

# Enhancing the treatment effect on melanoma by heat shock protein 70-peptide complexes purified from human melanoma cell lines

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**Abstract.** Dendritic cell (DC) vaccines are currently one of the most effective approaches to treat melanoma. The immunogenicity of antigens loaded into DCs determines the treatment effects. Patients treated with autologous antigen-loaded DC vaccines achieve the best therapeutic effects. In China, most melanoma patients cannot access their autologous antigens because of formalin treatment of tumor tissue after surgery. In the present study, we purified heat shock protein 70 (HSP70)-peptide complexes (PCs) from human melanoma cell lines A375, A875, M21, M14, WM-35, and SK-HEL-1. We named the purified product as M-HSP70-PCs, and determined its immunological activities. Autologous HSP70-PCs purified from primary tumor cells of melanoma patients (nine cases) were used as controls. These two kinds of tumor antigenic complexes loaded into DCs were used to stimulate an antitumor response against tumor cells in the corresponding patients. Mature DCs pulsed with M-HSP70-PCs stimulated autologous T cells to secrete the same levels of type I cytokines compared with the autologous HSP70-PCs. Moreover, DCs pulsed with M-HSP70-PCs induced CD8<sup>+</sup> T cells with an equal ability to kill melanoma cells from patients compared with autologous HSP70-PCs. Next, we used these PC-pulsed autologous DCs and induced autologous specific CD8<sup>+</sup> T cells to treat one patient with melanoma of the nasal skin and lung metastasis. The treatment achieved a good effect after six cycles. These findings provide

a new direction for DC-based immunotherapy for melanoma patients who cannot access autologous antigens.

## Introduction

Melanoma is a highly malignant tumor derived from melanin cells with an incidence rate of 1-3% among all tumors. Most melanoma patients have metastasis by the time of treatment. This disease is extremely resistant to radiotherapy and chemotherapy. The 5-year survival rate is <16% (1,2). Many new and effective treatment methods have emerged in recent years, such as tyrosine kinase inhibitors and immune checkpoint blockades (3). However, the vast majority of patients, even in developed countries, cannot afford these treatments.

Dendritic cell (DC)-based vaccine therapy is the earliest and most effective method among cellular immunotherapies for the treatment of melanoma. DCs are the most effective antigen-presenting cells (APCs) in the body and considered to be a primary part of the immune response. DCs play an extremely important role in the immune response because they activate cytotoxic T lymphocytes (4-6). In the last decade, DC-loaded tumor antigens to prepare cancer vaccines for treating melanoma have shown benefits in patients with melanoma.

The treatment effect of DC-based vaccine therapy is attributed to the loaded antigenic peptides. Access to effective tumor antigenic peptides is fundamental in determining the effect of treatment (7,8). Autologous tumor antigenic peptides from patients are undoubtedly the best choice. Most melanoma patients in China are unable to access fresh tumor tissue after surgery, making it impossible to obtain autologous tumor antigen peptides, which seriously affects treatment. Thus, finding an alternative for such patients has become a research focus.

Heat shock protein 70 (HSP70) has an important role as a molecular chaperone that binds to tumor antigen peptides in tumor cells (9-11). In our previous study, we purified HSP70-HER-2-peptide complexes (PCs) from the human breast cancer cell line SKBR-3, and showed that HSP70-HER-2-PCs contain more comprehensive tumor antigen peptides and induce stronger immune activity (12). Numerous studies

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show that human melanoma cell lines also contain a number of effective tumor antigen peptides (13,14). The aim of this study was to determine whether HSP70-PCs purified from human melanoma cell lines induce sufficient immune activity compared with autologous tumor antigen peptides.

In the current study, we purified HSP70-PCs using our method from human melanoma cell lines A375, A875, M21, M14, WM-35, and SK-HEL-1. The obtained antigens were named M-HSP70-PCs. Their immunological activities were determined by pulsing DCs and inducing specific CD8<sup>+</sup> T cells. Autologous HSP70-PCs purified from primary cells of melanoma patients were used as controls.

## Materials and methods

**Cell culture.** Human melanoma cell lines A375, A875, M21, M14, WM-35, and SK-HEL-1 were purchased from Peking Union Medical College (Beijing, China). The cells were cultured in RPMI-1640 medium or Dulbecco's modified Eagle's medium (both from Gibco, Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Media were supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone, Logan, UT, USA), 2 mol/l L-glutamine (Gibco, Invitrogen Co.), 100 U/ml penicillin G, and 100 µg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**Purification of M-HSP70-PCs.** The methods used to purify HSP70-PCs have been described previously (12). Human melanoma cell lines were heated in a water bath at 42°C for 12 h, followed by recovery for 2 h at 37°C in an atmosphere containing 5% CO<sub>2</sub>. After heat shock, the tumor cells were digested by 0.02% trypsin, and then 5x10<sup>6</sup> cells of each cell line were homogenized for 15 min on ice in a hypotonic buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), pH 7.2. After ultrasonication at 0°C for 30 min, the homogenate was centrifuged at 10,000 x g for 90 min at 4°C. The supernatant was dialyzed against buffer A (20 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 0.5 mM phenylmethylsulfonyl fluoride, and 15 mM β-mercaptoethanol, pH 7.4) overnight at 4°C. The sample was then loaded onto a Con A Sepharose column (Sigma Chemical Co.). Unbound proteins were collected at a flow rate of 12 ml/h. The fraction was dialyzed against buffer B (20 mM Tris-HCl, 20 mM NaCl, 3 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 0.5 mM phenylmethylsulfonyl fluoride and 15 mM β-mercaptoethanol, pH 7.4) overnight at 4°C. The sample was then applied to an ADP-agarose column (Sigma Chemical Co.) equilibrated with buffer B at a flow rate of 12 ml/h. The column was eluted with buffer B containing 0.5 M NaCl until proteins were not detected by the Bradford method. The target protein was eluted with buffer B containing 3 mM ADP (Sigma Chemical Co.). The endotoxin level in the preparations was determined by the Limulus Amebocyte Lysate (LAL) assay (Ocean Biologicals Co., China).

