Prognostic Significance of Tissue Transglutaminase in Drug Resistant and Metastatic Breast Cancer

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ABSTRACT

Purpose: Drug resistance and metastasis pose major impediments in the successful treatment of cancer. We previously reported that multidrug-resistant breast cancer cells exhibit high levels of tissue transglutaminase (TG2; EC 2.3.2.13). Because the drug-resistant and metastatic phenotypes are thought to share some common pathways, we sought to determine whether metastatic breast cancer cells express high levels of TG2.

Experimental Design: The metastatic breast cancer cell line MDA-MB-231 and the sublines derived from it were tested for TG2 expression. Similarly, several sublines derived from an immortal but normal breast epithelial cell line, MCF10A, representing various stages in breast cancer progression were studied for TG2 expression. The primary and nodal tumor samples from 30 patients with breast cancer were also studied for TG2 expression.

Results: The MDA-MB-231 cells expressed high basal levels of TG2. Two clones derived from this cell line, MDA231/cl.9 and MDA231/cl.16, showed a 10- to 15-fold difference in TG2 level. TG2-deficient MDA231/cl.9 cells exhibited higher sensitivity to doxorubicin and were less invasive than were the TG2-sufficient MDA231/cl.16 cells. The MCF10A-derived sublines had increased TG2 expression as they advanced from noninvasive to an invasive phenotype. Importantly, the metastatic lymph node tumors from patients with breast cancer showed significant higher levels of TG2 expression compared with the primary tumors from the same patients.

Conclusions: TG2 expression is up-regulated in drug-resistant and metastatic breast cancer cells, and it can serve as a valuable prognostic marker for these phenotypes.

INTRODUCTION

Despite continuous efforts to design new and effective treatments to improve the survival rates in patients with breast cancer, breast cancer has continued to be the second leading cause of cancer deaths in women after lung cancer (1). Currently, none of the known prognostic factors is capable of determining which patients with breast cancer have a high risk of relapsing disease. Therefore, identification of new factors predicting relapse, outcome, and response to systemic therapy is warranted.

The two factors that pose a continuing therapeutic challenge in the clinic and which are responsible for most of the cancer-related deaths in breast cancer patients are the ability of cancer cells to metastasize and their capacity to resist anticancer therapies. For example, most women who die of breast cancer do so when their tumor metastasizes; long-term survival is more likely if the cancer remains localized. Similarly, the ability of most cancer cells to become simultaneously resistant to different drugs—the phenomenon referred to as multidrug resistance—remains a significant problem in eradicating tumors with chemotherapy (2, 3). Therefore, development of new approaches that will accurately predict and circumvent drug resistance and treat metastatic tumors are likely to improve the disease outcome in breast cancer patients.

Although research efforts regarding metastasis and drug resistance have generally proceeded along separate tracks in the past, there are several reasons to believe that the two phenotypes may be linked and share some common pathways. For example, a growing body of evidence suggests that expression of certain dominantly acting oncogenes or altered expression of tumor-suppressor genes can enhance not only tumor aggressiveness but also make them more drug resistant (4, 5). Moreover, some tumor cell lines selected for resistance to drugs exhibit higher invasive or metastatic potential than their nonresistant parental cell lines (6). Conversely, in some instances metastatic tumors show more resistance to chemotherapeutic drugs than their primary counterparts (4). It is well known that chemotherapeutic agents can induce a series of cellular responses that can affect tumor cell proliferation and survival. Several drugs are known to kill tumor cells by activating common apoptotic pathways. Similarly, an important property of metastatic cancer cells is that they must survive and propagate within stressful microenvironments. Thus, mutations or alterations that disable apoptosis can provide cancer cells with both an intrinsic survival advantage and inherent resistance to chemotherapeutic drugs.

We have previously observed that the development of drug resistance in breast and other cancer cell lines is associated with high expression levels of tissue transglutaminase (TG2;
EC2.3.2.13; refs. 7–9). In this article, we demonstrate that the development of the metastatic phenotype in breast cancer cells is associated with a similar increase in TG2 protein expression. Possible implications of the increased TG2 expression in conferring drug resistance and metastatic phenotypes are discussed.

