

Polyclonal Antibody against Different Extracellular Subdomains of HER2 Induces Tumor Growth Inhibition *in vitro*

Reza Hosseini-Ghatar¹, Tahereh Soltantoyeh¹, Motahareh Bahadori², Jalal Khoshnoodi¹, Forough Golsaz-Shirazi¹, Mahmood Jeddi-Tehrani², Mohammad Mehdi Amiri^{1*}, Fazel Shokri^{1,2*}

¹Department of Immunology, School of Public Health, Tehran University of Medical Sciences, ²Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

ABSTRACT

Background: Human epidermal growth factor receptor 2 (HER2) has a crucial role in several malignancies. The extracellular domain of HER2 (HER2-ECD) has been extensively employed as an important target in passive and active immunotherapy. Isolated recombinant prokaryotic HER2-ECD subdomains were previously found to be ineffective in inducing anti-tumor antibody response. **Objective:** To employ recombinant eukaryotic HER2-ECD subdomains to raise anti-HER2 antibodies and determine their anti-tumor activity *in vitro*. **Methods:** Two paired subdomains of HER2-ECD (DI+II and DIII+IV), representing Pertuzumab and Trastuzumab binding domains, respectively, along with the full extracellular domain of HER2 were generated in CHO-K1 cells. Polyclonal antibodies were raised against these subdomains and characterized using ELISA, flow cytometry, and immunoblot and their anti-tumor activity was assessed by XTT assay. The cross-reactivity of these antibodies was specified along with other members of the human HER family. **Results:** Similar to Trastuzumab and anti-HER2-ECD antibody, anti-DI+II and DIII+IV polyclonal antibodies reacted with recombinant HER2-ECD and native HER2 expressed on tumor cells. These two polyclonal antibodies were able to inhibit the binding of Pertuzumab and Trastuzumab to HER2, respectively, and did not cross-react with other members of HER family. These antibodies were able to inhibit tumor cell growth *in vitro*, similar to Trastuzumab. **Conclusion:** The high immunogenicity of human HER2 DI+II and DIII+IV subdomains in rabbits and the tumor inhibitory activity of the purified specific antibodies imply that they might be suitable for active immunotherapy in formulation with appropriate adjuvants and in combination with other HER2 specific therapeutics.

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Keywords: Breast Cancer, HER2, Immunotherapy, Polyclonal Antibody, Subdomains of HER2

*Corresponding authors: Dr. Fazel Shokri and Dr. Mohammad Mehdi Amiri, Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, e-mails: fshokri@tums.ac.ir and m_amiri@tums.ac.ir

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) is a 185 KDa transmembrane protein overexpressed in 20-25 percent of patients with breast cancer (1,2). It has been shown that breast cancers overexpressing HER2 have an aggressive clinical course with poor prognosis (3). The HER family receptors are comprised of an N-terminal extracellular domain, a transmembrane domain and a C-terminal intracellular tyrosine kinase domain (4). The extracellular domain (ECD) of HER family members consists of four subdomain, namely DI, DII, DIII and DIV (1,5). Ligand mediated signaling through HER family receptors leads to the proliferation and growth of tumor cells (6,7). In contrast to other HER family members, HER2 is constitutively expressed in active conformation with no known ligands (7,8). Several studies have demonstrated the fact that the overexpression of HER2 results in the formation of HER2/HER2 homodimers and heterodimers with HER1, HER3 and HER4 (8,9). HER2 and ligand-activated HER3 form the most oncogenic heterodimer which potentially activates PI3K-Akt and MAPK pathways and subsequently improves tumor survival and proliferation (10-14).

