

Expression and identification of transmembrane and secretory human HER2 extracellular domain

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Abstract

HER2 extracellular domain (HER2_ECD) protein is a potential tumor vaccine for containing more antigenic epitopes that have more potent immunogenicity than the antigenic polypeptide. Transmembrane and secretory antigens are different in receptor binding, biological effects and action mechanisms. Therefore, it is necessary to prepare two kinds of HER2_ECD protein for its potential application. In this study, pDisplay and pCMV were used as vectors for the expression of transmembrane and secretory HER2_ECD. A sequence of transmembrane region (PDGFR Transmembrane Domain) is located behind the signal peptide and the cleavage site in the pDisplay. The PCR product HER2_ECD fragment was cloned into pDisplay to form recombinant plasmid pDisplay-HER2_ECD which was then transiently transfected in HEK293T cells. The expressed protein was confirmed to be 130kDa by Western Blotting, which was in agreement with the theoretical size of HER2_ECD. The positive rate of the transient cells in the experimental group was from 51.00% to 52.08% compared with the blank control group by flow fluorescence analysis. Moreover, green fluorescence (FITC) and red fluorescence (PE) were observed on the parallel cells of the experimental group compared with the blank control group by confocal microscopy fluorescence detection, which indicated that HER2_ECD was successfully expressed on the membrane. In order to facilitate the expression, purification and test, an Fc fragment was fused with HER2_ECD by overlap PCR then cloned into pCMV to get the recombinant plasmid pCMV-HER2_ECD-Fc which was then transiently transfected in HEK293T cells. Western Blotting analysis suggested that the expressed protein collected from the supernatant was 150 kDa, which was in accordance with the theoretical size of HER2_ECD-Fc. Purified by protein an affinity chromatography, the purity of recombinant HER2_ECD-Fc was confirmed to be 98.10% by SDS-PAGE and HPLC. In summary, transmembrane and secretory HER2_ECD proteins were successful prepared to be homogenesis by membrane-docking and secretory expression in mammalian cells.

Keywords

Umor Protein Vaccine, Transmembrane HER2_ECD, Secretory HER2_ECD, HEK293T Cell, Expression and Identification.

1. Introduction

Human epidermal growth factor receptor 2 (HER2), discovered in the 1980s^[1,2], is encoded by proto-oncogene c-erbB2. It is receptor tyrosine kinase binding to the surface of the cell membrane, known as ErbB-2, c-erbB2 or HER2. Her2 is 185kDa, including the signal peptide, intracellular domain, transmembrane region and extracellular domain of four parts [3]. Extracellular region of about 650 amino acid is the corresponding ligand binding sites, and there are abundant cysteines on it so that it can form homologous or heterodimers [4]. HER2 is mainly expressed in embryonic development. In

normal cells, HER2 plays an important role in all stages of cell growth, but overexpression of it is often associated with tumorigenesis. There have been a large number of studies and reports on the overexpression and effects of HER2 in breast cancer that it can induce the development and progression of breast cancer in vivo and in vivo [5,6]. HER2 overexpression in about 15-30% of breast cancer and 10-30% of gastric/gastroesophageal cancers, and the Her2 can be found in ovaries, endometrium, bladder, lung, colon and head and neck as well [7-9]. HER2 plays a signal transduction function that it accelerates proliferation of cells, formation and growth of tumors, only by forming the homologous or heterodimer which cannot be deprecating [10, 11]. HER2 dimer can activate intracellular signaling pathways in which AKT pathway plays the important role in the occurrence and development of tumors. Studies have shown that HER2 binds to the COX-2 gene promoter and up-regulates the expression of COX-2 by entering the nucleus as a transcription factor to promote tumor growth and proliferation [12]. The overexpression of HER2 increases the ability to metastasize, including cell mobility, invasiveness in vitro, experimental lung metastases, etc [13]. The overexpression of HER2 also affects the synthesis of certain adhesion molecules, such as epithelial cells cadherin (E-cadherin), and thus promotes metastasis [14].

