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Original Article

Cloning, expression of a truncated HSP110 protein that augments the activities of tumor antigen-specific cytotoxic and apoptosis via tHSP110-peptide complex vaccines

Xu Huang[1], Han Dong[1], Zhang Ting[2]

[1]*Jiaxing University Medical College, Jiaxing, Zhejiang, China;* [2]*College of Medical Technology, Zhejiang Chinese Medical University, Hangzhou, China*

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Abstract: The present study used a genetic engineering method to express a truncated heat shock protein 110 (tHSP110) isoform in *Escherichia coli* and verified its ability to bind to and present macromolecular antigens. Polymerase chain reaction (PCR) was used to obtain the truncated HSP110 gene, which was expressed in *E. coli*. The tHSP110 protein was non-covalently coupled to the intracellular domain (ICD) of human epidermal growth factor receptor 2 (HER2/Neu) in vitro to construct the antigen peptide complex tHSP110-ICD, which was identified by a co-immunoprecipitation assay. BALB/c mice were immunized 14-day interval for three times with the HSP110, tHSP110, HSP110-ICD, tHSP110-ICD, HSP110-$P_{851-859}$ (a complex formed by full-length HSP110 with a cytotoxic T lymphocyte (CTL) epitope peptide of the Her2/neu ICD) and tHSP110-$P_{851-859}$ complexes. Fourteen days after the last immunization, D_2F_2 cells were inoculated into BALB/c mice. The in vivo tumor volume of each group was measured every three days after cell inoculation to evaluate the immunization efficacy of the vaccine in each group. The level of the IFN-γ secreted by activated lymphocytes, the specific CTLs activity was detected. Immunohistochemical staining of bcl-2 and bax were measured on the tumor tissues of each group. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the expressed tHSP110 protein was 66 kDa in size. The non-covalent coupling of tHSP110 with ICD and peptide were confirmed by a co-immunoprecipitation assay. The in vivo tumor experiment results indicated no differences in the tumor volumes of the tHSP110-ICD and HSP110-ICD groups. In contrast, the tumor volume of the tHSP110-ICD group was significantly different compared with the tumor volume of the tHSP110-$P_{851-859}$ group. After the mice immunized with tHSP110-ICD, tHSP110-$P_{851-859}$ complexes, the complexes have potential immunogenicity, and can induce specific CTLs activity and apoptosis in BALB/c mice. As a tumor vaccine to inhibit in vivo tumor growth, the tHSP110 has the same ability to bind macromolecular antigens and activate tumor immune responses as full-length HSP110.

Keywords: Truncated HSP110, cloning, immune response, vaccine

Introduction

Heat shock protein 110 (HSP110) is a high molecular weight heat shock protein that has a strong ability to bind macromolecular antigens [1]. However, the HSP110 protein has a high molecular weight and contains complex functional domains. From the N terminus to the C-terminus, HSP110 contains four large functional domains (ATP-binding domain, peptide-binding domain, amino acid loop and helix region), but not every structural domain of the abovementioned functional domains is associated with its antigen-binding and antigen presentation functions [2-4]. Of the structural domains, the ATP-binding domain has the highest number of amino acids. This N-terminal domain is composed of 384 amino acids, and its molecular weight accounts for half of the total molecular weight of HSP110 [5]. Current in vivo studies showed that the major function of this domain was to regulate the binding and release of substrates from HSP through changing its binding to ATP and ADP [6-8]. To date, this domain has not been shown to play a role in binding and presenting antigen peptides. To

this end, the present study constructed a truncated HSP110 peptide that lacked the ATP-binding domain. The truncated HSP110 protein was coupled with the intracellular domain (ICD) of the macromolecular breast cancer antigen human epidermal growth factor receptor 2 (HER2/neu). After three vaccinations, D_2F_2 tumor cells were injected intraperitoneally to form a tumor. Tumor formation was observed in the mice, and the sizes of the tumor tissues were measured at different time points. The level of the IFN-γ secreted by activated lymphocytes, the specific CTLs activity was detected. Immunohistochemistry staining of bcl-2 and bax were measured on the tumor tissues of each group. The present study laid a foundation to reveal the functions of the different structural domains of HSP110 in the presentation of macromolecular antigens.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and calf serum were purchased from GIBCO (USA). The full length HSP110 plasmid BacPAK-HSP110 was provided by Dr. John R. Subjeck Roswell Park Cancer Institute. The mouse breast cancer cell line D_2F_2, which highly expressed a transfected full-length HER2/neu cDNA, was generously provided by Dr. Wei-Zen Wei at Wayne State University (USA). BALB/c mice were purchased from Zhejiang Academy of Medical Sciences (China). The luciferase assay kit was purchased from Beyotime Biotechnology (China). The HSP110 primary antibody was purchased from Santa Cruz Biotechnology (USA). The amino acids for peptide synthesis and the resins were purchased from GL Biochem (Shanghai) Co. Ltd. (China). Bcl-2 and bax immunohistochemistry detection kits were purchased from Beyotime, China. The genetically engineered her2/neu ICD and the truncated heat shock protein 110 (tHSP110) were constructed, expressed and purified in our lab.

