Original Article

Fusion of NY-ESO-1 epitope with heat shock protein 70 enhances its induced immune responses and antitumor activity against glioma *in vitro*

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Background: Glioma is the most common tumor originating in the brain and is difficult to cure. New York esophageal squamous cell carcinoma 1 (NY-ESO-1) is a promising cancer testis antigen (CTA) for tumor immunotherapy, and heat shock proteins (HSPs) can promote the antigen presentation of chaperoned peptides. This study investigates the therapeutic potential of HSP70 and NY-ESO-1 epitope fusion protein for glioma.

Methods: Recombinant HSP70 protein was purified and fused to NY-ESO-1 epitope to generate HSP70/NY-ESO-1 p86-94. NY-ESO-1 expression was induced in U251 glioma cells via 5-Aza-2'-deoxycytidine (5-Aza-CdR) treatment. Dendritic cells (DCs) loaded with HSP70/NY-ESO-1 p86-94 or NY-ESO-1 protein stimulated NY-ESO-1-specific cytotoxic T lymphocytes (CTLs). The killing effect of NY-ESO-1 specific CTLs on U251 cells was detected by lactate dehydrogenase (LDH).

Results: 5-Aza-CdR successfully induced NY-ESO-1 expression in U251 cells. NY-ESO-1-stimulated CTLs lysed more significantly with NY-ESO-1-positive U251 cells than with NY-ESO-1-negative cells. The immune response stimulated by a DC-based vaccine of HSP70/NY-ESO-1 p86-94 fusion protein was significantly enhanced compared with that induced by NY-ESO-1 alone.

Conclusions: These findings indicate that the HSP70/NY-ESO-1 p86-94 may significantly enhance CTLs-mediated cytotoxicity and targeting ability against NY-ESO-1-expressing tumors *in vitro*. 5-Aza-CdR treatment with HSP70 binding to tumor antigen is a new strategy for immunotherapy of the tumors with poor CTA expression.

Keywords: New York esophageal squamous cell carcinoma 1 (NY-ESO-1); heat shock protein 70 (HSP70); dendritic cell (DC); glioma; 5-Aza-2'-deoxycytidine (5-Aza-CdR)

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Introduction

Glioma is the most common form of primary tumor in the central nervous system with poor prognosis; thus, new effective treatment strategies for glioma are urgently needed. Recent studies have shown that glioblastoma cells express or can be induced to express some known cancer testis antigens (CTAs) (1,2). CTAs are potential targets for cancer immunotherapy due to the limited expression

in various tumors and normal tissues (3,4). Although more than 200 CTA group proteins have been documented in the CT database (www.cta.lncc.br) (5), only a limited number of them can induce both humoral and cellular immune responses.

New York esophageal squamous cell carcinoma 1 (NY-ESO-1) is a known CTA with prevalent expression in many cancer types (6,7). Because it can elicit robust humoral and cellular immune responses and has restricted expression patterns, NY-ESO-1 is an attractive candidate target for cancer immunotherapy (8). Multiple immunotherapeutic methods incorporating NY-ESO-1 have been developed recently (9-11). NY-ESO-1 vaccines have been tested to treat the NY-ESO-1-expressing tumors, including lung cancer, esophageal cancer, myeloma (12), and melanoma (6,13). A preclinical mouse model study on lung cancer immunization with a dendritic cell (DC)-targeting integration-deficient lentiviral vector showed promising results (14). However, only a tiny proportion of gliomas express NY-ESO-1 protein (15); consequently, basic CTA-based immunotherapy is unsuitable for treating brain tumors. To overcome this limitation and stimulate strong immune responses against gliomas, we need new strategies for CTA induction.

Epigenetic alteration, such as the hypermethylation of promoter CpG islands, is an important contributor to carcinogenesis in humans. The low NY-ESO-1 expression in gliomas could be the result of a lack of epigenetic regulation. Demethylating agents can increase the expression of tumor-associated antigens (TAAs), which consequently improves the presentation of TAAs to T cells (16). The DNA demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-CdR) can be incorporated into DNA during replication and may deregulate the DNA methylation level. 5-Aza-CdR can activate CTA transcription by silencing the hypermethylation of CTA promoters (17-19). Thus, 5-Aza-CdR treatment may enhance antitumor immune responses and 5-Aza-CdR can be a new target for cancer immunotherapy.

Tumor antigen peptides that are bound by heat shock proteins (HSPs), which can act as critical molecular chaperones of tumor antigens, may induce a stronger immune response compared with unbound tumor antigen peptides. The addition of HSP as a strategy to enhance the efficacy of cancer vaccines has been widely explored in tumor immunotherapy (20-22). HSP-peptide complexes were reported to successfully mediate the cross-presentation of antigen on human leukocyte antigen (HLA) class I- and class II-restricted epitopes (23).

Cytotoxic T lymphocytes (CTLs) are the main effector cells in anti-tumor immunity. CTLs can specifically kill target cells by recognizing antigenic peptide-MHC complexes on the surface of target cells. Cytotoxicity assays are used to determine the killing ability of CTLS.

