Int J Clin Exp Med 2016;9(2):1106-1116
www.ijcem.com /ISSN:1940-5901/IJCEM0018501

Original Article

Enhancement of Ki67 peptide-based vaccine potency by GP96 protein promotes anti-tumor effect in osteosarcoma

Bingnan Guo[1,2*], Lin Liu[2*], Huizhong Li[3], Liwei Ding[2], Zhuan Yan[2], Shuqun Hu[2], Xianliang Yan[2], Junnian Zheng[3], Tie Xu[2]

[1]Institute of Biology and Medical Sciences, School of Biology and Basic Medical Science, Soochow University, Suzhou, China; [2]Institute of Emergency & Rescue Medicine, Xuzhou Medical College, Xuzhou, China; [3]Jiangsu Center for The Collaboration and Innovation of Cancer Biotherapy, Cancer Institute, Xuzhou Medical College, Xuzhou, China. [*]Equal contributors.

Received October 26, 2015; Accepted January 9, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: Peptide-based vaccines enable the safe and precisely directed immune intervention in controlling human cancers. $Ki67_{280-288}$ peptide plus with GP96 protein is a promising vaccine for osteosarcoma (OS) immunotherapy. To improve the efficacy of $Ki67_{280-288}$ peptide vaccination, we exploited a heat shock protein, namely, GP96 as an adjuvant. In vitro, peptide-specific HLA-$A02^+$ lymphocytes from dendritic cell (DC) loading $Ki67_{280-288}$ plus with GP96 produced high perforin. Furthermore, the production of IFN-γ was significantly enhanced both in vitro and in vivo. Moreover, cytotoxicity assay demonstrated that peptide-specific HLA-$A02^+$ lymphocytes could enhance anti-OS activity in vitro. Finally, $Ki67_{280-288}$ plus GP96 was an efficient agent for OS immunotherapy in the prophylaxis transgenic mouse model. Results demonstrated that $Ki67_{280-288}$ plus GP96 could be a promising agent for OS immunotherapy.

Keywords: $Ki67_{280-288}$ peptide, GP96 protein, osteosarcoma (OS), HLA-$A02^+$, Dendritic cells (DCs)

Introduction

Osteosarcoma (OS) is the most common aggressive malignancy of the bone. OS accounts for 15% to 35% of all primary bone tumors [1, 2] and remains the second leading cause of cancer-related death in children and young adults [3]. Despite current treatments with wide surgical resection of the tumor and post-operative adjuvant chemotherapy, OS is prone to relapse and has a 20% chance of causing pulmonary metastases [4]. Once metastases have occurred, the prognosis will become poor and current therapeutic strategies will have limited efficacy. Furthermore, the prognosis of patients with recurrent disease is dismal. Therefore, new therapeutic approaches are required to treat OS. Tumor immunotherapy is an alternative treatment for achieving local cancer control and reducing the risk of recurrence. However, reports on immunotherapy in OS treatment are lacking.

Peptide-based immunotherapy strategies appear promising as an approach to successfully induce an anti-tumor immune response and prolong survival in patients with various cancers [5-7]. Ki67 is a non-histonic protein that was originally identified by in the early 1980s [8]. Ki67 is strictly associated with cell proliferation. The Ki67 antigen is a tumor growth marker that demonstrates the percentage of cells during division. The expression of Ki67 antigen can be used to assess survival, neoplastic progression, and disease relapse. Our previous study highlighted that $Ki67_{280-288}$ peptide as an immunogen could induce HLA-restricted CTL response in tumor patients [9]. GP96 as a heat shock protein (HSP) is a molecular chaperone in the ER and is capable of facilitating peptide loading and correct folding of major hisocompatibility complex (MHC) class I molecules [10, 11]. Increasing evidence confirmed that GP96 can be a favorable adjuvant to assist immunogen-enhancing immunogenicity [12-14].

In this study, we utilized $Ki67_{280-288}$ peptide plus with adjuvant GP96 vaccination to elucidate the effects and possible mechanisms involved in anti-tumor immune responses both in vivo and in vitro. Our results showed that $Ki67_{280-288}$ plus GP96 was an efficient agent for OS immunotherapy in prophylaxis transgenic mouse model. Furthermore, $Ki67_{280-288}$ plus GP96 enhanced tumor-specific CTL responses. $Ki67_{280-288}$ plus GP96 promoted IFN-γ production of lymphocytes both in vitro and in vivo. In conclusion, our result provided evidence that $Ki67_{280-288}$ plus GP96 could augment the anti-tumor effects of $Ki67_{280-288}$ application and could be a promising agent for OS immunotherapy.

Materials and methods

Regents, antibodies and animals

rhGM-CSF, IL-4, and TNF-α were purchased from PeproTech (USA). rhIL-2 was purchased from Beijing ShuangLu Pharmaceutical Co., LTD (China). APC-conjugated or FITC-conjugated anti-human HLA-A2, APC-conjugated anti-human HLA-DR, PE-CY5-conjugated anti-human CD80, FITC-conjugated anti-human CD86, and Percp-cy5.5-conjugated anti-perforin antibodies, human IL-1β, IL-6, IL-12, IFN-γ ELISA Kit and cell permeabilization kit were purchased from BD (USA). GP96 protein was purchased from ENZO (USA). Ki-67 antibody (H-300) and GP96 antibody were purchased from Santa Cruz (USA), ABCAM (UK) respectively. HRP-conjugated or FITC-conjugated goat anti-rabbit IgG was purchased from Sigma (USA). The C57BL/6-Tg (HLA-A2.1) mice, which were transgenic human HLA-A2 gene, purchased from model animal research center of Nanjing University.

Cell lines and cell culture

The human OS cell line U2OS was purchased from the cell bank of the Chinese Academy of Science (Shanghai China). U2OS cells were maintained in RPMI-1640 (Gibco) medium containing 10% FCS (Gibco) and stored at 37°C in a humidified atmosphere of 5% CO_2 in air.

$Ki67_{280-288}$ synthesis

The Ki-67 sequence was scanned for HLA-A*0201-binding peptides by the prediction software Bioinformatics and Molecular Analysis (BIMAS, NIH, USA) as described before and confirmed HLA-A*0201-binding Ki67 peptides (LQGETQLLV) [9] was synthesized by Shanghai Bioengineering Ltd. Co. (Shanghai, China) with a purity of >90% as verified by HPLC and MS analysis. Lyophilized peptides were dissolved in DMSO (Sigma) and the aliquot of peptides were stored at -80°C.