Construction of the tHSP110 gene expression vector

Two primers were designed using Primer Premier software (Premier Biosoft International, Canada) and the HSP110 cDNA sequence (1-2588 bp) was provided by gene database. The sequence of the upstream primer is 5'-AAGGCCATGGCTGATATCGGATCCATGTCGGTGGTGGGGTTGGACGTG-3'; the sequence of the downstream primer is 5'-TGGTGGTGGTGGTGGTGCTCGAGGTCCAAGTCCATATTAACAGAATTTTTCTCATTAG-3'. BamHI and XhoI restriction sites were included into the upstream and downstream primers, respectively. The truncated HSP110 fragment was obtained by polymerase chain reaction (PCR) with plasmid BacPAK-HSP110 as template. The digested tHSP110 gene was ligated to the pET32a plasmid vector, which was also double digested with BamHI and XhoI to obtain the truncated protein-expressing plasmid pET/His-tHSP110 (referred to as pET-tHSP110). Construction of the expression vector was confirmed by sequencing.

Transformation of the expression vector and protein expression

The protein-expressing plasmid pET-tHSP110 was transformed into *E. coli* BL21 to express the tHSP110. This engineered bacterium *E. coli* BL21 was inoculated into liquid LB medium containing 100 μg/mL ampicillin. The bacteria were cultured at 37°C with shaking at 250 r/min until the OD_{600} reached 0.6 to 1.0. Protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.8 mmol/L for 5 hours. One milliliter of culture was collected and centrifuged prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to verify protein expression. Western blotting was used to identify the expression of the truncated protein. The primary and secondary antibodies were a mouse monoclonal anti-His-tag antibody and a horseradish peroxidase-labeled goat anti-mouse IgG, respectively.

Luciferase assay

The luciferase assay was conducted after purification of the tHSP110 protein using a Ni Sepharose 6 Fast Flow column. Luciferase denaturing buffer (25 mmol/L HEPES, pH 7.6, 5 mmol/L DTT, and 6 mol/L guanidine hydrochloride) at a concentration of 0.5 μmol/L was added and incubated at 22°C for 60 min to denature the luciferase enzyme. The denatured luciferase was placed on ice for 30 min and then diluted 1:20 with denaturing buffer [2.0 μL of diluted solution was supplemented with 48

μL of refolding buffer (25 mmol/L HEPES, pH 7.6, 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 2 mmol/L DTT, and 2 mmol/L ATP) containing 2 μl of the HSP110 or tHSP110 protein (0.4 μmol/L)]. The renaturation buffer, which did not contain protein, was used as a negative control, and undenatured luciferase was used a positive control. The reaction system was incubated at 30°C. A total of 1.0 μL of the reaction mixture was collected at different time points, added to 50 μL of luciferase assay solution and mixed thoroughly. The luminescence was immediately measured for 10 s on an ultra-weak luminescence analyzer. The result was expressed as the percentage of the renatured enzymatic activity of the original enzymatic activity.

Synthesis of the CTL antigenic peptide

A fragment of the cytotoxic T lymphocyte (CTL) epitope peptide within the ICD of the her2/neu protein (named $P_{851-859}$) was chosen to synthesize the antigen peptide. A total of 0.468 g of fluorenylmethyloxycarbonyl (Fmoc)-Val-Wang resin was transferred to a reaction flask for polypeptide synthesis. Dimethylformamide (DMF) was added to the flask as a solvent to swell the resin. Diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) were used as coupling agents, and DMF was used as the solvent. The polypeptide chain was synthesized sequentially; the amino acid sequence was VLVKSPNHV (Val-Lys-Val-Gly-Ser-Leu-Ala-Phe-Leu). The amounts of Fmoc, Fmoc amino acid chloride, DIC, and HOBt were all equal to 3 times the amount of resin. After the synthesis was completed, a polypeptide cleavage reagent was used to cleave the polypeptide off the resin to obtain the crude product. The collected major peak polypeptide was dissolved in distilled water containing 0.1% trifluoroacetic acid (TFA), and the molecular weight was determined using a laser desorption/ionization time-of-flight mass spectrometer. After the crude peptide was purified by high-performance liquid chromatography (HPLC), the target polypeptide was collected and concentrated by vacuum rotary evaporation, lyophilized, and stored.

Construction of genetically engineered vaccines

Purified and accurately quantitated HSP110 and tHSP110 proteins were mixed with the her2 ICD macromolecular antigen and $P_{851-859}$, respectively, at a molar ratio of 1:10 in 10 mmol/L phosphate buffer containing 138 mmol/L NaCl, 2.7 mmol/L KCl and 1 mmol/L $MgCl_2$. The reaction took 90 min at 37°C and 30 min at room temperature. A Microcon-50 filter was used to remove the unbound antigen peptide. A co-immunoprecipitation assay was used to confirm the non-covalent coupling of the HSP110-ICD complex and the tHSP110-ICD complex. The HSP110-ICD and tHSP110-ICD complexes were mixed with a rabbit anti-mouse HSP110 antibody (1:200) and an irrelevant antibody (1:200), respectively.