Here, by prokaryotic expression and protein purification techniques, HSP70 and NY-ESO-1 p86-94 peptide were fused to stimulate peripheral blood monocyte DCs. NY-ESO-1 expression in U251 glioma cells was then induced by 5-Aza-CdR treatment, and the killing efficiency against these cells was measured. We aimed to determine if HSP70/NY-ESO-1 p86-94 could enhance the specific immune response against NY-ESO-1$^+$ U251 cells *in vitro*. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1476/rc).

Highlight box

Key findings

- HSP70/NY-ESO-1 p86-94 protein can significantly enhance the specific immune response against NY-ESO-1$^+$ U251 human glioma cells *in vitro*.

What is known and what is new?

- There are some studies on immunotherapy against some malignant tumors except human glioma with HSP70 or NY-ESO-1, but there are few related studies in glioma, especially peptide antigen vaccine.
- There are few related studies in glioma, especially peptide antigen vaccine. Our works suggest that NY-ESO-1 p86-94 antigen peptide could be used as a new immunotherapy target.

What is the implication, and what should change now?

- Combining 5-Aza-CdR treatment with the fusion of HSP70 to NY-ESO-1 p86-94 is a promising strategy for immunotherapy against glioma with poor cancer testis antigen expression.
- We will conduct experiments to further verify this conclusion, including other glioma cell lines and *in vivo*.

Methods

The cell line and plasmid

The human hepatocellular carcinoma (HCC) cell line MHCC-97H and human glioma cell line U251 were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Prokaryotic expression plasmid pET30a (Promega, Madison, WI, USA) was kept in our

laboratory. Cells were routinely cultured in humidified incubator with 5% CO_2 at 37 ℃.

Expression and purification of human HSP70 protein

The construction of human *HSP70* prokaryotic expression plasmid

RNA was extracted from HCC cell line MHCC-97H using Trizol (Invitrogen, Carlsbad, CA, USA). *HSP70* gene was cloned from RNA by reverse transcription-polymerase chain reaction (RT-PCR). The human *HSP70* gene sequence (GenBank: NM_005345.5) was used as a reference for primer design. The full length *HSP70* gene was amplified by forward (5'-cggaattcatggccaaagccgcggc-3') and reverse (5'-cccaagcttttaatctacctcctcaatgg-3') primers flanked by EcoR1 and Hind III restriction sites. The reaction program was composed of denaturation for 5 min at 95 ℃, 10 s at 98 ℃, 15 s at 61 ℃ and 2 min at 72 ℃ for 30 cycles, and a final extension for 10 min at 72 ℃. The obtained PCR product was cloned into pET30a vector, yielding the recombinant expression vector pET30a-*HSP70*, which was then verified by enzymatic digestion and DNA sequencing.

The purification of human HSP70 protein

The recombinant pET30a-*HSP70* vector was transformed into Escherichia coli Rosetta (DE3) (Novagen, Madison, WI, USA). After the bacterial culture grew to an optical density of about 0.5 at 600 nm, 0.1 mM isopropyl β-d-thiogalactoside (IPTG) (Sigma, St Louis, MO, USA) was added to the medium to induce protein expression. After incubation for 4 h at 32 ℃, the bacteria were collected by centrifugation. NP-40 was added to the cell suspension with final concentration of 0.1%, and lysozyme was added to the cell paste with final concentration of 45 kU/g. The resulting cell spheres were treated to suspend the cells and cut the DNA. Because the target protein had a His-tag, an affinity chromatography column with His-tag was used in accordance with the manufacturer's instructions (BugBuster Master Mix; Merck KGaA, Darmstadt, Germany) to purify the HSP70 protein. The quantification of HSP70 protein was determined by bicinchoninic acid (BCA).

Synthesis of HSP70 and NY-ESO-1 p86-94 complex

In previous experiments, CTLs induced by DCs loaded with NY-ESO-1 polypeptide p86-94 (RLLEFYLAM) had a strong killing ability for both HLA-A2-positive and NY-ESO-1-positive MHCC-97h HCC cells and on SW480 colon cancer cells. We selected NY-ESO-1 p86-94 to connect with HSP70 *in vitro*. Polypeptide NY-ESO-1 p86-94 was synthesized at GL Biochem Ltd. (Shanghai, China). The aggregation of HSP70 and NY-ESO-1 p86-94 epitope (HSP70/NY-ESO-1 p86-94) was synthesized according to the method of Li *et al.* (24). NY-ESO-1 p86-94 (1 mg/mL) and purified HSP70 protein (1.198 mg/mL) were mixed in a phosphate-buffered saline (PBS) solution containing 2 mM $MgCl_2$ at 37 ℃ for 1 h at a molar ratio of about 70:1. Then 100 mM ADP (final concentration of 0.5 mM) was added, and the mixture was allowed to react for 1 h at 37 ℃. Finally, HSP70/NY-ESO-1 p86-94 was purified and concentrated by using an ultrafiltration centrifuge (Millipore, Billerica, MA, USA).