Immunofluorescence

U2OS cell line was seeded and grown onto glass coverslips in 24-well culture plates (5×10^4 cells/cm^2). Cells were fixed with 4% paraformaldehyde in HPEM buffer (25 mM HEPES, 60 mM PIPES, 10 mM EGTA, 1 mM $MgCl_2$) for 20 min at room temperature (RT) overnight in a humidified atmosphere of 5% CO_2 in air and then further permeabilized in PBS/Triton X-100 0.1% for 20 min. Bovine serum albumin (5% in PBS/Tx-100 0.005%) was used for 30 min at RT as a blocking step prior to incubation with primary antibodies. Ki67 antibody was diluted in a blocking solution and incubated at the recommended periods, followed by incubation with secondary antibodies. DAPI (4,6-diamidino-2-phenylindole) was used to stain DNA. Special attention was taken to prevent fluorochrome fading by using a dark humidified chamber in all incubation steps.

SDS-PAGE and Western blot analysis

Approximately 2 μg GP96 protein were solubilized in SDS polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (250 mM Tris-HCl, pH 6.8, 0.5 M DTT, 10% SDS, 0.5% bromophenol blue, 50% Glycerol), boiled for 10 min, separated by SDS-PAGE, and/or transferred to PVDF membranes. Membranes were probed with anti-rabbit polyclonal GP96 antibodies diluted 1:1000 in 5% BSA and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence reaction.

Identification of PBMCs and U2OS cell line

5×10^5 PBMCs or U2OS cells were incubated with APC-conjugated anti-human HLA-A02 antibody at 4°C for 40 min in the dark. Another 5×10^5 PBMCs or U2OS cells were stained with APC-conjugated anti-human IgG as isotype control at 4°C for 40 min in the dark. After washing twice with PBS, the number of HLA-A*0201 positive cells was counted by FACS. For PBMCs,

we considered the one whose cell count was greater than 80% as belonging to HLA-A*0201 positive population. The HLA-A*0201 positive population was pooled together and/or frozen for further study.

Generation and maturation of human monocyte derived dendritic cells

Approximately 10 ml to 30 ml peripheral venous blood was drawn from healthy volunteers who underwent physical examination in the Affiliated Hospital of Xuzhou Medical College, Jiangsu, China. Consent from volunteers was obtained according to protocol approved by the review board of the Affiliated Hospital of Xuzhou Medical College. Identified HLA-A*0201$^+$ PBMCs were isolated from the blood by Ficoll-Urografin density gradient centrifugation and the monocytes were isolated by positive selection with CD14-conjugated microbeads (Miltenyi, Germany) and magnetic activated cell sorter. The rest of lymphocytes were cultured or frozen in RPMI-1640 medium with 30% FCS and 10% DMSO. Monocytes of more than 95% purity were cultured at a concentration of 1×10^6 cells/ml in a six-well plate (Becton Dickinson, USA) in DMEM containing 10% FBS and 1% penicillin/streptomycin supplemented with GM-CSF (50 ng/ml) and IL-4 (20 ng/ ml). On Day 5, immature dendritic cells (imDCs) were added with TNF-α (100 ng/ml) or PBS for 2 d.

Evaluation of cytokines secretion

For cultured DC supernatants, on Day 7, the supernatants were harvested to evaluate the concentrations of human IL-1β, IL-6, and IL-12. For IFN-γ, one week after the implantation, mouse serum was collected from the peripheral blood to evaluate the concentrations of serum IFN-γ. Cytokines level were determined by ELISA kit (BD, USA), according to manufacturer protocol.

Dendritic cell loading $Ki67_{280\text{-}288}$ plus with gp96

After the collection of mature dendritic cells (DCs), 5×10^6 in 2 ml RPMI supplemented with GM-CSF (50 ng/ml), IL-4 (20 ng/ml), and TNF-α (100 ng/ml) were incubated for 24 h with $Ki67_{280\text{-}288}$ (40 μg/ml) plus GP96 (40 μg/ml), $Ki67_{280\text{-}288}$ (40 μg/ml) alone, or GP96 alone (40 μg/ml), respectively. Negative controls were treated with PBS alone. Every 24 h, the cell medium was changed, the cells were washed, and repeated loading was conducted thrice.

Induction of peptide-specific lymphocytes with DCs loading peptides

Approximately 1×10^7 HLA-A*0201 positive lymphocytes were incubated with 5×10^6 DC loading $Ki67_{280\text{-}288}$ (40 μg/ml) plus with GP96 (40 μg/ml), $Ki67_{280\text{-}288}$ (40 μg/ml) alone, GP96 (40 μg/ml) alone, or PBS in RPMI-1640 medium at 37°C in humidified atmosphere containing 5% CO_2. Recombinant interleukin 2 (rhIL-2) at a concentration of 500 U/ml was added into the medium on the second day to stimulate lymphocytes for 7 d and repeated stimulation was conducted twice. After the second stimulation, the suspended lymphocytes were collected and percp-cy5.5-conjugated anti-perforin was stained with lymphocytes for analysis.

Cytotoxicity assays

CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) was used to measure the cytotoxic activity of lymphocytes, according to manufacturer protocol. Briefly, target cells U2OS (6×10^3 cells/well) were placed on 96-well U-bottom plates (Corning Costar, Cambridge, MA, USA), and lymphocytes (effectors) were added to a final volume of 100 μl in ratios of 100:1, 50:1, and 25:1 (E: T ratio). The plates were then incubated for 6 h in a humidified 5% CO_2 chamber at 37°C and centrifuged at 500×g for 5 min. Aliquots (50 μl) were transferred from all wells to fresh 96-well flat-bottom plates, and an equal volume of reconstituted substrate mix was added into each well. The plates were then incubated in the dark at RT for approximately 30 min. A 50 μl stop solution was added, and the absorbance was measured at 492 nm. The cell death percentages at each E: T ratio were calculated by using the following formula: (A492 nm [experimental]-A492 nm [effector spontaneous]-A492 nm [target spontaneous]) × 100/ (A492 nm [target maximum]-A492 nm [target spontaneous]).