Treatment pathways of anti-tumor to targeted HER2 are mainly anti-HER2 antibody and HER2 active immunotherapy-peptide vaccine, etc. HER2 is highly expressed in a variety of malignant tumors, so it is an ideal target for active immunotherapy of malignant tumors. Antigen peptide presented by MHC-I or MHC-II class molecular on cell surface is recognized by T cells and activated to produce a specific immune response, resulting in the corresponding antibody [15, 16]. Disis et al. [17] found that the complete HER2 protein was difficult to induce an effective immune response, but the extracellular epitope exposed to the extracellular domain could alter the immune tolerance of the organism. Esser man et al. [18] found that after HER2 transgenic mice immunized with HER2 exogenous proteins, HER2-specific humoral and cellular immunity were produced in mice. HER2 extracellular protein contains more antigenic epitopes and has significantly enhanced antigenicity than individual antigenic peptide molecules, it will also provide more T cell targets after antigen presenting cells (APC) processing. Studies have shown that transmembrane and secretory TNF- α are different in the receptor binding, biological effects and mechanism [19-21]. When these two types of antigen act as vaccine, the induced immune response, structure and biological activity of resulted antibody may have a difference. Therefore, we constructed transmembrane and secretory HER2 extracellular domain proteins, as the vaccine for HER2 active immunotherapy, preparing for the development of high effective anti-HER2 antibody.

2. Materials and methods

2.1 Materials and reagents

Expression vector pDisplay and pCMV, purchased from CHENDU TRANSVECTOR BIOTECHNOLOGY CO., kept in this laboratory. Cloning vector pEZ-M02-Her2 was purchased from Guangzhou Complex Genes limited company. pCMV-Fc was constructed and kept in this laboratory. *E. coli* DH5 α , HEK293T cells are provided in this laboratory. XmaI, SalI, Kpn I, Nhe I Fast Cutase purchased from Fementas. Prime STAR Max DNA Polymerase was purchased from Takara. HER293T cells were supplied by our laboratory, standard fetal bovine serum (FBS), 1640 medium were purchased from Gibico Corporation. Anti-HA mouse antibody, anti-human Her2 mouse antibody, goat anti-mouse IgG-HRP antibody, goat anti-mouse IgG-FITC antibody, goat anti-mouse IgG-PE antibody were purchased from abcam company.

2.2 Instruments

PCR amplification (China LJ). Metal bath (Hangzhou Ruicheng Instrument Co., Ltd.). Thermostat shaker (Shanghai Precision Instrument Co., Ltd.). SDS-PAGE electrophoresis (Bio-Rad). Gel imaging system (Bio-Rad). Spectral type laser scanning confocal microscope (manufactured by Leica, Germany). AKTA prime protein purifier (GE). SEC-HPLC (Thermo).

2.3 Construction of Expression Vectors

2.3.1 Construction of Expression Vector pDisplay-HER2_ECD

As shown in [Fig. 1A](#), there is a PDGFR Transmembrane Domain behind the signal peptide sequence in the expression vector pDisplay, so it was designed that the HER2_ECD gene was linked to the cleavage site on the plasmid to express HER2_ECD binded to the cell membrane. The primers were designed by making pEZ-M02-Her2 plasmid as template, XmaI and Sall as cleavage sites. The upstream primers were ATAAAACCCGGGACCCAAGTGTGCACCGGC, the downstream primers were ATAAAAGTCGACCGTCAGAGGGCTGGCTCT. Prime STAR Max DNA Polymerase was used to perform PCR. The amplification system (total volume 50uL) : F-primer (10uM), R-primer (10uM), Plasmid (1ng), Takara LA Taq (5U/uL, 0.5uL), 10×LA Taq Buffer II (Mg²⁺ plus), DNTP Mixture (2.5mM each) 8.0 uL. The reaction conditions were as follows: pre-denaturation: 94 °C, 3min; denaturation: 94 °C, 3sec; annealing: 55 °C, 3sec; extension: 72 °C, 1Kb / min; final extension: 72 °C, Insulation: 4 °C, ∞; reaction 30 cycles. The PCR product was digested with the plasmid pDisplay then the digested products were ligated overnight at 4 °C by T4 DNA ligase. Ligated products were transformed into super-competent DH5α, and the positive colonies were sent to sequence in Shanghai Sangon.