**MATERIALS AND METHODS**

**Cell Lines and Tumor Tissues.** Human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from American Type Culture Collection (Rockville, MD). The multidrug-resistant MCF-7 cell line (MCF-7/DOX) was obtained as described earlier (8). Clonal sublines from MDA-MB-231 were isolated by the limiting dilution technique. Briefly, the parental MDA-MB-231 cells, and the sublines derived from it (MDA231/cl.9 and MDA231/cl.16), were isolated by trypsinization, washed once with RPMI containing 5% FCS, and resuspended at 3 × 10^4 cells/mL in the medium. Aliquots (100 µL) of the cell suspension were added to each well of a 96-well plate. After 2 weeks, wells harboring single colonies were identified under the microscope and colonies were recovered by trypsinization and expanded in T-25 flasks. The MCF10A-derived sublines, representing various stages in the progression of breast cancer from benign hyperplasia to atypical hyperplasia to carcinoma in situ and fully malignant invasive tumors that can metastasize, were obtained as described earlier (10, 11). In the present study we refer to these cell lines as M1-M5. Properties and nomenclature of these cell lines are summarized in Table 1. The cell lysates from other estrogen receptor (ER)-positive (T47D, ZR75, and BT174) and ER-negative (SKBR-3, MDA-H3S, and MDA-MB-468) breast cancer cell lines were provided by Dr. Rakesh Kumar at M. D. Anderson Cancer Center (Houston, TX).

Paraffin-embedded xenografts of the MCF10A sublines (M2-M5) from nude mice were obtained as described previously (10). The specimens of patients in this study were obtained from the archives of the Pathology Department of M. D. Anderson Cancer Center after approval from the Institutional Review Board for the Welfare of Human Subjects. Archival tissue sections of primary breast cancer and axillary lymph node metastasis were randomly selected from 30 patients with newly diagnosed breast cancer. Clinicopathologic features of these patients are presented in Table 2.

**Cell Culture and Viability.** Ninety-six-well plates (Corning/Costar, Rochester, NY) were coated with either 10 µg/mL fibronectin (Sigma-Aldrich, St. Louis, MO) in PBS or 0.5% BSA (Sigma) in PBS for 16 hours at 4°C. The plates were then blocked with 0.5% BSA for 1 hour at room temperature. The parental MDA-MB-231 cells, and the sublines derived from it (MDA231/cl.9 and MDA231/cl.16), were isolated by trypsinization, washed once with RPMI containing 5% FCS, and resuspended at 3 × 10^4 cells/mL in the medium. Aliquots (100 µL) of the cell suspension were added to each well and incubated overnight at 37°C. Next day, the cells were either left untreated or treated in quadruplicate with 0.1 µg/mL or 0.3 µg/mL concentrations of doxorubicin (American Pharmaceutical Partners, Inc., Los Angeles, CA). Forty-eight hours later, the number of viable cells remaining in the well was determined by measuring their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) into a soluble formazan, as described earlier (7).

**Invasion Assay.** Invasive behavior of cells was determined in vitro by measuring the number of cells that invaded through Matrigel-coated Transwell polycarbonate membrane inserts, as described previously (12). Briefly, Transwell inserts

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Nomenclature and properties of MCF10A-derived breast cancer cell lines</th>
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<tbody>
<tr>
<td>Cell line</td>
<td>MCF10A (M-1)</td>
</tr>
<tr>
<td>Stage representing breast cancer development</td>
<td>Normal epithelium</td>
</tr>
<tr>
<td>Important features</td>
<td>Spontaneously immortalized line from nonmalignant human breast epithelium</td>
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<tr>
<td>Tumorigenicity in nude mice xenografts</td>
<td>Nontumorigenic</td>
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**Table 2 | Clinical features of the 30 patients studied**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>%</th>
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<tr>
<td>Tumor size</td>
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<tr>
<td>&lt;2 cm</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>≥2 cm</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Nuclear grade</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>26</td>
<td>87</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
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<td>3</td>
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<td>40</td>
</tr>
<tr>
<td>≥50 years</td>
<td>18</td>
<td>60</td>
</tr>
</tbody>
</table>

NOTE. All 30 patients had nodal-positive breast cancer. Abbreviation: PR, progesterone receptor.

