Expression of MAGE-A11 in breast cancer tissues and its effects on the proliferation of breast cancer cells

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Abstract. The melanoma-associated antigen (*MAGE*) genes are commonly expressed in tumors, but the function of many of these genes remains unknown. Here, we investigated the expression of one family member, MAGE-A11, in breast cancer tissues and corresponding adjacent normal tissues. MAGE-A11 expression was assessed in breast cancer tissues and adjacent normal tissues from 100 women by immunohistochemistry. MAGE-A11 was significantly more highly expressed in breast tumors (56% of samples) compared to normal tissues (0%; P<0.05). Additionally, its expression in breast tumors was investigated in relation to various clinicopathological features, including patient age, tumor stage and volume, and lymph node metastasis. MAGE-A11 protein expression was correlated with expression of the human epidermal growth factor receptor-2 (HER-2) and estrogen receptor (ER)-β (P<0.05), but not with age, pathological type, histological grade, clinical stage, tumor size or lymph node metastasis, or ER-α, progesterone receptor (PR) or amplified in breast cancer 1 (AIB-1) expression. To determine how its expression affects cancer cell proliferation *in vitro*, MCF-7 human breast cancer cells were transfected with pCMV-AC-MAGE-A11-GFP. MTT colorimetry and colony-forming assays indicated that MAGE-A11 overexpression significantly increased breast cancer cell proliferation and the ability to form colonies (P<0.05). These findings indicate that MAGE-A11, similarly to HER-2 and ER-β, may be an important diagnostic or prognostic indicator in breast cancer and potentially promotes tumor proliferation.

Introduction

In 1991 Van der Bruggen *et al* (1) isolated the first melanoma-associated antigen gene (*MAGE*) from melanoma cells using gene transfection. This gene, *MAGE-A1*, was identified as a testis tumor antigen gene. To date, more than 30 *MAGE* family members have been discovered, including *MAGE-A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *L2*, *Necidin*, *I* and *J*. Proteins in the MAGE family share an amino acid sequence containing a MAGE homology domain (MHD) and may be subdivided into groups I and II according to their protein expression patterns. Group I proteins, including MAGEs A, B and C, are expressed in many types of tumor tissues, but are not expressed in normal tissues, with the exception of placenta and adult testis (2).

MAGE-A encodes a tumor-specific antigen that is highly expressed (3-7) in the majority of tumor tissues. Currently, the MAGE-A subfamily includes MAGE-A1 through A15. All known genes of this subfamily are localized on chromosome Xq28. Each has a length of approximately 4.5 kb, contains three exons and encodes a protein approximately 309-319 amino acids long (369 amino acids for MAGE-A10) (8). The majority of the studies on this subfamily have focused on MAGE-A1 and MAGE-A3; particularly their involvement in tumorigenesis and their potential as therapeutic targets. The expression patterns and functions of most MAGE-A family members remain unclear, but it is likely that some of them also promote tumor development. Indeed, recent evidence demonstrates expression of both MAGE-A10 and MAGE-A11 in breast tumors (9).

To clarify the expression and role of MAGE-A11 in breast cancer, we compared MAGE-A11 protein expression by immunohistochemistry in breast cancer tissues and tumor-adjacent normal tissues. Further, MAGE-A11 expression was evaluated for potential correlations with the clinicopathological features of breast carcinomas. Additionally, we extended recent findings (9) by transfecting breast cancer cell lines with MAGE-A11 in order to determine the effect of its expression on tumor proliferation.

Materials and methods

Research participants. Tissues were collected from 100 patients who were undergoing surgery to remove breast tumors at The Affiliated Hospital of Yancheng Health Vocational and Technical College, Yancheng, China, between 2010 and 2011. Samples included cancer tissues and corre-