It has been shown that passive immunotherapy by monoclonal antibodies (mAbs) leads to tumor regression (15,16). However, acquired resistance to mAbs treatment has been shown as a consequence of several mechanisms such as mutation and the involvement of other compensatory signaling pathways (17,18). Among the current therapeutic anti-HER2 mAbs, Trastuzumab and Pertuzumab mAbs which target different subdomains of HER2-ECD have been approved for the treatment of HER2 overexpressed breast cancers (19,20). Many patients, on the other hand, have been reported to resist Trastuzumab (17,21). It has been demonstrated that targeting HER2-ECD with a cocktail of two or three mAbs, which recognize different subdomains similar to a polyclonal antibody, can breakdown the acquired resistance, leading to more significant anti-tumor activity compared with employing a single mAb (22-26). Recently, rabbit polyclonal antibody against human ovarian tumor cells has been found to induce significant anti-tumor effects (27), suggesting the implication of such antibodies in other types of cancer. Different fragments of HER2-ECD have been employed to induce anti-HER2 polyclonal antibody response and trigger cellular immunity in patients with HER2 overexpressing breast cancer (28). HER2-derived peptide vaccines were found to induce both humoral and cellular immune response against HER2 positive tumors (29-32). However, the magnitude and efficacy of immune response differ among these peptide vaccines. Also important is the eukaryotic or prokaryotic nature of the immunizing protein which can affect the functional activity of the specific polyclonal antibodies (33-37). In this study, we produced recombinant paired subdomains of DI+II and DIII+IV as well as full HER2-ECD proteins in CHO host cells. These proteins were employed to assess their antibody response in rabbits and to determine their inhibitory effects on tumor cell growth *in vitro*.

MATERIALS AND METHODS

Preparation and transfection of expression constructs. Full extracellular domain and DI+II and DIII+IV subdomains of HER2 constructs were generated via PCR amplification using specific primers (Table 1) and HER2-pCMV-XL4 construct (OriGene Technologies, Rockville, MD, USA) as template. The PCR products were

subsequently subcloned into pSecTag2/hygro eukaryotic expression vector (OriGene). All expression constructs were verified by automated sequencing prior to being applied to transfection.

Table 1. List of specific primers used for cloning and amplification of extracellular domain and subdomains of HER2.

| Domain/ Subdomain | Primer | Sequence | Amplicon size (bp) |
|----------------------|------------------|--|-----------------------|
| DI+II | DI-Psec-S: | 5'-GGTTTTTCTT <u>AAGCTT</u> GACCCAAGTGTGCACCG-3' | 1060 |
| | DII- Psec -AS: | 5'-AAGAAAAACCC <u>CTCGAG</u> CCTTGCTGCACTTCTC-3' | |
| DIII+IV | DIII- Psec -S: | 5'-GGTTTTTCTT <u>AAGCTT</u> GGACGTGGGATCCTGC-3' | 1156 |
| | DIV- Psec -AS: | 5'-AAGAAAAAAA <u>ACTCGAG</u> CCGTCAGAGGGC-3' | |
| Full ECD | Full ECD-Psec-S | 5'-GGTTTTTCTT <u>AAGCTT</u> GACCCAAGTGTGCACCG-3' | 2014 |
| | Full ECD-Psec-As | 5'-AAGAAAAAAA <u>ACTCGAG</u> CCGTCAGAGGGC-3' | |

S: sense; AS: anti-sense; underlined sequences denote restriction enzyme sites.

Protein expression. CHO-K1 cells (National Cell Bank of Iran, Pasteur Institute, Iran) were cultured in RPMI-1640 medium (Gibco, California, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, California, USA), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (Gibco). Cells were transiently transfected using Lipofectamine 3000 (Invitrogen, California, USA) as recommended by the manufacturer. In brief, a sub-confluent monolayer of CHO-K1 cells was washed with culture medium without antibiotics, and 150 µL of Opti-MEM (Invitrogen) was added. DNA constructs were diluted in 50 µL of Opti-MEM and 3 µL of P3000 reagent (Invitrogen). Next, the diluted DNA was added to 1.5 µL Lipofectamine 3000 pre-diluted in 50 µL of Opti-MEM and kept at room temperature for 5 min. The lipid-DNA complex was added to the wells and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cell culture supernatants were harvested 48 h later and assessed by ELISA. So as to select the stable transfectants, transfected cells were kept in 0.7 mg/ml Hygromycin for 2 weeks; the cells were then subcloned three times to select stable high producing clones. Ultimately, the cell culture supernatant was collected and recombinant proteins were purified by affinity chromatography through the use of anti-HER2 affinity column.

Purification of recombinant proteins. Recombinant HER2-ECD domain and subdomains were purified from supernatant using an affinity column packed with Sepharose 4B coupled with different anti-HER2 mAbs (1F2, 1H9, 1T0 and Trastuzumab) (38,39). HER2-ECD was purified and applied to rabbit immunization.