2.3.2 Construction of Expression Vector pCMV-HER2_ECD-Fc

As shown in [Fig. 1B](#), there is no transmembrane region sequence on the pCMV vector, so secrete HER2_ECD can be expressed? HER2_ECD and Fc fragments were amplified by PCR using the above method, pEZ-M02-Her2 and pCMV-Fc plasmids as templates. Then the HER2_ECD-Fc fragment was amplified by overlap PCR making HER2_ECD and Fc as templates, Kpn I and Nhe I as cleavage sites.

2.4 Expression of HER2_ECD and HER2_ECD-Fc by HEK293T cells

HEK293T cells were resuscitated from liquid nitrogen can and passed to density of 70-80%. The plasmid pDisplay-HER2_ECD and pCMV-Her2ED-Fc were transfected into HEK293T cells by PEI (Polyetherimide), PEI:DNA=1:5, and the blank control group without transfection was set. After 48 hours, the cells transfected by pDisplay-HER2_ECD were digested with trypsin and washed with 1×PBS then centrifuged at 4000r for 30 min. After 48 h, the supernatant of the cells transfected by pCMV-Her2ED-Fc was collected and centrifuged at -80°C for verification.

2.5 Identification of HER2_ECD protein on 293T transmembrane

2.5.1 Western Blotting test

The collected cells were digested with 1% SDS at 100 °C for 10 min, and the supernatant was used to detect the total protein concentration by BCA in order to adjust the same protein concentration in the samples. After SDS-PAGE, the protein was transferred to nitrocellulose membrane. Anti-human Her2 mouse antibody was used as primary antibody, and goat anti-mouse IgG-HRP antibody was made as secondary antibody. At last, the nitrocellulose membrane was developed with the developer through the gel imaging system, and the result was analysed.

2.5.2 Flow fluorescence analysis

After 48h, 5×10⁶ cells transfected by pDisplay-HER2_ECD were washed with 1×PBS, fixed with 5% paraformaldehyde. Anti-human Her2 mouse antibody was used as primary antibody, goat anti-mouse IgG-FITC antibody was made as secondary antibody. The cells of blank control group were set the fluorescence domain values, then the expression of HER2_ECD on HEK293T cell membrane was detected by Guava micro-capillary cell analyzer.

2.5.3 Spectral laser scanning confocal microscopy fluorescence detection

After 48h, 1×10⁵ transfected cells were washed with 1×PBS, fixed with 5% paraformaldehyde, increased the permeability with triton×100, and blocked with 5% BSA. Anti-HA mouse antibody was used as primary antibody, goat anti-mouse IgG-FITC antibody was made as secondary antibody. Moreover, anti-human Her2 antibody (with phycoerythrin PE) was directly incubated. Then the intracellular DNA was stained with DNA dye DAPI, and the treated cells were examined by

spectroscopic laser scanning confocal microscopy.

2.6 Identification and purification of HER2_ECD-Fc protein

2.6.1 Western Blotting test for the expressing of HER2_ECD-Fc

The supernatant of cells transfected by pCMV-HER2_ECD-Fc for 48h was collected and prepared for Western Blotting analysis. The method is the same as 2.5.1.

2.6.2 Purification and Identification of HER2_ECD-Fc

The cell supernatant with HER2_ECD-Fc was filtered with 0.45 filter and purified by protein A affinity chromatography column. The mixture of column was equilibrated with 20mMPB (sodium dihydrogen phosphate). Then the targeted protein was eluted with 100 mM glycine, whose pH was adjusted to 7.2 with Tris, the purity was analyzed by SDS-PAGE and HPLC.