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sponding tumor-adjacent tissues (>5 cm from cancer tissue edge). Fine needle aspiration cytology (FNAC) and frozen section (FS) were performed prior to surgery in order to confirm cancer. Patients had not received any radiotherapy, chemotherapy or endocrine therapy. The mean age of patients was 53.9±9.6 years, with ages ranging from 32-70 years. Samples included 78 cases of invasive ductal carcinoma and 22 cases of invasive lobular carcinoma; there were 4 cases of grade I, 69 cases of grade II and 27 cases of grade III cancer. TNM staging was performed according to the UICC in 1997: 16 cases were in stage I, 66 in stage II and 18 in stage III (10). Other tumor characteristics that were evaluated included tumor volume (28 cases <2 cm^3, 24 cases 2-5 cm^3 and 48 cases >5 cm^3) and metastasis (43 cases with lymph node metastasis, 57 without). Additionally, expression of breast cancer markers was investigated; these included estrogen receptor α (ER-α) (66 cases were positive), ER-β (44 cases were positive), human epidermal growth factor receptor 2 (HER-2) (51 cases were negative, 49 were positive), progestational hormone receptor (PR) (52 cases were positive) and amplification in breast cancer 1 (AIB-1) (54 cases were positive).

Experimental methods

Materials. Human breast cancer MCF-7 cell strains were purchased from Kunming Cell Bank (China Academy of Sciences). Lipofectamine 2000 transfection kits were purchased from Invitrogen (Carlsbad, CA, USA). Transfection plasmids (pCMV-AC-GFP) were purchased from Origene Technologies, Inc. (Rockville, MD, USA). Rabbit anti-human MAGE-A11 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and immunohistochemistry kits and DAB chromogenic agents were purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Rabbit anti-human MAGE-A11 polyclonal antibodies or rabbit anti-human β-actin (internal reference) monoclonal antibodies were purchased from Sigma (St. Louis, MO, USA). Electrochemistry light-emitting reagents were from Thermo Scientific (Rockford, IL, USA). The study was approved by the ethics committee of Yancheng Health Vocational and Technical College, Yancheng, China.

Immunohistochemistry. MAGE-A11 expression was detected in breast cancer sections by immunohistochemistry using the streptavidin-biotin-horseradish peroxidase complex method (SP method). Briefly, tissues were formalin-fixed, dehydrated and paraffin-embedded for sectioning at 4 μm. Tissue slices were dewaxed and rehydrated for antigen repair at a high temperature in a microwave. Cooled sections were treated with 3% hydrogen peroxide solution to block endogenous peroxidase activity, sealed with non-specific serum, then placed in a wet box and incubated at room temperature. Primary antibodies were added to the wet box, and sections were incubated at 4˚C overnight. Biotin-labeled rabbit anti-human MAGE-A11 polyclonal antibodies were added as the secondary antibody and placed at room temperature. Following three washes in PBS, streptococcus avidin-peroxidase was added to sections for incubation for 30 min at 37˚C. After washing with PBS three times, DAB chromogen was applied according to the manufacturer's instructions in order to develop color. Sections were counterstained with hematoxylin, dehydrated and mounted. Known positive tissue slices were used as a positive control and PBS was used as a negative control in place of primary antibodies.

Sections were examined under a light microscope. Protein staining appeared as yellow to brown granules in the cytoplasm and nucleus. To assess protein expression, 10 visual fields were selected at high power. Staining intensity was divided into four classes: uncolored (a score of 0), pale yellow (a score of 1), yellow (a score of 2) and brown-yellow (a score of 3). Additionally, staining frequency was divided into five classes according to the proportion of positively-stained cancer cells out of all tumor cells: ≤5% positive was scored as 0, 6-25% was scored as 1, 26-50% as 2, 51-75% as 3 and ≥76% as 4. Scores for staining intensity and staining frequency were added for each case; total scores of 0 were considered (-), total scores of 1-2 as (+), total scores of 3-5 as (++) and total scores of 6-7 as (+++).

Cells and cell culture. Human breast cancer MCF-7 cells were cultured in RPMI-1640 culture solution containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 8% $NaHCO_3$ at 37˚C under 5% CO_2 conditions.

MAGE-A11 transfection. pCMV-AC-MAGE-A11-GFP expression plasmid and null vector pCMV-AC-GFP (control) were transfected into MCF-7 cells during exponential growth, according to the methods included with the Lipofectamine 2000 transfection kits. Subsequent experiments were performed 48 h later.