Animal model

C57BL/6-Tg (HLA-A2.1) mice were maintained under SPF conditions and all procedures were conducted according to protocols approved by the Institutional Animal Care and Use

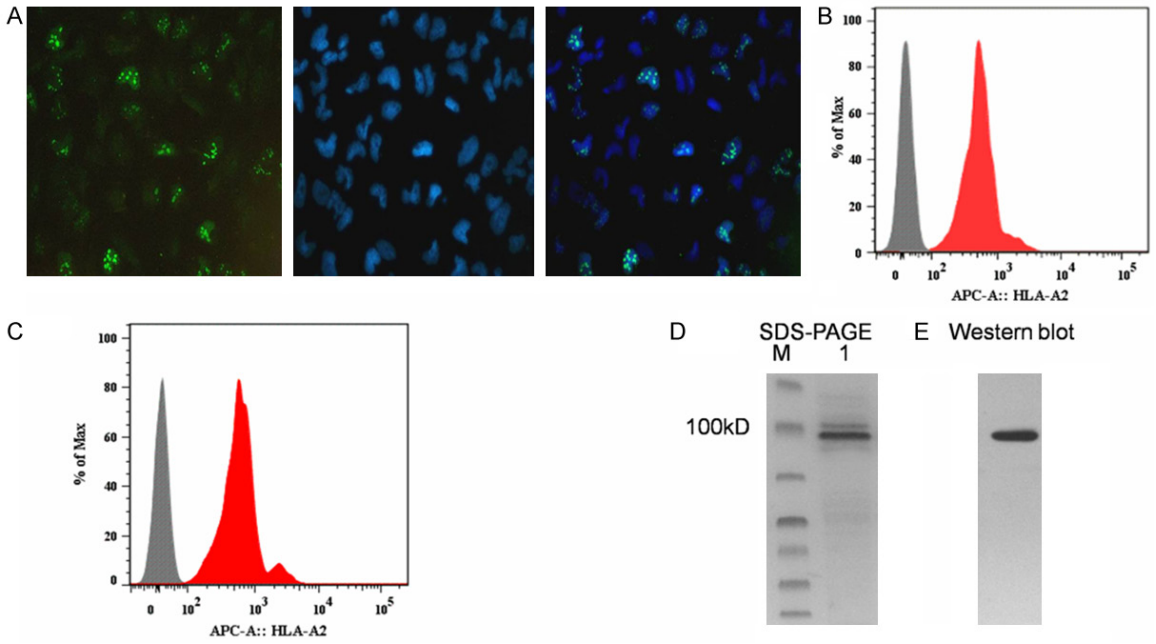

Figure 1. Characterization of U2OS cell line and PBMC. (A) Representative microscopy of Ki67 (Green) and DAPI (Blue) staining taken from human osteosarcoma cell line-U2OS in glass coverslips. (B, C) U2OS cell line (B) and PBMC (C) were cultured in medium with 10% FBS and collected cells of logarithmic growth phase were prepared to analyze frequencies of HLA-A2 expression by flow cytomerty. (D, E) GP96 (lane 1) was run in two identical SDS-PAGE 8% gels, one of which was stained with Coomassie blue (D). Markers (M) were loaded in the left-hand side lane. Protein bands in the unstained gel were transferred onto PVDF membrane for Western blotting with rabbit anti-GP96 Abs (1/1000 diluted) and HRP-conjugated goat anti-rabbit IgG for detection (E). The data shown are the representative of three experiments.

Committee of Soochow University and the Affiliated Hospital of Xuzhou Medical College. For prophylaxis experiments, the mice were immunized s.c. at the back with $Ki67_{280\text{-}288}$ (100 μg) plus with GP96 (100 μg), $Ki67_{280\text{-}288}$ (100 μg), GP96 (100 μg), or 200 μl PBS for a total of three times with 1-wk intervals. Two weeks after the last immunization, 1×10^7 U2OS cells were s.c. implanted into the right flank of mice. The tumor sizes were measured using a caliper every 3 days from day 3 post challenge. Tumor volumes were calculated using the formula $V=1/2\ (L \times W^2)$, where L is the length (longest dimension), and W is the width (shortest dimension).

Statistics

All data were analyzed by Student *t* test and were expressed as means ± SD or means ± SEM; data were analyzed using GraphPad Prism 6 software for Windows (GraphPad Software, San Diego, CA), and differences were considered statistically significant when $P<0.05$. The significance levels are marked $^*P<0.05$ and $^{**}P<0.01$.

Result

Osteosarcoma cell line U2OS expresses Ki67, HLA-A2, and highly detectable HLA-A2 on PBMCs

Whether Ki67, a promising molecular target in cancers [15, 16] and a novel immunogen [9], is expressed in OS is still unclear. Immunofluorescence assay was conducted to address it. **Figure 1A** shows that specific Ki67 antibody (the green) was strongly against Ki67 protein on human OS cell line U2OS and the nucleus was dyed by DAPI (blue). The presentation of peptides by MHC is important to the initiation of T-cell receptors of immune response. HLA-A2 is the most commonly expressed HLA class I allele among Caucasians and Asians (50%) [17]. Both U2OS and PBMCs highly expressed HLA-A2 (**Figure 1B** and **1C**). The selected HLA-$A2^+$ PBMCs were pooled together for further

