CTLA-4 blockade combined with 5-aza-2'-deoxycytidine enhances the killing effect of MAGE-A family common antigen peptide-specific cytotoxic T cells on breast cancer

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Abstract. Breast cancer is the leading cause of cancer-associated deaths in women. Combination immunotherapy attracts great interest as a treatment for breast cancer. However, there are no studies on the use of cytotoxic T-lymphocyte antigen 4 (CTLA-4) monoclonal antibody in combination with the melanoma-associated antigen A family (MAGE-As) co-antigen peptide (p248V9) for treating breast cancer, which should be explored. To this aim, in the present study, the samples of 115 patients with breast cancer were collected, and MAGE-As and CTLA-4 levels in breast cancer and adjacent normal tissues were assessed by immunohistochemical staining. The effect of 5-aza-2'-deoxycytidine (5DC) on the expression of MAGE-As in breast cancer cell lines was assessed by reverse transcription-quantitative PCR and western blot assay. Cytotoxic T cells (CTLs) were induced by MAGE-As co-antigen peptide. The specific lytic rate and IFN-γ level were examined by CCK-8 assay and ELISA, respectively. It was found that MAGE-As were highly expressed in breast cancer tissues. 5DC treatment promoted the expression of MAGE-As in breast cancer cells. The upregulation of the expression of MAGE-As specifically enhanced the ability of CTLs to kill breast cancer cells. CTLA-4 was highly expressed in breast cancer tissues and cells, and patients with breast cancer exhibiting high expression of CTLA-4 had low overall survival. CTLA-4 promoted the lytic efficiency of CTLs in breast cancer cells, and the combination of an anti-CTLA-4 antibody and 10 μM 5DC exhibited the highest cell lysis ability of CTLs. The present study demonstrated that MAGE-As co-antigen peptide-specific CTLs in combination with an anti-CTLA-4 monoclonal antibody and 5DC, have potent tumor cell-killing effects. It provides a novel theory for the development of breast cancer therapies.

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Introduction

Breast cancer is a disease that affects the health of women worldwide, and is the leading cause of cancer-associated deaths in women (1). The incidence of breast cancer has been increasing in China in recent years. It was estimated that there were 208,000 female patients with breast cancer in China in 2010. The incidence rate is highest in 50-year-old women, and the number of deaths due to breast cancer accounts for 9.6% of global deaths (2). Although surgery, radiotherapy, chemotherapy as well as other treatments have made significant progress, the clinical results remain frustrating. Numerous patients with breast cancer succumb to tumor recurrence or metastasis due to the systemic side effects of the current treatments (3). Tumor immunotherapies are different from traditional treatments, in which, the antitumor immune response of patients is activated to kill tumors. It is characterized by high specificity and minor side effects, which can help patients establish immune memory to exert a long-term 'monitoring' effect. Immunotherapy mainly utilizes the antigenicity of tumors; thus, finding a suitable antigen as a target is of importance to immunotherapy (4).

Cancer testis antigens (CTAs) are only expressed in tumor cells and germ cells (testis, ovarian and placental cells), while rarely expressed in somatic cells. This restricted expression makes them a promising tumor antigen of immunotherapy (5). Melanoma-associated antigen (MAGE) was identified by Professor Vander Bruggen, and belongs to the CTA subfamily (6). Among the numerous MAGE family members, the melanoma-associated antigen A family (MAGE-As) has energetical tumor antigen specificity, and can be recognized by autoimmune cells to induce specific antitumor humoral immunity and cellular immunity (7). The effect of antitumor immunotherapy depends on the specificity and expression level of tumor antigens (8). Breast cancer tissue can concurrently express multiple subtypes of the MAGE-A antigen, indicating that the MAGE-A antigen is a suitable target for breast cancer immunotherapy (9). However, the expression level of different subtypes of MAGE-A antigen

in breast cancer tissues is relatively low, which renders treatment based on tumor vaccines against specific MAGE-A antigen subtypes alone unable to induce strong cytotoxic T-cell antitumor responses (10). Increased expression of the MAGE-A antigen in tumor cells and identification of common antigenic peptides of MAGE-A antigen should be beneficial for the recognition and killing of tumor cells by the immune system. Our previous study revealed that demethylating drugs can increase the expression of MAGE-A11 in a breast cancer cell line, which enhanced the killing effects of CTLs on breast cancer (11). Therefore, the identification of additional drugs that can increase the expression of MAGE-As is necessary for immunotherapy against breast cancer.

At present, several studies have designed common antigen peptides (mostly polypeptides) of different subtypes of MAGE-A and verified their antitumor effectiveness in various types of tumor cells (12). However, a major disadvantage of polypeptides is their weak immunogenicity, which induces a weak immune response to $CD8^+$ T cells (13). Therefore, it is necessary to find novel approaches that can improve the immunogenicity of antigenic peptides to compensate the limitations of peptide immunity. The enhancement of immunogenicity of breast cancer by inducing an increase in the effect of T cell response and promotion of tumor suppressors is important in breast cancer immunotherapy. Notably, immunological checkpoint molecules are currently the most investigated, and promising treatment methods for a variety of tumors and their therapeutic effects have been demonstrated in animal experiments and in phase II and phase III clinical studies (14,15). The immunological checkpoint cytotoxic T-lymphocyte antigen 4 (CTLA-4) was found to inhibit dendritic cell (DC) function in breast cancer cells, which in turn rendered T cells unable to be effectively activated; thus, it greatly reduced tumor killing, and was one of the causes of cancer cell proliferation and metastasis (16). It has been suggested that breaking this immunosuppressive state may be useful for restoring DC and T-cell function (16).

To date, there are no studies using anti-CTLA-4 monoclonal antibody and MAGE-As common antigen peptide as a combination therapy for treating breast cancer. Therefore, in the present study, killing experiments on breast cancer were designed consisting of a co-antigen peptide of MAGE-A antigen combined with CTLA-4 blockade. Furthermore, the demethylating drug 5DC was used to pretreat cancer cells, which was found to increase the expression of MAGE-As and enhance the immunogenicity of the cells. Hence, MAGE-As co-antigen peptide-specific CTLs in combination with anti-CTLA-4 monoclonal antibody and 5DC have potent tumor cell killing effects, which provides a novel theory for the development of breast cancer therapies.

Materials and methods

Clinical samples. The breast cancer tissues and corresponding adjacent normal tissues of 115 patients (14 aged 30-40, 27 aged 40-50, 35 aged 50-60, 28 aged 60-70, 9 aged 70-80, 2 >80 years; 112 women and 3 men) with breast cancer who were hospitalized at the Fourth Hospital of Hebei Medical University (Shijiazhuang, Hebei, China) from 2012 to 2019 were collected. The patients were all onset for the first time, and no radiotherapy, chemotherapy or endocrine therapy were performed on the patients before this study. All patients were diagnosed with breast cancer by pathology. Pathological diagnosis and tumor staging criteria were performed in accordance with the standards of the World Health Organization and the Union for International Cancer Control. The present study was approved by the Ethics Committee of the Affiliated Hospital of Hebei Medical University. All patients involved in this study were informed of the experimental content, purpose and significance of the study, and signed the informed consent form.

Cell culture and treatment. MCF-7, MDA-MB-453, MDA-MB-231 and BT549 cells were provided by the Research Center of the Fourth Hospital of Hebei Medical University. Cells were cultured in RPMI-1640 medium (cat. no. A33823) containing 10% fetal bovine serum (FBS; cat. no. 16140071) and penicillin and streptomycin [P/S; penicillin, $5x10^5$ U/l; streptomycin 100 mg/l; (cat. no. 15070063; all from Thermo Fisher Scientific, Inc.] at 37˚C in a 5% CO_2 incubator.

