Correlation of tissue transglutaminase expression on breast cancer tissue with time to relapse, overall survival, and clinical and molecular prognostic factors: a preliminary report

E. Kyparidou\textsuperscript{1}, A. Athanasiadis\textsuperscript{2}, E. Xydakis\textsuperscript{3}, M. Papadakou\textsuperscript{3}, G. Panagos\textsuperscript{3}

\textsuperscript{1}Department of Pathology, "Agii Anargiri" Cancer Hospital of Kifisia, Athens, Greece; \textsuperscript{2}Department of Medical Oncology, Prefectorial General Hospital of Larisa, Larisa, Greece; \textsuperscript{3}Department of Medical Oncology, "Agii Anargiri" Cancer Hospital of Kifisia, Athens, Greece

Summary

\textbf{Purpose}: To correlate tissue transglutaminase (TTG) expression with the expression of molecules with prognostic significance in breast cancer patients and with classical clinical parameters (disease stage, histological grade, overall survival (OS), relapse rate, disease progression and time to treatment failure-TTF).

\textbf{Patients and methods}: Paraffin-embedded tissue specimens from 68 breast cancer patients were studied retrospectively for TTG expression, estrogen (ER) and progesterone (PG) receptors, c-erbB-2, p53, Bcl-2, and Ki-67. Sixty-seven patients were females (mean age 60.5 years). Histology was ductal carcinoma in 53 (inflammatory in 2 and mucinous in 1 of them), lobular in 13 and tubular in 2 cases. Grade was 1 and 2 in 45 cases and 3 in 23. Forty-six patients had early-stage disease (I - II B) and 22 advanced (III A - IV).

\textbf{Results}: Fifty patients had at least 1 favorable molecular prognostic factor while all but 3 had at least 1 unfavorable prognostic factor. Twenty-nine (42.6\%) patients have relapsed so far (mean TTF 31.4 months). Fifty-two (76.5\%) patients are still alive (mean OS 38.5 months). Of the 59 patients with nodal and/or metastatic disease 54 were expressing TTG and 32 Bcl-2. Five were not expressing either one while 22 were expressing both. Of the 9 patients without nodal and/or metastatic disease all but one were expressing both TTG and Bcl-2. Analyzing these subgroups of patients there was sufficient evidence that TTG expression was correlated with a trend for prolonged survival both in patients with localized and extensive disease, while the coexpression with Bcl-2 was correlated with a trend for prolongation of TTF and OS, both in relapsing and nonrelapsing patients. However, these differences did not reach statistical significance. Similar comparisons of TTG expression with the presence of adverse prognostic factors verified a beneficial effect of TTG expression on OS in all subgroups.

\textbf{Conclusion}: Our data suggest that TTG is an independent favorable prognostic factor for survival, possibly enhancing the apoptotic effect of chemotherapy.

\textbf{Key words}: Bcl-2, breast cancer, c-erbB-2, prognostic factors, tissue transglutaminase

Introduction

TTG is an enzyme that is expressed normally on endothelial cells, macrophages and skeletal mus-

cle cells. It is a member of the transglutaminases (TGs) family and its gene in humans is located on chromosome 20q12 [1].

The members of the TGs family differ biochemically as well as immunologically and are involved in different biological functions. Thus, plasma TG constitutes coagulation factor XIII, keratinocyte TG forms keratinocyte's differentiation factor and TTG seems to be involved in tissue reconstructive process (healing) via formation of stable extracellular matrix as well as in programmed cell death by forming insoluble protein cell coat [2,3]. TTG also regulates cell-to-cell and cell-to-matrix interactions by acting on cytokines and proteases of cell membrane and extra-
cellular matrix (e.g. activates inactive TGF-β, binds elafin and transforms plasminogen to functioning plasmin) [4-6].

TTG relation with functions such as apoptosis, extracellular matrix strengthening and cell-to-cell or cell-to-matrix connections led to the study of its role in carcinogenesis, stromal infiltration by neoplastic cells, drug resistance and metastatic capability of neoplasms. Many experimental studies on neoplastic cell cultures supported the initial hypothesis that TTG is involved in such mechanisms [7-11].

TTG expression in breast cancer is markedly increased in comparison with normal breast tissue, a fact suggesting an important role for TTG in regulating evolution and metastasis of breast cancer [12,13].