**Preliminary characterization of M-HSP70-PCs.** M-HSP70-PCs were subjected to sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining. For western blot analysis, separated proteins were blotted onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Billerica, MA, USA). Primary monoclonal antibodies included rabbit anti-Melan-A (ab51061; Abcam), mouse anti-NY-ESO-1 (sc-52869; Santa Cruz Biotechnology). Goat anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (sc-2030 and sc-2031). After secondary antibody incubation, proteins were visualized by autoradiography.

**Collection of fresh melanoma tumor tissue.** Surgical specimens were obtained from 15 patients who underwent resection of skin melanoma tumors at the Oncology Department, Affiliated Beijing Shi Ji Tan Hospital of Capital Medical University in 2011. No patients had received preoperative chemotherapy or radiotherapy. The age of the patients ranged from 35 to 61 years. Of the 15 patients, 10 were males and 5 females. All pathological types were malignant melanoma of the skin. The study was approved by the Human Research Ethics Committee of Beijing Shijitan Hospital. All patients provided informed consent for the collection of tissue samples. After the success of primary tumor cell culture, peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of patients.

**Primary melanoma cell culture and purification of autologous HSP70-PCs.** The methods of melanoma primary cell culture have been described previously with some modifications (15). The resected skin melanoma tissues were immediately placed in ice-cold RPMI-1640 medium containing 100 U/ml penicillin G and 100 µg/ml streptomycin, and transported to the laboratory within 10 min. After removal of necrotic tissues, the samples were rinsed with phosphate-buffered saline (PBS) twice and cut into small fragments. The fragments were incubated with 1% collagenase type II (Sigma Chemical Co.) in a gently shaking water bath for 1 h at 37°C. After passed through a 38-µm mesh sieve, the resulting cell suspension was washed twice and centrifuged at 300 x g for 10 min. The cells were diluted to 5x10<sup>5</sup> cells/ml and incubated in RPMI-1640 supplemented with 10% heat-inactivated FCS at 37°C with 5% CO<sub>2</sub>. To remove fibroblasts, the concentration of FCS in the medium was changed to 5% in the second week of culture, and then returned to 10% in the third week. Cells were passaged at 75% confluency.

The autologous HSP70-PCs of patients were purified from 5x10<sup>6</sup> primary cells according to the method described in 'Purification of M-HSP70-PCs'.

**Preparation of DCs and CD8<sup>+</sup> T cells.** DCs were generated as described previously (16). Briefly, PBMCs were isolated from heparinized venous blood of the patients by density gradient centrifugation using Ficoll-Hypaque (1.077 g; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) and cultured in RPMI-1640 medium containing 10% FCS for 2 h. Non-adherent cells were collected to generate CD8<sup>+</sup> T cells, and the adherent cells were cultured for 7 days in RPMI-1640 medium containing 10% FCS, 800 U/ml recombinant human

granulocyte-macrophage colony-stimulating factor (GM-CSF), and 500 U/ml recombinant human interleukin (IL)-4 (both from R&D Systems, Inc., USA) to generate DCs. Half medium volumes were replaced every other day, and 50 U/ml tumor necrosis factor- $\alpha$  (R&D Systems, Inc.) was added to the culture medium on the sixth day. The surface phenotype, include CD80, CD83, CD86 and HLA-DR, of all the DCs in this research were detected by flow cytometry, respectively.

CD8<sup>+</sup> T cells were harvested from the non-adherent fraction. Briefly, non-adherent cells were resuspended in RPMI-1640 medium containing 10% FCS, 100 U/ml penicillin G, and 100  $\mu$ g/ml streptomycin. Recombinant human interferon (IFN)- $\gamma$  (1,000 IU/ml) (PeproTech, Inc., USA) was added on day 0. After 24 h of incubation, 50 ng/ml mouse anti-human monoclonal antibody against CD3 (Becton, Dickinson and Co., USA), 100 U/ml recombinant human IL-1 $\beta$  (R&D Systems, Inc.), and 300 U/ml recombinant IL-2 (PeproTech, Inc., USA) were added. Cells were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and subcultured every third day in fresh complete medium with 300 U/ml IL-2 at 2x10<sup>6</sup> cells/ml.

