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Cancer–testis antigen expression in triple-negative breast cancer

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Background: Cancer–testis (CT) antigens, frequently expressed in human germline cells but not in somatic tissues, may become aberrantly reexpressed in different cancer types. The aim of this study was to investigate the expression of CT antigens in breast cancer.

Patients and methods: A total of 100 selected invasive breast cancers, including 50 estrogen receptor (ER) positive/HER2 negative and 50 triple negative (TN), were examined for NY-ESO-1 and MAGE-A expression by immunohistochemistry.

Results: A significantly higher expression of MAGE-A and NY-ESO-1 was detected in TN breast cancers compared with ER-positive tumors ($P = 0.04$). MAGE-A expression was detected in 13 (26%) TN cancers compared with 5 (10%) ER-positive tumors ($P = 0.07$). NY-ESO-1 expression was confirmed in nine (18%) TN tumor samples compared with two (4%) ER-positive tumors.

Conclusions: MAGE-A and NY-ESO-1 CT antigens are expressed in a substantial proportion of TN breast cancers. Because of the limited therapeutic options for this group of patients, CT antigen-based vaccines might prove to be useful for patients with this phenotype of breast cancer.

Key words: breast cancer, cancer–testis antigens, MAGE, NY-ESO 1

introduction

Cancer–testis (CT) antigens are encoded by a group of genes predominantly expressed in human germline cells. They are down-regulated in somatic adult tissues but may become aberrantly reexpressed in various malignancies [1]. To date, almost a 100 genes and gene families encoding CT antigens have been identified. CT antigens mapping to chromosome X are referred to as CT-X antigens and distinguished from non-X CT antigens located on other chromosomes [1–3]. The expression of CT-X antigens varies greatly between tumor types, being most frequent in melanomas, bladder, lung, ovarian and hepatocellular carcinomas and uncommon in renal, colon and hematological malignancies [4]. CT-X antigen expression is associated with a poorer outcome and is more prevalent in higher grade and advanced stage tumors [5–9]. Intensive research into their possible use in therapeutic vaccines is ongoing and several clinical vaccine trials employing CT-X antigens, in particular antigens of the MAGE-A family and NY-ESO-1, in patients with lung, ovarian cancers and melanoma are ongoing or have been completed [10–16]. However, few studies have explored the presence of CT antigens in breast cancer rendering contradictory results [17–20]. Interestingly, recent analysis in a limited number of patients indicated a higher incidence of CT-X antigen expression in triple-negative (TN) primary breast cancer [21]. Since TN breast cancer carries a worse clinical prognosis, presence of CT antigens would offer additional immunotherapeutic options. Consequently, in the present study, we analyzed a larger series of breast cancers for the presence of CT antigen. In order to elucidate the potential increased expression of CT antigens, we compared a larger series of TN breast cancer with a group of hormone-receptor-positive carcinomas.

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patients and methods

study population

The study is based on the breast database of the European Institute of Oncology, Milan, Italy, and contains medical history, concurrent diseases, type of surgery and pathological assessment including morphological and

biological features for all consecutive breast cancer patients who underwent surgery from January 1997 to December 2001. From this series of patients, a total of 100 invasive breast cancer cases—50 hormone-receptor-positive and 50 TN cases—were selected and corresponding paraffin blocks were retrieved from the archives of the Division of Pathology at the European Institute of Oncology. Tumor classification was done according to the World Health Organization Histological Classification of Breast Tumors, modified by Rosen and Obermann [22]. Tumor grade was assessed according to Elston and Ellis [23].

immunohistochemistry

Estrogen receptor (ER) and progesterone receptor (PgR) status as well as Ki-67-labeling index were assessed as previously reported [24, 25]. HER2 immunohistochemical (IHC) expression was evaluated using a 1 : 400 dilution of a polyclonal antiserum (Dako, Glostrup, Denmark). All tumors with equivocal (IHC 2+) results for HER2 were tested for gene amplification by FISH (Vysis PathVysion; Abbott, Chicago, IL). ER and/or PgR positivity was defined as tumors showing ≥50% expression in the neoplastic cells. TN tumors were characterized by a lack of immunoreactivity for ER as well as PgR and as negative by both IHC and FISH for HER2. All ER- and PgR-positive cases were centrally testes for HER2 expression. HER2 IHC expression was evaluated using a 1 : 400 dilution of a polyclonal antiserum (Dako). IHC expression was scored by two pathologists as follows: 0 (no staining or faint membrane staining), 1+ (faint membrane staining in >10% of tumor cells, incomplete membrane staining), 2+ (weak to moderate membrane staining in >10% of tumor cells) and 3+ (intense circumferential membrane staining in >10% of tumor cells). For this analysis, HER2 scores of 0 and 1+ were considered negative.

