

# Antibody Response to Human Extracellular HER2 Subdomain Proteins in Mice

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## ABSTRACT

**Background:** In addition to passive immunotherapy using anti-HER2 monoclonal antibodies, active immunotherapy via HER2 targeting is an interesting approach to inducing specific anti-tumor immune responses. We have recently reported the immunogenicity of HER2 subdomains following DNA immunization and HER2 protein boosting. In the present study, we evaluated the immunogenicity of different HER2 extracellular subdomains for the induction of anti-HER2 antibody response in BALB/c mice. **Objective:** To investigate and characterize antibody responses to human recombinant proteins of HER2 extracellular subdomains in immunized mice. **Methods:** Four subdomains of HER2 extracellular domain were expressed in E.coli; subsequently, purified recombinant proteins were intraperitoneally injected in BALB/c mice with Freund's adjuvant. The anti-HER2 antibody response was detected by ELISA, immunoblotting and flow cytometry. **Results:** All the four HER2 subdomains along with the full extracellular domain (fECD) were able to induce specific anti-HER2 antibodies. Although anti-HER2 subdomains antibodies could not react with eukaryotic recombinant fECD protein by ELISA, they were able to recognize this protein by immunoblotting under both reduced and non-reduced conditions. Furthermore, only the sera of mice immunized with fECD protein could recognize native HER2 on HER2 overexpressing tumor cells (>99%) by flow cytometry. Moreover, fECD immunized mice sera inhibited the proliferation of tumor cells by XTT assay. **Conclusion:** The prokaryotic recombinant proteins of HER2 extracellular subdomains are immunogenic, yet the induced specific antibodies do not react with the native HER2 protein due to the paucity of post-translation modifications and /or distortion of the native conformation of isolated HER2 extracellular subdomains which might be potentially effective for induction of cell mediated immune response against HER2.

*Sadri-Ardalani F, et al. Iran J Immunol. 2017; 14(2): 99-110.*

**Keywords: Breast cancer, HER2, Immunization, Recombinant protein**

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## INTRODUCTION

HER2 protein is normally expressed, in the embryonic tissues, at higher levels compared with their normal adult counterparts (1). This protein is overexpressed in 20-25% of breast cancer patients (2). Besides breast cancer, HER2 overexpression in colorectal, ovarian, pancreatic and prostate cancers has been reported to be associated with poor prognosis (3,4). Structurally, the HER2 molecule is a 185 kDa protein composed of extracellular, transmembrane and intracellular parts (5). Owing to its expression profile and roles in tumor biology, HER2 is considered as a therapeutic target for cancer immunotherapy (6). In this regard, both active and passive immunotherapy approaches have been employed for the prevention and treatment of HER2 positive human malignancies (7,8). Passive immunotherapy, in which approved therapeutic antibodies such as Trastuzumab and Pertuzumab are used, is currently employed to treat HER2+ breast cancer patients (9,10). Trastuzumab is the first humanized therapeutic monoclonal antibody approved by the US FDA in 1998 for the treatment of metastatic breast cancer (11). However, antibody based passive immunotherapy encounters several problems such as the need for repeated injections, tumor resistance and side effects of the antibodies. Such drawbacks imply the need for alternative modalities such as active immunotherapy (12,13).

The main goal of active immunotherapy is to activate the host immune system to eradicate tumor cells with minimal toxicity (14). Active immunotherapy against breast cancer in mice and humans has been partially successful with advantages including low toxicity, high specificity and immunologic memory (15). Owing to the spontaneous cellular and humoral immune responses to HER2 found in breast cancer patients, HER2 is considered as an appropriate candidate self-antigen for active immunotherapy (16,17). Accordingly, different forms of HER2 protein have been employed to induce cellular and humoral immune responses against HER2 molecule (18,19).

We have recently reported the immunogenicity of these subdomains following DNA immunization and HER2 protein boosting (20). In the present study, we employed the recombinant proteins of the HER2 extracellular subdomains so as to investigate their immunogenicity in mice.

## MATERIALS AND METHODS

**Construction of HER2 extracellular subdomains and eukaryotic fECD plasmids.** HER2-pCMV-XL4 (OriGene Technologies, Rockville, MD, USA) construct was employed as a template for the amplification of four HER2 extracellular subdomains and the full extracellular domain (fECD) using specific primers (Table 1). Domain I (DI) and DIII PCR products were separately cloned into pET-22b (+) after digestion with XhoI and HindIII enzymes, and DII and DIV were subcloned into pET-32a (+) following digestion with Sall and XhoI enzymes. The fECD PCR product was subcloned into pCMV6-Neo following digestion with HindIII and XbaI enzymes as previously described in more detail (20).