*Immunofluorescence staining.* DCs (1x10<sup>5</sup>) were pulsed with 10  $\mu$ g M-HSP70-PCs purified from the six melanoma cell lines at 37°C for 12 h. After culture, cytospin slides were prepared by centrifugation (100 x g for 5 min) using 100  $\mu$ l of cell suspension containing about 1x10<sup>6</sup> cells/ml. The cells were air-dried, immediately fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked in 3% bovine serum albumin (BSA) at 4°C for 1 h. The cells were then incubated with monoclonal rabbit anti-Melan-A and monoclonal mouse anti-NY-ESO-1 antibodies at a 1:100 dilution in PBS for 1 h at 37°C. After three washes in PBS, the cells were incubated with Texas Red-conjugated goat anti-rabbit IgG (ab6719) and FITC-conjugated goat anti-mouse IgG (ab6785; both from Abcam) at a 1:50 dilution in PBS for 30 min in the dark at 37°C. The cells were washed again three times in PBS and smeared onto the slides. Images were acquired using a Olympus DP71 microscope (Olympus) and analyzed by the included software.

*Enzyme-linked immunospot (ELISPOT) assay.* An ELISPOT assay was performed to assess the IFN- $\gamma$  production of autologous T cells using an IFN- $\gamma$  ELISPOT kit (R&D Systems, Inc.). DCs were divided into three groups (A-C) with 1x10<sup>5</sup> cells in each and pulsed for 12 h. Group A received GM-CSF and IL-4 only, group B received 10  $\mu$ g M-HSP70-PCs purified from the six melanoma cell lines, and group C received 10  $\mu$ g autologous HSP70-PCs purified from primary cells of each melanoma patient. After washing with PBS, the three groups of DCs were co-cultured with autologous T cells isolated using a nylon wool column at a 1:10 ratio in a 96-well culture plate (Nunc, Roskilde, Denmark) in the presence of 20 U/ml IL-2 for 7 days. Stimulated T cells (1x10<sup>4</sup> cells/well) as effector cells and primary melanoma cells (5x10<sup>3</sup> cells/well) as target cells were transferred to the ELISPOT plate, followed by incubation at 37°C for 18 h. The level of IFN- $\gamma$  was detected as described in the IFN- $\gamma$  ELISPOT kit manual with an automated ELISPOT reader system (Biosys Co., Germany).

*Induction of CD8<sup>+</sup> T cells by DCs pulsed with M-HSP70-PCs and in vitro cytotoxicity testing.* An LDH release assay was used to determine the specific cytolytic activities of CD8<sup>+</sup> T cells as described previously. Autologous DCs obtained using the method described above and autologous CD8<sup>+</sup> T cells of each melanoma patient were used in the following experiment. Cells were divided into three groups with 1x10<sup>6</sup> cells in each. Then, the three groups of CD8<sup>+</sup> T cells were co-cultured with the three groups of autologous DCs pulsed under the conditions mentioned above at a 10:1 ratio. The co-cultures were treated with 300 U/ml IL-2 in a 96-well plate for 1 week and named groups A, B and C correspondingly. Then, the three groups of CD8<sup>+</sup> T cells were used as effector cells in the assay using an LDH Cytotoxicity Detection kit (BioVision, Inc., USA). The corresponding primary melanoma cells were used as target cells in this assay. Briefly, target and effector cells were resuspended in assay medium (RPMI-1640 with 1% BSA), and then target cells (1x10<sup>4</sup> cells/well) were co-cultured with effector cells at various ratios (1:5, 1:10, and 1:20) in a 96-well round-bottomed culture plate at 37°C. After incubation for 4 h, the cells were centrifuged at 50 x g for 10 min, and the supernatant was collected and transferred to another 96-well for the LDH assay. LDH detection mixture (100  $\mu$ l/well) was added, followed by incubation in the dark for 30 min at room temperature. After addition of 50  $\mu$ l stop solution per well, the absorbance of the samples was measured by a microplate reader at 490 nm as the reference wavelength.

*Treatment of a patient with melanoma of the nasal skin and lung metastasis by M-HSP70-PCs.* A 47-year-old woman was admitted to the Department of Otolaryngology of Beijing Cancer Hospital for diagnosis of melanoma in her nasal skin in November 2011. Extensive resection of the tumor was performed in the same month. Postoperative pathological diagnosis confirmed the original diagnosis. One week after the operation, positron emission tomography and computed tomography (CT) showed no abnormalities, and the patient began chemotherapy (temozolomide + cisplatin + endostar). After four cycles of chemotherapy, the patient had a severe gastrointestinal reaction and some lung metastases were detected by enhanced chest CT in June 2012. Chemotherapy was stopped, and a review examination in July 2012 found that the lung metastases had become larger and increased. The patient came to our hospital for cellular immunotherapy, and we collected peripheral blood to culture autologous DCs and CD8<sup>+</sup> T cells. Because the patient could not obtain autologous tumor antigen peptides, after informed consent, she began M-HSP70-PC-loaded autologous DC and M-HSP70-PC-loaded autologous co-cultured DC-CD8<sup>+</sup> T-cell treatment in September 2012. The therapy was applied once every 6 months, and each cycle included upper arm subcutaneous injection of M-HSP70-PC (50  $\mu$ g)-pulsed DCs (5x10<sup>7</sup>) and intravenous reinfusion with co-cultured M-HSP70-PC (50  $\mu$ g)-pulsed DCs-CD8<sup>+</sup> T cells (4x10<sup>10</sup>).

