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## Possible predictive role of cancer/testis antigens in breast ductal carcinoma *in situ*

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**Abstract.** Cancer/testis antigens (CTAs) are a large family of tumor-associated antigens expressed in human tumors of different histological origin, but not in normal tissues, with the exception of the testes and placenta. Numerous immunohistochemical studies have reported associations between CTA expression and a negative estrogen receptor (ER) status in breast tumors, and demonstrated that CTAs are frequently expressed in tumors with higher nuclear grade. The expression of CTAs has not been studied as extensively in ductal carcinoma *in situ* (DCIS) as it has been in invasive breast cancer. The present retrospective study included archived paraffin-embedded specimens from 83 patients diagnosed with DCIS in the period between January 2007 and December 2014. The follow-up time for local recurrence ranged between 1 and 8 years (mean, 5.02 years). Antigens from the melanoma-associated antigen gene (MAGE) family, namely multi-MAGE-A, MAGE-A1, MAGE-A10 and New York esophageal squamous cell carcinoma 1 (NY-ESO-1) antigen, were evaluated by immunostaining and their subcellular location was investigated. Presence of tumor-infiltrating lymphocytes (TILs) was evaluated on all sections, together

with the histopathological variables of DCIS. Specific tested antigens exhibited associations with histopathological parameters for DCIS and all demonstrated statistically significant associations with nuclear staining, simultaneous cytoplasmic and nuclear staining, and local recurrence. Antigen MAGE-A10 demonstrated a significant association with higher expression of ER (P=0.005) and higher tumor nuclear grade (P=0.001), cytoplasmic staining (P=0.029) and antigen NY-ESO-1 with higher tumor size (P=0.001), expression of TILs (P=0.001) and R1 resection (P=0.001). A  $\chi^2$  test revealed significant associations between simultaneous cytoplasmic and nuclear staining and local recurrence (P=0.005), central necrosis (P=0.016), and the expression of ER (P=0.003) and progesterone receptor (PR) (P=0.010). Additional analysis revealed an association between antigen MAGE-A10 and TILs (P=0.05). Additional analysis of TILs indicated that they were significantly associated with tumor grade (P=0.023), central necrosis (P<0.001), ER (P=0.003) and PR (P=0.029). Overall, CTAs from the MAGE family (MAGE-A1, multi-MAGE-A and MAGE-A10) and NY-ESO-1 associate with histopathological predictive variables of DCIS. The expression of antigens NY-ESO-1 and MAGE-A10 could serve an important role in the treatment of patients with negative histopathological predictive variables, but further analysis is required. Simultaneous cytoplasmic and nuclear protein expression of MAGE-A family and NY-ESO-1 CTAs may represent an independent marker for local recurrence. Taken together, the present data suggest that CTAs are not perfect indicators of invasiveness for DCIS, but could inform treatment strategies for patients when taken in combination with other histopathological predictive variables. However, this was a small study and further larger studies will be necessary to confirm the current findings.

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*Abbreviations:* CTA, cancer/testis antigen; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; mAb, monoclonal antibody; PR, progesterone receptor; TILs, tumor-infiltrating lymphocyte; TN, triple-negative

*Key words:* ductal carcinoma *in situ*, cancer testis antigen, tumor-infiltrating lymphocytes, melanoma-associated antigen A10, New York esophageal squamous cell carcinoma 1 antigen

### Introduction

Ductal carcinoma *in situ* (DCIS) is a non-invasive type of breast cancer that evolves in the milk ducts of the breast and

remains located there. DCIS is a non-obligate precursor of invasive breast cancer and up to 40% of these lesions progress to invasive disease if untreated (1). The incidence of DCIS is rising, most likely due to increased use of mammographic screening and the transition from screen-film mammography to digital mammography (2). DCIS is not one entity but a heterogeneous group of at least four subtypes (luminal A, luminal B, Her 2 overexpressed and triple negative-very rare) (3). It remains unclear which type of DCIS is more likely to progress to invasive breast cancer and therefore will require more intensive treatment.

Cancer/testis antigens (CTAs) are a large family of tumor-associated antigens expressed in human tumors of different histological origin, but not in normal tissues, with the exception of the testis and placenta (4). This unique class of tumor-associated antigens was discovered in the early 1990s and the first to be identified was melanoma-associated antigen-1 (MAGE-1) in melanoma patients (5,6). CTAs may be divided into two large groups, depending on whether they are encoded on the X chromosome (X-CTA genes) or not (non-X-CTA genes) (7). X-CTA genes include the synovial sarcoma X (SSX) family, the GAGE/PAGE/XAGE super-families and the MAGE-A, MAGE-C and New York esophageal squamous cell carcinoma 1 (NY-ESO-1) multi-gene families, among others (7,8). Antigens in this group are widely and variably expressed among tumors of different histotypes (4). Expression of CTAs is highly variable and may be observed frequently in melanomas and bladder, lung, ovarian and hepatocellular carcinomas, but rarely in renal, colon and gastric cancer or hematological malignancies (9). In breast cancer, multiple immunohistochemical studies have reported an association between CTA expression and negative estrogen receptor (ER) status in breast tumors, and have demonstrated that CTAs are frequently expressed in tumors with higher nuclear grade (10,11). Spontaneous humoral and cell-mediated immune responses against several CTAs, including MAGE-A1 (6) and NY-ESO-1 antigens (12) has led to the proposal that CTAs could represent attractive cancer immunotherapy targets and has inspired research into the development of antigen-specific vaccines (9).