**Table 1. Specific primers used for subcloning of extracellular full domain (fECD) and subdomains of human HER2.**

| Domain specific primers | Sequence *                                  | Amplicon (bp) size |
|-------------------------|---|--------------------|
| fECD-forward            | 5'-AAAAGCTTGCCACCATGGAGCTGGCGGCCTTGTGC-3'   | 1956               |
| fECD -reverse           | 5'-AAATCTAGATCACGTCAGAGGGCTGGCTCTCTGCTCG-3' |                    |
| DI-forward              | 5'GGTTTTTCTTAAGCTTACCCAAGTGTGCACCG -3'      | 509                |
| DI-reverse              | 5'-AAGAAAAAAACTCGAGGGCGGTTGGTGTC -3'        |                    |
| DII-forward             | 5'-GTCGACTTCCCCAGCTCTGCTACCA-3'             | 548                |
| DII-reverse             | 5'-CTCGAGCTTGCTGCACTTCTCACAC -3'            |                    |
| DIII-forward            | 5'-GGTTTTTCTTAAGCTTGACGTGGGATCCTGC -3'      | 609                |
| DIII-reverse            | 5'-AAGAAAAACCCTCGAGCACACACTCGTCC -3'        |                    |
| DIV-forward             | 5'-GTCGACAATTCGTGCACACGGTGC -3'             | 545                |
| DIV-reverse             | 5'-CTCGAGCGTCAGAGGGCTGGCTCT -3'             |                    |

\*The restriction enzyme sites are underlined.

**Expression of HER2 extracellular subdomains.** Through heat shock method, the DI- and DIII-HER2-pET-22b (+) constructs were transformed into the competent BL21-DE3 *E. coli* strain (Novagen, Madison, WI, USA) and the DII- and DIV-HER2-pET-32a (+) constructs were transformed into the competent RosettaGami *E. coli* strain (Novagen) (21). The transformed colonies of the HER2 extracellular subdomains were selected by culturing on Luria–Bertani (LB) agar plates containing the selective antibiotics and were confirmed by colony PCR with specific primers. The selected colonies were cultured in LB broth medium containing ampicillin (100 µg/ml) for DI and DIII and ampicillin, kanamycin, tetracycline and streptomycin for DII and DIV; the recombinant protein expression was further induced by adding 1mM isopropyl-1-thio-β-D-galactoside (IPTG) (Sigma, St. Louis, USA). Expression of recombinant proteins was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

To optimize the expression conditions, we studied different expression vectors (pET22b (+), pET32a (+) and pET28b (+)), hosts (BL21-DE3 and RosettaGami), induction points (OD<sub>600</sub>: 0.5, 0.7 and 0.9), cultivation temperatures (18, 22 and 37°C) and cultivation times (1, 2, 3, 4, 5 and 12 hrs following induction); the most acceptable subdomain conditions were selected for a large scale protein production.

**Purification and characterization of recombinant proteins.** The pellets of transformed bacteria (related to DI, DII and DIII) were lysed with cold lysis buffer (NaH<sub>2</sub>PO<sub>4</sub> 100 mM, Tris base 30 mM and NaCl 100 mM, pH = 8) and incubated on ice for an hour, followed by sonication. The pellet of sonication was dissolved in the buffer A (NaH<sub>2</sub>PO<sub>4</sub> 100 mM, Tris base 10 mM, urea 8 M, NaCl 100 mM and imidazole 30 mM with pH = 8). The supernatant was filtered, mixed with Ni-NTA resin (Qiagen, Hilden, Germany) and shaken for 45-60 min on the platform shaker. The bound proteins were eluted with buffer A containing the gradually increasing concentrations of imidazole. The purified recombinant protein fractions were dialyzed against phosphate buffered saline (PBS) (20). DIV recombinant protein was soluble in the supernatant of sonication buffer, hence directly applied to the Ni-NTA resin. The purity of the purified proteins was determined by SDS-PAGE and verified with immunoblotting via polyclonal rabbit anti-His-tag antibody (Abcam, Cambridge, UK).