3. Results

3.1 The successful construction of pDisplay-HER2_ECD, pCMV-Her2ED-Fc vector

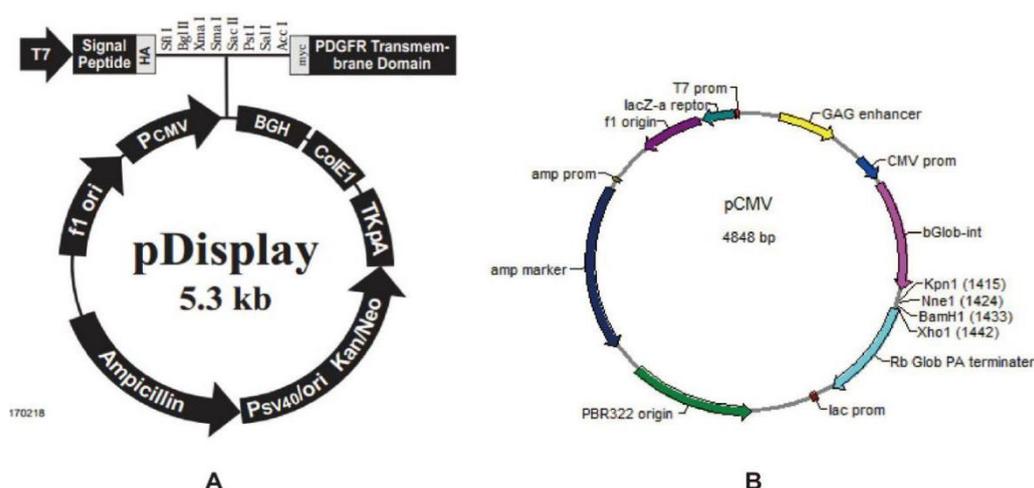


Fig. 1 The map of pDisplay and Pcmv

The gene fragment was amplified by PCR using pEZ-M02-Her2 plasmid as template, and detected by agarose gel electrophoresis. As shown in [Fig. 2A](#), the amplified fragment was about 1900bp, which was in accordance with the predicted size of HER2_ECD gene. Sequencing the positive colonies verified that the results was 100% matched with HER2_ECD gene sequence, indicating that pDisplay-HER2_ECD was constructed successfully. The gene product was amplified by overlapping PCR using HER2_ECD and Fc gene fragments as templates. As shown in [Fig. 2B](#), the amplified fragment was about 2600bp by agarose gel electrophoresis, consistent with predicted size of HER2_ECD-Fc gene. Sequencing the positive colonies verified that the results was 100% matched with HER2_ECD-Fc gene sequence, indicating that pDisplay-HER2_ECD was constructed successfully.

3.2 Western Botting test of HER2_ECD and HER2_ECD-FC

The supernatants of cell transfected by pDisplay-HER2_ECD and pCMV-Her2ED-Fc were analyzed by Western Blotting. The results of HER2_ECD were shown in [Fig. 2C](#). There were only one targeted band with a size of 130 kDa of HER2_ECD. Further measurement of the internal β -actin were carried out with a size of 43 kDa in line with its expected size. [Fig. 2D](#) showed the result of Her2ED-Fc that only one targeted band with a size of 150 kDa consistent with predicted protein size. The above results confirmed the successful experssing of HER2_ECD and HER2_ECD-FC.