Immunization, elispot assays

A total of 63 female 6-8 weeks old BALB/c mice were randomly divided into 7 groups with 9 mice per group. For the mice in each group, HSP10, tHSP10, HSP110-ICD, tHSP110-ICD, HSP110-$P_{851-859}$, and tHSP110-$P_{851-859}$ (all with a concentration of 0.1 $\mu g \cdot \mu L^{-1}$) were subcutaneously injected into the mice in a 100 μL volume between the 5th and 6th intercostal spaces of the left chest 14-day interval for three times. Phosphate-buffered saline (PBS) was injected into the negative control group. Fourteen days after the last immunization, D_2F_2 cells were inoculated into BALB/c mice. Twenty-eight days after the tumor challenge, 3 rats were randomly selected from each group, and were sacrificed by cervical vertebrae dislocation. Spleen was removed under sterile conditions, 8 mL of spleen cell suspension were slowly added to 4 mL Ficoll lymphocyte separation solution, and then the lymphocytes were isolated. 96-well plates were coated overnight with 10 μg/mL of anti-mouse IFN-γ antibody (100 μL per well), after washing 3 times with PBS, wells were blocked using 100 μL 2% skimmed milk powder for 2 hours at room temperature. After discarding the blocking solution, and washing the plates three times with PBS, lymphocytes (2×10^6/mL) were placed into 96-well plates (100 μL per well), 4 μL of HSP110, tHSP110, HSP110-$P_{851-859}$, tHSP110-$P_{851-859}$, HSP110-ICD and tHSP110-ICD complex were added to yield a final concentration of 20 μg/mL. For the control group, 4 μL of PBS were added. Three replicate wells were set up for each group, and then plates were incubated for 24 h at 37°C and 5% CO_2. Liquid medium in each well was then discarded, after drying, 100 μL of PBS containing 0.1% Tween-20 were added to wells, after standing for 10 min at 4°C, the wells were washed for 3 times in PBS containing 0.1% Tween-20, and dried. 100 μL of enzyme-labeled

anti-IFN-γ secondary antibody was added to each well and incubated at 4°C overnight, and then the wells were washed again for 3 times with PBS containing 0.1% Tween-20, 100 μL of alkaline phosphatase solution was added and incubated at 37°C for 1 h. Then the wells were washed again for 3 times with PBS containing 0.1% Tween-20. 100 μL of BCIP/NBT buffer was added to each well, and incubated at room temperature for 10 min. Data were analyzed by Elispot.

Determination of CTL activity

Twenty-eight days after the tumor challenge, spleen was taken, $CD8^+$ cells were isolated and purified from spleens, the $CD8^+$ cells were adjusted to a concentration of 1×10^6/mL, IL-2 was added to a 10 U/mL final concentration. $CD8^+$ cells were plated to 96-well plates (100 μL/well), 40 μL of HSP110, tHSP110, HSP-110-$P_{851\text{-}859}$, tHSP110-$P_{851\text{-}859}$, HSP110-ICD, tHSP110-ICD were added respectively to each well to a final concentration of 20 μg/mL, for the control group, PBS were added. After 5 days of culture, $CD8^+$ cells were used as effector cells. 50 μL 2×10^5/mL D_2F_2 cell were added to 96-well plates. 50 μL of $CD8^+$ cell suspension was added to each well, the maximum release was determined by adding 40 μL of complete medium and 10 μL of 1% Triton X-100 to target cells, spontaneous release was calculated by incubating the targets in 50 μL of complete medium alone. Three replicates were set up in each group, after 4 hours of culture, cells were centrifuged for 5 min, 50 μL supernatant was harvested and transferred into the centrifuge tube, and mixed with 950 μL of benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT). Samples were incubated in a 37°C water bath for 20 min, placed on the ice, then 10 μL of 0.1 mol/mL phenylmethanesulfonyl fluoride (PMSF) was immediately added. The absorption value was measured at a wavelength of 412 nm. The percentage of CTL killing activity was calculated using the following formula: (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100%.

Apoptosis assay for tumor

Twenty-eight days after the tumor challenge, the tumor tissues of three experimental mice in each group were taken, tumor tissue were taken from 3 rats of each group, and fixed in 10% formalin, embedded in paraffin. Continuous paraffin sections were prepared and endogenous peroxidase activity was blocked by peroxidase blocking agent. After being rinsed in PBS, antigen retrieval was performed in a microwave oven, followed by incubation with primary antibodies at 4°C overnight; the remaining steps were performed according to the manufacturer's instructions. Computer image analysis system was used to measure quantitative immunohistochemistry. Images were captured with a microscope camera system at 400 × magnification, and input into the computer image analysis system. Then Images were converting into grayscale images to separate positive stained area from background, the measurement window was selected as fixed area, the gray scale and area of the positive staining and background were detected. The absorbance values were automatically recorded, 3 fields were randomly selected from each section, and the mean value was obtained.

Tumor studies

The immunization was repeated three times at 14-day intervals. Fourteen days after the last immunization, 1×10^6 D_2F_2 cells were inoculated subcutaneously into the chests of the mice. Tumor volume was observed in the mice and recorded every three days starting from the day when the tumor cells were inoculated. The time when the tumor first appeared in each mouse was observed. During growth, the tumor volume was measured with a caliper and recorded every three days. The tumor volume was calculated according to the following formula: tumor volume = shorter diameter^2 × longer diameter × 3.14/6.

Statistical analysis

SPSS 18.0 was used for the statistical analysis. The data are expressed as the mean ± SD. The significance of differences was assessed with one-way analysis of variance (ANOVA), followed by Duncan's post hoc test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Cloning, expression and identification of the tHSP110 protein

A DNA fragment 1500 bp in size was obtained by polymerase chain reaction (PCR); this prod-