NY-ESO-1 and MAGE-A expression was assessed on whole tissue sections by IHC. For the analysis of NY-ESO-1, monoclonal antibody E978 (1 : 200) was used [26]. For the detection of MAGE-A antigens, an antibody cocktail consisting of monoclonal antibodies 6C1, MA454, M3H67 and 57B was employed (1 : 40) [27–29]. Tissue specimens were dewaxed and heated in an antigen retrieval solution [EDTA buffer (1 mM, pH 8.0)] for 15 min (NY-ESO-1) and 30 min (MAGE-A), respectively, at 99°C. The sections were then incubated with the primary antibodies overnight at 4°C. The EnVision Mouse (Dako) was used as a secondary detection system and diaminobenzidine tetrahydrochloride served as a chromogen. Sections of normal human testis with intact spermiogenesis were used as positive controls for both NY-ESO-1 and MAGE-A. The MAGE-A cocktail was tested for the first time on human samples.

scoring

NY-ESO-1 and MAGE-A IHC staining results were scored using a semiquantitative scoring system as previously described [30]. This method takes into account both the percentage of immunoreactive tumor cells and the staining intensity. The percentage of positive cells is then multiplied by the intensity of staining (1+, 2+ or 3+), and the final score ranges from 0 (no staining) to 300 (diffuse and strong immunostaining of all the tumor cells).

statistical methods

Fisher's exact test was used to test for difference of antigen expression between ER-positive responsive and TN breast cancers [31]. Different cut-offs of expression (i.e. 1+, 2+ and 3+)—as described by Domfeh et al. [30]—were considered to define the presence of antigen expression. Armitage's test for trend was also used, considering the degree of expression on an ordinal scale. All reported \$P\$ values were two sided.

results

From January 1997 to December 2001, a total of 5910 pT1-3 pN0-3 M0 patients with breast cancer were referred to the institute for clinical care and therapy and their data were included in the database. From this population, a total of 50 consecutive female patients with highly ER-positive and HER2-negative breast cancers (ER) and 50 patients with TN breast cancer were identified. The baseline pathological characteristics of ER and TN breast tumors are listed in Table 1. As expected, certain histopathological features differed among ER and TN breast cancer patients. All ER-positive patients were also HER2 negative at a central revision (see Table 1).

All samples were examined for MAGE-A and NY-ESO-1 expression by IHC (Figures 1 and 2). An heterogeneous staining pattern was present within specific tumor samples, ranging from 1+ to 3+. In Figure 2, the visual scale shows intensity (red for 3+, green for 2+ and blue for 1+) and percentage of staining for each of the tumor samples. Table 2 shows the overexpression of the two antigens in ER and TN tumors, according to different cut-offs. MAGE-A overexpression (score ≥2+) was detected in 13 (26%) TN cancers but only in 5 (10%) ER tumors ($P = 0.07$). NY-ESO-1 overexpression (score ≥2+) was documented in nine (18%) TN tumors but only in two (4%) ER lesions ($P = 0.05$).

We reviewed absolute number of positive cases, irrespective of intensity and/or extent of antigen expression. We observed 16 of 50 (32%) MAGE-A-positive TN cases. On the other hand, only 9 of 50 (18%) in the ER group were positive for MAGE-A. When considering intensity and/or extent of immunostaining,

Table 1. Pathological characteristics of TN and ER- and PgR-positive breast cancer

	ER and PgR positive		TN		*P* value
	N	*N*	%	%	
All samples	50	100	50	100	
Histology					
Ductal	49	98	41	82	0.02
Others	1	2	9	18	
Grade[a]					
1	8	16	1	2	0.03
2–3	41	82	48	96	
pT					
1	40	80	32	64	0.11
2–3	10	20	18	36	
pN					
0	26	52	35	70	0.10
1–3	24	48	15	30	
Ki-67					
<20%	28	56	5	10	<0.001
≥20%	22	44	45	90	
HER2/neu					
Negative	40	80	50	100	0.0012
1+	10	20	0	0	

[a]The sum does not add up to the total because of two missing values.
TN, triple negative; ER, estrogen receptor; PgR, progesterone receptor; pT, pathological T; pN, pathological N.

