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ORIGINAL PAPER

# High expression of MAGE-A10 cancer-testis antigen in triple-negative breast cancer

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**Abstract** Recent studies indicate that ER/PR/HER-2-negative (triple-negative, TN) breast cancers may be "CTA-rich" tumors, suggesting the possibility of CTA-based cancer vaccines as a treatment option for patients bearing these tumors. MAGE-A10 together with NY-ESO-1 is probably the most immunogenic CTA, representing a potentially highly attractive target of active specific immunotherapies. Paraffin-embedded tumor sections were collected retrospectively from 165 breast cancer patients diagnosed between 2002 and 2003. Immunohistochemical staining for MAGE-A10 and NY-ESO-1 was performed. The expression of MAGE-A10 and NY-ESO-1 was correlated with other clinicopathological variables. MAGE-A10 expression (score $\geq 2+$) was detected in 105/164 (64%), and NY-ESO-1 expression (score $\geq 2+$) was observed in 14/164 (8.5%) patients. No correlation between MAGE-A10 and NY-ESO-1 expression and tumor size, tumor grade, Ki-67 and lymph nodes status was detectable. MAGE-A10 expression was significantly associated with ER-negative ($P = 0.002$), PR-negative ($P = 0.002$) and HER-2-negative ($P = 0.044$) tumors. We clearly showed that MAGE-A10 is frequently expressed in the group of TN patients, where the majority (85.7%) of tumors express this CTA. Because of limited therapeutic options for the triple-negative breast cancer, the frequent expression of MAGE-A10 CTA in these cancers may offer the opportunity for a much needed additional treatment for this group of patients.

**Keywords** Breast cancer · MAGE-A10 · NY-ESO-1 · Cancer-testis antigens

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## Introduction

Cancer-testis antigens (CTA) are proteins with physiological expression restricted to adult testicular germ cells. They are down-regulated in somatic adult tissues but may be aberrantly re-expressed in various malignancies [1].

The first CTA was discovered by taking advantage of a newly developed DNA-cloning method to identify targets of T-cell recognition [2]. Cytotoxic T lymphocytes (CTL) recognizing autologous tumor cells were obtained from a patient bearing melanoma with an unusually favorable clinical course [3]. Using the melanoma cell line MZ2-MEL and autologous CTL clones cytolytic to this line, MAGE-1, subsequently renamed as MAGE-A1, was identified as the target antigen. This was the first molecularly characterized tumor antigen eliciting autologous CTL responses in a cancer patient [4].

Further analysis of the MAGE-A family revealed 12 closely related genes clustered at Xq28 [5]. A growing number of tumor-associated antigens (TAA) with similar characteristics, identified by cellular or serological screening techniques, have been reported since. Although some of them may be expressed in placenta as well, they are collectively referred to as CTA [6].

CTA presently include 44 distinct gene families, some comprising multiple members, such as MAGE-A and

GAGE1, as well as splice variants, such as XAGE1a and XAGE1b, for a total of 89 transcripts. CTA can be classified into those that are encoded on the X chromosome (CT-X antigens) and those that are not (non-X CT antigens) [7].

Although a possible role in chromosomal recombination, transcription, translation and signaling has been proposed, physiological functions of a large majority of CT-X antigens remain poorly elucidated [1, 7].

Expression of CTA in tumors of different histological origin is highly variable. RT-PCR analysis indicates that bladder, lung and ovarian cancers, hepatocellular carcinoma and melanoma frequently express CTA genes. In contrast, their expression is rarely observed in renal, colon and gastric cancers and in leukemia/lymphoma cells [1, 7, 8].

Spontaneous humoral and cell-mediated immune responses against several CTA, including MAGE-A1 [9] and NY-ESO-1 antigens [10], have been repeatedly demonstrated, thus raising the possibility that they could represent adequate cancer immunotherapy targets. This has led to intense research into the development of antigen-specific vaccines and their utilization in therapy [7]. Cancer vaccine trials performed by using MAGE-A and NY-ESO-1 CTA in different molecular forms have indeed demonstrated their ability to enhance or induce humoral and cell-mediated immune responses in some patients [11, 12].