Production and purification of polyclonal antibodies. The polyclonal antibodies were generated by intramuscularly immunizing three distinct New Zealand white rabbits with 50 µg of recombinant DI+II and DIII+IV extracellular subdomains of HER2, a full length of HER2-ECD, and an equal volume of complete Freund's adjuvant (Sigma, St Louis, MO, USA). Four additional booster doses were administered biweekly with 20 µg of recombinant antigens in incomplete Freund's adjuvant (Sigma). Immunized rabbits were bled one week following the fifth immunization and IgG were purified

from serum utilizing a protein G Sepharose 4B column (GE Healthcare, Buckingham, UK).

Screening of recombinant HER2 subdomains in the supernatant of transfected cells. Employing affinity-purified rabbit anti HER2-ECD antibody and HRP-conjugated anti-His tag antibody (SinaBiotech, Tehran, Iran), a sandwich ELISA was developed in order to screen recombinant HER2 subdomains in transfected cell culture supernatant. Briefly, 10 µg of anti-HER2 polyclonal antibody was coated in 96-well flat bottom microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) and incubated for 90 min at 37°C. After a three-time washing with PBS-Tween 20 (0.05%), plates were blocked for 90 min with PBS-Tween 20 (0.05%) containing 3% skim milk (Merck, Darmstadt, Germany) at 37°C. Plates were washed again, to which 50 µL of the supernatant of the transfected cells was further added. Following incubation at 37°C for 1 h, the plates were washed and incubated with an appropriate dilution of HRP-conjugated anti-His tag antibody. After incubation for 90 min and washing, the reaction was then revealed with tetramethylbenzidine (TMB) substrate (Pishtaz Teb, Karaj, Iran). Finally, the reaction was stopped with 30 µl of 1N HCL and the absorbance was measured by an ELISA reader (BioTek, Winooski, VT, USA) at 450 nm.

Titration of sera from rabbits immunized with recombinant proteins. An antigen-based ELISA was performed for the titration of immunized rabbit sera. In brief, plates were coated with 50 µl of 1 µg/ml DI+II, DIII+IV or HER2-ECD. After a three-time washing with 0.05% Tween 20/PBS, blocking was performed for 90 min with 50 µl of PBS-Skim milk 3%. Plates were once again washed three times, after which, the serial dilutions of rabbit sera were added and incubated for 90 min at 37°C. Finally, bound antibody was detected using sheep anti-rabbit IgG-HRP (SinaBiotech, Tehran, Iran) and TMB substrate (Pishtaz Teb, Tehran, Iran).

Flow cytometry analysis. Flow cytometry was conducted to assess binding of polyclonal antibodies to native HER2 protein expressed on the cell surface of BT-474 tumor cells (National Cell Bank of Iran). After harvesting the cells by trypsinization and twice washing with washing buffer (PBS, 2% FBS), 10⁶ cells were incubated for 1 h with 40 µg/mL of purified anti-DI+II, -DIII+IV and -HER2-ECD polyclonal antibodies at 4°C. Trastuzumab-FITC and purified normal rabbit IgG were included as positive and negative controls, respectively. After incubation, the cells were washed twice with washing buffer and then incubated for 1 h with FITC-conjugated donkey anti-rabbit immunoglobulin (Bio Legend, California, USA) at 4°C. Cells were then washed twice prior to scanning with a flow cytometer (Partec, Nuremberg, Germany). A total of 10,000 events were counted for each sample. Data analysis was further performed using FlowJo software (Tree Star, Inc, Ashland, OR, USA).

SDS-PAGE and immunoblotting analysis. For purity and structural characterization of DI+II, DIII+IV and HER2-ECD, 5 µg of affinity purified recombinant proteins were electrophoresed on 10% SDS-polyacrylamide gels under reduced and non-reduced conditions along with molecular weight marker and further stained with Coomassie Blue. Mouse IgG was used as a positive control under reducing and non-reducing conditions. For Immunoblotting, similar SDS-PAGE was conducted and bands transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After blocking with 5% skim milk (Merck, Germany), the membranes were incubated with 5 µg/mL of SPG affinity-purified 1F2 and 1H9 mouse mAbs, which bind to linear epitopes on DI and DIV subdomains of HER2, respectively (data has not been published yet), as well as anti-DI+II, DIII+IV and HER2-ECD specific polyclonal antibodies

overnight at 4°C; this was followed by extensive washing with PBS/Tween 20 (0.05%). The membranes were subsequently incubated 1.5 h with HRP-conjugated sheep anti-rabbit Ig (SinaBiotech, Tehran, Iran) at 37°C for. After extensive washing, the bands were visualized via ECL prime kit (Amersham Pharmacia Biotech, Chalfont, UK).

