealed elevated serum levels of ErbB2 ectodomain. In addition, 1 of 31 normal donor sera scored marginally positive (6.5 ng/ml). Prevalence of soluble ErbB2 in breast tumor patients was significant when compared to normal donors ($P = 0.019$). Mean soluble ErbB2 serum levels were significantly higher in metastatic disease when compared to stage I patients or normal donors ($P < 0.0001$) projecting a trend for increase of circulating ErbB2 antigen level with disease progression (Figure 3A).

As illustrated in Figure 3B, 12 of 15 patients with *in vivo* or *in vitro* evidence for existent ErbB2 immunity harbored elevated serum levels for soluble ErbB2 (ab^+/ag^+) ranging from 7.0 to 58.4 ng/ml (mean, 14.8 ng/ml). By contrast, only 4 of 56 antibody-negative sera displayed elevated ErbB2 antigen levels (ab^-/ag^+) in the range of 6.2 to 7.6 ng/ml. Conversely, 3 of 55 patients negative for soluble ErbB2 provided evidence for existent ErbB2 immunity (ab^+/ag^-), whereas 52 patients lacked both ErbB2 serum antibodies and circulating extracellular domain altogether (ab^-/ag^-). None of 31 normal donors exhibited simultaneous positivity of ErbB2 serum antibodies and soluble ErbB2, rendering an association highly selective for tumor patients ($P < 0.0001$). Concurrence of ErbB2 immunity and shed ectodomain associated with metastatic disease ($P < 0.0001$) and in addition to the individual parameters correlated with grade of differentiation (G2, $P = 0.0133$; G3, $P < 0.0001$).

To assess a direct association of ErbB2 immunity and release of ectodomain in individual tumor patients, we applied Fisher's exact test. As shown in Table 3, existent ErbB2 immunity significantly coincided with elevated levels of ErbB2 ectodomain, yielding a two-sided P value of <0.0001 (Table 3). The observed correlation between ErbB2 serum immunity and level of soluble extracellular domain supports the possibility that ErbB2 fragmentation and/or solubilization play a role in the development of an anti-ErbB2 response in breast cancer patients. Alternatively, concurrent segregation with an advanced disease condition might reflect a similar dependence on tumor burden of both ErbB2 serum immunity as well as release of soluble ErbB2 extracellular domain.

Discussion

We present evidence for preferential detection in advanced metastatic breast cancer of a native ErbB2 immune response as well as circulating ErbB2 ectodomain in patient sera. Moreover, a statistically significant asso-

A

B

Figure 3. Serum levels of ErbB2 ectodomain in breast cancer patients and normal donors. **A:** Serum levels of ErbB2 ectodomain in normal donor or primary and metastatic breast cancer patient populations by statistical box plot analysis. **Diamonds** mark the means and **horizontal lines** inside boxes mark the medians. Box heights depict range from the 25th to the 75th percentile; the vertical lines extend from them to the 10th and 90th percentiles. n, individuals tested. **B:** Individual soluble ErbB2 serum levels of 31 normal donors, 28 stage I and 43 stage II-IV breast cancer patients. **Open circles**, individuals lacking anti-ErbB2 immune response; **closed circles**, individuals harboring ErbB2-specific immune response.

ciation between presence of ErbB2 immunity and increase of circulating ErbB2 extracellular domain was observed among 71 patients representative of all stages of human breast cancer. Though also detectable at early stages of human breast cancer, both ErbB2 immune response and shed extracellular domain were significantly more prevalent after metastasis, in particular at stages III and IV. Concurrence of ErbB2 immunity and shed extracellular domain was restricted to cancer patients with metastatic disease. In addition, coincidence of both parameters correlated with a less differentiated tumor grade, an association that did not emanate from either parameter tested individually.

Evidence derived from animal models and human breast cancer implicates level of ErbB2 overexpression, tumor aggression, and tumor size in the detection of soluble ErbB2 extracellular domain.¹¹⁻¹⁸ Accordingly, se-

