Cancer Research

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β_1-Integrin Circumvents the Antiproliferative Effects of Trastuzumab in Human Epidermal Growth Factor Receptor-2–Positive Breast Cancer

David Lesniak,[1] Yaoxian Xu,[1] Jean Deschenes,[2] Raymond Lai,[2] John Thoms,[3] David Murray,[1] Sunita Gosh,[4] John R. Mackey,[5] Siham Sabri,[1] and Bassam Abdulkarim[1,3]

Departments of [1]Experimental Oncology, [2]Laboratory Medicine and Pathology, [3]Radiation Oncology, [4]Statistics and Epidemiology, and [5]Medical Oncology, Cross Cancer Institute and University of Alberta, Edmonton, Alberta, Canada

Abstract

Resistance to trastuzumab, the monoclonal antibody targeting human epidermal growth factor receptor 2 (HER-2), is a major concern for HER-2–positive metastatic breast cancer (MBC) patients. To date, HER-2 status is the only available biomarker for selecting patients for trastuzumab-based therapy. β_1-Integrin, an adhesion molecule involved in cell survival and drug resistance, shares common downstream signaling elements with HER-2, such as the phosphatidylinositol 3-kinase/Akt and extracellular signal-regulated kinase-1/2 (ERK1/2) pathways. The significance of β_1-integrin expression in HER-2–positive breast cancer and its involvement in a patient's response to trastuzumab-based therapy are unknown. We show here that overexpression of β_1-integrin is an independent negative prognostic factor for tumor progression of HER-2–positive MBC patients treated with trastuzumab-based chemotherapy. Enforced overexpression of β_1-integrin, its small interfering RNA–induced knockdown or treatment with a β_1-integrin–blocking antibody in HER-2–positive breast cancer cells, identified a strong inverse relationship between expression level of β_1-integrin and *in vitro* sensitivity to trastuzumab. Notably, β_1-integrin overexpression increased the phosphorylation of Akt-Ser473 and ERK1/2, thereby promoting survival and mitogenic signals to bypass the antiproliferative effects of trastuzumab. Our findings show that β_1-integrin provides a novel independent prognostic biomarker of trastuzumab response in HER-2–positive MBC patients and suggest a new target to augment the antiproliferative effects of trastuzumab. [Cancer Res 2009;69(22):8620–8]

Introduction

Human epidermal growth factor receptor 2 (HER-2/neu, c-erbB2), a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTK), is overexpressed in ~20% to 25% of invasive breast carcinomas. We and others have reported that the amplification or overexpression of HER-2 is an independent negative predictor for disease-free, brain metastasis-free, and overall survival (OS; refs. 1–3). Pivotal trials showing clinical benefit of the HER-2–targeted antibody trastuzumab (Herceptin) in combination with chemotherapy have led to a new standard of care for women with HER-2–positive metastatic (4) and early-stage (5) breast cancer. Nonetheless, nearly half of women with HER-2–positive metastatic breast cancer (MBC) fail to achieve a clinical response to trastuzumab combined with chemotherapy (6), and virtually all patients who achieve an initial response develop resistance to trastuzumab (7). Potential molecular mechanisms of trastuzumab resistance involve alterations of HER-2, such as truncated HER-2 receptors (8) or transactivation of HER-2 by other members of the HER family (9, 10). Other mechanisms involve downstream molecules such as deficiency of the tumor suppressor phosphatase and tensin homologue (PTEN; ref. 11), decreased levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (12), or overexpression of RTKs including EGFR (13), insulin-like growth factor type I receptor (14, 15), or Met RTK (16). Although these molecular mechanisms have been thoroughly investigated in preclinical models, most of them are not well validated in clinical samples (17, 18). Recently, using a cohort of 55 HER-2–positive MBC patients treated with trastuzumab-based therapy, Berns and colleagues showed that both PTEN deficiency and mutations in the *PIK3CA* gene, which encodes the p110-α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), were required to identify patients with significantly shorter time to tumor progression (TTP; ref. 18).

Given the proportion of HER-2–positive MBC patients exhibiting trastuzumab resistance (7, 9), we believe that additional mechanisms, yet to be identified, may also influence trastuzumab sensitivity. Increasing evidence suggests an interplay between HER-2 and integrins (19, 20), a large family of cell surface heterodimeric receptors composed of α and β subunits (21). The β_1 subunit, which is encoded by the *ITGB1* gene (also known as CD29 and VLAB), plays a major role in mediating cell-ECM interactions (22) and drug-induced (23–25) or radiation-induced resistance (26), as well as in tumor initiation (27), progression, and invasion (22). In this regard, increased expression of β_1-integrin was associated with poor prognosis in patients with small-cell lung cancer (28), melanoma (29), and invasive breast cancer (30). Several arguments support the tenet that the deleterious effects of increased β_1-integrin expression merit further investigation in the context of HER-2–positive breast cancer, notably, (*a*) binding of β_1-integrin to ECM induces similar downstream signaling pathways to HER-2 (31), such as the PI3K/Akt and extracellular signal-regulated kinase1/2 (ERK1/2) pathways (32); (*b*) reciprocal interactions (i.e., cross-talk) between HER-2 and integrins were reported in different cell types (19, 20); (*c*) β_1-integrin is overexpressed in JIMT-1 (33), a HER-2–positive breast cancer cell line isolated from a patient who did not respond to trastuzumab (34). Nonetheless, the prognostic relevance of

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S. Sabri and B. Abdulkarim contributed equally to this work.

Requests for reprints: Bassam Abdulkarim, Department of Oncology, Cross Cancer Institute and University of Alberta, 11560 University Avenue, Edmonton, Alberta, Canada T6G 1Z2. Phone: 780-432-8517; Fax: 780-432-8380; E-mail: bassamab@cancerboard.ab.ca.

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β_1-integrin expression in HER-2–positive breast cancer specimens and the functional consequences of its level of expression have not been investigated with respect to trastuzumab response.

Here, we show that β_1-integrin overexpression is an independent negative prognostic marker for TTP in HER-2–positive MBC patients treated with trastuzumab-based therapy. We investigated the clinical relevance of our findings in HER-2–positive breast cancer cell lines. Overexpression of β_1-integrin, as well as its targeted inhibition using small interfering RNA (siRNA) or a function-blocking antibody, revealed an inverse relationship between β_1-integrin expression and trastuzumab sensitivity. Regardless of HER-2 inhibition, β_1-integrin mediated an increase of Akt-Ser473 and ERK1/2 phosphorylation to provide an alternative signal circumventing the antiproliferative effects of trastuzumab. Taken together, our results highlight the role of β_1-integrin as a potential therapeutic target for HER-2–positive breast cancer, and its prognostic value should be considered in the application of HER-2–targeted therapies.