Induction and identification of DCs

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy staff in our laboratory by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) method according to the manufacturer's instruction. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Fujian Medical University (No. [2019(45)]). Informed consent was taken from all the donors. The expression of HLA-A2 on the surface of PBMCs was detected by flow cytometry, and HLA-A2 positive PBMCs were selected for DC induction and culture. The cells were inoculated at the density of $2–3\times10^6$ cells/mL on a 6-well plate for 2 h. Non-adherent cells were removed, and adherent cells were maintained in RPMI-1640 medium containing 20% fetal bovine serum (FBS), 1,000 U/mL GM-CSF (PeproTech, Rocky Hill, NJ, USA), and 500 U/mL interleukin (IL)-4 (PeproTech). The next day, fresh medium was added, and after 5 days of culture, 1/3 of the cells were collected as immature DCs (imDCs). The remaining cells were treated with HSP70/NY-ESO-1 p86-94 or 50 μg/mL IL-4 for 24 h, followed by 10 ng/mL of TNF-α for 48 hours to induce the formation of mature DCs (mDCs). The DCs were evaluated for the expression of HLA-DR, CD83, and CD86 by flow cytometry. The function of DCs was identified by performing mixed lymphocyte reaction assay.

Mixed lymphocyte reaction

T cells were separated from PBMCs using magnetic beads and then incubated in RPMI-1640 medium supplemented

with 20% FBS, 100 U/mL IL-2 (PeproTech), and 20 μg/mL phytohaemagglutinin (PHA). T cells were mixed with either imDCs or mDCs at a 20:1 ratio for 72 h. T-cell proliferation was monitored by MTT assay. The absorbance at 570 nm was measured with a Multi-Well Plate Reader (Beckman Coulter, Brea, CA, USA). The proliferation index (PI) was calculated according to the formula: PI = mixed lymphocyte reaction/lymphocyte reaction. The experiment was conducted 3 times.

Flow cytometry

The levels of HLA-A2 expression on PBMCs and HLA-DR, CD83, and CD86 expression on DCs were detected by flow cytometry performed in accordance with the instructions from BioLegend (San Diego, CA, USA). Briefly, 1×10^6 cells/mL suspended in PBS were incubated with the following anti-human antibodies (2 μg/10^6 cells): PE-HLA-A2, FITC-CD83, PE-CD86, and APC-HLA-DR (BioLegend). The cells were analyzed at room temperature in the dark for 30 min using the FACS Calibur software (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescein-conjugated isotype-matched unrelated antibodies were used as negative controls.

Induction of NY-ESO-1 in U251 human glioma cells

We confirmed that the human glioma cell line U251 does not express NY-ESO-1 by RT-PCR and immunohistochemistry. In order to induce NY-ESO-1 expression, U251 was cultured for 6 days in RPMI-1640 containing 1, 5, or 10 μmol/L 5-Aza-CdR (Sigma). Replace with fresh medium every 2–3 days. The expression level of NY-ESO-1 on U251 cells was detected by performing RT-PCR and immunocytochemical assays. Total RNA was extracted and reverse-transcribed into cDNA with the First Strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). The following primers were used for amplification: NY-ESO-1 forward (exon 1; 5'-cgcctgcttgagttctacctc-3') and NY-ESO-1 reverse (exon 3; 5'-agggaaagctgctggagacag-3'). The reaction was carried out for 35 cycles under the following conditions: 5 min at 95 ℃, 30 s at 94 ℃, 1 min at 60 ℃, and 45 s at 72 ℃, and a final 10 min extension at 72 ℃. β-actin was used as the positive control. The experiment was conducted at least three biological replicates.

Immunocytochemical staining

A suspension of U251 human glioma cells (1×10^5 cells/mL) was dropped onto each slide and incubated overnight at 37 ℃ and 5% CO_2, after which the cells were washed with PBS and fixed with cold acetone for 10 min. Then immunocytochemical staining was performed using streptavidin-peroxidase-complex method. The slides were incubated for 16 h at 4 ℃ in the presence of a mouse anti-human NY-ESO-1 monoclonal antibody (clonal code: E978; Zymed, San Francisco, CA, USA; dilution: 3 μg/mL). The primary antibody was visualized using EnVision+ System-HRP (Dako, Carpinteria, CA, USA) for 30 min with diaminobenzidine (DAB), and counterstained with hematoxylin. Normal testicular tissue was used as positive control. The sections treated with PBS in place of the primary antibody were served as negative controls.