The expression of CTAs in DCIS has not been studied as extensively as in invasive breast cancer. However, in two studies, the expression of CTAs in DCIS was studied and it was demonstrated that NY-ESO1 is expressed in a high proportion of DCIS tissues, particularly those that are ER-negative (10,11).

The present study investigated the expression of CTAs from the MAGE family (multi MAGE-A, MAGE-A1 and MAGE-A10) and NY-ESO-1 in DCIS, and their association with standard histopathological parameters for DCIS [tumor size, tumor grade, expression of ER and progesterone receptor (PR), necrosis and margin] and local recurrence. The evaluation of tumor-infiltrating lymphocytes (TILs) was also performed.

## Materials and methods

*Patients and samples.* This retrospective study included archived paraffin-embedded specimens from 83 patients diagnosed with DCIS who underwent segmentectomy

surgery at the University Hospital for Tumors, Sisters of Mercy University Hospital Center (Zagreb, Croatia) between January 2007 and December 2014. The patients were all female, aged between 40 and 70 years old (mean age 57.4 years). All cases of surgically resected DCIS were reviewed in the current study, and histopathological parameters (tumor size, histological tumor grade, ER and PR status, necrosis and margin) were routinely assessed and recorded in a database. All patients received radiotherapy following breast-conserving surgery (lumpectomy) and certain patients (those who were receptor-positive) received hormone therapy for 5 years. Follow-up ranged between 1 and 8 years (mean, 5.02 years). This study received ethical approval from the Sisters of Mercy University Hospital Center. Written informed patient consent was received at the time of the material collection.

*Histology and immunohistochemistry.* Tumors were fixed in 10% buffered formalin for approximately 24 h at 4°C, cut at 3-4 millimeters and sampled in 3-7 sections. The specimens were embedded in paraffin, routinely cut and stained with hematoxylin and eosin (H&E). In each case, the available H&E sections were reviewed and slides with the deepest portion of tumor penetration were selected for immunohistochemical analysis.

A total of 4 new 5- $\mu$ m sections were cut from the paraffin-embedded blocks of each sample for analysis. Tissue slides from paraffin-embedded breast cancer tumor samples were placed on Silane (3-aminopropyltriethoxysilane, A 3,648, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Following 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).

Four monoclonal antibodies were used to determine the expression of analyzed proteins in DCIS (antibodies are gift from Dr. Spagnoli, Basel, Switzerland, they are not commercial antibodies). Monoclonal antibodies (mAbs) recognizing the following CTAs were used: Anti-MAGE-A1 (clone 77B), Anti multi-MAGE-A (clone 57B), anti-MAGE-A10 (clone 3GA11) and anti-NY-ESO-1 (clone D8.38). These mouse monoclonal antibodies CTAs (77B, 57B, 3GA11 and D8.38) mAb were used undiluted (undiluted supernatants). 57B was generated on immunization of mice with recombinant MAGE-A3 (13). However, this antibody recognizes a variety of MAGE-A molecules, and it is considered a multi-MAGE-A-specific reagent. D8.38 antibody, recognizing NY-ESO-1 and its homologous LAGE-1 CTA, has been previously described (14). 3GA11 antibody recognizing MAGE-A10 (15) and 77B recognizing MAGE-A1 has also been previously described (16).

TMA staining was performed as described previously (17). Briefly, tissue slides from paraffin-embedded breast cancer tumor samples were placed on Silane (3-aminopropyltriethoxysilane, A 3648, Sigma, St. Louis MO, USA) and incubated for 20 min in a thermostat at 60°C. The sections were then deparaffinized and incubated for 3x5 min in 10 mmol/l of citrate buffer (pH 6.0) in a microwave oven at 800 W. Subsequently, tissue slides were washed with phosphate buffered saline (PBS) buffer (pH 7.2), and endogenous peroxidase activity was blocked by a 5-min treatment with hydrogen peroxide (No. S2023,

Table I. Frequency and percentage of study samples with given histopathological variables.

Parameters	n (%)	P-value
Tumor size, mm		0.078
<1.2	50 (60)	
>1.2	33 (40)	
Tumor grade		<0.001 <sup>a</sup>
1	18 (22)	
2, 3	65 (78)	
Central necrosis		0.124
No	34 (41)	
Yes	49 (59)	
TIL		0.510
No	45 (54)	
Yes	38 (46)	
Multi-MAGE-A		<0.001 <sup>a</sup>
No	13 (16)	
Yes	70 (84)	
MAGE-A10		1.000
No	42 (51)	
Yes	41 (49)	
MAGE-A1		<0.001 <sup>a</sup>
No	6 (7)	
Yes	77 (93)	
NY-ESO-1		<0.001 <sup>a</sup>
No	22 (27)	
Yes	61 (73)	
ER		<0.001 <sup>a</sup>
No	24 (29)	
Yes	59 (71)	
PR		0.028 <sup>a</sup>
No	31 (37)	
Yes	52 (63)	
R1		0.661
No	39 (47)	
Yes	44 (53)	
Cytoplasmic staining		0.004 <sup>a</sup>
No	28 (34)	
Yes	55 (66)	
Nuclear staining		<0.001 <sup>a</sup>
No	75 (90)	
Yes	8 (10)	
Cytoplasmic and nuclear staining		<0.001 <sup>a</sup>
No	68 (82)	
Yes	15 (18)	
Total staining		<0.001 <sup>a</sup>
No	59 (71)	
Yes	24 (29)	
Local recurrence		<0.001 <sup>a</sup>
No	76 (92)	
Yes	7 (8)	