For the culture of primary breast cancer cells, the adipose tissue and connective tissue around the breast cancer tissues were removed, and the breast cancer tissues were washed with PBS. The tissue was placed in P/S solution (penicillin, $5x10^5$ U/l; streptomycin 100 mg/l) for 20 min. Then, the tissues were cut into pieces (~1 mm^3) and placed into collagenase I solution ($2x10^5$ U/l) at 37˚C and 100 x g for 30 min. The dissociation solution was filtered through a 100-mesh cell sieve and centrifuged at 200 x g for 10 min, and then the supernatant was discarded. The precipitate was resuspended in RPMI-1640 medium supplemented with 5% FBS. Repeated differential adherence method was used to remove fibroblasts as thoroughly as possible and fibroblasts were collected as control cells according to a previous study (10).

For 5DC treatment, 2.5, 5 or 10 μM 5DC were added to the cell culture medium, and the cells were cultured at 37˚C for 72 h.

Immunohistochemical (IHC) staining. The tissue was embedded in paraffin and cut into 3-μm sections. The prepared tissue sections were placed in a 67˚C oven for 10 min. The tissue sections were immersed in xylene I and II for 15 min, and then sequentially placed in absolute ethanol I and absolute ethanol II for 20 min, and in 95 and 80% ethanol for 10 min. The sections were treated with high-pressure heat repair for 4 min, and then 6% methanol hydrogen peroxide solution was added dropwise on the sections, which were subsequently incubated at room temperature for 20 min in the dark. Next, 10% goat serum was added dropwise, and the sections were then incubated at 37˚C for 45 min. Incubation then took place with the primary antibody including anti-MAGE-A10 (1:500 dilution; product no. 81740) and anti-CTLA-4 (1:500 dilution; product no. 96399; both from Cell Signaling Technology, Inc.) overnight at 4˚C followed by incubation with the secondary antibody conjugated to HRP for DAB staining for 1 h at 37˚C. The nuclei were stained with hematoxylin and eosin (H&E) staining for 1 min. The slides were observed under an Olympus IX53 light microscope (magnification, x400; Olympus Corporation).

Ten fields were randomly selected from each section, and the percentage of positive cells and staining intensity were

evaluated, and the mean values were calculated. Positive cells were judged by cytoplasm or nucleus containing brownish yellow particles, and staining intensity scores were performed on positive cells. A score of 0 indicated no positive staining (similar to the background color); a score of 1 represented mild positive staining (light yellow); a score of 2 represented moderate positive staining (brownish yellow); and a score of 3 represented severe positive staining (tan). The positive cell percentage score was as follows: 0% corresponded to 0; 1-10% corresponded to 1; 11-50% corresponded to 2; and >51% corresponded to 3. The sum of the two scores was considered as the total score of the patient, and a total score <4 was defined as negative, while ≥4 was defined as positive.

Western blot assay. Total protein of breast cancer cells was extracted using RIPA lysis and extraction buffer (Thermo Fischer Scientific, Inc.). Quantification of proteins was performed by the Bradford protein concentration quantification method (Beyotime Institute of Biotechnology). In total, 50 μg proteins were electrophoresed on 10% SDS-PAGE, transferred to a PVDF membrane and blocked in 5% non-fat milk for 1 h. The membranes were then incubated with primary antibodies, anti-MAGE-A10 (1:1,000 dilution, product no. 81740) and anti-β-actin (1:5,000 dilution; product no. 4970; both from Cell Signaling Technology, Inc.) at 4˚C overnight. Then, the membranes were washed with PBS 3 times and incubated with secondary antibodies anti-rabbit IgG light chain (HRP) (1:10,000 dilution; product code ab99697; Abcam). The protein expression levels were measured with an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA of breast cancer cells was extracted using TRIzol (Thermo Fisher Scientific, Inc.). PrimeScript RT Reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd.) was used for RT. RT-qPCR was conducted using SYBR Green qPCR Master Mix Kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. GADPH was used as an internal standard. The RT-qPCR conditions for MAGE-As were as follows: 95˚C for 5 min, followed by 35 cycles at 95˚C for 30 sec, 65˚C for 30 sec and 72˚C for 30 sec, and 72˚C for 5 min. The RT-qPCR conditions for GAPDH were as follows: 95˚C for 5 min, followed by 22 cycles of 95˚C for 20 sec, 58˚C for 20 sec and 72˚C for 20 sec, and 72˚C for 5 min. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression level according to a previous study (17). The primers were as follows: MAGE-As forward, 5'-GTGGTCCTAAGATCT ACCAAGCA-3'; MAGE-As reverse, 5'-AGGGCAGCA GGTAGGAGTG-3'; GAPDH forward, 5'-AGCCACATCGCT CAGACAC-3' and GAPDH reverse, 5'-GCCCAATACGAC CAAATCC-3'.

MAGE-As-specific induction of cytotoxic T cells (CTLs). Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation at 400 x g for 30 min, and the middle white cell layer was aspirated after centrifugation. The cell concentration was adjusted to $1x10^6$ cells/ml with DC culture medium (RPMI-1640 medium containing 10% FBS, $5x10^5$ U/l penicillin and 100 mg/l streptomycin), and the cells were stimulated with IL-2 (50 ng/ml) and IL-17 (50 ng/ml) to become T cells. For DC induction, PBMCs were incubated with GM-CSF (100 ng/ml) and IL-4 (50 ng/ml), and their medium was changed every 12 h, followed by the addition of GM-CSF and IL-4. At 5 days post-culture, LPS (10 ng/ml) and TNF-α (20 ng/ml) were added, and the cells were cultured for another 2 days to stimulate DC maturation. MAGE-As polypeptide (10 μM) was added to the mature DCs, and antigen-loaded DCs were obtained by incubation at 37˚C for 24 h. Mature DCs and T cells were mixed at a ratio of 1:10. After adding IL-2 (1,000 U/ml), the cells were cultured for 2 days; then, half the volume was changed were every other day and supplemented with IL-2 (1,000 U/ml). This process was repeated 7 times to collect CTLs.

Flow cytometric assay. Mature DCs and CTLA-4 expression were detected by flow cytometry. For detection of mature DCs, PE-labeled anti-CD86 (1:1,000 dilution; product no. 60712; Cell Signaling Technology, Inc) + anti-CD80 (1:1,000 dilution; cat. no. 15416; Cell Signaling Technology, Inc.) and PE-labeled anti-CD1a (1:1,000 dilution; cat. no. NBP2-34731PE; Novus Biologicals) + anti-CD83 (1:1,000 dilution; product no. 99075S; Cell Signaling Technology, Inc.) were added to 100 μl cell suspension. Then, the mixture was incubated at 4˚C for 30 min in the dark and subjected to flow cytometry (BD Biosciences). For detection of CTLA-4 expression on the breast cancer cell surface, 5 μl PE-labeled anti-CTLA-4 monoclonal antibody (1:1,000 dilution; cat. no. 15132; Cell Signaling Technology, Inc.) was mixed with 100 μl cell suspension, and 5 μl PE-labeled IgG was added to the control group, followed by incubation at room temperature for 15 min and then assessment on a FACS machine (BD FACSCalibur).

CCK-8 assay. CCK-8 assay has been used for evaluating T cell lysis ability (17). The target cells included MCF-7 (ER^+, HLA-A*0201), MDA-MB-453 (HER-2 overexpression, HLA-A*0201), MDA-MB-231 and BT549 (triple negative, HLA-A*0201), while the effector cells included MAGE-As-specific CTLs. Four groups were established as follows: i) CTL group; ii) CTL + anti-CTLA-4 group; iii) CTL + 5DC group; and iv) CTL + anti-CTLA-4 + 5DC group. The target cells were seeded in 96-well plates at a density of $1x10^3$ cells/well and cultured overnight. Various concentrations of effector cells were added to the experimental wells to achieve different effector:target ratios (5:1, 10:1 and 20:1). After adding 10 μl CCK-8 solution per well and incubating at 37˚C for 3 h, the absorbance at 450 nm was detected using Multiskan FC (Thermo Fisher Scientific, Inc.). The specific lysis rate was calculated according to the following formula: Specific lysis rate (%)=optical density $[(OD)_{test\ group}-OD_{blank\ group})]/(OD_{control\ group}-OD_{blank\ group})$ x100.