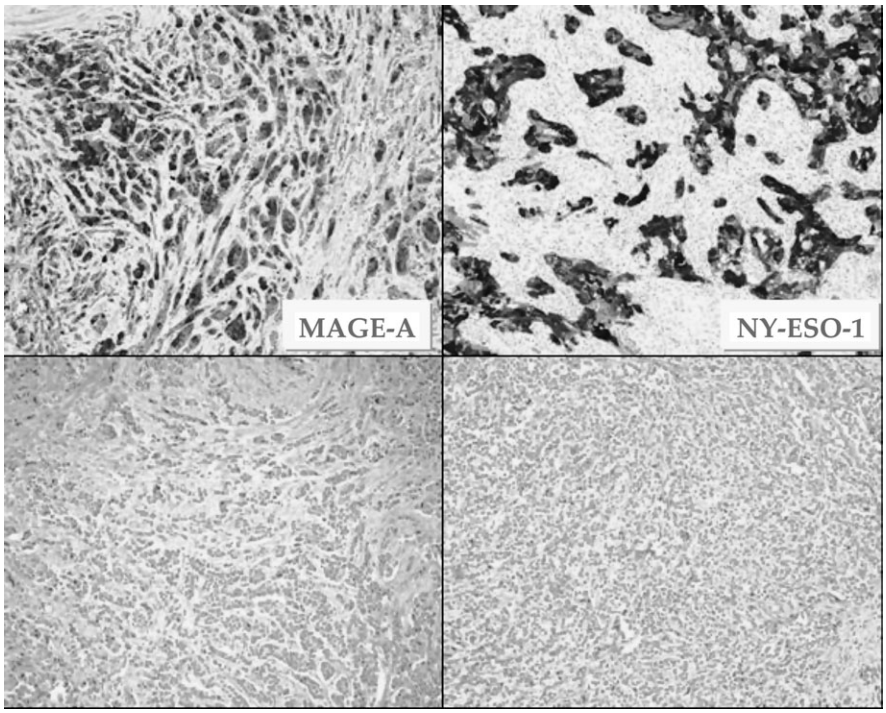

Figure 1. MAGE-A and NY-ESO-1 expression by immunohistochemistry in human breast cancer in comparison with negative samples.

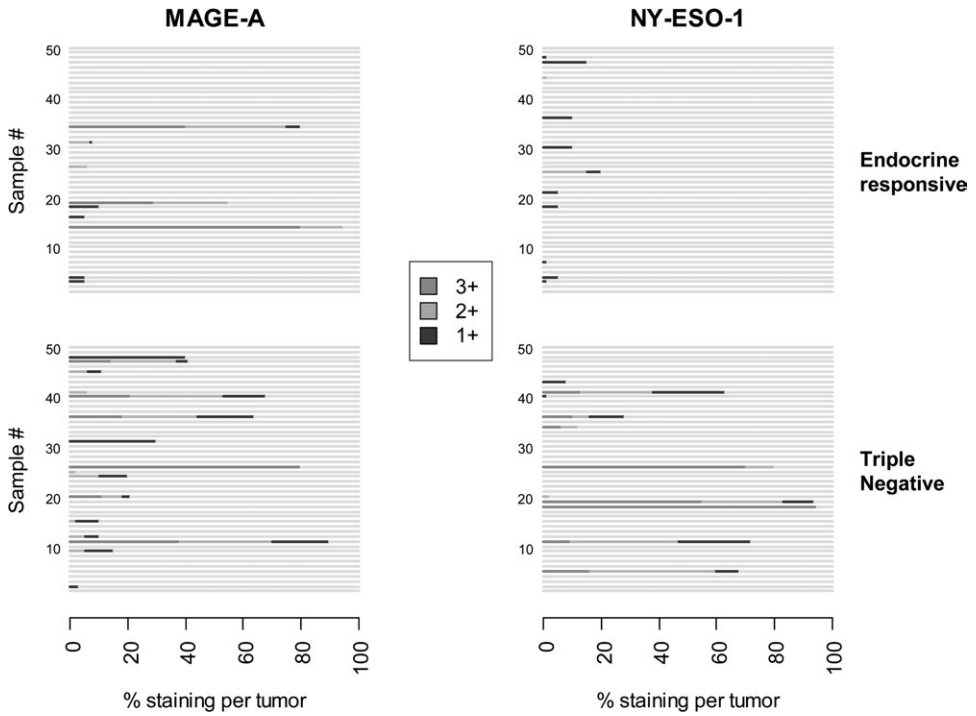

Figure 2. MAGE-A and NY-ESO-1 expression reported as intensity and percentage of stained cells in estrogen receptor- and progesterone receptor-positive and triple-negative breast cancer patients.

there is more striking difference between hormone responsive and TN cases.