Materials and Methods

Patients and specimens. We identified HER-2–positive women with MBC treated with trastuzumab-based chemotherapy (83 patients) or chemotherapy alone (30 patients) from the Alberta Cancer Registry (between 1998 and 2002). Our center began prospective HER-2 testing in a centralized laboratory in January 1998 (3). For this study, we reviewed the original HER-2 immunohistochemistry studies and performed chromogenic *in situ* hybridization on all cases, as per the published guidelines for HER-2 testing (35). Patients with 2+ immunohistochemistry scores and no *HER-2* amplification were excluded (n = 5). Clinical data were processed following the ethical guidelines implemented by the Alberta Cancer Board Ethics Review Board.

Immunohistochemistry. Formalin-fixed paraffin-embedded tumor tissue specimens were retrieved for 78 patients. Serial sections (4 μm) of paraffin-embedded whole tissue were processed for immunohistochemistry. After dewaxing and heat-induced epitope retrieval, slides were stained using anti–β_1-integrin mouse monoclonal antibody (supernatant, clone 7F10, 1:10, LabVision Corporation) on the automated immunostainer (Ventana, BenchMark XT) with Ventana kits (iVIEW DAB detection and amplifier). The immunoreaction was considered specific in the event of an intense brown chromogen deposition, without significant background. A trial set of 30 patients was initially assessed for both cytoplasmic and membrane staining of β_1-integrin. Whole tissue sections were scored (0 to 3+) based on the percentage of invasive tumor cells showing complete (circumferential) membrane staining. The circumferential membrane staining was found to correlate with response to trastuzumab-based therapy with the optimal cutoff established at 40% of positive tumor cells. Using these preliminary data, the entire cohort was assessed as follows: tumor with no membrane staining was scored as 0; 1% to 10%, scored 1+; 11% to 40%, scored 2+; and 41% to 100%, scored 3+. Normal breast epithelium and/or endothelial cells were used as positive internal controls. All slides were independently scored by two senior pathologists who were blinded to the clinical data. Cases for which initial readings straddled the 3+ cutoff were studied at the double-headed scope, and for all but one, the discrepancy was resolved. An average score was used for statistical analysis.

Statistical analysis. Patients were evaluated for a response after at least 9 wk of trastuzumab and then at 12-wk intervals using the Response Evaluation Criteria in Solid Tumors assessment (36). Complete response (CR) was defined as the disappearance of all target lesions. Partial response (PR) was defined as a decrease of more than 50% in the dimensions of all measurable lesions. Progressive disease was defined as an increase of more than 25% in the dimensions of any measurable lesion. Stable disease was defined by neither PR nor progressive disease criteria met, typically involving a small amount of growth or a small amount of shrinkage (<20%). The primary end point of this study was the TTP defined as the time from initiation of trastuzumab until the date of tumor progression. The secondary end points were the objective response rate (CR + PR) and OS. β_1-Integrin, tumor size, lymph node, estrogen receptor and progesterone receptor expression, lymphovascular invasion, and type of trastuzumab-based chemotherapy were analyzed by univariate analysis. Factors found to be significant by univariate analysis were then entered in a stepwise multivariate analysis to identify significant independent predictors. χ^2 tests and hazard ratios (HR), along with corresponding 95% confidence intervals (95% CI), were calculated. TTP and OS curves were constructed according to the Kaplan-Meier method. All reported P values are two sided. Differences were considered statistically significant when $P < 0.05$. Fisher's exact test was used to calculate the significance of the relationship between the dichotomous variables. The SAS program (version 9.1, SAS Institute, Inc.) was used for the statistical analysis.

In all *in vitro* experiments, statistical significance was assessed using two-tailed Student's t tests, with $P < 0.05$ considered to be significant in at least three independent experiments.

Cell culture and reagents. SKBR-3, BT-474, an JIMT-1 human breast cancer cells (American Type Culture Collection) and MCF-7/HER-2 cells (kindly provided by Dr. Dennis Slamon, University of California at Los Angeles, Los Angeles, CA) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen; complete medium) at 37°C in a 5% CO_2-humidified incubator. Trastuzumab (Genentech) was purchased from the Oncology Pharmacy at Cross Cancer Institute. AIIB2 was purchased from the Developmental Studies Hybridoma Bank (University of Iowa).

Stable transfectants. Human β_1-integrin cDNA was subcloned into the mammalian expression vector pIRES-hrGFPII (Stratagene). Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer's instructions. Plasmid (1 μg) was mixed with Lipofectamine 2000 (3 μL) in Opti-MEM (200 μL) for 15 min and added to the cells. After 5 h, the medium was replaced with fresh complete medium. After 48 h, green fluorescent protein (GFP)–positive cells were sorted using an Altra Hypersort cell sorter (Beckman Coulter). Stable transfectants were obtained by culturing cells in complete medium containing 2 mg/mL G418 for 1 wk, and then the concentration of G418 was reduced to 800 μg/mL and maintained in culture for 4 wk. GFP-positive colonies were selected using cloning rings.

siRNA transfection. The siRNA sequence targeting human β_1-integrin was cloned into the pSuper.retro.neo+gfp plasmid vector (Oligoengine); sense: 5′GATCACCGTGTAACCAACCGTAGCAAAGTTCAAGAGACTTTGCTACGGTTGGTTACATTTTTTC-3′, antisense: 5′TCGAGAAAAAATGTAACCAACCGTAGCAAAGTCTCTTGAACTTTGCTACGGTTGGTTACACGGT-3′. A nonspecific siRNA was used as a negative control; sense: 5′GATCACCGACTTCATAAGGCGCATGCACTTCAAGAGAGTGCATGCGCCTTATGAAGTCTTTTTTA-3′, antisense: 5′GCTTAAAAAAGACTTCATAAGGCGCATGCACTCTCTTGAAGTGCATGCGCCTTATGAAGTCGGT-3′.