Table 3. Correlation of ErbB2 Immunity and Soluble Ectodomain

ErbB2 immunity and shed ectodomain	Pos/total (%)	Correlation*
Antibody-positive (ab ⁺)	14/71 (20%)	ab ⁺ vs. ab ⁻ (<i>P</i> < 0.0001) [†]
Shed ectodomain-positive (ag ⁺ /ab ⁺)	11/14 (79%)	
Shed ectodomain-negative (ag ⁻ /ab ⁺)	3/14 (21%)	
Antibody-negative (ab ⁻)	57/71 (80%)	
Shed ectodomain-positive (ag ⁺ /ab ⁻)	5/57 (10%)	
Shed ectodomain-negative (ag ⁻ /ab ⁻)	52/57 (90%)	ag ⁺ vs. ag ⁻ (<i>P</i> < 0.0001) [‡]
Shed ectodomain-positive (ab ⁺ /ag ⁺)	16/71 (23%)	
Antibody-positive (ab ⁺ /ag ⁺)	11/16 (69%)	
Antibody-negative (ab ⁻ /ag ⁺)	5/16 (31%)	
Shed ectodomain-negative (ag ⁻)	55/71 (77%)	
Antibody-positive (ab ⁺ /ag ⁻)	3/55 (6%)	
Antibody-negative (ab ⁻ /ag ⁻)	52/55 (94%)	

*Fisher's exact test was used to determine association of ErbB2 immunity and shed ectodomain.

[†]Association of ErbB2 immune response with ErbB2 serum antigen.

[‡]Association of ErbB2 serum antigen with ErbB2 immune response.

rum levels of shed ErbB2 ectodomain can serve as indirect markers of tumor burden in ErbB2-positive malignancy. Determination of circulating ErbB2 ectodomain in the present study confirms previous observations in terms of its frequency, amount, and prevalence in advanced metastatic breast cancer.¹²⁻¹⁸ Statistical significance of an association with the presence of ErbB2 immunity supports the possibility that the amount and/or fragmentation of normal ErbB2 protein may play a role in the development of a syngeneic immune response in breast cancer patients. In this case, ErbB2 immunity is not necessarily directed against its extracellular domain. Detection of elevated ectodomain levels in serum might merely reflect overall ErbB2 self-antigen load of the organism as well as the degree of proteolytic ErbB2 fragmentation owing to growth rate, necrosis, and cell degeneration. Antigen fragmentation could be critical, since ErbB2 peptides, but not full length protein, gave rise to self-antigen immunity in animals.²⁵

ErbB2 gene amplification and overexpression represent early molecular lesions of spontaneous mammary carcinogenesis maintained during disease progression. Consequently, a similar incidence of these aberrations subsists in primary and metastatic tumors.^{5,9,31} On the contrary, both ErbB2 immune response and shed extracellular domain correlate with a progressed disease state, inferring a potential utility of these parameters for disease monitoring and design of immunotherapeutic approaches in ErbB2-associated malignancy. Particular relevance can be envisaged in the context of passive immunotherapy trials of advanced metastatic breast cancer involving humanized monoclonal antibodies targeting the extracellular domain of ErbB2.

Several studies have previously established ErbB2-specific immune responses in breast cancer patients.¹⁹⁻²² One prior study performed systematic analysis of different clinical stages and reported 2 of 26 stage I, 10 of 69 stage II, and none of 12 stage IV patients positive.²¹ Observations from the present study are comparable for stages I and II, with 1 of 28 and 4 of 30 patients displaying ErbB2-specific immunity, respectively. However, 5 of 8 stage IV patients scored positive here, in clear contrast to the preceding observation.²¹

Although small patient numbers may account for some differences, alternative possibilities cannot be excluded. ErbB2 antigen source in the prior study was SK-BR-3 in an ELISA capture assay, whereas we used denatured human recombinant ErbB2 protein expressed by NIH3T3 cells. SK-BR-3 in addition to an overexpressed ErbB2 protein harbors elevated ErbB3 and EGFR levels engaging ErbB2 in functionally active heterodimers.^{28,32} Thus, under conditions of incomplete denaturation certain ErbB2 epitopes might be masked. Furthermore, soluble ErbB2 extracellular domain levels increase with disease progression, as reported here and demonstrated previously,¹²⁻¹⁸ eventually neutralizing certain ErbB2 antibodies present in serum. This possibility rose with increasing disease stage and was more likely to occur under non-denaturing assay conditions.

In view of the possibility that high levels of ErbB2 ectodomain biased detection of ErbB2 immunity in serum analysis, we sought independent confirmation of existing ErbB2 immune responses by immortalization of PBL. Analysis included all 8 patients of stage IV, confirming the results of serum analysis in 7 patients including 3 negative and 4 positive cases. In addition, one patient (patient 6, stage IV) negative for ErbB2 serum antibodies in Western blot analysis had ErbB2-specific immunity associated with her B lymphocytes. It has not been resolved whether low serum titer of ErbB2 antibodies or the elevated ErbB2 ectodomain serum levels observed in this patient, precluded detection of an ErbB2 response in direct serum analysis. In the majority of cases, however, *in vitro* analysis of immortalized B lymphocytes confirmed the ErbB2 immune status determined in serum. Thirteen of 15 patients, including 6 positive and 7 negative for ErbB2 immunity, showed identical results on *in vivo* and *in vitro* analysis, whereas only 2 of 15 patients differed. This observation independently confirmed that in the present study sensitivity and specificity were adequate for serum detection of an existent ErbB2 immune response *in vivo*.