* ER/PR status for one patient was not available.
with 12-μmol/L pore size were coated with 0.78 mg/mL Matrigel in cold serum-free medium. Cells were recovered by trypsinization and washed once with serum-free medium. The cell pellets were resuspended in serum-free medium, and 0.5 mL of the cell suspension (0.5 × 10^6 cells) was added to duplicate wells. After 48 hours incubation, the cells that passed through the filter were stained with Hema-3 stain kit (Fisher Scientific, Houston, TX), and the cells in 10 random fields were counted under the microscope.

**Immunohistochemistry.** Sections of formalin-fixed, paraffin-embedded tumor samples (5 μmol/L thick) were heated to 60°C and dehydrated in xylene and graded alcohols. Antigen retrieval was done with 0.01 mol/L citrate buffer at pH 6.0 for 20 minutes in a 95°C steam bath. Slides were allowed to cool for 20 minutes at room temperature, followed by repeated rinsing with 0.1 mol/L PBS (pH 7.4) containing 0.1% Tween 20. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Each incubation step was conducted at room temperature and was followed by repeated rinsing with 0.1 mol/L PBS (pH 7.4) containing 0.1% Tween 20. Sections were incubated with a cocktail of two anti-TG2 monoclonal antibodies (mAb; Neomarkers, Fremont, CA) overnight at 4°C, followed by 30 minutes incubation each with biotinylated secondary antibody and peroxidase-labeled streptavidin. Antigen-antibody reaction was detected by 3,3′-diaminobenzidine and hydrogen peroxide chromogen substrate (Vector Labs, Burlingame, CA) for 3 to 5 minutes. Slides were counterstained with hematoxylin and mounted. The negative controls were incubated with nonimmune mouse IgG in place of primary antibody.

**TG2 Enzyme Activity.** Cell monolayers at 70 to 80% confluency were washed in PBS, collected, and resuspended in a minimal volume (100 to 300 μL) of lysis buffer [20 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA, 150 mmol/L NaCl, 14 mmol/L 2-mercaptoethanol, and 1 mmol/L phenylmethylsulfonyl fluoride]. Cells were then lysed in the same buffer by probe sonication for 8 to 10 pulses of 10 seconds each. The protein contents of the cell lysates were determined with the protein-dye reagent (Bio-Rad, Richmond, CA). Cell lysates were then assayed for TG2 activity by determining Ca^2+ -dependent incorporation of [3H]putrescine (specific activity, 14.3 Ci/mmol; Amersham-Pharmacia, San Francisco, CA) into dimethylcasein (7). The enzyme activity was expressed as nanomols of putrescine incorporated per hour per milligram of total lysate protein.

**Western Blot Analysis.** Thirty micrograms of protein were separated by SDS-PAGE on a 7.5% gel and electrophoretically transferred to a nitrocellulose membrane (Amersham-Pharmacia) with a semidry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA). The membrane was blocked overnight in 5% (wt/vol) nonfat dry milk in PBS (pH 7.4) containing 0.1% Tween 20. The membranes were incubated with either TG2 mAb (CUB7401; Neomarkers) at 1:3,000 dilution or with anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO) at 1:1,000 dilution. The enhanced chemiluminescence system (Amersham-Pharmacia) was used to detect the reaction between antigen and antibody.

**Immunofluorescence and Confocal Microscopy.** To examine the intracellular distribution of TG2 protein, we used scanning confocal laser microscopy (Molecular Dynamics,
Sunnyvale, CA). After 2 days of culture on coverslips, the cells were washed with PBS, fixed in 4% paraformaldehyde for 15 minutes, blocked with 5% normal goat serum for 1 hour, and then incubated at 4°C for 1 hour with CUB7401 mAb (0.1 µg/mL). The coverslips were washed three times with PBS (pH 7.4) containing 0.1% Tween 20 and incubated with antimouse IgG Alexa 488 (Molecular Probes, Eugene, OR) for 45 minutes. The stained coverslips were mounted on glass microscope slides in 80% glycerol. The cells were then visualized either under the Nikon Optiphot microscope or with a Zeiss Laser Scanning Microscope 510.