When evaluating the dominant intensity pattern of immunoreactivity, hormone-receptor-positive cases show more cases with predominant 1+ (blue) intensity [4 of 9 (44%) for MAGE-A and 9 of 11 (82%) for NY-ESO-1] than the TN cases [3 of 16 (19%) for MAGE-A and 2 of 11 (18%) for NY-ESO-1]. Consequently, 2+ and/or 3+ (green/red) intensity of immunostaining is present for the TN cases in >81% for MAGE-A and 82% NY-ESO-1, while for the ER cases, the numbers are 55% and 18%, respectively (Figure 1). If this is analyzed for all 50 cases of each group, MAGE-A expression

Table 2. MAGE-A and NY-ESO-1 expression in TN and ER- and PgR-positive breast cancer

Antigen	Expression	ER positive		TN		*P* value[a]
		N	%	*N*	%	
MAGE-A	≥1+	4	8	3	6	
	≥2+	2	4	7	14	
	3+	3	6	6	12	
	P trend: 0.07					
	Score[b]	12 (5, 270)		30 (3, 240)		0.09
NY-ESO-1	≥1+	9	18	2	4	
	≥2+	2	4	1	2	
	3+	0	0	8	16	
	P trend: 0.07					
	Score[b]	5 (1, 35)		114 (1, 285)		0.71

[a]Wilcoxon rank sums test, comparing score distributions among ER-positive and TN tumors, including those with no expression.
[b]Median (min, max): computed in patients with expression ≥1+.
TN, triple negative; ER, estrogen receptor; PgR, progesterone receptor.

Table 3. MAGE-A and NY-ESO-1 expression (score ≥2+) and pathological features of the breast cancers

	MAGE-A ≥2 positive		NY-ESO-1 ≥2 positive	
	%	*P* value	%	*P* value
All samples	18		11	
Histotype				
Ductal	17	1.00	10	0.30
Others	20		20	
Grade				
1	0	0.20	0	0.59
2–3	20		11	
pT				
1	18	1.00	12	1.00
2–3	18		7	
pN				
0	16	0.60	11	1.00
1–3	21		10	
Ki-67				
<20%	3	0.006	9	1.00
≥20%	25		12	

pT, pathological T; pN, pathological N.

with an intensity score ≥2+ is detected in 13 of 50 (26%) TN cancers but only in 5 of 50 (10%) ER-/PgR-positive tumors ($P = 0.07$). Similarly, NY-ESO-1 expression with an intensity score ≥2+ is documented in 9 of 50 (18%) TN tumors but only in 2 (4%) hormone-receptor-positive lesions ($P = 0.05$).

When analyzing the extent of tumor staining irrespective of the intensity score, there is a more homogeneous expression of CT antigens in the TN cases compared with the hormone-receptor-positive cases. The latter displayed immunostaining in >25% of the tumor in 3 of 9 (33%) MAGE-A-positive cases and in 0 of 11 NY-ESO-1-positive cases. On the contrary, TN cases show antigen expression in >25% of the tumor in 7 of 16 (56%) MAGE-A-positive cases and 7 of 11 (67%) of NY-ESO-1-positive cases (Figure 1 and 2). Taken together, the number and extent of staining are higher for the TN cases for the MAGE-A antigens and the expression is more homogeneous. While for NY-ESO-1 expression there is no difference in the absolute number between TN and hormone-receptor-positive cases, TN cases show a more homogeneous NY-ESO-1 expression and a higher intensity.

A combined analysis of CT-X antigen expression showed that MAGE-A and/or NY-ESO-1 were overexpressed (score ≥2+) more frequently in TN (34%) than in ER tumors (14%) ($P = 0.03$).

Overall, no association was found between pathological features of disease and MAGE-A and NY-ESO-1 overexpression (score ≥2+), except for the KI-67-labeling index and MAGE-A expression (Table 3).

discussion

Breast cancer is well recognized as a heterogeneous disease not only from a morphological and structural standpoint but also in its diverse functional features revealed through analysis of its genetic signatures and other indices detectable through IHC [32–37].

While such heterogeneity poses clinical problems, it also offers opportunities to develop therapies making use of such properties, as exemplified by the success of ER-directed therapies.

TN breast cancer represents a group of tumors, which are difficult to treat. TN cancers have been identified by gene array analysis revealing a higher expression of clusters of proliferation-related genes [32]. This is illustrated by a higher Ki-67-labeling index expression in TN tumors versus endocrine-responsive cancers [38]. Our cohort of patients showed a similar elevated Ki-67 labeling in the TN cases. TN tumors frequently express molecules that may drive these proliferative processes, such as epidermal growth factor receptor (EGFR) and vascular-related growth factors [39]. However, disappointing clinical responses to agents targeting EGFR have been reported [40]. On the other hand, *in vitro* chemosensitivity studies have shown that cells lacking BRCA1, such as TN breast cancer cells, may be sensitive to drugs that cause double-strand breaks in DNA [41], such as alkylating agents. Recently, biological agents such as poly(ADP-ribose) polymerase inhibitors (PARP inhibitors) have been studied [42].