ELISA. The supernatants of the co-cultured cells of each group were collected, and the IFN-γ level in the supernatant was analyzed by ELISA. The human IFN-γ ELISA kit was purchased from DAKEWE Biotech Co., Ltd. ELISA plates were coated with monoclonal antibodies (cat no. EA-0507; DAKEWE Biotech Co., Ltd.) and incubated overnight at 4˚C. After discarding the coating solution, the plates were washed three times with PBS-Tween-20 (0.05%). The samples were

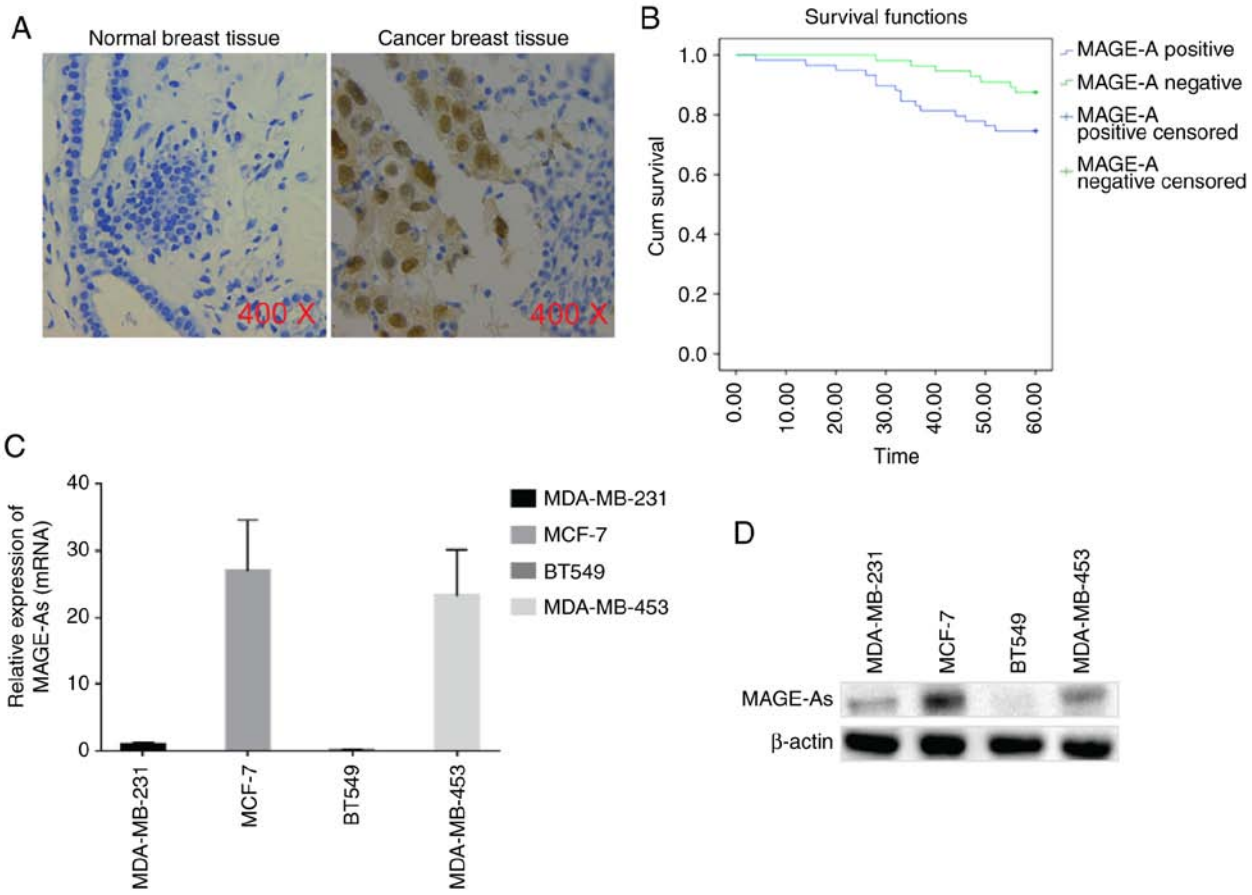

Figure 1. Expression of MAGE-As in breast cancer tissues and cells, and its influence on the prognosis of patients. (A) Immunohistochemical staining was used to measure MAGE-As expression in normal and cancerous breast tissues (n=4). (B) Kaplan-Meier analysis of the prognosis of patients with breast cancer exhibiting MAGE-As-high and low expression. (C) Reverse transcription-quantitative PCR (n=6) and (D) western blotting were performed to assess the protein and mRNA expression levels of MAGE-As in MDA-MB-231, MCF-7, BT549 and MDA-MB-453 cells. Data are presented as the mean ± standard deviation. MAGE-As, melanoma-associated antigen A family.

diluted (1:5 dilution) and incubated in the ELISA plates at 37˚C for 30 min. Next, biotinylated antibody (1:80 dilution; cat. no. EA-0507; DAKEWE Biotech Co., Ltd.) and avidin-labeled horseradish peroxidase (DAKEWE Biotech Co., Ltd.) were added to the ELISA plate, followed by incubation at 37˚C for 30 min. The chromogenic reagent (100 μl per well; DAKEWE Biotech Co., Ltd.) was added to the ELISA plate and incubated 37˚C for 15 min in the dark. Then, 50 μl termination solution (100 μl per well; DAKEWE Biotech Co., Ltd.) was added per well to stop the reaction. Multiskan FC (Thermo Fisher Scientific, Inc.) was used to measure the absorbance at 450 nm.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 5; GraphPad Software, Inc.). The association between the expression of MAGE-As and CTLA-4 and clinicopathological parameters was analyzed by χ^2 test. Survival analysis was performed using the Kaplan-Meier method with the log-rank test. Data are expressed as the mean ± standard deviation. Statistical analysis regarding comparison of different groups was performed by one-way analysis of variance (ANOVA) test followed by Tukey's multiple post hoc test. Student's t-tests were performed for comparison between two groups. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression of MAGE-As in breast cancer tissues and cells, and its influence on patient prognosis. To explore the role of MAGE-As in the progression of breast cancer, the expression of MAGE-As in breast cancer tissues and cells was evaluated in the present study. As assessed by immunohistochemical (IHC) staining, MAGE-As expression could not be detected in normal breast tissue, while it was positive in breast cancer tissues (both in the cytoplasm and nucleus), and the overall positive expression rate was 51.3% (Fig. 1A). The association between MAGE-As positive expression and the clinicopathological parameters of patients with breast cancer is presented in Table I. The results revealed that lymphatic metastasis as well as recurrence and metastasis were both associated with MAGE-As expression. The results of Kaplan-Meier analysis revealed that there was no significant difference in overall survival between patients with breast cancer who exhibited positive MAGE-As expression and those with negative MAGE-As expression (Fig. 1B). Furthermore, the expression levels of MAGE-As in breast cancer cell lines, including MDA-MB-231, MCF-7, BT549 and MDA-MB-453, was detected by RT-qPCR and western blotting. As revealed in Fig. 1C and D, the highest expression of MAGE-As was observed in MCF-7 cells, followed by MDA-MB-453 and

Table I. The associations between MAGE-As expression and clinicopathological parameters in breast cancer patients.