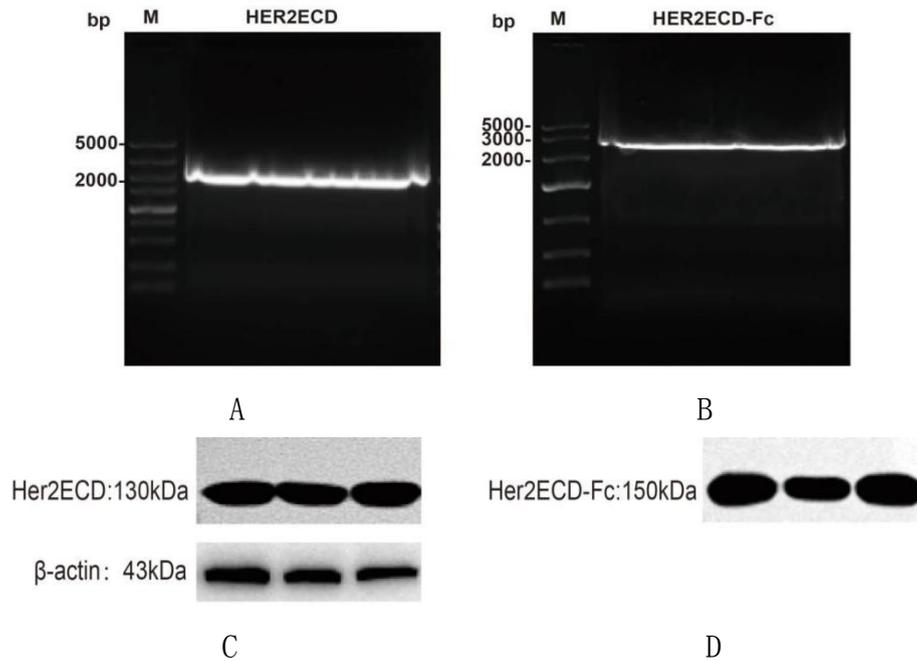
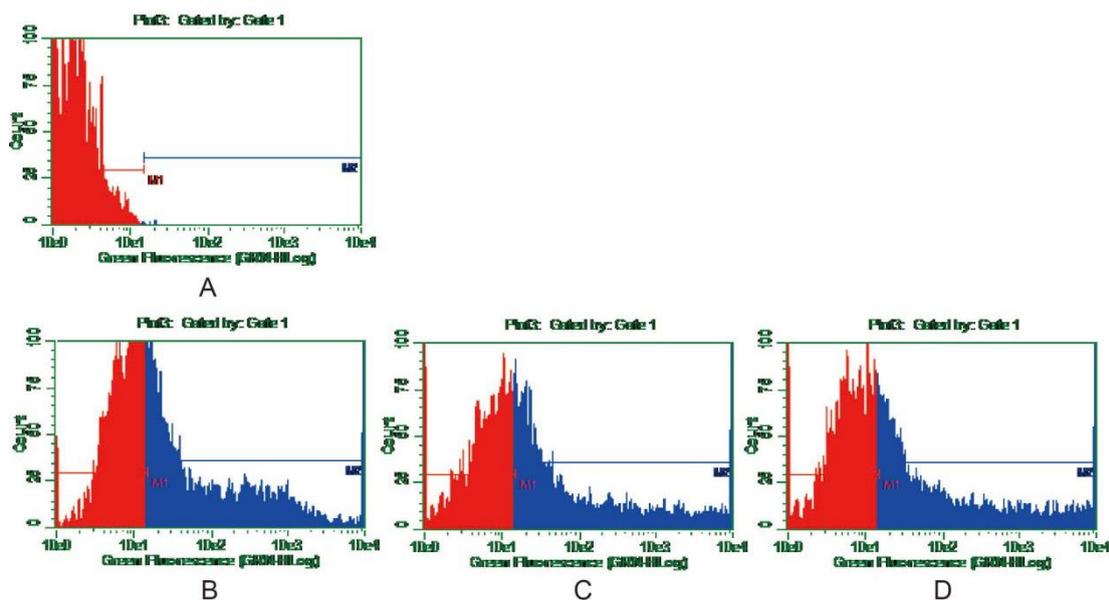


Fig. 2 Amplification and expression of HER2_ECD and HER2_ECD-FC
 A: PCR amplification of HER2_ECD gene. B: Overlap PCR amplified HER2_ECD-Fc gene.
 C: WB test of HER2_ECD expression. D: WB analysis of HER2_ECD-Fc expression.

3.3 Flow fluorescence analysis of expression of HER2_ECD on HEK293T cell membrane

The cells in the blank control group and experimental groups were analyzed by flow cytometry. As shown in Fig. 3, the three experimental groups (Fig. 3B-D) showed positive results compared with the blank control group (Fig. 3A). The positive rate respectively were 51.57%, 52.08%, 51.00%, indicating HER2_ECD was successfully expressed in the cell membrane.