Cross-reactivity of polyclonal antibodies with other human HER family members and Cynomolgus monkey HER2. Through indirect ELISA, we analyzed the cross-reactivity of the anti-DI+II, DIII+IV and HER2-ECD polyclonal antibodies with other members of the human HER family (HER1, HER3 and HER4), and Cynomolgus monkey HER2. Recombinant HER1, HER3 and HER4 (Speed BioSystems, Rockville, MD, USA) and Cynomolgus HER2 (Sino Biological Inc, Beijing, China) proteins were coated for 90 min at 1 µg/ml in 96-well flat bottom microtiter plates (Maxisorp, Nunc, Denmark) at 37°C. After washing, polyclonal antibodies elicited to DI+II, DIII+IV and HER2-ECD were added to wells at a final concentration of 10 µg/ml and incubated for 90 min at 37°C. In the end, HRP-conjugated sheep anti-rabbit Ig (SinaBiotech, Tehran, Iran) was added for an hour and the reaction was detected as previously described. All recombinant proteins contained a C-terminal 6X His-Tag, hence HRP anti-His tag antibody (SinaBiotech, Tehran, Iran) was used to check for the presence of coating layers.

***In-vitro* antitumor activity of polyclonal antibodies.** HER2 over-expressing BT-474 cells were seeded in 96 flat-bottom tissue culture plates (15000 cells/well) in RPMI-1640 medium containing 20% fetal bovine serum (Gibco). The cells were treated with different concentrations (5, 25, 100 µg/ml) of rabbit anti-DI+II, DIII+IV and HER-ECD polyclonal antibodies and incubated at 37°C in a humidified atmosphere of 5% CO₂. For combination treatment, half the concentration of each antibody (50, 12.5, 2.5 µg/ml) was made use of. Two different concentrations of Trastuzumab (5 and 50 µg/ml) and non-immune rabbit IgG (25 and 100 µg/ml) were included as positive and negative controls. Untreated cells were also considered as negative control. Following a 120 h incubation, 2, 3-bis (2 methoxy-4-nitro-5-sulfophenyl)-5-([phenylamino]-cabonyl)-2H-tetrazolium (XTT) solution was added to the wells according to the instruction of the manufacturer (Roche Diagnostics, Mannheim, Germany). Finally, the microtiter plates were read by ELISA reader at 450 nm along with 650 nm reference wavelength after a 3 h incubation with XTT. All experiments were performed in triplicate and tumor growth inhibition rate was estimated via the following formula:

$$\text{Tumor growth inhibition (\%)} = \frac{(\text{OD without antibody} - \text{OD with antibody}) \times 100}{\text{OD without antibody}}$$

Statistical analysis. Parametric OneWay ANOVA (Tukey test) was employed in order to compare the groups. P values of less than 0.05 were taken as significant.

RESULTS

Production and characterization of HER2 extracellular domain and subdomains.

The full extracellular domain of HER2 (HER2-ECD) together with paired subdomains I+II and III+IV were produced in CHO cells. The recombinant proteins were detected by ELISA and purified from culture supernatant by affinity chromatography, using Sepharose coupled with polyclonal anti-HER2-ECD antibody. The ELISA results are shown in Figure 1.

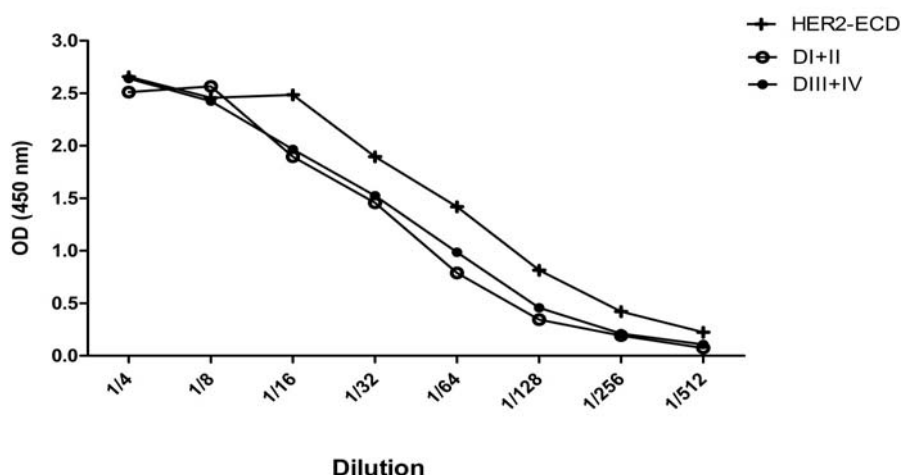


Figure 1. Detection of recombinant HER2 subdomains in culture supernatant of stable transfected CHO cells.