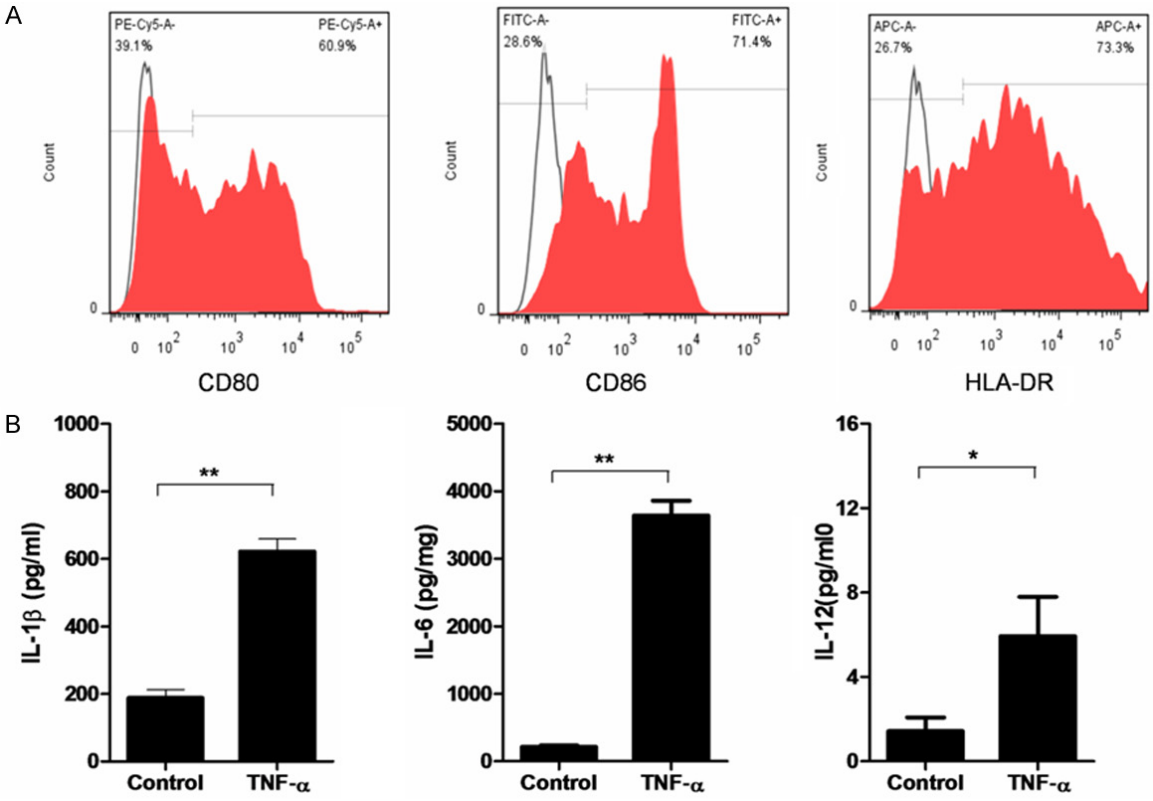

Figure 2. Immunophenotype of generated DCs by flow cytometry and cytokine release. A. After the 5 day incubation with GM-CSF and IL-4, subsequently TNF-α pulsed for another 2 days, DCs showed that the maturation marker CD80, CD86 and HLA-DR expression were enhanced by flow cytometry. B. In cytokine production assays, monocyte-derived DCs culture supernatant was then quantitatively assayed, in triplicate wells, for the presence of IL-1β, IL-6, and IL-12 using an ELISA kit. Values are presented as means ± SD. The data shown are the representative of three experiments. *P<0.05, **P<0.01.

study. Furthermore, we identified GP96 protein by SDS-PAGE and immunoblotting analysis (**Figure 1D** and **1E**). Taken together, both Ki67 and HLA-A2 were expressed on U2OS cell line and PBMCs were found to have high HLA-A2 expression.

DCs maturation and related cytokines released

DCs are professional APC with significant phenotypic heterogeneity and functional plasticity. DCs play important roles in initiating effective adaptive immune responses for elimination of invading pathogens [18]. On Day 7, cultured HLA-A2$^+$ monocytes-derived DCs surface marker CD80 (60.9%), CD86 (71.4%), HLA-DR (73.3%) were detected by FACS as shown in **Figure 2A**. Moreover, pro-inflammatory cytokines expression significantly increased, such as IL-1β, IL-6, and IL-12 in DCs supernatant (**Figure 2B**) compared with untreated DCs (no TNF-α) supernatant. The results suggested DC maturation.

Perforin secretion

As DCs maturation, they were loaded with $Ki67_{280\text{-}288}$ plus GP96 ($DC_{Ki67280\text{-}288\ plus\ GP96}$), $Ki67_{280\text{-}288}$ ($DC_{Ki67280\text{-}288}$), GP96 (DC_{GP96}), or PBS. The loading DCs were then cocultured with HLA-A2$^+$ lymphocytes for 7 d, which was repeated 2 times. Perforin secretion from the lymphocytes was analyzed by FACS. As shown in **Figure 3**, secreted perforin from lymphocytes was significantly increased in cocultured with $DC_{Ki67280\text{-}288\ plus\ GP96}$ group than those with $DC_{Ki67280\text{-}288}$, DC_{GP96}, or PBS alone group. Cocultured lymphocytes from $DC_{Ki67280\text{-}288}$ group, also, produced higher perforin than those from PBS or DC_{GP96} group. Perforin was mainly secreted by T-cells, especially CD8$^+$

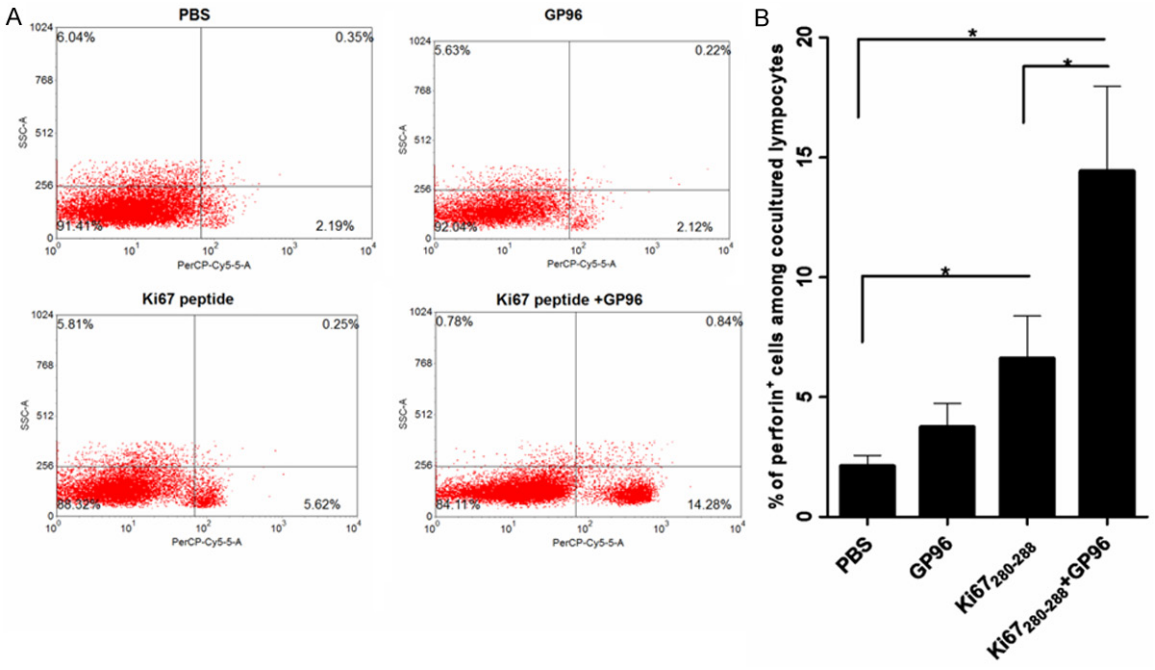

Figure 3. Activity of peptide-specific lymphocytes with mature DCs loading peptides. A. Intracellular perforin production was measured from lymphocytes, which were cocultured with $DC_{Ki67280\text{-}288\ plus\ GP96}$, $DC_{Ki67280\text{-}288}$, DC_{GP96} or PBS respectively. B. The percentages of perforin production in cocultured lymphocytes. Values are presented as means ± SD. The data shown are the representative of three experiments. *P<0.05.