JIMT-1 cells were transfected with siRNA (6 μg) using a Nucleofector device and corresponding kits according to the manufacturer's recommendations (Amaxa Biosystems, Inc.). After 48 h, GFP-positive cells were analyzed by fluorescence-activated cell sorting (FACS) for further experiments.

Cell proliferation assay. We used the sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent (XTT assay; Roche). Cells were seeded into 96-well microtiter plates at a density of 1,500 per well in complete medium (100 μL) with or without treatment. After 72 h, the XTT reagent was added as per the manufacturers' instructions. The absorbance was measured at 480 nm using a MultiSkan Spectrum microplate reader (Thermo Scientific). Cell viability (%) was calculated in relation to untreated control cells as follows: (experimental absorbance/untreated control absorbance) × 100.

Clonogenic assays. Cells were trypsinized in the exponential growth phase and single-cell suspensions were plated in triplicate in complete medium and allowed to adhere overnight. The following day, the medium was replaced with fresh complete medium without or with treatment and incubated for 12 to 14 d at 37°C. Surviving cells forming visible colonies (>50 cells) were stained with crystal violet (10% acetic acid) and counted automatically with a Colcount Colony Counter (Oxford Optronix). Surviving fraction was calculated in relation to the plating efficiency of the untreated

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control cells using the following formula (37): (number of colonies/number of cells plated) / (plating efficiency of untreated control cells).

Immunoblotting. Equal amounts of protein lysates (30 μg; Bio-Rad protein assay) were processed for immunoblotting as previously described (38). The following primary antibodies were used: HER-2 (clone Ab1, Calbiochem), β_1-integrin (clone 18, BD Transduction Laboratories), phospho-Akt (clone 193H12, Cell Signaling), phospho-ERK1/2 (Cell Signaling), total Akt (Cell Signaling), total ERK1/2 (Cell Signaling), and β-actin (clone AC-15, Sigma-Aldrich). Bands were scanned; normalized to total Akt, ERK1/2, or β-actin protein expression; and quantified with Adobe Photoshop CS2 software. Normalized band intensities were displayed as fold change relative to control conditions.

Results

Patient characteristics. We investigated the prognostic significance of β_1-integrin in HER-2–positive MBC patients treated with trastuzumab-based therapy (Supplementary Table S1). HER-2 testing, treatment, and follow-up of patients are all centralized in our institution within the auspices of a province-wide cancer care system. Data collected included standard prognostic factors including estrogen receptor and progesterone receptor status, tumor size, tumor grade (Nottingham modification of the Scarff-Bloom and Richardson grading scheme), lymphovascular invasion, treatment received, first metastatic site, date of last follow-up, and death. The median duration of follow-up was 28 months (range, 4–63 months).

Response to trastuzumab-based therapy. Patients received trastuzumab weekly until disease progression. Response to trastuzumab was evaluated after at least 9 weeks of trastuzumab-based therapy. An objective response rate of 37% (CR: 10% + PR: 27%) was observed after initial response evaluation. Thirty-four patients (43%) had stable disease and 15 (20%) had progressive disease. The median TTP was 7.9 months and the median OS was 15.4 months. Forty-four patients (56%) continued their therapy without disease progression at 6 months. At the time of last follow-up, 54 patients (70%) had died from disease progression.

β_1-Integrin is an independent prognostic factor for trastuzumab response. To determine the prognostic value of β_1-integrin in our cohort, we assessed β_1-integrin expression by immunohistochemistry in whole tissue sections. We assessed the specificity and sensitivity of the anti–β_1-integrin antibody (clone 7F10) by immunoblotting done on the HER-2–positive trastuzumab-sensitive breast cancer cell lines SKBR-3 and BT-474, which express lower levels of β_1-integrin than JIMT-1 cells (Fig. 1*A*; Supplementary Fig. S1: anti–β_1-integrin antibody clone 18). Paraffin-embedded formalin-fixed samples of JIMT-1 cells grown in the mammary fat pad of nude mice were used as positive controls of β_1-integrin staining by immunohistochemistry and showed an intense homogeneous membranous staining (Fig. 1*B*). In patients' tumors, endothelial cells exhibited strong membrane staining for β_1-integrin and were used as internal positive controls, whereas RBC, which do not express β_1-integrin, were used as internal negative controls (Fig. 1*C, inset*). β_1-Integrin membranous staining within epithelial tumor cells assessed in 78 tumors was scored as 0 in 33 tumors (42%, Fig. 1*C*), 1+ in 7 tumors (9%), 2+ in 15 tumors (19%), and 3+ in 23 tumors (30%; referred to as "β_1-integrin–overexpressing tumors"; Fig. 1*D*).

The prognostic value of β_1-integrin expression was compared with established histologic prognostic variables in univariate analysis. A strong significant association was found between β_1-integrin overexpression and short TTP [HR, 2.04 (95% CI,