MAGE-A10 together with NY-ESO-1 is probably the most immunogenic CTA [13–15], representing a potentially highly attractive target of active specific immunotherapies. However, the development of specific treatments has been limited by the lack of data regarding its expression at the protein level. Taking advantage of newly developed anti-MAGE-A10 monoclonal antibodies (mAbs), we have tested at the protein level, by immunohistochemistry, the expression of MAGE-A10 and NY-ESO-1 in 165 consecutive cases of breast carcinoma and their association with routinely assessed clinicopathological parameters.

## Materials and methods

### Specimens

Breast biopsies were obtained from patients with primary breast cancer operated at the University Hospital Center Zagreb between September 2002 and September 2003.

Representative blocks were retrieved from each case for the construction of a tissue microarray (TMA). The TMA used in this study comprises 165 patients. Tumor size, histological tumor type, histological grade, ER and PR status, HER-2 status, proliferative index, and lymph node status were routinely assessed and recorded in the database. Histological tumor type was determined using the Word Health Organization classification [16]. Histological grade was assessed using Elston–Ellis method [17]. ER and PR status were determined immunohistochemically by scoring percentages of positive nuclei. Positive staining for ER and PR was defined as nuclear staining in $\geq$10% of tumor cells [18].

HER-2 positivity was classified based on percentages of stained tumor cells and intensity of membrane staining using HercepTest guidelines according to manufacturers' recommendations. HER-2 immunostaining was considered positive when strong (+++) membranous staining was observed in $\geq$10% of tumor cells, whereas cases with 0 to + were regarded as negative. For patients with HER-2 ++ expression, confirmative FISH tests were additionally performed [19].

Proliferative index was measured by immunohistochemical staining with Ki-67 antibody and reported as positive nuclei out of 100 counted tumor cells [20].

The study was approved by the Institutional Review Board of our Hospital.

### Tissue microarrays and immunohistochemistry

Immunohistochemical staining of MAGE-A10 and NY-ESO-1 was performed on the breast cancer TMA. For the detection of MAGE-A10 protein, we used undiluted 3DA3 mAb, whereas [21] undiluted B9.8.1.1 mAb was utilized for NY-ESO-1 detection [22]. TMA staining was described in detail elsewhere [23]. Briefly, tissue slides from paraffin-embedded breast cancer tumor samples were places on Silane (3-aminopropyltriethoxysilane, A 3648, Sigma, St. Louis MO, USA). After deparaffinization, slides were heated in an 800-W microwave oven at maximum power for 8.5 min, held in 10 mmol/l citrate buffer (pH 6.0) for 5 min and then rinsed with a phosphate buffer solution (PBC, pH 7.2). To suppress endogenous peroxidase activity, slides were treated with $H_2O_2$. After additional rinsing with PBC, they were incubated for 20 min with a 1:10 dilution of normal rabbit serum (DakoX0902, Dako A/S) in a wet chamber at room temperature for 20 min to prevent non-specific binding of immunoglobulin. Slides were then treated with undiluted mAbs at room temperature for 90 min.

The EnVision (Dako) system was used as a secondary detection tool and diaminobenzidine tetrahydrochloride served as a chromogen. Slides were counterstained with hematoxylin prior to evaluation. Sections of normal human testis with intact spermiogenesis were used as positive controls for MAGE-A10 and NY mAbs.

### Scoring

MAGE-A10 and NY-ESO-1 staining results were scored using Allred scoring system [24]. This method takes into

account percentages of positive cells (scored on a 0–3 scale) and the intensity of their staining (scored on a 0–3 scale). The percentage of positive cells is then multiplied by the intensity of staining, and the final score ranges from 0 (no staining) to 9 (diffuse and strong staining). The final results were further classified as 0 (no staining), 1 (score 1, 2, 3), 2 (score 4, 5, 6) and 3 (score 7, 8, 9).

For statistical analysis, MAGE-A10 and NY-ESO-1 scores of 0 and 1 were considered negative, whereas scores 2 and 3 were considered positive.