mAbs directed against the ErbB2 extracellular domain have proven effective in preventing or reverting an ErbB2-mediated neoplastic phenotype *in vitro* and in animal models.³³⁻³⁵ Furthermore, a humanized anti-ErbB2 mAb has prompted objective antitumor effects in some

patients with ErbB2-associated metastatic breast cancer.^{7,8} In light of these encouraging results from clinical trial studies, it appears worthwhile to elucidate distinct pharmacodynamics affecting the other patients, despite comparable ErbB2 expression levels and similar pharmacokinetics of the monoclonal antibody.^{7,8} On one hand, ErbB2 activity state in distinct tumors might differ owing to heterodimer formation with other ErbB receptors.^{6,28,36} In this case, a heterodimerizing ErbB receptor or ligand may occlude the binding site for the monoclonal antibody *in vivo*. On the other hand, there is evidence that pre-existing immunity profiles to ErbB receptors are heterogeneous among patients harboring tumors with ErbB2 overexpression.²² Although soluble ErbB2 extracellular domain might interfere with pharmacokinetics in passive immunotherapy,⁷ investigation of pre-existent ErbB2 immunity allows assessment of whether a clinical response to ErbB2-mediated immunotherapy is predictable based on endogenous immunity. More importantly, given the heterogeneity of pre-existent anti-ErbB responses in ErbB2-positive tumor patients,²² definition of such responses in breast cancer patients may help to determine whether passive immunotherapy can be improved by either mimicking or avoiding a specific ErbB receptor self-immunity profile of individual patients.

Acknowledgments

We thank Dr. Angelo Del Nero, University "La Sapienza", Rome, for statistical analyses.