In our study we correlated TTG expression with the expression of other molecules with prognostic significance in breast cancer patients including both ER and PR, the antiapoptotic molecule Bcl-2, c-erbB-2, the oncosuppressive molecule p53 and the prolifera
tion factor Ki-67. We also searched for correlation between TTG expression and classical clinical parameters such as disease stage, histological grade, TTF (relapse and/or disease progression), and OS.

Materials and methods

The study population consisted of 67 women and 1 man diagnosed with breast cancer. Their mean age at diagnosis was 60.5 years (median 64, range 28-81, Table 1). Pathology material from the initial operation was available for review and further studies in all patients. In all cases tumor histology and histological grading were reconsidered as well as the co-existence of invasive intraductal (in situ) element. Clinical data including TTF and OS were collected in all cases.

All patients had invasive carcinoma and histology was ductal in 50 cases and lobular in 13, while of the remaining 5 cases 2 had inflammatory ductal carcinoma, 2 tubular carcinoma and 1 ductal mucinous carcinoma. Histological grading was low in 5 cases, intermediate in 40 and high in 23 cases (Table 1).

Disease stage was early in the majority of cases (9 with stage I and 37 with stage II B), while 11 cases had distant metastasis at diagnosis.

TTG expression was assessed in paraffin slices with immunohistochemistry (enhanced polymer – DAKO EnVision™ System) with specific monoclonal antibody (clone CUB7402) of DAKO as follows: paraffin-embedded tissue sections 3-4 microns thick were deparaffinized, rehydrated and incubated in hydrogen peroxide for 10-15 min. After washing in buffer, sections were pretreated in a microwave oven for 5 min x 2 and then incubated for 20 min with primary antibody. After washing in buffer, sections were incubated for 10 min with streptavidin peroxidase and then rinsed with buffer. Incubation for 5 min with DAB followed. Then, sections were counterstained with haematoxylin-eosin for 2 min and coversliped with permanent mounting media.

Cases where TTG was expressed only on endothelial cells were considered as negative. Cases where TTG was expressed either on stromal cells or on neoplastic cells were considered as positive.

The expression of molecules with prognostic significance was assessed with the same immunohistochemical technique. Monoclonal and polyclonal antibodies by DAKO were used. Assessments of the expression of all molecules were made by using intensity and extend of staining according to standard procedures.
Statistical considerations

Clinical data collected were tabulated according to TTG expression as well as to all molecules assessed. TTF and OS were analyzed using two-sided linear regression method and p-values were calculated in each pair of comparison.

Results

TTG positive expression was observed in all cases on small and large blood vessels. In 15 cases positive staining was found only on stromal cells, and in 11 cases positive staining was found only on neoplastic cells (Figure 1). In 27 cases positive staining was found both in stromal and neoplastic cells (Figure 2) while in 15 cases both stromal and neoplastic cells did not express TTG (Table 2).

Table 2. TTG expression

<table>
<thead>
<tr>
<th>TTG expression</th>
<th>Stromal cells</th>
<th>Neoplastic cells</th>
<th>Stromal and/or neoplastic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative staining</td>
<td>27</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>positive staining</td>
<td>41</td>
<td>39</td>
<td>53</td>
</tr>
</tbody>
</table>

In all cases where nests of in situ carcinoma coexisted, neoplastic cells of in situ element were negative for TTG expression even when infiltrating elements or the surrounding stroma were positive (Figure 3).

Twenty-two of the 53 TTG-positive patients (41.5%) relapsed, with a mean TTF of 27.3 months (range 4-125, median 14) and a mean disease-free survival (DFS) of 32.8 months from diagnosis (range 2-125+, median 21). Twelve of them (22.6%) died 9-54 months from diagnosis (mean 30.5, median 32). Mean OS was 40.6 months (range 2-140+, median 33) (Table 3). Relapsing patients had a mean OS of 46.1 months (range 9-140+, median 33), while patients still being in remission had a mean OS of 36.7 months (range 2-94+, median 29).

Seven of the 15 (46.7%) patients not expressing TTG relapsed or progressed within a mean TTF of 35.8 months (range 0-160, median 14) and a mean DFS or progression-free survival of 26.3 months (range 0-160, median 12). Four (26.7%) of them died 3-38 months from diagnosis (mean 25.6, median 31). Mean OS was 31.0 months (range 3-174+, median 22) (Table 3). Relapsing patients had a mean OS of 45.8 months (range 3-174+, median 33), while patients still being in remission had a mean OS of 18.0

Figure 1. TTG expression on neoplastic cells (arrows) (x250).