Parameters	n	MAGE-A positive	MAGE-A negative	χ^2	P-value
Age/years				0.257	0.621
>60	50	27	23		
≤60	65	32	33		
Sex				0.000	0.059
Female	112	57	55		
Male	3	2	1		
Tumor size (cm)				0.432	0.511
≥3 cm	45	25	20		
<3 cm	70	34	36		
Lymphatic metastasis				16.818	<0.001
Yes	85	52	33		
No	30	7	23		
Recurrence and metastasis				6.369	0.012
Yes	21	16	5		
No	94	43	51		
Vascular invasion				0.355	0.551
Yes	40	19	21		
No	75	40	35		
Histological grade				0.000	0.063
I	19	6	13		
II	69	35	34		
III	27	18	9		
TNM stage				0.000	0.364
I	45	22	23		
II	52	25	27		
III	18	12	6		
Tumor pathological type				1.932	0.165
Invasive ductal carcinoma	113	57	56		
Other	2	2	0		
Nipple invaded				0.149	0.700
Yes	15	7	8		
No	100	52	48		

MAGE-As, melanoma-associated antigen A family.

MDA-MB-231 cells, while the lowest expression of MAGE-As was detected in BT549 cells (n=6). Collectively, these data indicated that MAGE-As was highly expressed in breast cancer tissues and had no significant effect on the prognosis of patients with breast cancer. The expression levels of MAGE-As varied across different breast cancer cell lines.

5DC treatment promotes MAGE-As expression in breast cancer cells. An increase in the expression of MAGE-As proteins in breast cancer cells can enhance antigen-specific T cell killing (18); thus, the identification of drugs that can increase the expression of MAGE-As is important. For that aim, in the present study, breast cancer cells were treated with 2.5, 5 and 10 μM 5DC for 72 h. In the four breast cancer cell lines evaluated (MDA-MB-231, MDA-MB-453, BT549 and MCF-7 cell lines), the expression level of MAGE-As was significantly up-regulated in a dose-dependent manner (n=6; P<0.05 and P<0.01; Fig. 2A-H). The aforementioned data indicated that 5DC treatment upregulated MAGE-As expression in breast cancer cells.

Effect of MAGE-As on the immune response against tumors. To further explore the role of MAGE-As on immune responses involved in killing tumors, mature DCs were successfully obtained and verified by flow cytometry. As revealed in Fig. 3A-D, $CD80^+/CD86^+$ cells and $CD83^+/CD1a^+$ cells accounted for ~36 and 59% of mature DCs, respectively (n=6). The mature DCs were loaded with MAGE-As antigen peptide, and further co-incubated with T cells to induce CTLs. Subsequently, the effect of MAGE-As on the lysis efficiency of

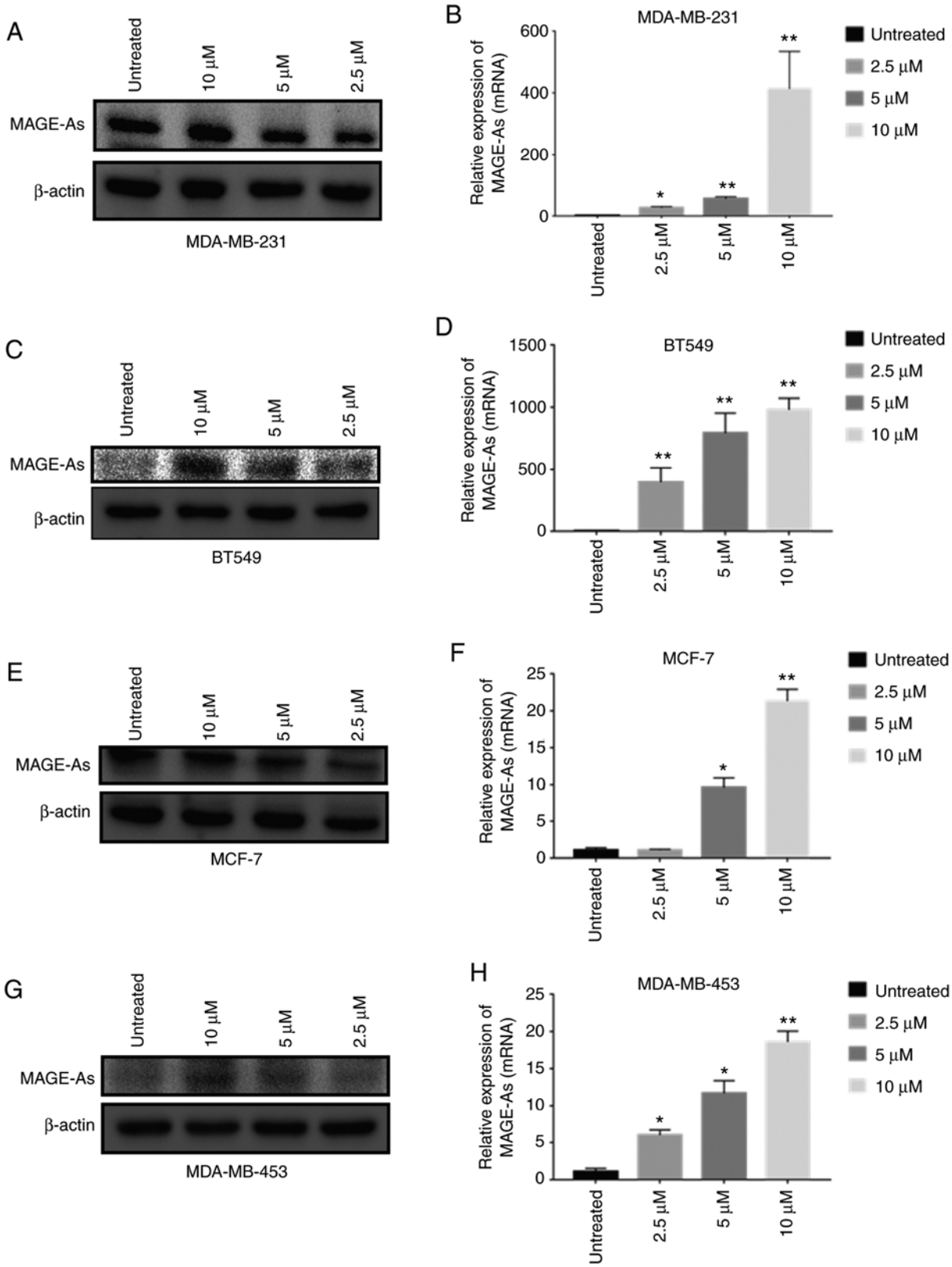

Figure 2. Treatment with 5-aza-2'-deoxycytidine promotes MAGE-As expression in breast cancer cells. Western blotting and reverse transcription-quantitative PCR were applied to assess the (A) protein and (B) mRNA expression levels of MAGE-As in MDA-MB-231 cells, the (C) protein and (D) mRNA expression levels of MAGE-As in BT549 cells, the (E) protein and (F) mRNA expression levels of MAGE-As in MCF-7 cells, and the (G) protein and (H) mRNA expression levels of MAGE-As in MDA-MB-453 cells (n=6). Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. the control (untreated cells). MAGE-As, melanoma-associated antigen A family.

breast cancer cells was examined by CCK-8 assay. The lysis efficiency of CTLs on breast cancer cells increased with the increase in CTL concentration (n=6; P<0.05; Fig. 3E-H). The lysis efficiency of the CTL + 10 μM 5DC group was higher