**Production and purification of eukaryotic recombinant fECD protein.** The CHO-K1 cells (Chinese hamster ovary cells), (National Cell Bank of Iran, Pasture Institute of Iran, Tehran, Iran) were transfected with the fECD-pCMV6-Neo construct and pCMV6-Neo mock vector without HER2 gene for negative control by JetPEI (PolyPlus Transfection, Illkirch, France). Briefly,  $75 \times 10^3$  cells/1000  $\mu$ l per well cells were grown, overnight, in RPMI-1640 culture medium (Gibco, life Technologies, Paisley, UK) containing 10% fetal bovine serum (Gibco) in a 12-well culture plate (Nunc, Denmark) at 37°C. The medium was then replaced with fresh culture medium. Four micrograms of fECD- pCMV6-Neo construct was added to 4  $\mu$ l JetPEI prepared reagent at 200  $\mu$ l total volume; the mixture was subsequently incubated at room temperature for 20-30 min. This DNA-JetPEI complex was added onto the cells. To specify the presence of fHER2, the supernatant of transfected cells was evaluated by ELISA following 48 hr incubation. The stable clones were selected under 700  $\mu$ g/ml G418 (Gibco) and high producer clones were chosen through limiting dilution method. After large scale cultivation, the supernatant was collected and purified using an affinity chromatography column constructed from 5 distinct anti-HER2 monoclonal antibodies (2A8, 1T0, 1F2, 1H9 and 1B5) (22) coupled with NHS activated Sepharose 4B. The recombinant proteins were eluted with Glycin-HCl buffer (0.2 M, pH= 2.5) and immediately dialyzed against PBS. The purified proteins were subsequently monitored by ELISA and characterized by SDS-PAGE and immunoblotting.

**Mice immunization protocol.** Five 6- to 8-weeks-old BALB/c female mice (Pasteur Institute of Iran, karaj, Iran) were allocated to six groups for immunization by HER2 extracellular subdomains (DI, DII, DIII, and DIV) and fECD recombinant proteins. Purified recombinant proteins were injected intraperitoneally together with complete Freund's adjuvant for the first injection; for other injections, on the other hand, incomplete Freund's adjuvant was employed at a dose of 50  $\mu$ g for the first injection and 25  $\mu$ g for other injections. Five doses of antigens were administered over 2-week intervals. PBS and Freund's adjuvant were applied to the control group. The animal study was approved by the Avicenna Research Institute and Tehran University of Medical Sciences Ethical Committees.

**Measurement of anti-HER2 antibody in the immunized mice by ELISA.** HER2 extracellular subdomains and fECD recombinant proteins (DI 10  $\mu$ g/ml, DII 2.5  $\mu$ g/ml, DIII 10  $\mu$ g/ml, DIV 1  $\mu$ g/ml and fECD 0.5  $\mu$ g/ml), were coated with 50  $\mu$ l/well in a 96-well ELISA plate (MaxiSorp, Nunc, Denmark) and incubated at 4°C overnight. After blocking with PBS-Tween 0.05% + skim milk 5%, serial dilutions of mice sera were added and incubated at 37°C for 1.5 hr. After that, horseradish peroxidase (HRP)-conjugated sheep anti-mouse immunoglobulin (Sina Biotec, Tehran, Iran) was added and incubated at 37°C for an hour. Tetramethylbenzidine (TMB) (Pishtazteb, Tehran, Iran) substrate was added and the reaction was halted by H<sub>2</sub>SO<sub>4</sub> 20%. The optical densities were measured at 450 nm by automatic ELISA reader (BioTek, Winooski, VT, USA).

**Reactivity of anti-HER2 subdomain antibodies with full HER2 ECD by immunoblotting.** After dissolving in sample buffer, the eukaryotic recombinant fECD HER2 protein was separated on 10% SDS-PAGE gel under reduced and non-reduced conditions and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, MA, USA) which was blocked with 3% skim milk and immunoblotted on the shaker with immunized mice sera at 1:100 dilutions for 1.5 hr at room temperature. After a plenary wash with PBS-Tween, an appropriate dilution of sheep-anti-mouse-Ig-

HRP (Sina Biotec) was added to the membrane as a secondary antibody and the bands were visualized by chemiluminescence using the ECL detection system (GE Healthcare, Uppsala, Sweden) (21).