Cytotoxicity assays

The cytotoxic function of activated CTLs was determined by performing a standard lactate dehydrogenase (LDH) cytotoxicity assay (Pierce, Waltham, MA, USA). T cells stimulated by mDCs were used as effector cells and divided into four groups. PBS, HSP70, NY-ESO-1, and HSP70/NY-ESO-1 p86-94. U251 (NY-ESO-1$^+$ or NY-ESO-1$^-$) cells were used as target cells. Effector cells were co-cultured in 96-well plates with 1×10^5 target cells at ratios of 5:1, 10:1, or 20:1 in a final volume of 200 μL at 37 ℃ for 6 h. Then 50 μL of supernatant per well was transfered to a new plate, and 50 μL of substrate mixture was added to each well. The plate was incubated in the dark at room temperature for 30 min. Fifty μL of stop solution was added to each well, and the optical densities were detected at a wavelength of 490 nm. For each effector: target cell ratio, the cytotoxicity was calculated as follows: specific lytic percentage = (effector/target release – spontaneous release)/(maximum release – spontaneous release) × 100%.

Statistical analyses

Statistical analyses were performed using the SPSS 23.0 software package (IBM, Armonk, NY, USA). Continuous variables with a normal distribution were presented as the mean ± standard deviations (SDs). Student's *t*-test was used to determine the significance of differences between two

experimental groups. The one-way analysis of variance, followed by Bonferroni *post-hoc* test, were used to determine statistical differences among three or four experimental groups. Differences were considered significant with a P value of <0.05.

Results

Expression and purification of HSP70 protein

We expressed recombinant full-length HSP70 to determine if NY-ESO-1 and HSP70 fusion protein can be effective for vaccination against tumors expressing NY-ESO-1. A 2,000-bp gene fragment was amplified by RT-PCR and then cloned into pET30a to construct the HSP70 prokaryotic expression plasmid pET30a-*HSP70* (*Figure 1A*). Successful construction of the pET30a-*HSP70* vector was confirmed by double enzyme digestion followed by DNA sequencing (*Figure 1B*). Two base mutations (221st base T mutated to C; 1,695th base G mutated to C) were found compared with the NCBI BLAST *HSP70* gene sequence (number: NM_005345.5), but they were synonymous mutations that did not change the translated HSP70. A high level of HSP70 expression was induced by IPTG, and a protein of the expected size (72 kD) was purified by His affinity chromatography (*Figure 1C*). A BCA assay revealed that the protein concentration was 1.198 mg/mL.

Induction and identification of DCs

HLA-A2-positive PBMCs collected using flow cytometry were treated and incubated for 5 days. Dendritic-like cells were then obtained and examined by light and electron microscopy and were observed to have irregular morphology and protrusions (*Figure 2*). After 24 h of incubation with HSP70/NY-ESO-1 p86-94 or NY-ESO-1 and 48 h after the addition of a tumor necrosis factor (TNF)-α, mDCs were induced and flow cytometry was performed. The levels of CD83, CD86, and HLA-DR expressed on mDCs surface induced by NY-ESO-1 (74.81%, 76.59%, 81.85%) or HSP70/NY-ESO-1 p86-94 (73.91%, 79.84%, 88.42%) were significantly higher than those in imDC group (2.50%, 17.58%, 32.66%) (P<0.01, *Figure 3*).

To determine the ability of mDCs on inducing T cells proliferation, the PIs of T cells incubated for 72 h with mDCs were calculated. PI of T cells incubated with HSP70/NY-ESO-1 p86-94-pulsed mDCs (3.60±0.86) was higher than that of T cells incubated with similarly treated imDCs (1.24±0.26) (P<0.05), indicating that HSP70/NY-ESO-1 p86-94-pulsed mDCs were more effective than imDCs at stimulating T-lymphocyte proliferation.

Induction of NY-ESO-1 expression by U251 glioma cells

In order to induce NY-ESO-1 expression by the glioma cell line U251, the cells were treated with a range of concentrations (1, 5, or 10 μmol/L) of 5-Aza-CdR. There were no significant differences in the tumor cell morphology or growth rate of 5-Aza-CdR-treated U251 cells compared with untreated cells. The results of RT-PCR indicated that NY-ESO-1 was not detected in 5-Aza-CdR-untreated U251 cells, whereas NY-ESO-1 was detected in 5-Aza-CdR-treated U251 cells. And the level of NY-ESO-1 in cells treated with 5 or 10 μmol/L 5-Aza-CdR was significantly higher than that in cells after 5-Aza-CdR (1 μmol/L) stimulation (P<0.01, *Figure 4A*,*4B*). Immunohistochemistry showed that 5-Aza-CdR-untreated U251 cells did not express NY-ESO-1 (*Figure 4C*), whereas positive expression of NY-ESO-1 in the cytoplasm of 5-Aza-CdR-untreated U251 was detected by immunohistochemistry (*Figure 4D*). This suggested that treatment with 5-Aza-CDR can induce the expression of NY-ESO-1 in tumor cells through demethylation.

