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ARTICLE

# Expression of Cancer-Testis Antigen CT7 (MAGE-C1) in Breast Cancer: An Immunohistochemical Study with Emphasis on Prognostic Utility

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**High expression of the cancer-testis antigen CT7, also referred to as MAGE-C1, has been recently described in a variety of malignant tumors, including breast carcinoma. To our knowledge, no data concerning the prognostic utility of CT7 expression in breast cancer are available. In this retrospective study, we evaluated the relationship between CT7 immunoreactivity and clinicopathological parameters as well as relapse-free survival (RFS) and metastasis-free survival (MFS) of 124 women with invasive breast cancer. A positive CT7 status, defined as immunoreactivity in more than 50% of tumor cells, was found in 18% of cases and correlated significantly with high tumor grade (p=0.004), but with no other clinicopathological parameter. In a univariate analysis, CT7 status showed an association with RFS by trend (p=0.107; relative risk [RR]: 1.85) and a significant association with MFS (p=0.043; RR: 2.02). In a multivariate analysis, tumor grade, stage, nodal status, angioinvasion, HER2 expression as well as estrogen and progesterone receptor expression were identified as significant independent prognostic factors of RFS and/or MFS. In this respect, CT7 expression showed a weak, statistically not significant trend towards an independent prognostic relevance concerning prediction of MFS (p=0.147; RR: 1.95). Our data suggest that estimation of CT7 immunoreactivity is of limited prognostic usefulness in breast cancer. It may provide additional information concerning assessment of MFS in selected cases.** (Pathology Oncology Research Vol 13, No 2, 91–96)

*Key words:* breast cancer, cancer-testis antigen, CT7, MAGE-C1, prognosis, immunohistochemistry

## *Introduction*

Cancer testis (CT) antigens are expressed in a variety of malignant tumors, but not in normal adult tissues except for testicular germ cells.[1,2] Since their expression is confined to neoplastic cells, they may represent ideal targets for antigen-based vaccination and antigen-directed immunotherapy against malignant tumors.[3,4] Until now, CT antigen-based immunotherapy has been applied with varying success in a lot of studies which mostly examined malignant melanoma,[5-11] whereas other malignant tumors like gastrointestinal carcinoma[12] or bladder cancer[9] were only exceptionally considered. These studies generally reported clinical responses only in a small minority of patients. Therefore, CT antigen-based immunotherapy still awaits establishment in clinical routine practice. Nevertheless, research about CT antigens is going on, and even other tumors than malignant melanomas are getting more and more into focus.

Expression of CT antigens of the MAGE family has been also reported in human breast carcinomas, although only to a limited degree.[13] Since the number of studies dealing with this issue is very small, only few data are currently available in the literature. Jungbluth and coworkers, who investigated the expression of the CT7 (MAGE-C1) antigen in a variety of human cancers using the highly specific CT7-33 antibody, found expression of the CT7 protein in 38% of breast carcinomas.[14] Unfortunately, the authors did not examine the relationship between CT7 expression and clinicopathological parameters or clinical outcome. Data concerning the

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prognostic value of CT7 in breast cancer do not exist so far. This fact prompted us to examine whether CT7 protein expression is associated with clinicopathological parameters, relapse-free survival (RFS) or metastasis-free survival (MFS) in patients with invasive breast carcinoma.

## *Material and Methods*

### *Patients*

A total of 124 patients (16 premenopausal and 108 postmenopausal) with invasive breast carcinoma were included. Age ranged between 32 and 91 years (median: 62 years). All patients were surgically treated at the municipal hospital of Lüneburg (Lower Saxony, Germany) between 1996 and 1998. Axillary lymph node dissection was done in all but two patients. Following surgery, radiotherapy was administered to 96 patients (77%), adjuvant hormonal therapy to 94 patients (76%), and adjuvant chemotherapy to 46 patients (37%). After completion of primary treatment, the patients were regularly monitored (median follow-up: 65.5 months). The occurrence of tumor relapse or metastatic tumor spread was regarded as positive events for the analyses of relapse-free survival (RFS) and metastasis-free survival (MFS), respectively.

Primary histological examination as well as immunohistochemical analysis of estrogen receptor (ER) and progesterone receptor (PR) status was performed at the Institute of Pathology of the municipal hospital of Lüneburg. Sixteen tumors (13%) were histologically identified as invasive lobular carcinomas, whereas all other tumors (87%) represented invasive ductal carcinomas. Histologic tumor slides were re-evaluated by two pathologists (S. K., V. O.), who graded all tumors according to the guidelines proposed by Elston and Ellis.[15] Details concerning tumor stage, nodal status, grading and other clinicopathological criteria are given in *Table 1*.