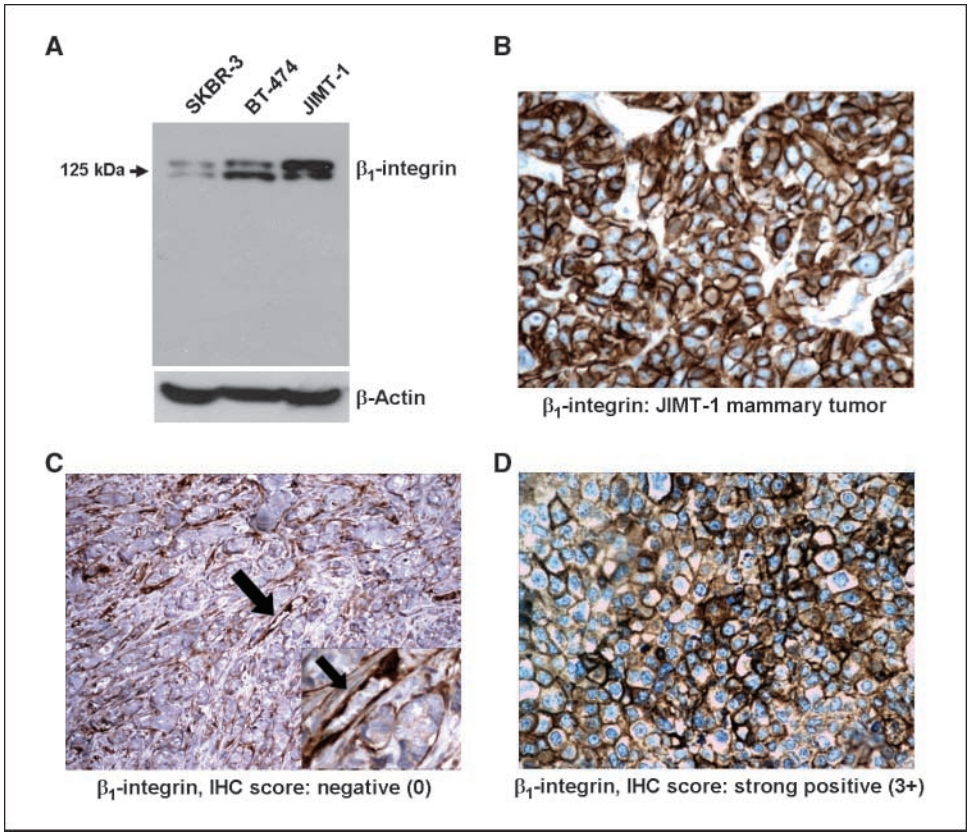

Figure 1. Immunohistochemical analysis of β_1-integrin membrane staining in paraffin-embedded whole tissue sections of HER-2–positive MBC patients treated with trastuzumab-based therapy. Anti–β_1-integrin antibody (clone 7F10) assessed by immunoblotting of lysates from cell lines with different levels of β_1-integrin (*A*; β-actin was used as a loading control) and by immunohistochemistry of JIMT-1 mammary tumor sections (*B*; positive control). Representative cases of β_1-integrin membrane staining in HER-2+ invasive ductal carcinoma scored as negative (0; *C*) or strong positive (3+; *D*). Endothelial cells and RBC are used as positive and negative internal controls, respectively (*C, arrows*; *inset*). Original magnification, ×200 (*B* and *D*), ×100 (*C*), ×400 (*inset*).

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Table 1. Multivariate analysis for TTP in patients receiving trastuzumab-based chemotherapy

Variable	HR (95% CI)	*P*
β_1-Integrin score 3+	2.6 (1.47–4.10)	0.0089
Tumor size >2 cm	1.63 (0.94–2.81)	0.011
Lymph node–positive	1.48 (0.83–2.6)	0.18
ER-positive	0.79 (0.49–1.27)	0.34
PR-positive	0.93 (0.52–1.24)	0.83
LVI-positive	0.7 (0.41–1.17)	0.17
Trastuzumab-based chemotherapy	0.89 (0.31–1.17)	0.13

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; LVI, lymphovascular invasion.

1.02–3.4); $P = 0.0081$], which is the primary end point of this study. There were no significant correlations between β_1-integrin expression and the other prognostic factors (estrogen receptor, progesterone receptor, and lymphovascular invasion). In multivariate analysis, β_1-integrin overexpression emerged as an independent prognostic factor for short TTP [4.8 versus 8.2 months; HR, 2.6 (1.47–4.10); $P = 0.0089$; Tables 1 and 2; Fig. 2*A*]. The level of β_1-integrin expression was the only variable significantly correlated to objective response rate (CR + PR), with only 4 of 23 (17%) patients with β_1-integrin–overexpressing tumors showing clinical response, as compared with 25 of 55 (45%) patients with tumors showing relatively low levels of β_1-integrin ($P = 0.02$, Table 2). Furthermore, a significantly higher proportion of patients with tumors overexpressing β_1-integrin [15 of 23 (65%)] progressed under trastuzumab in less than 6 months, as compared with patients not overexpressing β_1-integrin [19 of 55 (34%); Table 2]. Accordingly, long-term responders were exclusively not overexpressing β_1-integrin (scored 0–2+).

As expected, in multivariate analysis, both estrogen receptor and progesterone receptor status were independent positive prognostic factors for OS [HR, 0.55 (0.32–0.98); $P = 0.04$; HR, 0.69 (0.32–1.82); $P = 0.05$, respectively]. In contrast, β_1-integrin overexpression was significantly associated with reduced OS [13 versus 23 months (Table 2); HR, 2.82 (1.52–4.24); $P = 0.00081$].

To further validate the prognostic value of β_1-integrin, we studied a separate cohort of HER-2–positive MBC patients treated with chemotherapy (docetaxel, vinorelbine, or capecitabine) without trastuzumab ($n = 30$; Supplementary Table S2). In this cohort, β_1-integrin overexpression was not significantly associated with short TTP ($P = 0.75$) in univariate analysis, as illustrated in Kaplan-Meier analysis (Fig. 2*B*), suggesting that the prognostic value of β_1-integrin expression is restricted to trastuzumab-treated patients, which supports a mechanistic relationship to trastuzumab activity.

Overexpression of β_1-integrin circumvents the antiproliferative effects of trastuzumab. To investigate the biological relevance of our clinical findings, we analyzed the relationship between β_1-integrin overexpression and trastuzumab response in HER-2–positive cell lines. To generate a stable cell line overexpressing β_1-integrin, we transfected the trastuzumab-sensitive breast cancer cell line SKBR-3, which expresses barely detectable amounts of β_1-integrin, with a vector containing only GFP [referred to as empty vector (EV)] or GFP plus the Flag-tagged human integrin β_1-subunit (referred to as β1). Furthermore, to ensure that the association between β_1-integrin overexpression and decreased trastuzumab responsiveness is not limited to this one cell type, we also used the human breast carcinoma MCF-7 cell line with stable transfection of the *HER-2* gene (MCF-7/HER-2) to generate a stable cell line overexpressing both HER-2 and β_1-integrin (MCF-7/HER-2/β1). First, we analyzed the expression of β_1-integrin and HER-2 and the phosphorylation status of their common downstream

Table 2. Response to trastuzumab-based chemotherapy based on β_1-integrin staining by immunohistochemistry

	β_1-Integrin membranous staining by IHC		
	Score 3+, *n* = 23 (30%)	Score 0–2+, *n* = 55 (70%)	
	n (%)	*n* (%)	*P**
Response			
ORR	4 (17)	25 (45)	0.02
SD	12 (52)	22 (40)	0.07
PD	7 (30)	8 (45)	0.38
Site of progression			
Visceral	12 (53)	27 (49)	
Bone + soft tissue	4 (17)	12 (22)	
Brain	4 (17)	11 (20)	
Others	3 (13)	5 (9)	
Progression			
Median TTP (mo)	4.8	8.2	0.0089
Progression <6 mo	15 (65)	19 (34)	0.0146
No progression	0 (0)	5 (9)	
Survival			
Median survival (mo)	13	23	0.00081

Abbreviations: SD, stable disease; PD, progressive disease; ORR, objective response rate; IHC, immunohistochemistry.
**P* values: score 3+ versus 0-2+.