RESULTS

The starting point of this study was a recent report by Aoudjit and Vuori (13) in which these authors reported that culture of metastatic breast cancer cells (MDA-MB-231) on fibronectin-coated surfaces inhibited paclitaxel- and vincristine-induced apoptosis. On the basis of our earlier findings that drug-resistant breast cancer cells express high levels of TG2 (7–9, 14) and the observation by others that TG2 can act as a coreceptor for integrin-mediated binding of cells to fibronectin (15–17), we reasoned that MDA-MB-231 cells may have high levels of TG2 and participate in conferring resistance to chemotherapeutic drugs. To test this possibility, we first assayed cell lysates from several ER$^+$ and ER$^-$ cell lines, including MDA-MB-231, for TG2 expression. MDA-MB-231 cells, similar to the drug-resistant MCF-7 (MCF-7/DOX) cells, showed high levels of TG2 activity. The enzymatic activity was Ca$^{2+}$ dependent and could be completely inhibited by replacing Ca$^{2+}$ with EGTA in the reaction mixture (Fig. 1A). Western blot analysis of cell lysates also showed high levels of TG2 protein in the MDA-MB-231 cells (Fig. 1B). Another breast cancer cell line, SKBR-3, also showed a slight increase in TG2. Immunofluorescence staining of MDA-MB-231 cells with anti-TG2 mAb confirmed increased expression of TG2 in these cells (Fig. 1C).

To determine the implications of increased TG2 expression in MDA-MB-231 cells, we established 24 sublines from the parental cell line by the limiting dilution technique, as described in Materials and Methods. Characterization of these 24 sublines revealed substantial differences in TG2 activity and level compared with those of the parental cells. Three of the 24 sublines (MDA231/cl.2, MDA231/cl.9, and MDA231/cl.18) contained very low to undetectable levels of TG2. The remaining 21 sublines exhibited various levels of TG2. Fig. 2A compares TG2 activity in six selected MDA-MB-231 sublines. The enzyme activity in each subline correlated well with the level of the enzyme protein, as indicated by immunoblotting (Fig. 2B). Two sublines, MDA231/cl.9 and MDA231/cl.16, representing TG2-deficient and TG2-enriched cells, respectively, were additionally used to determine the importance of TG2 expression in drug-resistant and metastatic phenotypes. The extent of TG2 protein expression in these two sublines is shown in Fig. 2C.

Because MDA-MB-231 cells exhibit resistance to chemotherapeutic drugs when these cells are cultured on fibronectin-coated surfaces (13), we first sought to determine whether the MDA231/cl.9 and MDA231/cl.16 sublines would show similar behavior against cytotoxicity induced by doxorubicin, a chemo-
therapeutic drug commonly used to treat patients with breast cancer. MDA-MB-231 cells treated with 0.1 μg/mL doxorubicin and incubated on fibronectin-coated surfaces endured the doxorubicin-induced cytotoxic effects better than did cells incubated under similar conditions on BSA-coated surfaces (Fig. 3A). At a higher dose of doxorubicin (0.3 μg/mL), however, the parental cells were equally sensitive to doxorubicin whether cultured on BSA- or fibronectin-coated surfaces (Fig. 3A). MDA231/cl.9 cells failed to show any differential sensitivity to doxorubicin at either dose whether grown on fibronectin or BSA. MDA231/cl.16 cells, on the other hand, showed considerable resistance to doxorubicin-induced cytotoxicity when cultured on fibronectin-coated surfaces (Fig. 3A; P < 0.001). On BSA-coated surfaces, their sensitivity to doxorubicin was comparable with that of the MDA231/cl.9 cells. These results suggest that TG2 might protect the cells from doxorubicin-induced toxicity, especially when the cells are grown on fibronectin-coated surfaces.