Production and characterization of polyclonal anti-HER2 subdomain antibodies.

The purified recombinant proteins were used to immunize rabbits. The titration of the immunized rabbits sera, collected following each immunization dose, indicated an induction of high titers of antibody against fHER2-ECD domain as well as paired I+II and III+IV subdomains (Figure 2A). The titration of the protein G purified polyclonal antibodies against fHER2-ECD protein, showed that the reactivity of DI+II and DIII+IV specific antibodies was similar; however, both antibodies displayed a lower binding activity compared to fHER2-ECD specific polyclonal antibody (Figure 2B).

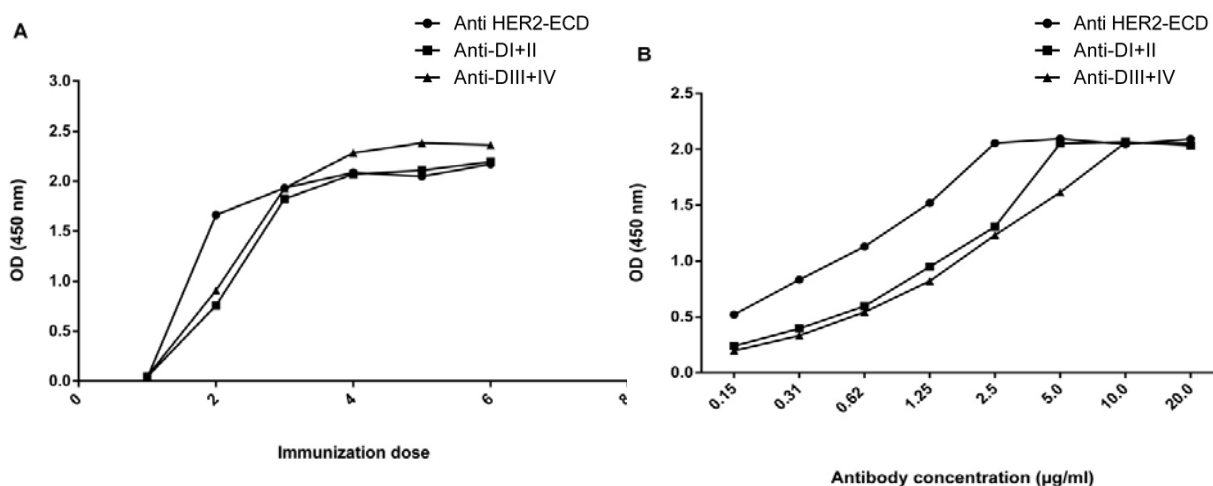


Figure 2. Titration of serum and purified anti-DI+II, anti-DIII+IV and anti-HER2-ECD polyclonal antibodies. (A) Serum samples collected from immunized rabbits after administration of each immunization dose were tested at 1/200 dilution against 5 µg/ml of the corresponding immunogen. (B) Purified antibodies were titrated against 5 µg/ml of fHER2-ECD.

SDS-PAGE analysis and immunoblotting of fHER2-ECD, DI+II and DIII+IV recombinant proteins using subdomain-specific polyclonal antibodies.

The SDS-PAGE results (Figure 3A) showed that the purified DI+II and DIII+IV were extensively polymerized in comparison with HER2-ECD under non-reducing condition. In addition, reducing the proteins partially leads to the depolymerization of these proteins, resulting in ~50 KD and ~95 KD monomer sizes, respectively pertaining to DI+II and DIII+IV and HER2-ECD. The higher molecular weight of these proteins compared to their expected amplicon size is most likely due to their glycosylation in the mammalian expression host (CHO cells).