**Reactivity of anti-HER2 subdomain antibodies with native HER2 by flow cytometry.** A human HER2 positive breast cancer cell line, BT474 (National Cell Bank of Iran), was utilized in order to evaluate the reactivity by flow cytometry of anti-HER2 antibodies in immunized mice. Monolayers of the cells were grown in RPMI-1640 culture medium containing 10% fetal bovine serum at 37°C and were detached by trypsin-EDTA (Gibco). Mice sera (1:100) were added to cells and incubated at 4°C over a period of an hour. After washing with PBS, FITC conjugated sheep anti-mouse immunoglobulin (Sina Biotec) was added and incubated in the dark at 4°C for 1 hr. Stained cells were detected by flow cytometry (Partec, Denmark) and data was analyzed with FlowJo software.

**The effect of anti-HER2 antibody on tumor cell proliferation by XTT assay.** BT474 cells ( $1 \times 10^4$ /150  $\mu$ l per well) were cultivated overnight with phenol red free RPMI-1640 culture medium (Gibco) containing 10% fetal bovine serum in a 96-well culture plate (Nunc) at 37°C. Wells were then replaced with 100  $\mu$ l/well serum free medium containing 1% fetal bovine serum. Subsequently, HER2 fECD immunized mice sera (1:400 and 1:800), normal unimmunized mice sera (1:400 and 1:800) as a negative control, sodium azide (0.5%) and Herceptin (Trastuzumab) (anti-HER2 monoclonal antibody, 10  $\mu$ g/ml) as positive controls were separately added (in triplicate) to the wells and incubated at 37°C for 48 hr. XTT (Sigma, St. Louis, MO USA, 1 mg/ml) was mixed with PMS (Sigma, 5 mM) at 1:200 ratio and 50  $\mu$ l of the solution was added to each well and incubated for 3-4 hr at 37°C. The optical densities were measured at 450 nm by automatic ELISA reader (BioTek). The percentage of growth inhibition was calculated by the following formula:

$$\text{Growth inhibition (\%)} = \left( \frac{(\text{OD Control} - \text{OD Treatment})}{\text{OD Control}} \right) \times 100$$

## RESULTS

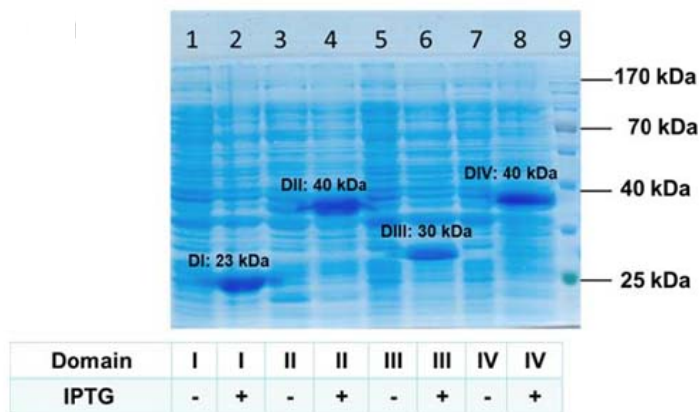
### Expression and purification of recombinant HER2 subdomains.

The recombinant DI-DIV HER2 extracellular subdomains were successfully expressed in *E. coli*. The expressed recombinant proteins displayed the expected molecular weight as shown by SDS-PAGE (DI=23, DII=40, DIII=30 and DIV=40 KDa) (Figure 1). As detected by immunoblotting, all purified proteins reacted with anti-His tag antibody (Figure 2). Different parameters were assessed to optimize the expression conditions. Our results showed that DII and DIV subdomains, ligated in pET28b (+), pET22b (+) and pET32a (+) vectors, were not expressed in BL21-DE3 host. However, these two subdomains ligated in pET32a (+) were successfully expressed in RosettaGami host. The optimal conditions for the expression of HER2 extracellular subdomains are summarized in Table 2.