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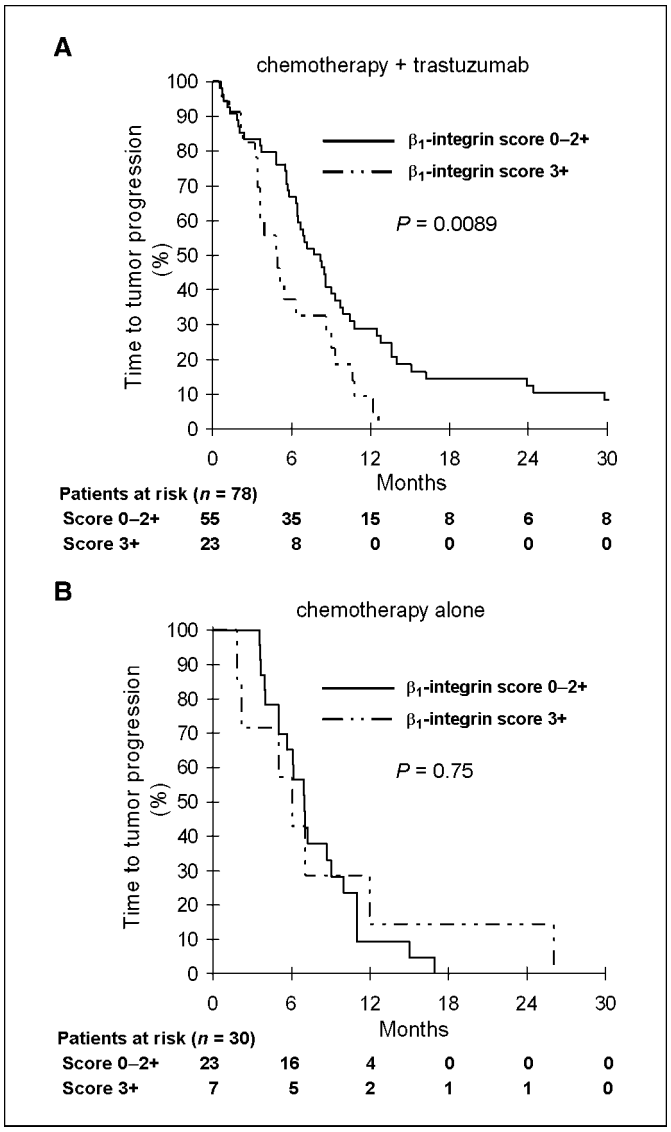

Figure 2. β_1-Integrin overexpression is an independent prognostic factor of short TTP for HER-2–positive MBC patients treated with trastuzumab-based therapy. Kaplan-Meier analysis for TTP of patients treated with trastuzumab-based therapy (*A*; $n = 78$) or chemotherapy alone (*B*; $n = 30$) stratified by β_1-integrin membranous staining.

signaling molecules Akt-Ser473 and ERK1/2 (Thr202 and Tyr204 on p42 and p44 ERK1/2) by immunoblotting. Compared with their respective EV cells, β_1-integrin was significantly increased in SKBR-3/β1-clone 1 (SKBR-3/β1-C1) and MCF-7/HER-2/β1 cells, with no alteration of HER-2 expression. Remarkably, increased phosphorylation of Akt-Ser473 and ERK1/2 was noticeable in SKBR-3/β1-C1 and MCF-7/HER-2/β1 cells (Fig. 3*A*).

Next, we assessed the relationship between β_1-integrin overexpression and the response to trastuzumab in β_1-integrin–overexpressing cells. Cells were exposed to increasing doses of trastuzumab and the extent of growth inhibition was assessed using the XTT viability/proliferation assay. As previously reported, trastuzumab inhibited the proliferation of trastuzumab-sensitive cells, SKBR-3/EV and MCF-7/HER-2/EV (39). Compared with EV cells, β_1-integrin overexpression significantly decreased the antiproliferative effects of trastuzumab (for both 1 and 10 μg/mL treatments) in SKBR-3/β1-C1 cells (1 μg/mL, $P = 0.0096$; 10 μg/mL, $P = 0.0098$) and MCF-7/HER-2/β1 cells (1 μg/mL, $P = 0.0009$; 10 μg/mL, $P = 0.0034$), respectively (Fig. 3*B*). These cells and other SKBR-3/β1 stable clones (data not shown) displayed similar response to trastuzumab as the trastuzumab-resistant JIMT-1 cell line (Fig. 3*B*). As expected, trastuzumab (24-hour continuous exposure) decreased the phosphorylation of both Akt-Ser473 and ERK1/2 in SKBR-3/EV cells. In contrast, in SKBR-3/β1-C1 cells, it decreased the phosphorylation of Akt-Ser473 to a lesser extent than for SKBR-3/EV cells, and ERK1/2 phosphorylation was actually increased by the drug (Fig. 3*C*).

To further investigate the relationship between β_1-integrin and trastuzumab response, we designed a specific β_1-integrin-siRNA construct to achieve the selective suppression of *ITGB1* gene expression and transfected JIMT-1, a HER-2–positive β1-overexpressing cell line (ref. 33; Fig. 1*A*; Supplementary Fig. S1). As shown by immunoblotting of FACS-sorted GFP-positive cells, β_1-integrin expression was markedly reduced (~85%) within 48 hours in cells treated with the β_1-integrin–specific siRNA (Fig. 3*D, lane 2*), compared with those treated with a random/nonspecific control siRNA (Fig. 3*D, lane 1*). siRNA-mediated suppression of β_1-integrin greatly decreased Akt Ser473 and ERK1/2 phosphorylation (>60%; Fig. 3*D*). Furthermore, as determined by the XTT assay, β_1-integrin knockdown was associated with reduced cell proliferation and viability ($P = 0.00006$) of JIMT-1 cells, whereas its overexpression provided a distinct survival and proliferative advantage to SKBR-3/β1/C1 cells ($P = 0.00017$; Fig. 3*D*). Because of this drastic decrease of cell viability, we were not able perform rigorous proliferation assays using JIMT-1/β_1-integrin/siRNA cultures treated with trastuzumab.