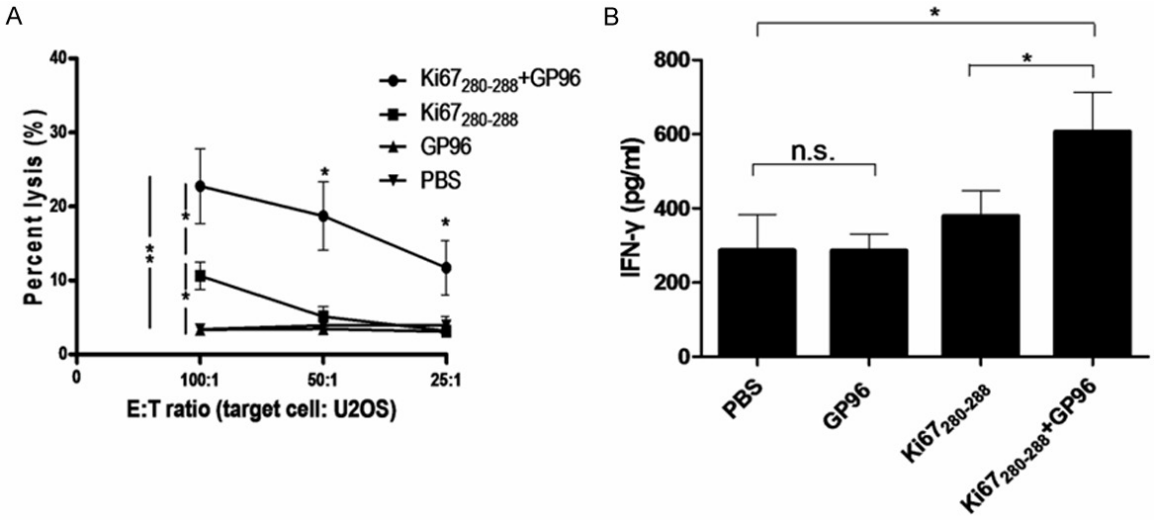

Figure 4. Anti-tumor response of Ki67 peptide-based vaccination in vitro. A. Cytotoxicity assay of lymphocytes from cocultured $DC_{Ki67280\text{-}288\ plus\ GP96}$, $DC_{Ki67280\text{-}288}$, DC_{GP96} or PBS respectively. Lymphocytes harvested from cocultured $DC_{Ki67280\text{-}288\ plus\ GP96}$, $DC_{Ki67280\text{-}288}$, DC_{GP96} or PBS and used as effector cells, and their killing capacity of U2OS targets was measured using CytoTox 96 nonradioactive cytotoxicity assay kit. The assays were done in triples. B. In cytokine production assays, cultured supernatant was then quantitatively assayed, in triplicate wells, for the presence of IFN-γ using an ELISA kit. Values are presented as means ± SD The data shown are the representative of three experiments. *P<0.05, **P<0.01.

T-cells, which suggested that highly secreted perforin in lymphocytes cocultured with $DC_{Ki67280\text{-}288\ plus\ GP96}$ group was perhaps due to T-cells, particularly $CD8^+$ T-cells.

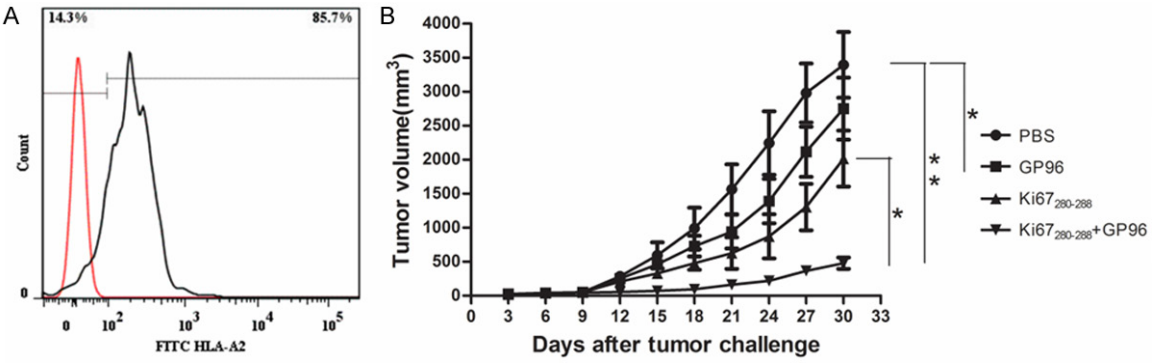

Figure 5. Anti-tumor response of Ki67 peptide-based vaccination in vivo. A. The phenotype of C57BL/6-Tg (HLA-A2.1) mice which were transgenic human HLA-A2 gene was determined by flow cytometry. B. Tumor growth curves of osteosarcoma model. Mice were immunized with $Ki67_{280-288}$ plus GP96, $Ki67_{280-288}$, GP96, or PBS, 3 times with 1-wk intervals, and then, the mice were challenged with 1×10^7 human osteosarcoma cell line-U2OS, 2 wk after the last immunization. The tumor volumes were monitored every 3 days. The experiments were performed with five to seven mice per group. The assays were done in triples. Values are presented as means ± SEM. The data shown are the representative of three experiments *P<0.05.