A: Blank control group. B, C, D: Three parallel experimental group.
 Fig. 3 Flow fluorescence analysis of expression of HER2_ECD on HEK293T cell membrane

3.4 Spectral laser scanning confocal microscopy fluorescence detection for expression of HER2_ECD on HEK293T cell membrane

The cells in Fig. 4A-D were with anti-HA mouse antibody as primary antibody, goat anti-mouse IgG-

FITC antibody as secondary antibody. The positive results will show the cell surface with green fluorescence. [Fig. 4A](#) is blank control group, the cell surface without any fluorescence imagination. [Fig. 4B-D](#) are three parallel experimental groups, the cell surface with green fluorescence. It indicates that HER2_ECD was successfully expressed on the cell membrane.

The cells in [Fig. 4E-H](#) were directly incubated with anti-human HER2 antibody (phycoerythrin PE). The positive results will show the cell surface with red fluorescence. [Fig. 4E](#) is blank control group, the cell surface without any fluorescence imagination. [Fig. 4F-H](#) are three parallel experimental groups, the cell surface with red fluorescence. It indicates that HER2_ECD was successfully expressed on the cell membrane again.

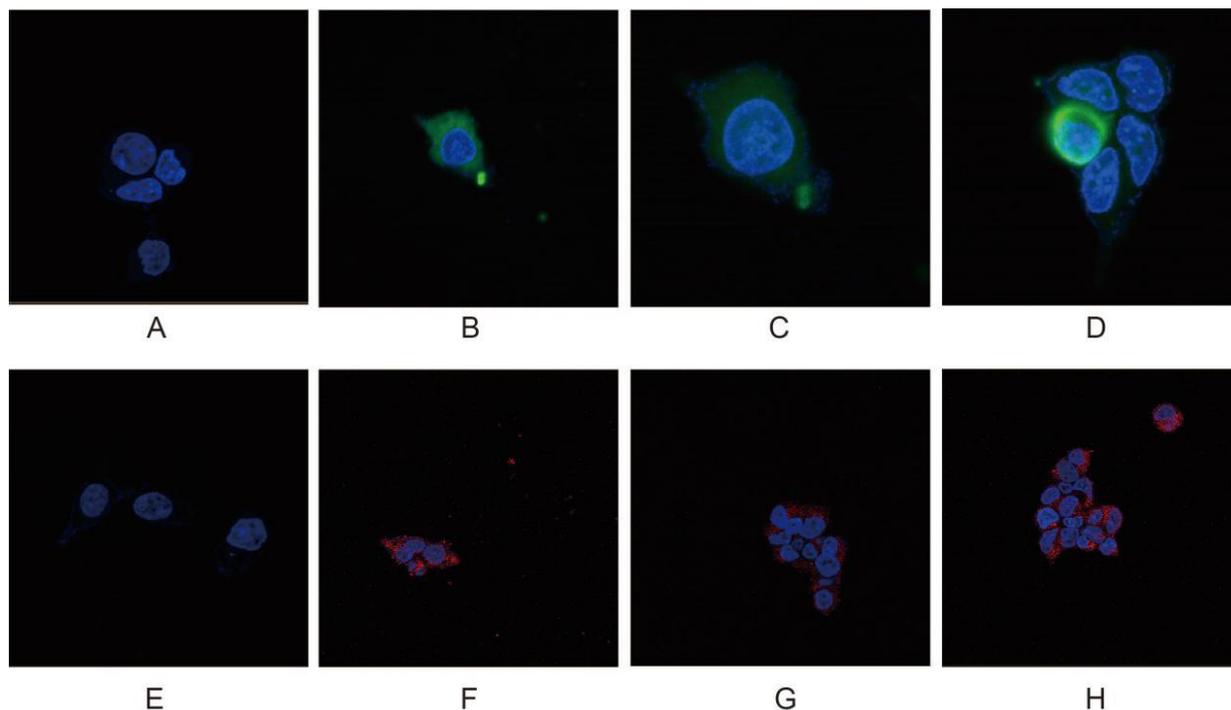


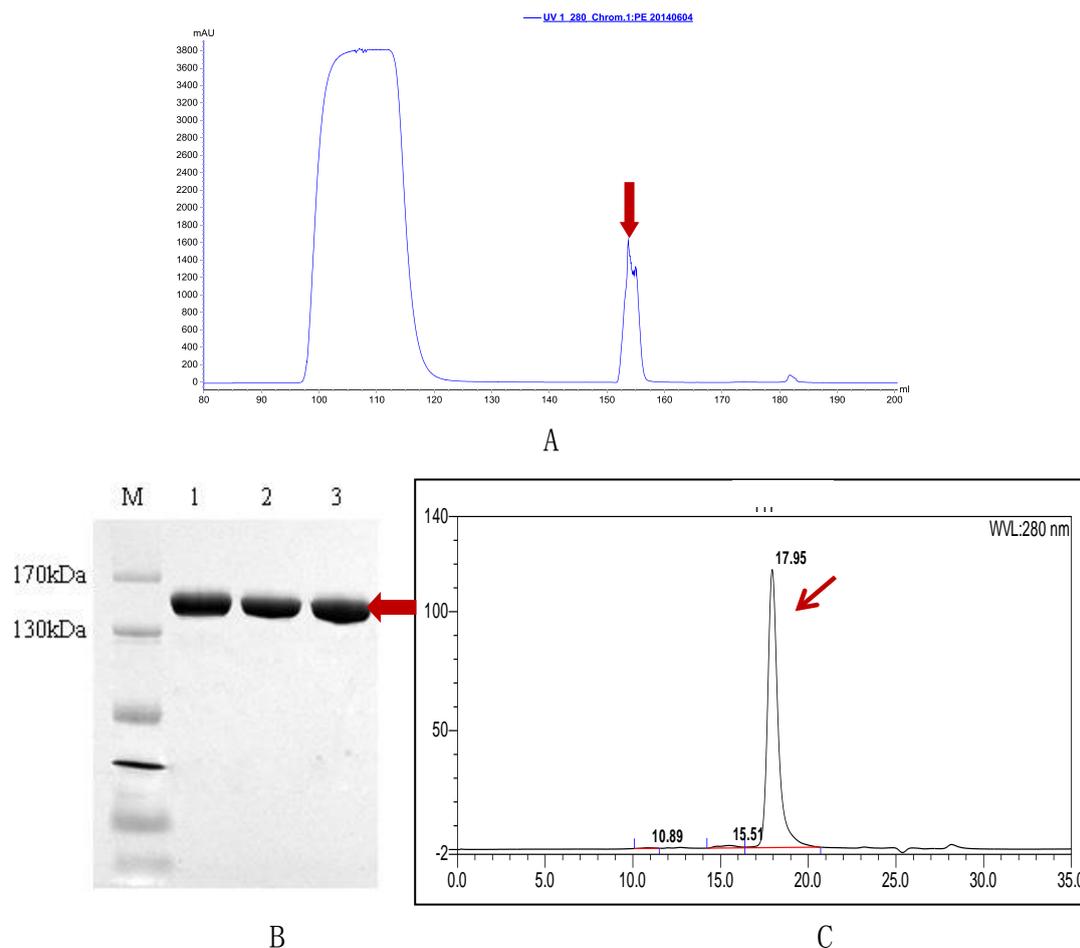
Fig. 4 Confocal microscopy detection for HER2_ECD expressed on the cell membrane

A: Blank control. B-D: Three parallel experimental groups.

E: Blank control. F-H: Three parallel experimental groups.

3.5 Identification and purification of HER2_ECD-Fc

After 48h of cell transfection, the supernatant was collected to purify by protein A affinity column. 20mM PB (sodium dihydrogen phosphate) was used to equilibrate, and 100 mM glycine was utilized to elute target protein whose pH was adjusted to 7.2 with Tris. [Fig. 5A](#) is the purification chromatography chart of HER2_ECD-Fc. The red arrow refers to the elution peak of the targeted protein. The purified protein was subjected to SDS-PAGE electrophoresis. As shown in [Fig. 5B](#) three lanes of SDS-PAGE were three parallel purified samples, and the amount of protein was 5 μ g. There were only one targeted band in the lanes, the size of 150kDa, consistent with the theoretical value and results of western blotting test. Moreover, it showed that the purified HER2_ECD-Fc protein was with high purity. The purity of HER2_ECD-Fc protein was 98.10% verified by HPLC([Fig. 5C](#)).