### *Immunohistochemistry*

Immunohistochemical staining was done on 5-mm-thick paraffin sections using a standard three-step immunoperoxidase technique and diaminobenzidine as chromogen.

***Table 1.*** **Clinicopathological parameters of 124 breast cancer patients, stratified according to CT7 status**

| *Parameter* | | | *CT7 negative n (%)* | *CT7 positive n (%)* | *p (Pearson's $c^2$ test)* |
|---|---|---|---|---|---|
| *Tumor grade* | G1 | (n=25) | 24 (96%) | 1 (4%) | **0.004** |
| | G2 | (n=73) | 62 (85%) | 11 (15%) | |
| | G3 | (n=26) | 16 (62%) | 10 (38%) | |
| *Tumor stage* | pT1 | (n=53) | 47 (89%) | 6 (11%) | 0.131 |
| | pT2 | (n=52) | 42 (81%) | 10 (19%) | |
| | pT3/4 | (n=19) | 13 (68%) | 6 (32%) | |
| *Lymph node status** | pN0 | (n=72) | 60 (83%) | 12 (17%) | 0.848 |
| | pN1/2/3 | (n=50) | 41 (82%) | 9 (18%) | |
| *Angioinvasion* | absent | (n=95) | 77 (81%) | 18 (19%) | 0.525 |
| | present | (n=29) | 25 (86%) | 4 (14%) | |
| *Tumor type* | lobular | (n=16) | 11 (69%) | 5 (31%) | 0.130 |
| | ductal | (n=108) | 91 (84%) | 17 (16%) | |
| *ER status** | negative | (n=19) | 15 (79%) | 4 (21%) | 0.497 |
| | positive | (n=101) | 86 (85%) | 15 (15%) | |
| *PR status** | negative | (n=27) | 23 (85%) | 4 (15%) | 0.869 |
| | positive | (n=93) | 78 (84%) | 15 (16%) | |
| *HER2 status** | Score 0/1+ | (n=82) | 70 (85%) | 12 (15%) | 0.225 |
| | Score 2/3+ | (n=38) | 29 (76%) | 9 (24%) | |
| *Ki-67 LI** | ≤median | (n=62) | 52 (84%) | 10 (16%) | 0.779 |
| | >median | (n=61) | 50 (82%) | 11 (18%) | |
| *Age* | ≤median | (n=62) | 52 (84%) | 10 (16%) | 0.638 |
| | >median | (n=62) | 50 (81%) | 12 (19%) | |

*data concerning Ki-67 LI, lymph node, ER, PR and HER2 status are unknown in a few cases; p levels with statistical significance (<0.05) are given in bold letters

Following microwave pre-treatment (10 minutes at 750 W), the slides were incubated with the mouse monoclonal antibody CT7-33 (dilution: 1:100; Dako, Glostrup, Denmark), which had been generated for CT7 antigen detection.[14] Moreover, HER2 and Ki-67 antigen immunostaining was performed using the HercepTest (Dako), which includes the prediluted polyclonal rabbit antibody A0485, and the MIB-1 antibody (dilution 1:20; Dako), respectively. In all staining runs, negative controls were included by omitting the primary antibody. Sections from a human melanoma with known high CT7 expression, from a tonsil (for Ki-67 antigen staining), and from a human breast carcinoma with known high HER2 immunoreactivity, respectively, served as positive controls.

Evaluation of immunohistochemical stainings was done without knowledge of clinical data. In cases where immunoreactivity was not homogeneous within the tumor, only areas with the strongest staining were considered. Ki-67 labeling index (LI) was calculated by estimating the proportion of tumor cells with stained nuclei within 1,000 representative tumor cells. The HER2 score was assessed semiquantitatively (0, 1+, 2+ or 3+) as proposed by the HercepTest manual. According to the recommendations of Chitale and coworkers,[16] CT7 expression was defined as positive if more than 50% of tumor cells were stained. The localization (cytoplasmatic and/or nuclear) and the degree of staining were not considered for immunohistochemical evaluation.