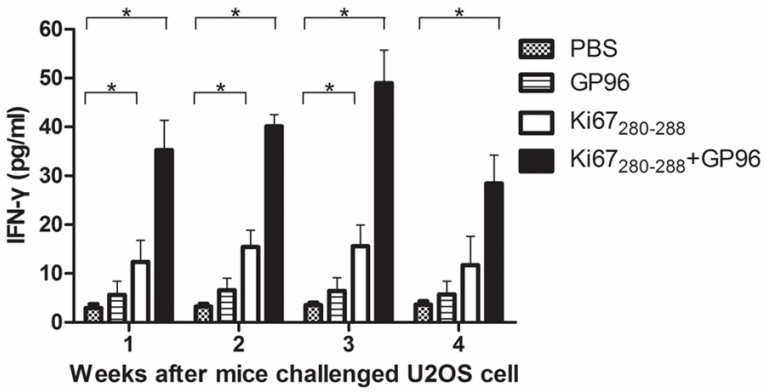

Figure 6. Concentrations of IFN-γ in tumor-burdened C57BL/6-Tg (HLA-A2.1) mice. Serum IFN-γ obtained from peripheral blood 1 week, 2 weeks, 3 weeks and 4 weeks after challenged with U2OS cells were determined by ELISA. Values are presented as means ± SEM. The data shown are the representative of three experiments. *P<0.05, n=5.

Cytotoxicity assay and IFN-γ production in vitro

$DC_{Ki67280-288\ plus\ GP96}$ may promote lymphocytes, especially T-cell maturation (perforin as a maturation marker). To test whether co-cultured lymphocytes was against human OS cell line-U2OS in vitro, the U2OS cell line was employed as target cell to address it. Stimulated lymphocytes were used in the cytotoxicity assay against U2OS targets. Lymphocytes from $DC_{Ki67280-288}$ stimulated exhibited significantly higher cytotoxicity against U2OS cells than DC_{GP96} and PBS treatment U2OS groups. Notably, cytotoxicity was further enhanced as lymphocytes from $DC_{Ki67280-288\ plus\ GP96}$ group (**Figure 4A**). IFN-γ is an essential cytokine for immunity against cancer and pathogens. We examined lymphocytes responses by in vitro stimulation with U2OS cell line and supernatant IFN-γ by ELISA. IFN-γ was significantly higher in lymphocytes from $DC_{Ki67280-288\ plus\ GP96}$ group than the other groups (P<0.05) (**Figure 4B**).

$Ki67_{280-288}$ plus GP96 vaccine induced osteosarcoma protection in vivo

To investigate protective effects of the DC vaccination loading with $Ki67_{280-288}$ plus GP96 in vivo, murine OS model was used. C57BL/6-Tg (HLA-A2.1) mice, which were transgenic human HLA-A2 gene (**Figure 5A**), were immunized with $Ki67_{280-288}$ plus GP96, $Ki67_{280-288}$, GP96, or PBS, 3 times with 1 wk intervals, and then, the mice were challenged with 1×10^7 human OS cell line U2OS two weeks after the last immunization and were monitored tumor size for 30 days. Mice immunized with $Ki67_{280-288}$ plus GP96 group and $Ki67_{280-288}$ group had significant reduction in tumor growth compared with mice immunized with PBS group (**Figure 5B**). Moreover, immunization with $Ki67_{280-288}$ plus GP96 group further reduced tumor growth compared with immunization

with $Ki67_{280-288}$ group. Serum IFN-γ, also, obtained from the mice' peripheral blood 1 week, 2 weeks, 3 weeks and 4 weeks after challenged with U2OS cells were determined by ELISA. During the period, the serum concentrations IFN-γ increase significantly in most mice. One week after last challenge, IFN-γ of $Ki67_{280-288}$ group was significantly higher than other groups except $Ki67_{280-288}$ plus GP96 group, reaching the highest in the 2nd week and decreased thereafter, while IFN-γ of $Ki67_{280-288}$ plus GP96 group witnessed a continuous elevation and reached almost the peak in the 3rd week but interestingly decreased in the 4th week (**Figure 6**).

Discussion

Immunotherapy has become an attractive therapeutic modality for cancer, which offers potentially targeted therapy with fewer adverse effects compared with conventional therapy [19]. Tumor associated antigens (TAAs) that can be targeted by CTL are fundamental concepts behind developing current immunotherapy. Three characteristics are required for an ideal TAA to elicit effective and safe T-cell-mediated anti-tumor immunity in cancer patients [20]: 1) Cancer-specific expression of TAA. An ideal TAA must be overexpressed in tumors, but not or low expressed on normal cells, such as cancer-testis antigen HER-2/neu, Ki67. 2) Oncogenic characteristics of TAA, which is involved in oncogenesis, are considered to be barely lost in the process of tumor progression, such as p53, Ki67. 3) Immunogenicity of TAA is required. Immune responses could be evaluated by detection of TAA-specific IgG or TAA-specific T-cell responses, such as IFN-γ, TNF-α, and IL-2 secretion. TAA-peptide-based immunotherapy is a promising approach to successfully induce anti-tumor immune responses and prolong survival in patients with various cancers [21-24]. However, without suitable adjuvants, a small synthetic peptide or a recombinant protein can elicit only a weak CTL response in this process. For instance, Rosenberg et al. [25] reported that the clinical effect of vaccination of TAA-derived CTL-epitope peptides (short peptides) alone was limited in a very small fraction of advanced melanoma patients. Therefore, antigens need to be combined with an efficient adjuvant for use in humans.

HSPs have been demonstrated to act as potent adjuvants in immunotherapy of malignant tumors [26] and infectious diseases [27]. GP96, as a HSP, has emerged as an attractive adjuvant component for vaccines. GP96, the major chaperon in the lumen of the endoplasmic reticulum involved in cross presentation of peptides to MHC class I and class II molecules, activates specific $CD8^+$ and $CD4^+$ T-cells [28, 29]. Moreover, GP96 can activate dendritic cells and macrophages through induction of proinflammatory cytokines via interaction with a subset of toll-like receptors [30]. Previous reports showed that GP96 or its N-terminal domain had an adjuvant function in tumor [31] and virus [32] specific CTL and humoral immune responses. HLA-A*0201 is an important HLA class I allele in Asian population. In our previous study, $Ki67_{280-288}$ peptide, which was identified to be well recognized by $CD8^+$ T-cells in cancer patients expressing HLA-A*0201 molecules, was shown to be targets for HLA-A*0201-restricted $CD8^+$ T-cells that produced generous IFN-γ.