Increased induction of NY-ESO-1-specific T-cell immune responses by HSP70/NY-ESO-1 p86-94

To determine whether HSP70 can enhance T-cell-mediated NY-ESO-1-specific immune responses, the cytotoxicity of CTLs (E) against U251 cells (T) was determined by performing LDH release assays with the following four groups: PBS group (T cells treated with PBS but not stimulated by mDCs), HSP70 group, NY-ESO-1 group, and HSP70/NY-ESO-1 p86-94 group (T cells stimulated by mDCs loaded with HSP70, NY-ESO-1, or HSP70/NY-ESO-1 p86-94, respectively). U251 cells (T) were divided into two groups: U251 cells treated with 5-Aza-CdR to induce NY-ESO-1 expression (NY-ESO-1$^+$ U251 cells) and untreated (NY-ESO-1$^-$ U251 cells). Regardless of the target cell type (NY-ESO-1$^+$ or NY-ESO-1$^-$ U251 cells), the percentage of NY-ESO-1-specific lysis by CTLs was less than 20% for PBS or HSP70-stimulated T cells. In contrast, with NY-ESO-1$^+$ U251 cells as target cells, the percentages of lysis by NY-ESO-1-stimulated and HSP70/NY-ESO-1 p86-94-stimulated T cells were higher than those of the controls (P<0.01); notably, there was more

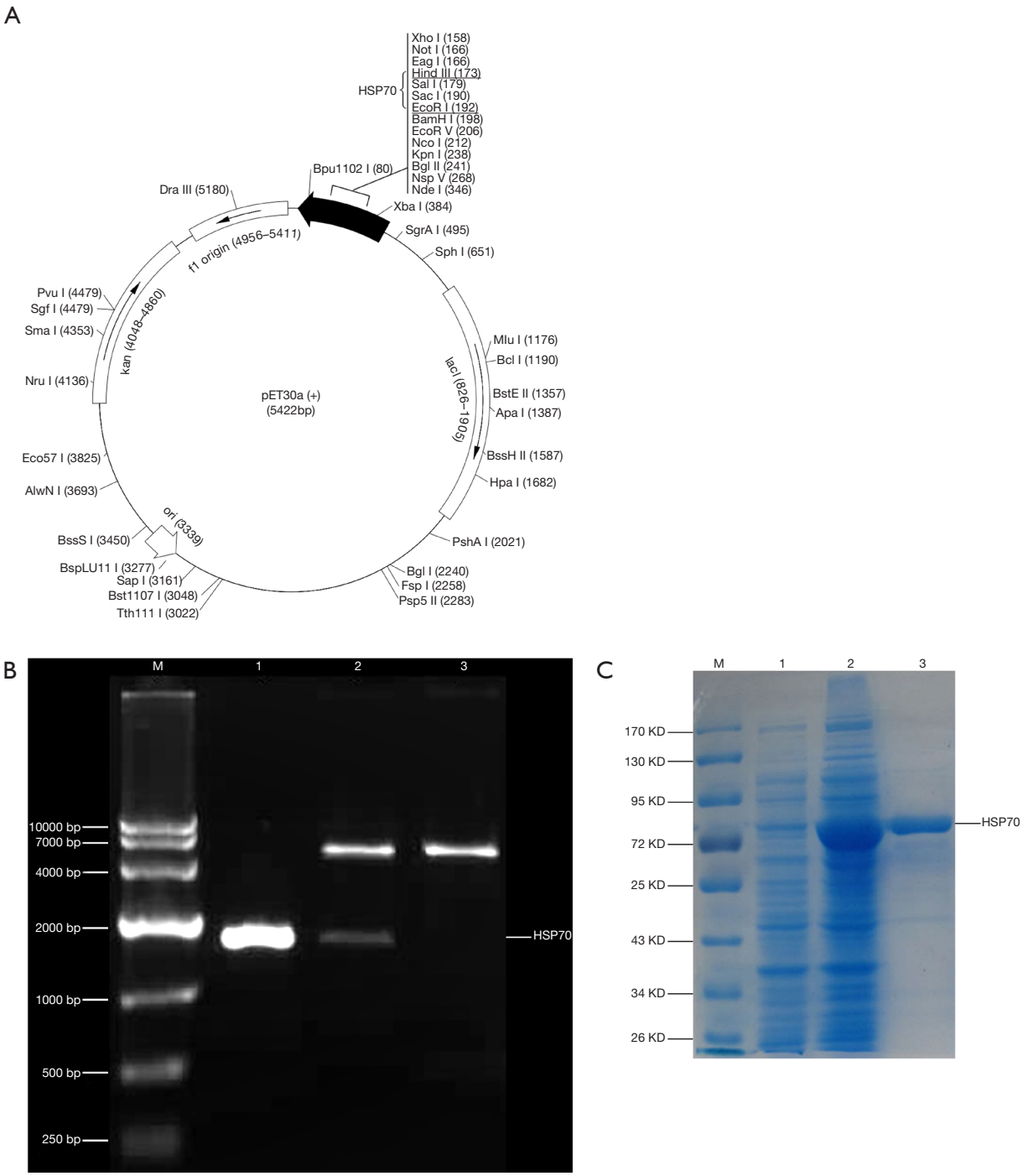

Figure 1 Construction of pET30a-*HSP70* vectors and recombinant protein expression and purification. (A) Schematic representation of the recombinant pET30a-*HSP70* vectors. The full-length *HSP70* gene was cloned into the vector pET30a using the restriction sites EcoR1 and Hind III. (B) RT-PCR confirmation of the recombinant *HSP70*. Lane 1: the full-length *HSP70*. Lane 2: double enzyme-digested 2,000-bp gene fragment. (C) HSP70 protein expressed by recombinant vector pET30-*HSP70*. Lane M: protein molecular weight markers. Lanes 1–2: whole cell lysate of *E. coli* Rosetta 2 transformed with pET30-*HSP70* without [1] and with [2] induction of IPTG. Lane 3: purified HSP70 protein at 1× loading concentration. HSP70, heat shock protein 70; RT-PCR, reverse transcription-polymerase chain reaction; IPTG, isopropyl β-d-thiogalactoside.

