My name is David Lesniak. I grew up and attended high school in St. Albert, Alberta, focusing more on athletics than academics throughout much of my upbringing. After completing high school in 2001, I moved to Southern California to pursue my passion for athletic competition. I played baseball for the College of the Desert Roadrunners between 2001 and 2003. These years were exciting and successful as we set a new school record for wins in 2003. More importantly, however, these two years taught me that success in any venue is achieved through hard work, dedication and teamwork. Although my commitment to athletics during this time was never in doubt, a strong underlying passion for medicine and science was becoming paramount. In 2003, I returned to Edmonton to begin pursuing a career in academia. I completed my BSc. in 2006 with a double major in Biology and Chemistry. My first exposure to research came as a summer student in 2006 at the Cross Cancer Institute (University of Alberta) under the supervision of Dr. Bassam Abdulkarim. Fascinated by the self-directed nature of scientific research, the complexities of cancer biology, and the translation aspects of my project in breast cancer, I entered the MSc. program in Oncology in September of 2006. In 2008 I transferred into the PhD program and in September 2009 I was accepted into the MD program here at the University of Alberta and have subsequently entered the MD/PhD program. I look forward to completing my PhD in Oncology and plan to use this knowledge as an active clinician scientist.
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. β1-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 β1-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 β1-integrin is an independent negative prognostic factor for tumor progression of HER-2–positive MBC patients treated with trastuzumab-based chemotherapy. Enforced overexpression of β1-integrin, its small interfering RNA–induced knockdown or treatment with a β1-integrin–blocking antibody in HER-2–positive breast cancer cells, identified a strong inverse relationship between expression level of β1-integrin and in vitro sensitivity to trastuzumab. Notably, β1-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 β1-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.

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 p27Kip1 (12), or overexpression of RTKs including EGFR (13), insulin-like growth factor type 1 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 β1 subunit, which is encoded by the ITGBI gene (also known as CD29 and VLAβ), 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 β1-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 β1-integrin expression merit further investigation in the context of HER-2–positive breast cancer, notably, (a) binding of β1-integrin to ECM induces similar downstream signaling pathways to HER-2 (31), such as the PI3K/Akt and extracellular signal-regulated kinase-1/2 (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) β1-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

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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β1-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 β1-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 β1-integrin, as well as its targeted inhibition using small interfering RNA (siRNA) or a function-blocking antibody, revealed an inverse relationship between β1-integrin expression and trastuzumab sensitivity. Regardless of HER-2 inhibition, β1-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 β1-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–β1-integrin mouse monoclonal antibody (supernatant, clone 7E10, 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 β1-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-reading level 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-reading level by two senior pathologists who were blinded to the clinical data.

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 initia-
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), β₁-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 β₁-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.

β₁-Integrin is an independent prognostic factor for trastuzumab response. To determine the prognostic value of β₁-integrin in our cohort, we assessed β₁-integrin expression by immunohistochemistry in whole tissue sections. We assessed the specificity and sensitivity of the anti-β₁-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 β₁-integrin than JIMT-1 cells (Fig. 1A, Supplementary Fig. S1: anti-β₁-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 β₁-integrin staining by immunohistochemistry and showed an intense homogeneous membranous staining (Fig. 1B). In patients’ tumors, endothelial cells exhibited strong membrane staining for β₁-integrin and were used as internal positive controls, whereas RBC, which do not express β₁-integrin, were used as internal negative controls (Fig. 1C, inset). β₁-Integrin membranous staining within epithelial tumor cells assessed in 78 tumors was scored as 0 in 33 tumors (42%, Fig. 1C), 1+ in 7 tumors (9%), 2+ in 15 tumors (19%), and 3+ in 23 tumors (30%; referred to as “β₁-integrin-overexpressing tumors”, Fig. 1D).

The prognostic value of β₁-integrin expression was compared with established histologic prognostic variables in univariate analysis. A strong significant association was found between β₁-integrin overexpression and short TTP [HR, 2.04 (95% CI, 2.04–4.08), P = 0.001].