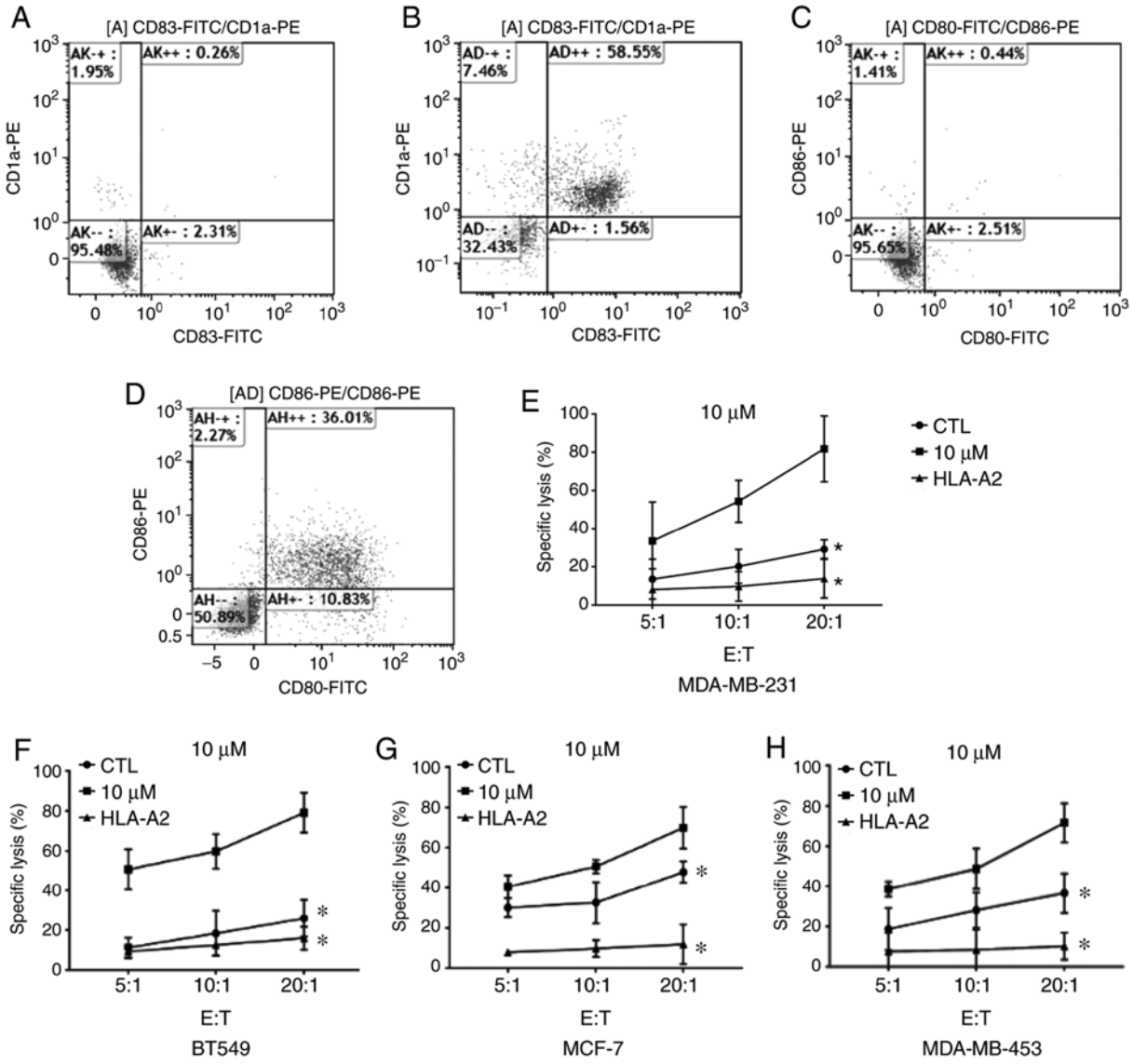

Figure 3. Effect of MAGE-As expression on the immune response in the tumor microenvironment. (A-D) Flow cytometry was used to detect the effects of (A) the control and (B) MAGE-As on CD83/CD1α dendritic cells, and the effects of (C) the control and (D) MAGE-As on CD80/CD86 dendritic cells. CCK-8 assays were applied to assess the lytic efficiency of CTLs on (E) MDA-MB-231, (F) BT549, (G) MCF-7 and (H) MDA-MB-453 cells. All of the aforementioned cell lines were divided into three groups: i) The CTL group; ii) the CTL + 10 μM 5-aza-2'-deoxycytidine group; and iii) the HLA-A2 group (n=6). Data are presented as the mean ± standard deviation. *P<0.05 vs. the CTL group. MAGE-As, melanoma-associated antigen A family; CTL, cytotoxic T cell.

than that of the CTL group under the same effector:target ratio (n=6; P<0.05; Fig. 3E-H). In addition, the lysis rate of CTLs on breast cancer cells in the HLA-A2 blockade group was significantly lower than that in the CTL and CTL + 10 μM 5DC groups, indicating that CTLs had HLA restriction on the lysis of breast cancer cells (n=6; P<0.05; Fig. 3E-H). Collectively, these data demonstrated that upregulation of MAGE-As expression specifically enhanced the ability of CTLs to kill breast cancer cells, and that MAGE-As-specific CTLs had HLA restriction on the lysis of breast cancer cells.

Expression of CTLA-4 in breast cancer tissues and cells, and its influence on patient prognosis. IHC staining revealed that the expression level of CTLA-4 in breast cancer tissues was higher than that in normal breast tissues, and it was mainly expressed in the cytoplasm and membrane of breast cancer cells (Fig. 4A). The association between CTLA-4 positive expression and clinicopathological parameters in patients with breast cancer is presented in Table II. The results revealed that tumor size, lymphatic metastasis, recurrence and metastasis as well as histological grade were associated with CTL-4 expression. Kaplan-Meier analysis indicated that the overall survival of patients with breast cancer who exhibited positive CTLA-4 expression was notably lower than that of patients with CTLA-4 negative expression (Fig. 4B). Flow cytometry was used to detect the expression of CTLA-4 in MDA-MB-231, BT549, MCF-7 and MDA-MB-453 cells. The positive expression rate of CTLA-4 in MDA-MB-231, BT549, MCF-7 and MDA-MB-453 cells was 97.79, 95.44, 93.86 and 94.19%, respectively (n=6; Fig. 4C-F). Overall, these results demonstrated that CTLA-4 was highly expressed in breast cancer tissues and cells, and that positive expression of CTLA-4 decreased the overall survival of patients with breast cancer.