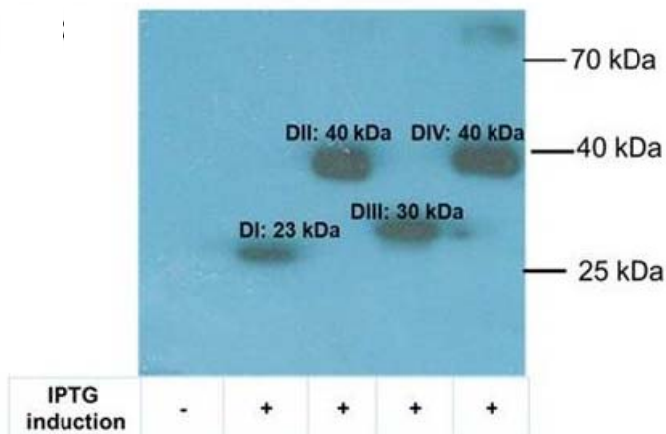
**Table 2. Optimized conditions for expression of human HER2-subdomains in *E. coli***

| Domain     | Vector     | Host ( <i>E. coli</i> strain) | OD600 | T (°C) | Time of induction (hr) | Sample for protein purification |
|------------|------------|-------------------------------|-------|--------|------------------------|---------------------------------|
| <b>I</b>   | pET-22b(+) | BL21-DE3                      | 0.5   | 37     | 5                      | pellet                          |
| <b>II</b>  | pET-32a(+) | RosettaGami                   | 0.5   | 37     | 12                     | pellet                          |
| <b>III</b> | pET-22b(+) | BL21-DE3                      | 0.5   | 37     | 5                      | pellet                          |
| <b>IV</b>  | pET-32a(+) | RosettaGami                   | 0.5   | 37     | 12                     | supernatant                     |

In the next step, large scale (1L) culture was carried out and the pellet of bacteria was collected by centrifugation; following sonication, the sonicated pellet and supernatant fractions were evaluated by SDS-PAGE for the availability of the recombinant proteins. The results demonstrate that most DI, DII and DIII recombinant proteins were found in the inclusion bodies of the pellet fraction, whereas DIV was mostly detected in the supernatant fraction (data not presented). Accordingly, DI-DIII proteins were purified under denaturation condition with 8 M urea, whereas the DIV subdomain was purified under native condition using Ni-NTA resin.



**Figure 1.** Electrophoresis profile of recombinant HER2 extracellular subdomains expressed in *E. coli*. DI- pET-22b (+) and DIII- pET-22b (+) were transformed and expressed in BL21-DE3, whereas DII- pET-32a (+) and DIV- pET-32a (+) were expressed in RosettaGami. Lanes 1, 3, 5 and 7 represent pre-induction samples and lanes 2, 4, 6 and 8 show post-induction samples. Lane 9 is prestained protein ladder.