### Statistical analysis

Association between immunohistochemical data and different clinicopathological parameters was evaluated by $\chi^2$ test. A $<0.05$ $P$-value of was considered significant. For all statistical analyses, computer program SPSS 11.5.0 (SPSS for Windows, 2002) was used.

## Results

### Patient population

A total of 165 pT1-3 pN0-3 M0 breast cancer patients operated at our institution between September 2002 and September 2003 were included in the study. Data on tumor type, size, grade, ER and PR status, HER-2/neu, Ki-67 and axillary lymph node status are summarized in Table 1.

### MAGE-A10 and NY-ESO-1 expression

One hundred and sixty-four samples were examined for MAGE-A10 and 165 for NY-ESO-1 by IHC. Table 2 summarizes IHC staining results. MAGE-A10 expression (score $\geq 2+$) was detected in 104/164 (63%), and NY-ESO-1 expression (score $\geq 2+$) was detected in 14/164 (8.5%) of patients.

### Association between MAGE-A10 and NY-ESO-1 expression and different clinicopathological parameters

Table 3 presents associations between MAGE-A10 and NY-ESO-1 expression with clinicopathological variables. Expression of MAGE-A10 was significantly associated with HER-2-negative ($P = 0.044$), ER-negative ($P = 0.002$) and PR-negative ($P = 0.002$) breast cancers. No association was found between MAGE-A10 expression and tumor size, grade, proliferative index and lymph node status (data not shown). Of all ER-negative patients (either HER2+ or HER2−), 51/67 (75%) were MAGE-A10 positive (score $\geq 2$), while only 16/68 (23.5%) were MAGE-A10 negative ($P = 0.002$).

**Table 1** Frequency and percentage of tumor size, histological tumor types, grade, ER/PR status, HER-2 status, Ki-67, LN stage

| | Number | % |
|---|---|---|
| Tumor size | | |
| <2 cm | 90 | 54.5 |
| 2–5 cm | 66 | 40.0 |
| >5 cm | 9 | 5.5 |
| Histological tumor type | | |
| Ductal | 109 | 66 |
| Lobular | 33 | 20 |
| Other | 23 | 14 |
| Estrogen receptors | | |
| Positive | 96 | 58.5 |
| Negative | 68 | 41.5 |
| Progesterone receptors | | |
| Positive | 79 | 47.9 |
| Negative | 86 | 52.1 |
| HER-2/neu | | |
| Negative | 134 | 81.2 |
| Positive | 31 | 18.8 |
| Tumor grade | | |
| 1 | 28 | 17.1 |
| 2 | 80 | 48.8 |
| 3 | 56 | 34.1 |
| Ki-67 | | |
| <15% | 124 | 77 |
| >15% | 37 | 23 |
| Lymph nodes | | |
| Positive | 53 | 32.1 |
| Negative | 83 | 50.3 |
| Unknown | 29 | 17.6 |

**Table 2** Frequency and percentage of MAGE-A10 and NY-ESO-1 expression

| Antigen | Expression score | Number | % |
|---|---|---|---|
| MAGE-A10 | 0 | 4 | 2.4 |
| | 1 | 56 | 34.1 |
| | 2 | 76 | 46.3 |
| | 3 | 28 | 17.1 |
| NY-ESO-1 | 0 | 135 | 81.8 |
| | 1 | 16 | 9.7 |
| | 2 | 4 | 2.4 |
| | 3 | 10 | 6.1 |

Expression of NY-ESO-1 (score $\geq 2$) was significantly associated with ER-negative ($P = 0.018$) and HER-2-positive ($P = 0.001$) breast cancer. No association was found between NY-ESO-1 expression and PR status, tumor size, grade, proliferative index and lymph node status.