<sup>a</sup>Statistically significant result. TIL, tumor-infiltrating lymphocyte; MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1 antigen; ER, estrogen receptor; PR, progesterone receptor; R1, positive surgical margin.

Table II. Sensitivity, specificity and cutoff values for antigen and receptor detection.

Parameters	Sensitivity, (%)	Specificity, (%)	Cutoff value, (%)
ER	100	100	>20
PR	100	100	>30
Multi-MAGE-A	100	100	>0
MAGE-A10	100	100	>0
MAGE-A1	100	100	>0
NY-ESO-1	98	91	>0

MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1 antigen; ER, estrogen receptor; PR, progesterone receptor.

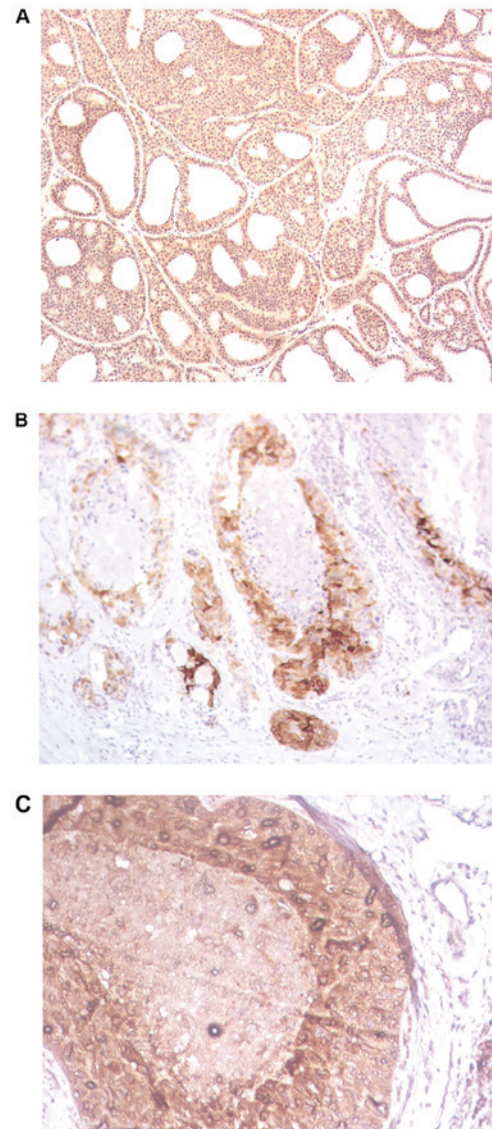


Figure 1. Immunohistochemistry staining of DCIS samples using mAb specific to NY-ESO-1 (clone D8.38) (observed in brown) and MAGE-A1 (mAb clone 77B). Sections presented variable (A) nuclear NY-ESO-1 staining, and (B) cytoplasmic and (C) nuclear MAGE-A1 staining. Original magnification, x10. DCIS, ductal carcinoma *in situ*; NY-ESO-1, New York esophageal squamous cell carcinoma 1 antigen; MAGE-A1, melanoma-associated antigen A1; mAb, monoclonal antibody.



Table III. Mann-Whitney U test results of the expression of cancer/testis antigens and histopathological variables.