Taken together, our results strongly support a direct relationship between overexpression of β_1-integrin and the decreased antiproliferative effects of trastuzumab. These data suggest an alternative mechanism—β_1-integrin overexpression—through which HER-2–positive breast cancer cells might circumvent responsiveness to trastuzumab.

Combination of β_1-integrin–blocking antibody and trastuzumab restored the antiproliferative effects of trastuzumab. To establish proof-of-concept of a potential targeted treatment, we investigated the biological effect of β_1-integrin inhibition on response to trastuzumab using a monoclonal function-blocking antibody (clone AIIB2), which binds to the extracellular domain of β_1-integrin and inhibits β_1-integrin–induced signaling (40). We tested the effect of combining AIIB2 and trastuzumab in HER-2–positive cells with different levels of β_1-integrin expression and a well-known trastuzumab-sensitive (SKBR-3 and BT-474) or trastuzumab-resistant (JIMT-1) phenotype. As shown by immunoblotting, in trastuzumab-sensitive cells, as compared with the isotype control, trastuzumab, but not AIIB2 alone, decreased Akt-Ser473 phosphorylation of SKBR-3 cells within 1 hour, and this decrease was sustained up to 24 hours (Fig. 4*A*). Decreased Akt-Ser473 phosphorylation was apparent in BT-474 cells within 24 hours and was accompanied by decreased phosphorylation of ERK1/2 (Supplementary Fig. S2). In both cell lines, the combination of trastuzumab and AIIB2 induced similar effects as trastuzumab alone.

In JIMT-1 cells, the combination of trastuzumab and AIIB2, but not trastuzumab alone, decreased both the Akt-Ser473 and ERK1/2 phosphorylation within 1 hour (>35% and >50%, respectively; Fig. 4*B*). Interestingly, this decrease was sustained up to 24 hours and was accompanied by decreased expression of β_1-integrin (Fig. 4*B*). This effect was more pronounced than for either treatment alone, suggesting that HER-2 and β_1-integrin act in a dependent manner through activation of the Akt and ERK1/2 pathways in HER-2–positive cells overexpressing β_1-integrin.

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Next, we analyzed the ability of the combination of AIIB2 and trastuzumab to inhibit cell contact–dependent growth. When compared with each antibody alone, the combination of AIIB2 and trastuzumab (10 μg/mL) significantly inhibited the colony-forming ability of SKBR-3/β1-C1 (0.00273) and JIMT-1 cells ($P = 0.0034$), but not of SKBR-3/EV cells ($P = 0.5573$) that express low levels of β_1-integrin (Fig. 4*C*). The combined treatment restored the trastuzumab sensitivity of SKBR-3/β1-C1 and JIMT-1 cells to the same level as SKBR-3/EV, suggesting that both HER-2 and β_1-integrin inhibition is required to restore the trastuzumab responsiveness of SKBR-3/β1-C1 and JIMT-1 cells (Fig. 4*C*). We also evaluated the synergistic or additive effect of AIIB2 and trastuzumab using the XTT assay and the drug-effect equation of Chou and Talalay (41). Isobologram analysis showed a highly synergistic effect of trastuzumab (1 or 10 μg/mL) and AIIB2 (10 μg/mL) combination in JIMT-1 cells (Supplementary Fig. S3). Using the XTT assay, the effect of AIIB2 and/or trastuzumab was further tested in SKBR-3/β1-C1 compared with SKBR-3/EV cells. The combination of trastuzumab and AIIB2 decreased the proliferation of SKBR-3/β1-C1 cells to a significantly greater extent than either trastuzumab or AIIB2 alone ($P = 0.0029$ and $P = 0.0303$, respectively; Fig. 4*D*). Hence, concomitant inhibition of β_1-integrin and HER-2 synergistically increased the cytotoxic or antiproliferative effects of trastuzumab and preferentially suppressed the survival or growth of HER-2–positive cells expressing β_1-integrin.

Discussion

The occurrence of trastuzumab resistance in HER-2–positive MBC patients reflects the complexity and heterogeneous nature of this disease. Our study highlights the clinical relevance of β_1-integrin as a biomarker of prognostic value for HER-2–positive MBC patients receiving trastuzumab-based therapy, among whom overexpression of β_1-integrin was clearly associated with a shorter duration of response. To our knowledge, this is the first study showing a correlation between β_1-integrin expression and clinical outcome in the subset of HER-2–positive breast cancer. In a cohort