**Table 3** MAGE-A10 and NY-ESO-1 expression in relation to HER-2, ER and PR status ($\chi^2$)

| Variable | MAGE-A10 | | | | | NY-ESO-1 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Negative | | Positive | | $\chi^2$, $P$ value | Negative | | Positive | | $\chi^2$, $P$ value |
| | 0 | 1 | 2 | 3 | | 0 | 1 | 2 | 3 | |
| HER-2 | | | | | | | | | | |
| Negative | 3 | 41 | 64 | 25 | $\chi^2 = 4.058$ | 116 | 13 | 1 | 4 | $\chi^2 = 20.566$ |
| Positive | 1 | 15 | 12 | 3 | $P = 0.044$ | 19 | 3 | 3 | 6 | $P < 0.001$ |
| Total | 4 | 56 | 76 | 28 | | 135 | 16 | 4 | 10 | |
| ER | | | | | | | | | | |
| Negative | 2 | 14 | 37 | 14 | $\chi^2 = 9.392$ | 50 | 8 | 2 | 8 | $\chi^2 = 5.560$ |
| Positive | 2 | 42 | 38 | 14 | $P = 0.002$ | 84 | 8 | 2 | 2 | $P = 0.018$ |
| Total | 4 | 56 | 75 | 28 | | 134 | 16 | 4 | 10 | |
| PR | | | | | | | | | | |
| Negative | 2 | 20 | 46 | 17 | $\chi^2 = 9.730$ | 67 | 11 | 2 | 6 | $\chi^2 = 0.136$ |
| Positive | 2 | 36 | 30 | 11 | $P = 0.002$ | 68 | 5 | 2 | 4 | $P = 0.712$ |
| Total | 4 | 56 | 76 | 28 | | 135 | 16 | 4 | 10 | |

**Table 4** MAGE-A-10 and NY-ESO-1 expression in ER+/PR+/HER2- and ER-/PR-/HER2-breast cancer patients

| Antigen | | Expression score | ER+/PR+/HER2-N % | ER-/PR-/HER2-N % | $P$ |
|---|---|---|---|---|---|
| MAGE-A10 | Negative | 0 | 2 2.4 | 1 2.0 | 0.004 |
| | | 1 | 35 42.4 | 6 12.0 | |
| | Positive | 2 | 34 41.0 | 29 59.2 | |
| | | 3 | 12 14.5 | 13 26.5 | |
| | | Total | 83 100 | 49 100 | |
| NY-ESO-1 | Negative | 0 | 77 92.8 | 38 76 | 0.013 |
| | | 1 | 6 7.2 | 7 14 | |
| | Positive | 2 | 0 0.0 | 1 2 | |
| | | 3 | 0 0.0 | 4 8 | |
| | | Total | 83 | 50 100 | |

To exclude a potential influence of HER-2 positivity, we performed additional analysis only for the HER-2-negative patients. HER-2-negative patients ($n = 133$) were further subdivided into two groups according to ER status: ER+/PR+/HER2- ($n = 83$) and ER-/PR-/HER2- ("triple-negative") group ($n = 50$). For both subgroups, we additionally checked association with MAGE-A10 and NY-ESO-1 expression using $\chi^2$-test (Table 4). MAGE-A10 expression (score $\geq 2$) was detected in 42/49 (85.7%) of TN breast cancer patients and only in 46/83 (55.5%) of ER+/PR+/HER2 − tumors. The difference is highly statistically significant ($P = 0.004$).

NY-ESO-1 expression (score $\geq 2$) was detected in only 5/50 (10%) of TN breast cancer patients and in 0/83 (0%) of ER+/PR+/HER-2− patients ($P = 0.013$). Although there are more NY-ESO-1-positive patients in the TN subgroup of patients and the difference is statistically significant, these results should be interpreted cautiously due to the low number of cases.

## Discussion

The search for human tumor antigens as potential immunotherapeutic targets, either for antibody-based therapy or for cancer vaccines, represents an appealing therapeutic concept since decades. Recent advances in molecular characterization of human TAA have opened the way toward active specific immunotherapy of cancer [26]. Such antigens should not be expressed or should display a highly restricted expression in normal tissues in order to minimize the effects of eventual autoimmune reactions. Several markers fulfilling these criteria, including human papilloma virus antigen in cervical cancer and CD20 in B-cell lymphoma, have been identified [25]. CTA are of particular interest, because they are expressed in a very limited number of healthy tissues typically including HLA class I negative spermatogonia, while they are expressed in a wide range of malignancies [25].