References

- Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes E, Whittle N, Waterfield MD, Seeburg PH: Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 1984, 309: 418–425
- Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, Saito T, Toyoshima K: Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor. *Nature* 1986, 319:230–234
- Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA: Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci USA* 1989, 86:9193–9197
- Plowman GD, Whitney GS, Neubauer MG, Green JM, McDonald VL, Todaro GJ, Shoyab M: Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc Natl Acad Sci USA* 1990, 87:4905–4909
- Hynes NE, Stern DF: The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* 1994, 1198:165–184
- Graus-Porta D, Beerli RR, Daly JM, Hynes NE: ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *Embo J* 1997, 16:1647–1655
- Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L: Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol* 1996, 14:737–744
- Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga JM, Tripathy D, Baly D, Baughman SA, Twaddell T, Glaspy JA, Slamon DJ: Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J Clin Oncol* 1998, 16:2659–2671
- Lacroix H, Iglehart JD, Skinner MA, Kraus MH: Overexpression of erbB-2 or EGF receptor proteins present in early stage mammary carcinoma is detected simultaneously in matched primary tumors and regional metastases. *Oncogene* 1989, 4:145–151
- Iglehart JD, Kraus MH, Langton BC, Huper G, Kerns BJ, Marks JR: Increased erbB-2 gene copies and expression in multiple stages of breast cancer. *Cancer Res* 1990, 50:6701–6707
- Langton BC, Crenshaw MC, Chao LA, Stuart SG, Akita RW, Jackson JE: An antigen immunologically related to the external domain of gp185 is shed from nude mouse tumors overexpressing the c-erbB-2 (HER-2/neu) oncogene. *Cancer Res* 1991, 51:2593–2598
- Leitzel K, Teramoto Y, Sampson E, Mauceri J, Langton BC, Demers L, Podczaski E, Harvey H, Shambaugh S, Volas G, Weaver S, Lipton A: Elevated soluble c-erbB-2 antigen levels in the serum and effusions of a proportion of breast cancer patients. *J Clin Oncol* 1992, 10:1436–1443
- McKenzie SJ, DeSombre KA, Bast BS, Hollis DR, Whitaker RS, Berchuck A, Boyer CM, Bast RC Jr: Serum levels of HER-2 neu (C-erbB-2) correlate with overexpression of p185neu in human ovarian cancer. *Cancer* 1993, 71:3942–3946
- Narita T, Funahashi H, Satoh Y, Takagi H: C-erbB-2 protein in the sera of breast cancer patients. *Breast Cancer Res Treat* 1993, 24:97–102
- Pupa SM, Menard S, Morelli D, Pozzi B, De Palo G, Colnaghi MI: The extracellular domain of the c-erbB-2 oncoprotein is released from tumor cells by proteolytic cleavage. *Oncogene* 1993, 8:2917–2923
- Andersen TI, Paus E, Nesland JM, McKenzie SJ, Borresen AL: Detection of c-erbB-2 related protein in sera from breast cancer patients: relationship to ERBB2 gene amplification and c-erbB-2 protein overexpression in tumour. *Acta Oncol* 1995, 34:499–504
- Molina R, Jo J, Filella X, Zanon G, Pahisa J, Munoz M, Farrus B, Latre ML, Gimenez N, Hage M, Estape J, Ballesta AM: C-erbB-2 oncoprotein in the sera, and tissue of patients with breast cancer: utility in prognosis. *Anticancer Res* 1996, 16:2295–2300
- Revillion F, Hebbard M, Bonnetterre J, Peyrat JP: Plasma c-erbB2 concentrations in relation to chemotherapy in breast cancer patients. *Eur J Cancer* 1996, 32A:231–234
- Pupa SM, Menard S, Andreola S, Colnaghi MI: Antibody response against the c-erbB-2 oncoprotein in breast carcinoma patients. *Cancer Res* 1993, 53:5864–5866
- Cheever MA, Disis ML, Bernhard H, Gralow JR, Hand SL, Huseby ES, Qin HL, Takahashi M, Chen W: Immunity to oncogenic proteins. *Immunol Rev* 1995, 145:33–59
- Disis ML, Pupa SM, Gralow JR, Dittadi R, Menard S, Cheever MA: High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. *J Clin Oncol* 1997, 15:3363–3367
- Bei R, Masuelli L, Moriconi E, Visco V, Moretti A, Kraus MH, Muraro R: Immune responses to all ErbB family receptors detectable in serum of cancer patients. *Oncogene* 1999, 18:1267–1275
- Nanda NK, Sercarz EE: Induction of anti-self-immunity to cure cancer. *Cell* 1995, 82:13–17
- Hayakawa K, Asano M, Shinton SA, Gui M, Allman D, Stewart CL, Silver J, Hardy RR: Positive selection of natural autoreactive B cells. *Science* 1999, 285:113–116
- Disis ML, Gralow JR, Bernhard H, Hand SL, Rubin WD, Cheever MA: Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, oncogenic self-protein. *J Immunol* 1996, 156:3151–3158
- Lemoine NR, Staddon S, Dickson C, Barnes DM, Gullick WJ: Absence of activating transmembrane mutations in the c-erbB-2 proto-oncogene in human breast cancer. *Oncogene* 1990, 5:237–239
- Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA: ErbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science* 1987, 237:178–182
- Alimandi M, Romano A, Curia MC, Muraro R, Fedi P, Aaronson SA, Di Fiore PP, Kraus MH: Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene* 1995, 10:1813–1821
- Visco V, Dolecek C, Denepoux S, Le Mao J, Guret C, Rousset F, Guinépain MT, Kraft D, Valenta R, Weyer A, Banchereau J, La-

- becque S: Human IgG monoclonal antibodies that modulate the binding of specific IgE to birch pollen Bet v 1. *J Immunol* 1996, 157:956–962
30. Garrone P, Neidhardt EM, Garcia E, Galibert L, van Kooten C, Banchereau J: Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J Exp Med* 1995, 182:1265–1273
31. King CR, Swain SM, Porter L, Steinberg SM, Lippman ME, Gelmann EP: Heterogeneous expression of erbB-2 messenger RNA in human breast cancer. *Cancer Res* 1989, 49:4185–4191
32. King CR, Borrello I, Bellot F, Comoglio P, Schlessinger J: Egf binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB-2 protein in the mammary tumor cell line SK-BR-3. *Embo J* 1988, 7:1647–1651
33. Drebin JA, Link VC, Stern DF, Weinberg RA, Greene MI: Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell* 1985, 41: 697–706
34. Kasprzyk PG, Song SU, Di Fiore PP, King CR: Therapy of an animal model of human gastric cancer using a combination of anti-erbB-2 monoclonal antibodies. *Cancer Res* 1992, 52:2771–2776
35. Katsumata M, Okudaira T, Samanta A, Clark DP, Drebin JA, Jolicoeur P, Greene MI: Prevention of breast tumour development in vivo by downregulation of the p185neu receptor. *Nat Med* 1995, 1:644–648
36. Klapper LN, Glathe S, Vaisman N, Hynes NE, Andrews GC, Sela M, Yarden Y: The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc Natl Acad Sci USA* 1999, 96:4995–5000