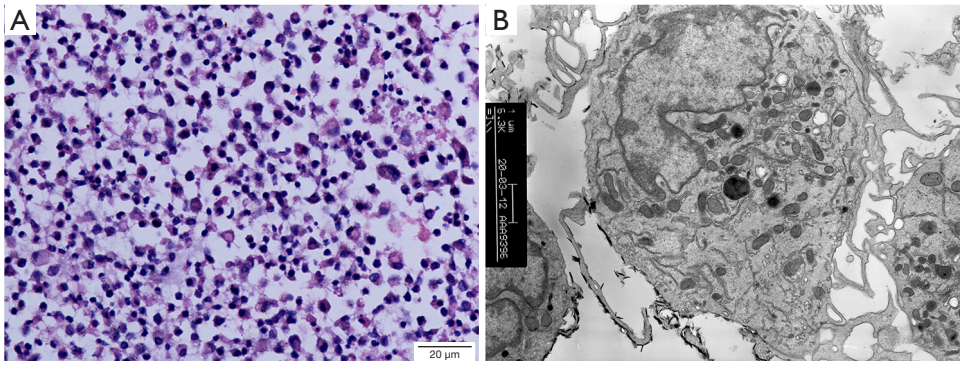

Figure 2 Morphological changes of DCs. Representative light microscopy by hematoxylin-eosin staining at 400× magnification (A) and transmission electron microscopy at 25,000× magnification (B) images showing the shape of DCs. DCs have irregular morphology and protrusions. DCs, dendritic cells.

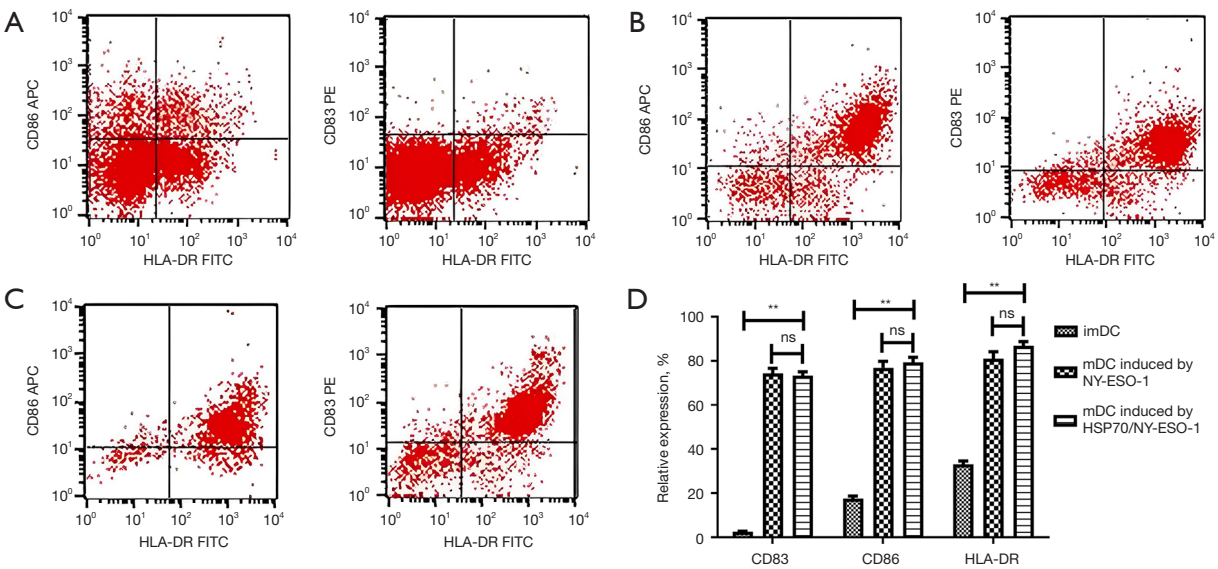

Figure 3 Flow cytometry assessment of DCs surface molecule expression. The levels of CD83, CD86, and HLA-DR expressed on mDCs surface induced by NY-ESO-1 (B) or HSP70/NY-ESO-1 p86-94 (C) were significantly higher than those in imDCs group (A) (P<0.01), as shown in the bar graph (D). **, P<0.01; ns, no significant difference. DCs, dendritic cells; mDCs, mature dendritic cells; NY-ESO-1, New York esophageal squamous cell carcinoma 1; HSP70, heat shock protein 70; imDCs, immature dendritic cells.

