Heterogeneous Expression of CT10, CT45 and GAGE7 Antigens and their Prognostic Significance in Human Breast Carcinoma

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Objective: The goal of this study was to detect the intertumoral heterogeneity of CT10, CT45 and GAGE7 expression and further to analyze their prognostic value.

Methods: The intertumoral heterogeneity of three cancer/testis antigens was examined by immunohistochemistry using 120 samples from patients with infiltrating ductal breast carcinoma. The expression patterns were classified and correlated with the clinicopathologic variables and outcome of the patients.

Results: CT10 showed punctate, focal and diffuse expression patterns according to the characteristic of its distribution. CT45 showed cytoplasmic, nuclear or combined cytoplasmic and nuclear expression patterns according to its subcellular location. GAGE7 exhibited nuclear, cytoplasmic and nucleolar expression patterns. Three cancer/testis antigens were also observed coordinately expressed in infiltrating ductal breast carcinoma. Patients with tumors with CT10 expression was significantly correlated with nodal metastases \( (P = 0.001) \) and advanced clinical stages \( (P = 0.001) \). Patients with tumors with cytoplasmic GAGE7 and with the expression of two or more cancer/testis antigens were significantly correlated with advanced clinical stages \( (P = 0.001 \text{ and } P = 0.030) \). No significant difference was identified between the different expression patterns of CT45 and clinicopathologic variables. In addition, Kaplan–Meier analysis revealed that diffuse CT10 expression and coexpression of three cancer/testis antigens were related to the poor prognosis of patients with infiltrating ductal breast carcinoma.

Conclusions: Diffuse CT10 expression and the coexpression of three cancer/testis antigens can be used as a biomarker to distinguish patients with a poorer outcome of the breast carcinoma. Our finding may provide useful data for evaluating the prognosis of this disease and improving the effectiveness of therapeutic application based on the three cancer/testis antigens.

Key words: heterogeneity – different expression pattern – CT antigen – prognosis – breast carcinoma

INTRODUCTION

Cancer/testis antigens (CTAs) are protein antigens with the normal expression restricted to adult testicular germ cells, and yet are aberrantly activated and expressed in a proportion of various types of human cancer \( (1) \). The limited expression and their \textit{in vivo} immunogenicity make CTAs to be...
vigorously pursued as targets for therapeutic cancer vaccines (2). In addition, a close association of the protein expression of CTAs with the advanced disease and poor outcome of the malignancy make them also to be hunted as a potential prognostic biomarker of cancer (3,4).

However, immunohistochemical data have shown that the protein expression of CTAs in tumors were often heterogeneous. The heterogeneous expression of CTAs in human cancer can be found between different tumor types, different tumors of the same type (intertumor heterogeneity) and different cells or regions of the same tumor (intratumor heterogeneity) (5). The intertumoral heterogeneity of CTA protein expression is likely to have a dramatic impact on the clinical outcome and on the design of more effective CTA-based immunotherapeutic approaches (6).

CT10, CT45 and GAGE7 are all promising vaccine candidates in immunotherapy approaches to cancer, since spontaneous humoral and cellular responses against CT10 and GAGE7 have been detected in cancer patients (7–12), and anti-CT45 antibodies have also been found in patients with Hodgkin lymphoma recently (13). CT10, CT45 and GAGE7 are also the potential diagnostic markers of the cancer, because their expression have been reported to correlate with advanced clinical stage of lymph node metastasis and the poor survival of the malignancies (14–19). Despite their immunogenic property in cancer patients and the obvious impact on clinical outcome, to the best of our knowledge, not all patients with malignancy can induce spontaneous humoral and/or cellular immune responses against CT10, CT45 and GAGE7 or have poor survival with their protein expression. For example, the expression of CT10 correlated with better disease-free and better overall survival of human urothelial carcinoma (17) and the expression of GAGE7 had no significant correlation with the outcome of esophageal cancer (20). The intertumoral heterogeneity of their protein expression may be responsible for these discrepancies. However, so far no study has systematically analyzed the correlation between the intertumoral heterogeneity of CTA expression and the prognosis of the malignancy.

In the present study, we systematically analyzed the heterogeneous protein expression of CT10, CT45 and GAGE7 in a large series of breast carcinoma samples and further classified the heterogeneity of their protein expression into three different patterns. We also sought to assess for correlations between the different expression patterns of each CTA with the overall survival of patients with breast carcinoma. These information is crucial to increase the effectiveness of prognostic values and therapeutic application of CT10, CT45 and GAGE7.