*Statistical analysis*

All analyses were done using the Statistical Package for Social Sciences software (SPSS, Chicago, Illinois, USA). A p value of <0.05 defined statistical significance. Associations between categorized parameters were examined using Pearson's $\chi^2$ test. Numerical parameters (age and Ki-67 LI) were categorized into two groups on the basis of their median values. Kaplan-Meier curves were plotted from data of RFS and MFS, respectively. Data from patients who were lost to follow-up were treated as censored data. The Cox proportional regression hazard model was used for survival analyses. In a first step, CT7 status and other parameters with potential prognostic significance were tested by univariate analysis. In a second step, a multivariate analysis was performed, in which all parameters that yielded a p value of <0.10 by univariate analysis were included. Of the prognostic parameters that contributed significantly to the model, the effect was calculated in terms of relative risk (RR) and associated 95% confidence intervals (CI).

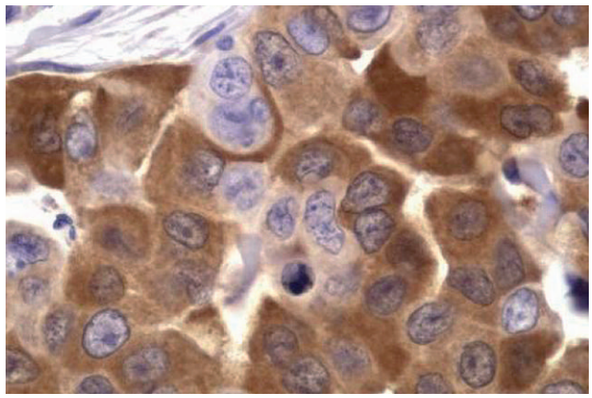

***Figure 1.*** *CT7 expression in an invasive ductal breast carcinoma (magnification: 400x). Immunoreactivity (indicated by dark color) is found in the cytoplasm of many tumor cells.*

## *Results*

A positive CT7 status was registered in 22 tumors (18%). CT7 expression was generally found in the cytoplasm of tumor cells (*Fig. 1*), and only few cases displayed

***Table 2.*** **Univariate Cox regression analysis for RFS and MFS in 124 breast cancer patients**

| *Parameter* | *RFS* | | | *MFS* | | |
|---|---|---|---|---|---|---|
| | *p* | *RR* | *95% CI* | *p* | *RR* | *95% CI* |
| *CT7 status* | 0.107 | 1.85 | 0.88–3.90 | **0.043** | 2.02 | 1.02–4.02 |
| *Tumor grade* | **0.002** | 2.30 | 1.37–3.84 | **0.002** | 2.11 | 1.31–3.38 |
| *Tumor stage* | **0.004** | 1.83 | 1.21–2.77 | **0.0001** | 2.39 | 1.59–3.59 |
| *Lymph node status* | **0.011** | 1.51 | 1.10–2.09 | **0.0001** | 2.02 | 1.51–2.72 |
| *Angioinvasion* | 0.141 | 1.67 | 0.84–3.31 | **0.002** | 2.68 | 1.45–4.98 |
| *Tumor type* (ductal vs. lobular) | 0.307 | 1.85 | 0.57–6.01 | 0.978 | 0.99 | 0.42–2.35 |
| *ER status* | **0.001** | 3.49 | 1.71–7.13 | **0.048** | 2.07 | 1.01–4.27 |
| *PR status* | **0.011** | 2.41 | 1.22–4.77 | **0.013** | 2.29 | 1.19–4.40 |
| *HER2 status* | **0.029** | 2.07 | 1.08–3.98 | **0.004** | 2.41 | 1.33–4.36 |
| *Ki-67 LI* (≤ vs. > median) | 0.129 | 1.63 | 0.87–3.08 | **0.018** | 2.10 | 1.14–3.89 |
| *Age* (> vs. ≤ median) | 0.142 | 1.62 | 0.85–3.09 | 0.211 | 1.46 | 0.81–2.65 |

p levels with statistical significance (<0.05) are given in bold letters

additional nuclear staining of some tumor cells. In cases which also contained non-neoplastic breast tissue, no CT7 immunoreactivity was observed in normal breast epithelia. Thus, CT7 expression was generally confined to the tumor cells of breast carcinomas.

Analysis of the relationship between CT7 expression and clinicopathologic parameters revealed that CT7 status was significantly correlated only with tumor grade (p=0.004), but with no other parameter (*Table 1*). A weak, but statistically not significant association was observed between CT7 status and tumor stage (p=0.131). A positive CT status was observed more frequently in invasive lobular carcinomas (31%) compared to invasive ductal carcinomas (16%), but this difference was not statistically significant.