Western blotting. MCF-7 cells were collected 48 h following transfection and washed twice with PBS. Suspensions were centrifuged and supernatants were discarded. Cell pellets were sonicated in order to quantify the total proteins with bicinchoninic acid (BCA). Samples of total protein (30 μg/well) were loaded on a 10% gel for SDS-PAGE. Proteins were transferred to polyvinylidene difluoro ethylene film (PVDF). The membrane was washed with 5% non-fat milk powder at 4˚C overnight. Rabbit anti-human MAGE-A11 polyclonal antibody (1:1000) or rabbit anti-human β-actin (reference control) monoclonal antibody were added and incubated with the membrane at room temperature for 1 h. After the membrane was washed three times in PBS, 1:3000 horseradish peroxidase-labeled anti-rabbit secondary antibody was added at room temperature for 1 h. Additional PBS washes were performed prior to the addition of the electrochemical luminescence reagent. Infrared fluorescence scan imagery was used to develop and analyze blots. The relative molecular mass of MAGE-A11 protein carrying green fluorescent protein (GFP) labels was $7x10^4$. The experiment was repeated three times.

MTT colorimetry. MCF-7 cells were inoculated in 6 cm petri dishes. At 60% confluence, cells were transfected with the pCMV-AC-MAGE-A11-GFP expression plasmid or null vector (pCMV-AC-GFP) as mentioned previously. Forty-eight hours after transfection, cells were inoculated in 96-well plates at a density of 5000 cells/well and incubated overnight to promote adherence. Adherent cells were continuously cultured for 0, 24 or 48 h; at the end of each time point, 10 μl of MTT (100 mg/ml) was added per well. Subsequently, absorbance (OD) values were measured for each well at a 490-nm wavelength using a Thermo Labsystems colorimeter. Proliferation rates were calculated 24 and 48 h after cell adherence according to the following formula: cellular

Table I. Expression of MAGE-A11 in breast cancer and adjacent normal tissue [n (%)].

Groups	n	-	+	++	+++
Breast cancer tissues	100	44 (44.0)	23 (23.0)	20 (20.0)	13 (13.0)
Adjacent normal tissues	100	100 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	200	144 (72.0)	23 (11.5)	20 (10.0)	13 (6.5)

MAGE, melanoma-associated antigen. χ^2=77.778, P=0.001.

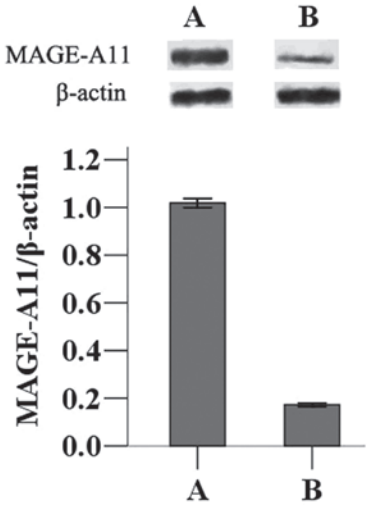

Figure 1. Expression of MAGE-A11 protein in MCF-7 cells by western blotting. (A) pCMV-AC-MAGE-A11-GFP and (B) pCMV-AC-GFP. MAGE, melanoma-associated antigen.

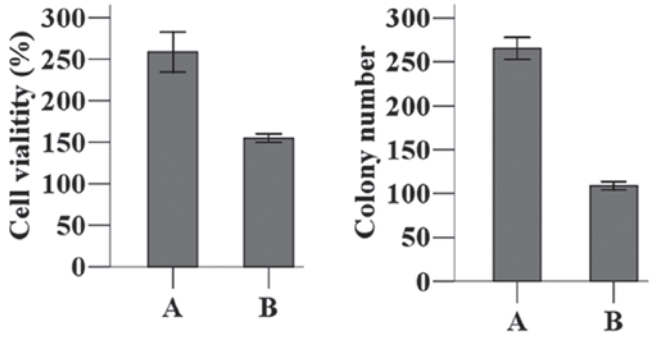

Figure 2. Promoting effects of MAGE-A11 on proliferation of MCF-7 cells. (A) pCMV-AC-MAGE-A11-GFP and (B) pCMV-AC-GFP. MAGE, melanoma-associated antigen.

proliferation rate = average OD value in experimental group / average D value of 0 h group x 100%. The experiment was repeated three times.