The early identification of features associated with response or resistance to primary therapy is important in the development of the most effective multimodal approaches and identifying cohorts of patients most likely to benefit from chemotherapy. Features predictive of response and outcome include steroid hormone receptor expression. Pathological complete remission (pCR) rate are significantly higher following neoadjuvant chemotherapy for patients with TN tumors compared with the hormone-receptor-positive cohort [43]. Regardless of the higher likelihood of pCR for patients with TN disease, the 5-year disease-free survival is significantly worse for this cohort compared with the ER-positive cohort in several studies [43]. Importantly, patients with ER-positive residual tumors fare remarkably better than patients with ER-negative tumors not achieving a pCR [43].

In recent years, CT antigens have emerged as new therapeutic options for the treatment of cancer. They have been identified in a wide variety of malignant tumors but in normal adult tissues, CT antigens are solely present in testicular germ cells. Due to the lack of major histocompatibility complex molecules, male germ cells are not subjected to any potential T-cell response and no associated side-effects have been observed in any of the previous clinical trials employing CT antigens [10–16]. Though a wide variety of tumors have been studied, knowledge about presence of CT antigens on a protein level in breast cancer is comparably limited and contradictory. In one study of ductal carcinomas, NY-ESO-1 and/or MAGE-A antigens were found in up to 50% and 90%, respectively [18], while others found a much low incidence [17, 19, 20]. A more recent study suggested an elevated expression of CT antigens in the recently identified subtype of TN breast carcinomas [21]. However, this study was focused on messenger RNA expression and IHC data were restricted to tissue microarray (TMA) tissues. Our present analysis employed full sections in order to adequately address issues such heterogeneity and intensity of antigen expression. The previous TMA analysis did not evaluate extent of staining and intensity, as it was done in the present study. We show that the incidence of MAGE-A and NY-ESO-1 expression for the common hormone-receptor-positive ductal breast is ~20% for both antigens. This is below the incidence, which has been reported in other tumors, such as melanoma and lung cancer [5, 6]. Nevertheless, it is higher than in previous other studies in breast cancer [17–19]. Most interestingly is the altered expression of MAGE-A and NY-ESO-1 in TN breast cancer of our series. There is a disparate change of incidence for both antigens. While MAGE-A shows a higher incidence in TN cases, in NY-ESO-1, the number of positive cases does not change. However, the most significant finding of our study is the increase of antigen intensity and the extent of tumor staining. For both MAGE-A and NY-ESO-1, there is an increased intensity and homogeneity in antigen expression. In hormone-receptor-positive cases, the expression of both antigens was predominantly present in a limited fashion mostly involving <25% of the tumor. Moreover, this expression showed a rather low intensity. In TN breast cancer of our series, there was not only a more homogeneous antigen expression involving larger areas of the tumor but expression was also more intense. Expression homogeneity and intensity have not been properly addressed in previous analyses of breast cancer, some of which were based on TMA slides [21]. TMA analysis is limited as to the informative value regarding extent of antigen expression.

The fact that MAGE-A and NY-ESO-1 expression is increased in TN breast cancer is of potential clinical relevance specifically in the adjuvant setting of treatment. It is our current thinking that patients with TN breast cancer and minimal residual disease after preoperative chemotherapy are the ideal setting to test the efficacy of a vaccination strategy. To date, vaccines for breast cancer have been mainly used in end-stage disease. Several clinical studies have been completed with vaccines against antigens, such as MUC1, CEA, HER2 and the carbohydrate antigens with varying results [44]. However, immunotherapy might be most effective when patients have only minimal residual disease after initial treatment. CT-X antigens offer a novel opportunity for fostering vaccine development and therapy. Vaccines including members of the MAGE-A and NY-ESO-1 families are currently being tested in clinical trials for patients with melanoma and lung cancer, where such antigens are frequently expressed [10–16]. Our study demonstrates MAGE-A and NY-ESO-1 antigen expression in a group of patients for which therapeutic options are limited. Analysis of MAGE-A and NY-ESO-1 antigen expression in breast cancer patients after surgery may allow identification of patients who are potential candidates for adjuvant therapeutic vaccines.

In conclusion, MAGE-A and NY-ESO-1 may be of therapeutic value as a vaccine-based treatment in TN breast cancers.

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disclosure

None of the authors declare conflicts of interest.

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