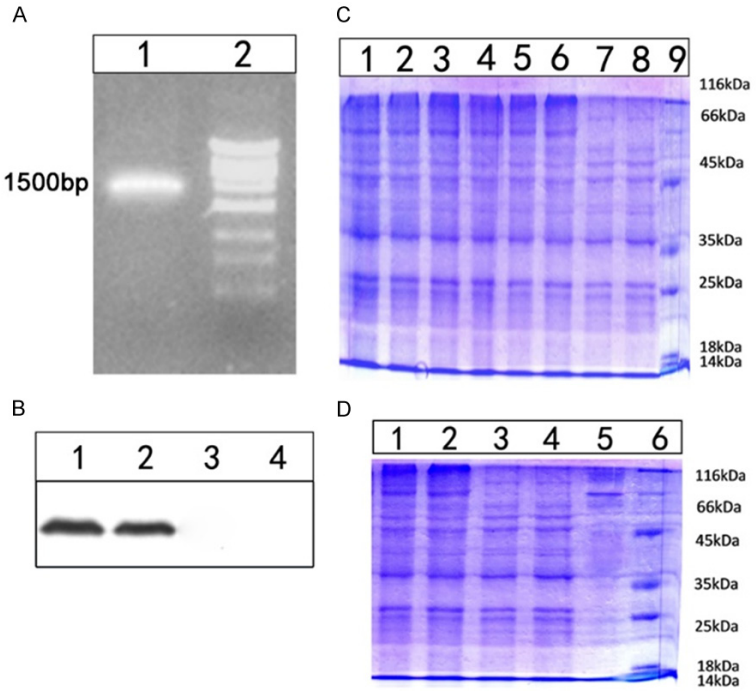

Figure 1. Cloning and expression of tHSP110 in *E. coli*. A: PCR amplification of tHSP110 gene. Lane 1: DNA marker. Lane 2: fragment of tHSP110 gene. B: Western blot of the *E. coli* cells after transfection with PET32-tHSP110 plasmids, and the supernatant immunoblotted for tHSP110. Lane 1-2: supernatant of tHSP110 *E. coli* Lane 3: untransfected control. Lane 4: control antibody. C: SDS-PAGE analysis of the tHSP110 expression. The band about 66Kda represents the produced tHSP110. Lane 1-7, total protein after induction with IPTG. (Lane 1: supernatant from the 5-hour induction; Lane 2: supernatant from the 4-hour induction; Lane 3: supernatant from the 3-hour induction; Lane 4: supernatant from the 2-hour induction; Lane 5: supernatant from the 1-hour induction; Lane 6: supernatant from the 0.5-hour induction; Lane 7: supernatant from uninduced cells.) Lane 8: supernatant from uninduced cells. Lane 9: protein marker. D: SDS-PAGE analysis of the tHSP110 expression. Lane 1-2: total protein after induction with IPTG. Lane 3-4: total protein without induction. Lane 5: tHSP110 after purification. Lane 6: protein marker.

uct size was consistent with the expected result and indicated that the target gene had been obtained (**Figure 1A**). After digestion with BamHI and XhoI restriction enzyme, the fragment was ligated to the PET32 plasmid, which was double digested with the same enzymes, and the recombinant plasmid was obtained. Sequencing (data not shown) confirmed the correct construction of the recombinant plasmid. The SDS-PAGE results showed that the induced sample had an obvious extra protein band compared with the uninduced sample (**Figure 1C**). By comparing the expressed protein band from the induction with the marker, we determined that the molecular weight of tHSP110 was 66 kDa. Purification of tHSP110 was achieved by Ni^{2+} affinity chromatography (**Figure 1D**). A rabbit anti-human HSP110 antibody was used as the primary antibody, and a horseradish peroxidase-labeled goat anti-rabbit antibody was used as the secondary antibody in the western blot assay. A specific protein band appeared after incubation with the HSP110-specific antibody (**Figure 1B**).

Luciferase binding experiment

The chaperone activity of HSP110 might help denatured proteins refold and allow denatured proteins to regain their activity. The present experiment showed (**Figure 2C**) that denatured luciferase gradually refolded proteins in a certain amount of time in the presence of the HSP110 or tHSP110 protein. HSP110 helped luciferase restore approximately 80% of protein activity in 25 min, which was also done by tHSP110. This result indicated that the ATP-binding domain was not necessary for the molecular chaperone function of HSP110.

Mass spectrometry analysis

The synthesized antigen peptide was subjected to a mass spectrometry assay (**Figure 2A**). The assay showed the existence of a target synthetic peptide with a molecular weight of 996 Da, indicating that the target peptide was synthesized using the solid phase synthesis method. A synthetic peptide with a molecular weight of 996 Da was consistent with the expected result. The synthetic peptide was further purified by HPLC. The HPLC result is shown in **Figure 2B**. The structure identification indicated that the purity of the synthetic peptide was 81%.

Co-immunoprecipitation assay

The genetically engineered tHSP110 and ICD proteins were mixed at a 1:1 molar ratio and