*Statistical analysis.* Values are expressed as the means  $\pm$  standard deviation (SD) or a percentage. All analyses were conducted using SPSS 17.0 software. The results were considered statistically significant at P<0.05.

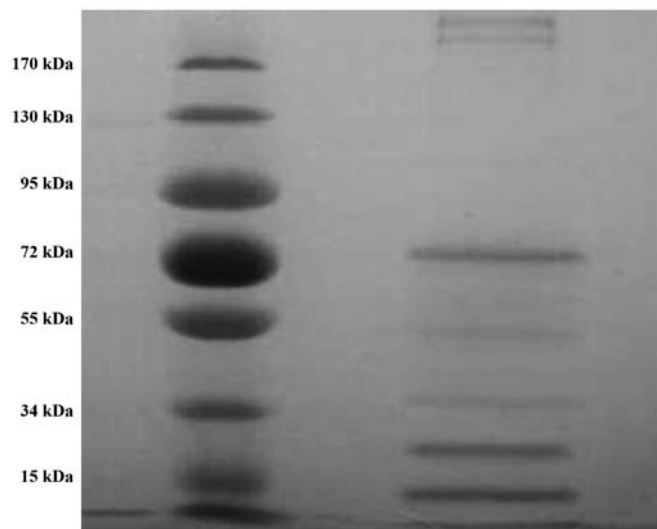


Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of M-HSP70-PCs. Lane 1, the protein marker; lane 2, M-HSP70-PCs.

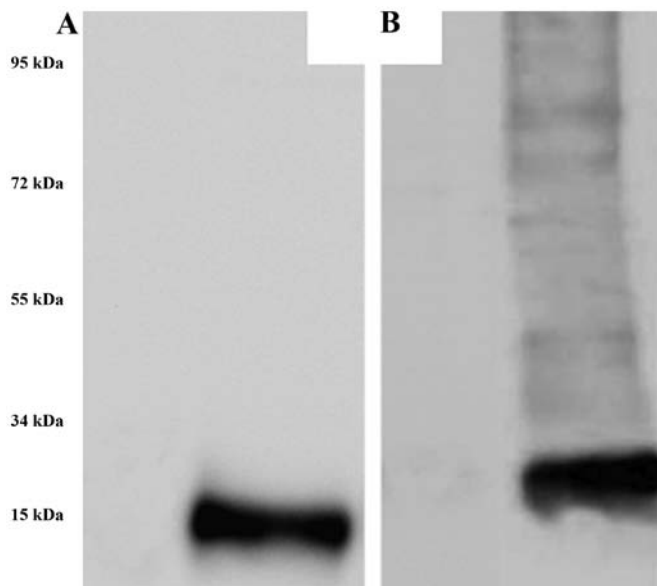


Figure 2. Western blot analysis of Melan-A and NY-ESO-1 proteins in M-HSP70-PCs. (A) Analysis of Melan-A protein in M-HSP70-PCs. (B) Analysis of NY-ESO-1 protein in M-HSP70-PCs.

## Results

**Preliminary characterization of M-HSP70-PCs.** M-HSP70-PCs were purified from six melanoma cell lines and analyzed by SDS-PAGE. The purified products contained various proteins according to the different molecular weights (Fig. 1). The 13- and 21-KDa proteins were especially striking. In western blot analysis, Melan-A- and NY-ESO-1-specific antibodies bound to M-HSP70-PCs (Fig. 2), demonstrating that the obtained complex contained Melan-A and NY-ESO-1 proteins that are the most important melanoma antigen peptides. Other proteins may be melanoma antigen peptides bound by HSP70.

Quantitative detection was performed using the Bradford standard curve method. The protein content of M-HSP70-PCs

Table I. Information on malignant melanoma patients (9 cases).

Patient no.	Age	Gender	Tumor site	Tumor size (mm)
1	51	Male	Abdominal skin	52x44
2	37	Male	Upper extremity skin	35x30
3	59	Female	Abdominal skin	32x28
4	53	Male	Facial skin	63x46
5	37	Male	Abdominal skin	41x30
6	40	Male	Lower limb skin	57x33
7	35	Female	Upper extremity skin	50x25
8	61	Male	Upper extremity skin	31x26
9	42	Female	Back skin	44x31