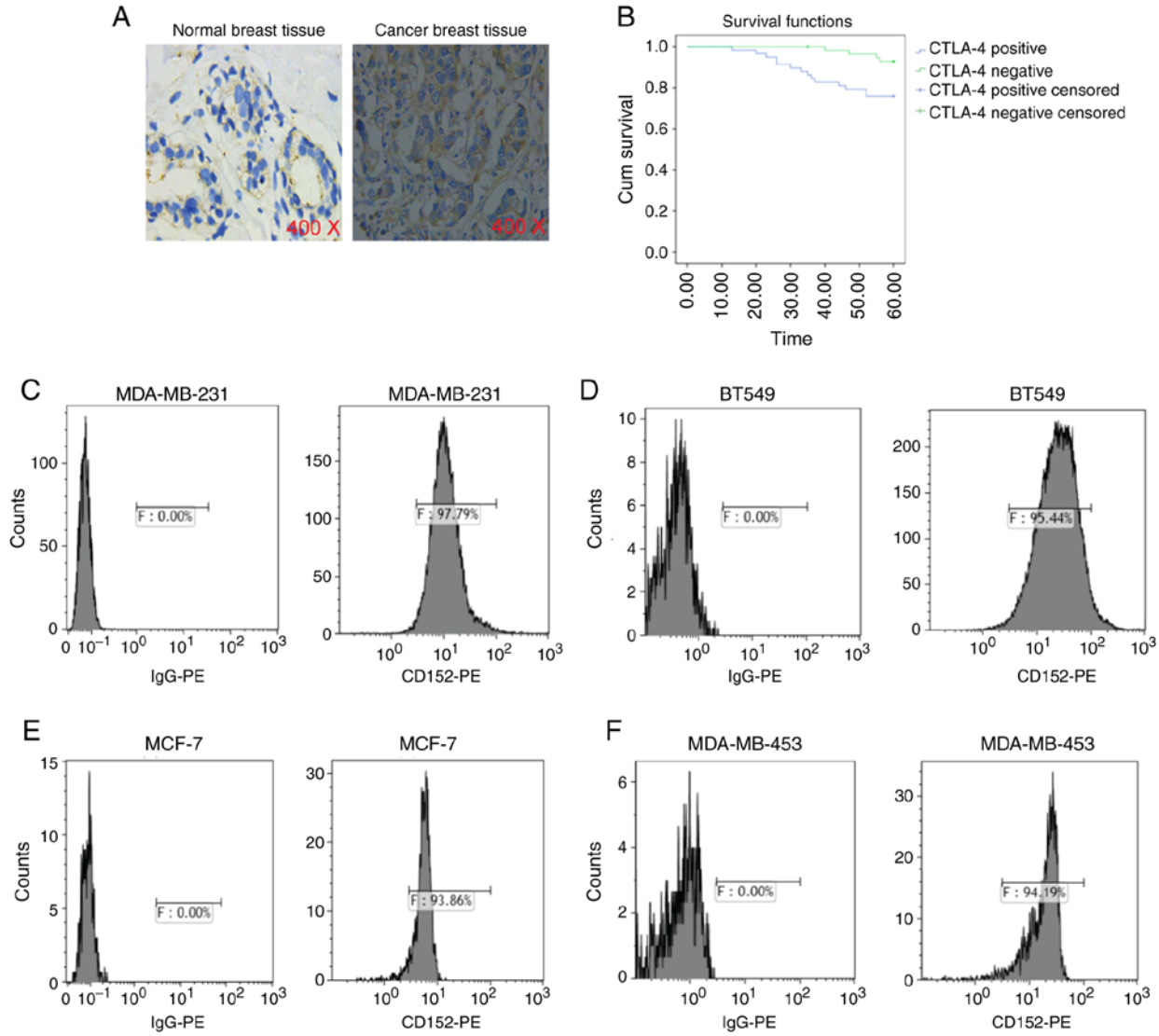

Figure 4. Expression of CTLA-4 in breast cancer tissues and cells, and its influence on the prognosis of patients. (A) Immunohistochemical staining was performed to detect the CTLA-4 levels in normal breast tissues and in breast cancer tissues. (B) Kaplan-Meier analysis of patients with breast cancer exhibiting positive or negative CTLA-4 expression. Flow cytometry was used to assess the CTLA-4 levels in (C) MDA-MB-231, (D) BT549, (E) MCF-7 and (F) MDA-MB-453 cells (n=6). Data are presented as the mean ± standard deviation. CTL, cytotoxic T cell.

Effects of anti-CTLA-4 monoclonal antibody alone and anti-CTLA-4 monoclonal antibody combined with 5DC on the cytotoxic function of MAGE-As-specific CTLs. To demonstrate the effects of anti-CTLA-4 monoclonal antibody alone and in combination with 5DC on the cytotoxic function of CTLs, the specific lysis rate of MDA-MB-231, MDA-MB-453, BT549 and MCF-7 cells that underwent different treatments (including control, 5DC alone treatment, anti-CTLA antibody alone treatment, and combined treatment of 5DC and anti-CTLA antibody) was determined by CCK-8 assay. The specific lysis rate of the CTL + anti-CTLA-4 group was significantly higher than that of the CTL group (n=6; P<0.05; Fig. 5A-D). By comparing the specific lysis rates of different treatments at the same effector:target ratio, it was revealed that the CTL + anti-CTLA-4 + 10 μM 5DC group had the highest cell lysis rate (n=6; P<0.05; Fig. 5A-D). However, at an effector:target ratio of 20:1, CTLs exhibited no significant difference in the lysis rate of MDA-MB-231, MDA-MB-453 and MCF-7 cells compared with that of the CTL + anti-CTLA-4 + 10 μM 5DC and CTL + 10 μM 5DC groups, while no significant difference was found in the lysis rate of BT549 cells between the CTL + anti-CTLA-4 + 10 μM 5DC, CTL + anti-CTLA-4 and CTL + 10 μM 5DC groups (n=6; Fig. 5A-D). Subsequently, the level of IFN-γ in the supernatant of each group was assessed by ELISA. As revealed in Fig. 5E-H (n=6; P<0.05, P<0.01 and P<0.001), it was revealed that the level of IFN-γ in the cell culture supernatant was, from high to low, as follows: CTL + anti-CTLA-4 + 10 μM 5DC group, CTL + 10 μM 5DC group, CTL + anti-CTLA-4 group and CTL group. Collectively, CTLA-4 promoted the lysis efficiency of CTLs on breast cancer cells, and the combination of anti-CTLA-4 and 10 μM 5DC enhanced the lytic ability of CTLs.

Combination of anti-CTLA-4 antibody and 5DC enhances the killing effect of CTLs on primary breast cancer cells. To further verify the effects of the combination of anti-CTLA-4 antibody and 5DC on the killing effect of CTLs in primary

Table II. The associations between CTLA-4 expression and clinicopathological parameters in breast cancer patients.

Parameters	n	CTLA-4 positive	CTLA-4 negative	χ^2	P-value
Age/years				1.096	0.295
>60	50	27	23		
≤60	65	41	24		
Sex				0.747	0.388
Female	112	65	47		
Male	3	3	0		
Tumor size (cm)				4.391	0.036
≥3 cm	45	32	13		
<3 cm	70	36	34		
Lymphatic metastasis				9.606	0.002
Yes	85	56	29		
No	30	12	18		
Recurrence and metastasis				10.446	0.001
Yes	21	19	2		
No	94	49	45		
Vascular invasion				0.874	0.350
Yes	40	26	14		
No	75	42	33		
Histological grade				0.000	0.022
I	19	6	13		
II	69	43	26		
III	27	19	8		
TNM stage				1.398	0.497
I	45	23	22		
II	52	34	18		
III	18	11	7		
Tumor pathological type				0.212	0.645
Invasive ductal carcinoma	113	66	47		
Other	2	2	0		
Nipple invaded				2.463	0.117
Yes	15	11	4		
No	100	57	43		

CTLA-4, cytotoxic T-lymphocyte antigen 4.

breast cancer cells, fibroblasts and primary breast cancer cells were cultured, and the expression levels of MAGE-As in fibroblasts and primary cells were assessed by western blotting and RT-qPCR. As revealed in Fig. 6A and B (n=6; P<0.05), MAGE-As levels in primary cells were notably higher than those in fibroblasts. In addition, 10 μM 5DC treatment successfully induced the expression of MAGE-As in primary breast cancer cells (n=6; P<0.05; Fig. 6C and D). A CCK-8 assay revealed that the specific lysis efficiency of CTLs in the CTL + 10 μM 5DC and CTL + anti-CTLA-4 groups was higher than that in the CTL group, and the lysis rate was highest in the CTL + anti-CTLA-4 + 10 μM 5DC group (n=6; P<0.05; Fig. 6E). Furthermore, the level of IFN-γ in the culture medium of the CTL + anti-CTLA-4 + 5DC group was the highest, followed by the CTL + 10 μM 5DC, CTL + anti-CTLA-4, and CTL groups, respectively (n=6; P<0.05; Fig. 6F). These results were consistent with the trend observed in MDA-MB-231, MDA-MB-453, BT549 and MCF-7 cells. In summary, the combination of anti-CTLA-4 antibody and 5DC enhanced the killing effect of CTLs on primary breast cancer cells.