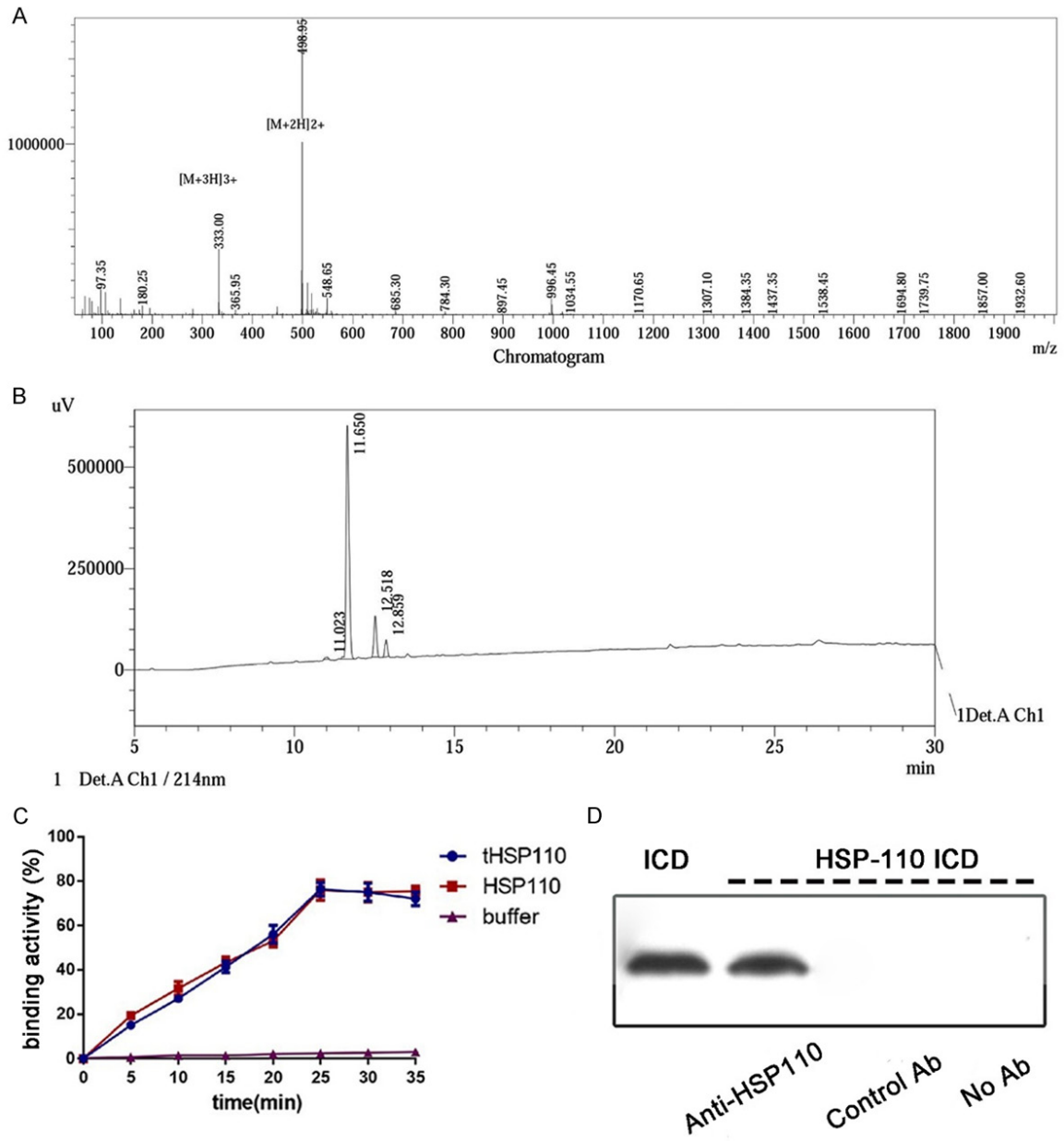

Figure 2. Identification and purification of the synthetic (VLVKSPNHV) peptide. A: Mass spectrum analysis of the synthetic (VLVKSPNHV) peptide. B: Mass spectrometer spectra of the collected fraction of synthetic purified VLVKSPNHV the molecular weights were 966 Da obtained by deconvolution of the MS spectra. C: Luciferase binding experiment of HSP110 and tHSP110 protein. The result was expressed as the percentage of the renatured enzymatic activity of the original enzymatic activity. D: Western-bolt analysis of the HSP-ICD complexes.

incubated in vitro for 90 min at 37°C. The western blot analysis indicated that HSP110 and the macromolecular antigen her2 ICD formed an HSP110-antigen peptide complex in vitro after a 30 min reaction at room temperature. Full-length HSP110 and the her2 ICD formed an HSP110-antigen peptide complex also. The western blot result was shown in **Figure 2D**.

Truncated HSP110-ICD complex-induced CTL activity assay

Specific CTL assay showed that the rate of killing of target cells by CTL in PBS, HSP110, tHSP110, HSP110-$P_{851\text{-}859}$, tHSP110-$P_{851\text{-}859}$, HSP110-ICD, tHSP110-ICD groups were (2.17±1.17)%, (11.33±3.61)%, (10.33±3.50)%,