CTL lysis in the HSP70/NY-ESO-1 p86-94-stimulated group compared with the NY-ESO-1-stimulated group at the effector: target ratio of 10:1 and 20:1 (P<0.01) (*Figure 5A*). Moreover, the percentages of lysis of NY-ESO-1$^+$ U251 target cells by NY-ESO-1-stimulated and HSP70/NY-ESO-1 p86-94-stimulated T cells were higher than those of NY-ESO-1$^-$ U251 target cells (P<0.01) (*Figure 5B*). These results showed that mDC stimulation with NY-ESO-1 protein induced a specific immune response and that mDC stimulation with the fusion protein HSP70/NY-ESO-1 p86-94 induced an enhanced specific immune response.

Discussion

NY-ESO-1 has strong immunogenicity, and cancer immunotherapy targeting NY-ESO-1 has achieved good results. We previously identified that NY-ESO-1 loaded DCs can stimulate antigen specific T cell responses against HCC (25). This study aimed to make a fusion protein of

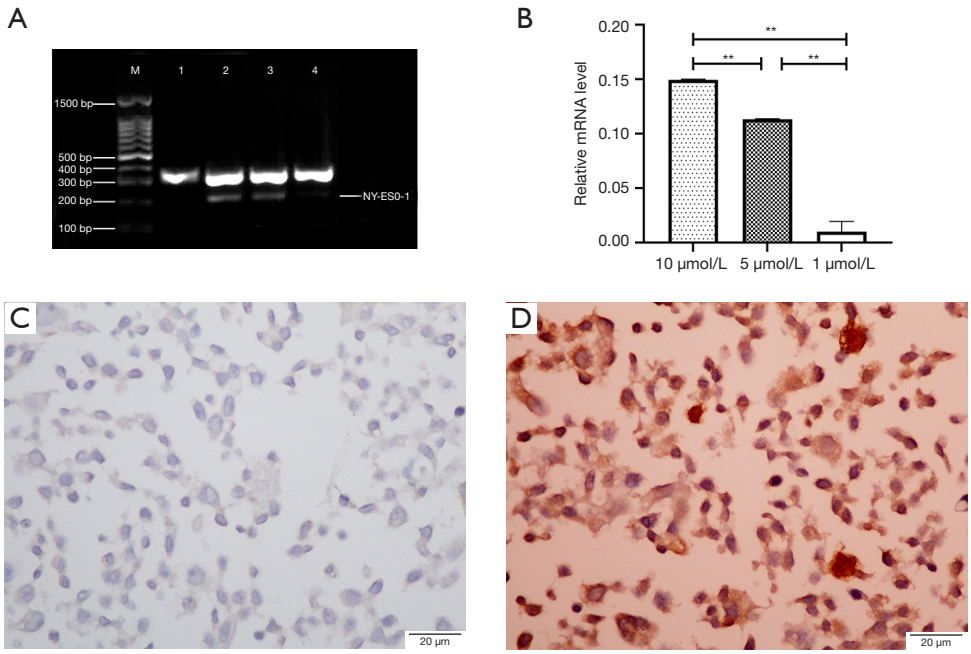

Figure 4 NY-ESO-1 expression in U251 glioma cells after 5-Aza-CdR treatment. (A) The expression of NY-ESO-1 mRNA in U251 cells as assessed by RT-PCR. Lane M: 100-bp DNA ladder. Lane 1: cells not subjected to 5-Aza-CdR treatment. Lanes 2–4: cells treated with 10, 5, or 1 μmol/L 5-Aza-CdR, respectively. (B) The level of NY-ESO-1 in cells treated with 5 or 10 μmol/L 5-Aza-CdR was significantly higher than that in cells after 5-Aza-CdR (1 μmol/L) stimulation as assessed by RT-PCR (P<0.01). (C) The negative expression of NY-ESO-1 protein in 5-Aza-CdR-untreated U251 cells as assessed by immunohistochemical assay at 400× magnification. (D) The positive expression of NY-ESO-1 protein in 5-Aza-CdR-treated U251 cells as assessed by immunohistochemical assay at 400× magnification. **, P<0.01. NY-ESO-1, New York esophageal squamous cell carcinoma 1; 5-Aza-CdR, 5-Aza-2'-deoxycytidine; RT-PCR, reverse transcription-polymerase chain reaction.