Parameters	Multi-MAGE-A	MAGE-A10	MAGE-A1	NY-ESO-1
Tumor size				
U	1,909	3,112	1,618	2,282
P-value	0.001 <sup>a</sup>	0.216	0.001 <sup>a</sup>	0.001 <sup>a</sup>
Tumor grade				
U	3,237	2,448	2,946	3,278
P-value	0.327	0.001 <sup>a</sup>	0.010 <sup>a</sup>	0.474
Central necrosis				
U	2,573	3,112	2,282	2,946
P-value	0.001 <sup>a</sup>	0.217	0.001 <sup>a</sup>	0.051
TILs				
U	2,116	3,320	1,826	2,490
P-value	0.001 <sup>a</sup>	0.644	0.001 <sup>a</sup>	0.001 <sup>a</sup>
ER				
U	2,988	2,697	2,697	3,361
P-value	0.043 <sup>a</sup>	0.005 <sup>a</sup>	0.001 <sup>a</sup>	0.732
PR				
U	2,697	2,988	2,407	3,071
P-value	0.002 <sup>a</sup>	0.088	0.001 <sup>a</sup>	0.138
R1				
U	2,365	3,320	2,075	2,739
P-value	0.001 <sup>a</sup>	0.644	0.001 <sup>a</sup>	0.001 <sup>a</sup>
Cytoplasmic staining				
U	2,822	2,863	2,531	3,195
P-value	0.008 <sup>a</sup>	0.029 <sup>a</sup>	0.001 <sup>a</sup>	0.315
Nuclear staining				
U	871	2,075	581	1,245
P-value	0.001 <sup>a</sup>	0.001 <sup>a</sup>	0.001 <sup>a</sup>	0.001 <sup>a</sup>
Cytoplasmic and nuclear staining				
U	1,162	2,365	871	1,535
P-value	0.001 <sup>a</sup>	0.001 <sup>a</sup>	0.001 <sup>a</sup>	0.001 <sup>a</sup>
Total staining				
U	1,535	2,739	1,245	1,909
P-value	0.001 <sup>a</sup>	0.007 <sup>a</sup>	0.001 <sup>a</sup>	0.001 <sup>a</sup>
Local recurrence				
U	830	2,033	539	1,203
P-value	0.001 <sup>a</sup>	0.001 <sup>a</sup>	0.001 <sup>a</sup>	0.001 <sup>a</sup>

<sup>a</sup>Statistically significant result. U, Mann-Whitney U value; MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1 antigen; TIL, tumor-infiltrating lymphocyte; ER, estrogen receptor; PR, progesterone receptor; R1, positive surgical margin.

Dako, Carpinteria, CA, USA). Slides were then washed with PBS-buffer and incubated for 90 min with multi-MAGE-A 57B, MAGE-A1 77B, MAGE-A10 3GA11 or NY-ESO-1 D8.38 undiluted supernatants at room temperature. After washing in PBS, bound primary antibodies were detected using biotinylated anti-mouse secondary antibody (EnVision FLEX, High pH Kit, catalogue number 8010; Dako, ready for use) for 45 min and visualized with diaminobenzidine as chromogen on Autostainer Link 48 (Dako). Slides were counterstained with hematoxylin, dehydrated, cleared and cover-slipped.

Melanomas and testicular tissues expressing CTAs from University Hospital for Tumors, Sisters of Mercy University Hospital Center (Zagreb, Croatia) were used as positive controls throughout the study, and healthy skin tissue and unstained tumor cells served as the negative control. The specimens were described as positive or negative for TIL according to their presence in the samples. The positive cells were scored in whole tumor at x200 magnification using a light microscope on selected slides. All samples were examined independently by three observers and any difference was resolved by a joint review.

Table IV. Multivariate logistic regression for cancer/testis antigens and histopathological parameters.

Parameters	Multi-MAGE-A	MAGE-A10	MAGE-A1	NY-ESO-1
Cytoplasmic staining				
P-value	0.363	0.427	0.006 <sup>a</sup>	0.181
OR	0.363	0.651	74.761	0.990
95% CI	0.041-3.223	0.226-1.877	3.344-1674.250	0.976-1.005
Nuclear staining				
P-value	0.996	0.873	0.997	0.425
OR	25.2x10 <sup>6</sup>	1.135	2.97x10 <sup>6</sup>	1.01
95% CI	0.000-0.000	0.234-5.370	0.000-0.000	0.986-1.034
Cytoplasmic and nuclear staining				
P-value	0.994	0.503	0.996	0.386
OR	28.3x10 <sup>-6</sup>	1.522	3.51x10 <sup>6</sup>	1.007
95% CI	0.000-0.000	0.445-5.206	0.000-0.000	0.991-1.075
Total staining				
P-value	0.936	0.163	0.043 <sup>a</sup>	0.111
OR	1.080	2.240	0.070	1.014
95% CI	0.180-6.450	0.720-65.930	0.005-0.91	0.628-6.79
Tumor size				
P-value	0.174	0.619	0.685	0.915
OR	3.258	0.788	0.617	1.065
95% CI	0.592-17.926	0.308-2.013	0.059-6.370	0.333-3.400
Tumor grade				
P-value	0.478	0.348	0.310	0.108
OR	1.830	1.733	4.298	0.169
95% CI	0.344-9.735	0.549-5.465	0.257-71.641	0.019-1.472
Central necrosis				
P-value	0.318	0.196	0.487	0.331
OR	2.096	1.877	2.185	0.542
95% CI	0.490-8.962	0.721-4.883	0.240-19.834	0.158-1.861
TILs				
P-value	0.089	0.106	0.067	0.328
OR	6.572	2.203	0.075	1.850
95% CI	0.750-57.578	0.845-5.740	0.004-1.195	0.539-6.346
ER				
P-value	0.253	0.066	0.991	0.741
OR	2.472	0.356	0.987	1.233
95% CI	0.524-11.658	0.118-1.070	0.100-9.663	0.355-4.273
PR				
P-value	0.149	0.088	0.851	0.929
OR	2.992	0.416	1.229	1.055
95% CI	0.675-13.263	0.152-1.138	0.141-10.687	0.323-3.448
R1				
P-value	0.575	0.604	0.894	0.344
OR	1.508	1.276	1.154	0.572
95% CI	0.357-6.361	0.507-3.214	0.139-9.591	0.180-1.817
Local recurrence				
P-value	0.997	0.71	0.999	0.996
OR	38.4x10 <sup>6</sup>	0.757	0.000	1.298
95% CI	0.000-0.000	0.146-3.705	-	8.35x10 <sup>-49</sup> -2.02x10 <sup>48</sup>

<sup>a</sup>Statistically significant result. MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1 antigen; OR, odds ratio; TIL, tumor-infiltrating lymphocyte; ER, estrogen receptor; PR, progesterone receptor; R1, positive surgical margin.