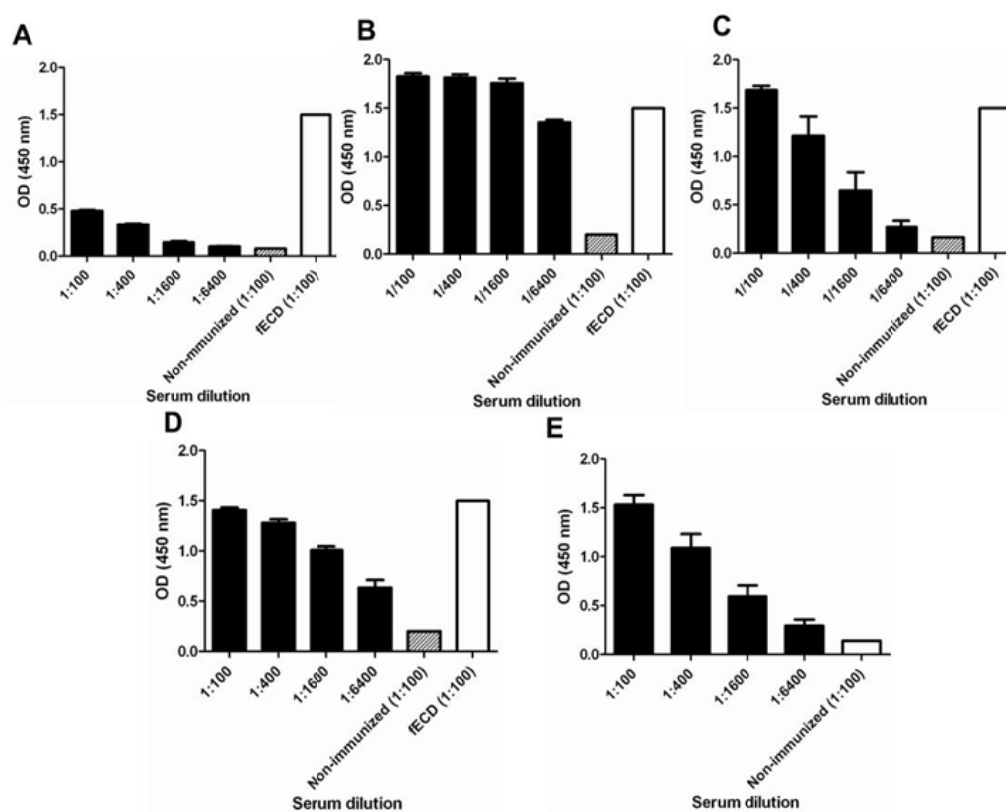


**Figure 2.** Detection of recombinant HER2 extracellular subdomains with anti-His tag antibody by immunoblotting. DI- pET-22b (+) and DIII- pET-22b (+) were transformed and expressed in BL21-DE3, whereas DII- pET-32a (+) and DIV- pET-32a (+) were expressed in RosettaGami.

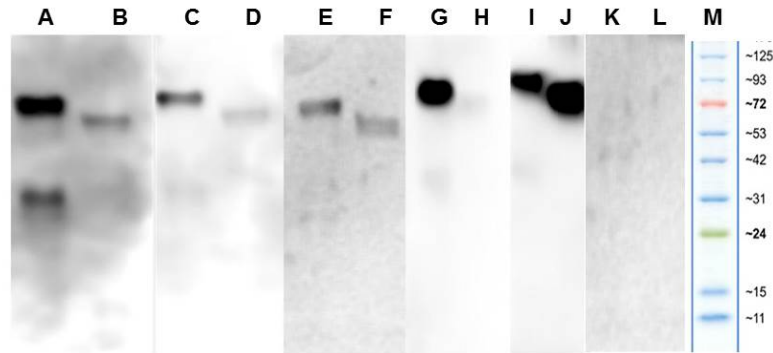
### Assessment of anti-HER2 antibody response in immunized mice by ELISA and immunoblotting.

Serum samples belonging to the HER2 subdomains of immunized mice were titrated against eukaryotic fECD and prokaryotic DI-DIV subdomains of HER2 by ELISA. All the four prokaryotic HER2 subdomains as well as fECD could induce anti-HER2 antibody specific to the corresponding immunogen (Figure 3 A-E).

However, none of the anti-subdomain antibodies reacted with the eukaryotic recombinant fECD. The reactivity of these anti-subdomain antibodies was also tested by immunoblotting with the eukaryotic fECD protein under reduced and non-reduced conditions. The results illustrated that all anti-subdomain antibodies recognized fECD protein under both non-reduced and particularly reduced conditions, except anti-DIV antibody which did not react with fECD protein under non-reduced condition (Figure 4).



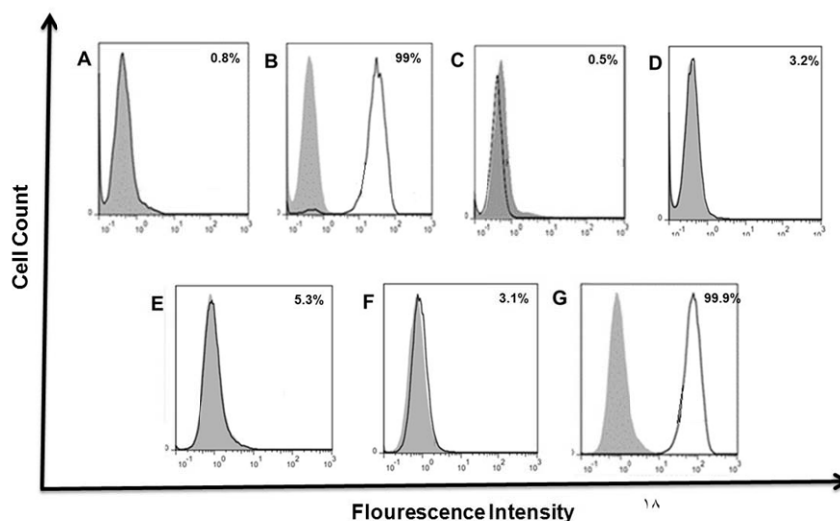
**Figure 3. Reactivity of immunized mice sera with HER2 recombinant domain and subdomains by ELISA.** Serum from mice immunized with DI (A), DII (B), DIII (C), DIV (D) and fECD (E) recombinant proteins was titrated on plates precoated with the corresponding proteins. Serum from non-immunized mice served as negative control. The solid bars present the standard error of the mean (SEM).