Figure 2. TTG expression on neoplastic cells (horizontal arrow) and stromal cells (vertical arrow) (x250).

Figure 3. TTG expression in invasive elements (multiple arrows pointing down and left) while intraductal elements are negative (single arrow pointing down) (x250).
Table 3. Time to treatment failure (TTF) and overall survival (OS) depending on TTG expression

| TTG+ | 41.5 | 32.8 (2-125) | 22.6 | 40.6 (2-140) | 36.7 (2-94) |
| TTG- | 46.7 | 26.3 (0-160) | 26.7 | 31.0 (3-174) | 18.0 (3-68) |

All p-values are nonsignificant

months (range 3-68+, median 9). It is worth noticing that 2 patients surviving for 69 and 174 months in this subgroup of patients had initially low disease stage (II) as well as low histological grading.

Relapse or progression-free survival according to TTG expression is shown on Figure 4. The extend of TTG expression on stromal or neoplastic cells did not affect DFS and OS (Table 3).

Thirty-seven of the 53 (69.8%) patients expressing TTG were also expressing ER and/or PR on their neoplastic cells, while the remaining 16 were receptors-negative. Of the 15 patients not expressing TTG 8 (53.3%) were expressing ER and/or PR (Table 4). Analysis of relapse and survival data according to TTG and hormone receptors expression (Table 5) showed that coexpression of them was correlated with lowest relapse and death rates as well as longer progression-free and OS (Figure 5).

Table 4. Expression of ER, PR, c-erbB-2, p53, Bcl-2 and Ki-67

<table>
<thead>
<tr>
<th>Expression</th>
<th>ER</th>
<th>PR</th>
<th>c-erbB-2</th>
<th>p53</th>
<th>Bcl-2</th>
<th>Ki-67</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive staining</td>
<td>41</td>
<td>39</td>
<td>26</td>
<td>40</td>
<td>40</td>
<td>63</td>
</tr>
<tr>
<td>negative staining</td>
<td>27</td>
<td>29</td>
<td>42</td>
<td>28</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>ER+ or PR+</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER- and PR-</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Twenty-three of the 53 TTG-positive patients expressed c-erbB-2, while of the 15 TTG-negative patients only 3 were expressing c-erbB-2 (Table 4). Comparison of relapse and survival data according to TTG and c-erbB-2 expression revealed that TTG expression correlated with improved relapse and death rates in both c-erbB-2-positive and negative subgroups (Table 6). DFS and OS were better in the presence of TTG in the c-erbB-2-negative group.

Thirty-one of the 53 patients expressing TTG were also expressing Bcl-2, while of the 15 TTG-negative patients 9 were expressing Bcl-2 (Table 4). Comparison of relapse and survival data according to TTG and Bcl-2 expression revealed that TTG expression correlated with improved relapse and death rates in both Bcl-2-positive and negative subgroups (Table 7), as well as DFS and OS in the Bcl-2-positive subgroup. In the Bcl-2-negative group, TTF were better in the presence of TTG expression (Figure 6).

Comparison of clinical data according to disease stage at diagnosis and TTG expression failed to produce a consistent trend. OS in TTG-positive patients

Table 5. Time to treatment failure (TTF) and overall survival (OS) depending on hormone receptors and TTG expression

| TTG+ ER+/PR+ | 37.8 | 38.6 (2-125) | 16.2 | 45.2 (2-140) | 39.4 (2-94) |
| TTG- ER+/PR+ | 50.0 | 31.4 (4-160) | 25.0 | 34.8 (4-174) | 6.4 (4-10) |
| TTG+ ER-/PR- | 50.0 | 19.5 (4-56) | 37.5 | 30.1 (6-56) | 29.0 (6-56) |
| TTG- ER-/PR- | 42.9 | 20.4 (0-68) | 28.6 | 26.6 (3-68) | 29.3 (3-68) |

All p-values are nonsignificant
Table 6. Time to treatment failure (TTF) and overall survival (OS) depending on c-erbB-2 and TTG expression