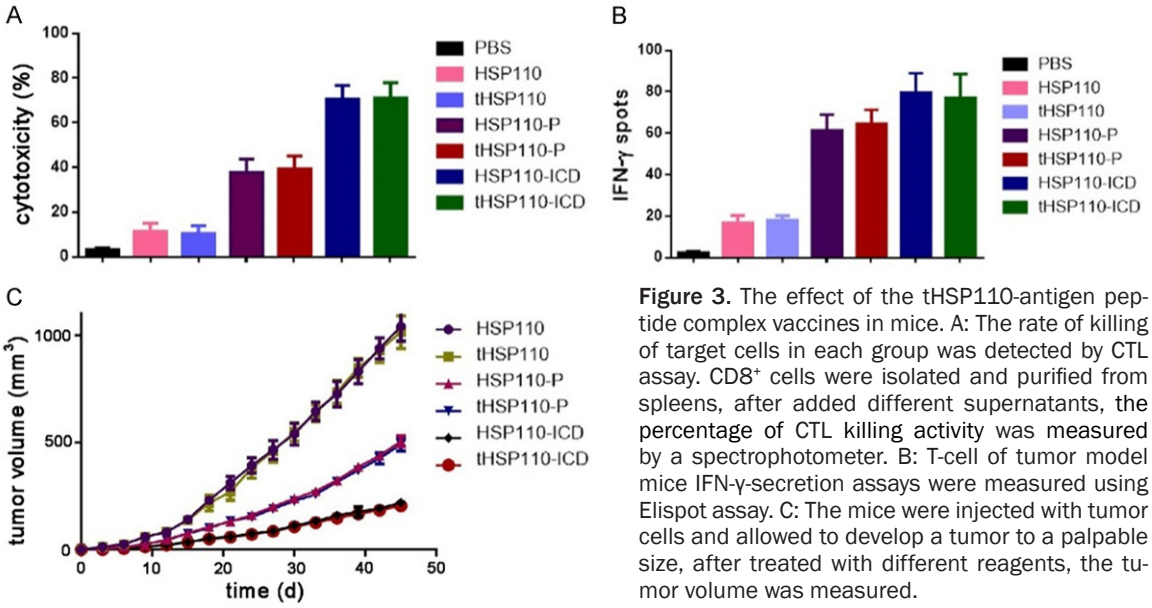

Figure 3. The effect of the tHSP110-antigen peptide complex vaccines in mice. A: The rate of killing of target cells in each group was detected by CTL assay. $CD8^+$ cells were isolated and purified from spleens, after added different supernatants, the percentage of CTL killing activity was measured by a spectrophotometer. B: T-cell of tumor model mice IFN-γ-secretion assays were measured using Elispot assay. C: The mice were injected with tumor cells and allowed to develop a tumor to a palpable size, after treated with different reagents, the tumor volume was measured.

(36.00±7.26)%, (38.50±6.83)%, (70.5±6.22)% and (71.17±6.74)% respectively. There were differences in the rate of target cell killing between groups (P<0.05). Furthermore, Statistical analysis indicated that there was no significant difference in the rate of target cell killing by specific CTL between tHSP110-$P_{851\text{-}859}$ and HSP-110-$P_{851\text{-}859}$ groups (P=0.435, P>0.05), tHS-P110-ICD and HSP110-ICD groups (P=0.834, P>0.05), while there were significant differences between tHSP110-$P_{851\text{-}859}$ and tHSP110-ICD groups (P<0.05). The CTL assay results were shown in **Figure 3A**.

Elispot assay

T-cell IFN-γ-secretion assays showed that the number of blue spots in PBS, HSP110, tHS-P110, HSP110-$P_{851\text{-}859}$, tHSP110-$P_{851\text{-}859}$, HS-P110-ICD and tHSP110-ICD groups were 2.33±0.52, 16.67±3.56, 17.83±2.32, 61.33± 7.69, 64.5±6.83 79.5±9.46 and 77.38±11.51 respectively. There were differences in the number of IFN-γ-secreting T cell between each group, while there was no significant difference in the number of IFN-γ-secreting spleen cells between tHSP110-$P_{851\text{-}859}$ and HSP110-$P_{851\text{-}859}$ groups (P=0.441, P>0.05), tHSP110-ICD and HSP110-ICD groups (P=0.542, P>0.05). Furthermore, there were significant difference between tHSP110-$P_{851\text{-}859}$ and tHSP110-ICD groups (P<0.05). The IFN-γ-secretion assays results were shown in **Figure 3B**.

Tumor volume

The tumor volume was measured once every three days, and the tumor volumes were shown in **Figure 3C**. A solid mass could be felt at the injection location approximately 7-10 d after subcutaneous injection of D_2F_2 cells into the mice. Approximately 12-20 d after inoculation, the tumor was in the fast growing phase. The tumor volume increased continuously with tumor growth. The tumor volume did not significantly differ between the HSP110 and tHSP110 groups. Similarly, the tumor volumes did not significantly differ between the HSP110-$P_{851\text{-}859}$ and tHSP-$P_{851\text{-}859}$ groups or between the HSP-110-ICD and tHSP110-ICD groups. In contrast, there were significant differences between the tHSP110, tHSP110-$P_{851\text{-}859}$ and tHSP110-ICD groups or between the HSP110, HSP110-$P_{851\text{-}859}$ and HSP110-ICD groups. These results indicated that tHSP110 had the same function as HSP110 in macromolecular antigen presentation and activation of tumor immunity.

Apoptosis assay for tumor

Positive reaction was found in the cytoplasm of the tumor cells under a microscope, the positive rates of Bcl-2 protein expression in PBS, HSP110, tHSP110, HSP110-$P_{851\text{-}859}$, tHSP110-$P_{851\text{-}859}$, HSP-ICD and tHSP110-ICD groups were (55.29±6.17)%, (38.63±5.49)%, (36.96± 6.12)%, (22.75±3.16)%, (22.75±3.32)%, (9.93±