Colony-forming assay. MCF-7 cells were inoculated in 6-cm petri dishes. At 60% confluence, cells were transfected with pCMV-AC-MAGE-A11-GFP expression plasmid or the null vector pCMV-AC-GFP, as mentioned previously. Forty-eight hours later, cells were inoculated in 10-cm petri dishes at a density of 3000 cells/dish and incubated overnight for adherence. G418 was added at a final concentration of 600 pg/ml for selection; the solution was replaced once a week. Two weeks later, Wright-Giemsa staining was performed and cell colonies (diameter >1 mm) were counted. The experiment was repeated three times with three culture dishes per group; the mean number of colonies was determined.

Statistical methods. SPSS 17.0 statistical software was used for statistical analysis. A χ^2 test was used to compare expression of MAGE-A11 protein; for measurement data, two-tailed Student's t-test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

MAGE-A11 expression in breast cancer and corresponding tumor-adjacent tissue. Immunohistochemistry of MAGE-A11 in breast cancer and normal adjacent tissues showed that, of 100 cases, the protein was expressed in 56.0% of breast cancer samples and 0.0% of normal adjacent tissues (Table I). This difference in expression rate was significantly different (P<0.05).

MAGE-A11 expression and clinicopathological features of breast cancer. MAGE-A11 expression in breast tumors was related to expression of both HER-2 and ER-β (P<0.05), but there were no correlations with patient age, pathology, differentiation, clinical staging, tumor size or lymphatic metastasis, or ER-α, PR or AIB-1 expression (Table II).

Effect of MAGE-A11 in transfected MCF-7 cells. The MAGE-A11 gene was transfected into MCF-7 cells to determine the effects of its expression on tumor progression. Following transfection of the pCMV-AC-MAGE-A11-GFP expression plasmid or the pCMV-AC-GFP control plasmid, western blot analysis was used to measure expression in MCF-7 cells. Cells transfected with pCMV-AC-MAGE-A11-GFP displayed higher expression of MAGE-A11 than those transfected with the control plasmid (Fig. 1).

Subsequently, MTT colorimetry was used to determine proliferation rates of MCF-7 cells transfected with either the MAGE-A11 expression construct or the control plasmid. Transfection of pCMV-AC-MAGE-A11-GFP expression plasmid resulted in a significantly higher proliferation rate than did transfection of the null vector (P<0.05). Furthermore, a colony-forming assay demonstrated that, after transfecting the pCMV-AC-MAGE-A11-GFP expression plasmid, MCF-7 cells formed significantly more colonies than cells not overexpressing MAGE-A11 (Fig. 2; P<0.05).

Table II. Correlation between expression of MAGE-A11 protein and clinicopathological parameters in breast cancer [n (%)].