![Image](https://example.com/image1.png)

**Figure 1.** Immunohistochemical analysis of β₁-integrin membrane staining in paraffin-embedded whole tissue sections of HER-2-positive MBC patients treated with trastuzumab-based therapy. Anti-β₁-integrin antibody (clone 7F10) assessed by immunoblotting of lysates from cell lines with different levels of β₁-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 β₁-integrin membrane staining in HER-2-expressing ductal carcinoma scored as negative (0; C, arrows; inset), 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).
1.02–3.4; \( P = 0.0081 \), which is the primary end point of this study. There were no significant correlations between \( \beta_1 \)-integrin expression and the other prognostic factors (estrogen receptor, progesterone receptor, and lymphovascular invasion). In multivariate analysis, \( \beta_1 \)-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. 2A]. The level of \( \beta_1 \)-integrin expression was the only variable significantly correlated to objective response rate (CR + PR), with only 4 of 23 (17%) patients with \( \beta_1 \)-integrin–overexpressing tumors showing clinical response, as compared with 25 of 55 (45%) patients with tumors showing relatively low levels of \( \beta_1 \)-integrin (\( P = 0.02 \), Table 2). Furthermore, a significantly higher proportion of patients with tumors overexpressing \( \beta_1 \)-integrin [15 of 23 (65%)] progressed under trastuzumab in less than 6 months, as compared with patients not overexpressing \( \beta_1 \)-integrin [19 of 55 (34%); Table 2]. Accordingly, long-term responders were exclusively not overexpressing \( \beta_1 \)-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, \( \beta_1 \)-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 \( \beta_1 \)-integrin, we studied a separate cohort of HER-2–positive MBC patients treated with chemotherapy (docetaxel, vinorelbine, or capecitabine) without trastuzumab \( (n = 30; \text{Supplementary Table S2}) \). In this cohort, \( \beta_1 \)-integrin overexpression was not significantly associated with short TTP \( (P = 0.75) \) in univariate analysis, as illustrated in Kaplan-Meier analysis (Fig. 2B), suggesting that the prognostic value of \( \beta_1 \)-integrin expression is restricted to trastuzumab-treated patients, which supports a mechanistic relationship to trastuzumab activity.

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta_1 )-Integrin score 3+</td>
<td>2.6 (1.47–4.10)</td>
<td>0.0089</td>
</tr>
<tr>
<td>Tumor size &gt;2 cm</td>
<td>1.63 (0.94–2.81)</td>
<td>0.011</td>
</tr>
<tr>
<td>Lymph node–positive</td>
<td>1.48 (0.83–2.6)</td>
<td>0.18</td>
</tr>
<tr>
<td>ER-positive</td>
<td>0.79 (0.49–1.27)</td>
<td>0.34</td>
</tr>
<tr>
<td>PR-positive</td>
<td>0.93 (0.52–1.24)</td>
<td>0.83</td>
</tr>
<tr>
<td>LVI-positive</td>
<td>0.7 (0.41–1.17)</td>
<td>0.17</td>
</tr>
<tr>
<td>Trastuzumab–based chemotherapy</td>
<td>0.89 (0.31–1.17)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

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

**Table 2. Response to trastuzumab–based chemotherapy based on \( \beta_1 \)-integrin staining by immunohistochemistry**

<table>
<thead>
<tr>
<th>( \beta_1 )-Integrin membranous staining by IHC</th>
<th>Score 3+, ( n = 23 (30%) )</th>
<th>Score 0–2+, ( n = 55 (70%) )</th>
<th>( P^\dagger )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response</td>
<td>( % )</td>
<td>( % )</td>
<td></td>
</tr>
<tr>
<td>ORR</td>
<td>4 (17)</td>
<td>25 (45)</td>
<td>0.02</td>
</tr>
<tr>
<td>SD</td>
<td>12 (52)</td>
<td>22 (40)</td>
<td>0.07</td>
</tr>
<tr>
<td>PD</td>
<td>7 (30)</td>
<td>8 (45)</td>
<td>0.38</td>
</tr>
<tr>
<td>Site of progression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visceral</td>
<td>12 (53)</td>
<td>27 (49)</td>
<td></td>
</tr>
<tr>
<td>Bone + soft tissue</td>
<td>4 (17)</td>
<td>12 (22)</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>4 (17)</td>
<td>11 (20)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>3 (13)</td>
<td>5 (9)</td>
<td></td>
</tr>
<tr>
<td>Progressation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median TTP (mo)</td>
<td>4.8</td>
<td>8.2</td>
<td>0.0089</td>
</tr>
<tr>
<td>Progression &lt;6 mo</td>
<td>15 (65)</td>
<td>19 (34)</td>
<td>0.0146</td>
</tr>
<tr>
<td>No progression</td>
<td>0 (0)</td>
<td>5 (9)</td>
<td></td>
</tr>
<tr>
<td>Survival</td>
<td>13</td>
<td>23</td>
<td>0.00081</td>
</tr>
</tbody>
</table>