A: Chromatography of HER2_ECD-Fc purified by Protein A.
 B: SDS-PAGE of HER2_ECD-Fc. C: HPLC for HER2_ECD-Fc.
 Fig. 5 Identification and purification of HER2_ECD-Fc protein

4. Discussion

Studies have shown that transmembrane and secretory antigens such TNF α were different in the receptor binding, biological effects and mechanisms. In this study, the transmembrane and secretory HER2_ECD were made, which was the first step of obtaining the high biological activity anti-HER2 antibody through immune response. pDisplay and pCMV were used as vectors for the expression of transmembrane and secretory HER2_ECD, respectively. There is a PDGFR Transmembrane Domain in pDisplay. The PCR products of HER2_ECD and HER2_ECD-Fc were respectively cloned to pDisplay and pCMV to form the recombinant plasmids of pDisplay-HER2_ECD and pCMV-Her2ED-Fc. At present, the ligand binding area of HER2 extracellular domain has been expressed in *Escherichia coli*, but the expressed protein was in the form of inclusion bodies. Purification and refolding for bioactive soluble protein is necessary. However, the yield of renatured protein significantly reduces. Therefore, we chose HEK293T eukaryotic expression system, which originated from HEK293T of human embryonic kidney cells and modified by inserting T antigen, to reach high expression level. The HEK293T cells transfected by pDisplay-HER2_ECD were detected by Western Blotting, flow cytometry and confocal microscopy, and showed that HER2_ECD was successfully expressed on the membrane. The supernatant of HEK293T cells transfected by pCMV-HER2_ECD-Fc was confirmed by Western Blotting, purified by protein A affinity chromatography, identified by SDS-PAGE and HPLC, and the HER2_ECD-Fc with high purity of 98.10% was obtained.

Antitumor immunity is that T cells identify antigen peptide presented by MHC I or MHC II class molecules on cell surface, then T cells can be activated to produce a specific immune response [22-27].

Extraction of antigens from tumor tissue is subject to many objective conditions, it is difficult to obtain a large number of tumor antigens for medical research. At present, most of the tumor vaccines are directly synthesized epitope antigen peptide of T cell. The advantages of synthetic peptide vaccines are simple, convenient operation, and the high purity of vaccine. However, the type of antigen peptide is often such single that immune response confine to a specific epitope, prone to the body immune tolerance to the tumor. In this study, complete HER2 extracellular domain was cloned and expressed to obtain all the antigenic epitopes of HER2_ECD. It is reported that the antigen peptides of specific anti-T lymphocyte (CTL) response, many helper T lymphocyte (Th) and B cell epitopes all exist in the extracellular region of HER2^[28-31]. HER2_ECD protein contains more antigenic epitopes and significantly enhancing immunogenicity than the antigenic peptide. It will provide more T cell target antigen after presenting cells (APC) processing, which is a potential tumor protein vaccine.

It need to solve the problem of immunogenicity that making HER2_ECD as the tumor protein vaccine. HER2_ECD with weak immunogenicity must be used in combination with immune adjuvant, to be uptook and processed by antigen-presenting cells. In recent years, it has been found that the complex of HSPs in tumor cells and tumor antigenic proteins was used to immunize homologous animals to induce specific anti-tumor cell immunity^[32]. Therefore, the HER2 extracellular domain protein is a promising tumor protein vaccine. The transmembrane and secretory HER2_ECD proteins expressed in this study were prepared for the preparation of the HER2 extracellular vaccine. It is expected that through vaccine immunization the anti-HER2 antibody with better properties of physical and chemical and higher biological activity will be obtained, it is conducive to the further development of immune biology diagnosis and treatment research of HER2 high expressed positive tumors.

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