<table>
<thead>
<tr>
<th>Relapse</th>
<th>TTF (months (range))</th>
<th>Death</th>
<th>OS, months (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>All patients</td>
<td>Non-relapsing</td>
</tr>
<tr>
<td>TTG+ c-erbB-2+</td>
<td>47.8</td>
<td>23.7 (2.94)</td>
<td>34.8</td>
</tr>
<tr>
<td>TTG- c-erbB-2+</td>
<td>100.0</td>
<td>61.2 (0, 23, 160)*</td>
<td>66.7</td>
</tr>
<tr>
<td>TTG+ c-erbB-2-</td>
<td>36.7</td>
<td>39.8 (3-125)</td>
<td>13.3</td>
</tr>
<tr>
<td>TTG- c-erbB-2-</td>
<td>33.3</td>
<td>17.6 (3-68)</td>
<td>16.7</td>
</tr>
</tbody>
</table>

All p-values are nonsignificant
*Figures in parenthesis represent individual TTF and OS values for the 3 cases forming the TTG+/c-erbB-2+ group

Figure 5. Time to treatment failure according to TTG and ER/PR expression.

was longer in all stage groups, but these differences were not statistically significant. Although relapse rate was lower and DFS longer in the presence of TTG in stages I, II and III, patients in stage IV showed an opposite trend (Table 8). These differences, however, were not statistically significant.

Of the remaining molecular prognostic factors, 40 patients were expressing p53 and all but 5 were expressing Ki-67 (Table 4). TTG was evenly expressed in all these subgroups (33 positive versus 7 negative in the p53-expressing group and 20 positive versus 8 negative in p53-nonexpressing group; 49 positive versus 14 negative in the Ki-67-expressing group and 4 positive versus 1 negative in the Ki-67 nonexpressing group). Clinical data analysis of these subgroups gave results consistent with those of the whole study population, e.g., lower relapse and death rates as well as longer OS and DFS for TTG-expressing patients, but differences were not statistically significant.

Fifty patients had at least one favorable molecular prognostic factor (ER, PR, Bcl-2) and 40 of them were expressing TTG as well. The presence of TTG expression was related with both improved relapse and death rates and DFS and OS. Among the 18 pa-

Figure 6. Time to treatment failure according to TTG and bcl-2 expression.

Table 7. Time to treatment failure (TTF) and overall survival (OS) depending on Bcl-2 and TTG expression

<table>
<thead>
<tr>
<th>Relapse</th>
<th>TTF (months (range))</th>
<th>Death</th>
<th>OS, months (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>All patients</td>
<td>Non-relapsing</td>
</tr>
<tr>
<td>TTG+ Bcl-2+</td>
<td>38.7</td>
<td>40.4 (2-125)</td>
<td>19.4</td>
</tr>
<tr>
<td>TTG- Bcl-2+</td>
<td>55.6</td>
<td>26.9 (0-160)</td>
<td>22.2</td>
</tr>
<tr>
<td>TTG+ Bcl-2-</td>
<td>45.5</td>
<td>22.2 (2-57)</td>
<td>27.3</td>
</tr>
<tr>
<td>TTG- Bcl-2-</td>
<td>33.3</td>
<td>25.4 (3-68)</td>
<td>33.3</td>
</tr>
</tbody>
</table>

All p-values are nonsignificant
Table 8. Time to treatment failure (TTF) and overall survival (OS) depending on disease stage at diagnosis and TTG expression

<table>
<thead>
<tr>
<th></th>
<th>Relapse</th>
<th>TTF months (range)</th>
<th>Death</th>
<th>OS, months (range)</th>
<th>Non-relapsing</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTG+ I - II</td>
<td>32.4</td>
<td>39.9 (3-125)</td>
<td>16.2</td>
<td>44.2 (3-140)</td>
<td>39.2 (3-94)</td>
</tr>
<tr>
<td>TTG- I - II</td>
<td>11.1</td>
<td>33.8 (3-160)</td>
<td>0.0</td>
<td>35.3 (3-174)</td>
<td>18.0 (3-68)</td>
</tr>
<tr>
<td>TTG+ III</td>
<td>55.6</td>
<td>19.3 (2-56)</td>
<td>33.3</td>
<td>36.9 (2-76)</td>
<td>29.7 (2-56)</td>
</tr>
<tr>
<td>TTG- III</td>
<td>100.0</td>
<td>18.7 (14-23)</td>
<td>50.0</td>
<td>35.7 (14-23)</td>
<td>–</td>
</tr>
<tr>
<td>TTG+ IV</td>
<td>71.4</td>
<td>12.7 (6-25)</td>
<td>42.9</td>
<td>26.6 (11-54)</td>
<td>20.0 (15-25)</td>
</tr>
<tr>
<td>TTG- IV</td>
<td>100.0</td>
<td>13.2 (0-35)</td>
<td>75.0</td>
<td>18.8 (3-38)</td>
<td>–</td>
</tr>
</tbody>
</table>