In the current study, HLA-A*0201 expression, firstly, was assessed with both PBMCs of healthy donors and OS cell line-U2OS. As expected, HLA-A*0201 was highly expressed in U2OS cell line and PBMCs of partial volunteers that were pooled together upon identification of HLA-A*0201$^+$ (**Figure 1B** and **1C**). As a TAA, Ki67 was confirmed overexpressed in U2OS cell line by immunofluorescence assay in this study (**Figure 1A**). Our result suggested that the U2OS cell line, as an OS model, could be further studied in our system. Our hypothesis was that DCs maturation could facilitate $Ki67_{280-288}$ peptide presentation. Thus, human monocyte derived DC was cultured in vitro with GM-CSF and IL-4. On Day 5, TNF-α instead of LPS was added for DC maturation. Immunophenotype of maturation marker was up-regulated, such as CD80, CD86, and HLA-DR (**Figure 2A**) on Day 7. Moreover, pro-inflammatory cytokines, which were produced by DC maturation, produced IL-1β, IL-6, and IL-12 which were enhanced on Day 7 (**Figure 2B**).

In addition, our findings indicated that DCs loading $Ki67_{280-288}$ plus GP96 could affect the quality of induced $Ki67_{280-288}$-specific cellular immune responses in vitro. We showed that DC loading $Ki67_{280-288}$ peptide resulted in enhance-

ment of the function of CTL responses, perforin (**Figure 3A**) and IFN-γ upregulation (**Figure 4B**). Strikingly perforin (**Figure 3A**) and IFN-γ upregulation (**Figure 4B**) in $DC_{Ki67280\text{-}288\ plus\ GP96}$ group was significantly higher compared with those in $DC_{Ki67280\text{-}288}$ group. Notably, lymphocytes form DC loading $Ki67_{280\text{-}288}$ peptide displayed significantly high cytotoxicity against U2OS in vitro, which was future enhanced as lymphocytes from $DC_{Ki67280\text{-}288\ plus\ GP96}$ group (**Figure 4A**). The results are consistent with the findings that C-terminal fusion of GP96 to Her2 could enhance efficacy of Her2 DNA vaccine [33]. Furthermore, Leila et al. [34] found that an N-terminal fusion of GP96 fragment plus HCV vaccine enhanced HCV-specific CTL immune responses. However, both Pakravan and Leila performed assay with DNA vaccine. In our experiments, we directly employed DC loading GP96 protein and $Ki67_{280\text{-}288}$ peptide as a vaccine. Overall, enhanced immune responses observed for an immunogen plus GP96 were consistent with others. Remarkably, in prophylaxis transgenic mouse model, mice immunized with $Ki67_{280\text{-}288}$ peptide plus with GP96 group revealed significant tumor reduction than other groups (**Figure 5B**). Mounting data showed that T cell-mediated tumor rejection depend not only upon perforin, granzyme and Fas/FasL but on some soluble cytokines such as IFN-γ [35]. During our treatment in vivo, IFN-γ production of $Ki67_{280\text{-}288}$ peptide plus GP96 group is the highest than that of other groups, suggesting they promote DC-primed T cell polarization to Th1 (**Figure 6**). $Ki67_{280\text{-}288}$ vaccinated mice as well as has high level IFN-γ. However, the precise mechanism of protection effect in vivo, which our efforts will focus on, should be further investigated.

In conclusion, we utilized $Ki67_{280\text{-}288}$ peptide plus with adjuvant GP96 vaccination to elucidate effects and possible mechanisms involved in anti-tumor immune responses both in vivo and in vitro. Our results showed that $Ki67_{280\text{-}288}$ plus GP96 was an efficient agent for OS immunotherapy in prophylaxis transgenic mouse model, which perhaps enhanced tumor-specific CTL responses (IFN-γ production). The results showed promotion of IFN-γ and perforin production of lymphocytes and enhanced cytotoxicity against U2OS in vitro. Our results warrant future studies for the development of an effective cancer vaccine based on the current strategies.

Acknowledgements

This study was supported by grants from National Foundation of Nature Science of China (81202015), the Science Foundation of Xuzhou Science & Technology bureau (KC15J0060, KC15SX009, XM12B025, KC14-SH087).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Tie Xu, Institute of Emergency & Rescue Medicine, Xuzhou Medical College, Xuzhou 221002, China. E-mail: xutie889@163.com

References

[1] Mirabello L, Troisi RJ and Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer 2009; 115: 1531-1543.

[2] Moore DD and Luu HH. Osteosarcoma. Cancer Treat Res 2014; 162: 65-92.

[3] Goldsby R, Burke C, Nagarajan R, Zhou T, Chen Z, Marina N, Friedman D, Neglia J, Chuba P and Bhatia S. Second solid malignancies among children, adolescents, and young adults diagnosed with malignant bone tumors after 1976: follow-up of a Children's Oncology Group cohort. Cancer 2008; 113: 2597-2604.

[4] Bielack SS, Carrle D, Hardes J, Schuck A and Paulussen M. Bone tumors in adolescents and young adults. Curr Treat Options Oncol 2008; 9: 67-80.

[5] Lu W, Qiu L, Yan Z, Lin Z, Cao M, Hu C, Wang Z, Wang J, Yu Y, Cheng X, Cao P and Li R. Cytotoxic T cell responses are enhanced by antigen design involving the presentation of MUC1 peptide on cholera toxin B subunit. Oncotarget 2015; 6: 34537-48.

[6] Derouazi M, Di Berardino-Besson W, Belnoue E, Hoepner S, Walther R, Benkhoucha M, Teta P, Dufour Y, Yacoub Maroun C, Salazar AM, Martinvalet D, Dietrich PY and Walker PR. Novel Cell-penetrating peptide-based vaccine induces robust CD4+ and CD8+ T cell-mediated antitumor immunity. Cancer Res 2015; 75: 3020-3031.

[7] Parmiani G, Russo V, Maccalli C, Parolini D, Rizzo N and Maio M. Peptide-based vaccines for cancer therapy. Hum Vaccin Immunother 2014; 10: 3175-3178.