Because MDA-MB-231 cells are highly metastatic, we next sought to determine whether high levels of TG2 in MDA231/cl.16 cells would in some way affect their metastatic properties. To test this, we compared the invasive functions of MDA231/cl.9 and MDA231/cl.16 cells in an in vitro assay by measuring under identical conditions the number of cells that invaded through Matrigel Transwell filters. We found a substantial difference in the invasion properties of the two clones (Fig. 3, B and C). The TG2-rich MDA231/cl.16 cells were 5- to 6-fold more invasive than the TG2-deficient MDA231/cl.9 cells. Similarly, the drug-resistant MCF-7/DOX cells, which express elevated levels of TG2 (7 to 9; Fig. 1, A and B) were also more invasive than the parental drug-sensitive TG2-deficient MCF-7 cells. These results suggested that expression of TG2, in addition to conferring the drug-resistant phenotype, might also confer metastatic potential on the breast cancer cells.

To test this possibility additionally, we sought to determine the status of TG2 expression in a series of genetically related human breast epithelial cell lines, representing various stages in the progression process from benign hyperplasia to atypical hyperplasia to carcinoma in situ and highly malignant metastatic cancer (Table 1). These cell lines were derived from the immortal but otherwise normal MCF10A cell line (10, 11). An enzy-

**Fig. 3** Drug-resistant and invasive phenotypes in breast cancer cell lines correlate with high TG2 expression. A. The parental MDA-MB-231 (WT) cell line and two subclones representing TG2-deficient (MDA231/cl.9) and TG2-rich (MDA231/cl.16) sublines were cultured on BSA-coated or fibronectin-coated wells of 96-well plates. After overnight incubation, cells were left either untreated or treated with either 0.1 μg/mL or 0.3 μg/mL doses of doxorubicin. After 48 hours of treatment, viable cells were determined by MTS assay and plotted as a percentage of controls (untreated). Values shown are from quadruplicate samples; bars, ±SD. B. TG2-deficient (MCF-7/WT and MDA231/cl.9) and TG2-rich (MCF-7/DOX and MDA231/cl.16) human breast cancer cell lines were compared for their invasive properties, with Matrigel-Transwell membranes. Representative fields with cells that migrated under the membrane through the Matrigel were photographed. C. Ten fields were counted randomly under the microscope for the number of cells that had migrated through the Matrigel and were plotted as an average number of cells per field.
matic assay of cell extracts from the parental MCF10A (M-1) and four sublines derived from it (M2 to M5; Table 1) revealed very low levels (2.3 ± 1.4 nmol/hour/mg) of enzymatic activity in the M-1 and M-2 sublines (Fig. 4A). In contrast, the M-3 and M-4 sublines showed considerable enzyme activity (21.8 ± 3.4 nmol/hour/mg). Similarly, the highly aggressive M-5 cells had very high TG2 activity (41.2 ± 4.6 nmol/hour/mg; Fig. 4A). A Western blot analysis of the MCF10A sublines indicated a good correlation between enzymatic activity and TG2 protein levels in all of the MCF10A-derived sublines (Fig. 4B).

To determine in vivo expression of TG2 during various stages of breast cancer progression, all five cell lines (M1 to M5) were xenografted into athymic nude mice. The tumors were resected at the times indicated in Fig. 4C, fixed immediately, and processed for paraffin-embedded tissue sections. Tumor sections (5 μmol/L thick) were then immunostained with an anti-TG2 mAb. The M-1 normal breast epithelial cells (MCF10A) did not form any lesions in the nude mice. The M-2 oncogenic H-RAS–initiated premalignant cells formed no palpable lesions but formed normal-looking bilayered ducts that did not express TG2 (Fig. 4C). In contrast, the aggressive M-3, M-4, and M-5 cells were strongly positive for TG2 expression (Fig. 4C). These results suggested a correlation between TG2 expression and the development of a metastatic phenotype in breast cancer cells.