The clinical course of breast carcinomas was characterized by local tumor relapse in 39 patients (31%) after a median period of 23 months and by metastatic tumor spread in 45 patients (36%) after a median period of 33 months. Univariate Cox regression analysis revealed that tumor grade, stage, lymph node status, angioinvasion, ER status, PR status, HER2 status and Ki-67 LI were significantly associated with RFS and/or MFS, respectively (*Table 2*). CT7 status correlated significantly with MFS (p=0.043) and showed a weak trend of correlation with RFS (p=0.107). The corresponding Kaplan-Meier survival curves are shown in *Fig. 2*.

In order to identify independent predictors of RFS and MFS, multivariate analyses were performed, in which all parameters that yielded a p value of <0.10 by univariate analysis were included. Thus, an independent prognostic relevance was found for tumor grade, stage and ER status concerning RFS and for stage, lymph node status, angioinvasion, PR status and HER2 status concerning MFS (*Table 3*). CT7 status displayed only a weak, but statistically not significant trend of independent prognostic value concerning MFS (p=0.147).

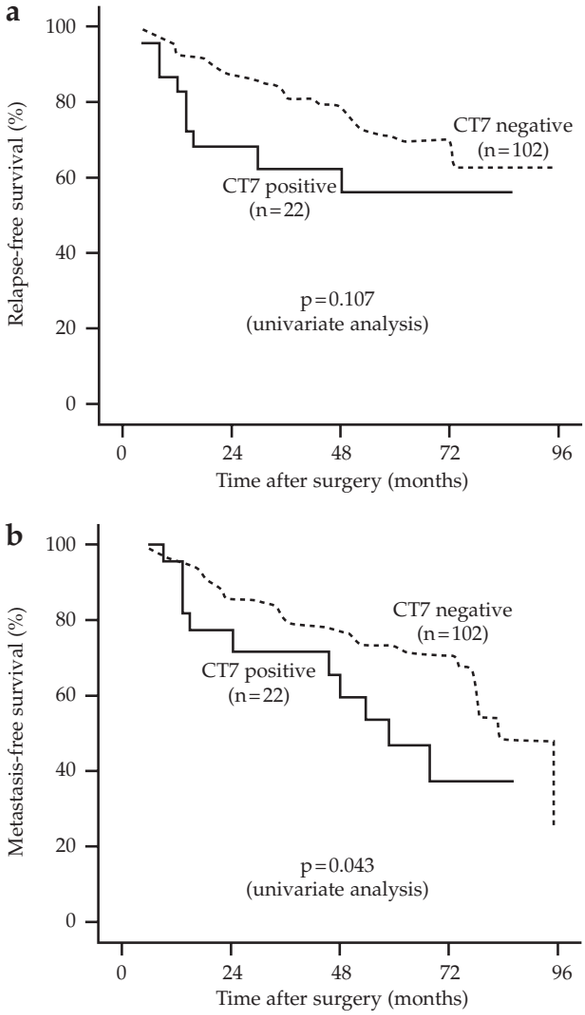


***Figure 2.*** *Kaplan-Meier curves illustrating the influence of CT7 expression on RFS* ***(a)*** *and MFS* ***(b).*** *CT7-positive tumors show a trend towards a worse RFS (p=0.107) and a significantly worse MFS (p=0.043).*

## *Discussion*

Breast cancer is among the leading causes of death in women worldwide. In industrialized countries, it represents the most common type of cancer. About one-third of women with node-negative breast carcinoma and approximately two-third of women with node-positive breast carcinoma experience a tumor relapse within ten years after local-regional therapy.[17,18] Once a tumor relapse has occurred, distant metastases are observed in up to 64% of patients.[19] These data underline a great need for more sensitive and specific prognostic markers in breast cancer. Such markers may represent helpful tools for better identification of those patients who have a high risk of tumor relapse or metastatic tumor spread, and who may therefore profit from a more aggressive therapy.

A major aim of the present study was to examine the prognostic value of CT7 expression in breast carcinomas. Interestingly, we found a significant correlation between CT7 immunoreactivity and MFS of breast cancer patients. Although CT7 status was not identified as an independent prognostic factor in a multivariate analysis, our data point to some prognostic relevance of this parameter, and therefore we suggest that its prognostic value in breast cancer should be further validated in a prospective study.