[8] Gerdes J, Schwab U, Lemke H and Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983; 31: 13-20.
[9] Xu W, Li HZ, Liu JJ, Guo Z, Zhang BF, Chen FF, Pei DS and Zheng JN. Identification of HLA-A*0201-restricted cytotoxic T lymphocyte epitope from proliferating cell nuclear antigen. Tumour Biol 2011; 32: 63-69.
[10] Zhao R, Davey M, Hsu YC, Kaplanek P, Tong A, Parsons AB, Krogan N, Cagney G, Mai D, Greenblatt J, Boone C, Emili A and Houry WA. Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. Cell 2005; 120: 715-727.
[11] Taipale M, Krykbaeva I, Koeva M, Kayatekin C, Westover KD, Karras GI and Lindquist S. Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. Cell 2012; 150: 987-1001.
[12] Ju Y, Fan H, Liu J, Hu J, Li X, Li C, Chen L, Gao Q, Gao GF and Meng S. Heat shock protein gp96 adjuvant induces T cell responses and cross-protection to a split influenza vaccine. Vaccine 2014; 32: 2703-2711.
[13] Crane CA, Han SJ, Ahn B, Oehlke J, Kivett V, Fedoroff A, Butowski N, Chang SM, Clarke J, Berger MS, McDermott MW, Prados MD and Parsa AT. Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein. Clin Cancer Res 2013; 19: 205-214.
[14] Guo QY, Yuan M, Peng J, Cui XM, Song G, Sui X and Lu SB. Antitumor activity of mixed heat shock protein/peptide vaccine and cyclophosphamide plus interleukin-12 in mice sarcoma. J Exp Clin Cancer Res 2011; 30: 24.
[15] Li XQ, Pei DS, Qian GW, Yin XX, Cheng Q, Li LT, Li HZ and Zheng JN. The effect of methylated oligonucleotide targeting Ki-67 gene in human 786-0 renal carcinoma cells. Tumour Biol 2011; 32: 863-872.
[16] Li LT, Jiang G, Chen Q and Zheng JN. Ki67 is a promising molecular target in the diagnosis of cancer (review). Mol Med Rep 2015; 11: 1566-1572.
[17] Li Y, Zhou L, Li Y, Zhang J, Guo B, Meng G, Chen X, Zheng Q, Zhang L, Zhang M and Wang L. Identification of autoreactive CD8+ T cell responses targeting chromogranin A in humanized NOD mice and type 1 diabetes patients. Clin Immunol 2015; 159: 63-71.
[18] Liu J and Cao X. Regulatory dendritic cells in autoimmunity: A comprehensive review. J Autoimmun 2015; 63: 1-12.
[19] Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. Science 2013; 342: 1432-1433.
[20] Nishimura Y, Tomita Y, Yuno A, Yoshitake Y and Shinohara M. Cancer immunotherapy using novel tumor-associated antigenic peptides identified by genome-wide cDNA microarray analyses. Cancer Sci 2015; 106: 505-511.
[21] Shariat S, Badiee A, Jalali SA, Mansourian M, Yazdani M, Mortazavi SA and Jaafari MR. P5 HER2/neu-derived peptide conjugated to liposomes containing MPL adjuvant as an effective prophylactic vaccine formulation for breast cancer. Cancer Lett 2014; 355: 54-60.
[22] Kumari S, Walchli S, Fallang LE, Yang W, Lund-Johansen F, Schumacher TN and Olweus J. Alloreactive cytotoxic T cells provide means to decipher the immunopeptidome and reveal a plethora of tumor-associated self-epitopes. Proc Natl Acad Sci U S A 2014; 111: 403-408.
[23] Shen H, Shao HW, Chen XH, Wu FL, Wang H, Huang ZL, Shen J, Wang T, Zhang WF and Huang SL. Identification of a novel HLA-A2-restricted mutated Survivin epitope and induction of specific anti-HCC CTLs that could effectively cross-recognize wild-type Survivin antigen. Cancer Immunol Immunother 2013; 62: 393-403.
[24] Tomita Y, Imai K, Senju S, Irie A, Inoue M, Hayashida Y, Shiraishi K, Mori T, Daigo Y, Tsunoda T, Ito T, Nomori H, Nakamura Y, Kohrogi H and Nishimura Y. A novel tumor-associated antigen, cell division cycle 45-like can induce cytotoxic T-lymphocytes reactive to tumor cells. Cancer Sci 2011; 102: 697-705.
[25] Rosenberg SA, Yang JC and Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004; 10: 909-915.
[26] Bolhassani A and Rafati S. Heat-shock proteins as powerful weapons in vaccine development. Expert Rev Vaccines 2008; 7: 1185-1199.
[27] Wood CG and Mulders P. Vitespen: a preclinical and clinical review. Future Oncol 2009; 5: 763-774.
[28] Doody AD, Kovalchin JT, Mihalyo MA, Hagymasi AT, Drake CG and Adler AJ. Glycoprotein 96 can chaperone both MHC class I- and class II-restricted epitopes for in vivo presentation, but selectively primes CD8+ T cell effector function. J Immunol 2004; 172: 6087-6092.
[29] Javid B, MacAry PA and Lehner PJ. Structure and function: heat shock proteins and adaptive immunity. J Immunol 2007; 179: 2035-2040.
[30] Yang Y, Liu B, Dai J, Srivastava PK, Zammit DJ, Lefrancois L and Li Z. Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. Immunity 2007; 26: 215-226.
[31] Baker-LePain JC, Sarzotti M, Fields TA, Li CY and Nicchitta CV. GRP94 (gp96) and GRP94 N-terminal geldanamycin binding domain elicit

tissue nonrestricted tumor suppression. J Exp Med 2002; 196: 1447-1459.
[32] Li H, Zhou M, Han J, Zhu X, Dong T, Gao GF and Tien P. Generation of murine CTL by a hepatitis B virus-specific peptide and evaluation of the adjuvant effect of heat shock protein glycoprotein 96 and its terminal fragments. J Immunol 2005; 174: 195-204.
[33] Pakravan N, Hashemi SM and Hassan ZM. Adjuvant activity of GP96 C-terminal domain towards Her2/neu DNA vaccine is fusion direction-dependent. Cell Stress Chaperones 2011; 16: 41-48.
[34] Pishraft-Sabet L, Kosinska AD, Rafati S, Bolhassani A, Taheri T, Memarnejadian A, Alavian SM, Roggendorf M and Samimi-Rad K. Enhancement of HCV polytope DNA vaccine efficacy by fusion to an N-terminal fragment of heat shock protein gp96. Arch Virol 2015; 160: 141-152.
[35] Peng L, Krauss JC, Plautz GE, Mukai S, Shu S and Cohen PA. T cell-mediated tumor rejection displays diverse dependence upon perforin and IFN-gamma mechanisms that cannot be predicted from in vitro T cell characteristics. J Immunol 2000; 165: 7116-7124.