To additionally validate this possibility, we studied TG2 expression in 30 paired samples of primary and lymph node metastasis from women with breast cancer. The clinicopathologic features of these patients are summarized in Table 2; the data on TG2 expression are shown in Table 3 and Fig. 5. Significantly more lymph node tumors expressed TG2 (75%) than did primary tumors (32%; P < 0.001). Additionally, the extent of TG2 expression was much stronger in the lymph node metastasis than in the primary breast cancer (Fig. 5). However, with regard to the association of TG2 expression in ER/progesterone receptor or Her2-positive or -negative tumors, no evidence of difference in TG2 expression was observed.

In conclusion, we demonstrate that TG2 expression is up-regulated in drug-resistant and metastatic tumors and that increased expression of TG2 could serve as a prognostic marker for tumors with these phenotypes.
DISCUSSION

The development of drug resistance and metastasis pose major clinical problems in treatment of breast cancer. Understanding molecular mechanisms that underlie the development of drug resistance and metastatic phenotypes is imperative for developing effective therapies against breast cancer. The present study provides evidence that TG2 expression is up-regulated in drug-resistant and metastatic breast cancer cells. Thus, increased expression of TG2 may serve as a prognostic marker for drug-resistant and metastatic tumors and play a role in the development of these phenotypes.

The two clones MDA231/cl.9 and MDA231/cl.16, isolated from a metastatic breast cancer cell line MDA-MB-231 on the basis of their differences in the basal expression of TG2, exhibited a substantial difference in their ability to invade through the Matrigel-Transwell membranes (Fig. 3, B and C) and sensitivity to doxorubicin (Fig. 3A). The highly invasive and chemoresistant MDA231/cl.16 cells expressed 12- to 15-fold higher TG2 than the drug-sensitive, noninvasive MDA231/cl.9 cells (Fig. 2). Previously, we reported that irrespective of their source and type, cancer cells selected for resistance against chemotherapeutic drugs exhibit an inordinate increase in TG2 levels (7–9, 14). Although the consensus is that drug-resistance and metastasis represent different phenotypes, there are several reasons to believe that some common pathways may operate during these processes. For example, during advanced stages, cancer cells accumulate many genetic alterations that can render them more resistant to apoptosis (18). This property endows the tumor cells with not only an increased ability to grow and survive in foreign tissue environments (i.e., metastasize) but also an ability to develop a drug-resistant phenotype. Moreover, cancer cell lines selected in vitro for resistance to drugs are more metastatic in vivo (6), whereas cancer cells isolated from metastatic sites, in general, exhibit higher resistance to chemotherapeutic drugs (4, 5). On the basis of previous observations and current results that drug-resistant and metastatic breast cancer cells express high levels of TG2, it is tempting to speculate that TG2 may protect cancer cells from chemotherapy and stress-induced apoptosis. Indeed, a reduction in TG2 levels by stable transfection with TG2-specific antisense or ribozyme has been shown to reverse the resistance of PC-14/ADR lung cancer cells against doxorubicin and other chemotherapeutic drugs, suggesting that TG2 plays a role in the acquisition of drug resistance (19).

TG2 is an important member of the transglutaminase family of enzymes that catalyze the Ca\(^{2+}\)-dependent post-transla-