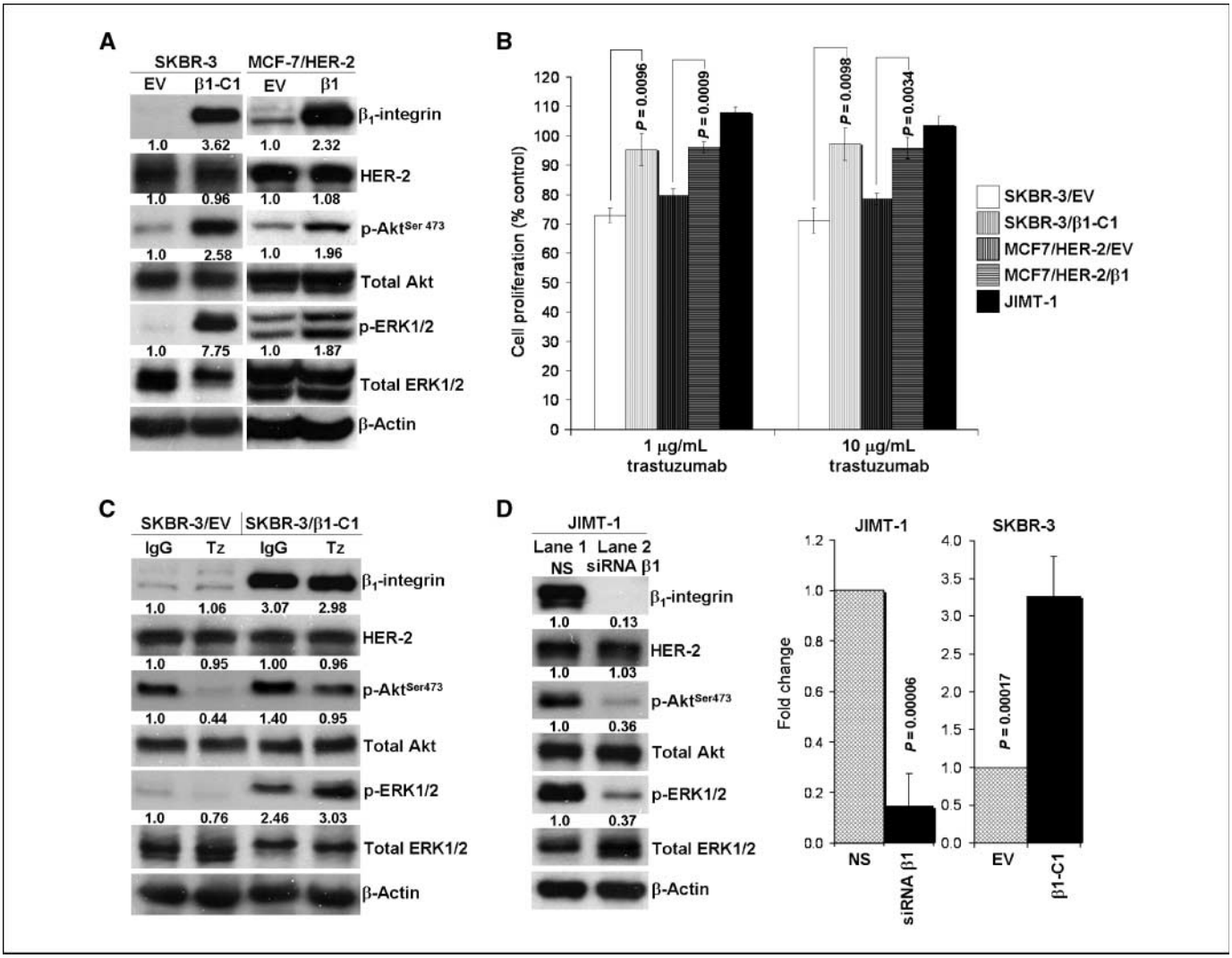

Figure 3. β_1-Integrin–induced Akt-Ser473 and ERK1/2 phosphorylation circumvents the antiproliferative effects of trastuzumab in HER-2–positive cell lines. *A,* immunoblotting analysis of stable SKBR-3/EV (*EV*), SKBR-3/β_1-C1 (*β1-C1*), MCF-7/HER-2/EV (*EV*), and MCF-7/HER-2/β1 (*β1*) using the indicated antibodies. *B,* XTT analysis of SKBR-3/β1-C1 and MCF-7/HER-2/β1 cells compared with SKBR-3/EV, MCF-7/HER-2/EV, or JIMT-1 following 72 h of continuous trastuzumab treatment. *C,* immunoblotting of SKBR-3/EV or SKBR-3/β1-C1 cells treated with IgG or trastuzumab for 72 h. *D,* immunoblotting analysis of GFP-positive JIMT-1 cells 48 h posttransfection with nonspecific (*NS*) or β_1-integrin targeting sequences (*siRNAβ1*). Band intensities were normalized to total ERK1/2, Akt, or β-actin (*left*). XTT analysis of GFP-positive JIMT-1 or SKBR-3 cells grown in complete medium for 72 h (*right*). *Columns,* mean; *bars,* SEM.

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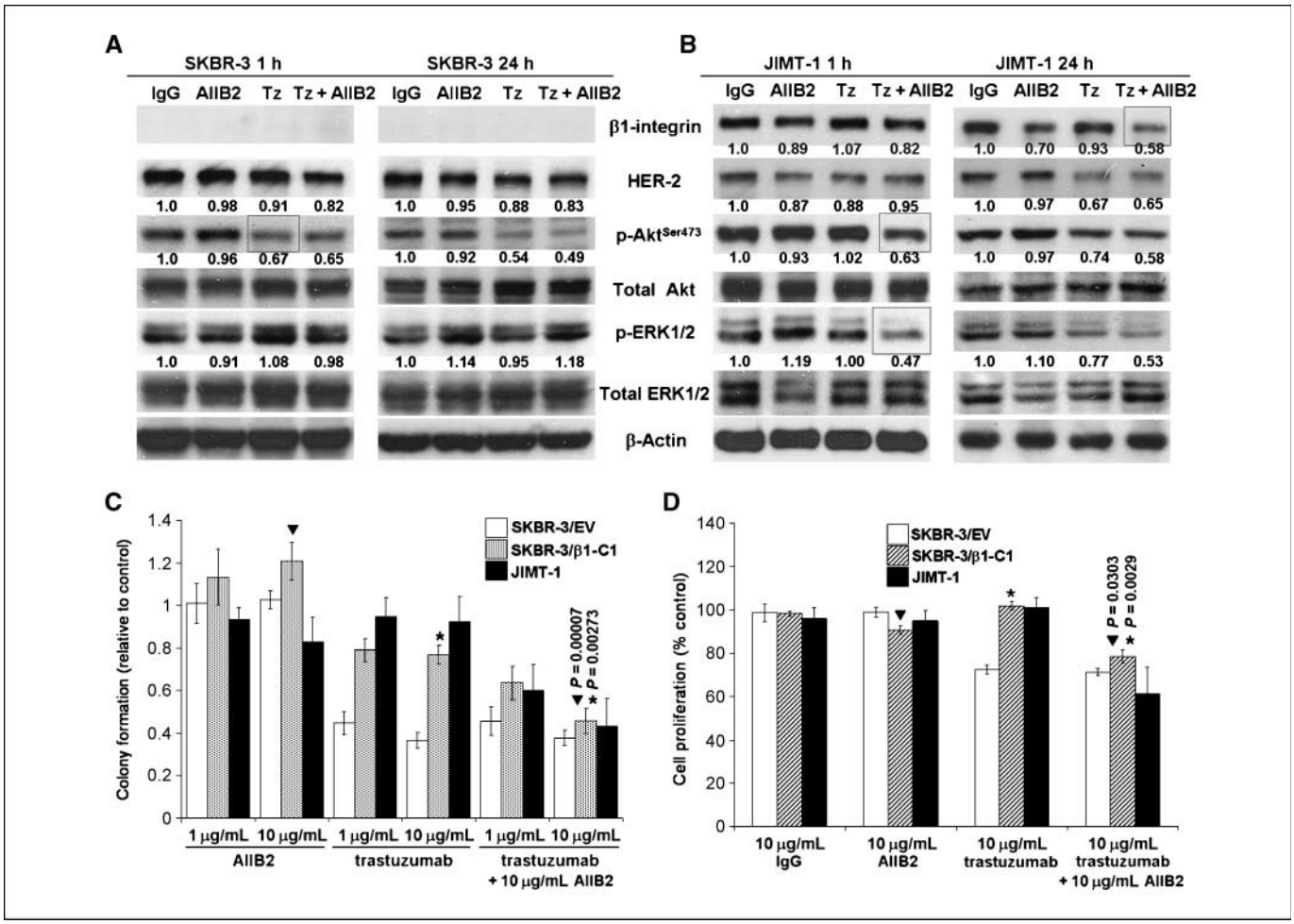