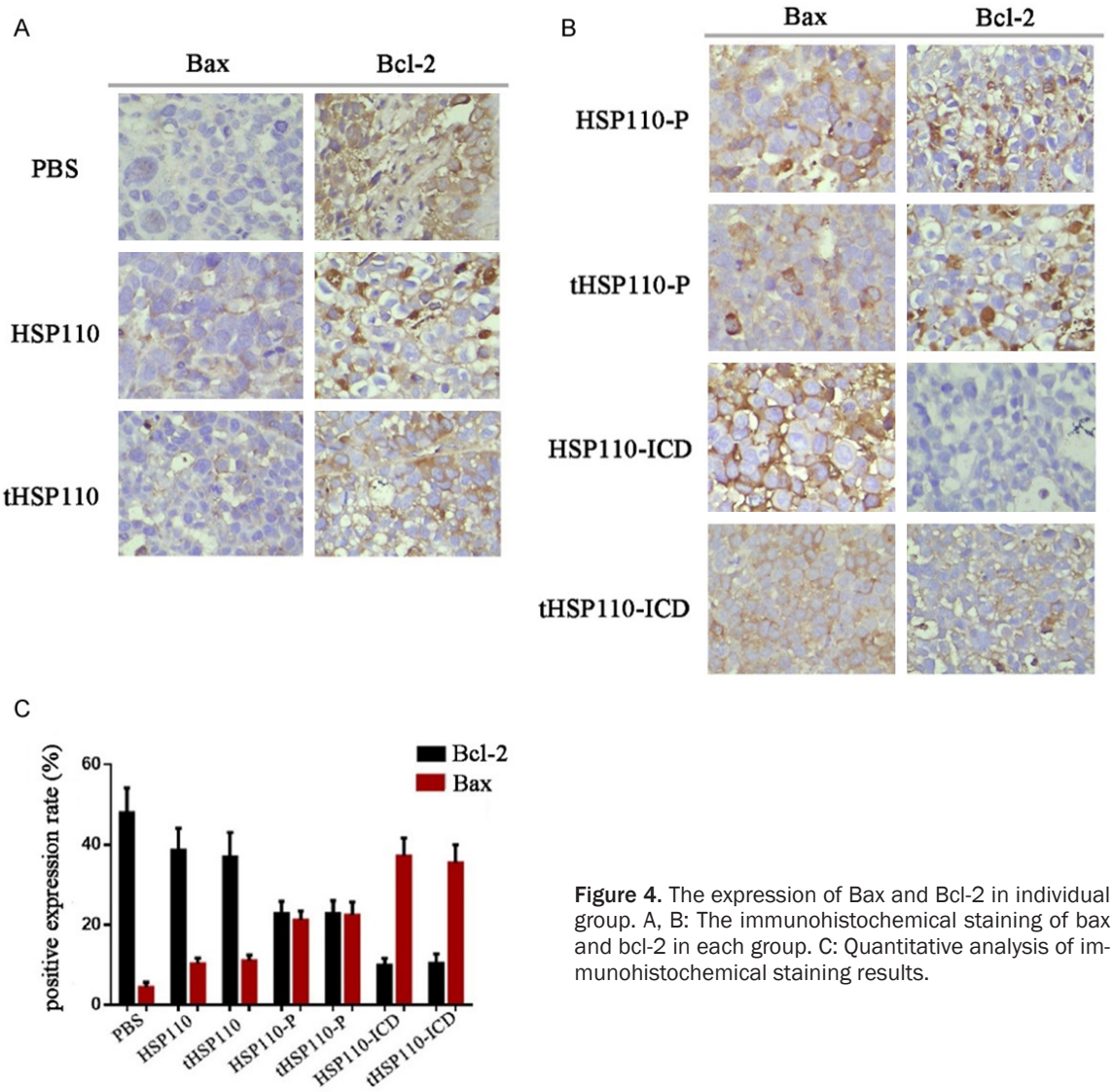

Figure 4. The expression of Bax and Bcl-2 in individual group. A, B: The immunohistochemical staining of bax and bcl-2 in each group. C: Quantitative analysis of immunohistochemical staining results.

1.77)% and (10.42±2.36)% respectively. The positive rates of Bax protein expression were (4.44±1.28)%, (10.32±1.47)%, (11.08±1.42)%, (21.20±2.27)%. (22.41±3.33)%, (37.18±4.53)% and (35.52±4.49)% respectively (**Figure 4A**, **4B**). Results showed that the expression of Bcl-2 protein was successively decreased, and the expression of Bax protein was increased in HSP110, HSP110-$P_{851-859}$, HSP110-ICD groups, and there were significant differences in the expression of Bcl-2 and Bax protein between groups (**Figure 4C**). But in tHSP110, tHSP110-$P_{851-859}$ and tHSP110-ICD groups, the expression of Bcl-2 protein were markedly increased, the expression of Bax protein was decreased, and there were significant differences between groups. There was no difference in the expression of Bcl-2 and Bax protein between HSP110-ICD and HSP110-ICD groups, HSP110-$P_{851-859}$ and tHSP110-$P_{851-859}$ groups, HSP110 and tHSP110 groups (**Figure 3D**).

Discussion

HSPs extracted from tumor cells can initiate a CTL response [9, 10]. Recent studies confirmed that the expression of HSP70 in tumor cells had good immunogenicity and tumor specificity [11-13]. HSP70 from tumor cells binds to antigen peptides, presents them on the cell surface to activate T cells, and induce the production of CTLs. HSP70-bound antigen peptides can activate specific immunity against almost all tumor cells. Using this feature, HSP70 antigen peptide

complexes have been used in multiple anti-tumor immunizations [14-16]. HSP110 is a high molecular weight heat shock protein [17]. Similar to HSP70, it can activate specific immunization against tumor cells [18-20]. Studies found that HSP110 exhibited a stronger antigen-binding ability than HSP70, with an even stronger immune activation ability [21, 22]. The basic reason for the difference in the immune activation ability between HSP110 and HSP70 is that HSP110 can bind to macromolecular antigens (which may contain up to several hundred amino acids), whereas HSP70 can only bind to small molecule antigen peptides (typically approximately 10 amino acids in length) [23, 24]. Thus, when the same number of HSP110 molecules is compared with HSP70, the antigen peptide information carried by HSP110 is much higher than that by HSP70. This finding suggests that the antigen presentation efficiency of HSP110 is several folds greater than the antigen presentation efficiency of HSP70. HSP110 can be considered a "super HSP70" in terms of the activation of CTL responses that kill tumor cells [25].