**Figure 4. Reactivity of immunized mice sera with eukaryotic recombinant fECD HER2 protein by immunoblotting.** Recombinant fECD-HER2 protein was electrophoresed under reduced (A, C, E, G, I and K) and non-reduced (B, D, F, H, J and L) conditions. Serum from mice immunized with DI (A, B), DII (C, D), DIII (E, F), DIV (G, H) and fECD-HER2 (I, J) and non-immunized (K, L) was applied on electroblotted PVDF membrane. Prestained protein ladder is shown in lane M.

#### Reactivity of HER2 subdomain specific antibodies with native HER2 by flow cytometry.

The reactivity of HER2 subdomain specific antibody in the serum of immunized mice with native HER2 protein expressed on BT474 breast cancer cell line was evaluated by flow cytometry. Only the sera of mice immunized with fECD HER2 protein were able to recognize the native HER2 on BT474 cells (>99%) (Figure 5 G). None of the HER2 subdomain specific antibodies reacted with BT474 cells (Figure 5 C-F), compatible with the ELISA results.

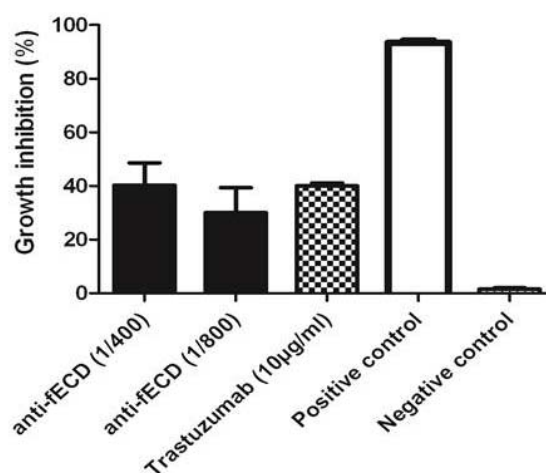


**Figure 5. Flow cytometry profiles of HER2 subdomains induced antibodies with HER2 expressing BT474 tumor cells.** (A) normal mouse serum (negative control); (B) Trastuzumab; (C) anti-DI antibody; (D) anti-DII antibody; (E) anti-DIII antibody; (F) anti-DIV antibody; (G) anti-fECD antibody. All mice in each group have been checked for the reactivity with BT474 by flow cytometry. One representative experiment of each group is shown. Figures represent percent of positive cells.



### The effect of anti-fECD antibody on tumor cell growth.

Only the fECD HER2 induced antibody reacted with native HER2 on tumor cells, hence the fact that its effect on the proliferation of BT474 cell line was evaluated by XTT assay. Anti-HER2 antibody of immunized mice sera induced 40% inhibition (1:400 dilution) and 30% inhibition (1:800 dilution) on the growth rate of BT474 cells following 48 hr incubation, an inhibition rate similar to that induced by 10 µg/ml of the commercial anti-HER2 monoclonal antibody, Trastuzumab (Figure 6).



**Figure 6. Anti-proliferative effect of serum anti-fECD-HER2 antibody on BT474 cells.** The results represent percent of cell growth inhibition obtained by XTT assay. The growth inhibition rates were calculated by formula presented in materials and methods. Normal mouse serum (1:400 dilution) and 0.5% sodium azide were employed as negative and positive controls, respectively. The solid bars present the standard error of the mean (SEM).

## DISCUSSION

Two FDA-approved humanized anti-HER2 monoclonal antibodies, Trastuzumab and Pertuzumab, specific for extracellular subdomains 4 and 2 of HER2, respectively, are currently being made use of the treatment of breast cancer patients with HER2 overexpression (23,24). In addition to passive immunotherapy, active immunization by HER2 molecule is an interesting approach to inducing specific anti-tumor immune responses. In this context, active immunotherapy may induce more effective therapeutic responses comparisons to passive immunization with monoclonal antibodies. Because of the short half-life of antibodies, they are cleared from circulation within a short period of time, curbing their therapeutic potential. However, active immunization can be a steady source of humoral and cellular immune response for patients (2). Furthermore, the immunogenicity of monoclonal antibodies hampers prolonged and repeated injections, while endogenous antibodies, generated following vaccination, are not expected to be immunogenic (25). These advantages have made active immunotherapy

more appealing. In the current research, we evaluated the immunogenicity of different HER2 extracellular subdomains with the objective of inducing anti-HER2 antibody responses in BALB/c mice.