Table V. Associations between histopathological variables and staining ( $\chi^2$  test).

Parameters	ER	PR	R1	Cytoplasmic staining	Nuclear staining	Nuclear and cytoplasmic staining	Total staining
Tumor size	0.224	0.088	0.449	0.174	0.167	0.546	0.821
Tumor grade	0.014 <sup>a</sup>	0.002 <sup>a</sup>	0.175	0.968	0.254	0.119	0.479
Central necrosis	0.004 <sup>a</sup>	<0.001 <sup>a</sup>	0.072	0.101	0.834	0.016 <sup>a</sup>	0.163
TILs	0.003 <sup>a</sup>	0.029 <sup>a</sup>	0.413	0.138	0.621	0.073	0.623
ER	-	-	-	0.012 <sup>a</sup>	0.797	0.003 <sup>a</sup>	0.007 <sup>a</sup>
PR	-	-	-	0.029 <sup>a</sup>	0.447	0.010 <sup>a</sup>	0.012 <sup>a</sup>
R1	0.726	0.797	-	0.053	0.572	0.081	0.038 <sup>a</sup>
Local recurrence	0.395	0.753	0.308	0.171	0.367	0.005 <sup>a</sup>	0.085

<sup>a</sup>Statistically significant result. ER, estrogen receptor; PR, progesterone receptor; R1, positive surgical margin; TIL, tumor-infiltrating lymphocyte.

**Scoring.** Multi-MAGE-A, MAGE-A1, MAGE-A10 and NY-ESO-1 staining results were scored using the Allred scoring system (18). 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). If the expression of CTAs was detectable in <10% of tumor cells it was scored as 1, in 10-50% of tumor cells it was scored as 2, or in >50% of tumor cells it was scored as 3. Score 0 was attributed to negative samples. The percentage of positive cells was then multiplied by the intensity of staining, 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction and the final score ranged between 0 (no staining) and 9 (diffuse and strong staining). The final results were further classified as 0 (no staining), 1 (score 1, 2 or 3), 2 (score 4, 5 or 6) and 3 (score 7, 8 or 9). Staining was considered positive (score 2 or 3) where all or a majority of the tumor cells were stained.

**Statistical analysis.** Data were analyzed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA), MedCalc (version 18.2.1; MedCalc Software bvba, Ostend, Belgium) and IBM SPSS Statistics (version 21.0; IBM Corp., Armonk, NY, USA). Descriptive statistics were calculated for the expression of proteins from the MAGE family (multi-MAGE-A, MAGE-A1 and MAGE-A10), NY-ESO-1, standard histopathological parameters for DCIS (tumor size, tumor grade, expression of ER and PR, necrosis, margin and TILs) and local recurrence. Cutoffs were established by receiver-operating characteristic curve analysis. Associations between variables were analyzed with the Mann Whitney U signed rank test and with Fisher's exact test. The  $\chi^2$  test was used for the analysis of associations between histopathological variables, staining and TILs. Furthermore, a multivariate logistic regression model was used to predict the effect of a series of variables (antigens) on a binary response variable (histopathological parameters for DCIS).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Patient population.** This retrospective study included a total of 83 patients who were diagnosed with DCIS and underwent surgery between January 2007 and December 2014. Data on tumor size, tumor grade, expression of ER and PR, necrosis,

margin, TILs, and expression of CTAs multi-MAGE-A, MAGE-A1, MAGE-A10 and NY-ESO-1 in samples are summarized in Table I. Antigens MAGE-A1, multi-MAGE-A, NY-ESO-1 and MAGE-A10 were expressed in 93, 84, 73 and 49% of cases, respectively (Table I). The cutoff values for the detection of ER, PR and the CTAs were calculated (Table II). Since expression of CTAs has been previously detected in different intracellular locations (19), the present study focused on three different staining patterns, namely nuclear, cytoplasmic, and simultaneous cytoplasmic and nuclear expression of multi-MAGE-A, MAGE-A1, MAGE-A10 and NY-ESO-1 in breast DCIS cells (Fig. 1). This specifies what was focused on, but not what was found.