Abbreviations: SD, stable disease; PD, progressive disease; ORR, objective response rate; IHC, immunohistochemistry.

\( ^\dagger \) \( P \) values: score 3+ versus 0–2+.
signaling molecules Akt-Ser473 and ERK1/2 by immunoblotting. Compared with their respective EV cells, β1-integrin was significantly increased in SKBR-3/β1-c1 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 BT-474 cells within 24 hours and was sustained up to 24 hours (Fig. 4A). 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.

Next, we assessed the relationship between β1-integrin overexpression and the response to trastuzumab in β1-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, β1-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 = 0.0009; 10 μg/mL, P = 0.0034), respectively (Fig. 3B). 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. 3B). 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. 3C).

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

Taken together, our results strongly support a direct relationship between overexpression of β1-integrin and the decreased antiproliferative effects of trastuzumab. These data suggest an alternative mechanism—β1-integrin overexpression—through which HER-2–positive breast cancer cells might circumvent responsiveness to trastuzumab.

**Combination of β1-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 β1-integrin inhibition on response to trastuzumab using a monoclonal function-blocking antibody (clone AIIB2), which binds to the extracellular domain of β1-integrin and inhibits β1-integrin–induced signaling (40). We tested the effect of combining AIIB2 and trastuzumab in HER-2–positive cells with different levels of β1-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. 4A). 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. 4B). Interestingly, this decrease was sustained up to 24 hours and was accompanied by decreased expression of β1-integrin (Fig. 4B). This effect was more pronounced than for either treatment alone, suggesting that HER-2 and β1-integrin act in a dependent manner through activation of the Akt and ERK1/2 pathways in HER-2–positive cells overexpressing β1-integrin.

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**Figure 2.** β1-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 β1-integrin membranous staining.
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 β1-integrin (Fig. 4C). 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 β1-integrin inhibition is required to restore the trastuzumab responsiveness of SKBR-3/β1-C1 and JIMT-1 cells (Fig. 4C). 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. 4D). Hence, concomitant inhibition of β1-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 β1-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 β1-integrin as a biomarker of prognostic value for HER-2–positive MBC patients receiving trastuzumab-based therapy, among whom overexpression of β1-integrin was clearly associated with a shorter duration of response. To our knowledge, this is the first study showing a correlation between β1-integrin expression and clinical outcome in the subset of HER-2–positive breast cancer. In a cohort

Figure 3. β1-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/β1-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 β1-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.
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 $\beta_1$-integrin (immunohistochemistry score 3+) with significantly increased risk of short TTP. In multivariate analysis, $\beta_1$-integrin emerged as the strongest independent prognostic factor for short TTP ($P = 0.0089$).

To determine whether $\beta_1$-integrin expression carried only prognostic information, or whether $\beta_1$-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 $\beta_1$-integrin status and response to chemotherapy, further suggesting that $\beta_1$-integrin status might be a potential predictive marker of trastuzumab response. However, to unequivocally confirm the predictive value of $\beta_1$-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 $\beta_1$-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 $\beta_1$-integrin overexpression and decreased antiproliferative effects of trastuzumab. Our studies involving the overexpression of $\beta_1$-integrin, its knockdown using siRNA, or its inhibition using AIIB2 antibody collectively support the hypothesis that $\beta_1$-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
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 β1-integrin. An intriguing aspect of β1 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 β1 integrin, was significantly correlated with increased β1-integrin expression in invasive breast cancer (30). Indeed, aberrant β1-integrin expression and/or tumor microenvironment may concomitantly resist 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 β1-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 β1-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 β1-integrin.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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