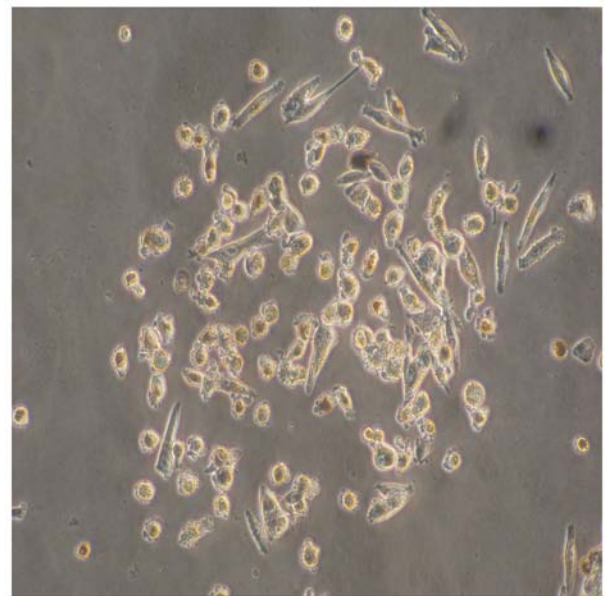


Figure 3. Primary tumor cells of one patient with melanoma. Passage 2 cells cultured for 3 days are shown (magnification, x400). Cells showed a rapid growth rate and high purity. (Cells from the patient no. 2 were used).

purified from the six melanoma cell lines ( $3 \times 10^7$  cells) was  $917.03 \mu\text{g}$ . The endotoxin levels in the preparations were lower than  $0.03 \text{ EU/mg}$  as determined by the LAL assay.

**Primary melanoma cell culture.** Of the 15 primary melanoma cell cultures obtained from surgical specimens, four became contaminated during culture and two underwent senescence. The remaining cultures (9 cases) were passaged 4-5 times, and the number of cells reached  $1 \times 10^7$  usable for preparation of autologous HSP70-PCs, and as target cells for further experiments (Fig. 3). Detailed information on the 9 cases of melanoma patients are shown in Table I.

**Immunofluorescence staining.** Next, we determined the ability of DCs to take up M-HSP70-PCs purified from melanoma cell lines. DCs were pulsed with M-HSP70-PCs at  $37^\circ\text{C}$  for 12 h. After the numerous aforementioned treatments and extensive washing to remove unbound proteins, the DCs were

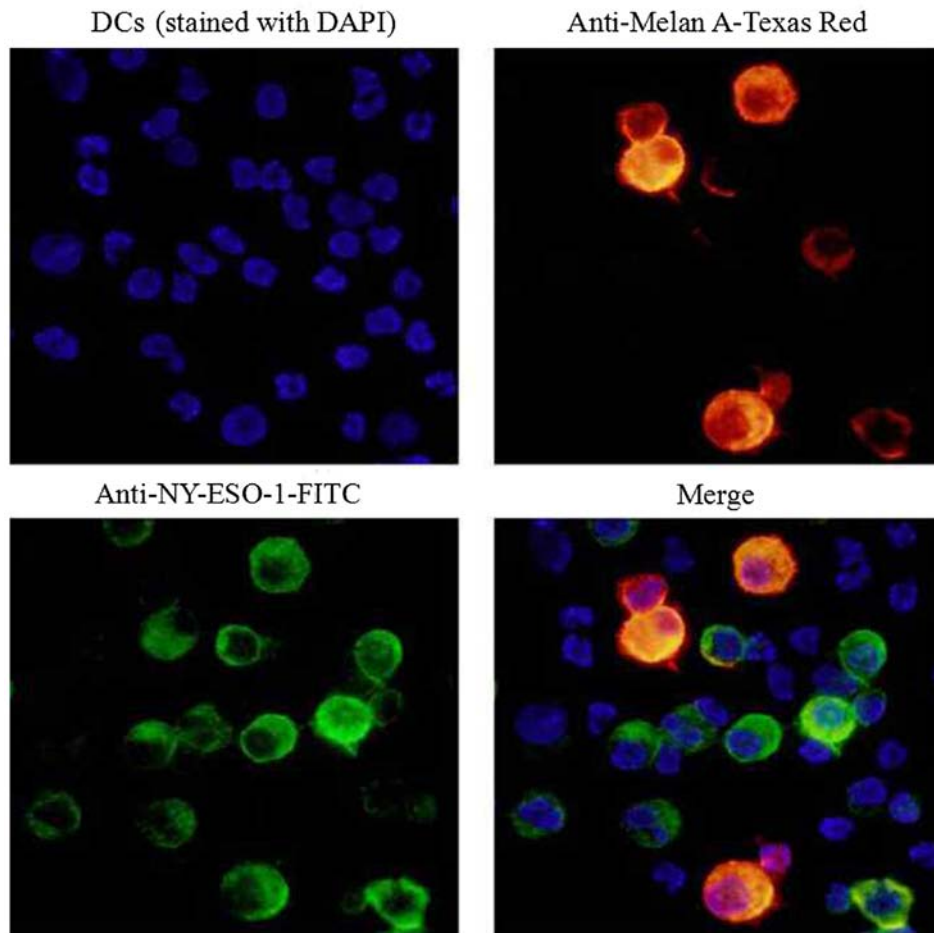


Figure 4. Dendritic cell (DC) uptake of M-HSP70-PCs. DCs were pulsed with M-HSP70-PCs at 37°C for 12 h. The cells were then incubated with specific monoclonal antibodies against Melan-A and NY-ESO-1, and visualized by fluorescence microscopy. Melan-A (red) and NY-ESO-1 (green) proteins were observed on the DC surface. (DCs from patient no. 4 were used).

analyzed under an Olympus DP71 microscope. Fig. 4 shows that DCs contained Melan-A protein (red) and NY-ESO-1 protein (green). Evidently, the M-HSP70-PCs were taken up by the DCs.