According to our own findings (*Table 1*), a positive CT7 status correlated significantly with tumor grade (p=0.004) and displayed a weak trend of correlation with tumor stage (p=0.131) in breast carcinomas. Even in other human malignancies like renal cell carcinoma,[20] bladder carcinoma,[21] or esophageal carcinoma,[22] a positive correlation

*Table 3.* **Multivariate Cox regression analysis for RFS and MFS in 124 breast cancer patients**

| *Parameter* | *RFS* | | | *MFS* | | |
|---|---|---|---|---|---|---|
| | *p* | *RR* | *95% CI* | *p* | *RR* | *95% CI* |
| *CT7 status* | –* | –* | -* | 0.147 | 1.95 | 0.79–4.80 |
| *Tumor grade* | **0.036** | 1.82 | 1.04–3.20 | 0.497 | 1.25 | 0.66–2.34 |
| *Tumor stage* | **0.013** | 1.87 | 1.14–3.06 | **0.030** | 1.67 | 1.05–2.65 |
| *Lymph node status* | 0.236 | 1.27 | 0.86–1.88 | **0.019** | 1.53 | 1.07–2.18 |
| *Angio-invasion** | –* | –* | –* | **0.035** | 2.20 | 1.06–4.59 |
| *ER status* | **0.009** | 2.83 | 1.30–6.18 | 0.751 | 1.17 | 0.45–3.01 |
| *PR status* | 0.468 | 1.38 | 0.58–3.28 | **0.011** | 2.46 | 1.23–4.94 |
| *HER2 status* | 0.453 | 1.34 | 0.63–2.85 | **0.013** | 2.27 | 1.19–4.36 |
| *Ki-67 LI** (≤ vs. > median) | –* | –* | –* | 0.173 | 1.61 | 0.81–3.19 |

*CT7 status, angioinvasion and Ki-67 LI were not included in the multivariate analysis for RFS because their p values were >0.10 in the univariate analysis; p levels with statistical significance (<0.05) are given in bold letters

between expression of CT antigens (at least on the mRNA level) and tumor stage has been described. Furthermore, immunohistochemical expression of several CT antigens (among them CT7) has been found in a higher percentage of endometrial carcinomas of high grade compared to those of low grade.[16] In the light of these data, it seems as if there was an association between CT7 expression and features of a more aggressive clinical behavior in some human epithelial cancers. However, on the contrary, some other studies are pointing to a tumor-suppressing effect of CT antigen expression. Thus, MAGE-A4 has been demonstrated to induce suppression of tumorigenic activity in liver carcinoma cells[23] and to promote apoptosis in non-small cell lung cancer cells.[24] On the other hand, a recent study reported that high MAGE-A expression was associated with chemoresistance to etoposide in melanoma cells in vitro, which may be presumably caused by strong down-regulation of the p53 transactivation function due to impaired acetylation of both p53 and histones surrounding p53-binding sites.[25] Although the exact biological function of CT antigens is presently still unknown,[14,25] future studies will hopefully allow more insights into the activities of CT antigens in tumor cells on the molecular level.

The role of CT antigens as targets for vaccination-based immunotherapy in malignant tumors has been a matter of debate for many years. The fact that CT antigen expression is found solely in neoplastic tumor cells, but not in normal tissue (except testis), renders them appropriate candidates for targets of antigen-based therapy.[3,4] In this context, high expression of the candidate antigen by all cells of the primary and metastatic tumor would represent an ideal setting for antigen-directed immunotherapy.[13] However, this situation is not fulfilled in most tumors because of the heterogeneity of tumor antigen expression. This also applies to CT7 expression in breast cancer where, according to Jungbluth et al[14] and our data, only a minority of tumors (between 18% and 38%) display CT7 antigen expression in at least 50% of tumor cells. Nevertheless, this heterogeneity of tumor antigen expression does not automatically exclude breast carcinomas from the possibility of antigen-based immunotherapy. In this context, we regard it noteworthy that a study examining pretreatment antigen expression in tumor cells from melanoma patients treated by peptide vaccination reported that a significant part of responders showed antigen expression only in less than 50% of tumor cells.[26] Moreover, tumors completely negative for the immunization antigen may respond to single antigen-based therapies due to the mechanism of epitope spreading.[27,28] In the light of these reports, antigen-specific therapy in breast carcinoma patients appears to be not out of sight at present, but rather seems to be worth further investigation.

To summarize, our data demonstrated significant correlation between the expression of the MAGE-1 family protein CT7 and the clinical outcome of breast carcinomas inasmuch as CT7-positive tumors displayed a worse MFS than CT7-negative tumors. Although the CT7 status was not identified as an independent prognostic factor by multivariate analysis, we suggest that estimation of CT7 immunoreactivity may bear some limited prognostic relevance in breast cancer and could serve as an additional prognostic marker in selected cases in which conventional prognostic factors point to divergent prognostic outcomes.

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