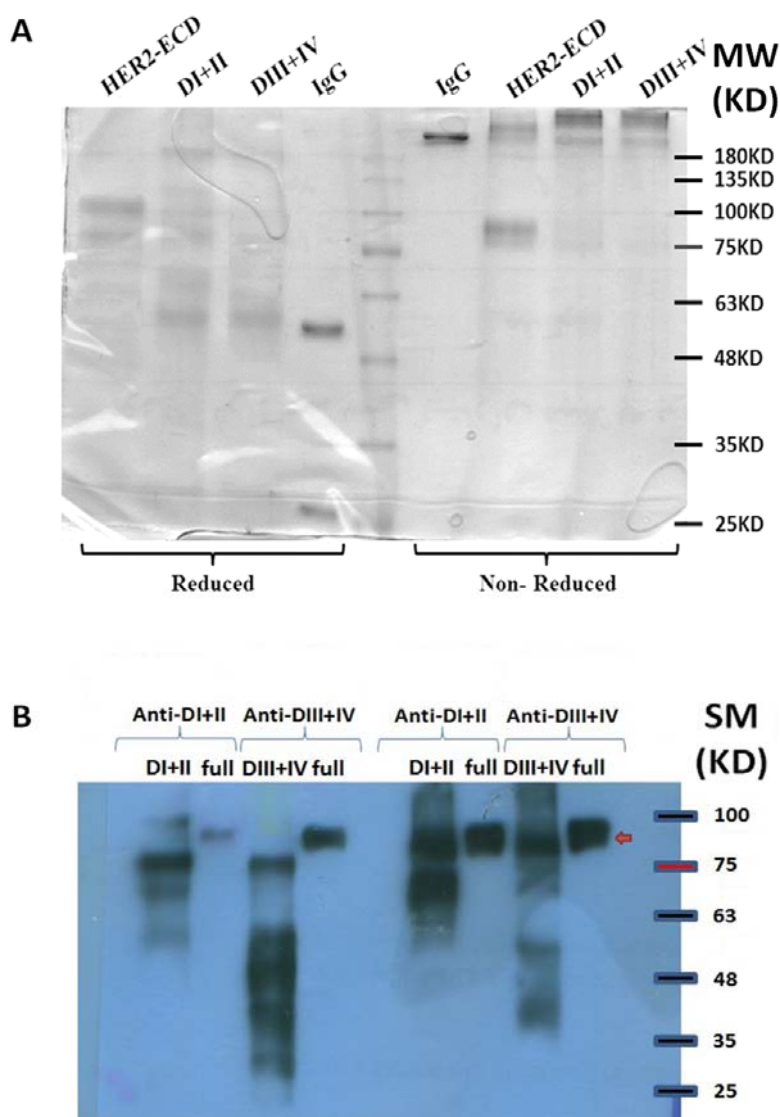


Figure 3. Electrophoresis and immunoblot profiles of the recombinant extracellular subdomains of HER2. Recombinant DI+II, DIII+IV and HER2-ECD proteins were resolved on 10% SDS-PAGE under reducing and non-reducing conditions and then stained with Coomassie blue (A) or transferred to a nitrocellulose membrane and immunoblotted with polyclonal anti-DI+II and anti-DIII+IV antibodies (B). SM, size marker (kilodalton). Arrowhead shows HER2-ECD monomer band.

The purified proteins were characterized by immunoblotting using polyclonal anti-subdomains antibodies (Figure 3B). Both anti-DI+II and anti-DIII+IV antibodies detected a single band (~95 KD), representing the full HER2-ECD in reduced and non-reduced conditions. Nonetheless, different bands were detected by these antibodies during the purified preparations of DI+II and DIII+IV, suggesting the dimerization and polymerization of these proteins, in accordance with SDS-PAGE results.

Binding of HER2 subdomain specific polyclonal antibodies to HER2-expressing tumor cells.

Polyclonal antibodies produced against full HER2-ECD as well as DI+II and DIII+IV paired subdomains bound to native HER2 expressed on the surface membrane of the tumor cells. FITC-labeled Trastuzumab and non-immune rabbit IgG were used as positive and negative controls. The mean fluorescence intensity (MFI) of Trastuzumab and anti-HER2-ECD antibodies was higher, in comparison to subdomain specific antibodies (Figure 4).