*Antigen-specific IFN- $\gamma$  production induced by DCs pulsed with M-HSP70-PCs.* Cell-mediated immunity, which is particularly important for tumor suppression, is characterized by the production of type I cytokines. Therefore, we explored the ability of DCs pulsed with M-HSP70-PCs to stimulate autologous T cells and induce IFN- $\gamma$  secretion. Autologous T cells co-cultured with the three DC groups were used as effector cells, and primary melanoma cells, which were used to obtain the autologous HSP70-PCs, were applied as target cells. The cells were then analyzed by an IFN- $\gamma$  ELISPOT. Fig. 5 shows a significantly higher IFN- $\gamma$  levels in groups B and C than in group A ( $P < 0.05$ ). There was no significant difference between groups B and C ( $P > 0.05$ ). This result confirmed that the DCs pulsed with M-HSP70-PCs stimulated T cells to secrete the same levels of type I cytokines compared with DCs pulsed with autologous HSP70-PCs.

*Cytotoxicity in vitro.* The three groups of autologous CD8<sup>+</sup> T cells were induced by co-culturing with the three

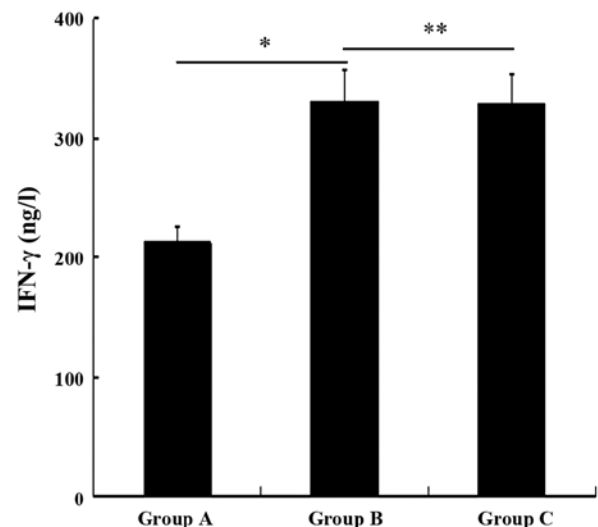


Figure 5. Secretion of interferon (IFN)- $\gamma$  by T cells induced with autologous dendritic cells (DCs). Primary tumor cells of melanoma patients were used as target cells, and three groups of effector cells were used. Group A, autologous DCs that received only granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. Group B, autologous DCs pulsed with 10  $\mu$ g M-HSP70-PCs. Group C, autologous DCs pulsed with 10  $\mu$ g autologous heat shock protein 70 (HSP70)-peptide complexes (PCs). Assays were performed in triplicate. The results are expressed as the means  $\pm$  standard deviation (SD) (n=3). \* $P < 0.05$ , \*\* $P > 0.05$ . (Data from patient no. 3 were used).

groups of autologous DCs. The specific cytolytic activities of CD8<sup>+</sup> T cells against primary melanoma cells was examined by detecting the level of LDH release after 4 h of co-culturing effector cells (CD8<sup>+</sup> T cells) with target cells (autologous primary melanoma cells) at 5:1, 10:1, and 20:1. The LDH release level was not significantly different between groups B and C ( $P>0.05$ ), but significantly higher in group A ( $P<0.05$ ) (Fig. 6). This result indicated that M-HSP70-PCs had an equivalent ability as autologous HSP70-PCs to induce autologous CD8<sup>+</sup> T cells to kill the melanoma cells of patients. Therefore, M-HSP70-PCs may be an efficient and general melanoma antigen complex.

*M-HSP70-PC-induced specific DCs and CD8<sup>+</sup> T-cell treatment of a patient with melanoma of the nasal skin and lung metastasis.* A patient with melanoma of the nasal skin and lung metastasis began treatment with autologous DCs and CD8<sup>+</sup> T cells induced by M-HSP70-PCs. The patient had not received any other therapy since September 2012. The DCs and CD8<sup>+</sup> T cells were induced from autologous peripheral blood (Fig. 7A and B), and the treatment was performed once every 6 months. After six cycles of treatment, the primary tumor of the nasal skin had not relapsed and no new metastases had appeared. An enhanced chest CT in March 2015 showed that some lung metastases had shrunk and others had disappeared (Fig. 8).

## Discussion

In recent years, with the rapid development of modern tumor immunology, immunotherapy of melanoma has achieved great success. Many new treatments, such as cytotoxic T lymphocyte-associated antigens, B-type Raf kinase inhibitors, and anti-programmed cell death 1/ligand antibodies, have greatly extended progress-free and overall survival of melanoma patients (3,17-19). However, there are huge costs associated with these treatments.