Discussion

In recent years, although various treatments against breast cancer have progressed, the prognosis is still not optimistic, and a considerable proportion of patients experience recurrence and even death (2). Unlike traditional chemoradiotherapy, tumor immunotherapy is aimed to stimulate the immune system in the body, and has less systemic side effects than

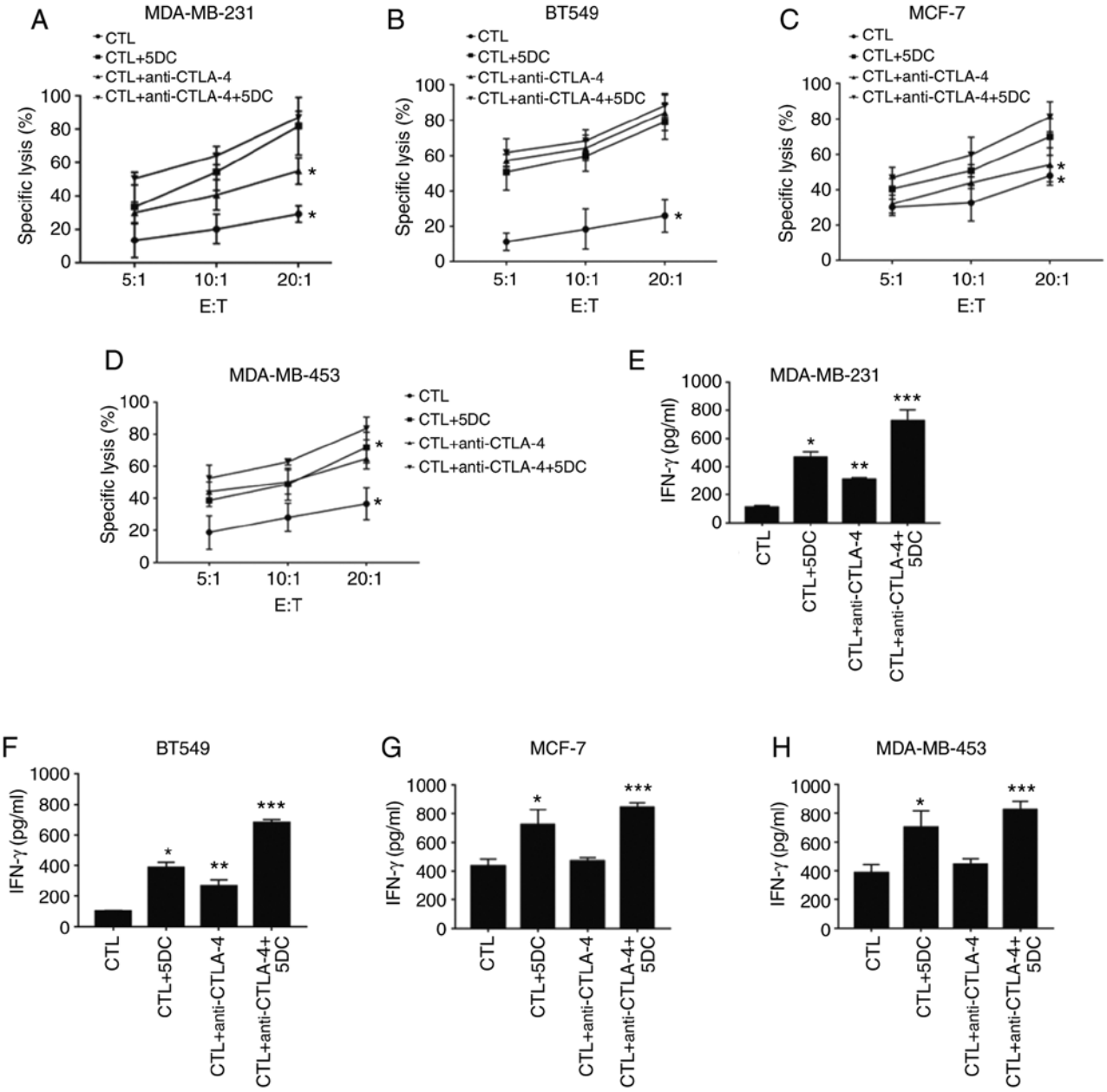

Figure 5. Effects of anti-CTLA-4 monoclonal antibody and anti-CTLA-4 monoclonal antibody combined with 5DC on the cytotoxic function of MAGE-As-specific CTLs. MDA-MB-231, BT549, MCF-7 and MDA-MB-453 cells were divided into 5 groups: i) the CTL group; ii) the CTL + 10 μM 5DC group; iii) the CTL + anti-CTLA-4 group; and iv) the CTL + anti-CTLA-4 + 10 μM 5DC group. CCK-8 assays were used to detect the specific lytic efficiency of CTLs in various cells including (A) MDA-MB-231, (B) BT549, (C) MCF-7, and (D) MDA-MB-451 when the effector:target ratio was 5:1, 10:1 and 20:1, respectively. ELISA was performed to assess the level of IFN-γ in the supernatant of (E) MDA-MB-231, (F) BT549, (G) MCF-7 and (H) MDA-MB-453 cells (n=6). Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001 vs. the CTL group. MAGE-As, melanoma-associated antigen A family; CTL, cytotoxic T cell; 5DC, 5-aza-2'-deoxycytidine.

chemoradiotherapy (18). The most critical point of immunotherapy is finding the right target antigen. MAGE-A is the first human tumor-associated antigen found at the molecular level, and belongs to the CTA antigen family (10). MAGE-A was considered an ideal target antigen in immunotherapy due to its characteristics of high expression in tumor tissues, no expression in normal somatic cells and restricted expression in germ cells (6). In the present study, it was revealed that MAGE-As was highly expressed in breast cancer tissues, and that 5DC treatment promoted MAGE-As expression in breast cancer cells. There was no significant difference in overall survival between patients with breast cancer who exhibited positive MAGE-As expression and those with negative MAGE-As expression, which may be due to the limited number of patients in our study. Notably, CTLA-4 was highly expressed in breast cancer tissues and cells. In addition, the present study revealed that the combination of anti-CTLA-4 antibody and 5DC enhanced the killing effect of CTLs on primary breast cancer cells.

The MAGE-As antigen belongs to the MAGE family, and includes -A1, -A2, -A3, -A4, -A6, -A10 and -A12. The MAGE-A gene consists of 12 highly homologous genes located on the Xq28 chromosome (19,20). MAGE-As is widely expressed in a range of cancer types of different tissue origin, including