Figure 4. Combination of trastuzumab and AIIB2 decreases phosphorylation of Akt-Ser473 and ERK1/2 and sensitizes HER-2–positive cells to trastuzumab. Immunoblotting analysis of SKBR-3 (*A*) or JIMT-1 (*B*) cells treated with 10 μg/mL IgG, AIIB2, trastuzumab (*Tz*), or Tz + AIIB2 for 1 or 24 h, using the indicated antibodies. *C,* colony-forming assay for SKBR-3/EV, SKBR-3/β1-C1, and JIMT-1 cells treated as indicated for 12 to 14 d. SKBR-3/EV: $P = 0.000019$, 10 μg/mL Tz + AIIB2 versus 10 μg/mL AIIB2; $P = 0.5573$, 10 μg/mL Tz + AIIB2 versus 10 μg/mL Tz. JIMT-1: $P = 0.0081$, 10 μg/mL Tz + AIIB2 versus 10 μg/mL AIIB2; $P = 0.0034$, 10 μg/mL Tz + AIIB2 versus 10 μg/mL Tz. *D,* XTT analysis of SKBR-3/EV, SKBR-3/β1-C1, and JIMT-1 cells after 72 h of continuous treatment. Compared with AIIB2 or trastuzumab alone, the combination significantly decreased proliferation of SKBR-3/β1-C1 and JIMT-1 cells. JIMT-1: $P = 0.0368$, 10 μg/mL Tz + AIIB2 versus 10 μg/mL AIIB2; $P = 0.0371$, 10 μg/mL Tz + AIIB2 versus 10 μg/mL Tz.

of 78 HER-2–positive MBC patients treated with trastuzumab-based therapy in a single institution with standardized diagnostic and response assessments, we identified a subpopulation (30%) of patients overexpressing β_1-integrin (immunohistochemistry score 3+) with significantly increased risk of short TTP. In multivariate analysis, β_1-integrin emerged as the strongest independent prognostic factor for short TTP ($P = 0.0089$).

To determine whether β_1-integrin expression carried only prognostic information, or whether β_1-integrin expression could potentially represent a predictive marker of trastuzumab response, we assembled a limited series of clinically matched trastuzumab-naïve chemotherapy-treated HER-2–positive MBC patients. This population was derived from women prospectively tested as having HER-2–positive disease whose clinical management predated the regulatory approval of trastuzumab. In this group, we did not observe a correlation between β_1-integrin status and response to chemotherapy, further suggesting that β_1-integrin status might be a potential predictive marker of trastuzumab response. However, to unequivocally confirm the predictive value of β_1-integrin overexpression, evaluation of this putative biomarker in samples derived from controlled trials in which large cohorts of patients have been randomized to receive trastuzumab-based chemotherapy versus chemotherapy alone is required.

We further validated the biological relevance of this biomarker using *in vitro* models. As shown in our *in vitro* data and by other authors (32), the effect of β_1-integrin overexpression per se may stem from its ability to promote tumor cell proliferation and survival through increased activity of the PI3K/Akt and ERK1/2 pathways. We provide evidence to support the functional link between β_1-integrin overexpression and decreased antiproliferative effects of trastuzumab. Our studies involving the overexpression of β_1-integrin, its knockdown using siRNA, or its inhibition using AIIB2 antibody collectively support the hypothesis that β_1-integrin elicits regulatory mechanisms for Akt and ERK1/2 phosphorylation, which bypass the antiproliferative effects of trastuzumab through the HER-2/PI3K/Akt signaling axis. Our findings are supported by the results of previous studies showing that phosphorylation of Akt functionally inactivates several proapoptotic and cell cycle regulatory molecules. Constitutive PI3K/Akt activity inhibits apoptosis and cell cycle arrest mediated by

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trastuzumab in HER-2–positive breast cancer cells (42). From a molecular perspective, other mechanisms may contribute to the decreased response to trastuzumab seen in patients overexpressing β_1-integrin. An intriguing aspect of β_1-integrin signaling through ECM interactions, previously described as cell adhesion–mediated drug resistance, also provides a prosurvival signal (via the PI3K/Akt pathway) that protects breast cancer cells from apoptosis induced by chemotherapy (24) or ionizing radiation (26). In addition, increased expression of ECM components such as fibronectin, an extracellular ligand of β_1-integrin, was significantly correlated with increased β_1-integrin expression in invasive breast cancer (30). Indeed, aberrant β_1-integrin expression and/or tumor microenvironment may concomitantly promote resistance to anchorage-dependent apoptosis (anoikis), increased cell survival, tumor growth, and drug resistance. Integrin-mediated transactivation of growth factor receptors (43, 44) may further trigger alternative survival signaling mechanisms and counteract the antitumor effects of trastuzumab.

In summary, our study sheds light into the mechanisms of trastuzumab resistance in HER-2–positive MBC patients, which may optimize the use of trastuzumab and other novel HER-2–targeted therapies (10, 42). The identification of an inexpensive and technically routine test has enabled us to propose β_1-integrin as a molecular prognostic biomarker of trastuzumab response in women with HER-2–positive MBC. Current guidelines recognize HER-2 as the only biomarker for selection of both metastatic and early-stage breast cancer patients eligible for trastuzumab-based therapy. A more rigorous selection of patients who are most likely to benefit from trastuzumab may avoid unnecessary toxicities such as cardiac events (45) and reduce costs of drug acquisition and administration of ineffective therapy (46). For those patients with tumors overexpressing β_1-integrin, our study provides a rationale to explore novel alternative therapeutic strategies, such as the combination of trastuzumab with small-molecule or antibody inhibitors of β_1-integrin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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