The HER2 subdomains were cloned, expressed and purified from a prokaryotic expression system. Based on the present findings, the prokaryotic HER2 subdomains and eukaryotic fECD were immunogenic and triggered anti-HER2 antibody. The anti-HER2 subdomains antibodies were not capable of reacting with the native or eukaryotic recombinant fECD protein in ELISA and flow cytometry. Nevertheless, all HER2 subdomain- and fECD-induced antibodies reacted with the eukaryotic fECD protein under both reduced and non-reduced conditions by immunoblotting. The reactivity with non-reduced protein was weak, particularly for the subdomain IV-specific antibody. These observations could be interpreted based on the three-dimensional structure of fECD HER2 protein which seems to be either lost or largely modified in the prokaryotic extracellular subdomains (26,27). Accordingly, these HER2 subdomains induce antibodies which do not react with native HER2 protein. Further corroborating our findings are the absence of post translational modifications in prokaryotic subdomains and/or partial denaturation of these proteins during solubilization and purification with 8M urea or the distortion of the native conformation of the isolated subdomains, and the reactivity of these antibodies with eukaryotic fECD HER2 under both reduced and non-reduced conditions. Boiling fECD protein in SDS entails its partial linearization and denaturation with subsequent exposure of certain epitopes, accessible to these antibodies. Moreover, reactivity with the reduced fECD protein was better than the non-reduced preparation. In line with our findings, Rockberg *et al.* showed that rabbit immunization with four HER2 extracellular (residues 42–186, 236–363, 324–530, 531–626) recombinant proteins, expressed in prokaryotic host, induced polyclonal antibodies which were not attached to the native HER2 on BT474 cell line by flow cytometry (28). In addition, they noted that only rabbit polyclonal antibody raised against residues spanning the DII and DIII subdomains (amino acids 347-492) could recognize HER2 protein expressed on this cell line and cancer tissues by flow cytometry and immunohistochemistry, respectively (28). The inability of the antibodies (generated against prokaryotic HER2 extracellular subdomains) to bind with the native eukaryotic HER2 protein could be due to the paucity of post translational modifications and/or distortion of the native conformation of HER2 extracellular subdomains. As a matter of fact, dissociation of each subdomain impacts their tertiary and quaternary structures leading to substantial changes in their conformation.

HER2 active immunotherapy has been studied using DNA, peptides, whole protein, and extracellular and intracellular domains of HER2 in animal models and clinical trials (2,18,29,30). For instance, HER2 DNA immunization in combination with anti-HER2 monoclonal antibody thwarted the tumor growth in a breast cancer mouse model (31). In yet another study, HER2 peptide vaccine along with GM-CSF, as an adjuvant, retarded tumor growth and tolerance break down in transgenic immunized mice (32). Furthermore, vaccination with the fECD of HER2 protein along with anti-HER2 antibody fused to IL2, IL12 or GM-CSF reduced tumor growth and prolonged the life of the immunized mice (2). Desis *et al.* demonstrated that fECD of HER2 protein together with GM-CSF as an adjuvant, with no further toxic responses, induced specific T-cell responses in patients with breast and ovarian cancers (18). In another clinical trial conducted on breast cancer patients (stage II and III), active immunotherapy using

incomplete forms of intracellular and extracellular domains of HER2 led to tumor regression in a number of patients (19).

We have recently studied these subdomains following DNA immunization. Last but not least, specific antibody responses were triggered when DNA primed mice were boosted with recombinant HER2 subdomain proteins (20).

It was demonstrated that the recombinant prokaryotic subdomain proteins are immunogenic, yet the induced antibodies do not bind with the native HER2 molecule due to protein denaturation, lack of post-translation modifications and/or loss of native conformation of the isolated subdomains. Since these alterations do not substantially affect antigen presentation and T lymphocyte stimulation, these subdomains might be effective in activating cell mediated immunity and immunoprotection against tumor. We are currently investigating these aspects along with analyzing the production of eukaryotic HER2 subdomains.

## ACKNOWLEDGEMENTS

This study was partially supported by grants from Avicenna Research Institute and Tehran University of Medical Sciences (grant number: 23315).

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