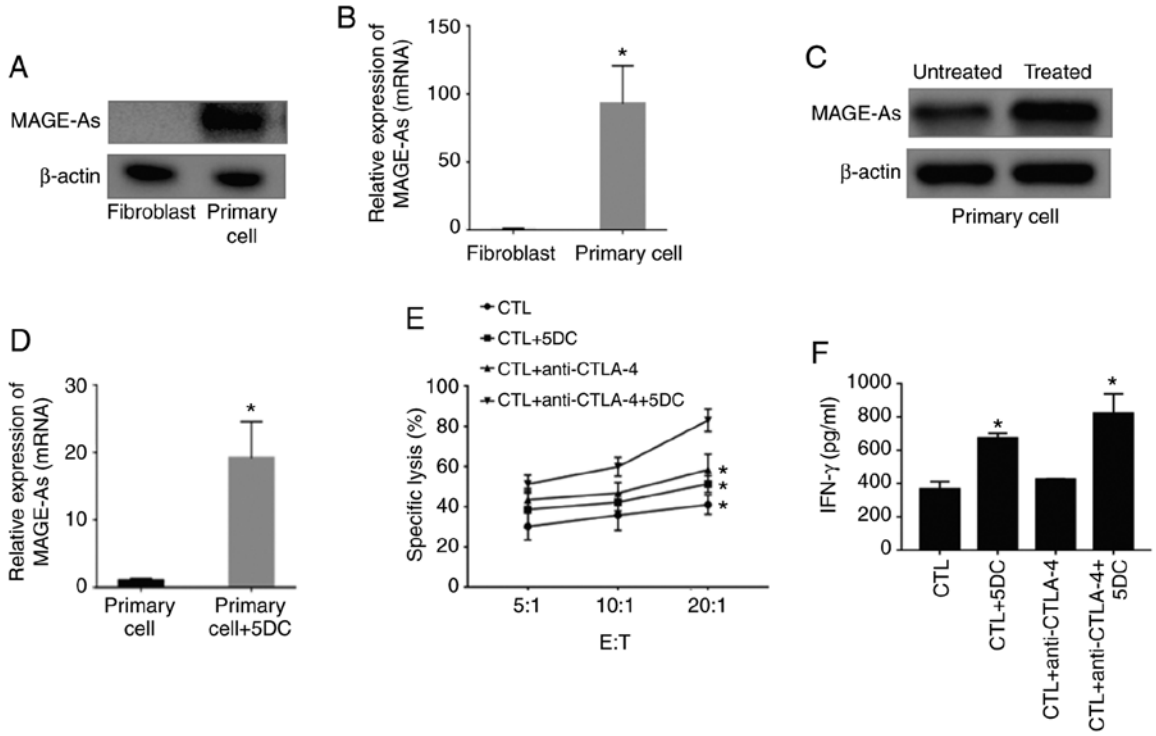

Figure 6. Combination of anti-CTLA-4 antibody and 5DC enhances the killing effect of CTLs on primary breast cancer cells. (A) Western blotting and (B) RT-qPCR were used to assess MAGE-As expression levels in fibroblasts and primary breast cancer cells. (C) Western blotting and (D) RT-qPCR were used to assess the expression levels of MAGE-As in primary cells with or without 10 μM 5DC treatment. (E) A CCK-8 assay was performed to assess the specific lysis rate of CTLs on primary breast cancer cells in the CTL, CTL + anti-CTLA-4, CTL + 10 μM 5DC and CTL + anti-CTLA-4 + 10 μM 5DC groups. (F) ELISA was applied to detect the levels of IFN-γ in the supernatant of primary breast cancer cell cultures in the CTL, CTL + anti-CTLA-4, CTL + 10 μM 5DC and CTL + anti-CTLA-4 + 10 μM 5DC groups (n=6). Data are presented as the mean ± standard deviation. *P<0.05 vs. the CTL group. CTL, cytotoxic T cell; 5DC, 5-aza-2'-deoxycytidine; MAGE-As, melanoma-associated antigen A family; RT-qPCR, reverse transcription-quantitative PCR.

breast, ovarian, bladder, lung, prostate and thyroid cancer, and is associated with poor prognosis of patients (21-23). Aberrant high expression of MAGE-A3 and -A6 in breast cancer was revealed to be associated with estrogen receptor (ER), progesterone receptor (PR), tumor size and adverse outcome (24,25). Consistently, the results of the present study revealed that MAGE-As were not expressed in normal breast tissue, but were abnormally highly expressed in breast cancer tissues, and were associated with lymph node metastasis, recurrence and metastasis. Abd-Elsalam and Ismaeli revealed that the positive expression rate of MAGE-A1-A6 and MAGE-A12 mRNA in venous blood of patients with breast cancer was associated with the TNM stage of tumors (26). Although it appears that the present study has a certain discrepancy with the aforementioned studies, it is certain that the expression of MAGE-As detected by the two experimental methods was closely associated with the clinical parameters of the patients.

The expression of the MAGE-A family genes is regulated by promoter methylation. Demethylating agents can increase the expression of MAGE-A antigen, thereby enhancing the killing function of tumor-specific T cells (26). Two currently used demethylating drugs, decitabine and 5-azacytidine, have been demonstrated to promote the expression of MAGE-A antigen (27,28). The positive expression of antigen on the surface of cancer cells is a prerequisite for peptide-based immunotherapy. Our previous study found that the novel methyl inhibitor 5DC can induce the expression of MAGE-A11 in breast cancer cell lines (11). Consistently, in the present study, it was revealed that treatment of breast cancer cell lines with the demethylating drug 5DC could induce the expression of MAGE-As antigen in breast cancer cells. Theoretically, the combination of other methods could improve the immunogenicity of antigenic peptides, thereby overcoming some limitations of peptide immunity. The present results indicated that the increased expression level of MAGE-As by 5DC treatment could improve the immunotherapeutic effect of the MAGE-As common antigen peptide in breast cancer cells.

CTLA-4 is an immunological checkpoint molecule and is an important T lymphocyte surface molecule. It is mainly expressed on the surface of activated immune effector T cells and regulatory T cells (28). Previous studies have found that CTLA-4 is associated with immune disorders in patients with breast cancer, and have reported that breast tumor cells highly express CTLA-4, and that the expression of CTLA-4 in the peripheral blood mononuclear cells (PBMCs) of patients with breast cancer is higher than that that of the normal control group (29,30). *In vitro* experiments revealed that CTLA-4 can inhibit the response of T cells to antigen, and blocking soluble CTLA-4 can significantly enhance the response of PBMCs to antigen (31). The present study revealed that CTLA-4 exhibited low expression in normal breast tissue, but was abnormally highly expressed in breast cancer tissues, and that CTLA-4 positive expression was associated with tumor size, lymph node metastasis, recurrence and metastasis. Patients with breast cancer exhibiting positive CTLA-4 expression had a lower overall survival rate at 60 months.

It is well known that cellular immunity is one of the main antitumor processes (32); thus, another important goal of immunotherapy is to stimulate tumor-reactive T cells. The combined application of multiple immunotherapeutic methods against specific tumors can improve antitumor efficacy, delay the development of the disease and prolong patient survival (32). Immunological checkpoint inhibitors have demonstrated efficacy in immunotherapy against melanoma and lung cancer, thus laying the foundation for the clinical development of agents that target these immune escape mechanisms in various solid tumors (32). The present study revealed that the combination of anti-CTLA-4 antibody and 10 μM 5DC exhibited marked enhancement on the lytic ability of CTLs in breast cancer cell lines. Similar observations were further verified in primary breast cancer cells.

In summary, the present study demonstrated that MAGE-As common antigen peptide-specific CTLs in combination with anti-CTLA-4 monoclonal antibody and a demethylating drug (5DC) can produce potent tumor cell killing function on breast cancer. It provides important insights into the future research of breast cancer-targeted therapy. However, there are some limitations in the present study. First, the limited number of IHC analyses may affect the association between the positive expression of MAGE-As and the prognosis of patients with breast cancer. Second, the immune environment *in vivo* is complex, which may affect the specific CTL killing function of MAGE-As thus affecting the results of this study. The present results revealed that CTLA-4 blockade, demethylating drug 5DC or a combination of both can improve the killing effect of MAGE-As-specific CTLs. However, despite CTLA-4 exhibiting a highly positive expression rate in the four breast cancer cell lines evaluated in the present study, there was no significant difference between the CTL + anti-CTLA-4 + 5DC group and the CTL + 5DC group. Further investigation should be conducted to clarify the reason for these results. One possible explanation may be the difference between *in vitro* and *in vivo* environments. For example, in the body, an anti-CTLA-4 monoclonal antibody may directly act on the tumor microenvironment, which can act on activated T cells and on CTLA-4-positive cancer cells, and relieve the immunosuppression of DCs by CTLA-4 molecules in the tumor microenvironment. Second, the interaction time between an anti-CTLA-4 monoclonal antibody and T cells *in vitro* is limited by the culture time of cells, which is different from the mechanism of repeated administration after a period of time *in vivo*. Therefore, further studies on the effectiveness of CTLA-4 combined with MAGE-As common antigenic peptide in antitumor therapy, and the new combined therapy mode and appropriate drug concentration are required. Finally, combined treatment based on MAGE-As and anti-CTLA-4 antibodies may be a simple, effective and safer therapeutic method against cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WL and BS conceived and designed the experiments. WL, MS, XH and YW performed the majority of the experiments. WL performed the data analysis. BS wrote the manuscript. All authors read the manuscript and approved the final version.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Hospital of Hebei Medical University. All patients involved in this study were informed of the experimental content, purpose and significance of the study, and signed the informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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