There are some reports of recent studies abroad that investigated the anti-tumor function of HSP110 [26]. However, these studies all focused on binding of HSP110 with different tumor antigens to play an anti-tumor role. Subjeck non-covalently coupled HSP110 with a breast cancer antigen, melanoma antigen and many other tumor antigens and used the HSP110-antigen peptide complexes to immunize mice; the anti-tumor effect was confirmed in these studies [27, 28]. Kim HL et al. used HSP110 and tumor protein IX from kidney cancer (carbonic anhydrase IX, CA9) to form a complex; then, the authors conducted in vivo and in vitro anti-tumor experiments. A solid-phase enzyme-linked immunospot (ELISPOT) assay confirmed that the HSP110 and CA9 complex induced an IFN-γ response. Animal experiments confirmed that this vaccine inhibited the growth of kidney cancer in BALB/c mice [29].

HSP70 and HSP110 have structural similarities [30, 31]. For example, both HSP70 and HSP110 have an ATP-binding domain, peptide-binding domain, amino acid loop and helix region [32]. The ATP-binding domain has the highest molecular weight and contains the most amino acids. In terms of the preparation of a genetically engineered vaccine, a smaller molecular weight vaccine is relatively easier to express and secrete and is more economical and easier to purify. Studies found that the ATP-binding domain of HSP only functioned in substrate binding and release in vivo through the alternative binding of ATP and ADP [33]. Therefore, demonstration of the peptide-binding ability of the truncated HSP110 constructed in vitro should help to further localize the antigen peptide-binding domain of HSP110 and promote the production of simpler and more effective tumor vaccines [34].

Bcl-2/Bax plays an important role in the regulation of apoptosis in breast cancer tissues, and has direct relation with apoptosis. Bcl-2 is recognized as anti-apoptotic gene, exhibits inhibitory effects on apoptosis, Bcl-2 can form heterodimers with Bax, and antagonize Bax action. Bax can promote apoptosis. Our results showed that tHSP110 and HSP110 had the same ability to bind to the same large macromolecule antigens, and activated the apoptosis signal transduction which ultimately leading to apoptosis of the tumor cells.

To this end, we constructed a truncated HSP110 in which the ATP-binding domain was removed. Purified tHSP110 was non-covalently coupled with a tumor antigen ICD in vitro. We synthesized a CTL antigen peptide of the her2/neu protein as a control for immunotherapy experiments of the vaccine using a mouse model that highly expressed the HER2/neu protein. The experiments confirmed that the antigen binding ability and the tumor immunity of tHSP110 were consistent with those of HSP110 with no significant differences. Thus, tHSP110 had an anti-tumor ability similar to HSP110, whereas the tumor immunity of the tHSP110-ICD group was significantly different from the tHSP110-$P_{851\text{-}859}$ group. Because $P_{851\text{-}859}$ is a CTL epitope of the her2 ICD, the current experimental results indicated that the tHSP110-ICD complex could produce even more powerful tumor immunity than tHSP110-$P_{851\text{-}859}$; in other words, tHSP110 had the same powerful macromolecular presentation ability as HSP110. We confirmed that the ATP-binding domain of HSP110 was not necessary for antigen presentation. tHSP110 has a reduced molecular weight compared with HSP110 and maintains its polypeptide binding and antigen presentation abilities. This genetically engineered truncated HSP110 vaccine has a smaller molecular

weight and is easier to administer. Moreover, the same dosage of truncated peptide carried more tumor antigen with a higher CTL activation efficiency. Next, we will study the antitumor activities of different structural domains of HSP110 to construct a functional HSP110 vaccine with the highest immune effect using the smallest fragment.

In our study, we found that tHSP110 had the same antigen-binding capacity and the intensity of CTL response as HSP110, which had the same antitumor ability as full-length hsp110. While compared with tHSP110-$P_{851-859}$ group, there were significantly differences in rate of CTL killing and the number of splenocytes secreting IFN-γ in tHSP110-ICD group. Because $P_{851-859}$ is a CTL epitope of her2 ICD, HER2-ICD contains the CTL epitopes, including $P_{789-797}$, $P_{851-859}$, $P_{1172-1180}$. The results showed that the tHSP110-ICD complex induced potent activation of CTL response than tHSP110-$P_{851-859}$, which indicated that tHSP110 had the same powerful ability to deliver macromolecule antigen as THSP110. This indicated that ATP-binding domain of Hsp110 was a non-essential region for delivery of macromolecule antigen. tHSP110 had a lower molecular weight than HSP110, and retained the ability to bind peptides and deliver antigens as HSP110. Truncated HSP110 genetically engineered vaccines have a low molecular weight, can be more convenient to administer. The same dose of truncated peptide carried more tumor antigens and had higher efficiency to activate CTL. As the next step, we will further investigate the antitumor activities of different structure and function of Hsp110, and expect to construct the HSP110 vaccine with the smallest segment that being more effective in immunity.

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Disclosure of conflict of interest

None.

Address correspondence to: Han Dong, Biochemistry Department of Medical College, Jiaxing University, Jiaxing 314001, Zhejiang, China. Tel: +86 13957369030, +86 573-83643819; E-mail: hdd-4426@163.com

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