PATIENTS AND METHODS

PATIENTS

This study was approved by the Ethics Committee of the Fourth Military Medical University. A total of 120 patients with infiltrating ductal breast carcinoma (IDBC) undergoing curative surgical resection for breast cancer at the Xijing Hospital, the First Affiliated Hospital of the Fourth Military Medical University during 2005. Patients who had not received any form of preoperative chemotherapy and/or radiation therapy were included in this study. Tumor samples were collected as paraffin-embedded tissues from the archives of the Department of Pathology of the Xijing Hospital. Informed consent was obtained from all patients. All the patients had the follow-up records for over 5 years (mean, 44.85).

MONOCLONAL ANTIBODIES

All monoclonal antibodies LX-CT10.9, LX-GAGE7.4 and LX-CT45.8 specific to CT10, GAGE7 and CT45 were generated in our laboratory using BALB/c mice immunized with purified CT10, CT45 and GAGE7 recombinant proteins which were provided by the Ludwig Institute for Cancer Research, New York, USA. All monoclonal antibodies were identified to be satisfactory in testicular tissues (21,22) by immunohistochemistry. The selected hybridoma clones were cultured in serum-free medium and the hybridoma cultured supernatants with secreted monoclonal antibodies were collected. The optimal dilutions of the three hybridoma supernatants for immunohistochemistry staining were 1:10 (LX-CT10.9), 1:1000 (LX-CT45.3) and 1:10 (LX-GAGE7.4), respectively.

IMMUNOHISTOCHEMISTRY

Formalin-fixed paraffin-embedded tissues were cut into 5-μm sections and the sections were deparaffinized in xylene and ethanol and rehydrated in a series of graded alcohols and were treated with 3% H2O2 to block the endogenous peroxidase activity and subjected to antigen retrieval with autoclaving for 2 min in 10 mM citrate buffer, pH 6.0. The sections were incubated with the primary antibody for 1 h at room temperature. The immunostaining was carried out using DAKO Envision + horseradish peroxidase mouse detection system (DakoCytomation, Carpinteria, CA) and DAB as the chromogen. The slides were counterstained with hematoxylin and evaluated.

Normal adult testis with intact spermatogenesis served as positive controls and negative controls were prepared by omission of the primary antibody.

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS version 16.0 software. Associations between the different expression patterns of CT10, CT45 or GAGE7 and the coexpression of CTAs with clinicopathologic variables of the breast carcinoma was assessed using Pearson’s χ2 test and Fisher’s exact test. The correlation of different expression pattern with survival was evaluated using the Kaplan–Meier method, and
the differences between the groups were compared using the log-rank test. Results were considered significant if \( P < 0.05 \).

**RESULTS**

**DIFFERENT EXPRESSION PATTERN OF CT10, CT45 AND GAGE7 IN BREAST CARCINOMA**

Of the 120 samples subjected to immunohistochemical analysis, CT45 was the most frequently expressed CTA, being present in 59/120 (49.2%) cases, followed by CT10 (40/120; 33.3%) and GAGE7 (37/120; 30.8%). Three CTAs showed heterogeneous expression in the tissues of IDBC. CT10 showed a confined nuclear staining and the distribution of the positive cells ranged from very focal to diffuse. According to the characteristic of its distribution, we classified the expression patterns of CT10 into punctate (the proportion of CT10-positive staining \(<5\%\) and scattered distribution), focal (the proportion of CT10-positive staining were \(5\%–25\%\) and aggregate distribution) and diffuse (the proportion of CT10-positive staining \(>25\%\) and distributed in all tumoral tissues) expression. There are 12 cases with punctate CT10 staining, 18 cases with focal CT10 staining and 10 cases with diffuse CT10 staining (Fig. 1B–D). CT45 exhibited either a cytoplasmic, nuclear or combined cytoplasmic and nuclear expression patterns. CT45 cytoplasmic or both cytoplasmic and nuclear-positive cells occupied \(~75–100\%\) of tumor cells but nuclear-positive cells occupied only \(~30–50\%\) of tumor cells. There are 34 cases with CT45 cytoplasmic staining, 8 cases with nuclear CT45 staining and 17 cases with CT45 both nuclear and cytoplasmic staining (Fig. 1E–H). GAGE7-positive cells occupied \(~5–20\%\) of the tumor cells. Similar to CT45, the expression patterns of GAGE7 were classified into 13 nuclear staining, 19 weak cytoplasmic staining and 5 nucleolar staining (Fig. 1I–K). In addition, we also observed a coordinated expression pattern of the three CTAs. Thirty-four cases showed immunoreactivity with 1 of the 3 CTAs, 35 samples immunostaining with 2 of the 3 CTAs and 10 tumors were positive for all 3 CTAs.