**Associations between multi-MAGE-A, MAGE-A1, MAGE-A10 and NY-ESO-1 expression and histopathological parameters of DCIS.** All the tested antigens exhibited associations with histopathological parameters for DCIS, and they all demonstrated statistically significant associations with nuclear staining, simultaneous cytoplasmic and nuclear staining, and local recurrence (Table III). Antigen MAGE-A10 was significantly associated with higher expression of ER ( $P=0.005$ ), higher tumor grade ( $P=0.001$ ) and cytoplasmic staining ( $P=0.029$ ), and antigen NY-ESO-1 with larger tumor size ( $P=0.001$ ), expression of TILs ( $P=0.001$ ) and R1 resection ( $P=0.001$ ). The multivariate logistic regression model, antigen MAGE-A1 expression demonstrated a significant association with cytoplasmic staining ( $P=0.006$ , Table IV). The association between the subcellular expression pattern of CTA and histopathological parameters was analyzed. The  $\chi^2$  test identified a significant association between the cytoplasmic staining pattern and the expression of ER ( $P=0.012$ ) and PR ( $P=0.029$ ). It also identified a significant association between local recurrence and cytoplasmic and nuclear CTA expression pattern in breast DCIS cells ( $P=0.005$ ). Simultaneous cytoplasmic and nuclear staining was also significantly associated with central necrosis ( $P=0.016$ ), and the expression of ER ( $P=0.003$ ) and PR ( $P=0.010$ ) (Table V). The analysis of association between cytoplasmic staining, nuclear staining, cytoplasmic and nuclear staining, and total staining with histopathological variables showed similar results (Table VI).

Table VI. Cytoplasmic staining, nuclear staining, cytoplasmic and nuclear staining, and total staining in association with histopathological variables.

Parameters	n (%)	Cytoplasmic staining			Nuclear staining			Cytoplasmic and nuclear staining			Total staining		
		Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value
Tumor size, mm													
<1.2	50 (60)	14 (28)	36 (72)	0.131	47 (94)	3 (6)	0.158	42 (84)	8 (16)	0.373	36 (72)	14 (28)	0.505
>1.2	33 (40)	14 (42)	19 (58)		28 (85)	5 (15)		26 (79)	7 (21)		23 (70)	10 (30)	
Tumor grade													
1	18 (22)	6 (33)	12 (67)	0.601	15 (83)	3 (17)	0.234	17 (94)	1 (6)	0.107	14 (78)	4 (22)	0.347
2, 3	65 (78)	22 (34)	43 (66)		60 (92)	5 (8)		51 (78)	14 (22)		45 (69)	20 (31)	
Central necrosis													
-	34 (41)	8 (24)	26 (76)	0.079	31 (91)	3 (9)	0.574	32 (94)	2 (6)	0.014 <sup>a</sup>	27 (79)	7 (21)	0.125
+	49 (59)	20 (41)	29 (59)		44 (90)	5 (10)		36 (74)	13 (26)		32 (65)	17 (35)	
TILs													
-	45 (54)	12 (27)	33 (73)	0.106	40 (89)	5 (11)	0.456	40 (89)	5 (11)	0.066	33 (73)	12 (27)	0.401
+	38 (46)	16 (42)	22 (58)		35 (92)	3 (8)		28 (74)	10 (26)		26 (68)	12 (32)	
Multi-MAGE-A													
-	13 (16)	5 (38)	8 (62)	0.461	13 (100)	0 (0)	0.239	13 (100)	0 (0)	0.059	32 (82)	7 (18)	0.555
+	70 (84)	23 (33)	47 (67)		62 (89)	8 (11)		55 (79)	15 (21)		27 (61)	17 (39)	
MAGE-A10													
-	42 (51)	13 (31)	19 (69)	0.378	39 (93)	3 (7)	0.343	37 (88)	5 (12)	0.116	33 (79)	9 (21)	0.100
+	41 (49)	15 (37)	26 (63)		36 (88)	5 (12)		31 (76)	10 (24)		26 (63)	15 (37)	
MAGE-A1													
-	6 (7)	5 (83)	1 (17)	0.015 <sup>a</sup>	6 (100)	0 (0)	0.533	6 (100)	0 (0)	0.290	3 (50)	3 (50)	0.229
+	77 (93)	23 (30)	54 (70)		69 (90)	8 (10)		62 (80)	15 (20)		56 (73)	21 (27)	
NY-ESO-1													
-	22 (27)	7 (32)	15 (68)	0.522	22 (100)	0 (0)	0.075	20 (91)	2 (9)	0.171	17 (77)	5 (23)	0.324
+	61 (73)	21 (34)	40 (66)		53 (87)	8 (13)		48 (79)	13 (21)		42 (69)	19 (31)	
ER													
-	24 (29)	13 (54)	11 (46)	0.013 <sup>a</sup>	22 (92)	2 (8)	0.579	15 (62)	9 (38)	0.006 <sup>a</sup>	12 (50)	12 (50)	0.008 <sup>a</sup>
+	59 (71)	15 (25)	44 (75)		53 (90)	6 (10)		53 (90)	6 (10)		47 (80)	12 (20)	
PR													
-	31 (37)	15 (48)	16 (52)	0.027 <sup>a</sup>	29 (93)	2 (7)	0.364	21 (68)	10 (32)	0.012 <sup>a</sup>	17 (55)	14 (45)	0.012 <sup>a</sup>
+	52 (63)	13 (25)	39 (75)		46 (88)	6 (12)		47 (90)	5 (10)		42 (81)	10 (19)	



Table VI. Continued.