All p-values are nonsignificant.

tients not expressing even one favorable molecular prognostic factor 13 were expressing TTG. In this subgroup the presence of TTG expression was not associated with DFS or OS benefit.

Discussion

Although it is recognized that TTG is very often expressed in breast cancer [12,13], its significance in breast cancer biology has not been elucidated so far. Few published studies investigate TTG expression in human breast cancer and mostly they are usually concerned about the distribution of stain in tissues (neoplastic cells, stromal cells, etc), credibility of immunohistochemical techniques, and the nature of cells able to produce it. Few of the published studies deal with its probable correlation with prognosis or correlate it with other recognized molecular prognostic factors in vivo [13,14] or in vitro [15,16].

On the contrary, there are studies crediting with prognostic significance the increased expression of TTG on glioma cells [17], as marker of effectiveness for hormonal treatment in prostate cancer [18], as probable marker for anticipated risk of developing non small cell lung cancer [19], and as probable marker for invasive evolution of bladder cancer [20]. In any case, TTG significance is emphasized in stromal invasion, metastatic evolution, apoptotic cell death and drug resistance [7].

Some investigators report correlation between TTG and Bcl-2 expression in the form of an inverse relation of the 2 markers [17]. In our study we were unable to confirm that inverse relation.

Our results suggest that both TTG and Bcl-2 are favorable prognostic factors and when they are both expressed there is an additive beneficial effect on relapse rate, time to relapse and death rate, a fact not previously observed (Table 7, Figure 6).

Our results also suggest that TTG expression is related with longer DFS and OS, especially in II and III disease stages. When metastatic disease is present at diagnosis, the TTG prognostic significance is restricted to lower death rate and longer OS.

TTG ability to strengthen extracellular matrix around neoplastic cells by forming stable bonds between stromal proteins explains its favorable prognostic effect in cases with early-stage disease by interfering with tumor development and blood vessels’ infiltration by tumor cells [21]. Also, TTG favorable action on apoptotic process explains the longer survival of patients with advanced disease by facilitating treatment’s apoptotic action [14].

This frequent coexpression of TTG with unfavorable prognostic factors like p53, Ki-67 and c-erbB-2 is explained on the basis of TTG relation with the apoptotic process given that: 1) p53 is well related to apoptosis; 2) Ki-67 marks cell populations that are rapidly proliferating and, because of that, are unstable, have higher rate of apoptosis, and are more sensitive to chemotherapy; 3) c-erbB-2 is classified as an adverse prognostic marker; when it is strongly expressed in patients with advanced disease treatment with the specific monoclonal antibody (herceptin) may be favored when TTG is coexpressed in the same cell population.

With regard to Bcl-2, that is considered as favorable prognostic marker despite its anti-apoptotic action, our results indicate that its presence is correlated with prolongation of time to relapse, especially when TTG is coexpressed in the same cell population. This can be probably attributed to the fact that tumors of high differentiation express Bcl-2 frequently (expressed in 68% of our study population with histo-
logical grade 1 and 2), while tumors of low differentiation express Bcl-2 less often (expressed in 39% of our study population with histological grade 3).

The fact that our study population is rather small to form large homogeneous groups with respect to disease stage at diagnosis, histological grade, treatment schedule etc, does not permit to draw firm statistical conclusions of our results in order to prove our initial working hypothesis and convert the trends observed to statistically significant differences. Nevertheless, the apparent trend for longer OS, progression-free and relapse-free survival, and lower relapse and death rates among patients with TTG expression encourage us to continue our investigation in order to increase our study population and prolong follow up time.

Acknowledgement

The study was supported by a Grant from the Greek Ministry of Health (Grant no. 027/98, ministerial resolution A2a/3608/15-11-99).

References