**THE FREQUENCIES OF EACH CTA’S EXPRESSION PATTERNS WERE DIFFERENT IN DIFFERENT CLINICAL STAGES OF BREAST CANCER**

In 79 cases presenting early stage (stage I + II), the frequency of CT10 expression was 25.3% \((n = 20)\) among which punctate, focal and diffuse staining occupied 10.1% \((n = 8)\), 13.9% \((n = 11)\) and 1.3% \((n = 1)\) respectively, whereas in 41 cases with advanced stage III + VI, the

![Figure 1](https://example.com/figure1.png)

**Figure 1** Different immunohistochemical staining of CT10, CT45 and GAGE7 in breast cancer tissues. (A) CT10 expressed in normal testis as a positive control. (B–D) Tumor cells showed punctate, focal and diffuse expression of CT10. (E) CT45 expressed in normal testis as a positive control. (F–H) Tumor cells showed nuclear, cytoplasmic, and both cytoplasmic and nuclear staining of CT45. (I) GAGE7 expressed in normal testis as a positive control. (J–L) Tumor cells showed nuclear, cytoplasmic and nucleolar staining of GAGE7.
overall frequency increased to 48.8% (n = 20) with punctate, focal and diffuse staining increased to 9.8% (n = 4), 17% (n = 7) and 22% (n = 9), respectively (Fig. 2A). The frequency of CT45-positive staining was 45.6% (n = 36) in Stage I + II, among which cytoplasmic staining was 24.1% (n = 19), nuclear staining was 10.1% (n = 8) and both cytoplasmic and nuclear staining was 11.4% (n = 9). The overall frequency of CT45 increased to 56.1% (n = 23) in Stage III + VI, with cytoplasmic staining increased to 36.6% (n = 15). The frequency of both cytoplasmic and nuclear-positive staining was increased to 19.5% (n = 8), while no nuclear staining was found in Stage III + VI (Fig. 2B). In all early stage (Stage I + II) samples, the overall frequency of GAGE7 expression was 24% (n = 19) among which the frequency of cytoplasmic-positive staining was 6.3% (n = 5), nuclear staining was 12.7% (n = 10) and nucleolar-positive staining was 5% (n = 4). In the advanced stage (Stage III + VI), the overall frequency of GAGE7 was 43.9% (n = 18), cytoplasmic positive increased to 34.1% (n = 14), while the frequency of nuclear staining and nucleolar staining decreased to 7.3% (n = 3) and 2.4% (n = 1). The positive samples with at least one of the three CTA occupied 26.6% (n = 21) in Stage I + II and 31.7% (n = 13) in Stage III + VI (Fig. 2C). The number of CTA coexpression increased with the development of the tumor. The frequency of the positive samples with coexpression of two and three CTAs increased from 25.3% (n = 20) and 6.3% (n = 5) in Stage I + II to 36.6% (n = 15) and 12.2% (n = 5) in Stage III + VI indicating that the more CTA coexpression, the more serious the progression of breast carcinoma (Fig. 2D).

Relationship between different expression patterns of three CTAs and clinicopathological data

Table 1 summarises the associations between different expression patterns of three CTAs and clinicopathological variables. Significant association was observed between the different expression patterns of CT10 with lymph node stage ($P < 0.001$) and clinical stage ($P = 0.001$). Patients with tumors with focal or diffuse CT10 expression were more frequently with nodal metastases ($P < 0.001$) than those with punctate or no expression, and at patients with tumors with diffuse CT10 expression were more advanced clinical stages ($P = 0.001$) than those with focal/punctate/no CT10 expression. In addition, tumors with cytoplasmic GAGE7 expression or with expression of two or more CTAs were significantly correlated with advanced clinical stages ($P = 0.001$ and $P = 0.030$, respectively) Different expression

Figure 2  The frequency of different expression patterns of CT10, CT45 and GAGE7 changed with the clinical stages of the breast carcinoma. (A) The percentage of different expression patterns of CT10 in different clinical stages. (B) The percentage of different expression patterns of CT45 in different clinical stages. (C) The percentage of different expression patterns of GAGE7 in different clinical stages. (D) The percentage of different coexpression patterns of the three CTAs in different clinical stages (chi-square tests, negative vs. positive, *$P < 0.05$, **$P < 0.05$).
of CT45 showed no significant association with any clinicopathological variables.

**Prognostic Significance of Different Expression Pattern of CT10, CT45 and GAGE7**

Kaplan–Meier analysis was done to analyze the prognostic value (Fig. 3). To analyze a more homogeneous group of patients, the relationship between different expression patterns of three CTAs and clinical outcome was analyzed within 42 patients with advanced clinical stage. The results showed that patients with diffuse CT10 expression had a significantly shorter survival time compared with those with negative CT10 expression \((P < 0.001)\). Patients with three CTAs coordinate expression had a significantly shorter survival time compared with those with CTA-negative expression \((P = 0.007)\). The overall survival of patients with different expression patterns of CT45 and GAGE7 were also compared, but no prognostic value was found.