Clinicopathological characteristics	n	-	+	++	+++	χ^2	P-value
Age (years)							
<55	53	26 (49.1)	12 (22.6)	7 (13.2)	8 (15.1)	3.643	0.303
≥55	47	18 (38.3)	11 (23.4)	13 (27.7)	5 (10.6)		
Pathological type							
Invasive ductal carcinoma	78	34 (43.6)	17 (21.8)	16 (20.5)	11 (14.1)	0.616	0.893
Lobular carcinoma	22	10 (45.5)	6 (27.3)	4 (18.2)	2 (9.1)		
Histological grade							
Grade I	4	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	8.159	0.227
Grade II	69	31 (44.9)	17 (24.6)	14 (20.3)	7 (10.1)		
Grade III	27	9 (33.3)	6 (22.2)	6 (22.2)	6 (22.2)		
TNM staging							
Stage I	16	10 (62.5)	3 (18.8)	3 (18.8)	0 (0.0)	5.089	0.532
Stage II	66	28 (42.4)	16 (24.2)	12 (18.2)	10 (15.2)		
Stage III	18	6 (33.3)	5 (22.2)	5 (27.8)	3 (16.7)		
Carcinoma diameter (cm^3)							
≤2	28	14 (50.0)	5 (17.9)	5 (17.9)	4 (14.3)	1.359	0.968
2-5	24	11 (45.8)	6 (25.0)	4 (16.7)	3 (12.5)		
≥5	48	19 (39.6)	12 (25.0)	11 (22.9)	6 (12.5)		
Lymph node metastasis							
Yes	34	15 (44.1)	9 (26.5)	6 (17.6)	4 (11.8)	1.177	0.759
No	66	29 (43.9)	14 (21.2)	14 (21.2)	9 (13.6)		
ER-α							
Negative	34	10 (40.0)	10 (40.0)	3 (12.0)	2 (8.0)	0.473	0.925
Positive	66	2 (13.3)	3 (20.0)	4 (26.7)	6 (40.0)		
ER-β							
Negative	56	35 (62.5)	13 (23.2)	5 (8.9)	3 (5.4)	23.421	0.001
Positive	44	9 (20.5)	10 (22.7)	15 (34.1)	10 (22.7)		
HER-2							
Negative	51	34 (66.7)	12 (23.5)	5 (9.8)	0 (0.0)	31.107	0.001
Positive	49	10 (20.4)	11 (22.4)	15 (30.6)	13 (26.5)		
PR							
Negative	48	19 (39.6)	11 (22.9)	11 (22.9)	7 (14.6)	0.980	0.806
Positive	52	25 (48.1)	12 (23.1)	9 (17.3)	6 (11.5)		
AIB-1							
Negative	46	16 (34.8)	10 (21.7)	14 (30.4)	6 (13.0)	6.342	0.096
Positive	54	28 (51.9)	13 (24.1)	6 (11.1)	7 (13.0)		

MAGE, melanoma-associated antigen; ER, estrogen receptor; HER-2, human epidermal growth factor receptor-2; PR, progesterone receptor; AIB-1, amplified in breast cancer 1.

Discussion

Many studies have described the expression of MAGE-A family members in tumors. However, the majority of these have relied on RT-PCR, gene microarray and RNA *in situ* hybridization (11,12), as the detection of specific protein members of this family is difficult. High conservation of protein sequences among MAGE-A family members creates challenges in the development of specific antibodies. The availability of a specific antibody for MAGE-A11 allowed us to perform protein expression studies of this family member.

We found that the majority of breast cancers expressed MAGE-A11, while none of the normal adjacent breast tissues expressed the protein. Therefore, MAGE-A11, similarly to other MAGEs, appears to be a tumor-specific antigen in breast cancer. Additionally, the expression of MAGE-A11 within

breast tumors was correlated with the expression of both HER-2 and ER-β. These results confirm recent findings (9). HER-2 expression is an important index of poor prognosis for breast cancer patients (13), therefore, MAGE-A11 expression may also indicate poorer prognosis for breast cancer patients. Similarly, ER is commonly used as diagnostic indicator for, and may help to guide the treatment of, breast cancer patients. For example, patients with ER-positive tumors often receive tamoxifen endocrine therapy (14). The correlation of ER-β expression with MAGE-A11 expression may indicate the usefulness of MAGE-A11 as a diagnostic indicator. However, the lack of correlation between MAGE-A11 and ER-α expression requires further research.

Our study builds on the previous report of MAGE-A11 expression in breast tumors by evaluating the effects of MAGE-A11 overexpression in breast cancer cells. Transfection of a GFP-MAGE-A11 construct into MCF-7 breast cancer cells resulted in increased proliferation of cells and higher rates of colony-formation. Previous research may hint at the mechanism by which MAGE-A11 promotes tumor cell proliferation. MAGE-A11 reportedly is capable of combining with a specific amino acid radical sequence FXXLF at the amino terminus of the androgen receptor (AR), increasing AR transcriptional activity (15) by phosphorylation and ubiquitination of the epithelial growth factor-dependent MAGE-A11. Overexpression of MAGE-A11 appears to promote the growth of prostate cancer by AR-dependent cell proliferation (16). A similar phenomenon may occur in breast and other tumors.

In conclusion, in breast cancer tissue expression of MAGE-A11 protein is related to the expression of HER-2 and ER-β. Expression of this tumor-specific antigen promotes proliferation of human breast cancer cells *in vitro*. These results indicate that MAGE-A11 may be a new treatment target for breast cancer due to its effects on the occurrence and development of breast cancer.

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