Parameters	Cytoplasmic staining			Nuclear staining			Cytoplasmic and nuclear staining			Total staining			
	n (%)	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value
R1													
-	39 (47)	9 (23)	30 (77)	0.044 <sup>a</sup>	36 (92)	3 (8)	0.427	35 (90)	4 (10)	0.071	32 (82)	7 (18)	0.032 <sup>a</sup>
+	44 (53)	19 (43)	25 (57)		39 (89)	5 (11)		33 (75)	11 (25)		27 (61)	17 (39)	
Local recurrence													
-	76 (92)	24 (32)	52 (68)	0.170	68 (89)	8 (11)	0.478	65 (85)	11 (15)	0.018 <sup>a</sup>	56 (74)	20 (26)	0.103
+	7 (8)	4 (57)	3 (43)		7 (100)	0 (0)		3 (43)	4 (57)		3 (43)	4 (57)	

<sup>a</sup>Statistically significant result. TIL, tumor-infiltrating lymphocyte; MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1 antigen; ER, estrogen receptor; PR, progesterone receptor; R1, positive surgical margin.

Additional analysis of the association between the expression of CTA and standard histopathological parameters with Fisher's exact test indicated that expression of MAGE-A10 antigen was associated with TILs (P=0.05; Table VII). The additional analysis of TILs and histopathological variables indicated a significant association with tumor grade (P=0.023), central necrosis (P<0.001) and expression of ER (P=0.003) and PR (P=0.029) (Table VIII). Multivariate logistic regression models indicated an association between TILs and tumor size (P=0.038), tumor grade (P=0.030), central necrosis (P=0.001), and expression of ER (P=0.005) and PR (P=0.031) (Table IX).

## Discussion

The role of CTAs has not been studied as extensively in DCIS as it has in invasive breast cancer. A small number of studies have analyzed the expression of CTAs in DCIS, but none have analyzed the associations between the expression of CTAs and histopathological predictive variables. In the current study, associations between histopathological predictive variables and the expression of CTAs from the MAGE family (multi-MAGE-A, MAGE-A1 and MAGE-A10) and NY-ESO-1 were evaluated. Caballero *et al* (10) recently reported that antigen NY-ESO-1 is a predictor of good prognosis in patients with DCIS. In this study, NY-ESO-1 was predominantly expressed in ER-negative DCIS and patients who expressed NY-ESO-1 antigen did not suffer from recurrence over a 10-year period. Therefore, it was concluded that NY-ESO-1 has a 'protective effect' and is expressed in patients who will not subsequently develop invasive breast cancer.

In the present study, all examined antigens were demonstrated to be associated with histopathological predictive variables of DCIS. Different staining patterns (cytoplasmic, nuclear, or cytoplasmic and nuclear) and nuclear protein expression of MAGE-A family and NY-ESO-1 CTAs were observed. All tested antigens were significantly associated with nuclear staining, simultaneous cytoplasmic and nuclear staining, and local recurrence. Using the multivariate logistic regression model, antigen MAGE-A1 expression demonstrated a significant association with cytoplasmic staining (P=0.006). A similar staining pattern for MAGE-A antigen has been reported previously in DCIS and in invasive breast cancer, as well as in other malignant tumors (9,12,20). Notably, simultaneous cytoplasmic and nuclear staining was significantly associated with local recurrence, central necrosis, and the expression of ER and PR. This was also previously observed in head and neck carcinoma, where simultaneous cytoplasmic and nuclear protein expression of MAGE-A family and NY-ESO-1 CTAs represented an independent marker for poor survival (19). In the present study, antigen MAGE-A10 was revealed to be significantly associated with higher expression of ER and higher tumor grade, and antigen NY-ESO-1 with higher tumor size, expression of TILs and R1 resection. Antigen NY-ESO-1 was predominantly expressed in ER-negative DCIS, which is consistent with the results from a previous study (10). An association between the expression of MAGE-A10 and TILs was also observed (P=0.05). In the current analysis of TILs and their significance in DCIS, a significant association was identified between TILs and tumor grade, central

Table VII. Fisher's exact test results of the expression of cancer/testis antigens against histopathological variables.