**DISCUSSION**

Previous studies have analyzed the expression of CT10, CT45 and GAGE7 in malignancies by immunohistochemistry. However, all of these studies have focused their attention...
on the positive staining but ignored the heterogeneity of their expression. To avoid this limitation, this study focused on analyzing the correlation of the intertumoral heterogeneity of CT10, CT45 and GAGE7 expression with the prognosis of breast carcinoma.

The expression patterns of CT10 were classified into punctate, focal and diffuse expression in accordance with the previous study (15). Epigenetic regulation is a key mechanism in the transcriptional regulation of CTA genes (23), which refer to the covalent modifications of DNA and core histones that are heritable and affect genome function without altering the DNA nucleotide sequence (24). The epigenetic changes can be transmitted and maintained through cell divisions. The punctate expression of CT10 might be induced by the epigenetic regulation during the tumorigenesis, and the cancer cell clone with these epigenetic changes acquired a growth advantage within the tumor. Hence, more and more cells were positive for CT10 until diffuse CT10 expression appeared. Previous studies have addressed the nuclear staining of CT45 in breast carcinoma (15); however, they did not describe the cytoplasmic as well as both nuclear and cytoplasmic staining except that Chen found the diffused cytoplasmic distribution of CT45 in mitotic lung cancer cell (15,21). CT45 is a protein of 189 amino acids with a theoretical molecular mass of 21 159 Da containing two nuclear localization sites, possible N-glycosylation sites and several possible phosphorylation sites (19). These characteristics suggest that CT45 could potentially be a transcriptional factor which may translocate between the nucleus and cytoplasm. Then it was not surprising that the protein expression of CT45 was found in the cytoplasm and in both the cytoplasm and nucleus of cells. The overall frequency of CT45 expression in our study was over 45% which was much higher than the previous. This discrepancy might be due to the different tissue samples used in our study since CT45 were more frequently expressed in the estrogen receptor-negative patients (15). And the monoclonal antibody against CT45 used in our study was not the same clone as in previous studies which might recognize different epitopes of the CT45 proteins (21). The novel finding in present study was the nucleolar location of GAGE7. GAGE7 can interact with nucleophosmin (NMP)/B23, a kind of multifunctional nuclear protein which plays important roles in resistance to apoptosis induced by IFN-γ (25). Our finding may support this view from the morphological aspects. The coordinate expression of CT10, CT45 and GAGE7 in patients with

Figure 3. Kaplan–Meier survival curves stratified according to the different expression patterns of CT10, CT45, GAGE7 and coexpression of three CTAs (log-rank test, negative vs. positive).

(15,21).
breast carcinomas may suggest a common molecular mechanism of those genes activation due to global DNA hypomethylation during the tumorigenesis (23).

Previous studies have found that CT10 were preferentially expressed in advanced prostate carcinoma (26) and classical Hodgkin’s lymphoma (27). Compatible with previous studies, our results showed that tumors with focal or diffuse CT10 expression had more lymph node metastases, and tumors with diffuse CT10 expression were at more advanced clinical stages. CT10 is a member of the type I MAGE protein which has a conserved domain known as the MAGE homology domain (MHD) (28). The MHD can bind to KAP1, a corepressor of p53, to facilitate KAP1/p53 complex formation and suppress p53-dependent apoptosis (29). The combination of MHD and KAP1 also can promote the expression of the oncogene ID1 (inhibitor of differentiation-1) which can induce cell invasiveness (30). These results suggest that diffuse CT10 expression contributes to tumor development and progression by providing survival advantage and promoting metastasis of tumor cells.

Patients with cytoplasmic GAGE7 expression were at more advanced clinical stages. A previous study has discovered NPM/B23 as a binding partner of GAGE7. GAGE7 can increase the stabilization and abundance of NPM/B23 protein in the cytoplasm. Increased level of NPM/B23 is associated with tumorigenesis and malignant transformation (31). Patients with tumors with two or more CTA expressions were more frequently at advanced clinical stages than those with tumors with no CTA expression. CTAs are normally expressed only in the human germ line but aberrantly activated during tumorigenesis. An aberrant expression of CTAs in cancer reflects the activation of the silenced gametogenic programme in somatic cells, and that this programmatic acquisition is one of the driving forces of tumorigenesis (5). The more coordinate expression of CTAs will endue cells with the stronger driving forces of tumorigenesis.

Furthermore, diffuse expression of CT10 and coexpression of three CTAs were significantly associated with a poorer outcome of the patients which were in accordance with the previous finding that during tumor progress, the level and number of CTAs genes expression are likely to increase (2).

In conclusion, this study analyzed the heterogeneous expression of CT10, CT45 and GAGE7 in breast carcinoma and revealed the correlation between the different expression patterns of the three CTAs with the clinicopathologic variables and overall survival of the patients. Our results may provide useful data for improving the effectiveness of diagnosis and immunotherapy of breast carcinoma based on the three CTAs.

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**Conflict of interest statement**

None declared.

**References**