### Table 3 TG2 expression in paired samples of primary and lymph node metastatic breast tumors

<table>
<thead>
<tr>
<th>TG2 expression</th>
<th>Primary tumor (n)</th>
<th>LN metastasis (n)</th>
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<tbody>
<tr>
<td></td>
<td>19</td>
<td>7</td>
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<tr>
<td>+</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>++</td>
<td>4</td>
<td>16</td>
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NOTE. Total 30 patients, 28 were evaluable.
Abbreviation: LN, lymph node.
tional modification of proteins by inserting covalent isopeptide bonds (20). Many reports have supported the involvement of TG2 in apoptosis. Its overexpression primes cells for suicide, and inhibition of its expression with an antisense strategy results in decreased cell death (21–24). Nevertheless, expression of TG2 and apoptosis do not completely overlap. Direct evidence of this comes from TG2 (−/−) knockout mice, which show no phenotype suggestive of any perturbed apoptosis (25, 26). More recent studies have provided direct evidence and suggested that increased expression of TG2 prolongs cell survival by preventing apoptosis (27–29). It has been proposed that proapoptotic and antiapoptotic effects of TG2 strongly depend on its location within the cell (30). We recently showed that drug-resistant MCF-7 (MCF-7/DOX) cells sustain the expression of TG2 because of their defective or deficient intracellular calcium pools (7). Activation of endogenous TG2 by elevating cytosolic calcium resulted in a massive and spontaneous apoptosis of MCF-7/DOX cells (31). It is conceivable that under extreme conditions the massive levels of Ca^{2+} that are released from intracellular stores or recruited from outside of the cell, activate TG2, resulting in post-translation modifications of some key proteins that, in turn, may facilitate the onset of programmed cell death (32, 33).

Although predominantly a cytosolic protein, TG2 can also localize in the plasma and nuclear membranes. In the nucleus TG2 can interact with retinoblastoma protein and protect cells from apoptosis (29, 30). Similarly, TG2 on the cell surface can serve as a coreceptor for fibronectin (15, 17). TG2 exerts this function by associating with β1 and β3 integrins while simultaneously binding to fibronectin through the gelatin-binding domain (M, 42,000 fragment, modules I_{1,16},I_{1,7,9}). The integrin-mediated interaction of TG2 is able to promote adhesion, migration, and spreading of cells on fibronectin-coated surfaces and is independent of the enzymatic activity (15, 17). It is likely that TG2 in drug-resistant and metastatic tumor cells plays a similar role and protect cells from apoptosis by interacting with retinoblastoma protein and promoting interaction between cell-surface integrins and fibronectin. Indeed, factors that promote interaction between cell-surface integrins and their extracellular matrix ligands, including fibronectin, have been shown to affect signaling pathways that can influence not only the cell adhesive, migratory, and invasive functions but also the survival and proliferation in tumor cells (34–37). Our preliminary results suggest that TG2 closely associates with β1 and β2 integrins on the surface of drug-resistant and MDA231/cl.16 cells and that culture of TG2-positive breast cancer cells on fibronectin-coated surfaces induces strong tyrosine phosphorylation of focal adhesion kinase,5 an upstream event that leads to the activation of downstream antiapoptotic and cell survival signaling pathways (38, 39). A similar activation in focal adhesion kinase was observed by Akimov et al. (17) in TG2-transfected fibroblasts, after their culture on fibronectin or its gelatin-binding domain.

Two recent reports have documented a direct involvement of TG2 in normal cells. The first study (40) suggested that inhibition of TG2 could effectively block transmigration of T lymphocytes across intercellular and tumor necrosis factor α-treated endothelial cells. The second study (41) observed that the transforming growth factor β-induced increase in cell-surface expression of TG2 was responsible for augmenting the attachment and migration of retinal pigment epithelial cells on fibronectin-containing matrices. Previous studies from our own laboratory suggest that, once activated, macrophages can accumulate large amounts of TG2 (42). It is likely that increased level of TG2 by activated macrophages is involved in their migration to inflammatory sites and in protecting these cells from toxic effects of soluble mediators that they produce to kill the neoplastic and infectious agents. Interestingly, Jiang et al. (43), in an attempt to identify metastasis-associated proteins by proteomic analysis, identified TG2 as one of the 11 proteins that were selectively amplified in metastatic human lung carcinoma. In conclusion, our data demonstrate that TG2 expression is up-regulated in drug-resistant and metastatic breast cancer cells, and it could serve as a prognostic marker for the development of these phenotypes.

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6. Bae SN, Arand G, Azzam H, et al. Molecular and cellular analysis of drug-resistant and metastatic phenotype suggestive of any perturbed apoptosis (25, 26). More recent studies have provided direct evidence and suggested that increased expression of TG2 prolongs cell survival by prevent-
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