Parameters	Multi-MAGE-A			MAGE-A10			MAGE-A1			NY-ESO-1			
	n (%)	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value	Negative, n (%)	Positive, n (%)	P-value
Tumor size, mm													
<1.2	50 (60)	10 (20)	40 (80)	0.151	25 (50)	25 (50)	0.536	4 (8)	46 (92)	0.550	14 (28)	36 (72)	0.453
>1.2	33 (40)	3 (9)	30 (91)		17 (51)	16 (49)		2 (6)	31 (94)		8 (24)	25 (76)	
Tumor grade													
1	18 (22)	4 (22)	14 (78)	0.297	11 (61)	7 (39)	0.230	2 (11)	16 (89)	0.387	3 (17)	15 (83)	0.226
2, 3	65 (78)	9 (14)	56 (86)		31 (48)	34 (52)		4 (6)	61 (94)		19 (29)	46 (71)	
Central necrosis													
-	34 (41)	8 (23)	26 (77)	0.092	21 (62)	13 (68)	0.070	4 (12)	30 (88)	0.184	9 (26)	25 (74)	0.600
+	49 (59)	5 (10)	44 (90)		21 (43)	28 (57)		2 (4)	47 (96)		13 (26)	36 (74)	
TILs													
-	45 (54)	10 (22)	35 (78)	0.067	27 (60)	18 (40)	0.050	3 (7)	42 (93)	0.578	14 (31)	31 (69)	0.217
+	38 (46)	3 (8)	35 (92)		15 (40)	23 (60)		3 (8)	35 (92)		8 (21)	30 (79)	
ER													
-	24 (29)	5 (21)	19 (79)	0.303	9 (37)	15 (63)	0.100	2 (8)	22 (92)	0.562	7 (29)	17 (71)	0.462
+	59 (71)	8 (14)	51 (86)		33 (56)	26 (44)		4 (7)	55 (93)		15 (25)	44 (75)	
PR													
-	31 (37)	7 (23)	24 (77)	0.152	13 (42)	18 (58)	0.161	3 (10)	28 (90)	0.399	9 (29)	22 (71)	0.438
+	52 (63)	6 (11)	46 (89)		29 (56)	23 (44)		3 (6)	49 (94)		13 (25)	39 (75)	
R1													
-	39 (47)	7 (18)	32 (82)	0.405	21 (54)	18 (46)	0.368	3 (8)	36 (92)	0.603	9 (23)	30 (77)	0.339
+	44 (53)	6 (14)	38 (86)		21 (48)	23 (52)		3 (7)	41 (93)		13 (29)	31 (71)	
Local recurrence													
-	76 (92)	13 (17)	63 (83)	0.289	39 (51)	37 (49)	0.486	6 (8)	70 (92)	0.579	22 (29)	54 (71)	0.105
+	7 (8)	0 (0)	7 (100)		3 (43)	4 (57)		0 (0)	7 (100)		0 (0)	7 (100)	

MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1 antigen; TIL, tumor-infiltrating lymphocyte; ER, estrogen receptor; PR, progesterone receptor; R1, positive surgical margin.

Table VIII. Analysis of associations between tumor-infiltrating lymphocytes and histopathological variables ( $\chi^2$  test).

Parameters	P-value
Tumor size	0.618
Tumor grade	0.023 <sup>a</sup>
Central necrosis	<0.001 <sup>a</sup>
ER	0.003 <sup>a</sup>
PR	0.029 <sup>a</sup>
R1	0.413
Local recurrence	0.528

<sup>a</sup>Statistically significant result. ER, estrogen receptor; PR, progesterone receptor; R1, positive surgical margin.

Table IX. Multivariate logistic regression analysis of tumor-infiltrating lymphocytes against histopathological variables.

Parameters	P-value	OR	95% CI
Tumor size	0.038 <sup>a</sup>	0.3	0.1-0.9
Tumor grade	0.030 <sup>a</sup>	3.9	1.1-12.9
Central necrosis	0.001 <sup>a</sup>	8.8	3.0-25.4
ER	0.005 <sup>a</sup>	0.2	0.1-0.6
PR	0.031 <sup>a</sup>	0.4	0.1-0.9
R1	0.413	1.4	0.6-3.4
Local recurrence	0.532	1.6	0.3-7.9

<sup>a</sup>Statistically significant result. OR, odds ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; R1, positive surgical margin.

necrosis, and negative ER and PR status. These findings are consistent with previous studies (21-23).

In summary, associations between CTAs from the MAGE family (MAGE-A1, multi-MAGE-A and MAGE-A10) and NY-ESO-1, and histopathological predictive variables of DCIS, were revealed. An association was also observed between the MAGE-A10 antigen and the presence of TILs. These results indicate that MAGE-A10 and NY-ESO-1 may serve a function in DCIS and could present a potential target for a novel treatment strategy. Additional analysis in a larger group of patients will be required to evaluate this further. Simultaneous cytoplasmic and nuclear protein expression of MAGE-A family and NY-ESO-1 CTAs may represent an independent marker for local recurrence. In conclusion, CTAs are not perfect indicators of invasiveness for DCIS, but in combination with other histopathological predictive variables, they could inform treatment strategies for patients. However, the present study was small and fresh-frozen tissue samples were not available, therefore the additional analysis on mRNA and protein level was not performed. Further larger studies are warranted to expand the cohort of patients under investigation and further support the present data at the gene expression level.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

AR, GS and AJ analyzed and interpreted the patient data regarding the expression of CTAs from the MAGE family (multi MAGE-A, MAGE-A1 and MAGE-A10) and NY-ESO-1 in DCIS, and their association with standard histopathological parameters for DCIS [tumor size, tumor grade, expression of ER and progesterone receptor, necrosis and margin. and local recurrence. BS performed the histological examination of the samples. MB contributed in statistical analysis of data. LBO read and revised the article, and contributed to data interpretation and conception of the present study. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study received ethical approval from the Sisters of Mercy University Hospital Center. Written informed patient consent was received.

## Patient consent for publication

Patients provided their consent for the publication of any data/associated images.

## Competing interests

The authors declare that they have no competing interests.

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