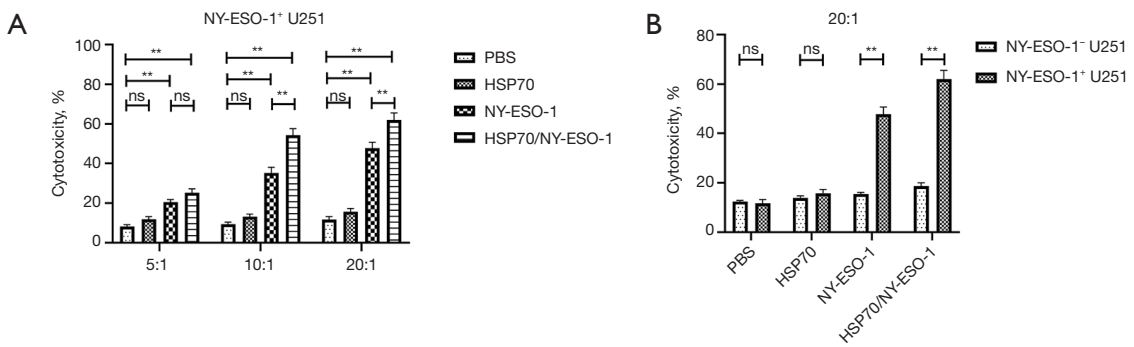

Figure 5 The specific cytotoxic lysis of U251 (NY-ESO-1⁺) glioma cells by T cells. With NY-ESO-1⁺ U251 cells as target cells, the percentages of lysis by NY-ESO-1-stimulated and HSP70/NY-ESO-1 p86-94-stimulated T cells were higher than those of the controls (P<0.01); notably, there was more CTL lysis in the HSP70/NY-ESO-1 p86-94-stimulated group compared with the NY-ESO-1-stimulated group at the effector: target ratio of 10:1 and 20:1 (P<0.01) (A). Moreover, the percentages of lysis of NY-ESO-1⁺ U251 target cells by NY-ESO-1-stimulated and HSP70/NY-ESO-1 p86-94-stimulated T cells were higher than those of NY-ESO-1⁻ U251 target cells (P<0.01) (B). **, P<0.01; ns, no significant difference. NY-ESO-1, New York esophageal squamous cell carcinoma 1; PBS, phosphate-buffered saline; HSP70, heat shock protein 70; CTL, cytotoxic T lymphocytes.

HSP70 and NY-ESO-1, and investigated the therapeutic potential of the fusion protein HSP70/NY-ESO-1 p86-94 for glioma. We first constructed a prokaryotic plasmid containing the gene for human HSP70, then purified the expressed HSP70 protein, and finally synthesized the fusion protein HSP70/NY-ESO-1 p86-94. The NY-ESO-1-specific lysis activity against NY-ESO-1$^+$ U251 glioma cells *in vitro* was assessed. It was demonstrated that HSP70/NY-ESO-1 p86-94 can induce a stronger immune response against NY-ESO-1-expressing glioma cells compared to using NY-ESO-1 alone. Thus, combining HSPs with CTAs could be used as a strategy for increasing the immunogenicity of glioma.

Research on various NY-ESO-1-based vaccines (8,26), such as NY-ESO-1 protein, polypeptide, and DNA, has progressed rapidly in recent years. Because the frequency of HLA-A2 phenotype is high in China, we synthesized several HLA-A2-restrictive NY-ESO-1 polypeptides and performed preliminary experiments to identify their specific lysis activity against tumor cells *in vitro*. The nine-peptide fragment p86-94 (RLLEFYLAM) was found to elicit NY-ESO-1-specific immune responses, so HSP70/NY-ESO-1 p86-94 was used in our studies for glioma immunotherapy.

Because of the restricted expression of NY-ESO-1 on gliomas, we need to explore new induction strategies. 5-Aza-CdR is a methylation inhibitor and has been successfully used to reverse the methylation status of multiple gene promoters (27,28). Here, 5-Aza-CdR successfully induced NY-ESO-1 expression in glioma cell line U251 lacking NY-ESO-1 protein. This finding confirmed that NY-ESO-1 expression is related to gene hypermethylation and that 5-Aza-CdR is an effective inducer of NY-ESO-1 expression.

HSP70 serves as an adjuvant to augment tumor-specific immune responses. To study the ability of HSP70 to act as an adjuvant for enhancing an NY-ESO-1-based vaccine, we generated the fusion protein HSP70/NY-ESO-1 p86-94 and assessed its effectiveness in inducing a specific CTL response against glioma. The data demonstrated that, compared to vaccination with NY-ESO-1 alone, vaccination with HSP70/NY-ESO-1 p86-94 results in a greater CTLs response against NY-ESO-1$^+$ U251 cells.

Various strategies have been developed to target DCs in tumor immunotherapy (29,30). The specific immune response against glioma induced by our HSP70/NY-ESO-1 p86-94 DC-based vaccine was significantly enhanced *in vitro*. Thus, it is worthwhile to further investigate the anti-tumor activity of DCs stimulated by the polypeptide complex HSP70/NY-ESO-1 p86-94.

Conclusions

In comparison to NY-ESO-1, the fusion protein HSP70/NY-ESO-1 p86-94 can induce a stronger NY-ESO-1-specific immune response *in vitro*. Thus, combining demethylation with the fusion of HSP70 to tumor antigens is a promising strategy for designing NY-ESO-1-based vaccines for immunotherapy against malignant tumors expressing NY-ESO-1 antigens.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Fujian Medical University (No. [2019(45)]). Informed consent was taken from all the donors.

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