Cellular immune therapy such as DC-based immunotherapy has been recognized worldwide in recent years. DCs are professional APCs bridging innate and adaptive immunities. Their role in the periphery is to take up antigenic proteins that are processed and presented to T lymphocytes at lymphoid organs in combination with major histocompatibility molecules (4,20,21). Many clinical trials of immunotherapy have been performed using DC-based vaccines against melanoma since Nestle *et al* (22) reported the efficacy of a melanoma lysate or peptide-treated DC vaccine in 1998 (23,24). These studies have confirmed that DC-based vaccines are an efficient, safe and inexpensive method of melanoma treatment. Therefore, DC therapy has become one of the most widely used and effective treatments for melanoma in China as well as worldwide.

The type of antigens pulsed into DCs determines the effect of DC-based vaccines, and purified autologous tumor antigens are no doubt the best choice. However, for various reasons, some patients cannot obtain autologous tumor antigens. Early studies revealed many important antigens in human tumor cell lines. They used lysates of human tumor cell lines to pulse DCs for treatment of the same kind of tumor and achieved a certain therapeutic effect (4,12,13,24,25).

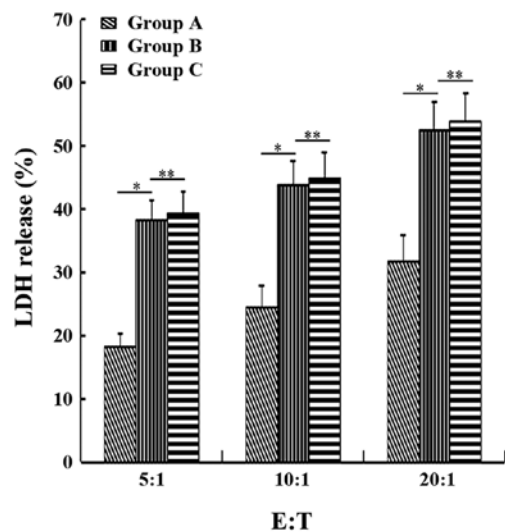


Figure 6. LDH release by CD8<sup>+</sup> T cells induced with autologous dendritic cells (DCs). Primary tumor cells of melanoma patients were used as target cells, and three groups of effector cells were used. Group A, autologous DCs that received only granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. Group B, autologous DCs pulsed with M-HSP70-PCs. Group C, autologous DCs pulsed with autologous heat shock protein 70 (HSP70)-peptide complexes (PCs). Assays were performed in triplicate. Results are expressed as the means  $\pm$  standard deviation (SD) ( $n=3$ ). \* $P<0.05$ , \*\* $P<0.005$ . (Data from patient no. 9 were used).

HSP70 is overexpressed in many tumors and acts at a crossroad of key intracellular processes in its role as a molecular chaperone. It associates with numerous tumor antigenic peptides and forms immunogenic complexes called HSP70-PCs (9,10,26). Our previous study revealed that HSP70-PCs purified from tumor cells have better immunocompetence than lysates of the same cells. Moreover, we established a new method using the detergent CHAPS to purify HSP70-PCs containing more efficient tumor peptides from human cancer cells (12).

We believe that a different cell line may contain different tumor antigen peptides which could be combined with HSP70. The six human melanoma lines we used were the total we were able to obtain at that time. In this study, we purified M-HSP70-PCs from human melanoma cell lines using the new method. To determine their immune activity, autologous HSP70-PCs purified from primary tumor cells of melanoma patients were used as a control. The M-HSP70-PCs had the same immunocompetence to induce DCs and CD8<sup>+</sup> T cells to specifically kill tumor cells from which the autologous HSP70-PCs were purified. This result suggests that M-HSP70-PCs may be an effective and general antigen complex of melanoma. In SDS-PAGE and western blot analyses, Melan-A and NY-ESO-1 were detected in M-HSP70-PCs.

We applied M-HSP70-PCs as antigens to treat one patient with melanoma and lung metastasis, and achieved a good therapeutic effect. The success of the treatment verified the safety and effectiveness of M-HSP70-PCs. It is worth mentioning that we used both autologous DCs and co-cultured DCs-CD8<sup>+</sup> T cells as the therapeutic cells, because we believe there is some immune suppression in cancer patients and intravenous reinfusion of a large number of CD8<sup>+</sup> T cells co-cultured with DCs *in vitro* might enhance the treatment effect.