Relatively few studies have examined CTA expression in breast cancer. Given its relatively high immunogenicity,

MAGE-A10 CTA appears to represent a particularly attractive target for antigen-specific immunotherapy. However, due to the lack of specific reagents, this TAA could not reliably be identified in tumor sections so far. In this study, we analyzed expression of MAGE-A10 and NY-ESO-1 antigens on archival paraffin-embedded samples of breast cancer tissue from 165 patients and correlated their expression with other clinicopathological variables. To our knowledge, this is the first report specifically examining expression of MAGE-A10, at the protein level, in breast cancer.

MAGE-A10 expression (score $\geq 2+$) was detectable in 64% of patients and that of NY-ESO-1 in 8.5% patients. Data on MAGE-A and NY-ESO-1 expression in literature are highly variable. The frequency of multispecific MAGE-A and NY-ESO-1 positivity in published studies ranges between 17 and 74% and 2–40%, respectively. In the largest series of 1,355 patients, Theurillat et al. [27] observed a low rate of NY-ESO-1 protein expression in only 2.1% (28/1355). In the study of recurrent ductal breast cancer, Bandic found 74% of MAGE-A-positive and 40% of NY-ESO-1-positive breast cancer [28]. Different results observed between studies may be due to different antibodies used for CT detection or difference in scoring system. Some authors define positive CT-X antigen expression according to percentage of cells [21, 28, 29], while others combine the extent and intensity of CT expression using semi-quantitative scoring systems [30, 31]. In addition, TMA-based analyses are likely to have a higher false-negative rate due to sampling error, since CTA expression in tumors is frequently focal.

Since identification of CTA-positive tumor cells in clinical specimens is a key prerequisite for the development of specific immunotherapy strategies and for their monitoring, standardization of the procedure is necessary to establish clear indication for treatment and monitoring of their impact on tumor progression.

CTA expression in other tumors is frequently associated with poor outcome, high-grade lesions and advanced disease [32–35]. For example, NY-ESO-1 has been found to be expressed in 40% of grade 3 bladder tumors and 23% of grade 2 tumors, but in no grade 1 tumor [36]. Similarly, MAGE-A1 expression has been found in 48% of metastatic melanoma versus 16% of primary melanoma [37]. In NSCLS, expression of MAGE-A3 and NY-ESO-1 is a marker of poor prognosis, associated with advanced disease [32].

Studies exploring potential prognostic significance of CTA expression in breast cancer have yielded contradictory findings. Some authors found that expression of CTA is associated with poorly differentiated histological phenotypes [29, 31]. Others found no association between their expression and various pathological parameters [28] or only an association between MAGE-A1 and Ki-67 labeling index [30]. In our study, the expression of MAGE-A10 and NY-ESO-1 was not associated with typical adverse clinicopathological features, including larger tumor size, higher histological grade, frequency of Ki67-positive cells or cancer-positive lymph nodes.

Recent analyses indicate a higher incidence of CT-X antigen expression in TN breast cancer [30, 31, 38]. Since TN breast cancer carries a worse clinical prognosis and treatment options are limited for this group of patients, innovative treatments for this group of patients are of potentially high clinical relevance. Currently, vaccines comprising members of MAGE-A and NY-ESO-1 families are being investigated in clinical trials in patients with melanoma, ovarian and lung cancer, where such antigens are frequently expressed [39–41]. Since breast cancer was considered as "CTA-poor" tumor type, CTA-based cancer vaccine trials have not included patients bearing these tumors.

Here, we addressed the question of a potential association of CT-X antigen expression with hormone receptor and/or HER-2 status. We clearly showed that MAGE-A10 is frequently expressed in the group of TN patients, where the majority (85.7%) of tumors express this CTA. Because of limited therapeutic options for the triple-negative breast cancer, the frequent expression of MAGE-A10 CTA in these cancers may offer the opportunity for a much needed additional treatment for this group of patients.

**Conflict of interest** None.

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