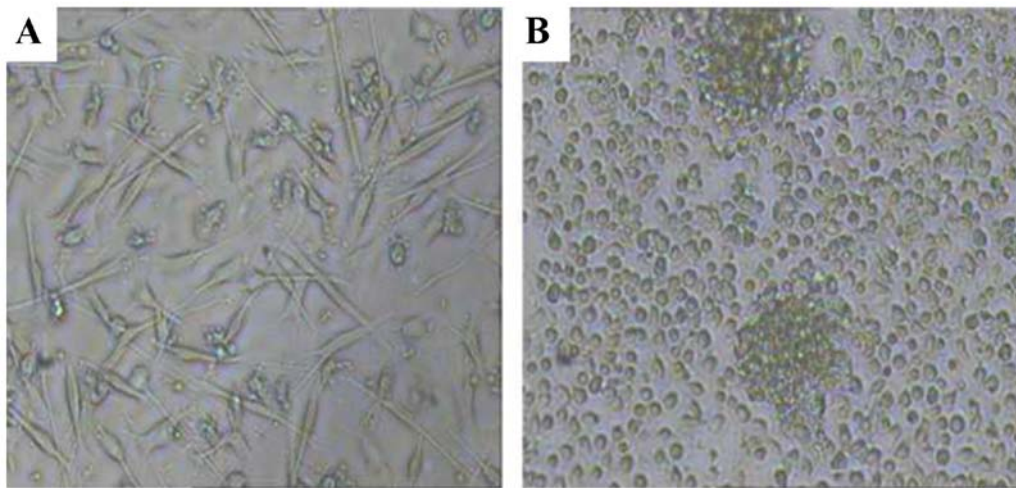


Figure 7. Autologous dendritic cells (DCs) and CD8<sup>+</sup> T cells of a patient with melanoma and lung metastasis. (A) Autologous DCs cultured for 5 days (magnification, x400). (B) Autologous CD8<sup>+</sup> T cells cultured for 11 days (magnification, x400). (Cells from the patient no. 4 were used).

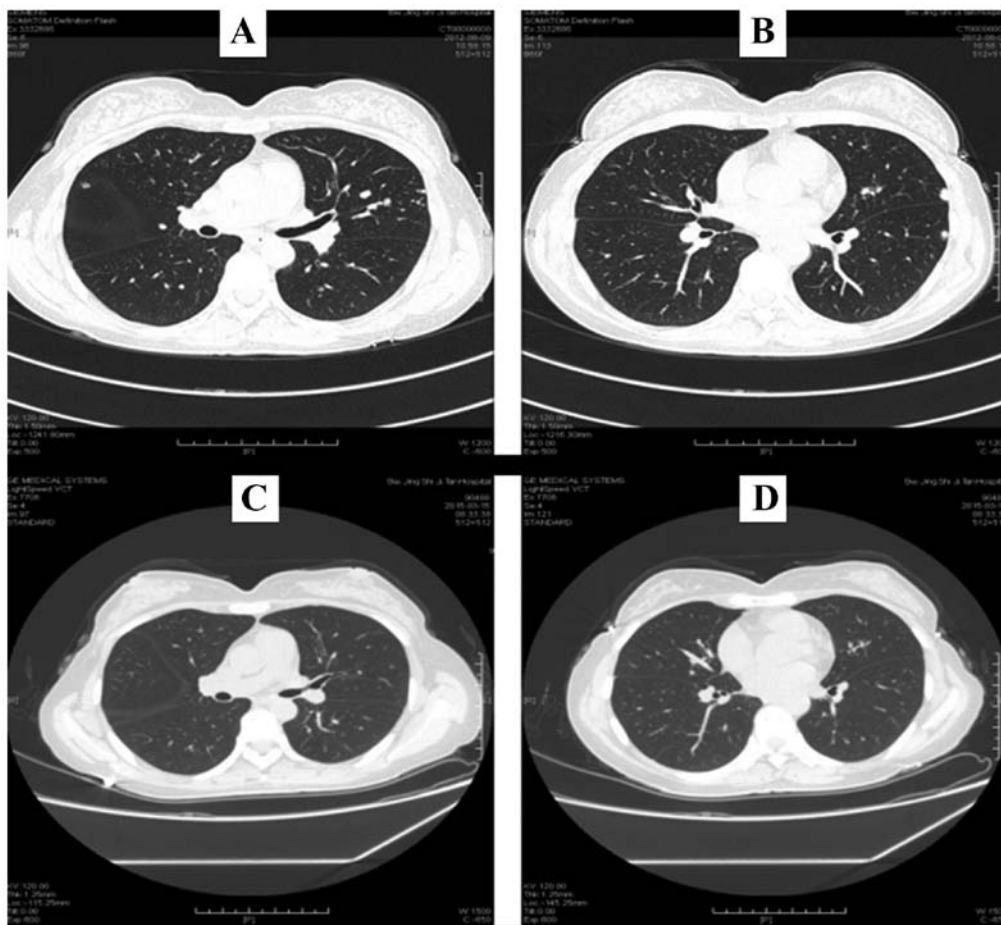


Figure 8. Enhanced chest computed tomography (CT) contrast images. (A and B) Different levels of images of the patient July 9th, 2012. (C and D) Images corresponding to (A) and (B) March 15th, 2015. (Patient information have been removed).

In summary, we prepared an effective and general antigen complex for melanoma treatment. The product, M-HSP70-PCs, may contain comprehensive and efficient melanoma antigens with the same immunocompetence against human melanoma cells as autologous antigens. We will explore the identity of other antigens in M-HSP70-PCs in further studies to reveal

new melanoma-associated antigen peptides. Simultaneously, we will continue to conduct clinical trials to confirm the treatment effect of this therapy. The findings of this study provide a better therapeutic approach for DC-based cellular immunotherapy of melanoma patients who cannot access autologous tumor antigens.

## Acknowledgements

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