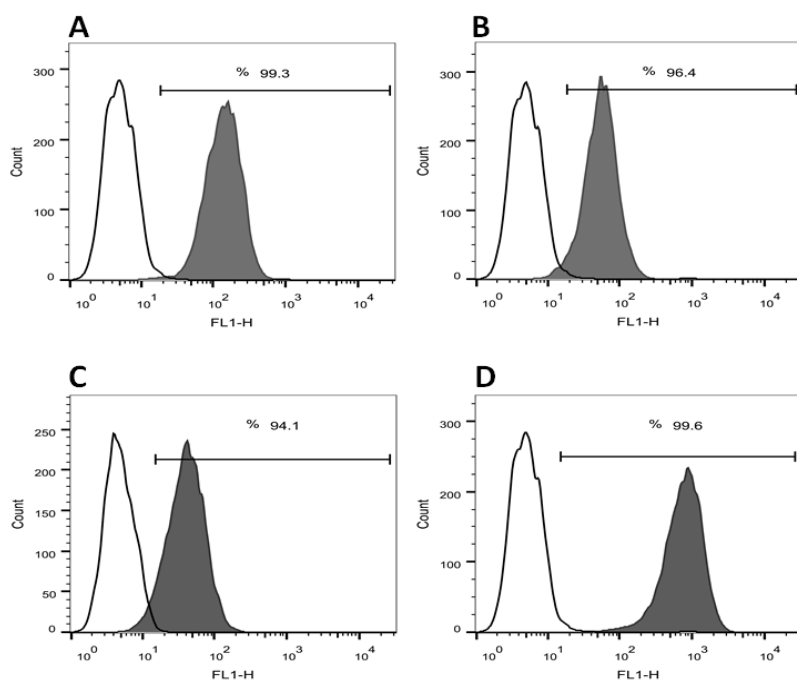


Figure 4. Binding of anti HER2-ECD (A), anti-DI+II (B) and anti-DIII+IV (C) antibodies to HER2 positive BT-474 cells. FITC-labeled Trastuzumab (D) was included as a positive control. Purified non-immune rabbit IgG (A, B, C) and human IgG (D) were used to account for background binding (light histogram). The figures represent percent of positive cells detected by flow cytometry.

Cross-reactivity of polyclonal antibodies with HER family members and Cynomolgus monkey HER2.

None of the three polyclonal antibodies reacted with human recombinant HER1, HER3 and HER4 proteins by ELISA. However, all three antibodies were robustly reactive to recombinant Cynomolgus HER2 protein, similar to human HER2 (Figure 5). Since all HER2 recombinant proteins were terminated with a His-tag, an anti-His antibody was used as a control to confirm the coating protein.

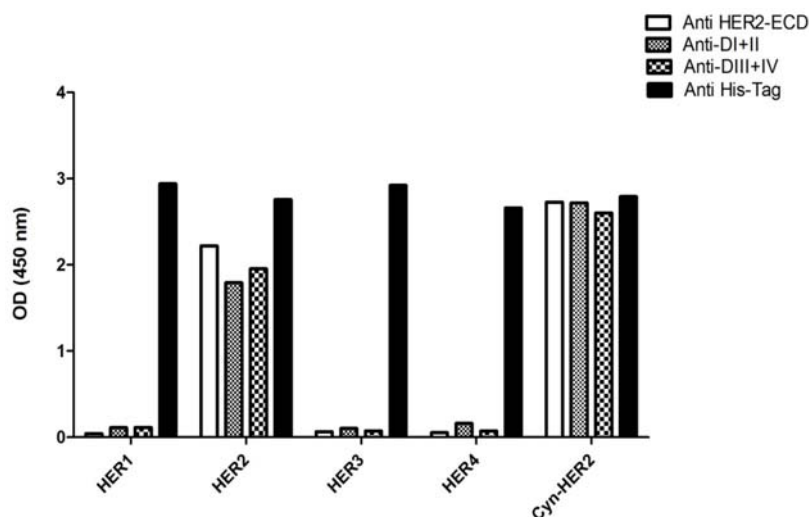


Figure 5. Cross-reactivity of polyclonal anti-DI+II, anti-DIII+IV and anti-HER2-ECD antibodies with HER family members and Cynomolgus monkey HER2. Anti His-tag mAb is included as a control to confirm presence of the coating proteins. Cyn-HER2: Cynomolgus monkey HER2.

Localization of overlapping epitopes recognized by polyclonal antibodies and Trastuzumab or Pertuzumab.

ELISA results of cross-inhibition showed that, dose dependently, anti-HER2-ECD antibody was capable of obstructing both Trastuzumab and Pertuzumab (Figure 6). Furthermore, anti-DI+II antibody inhibited Pertuzumab, dose dependently, while it was not able to hinder Trastuzumab which binds to DIV of HER2-ECD (40). Contrary to anti-DI+II antibody, anti-DIII+IV antibody strongly inhibited Trastuzumab, yet failed at inhibiting Pertuzumab which binds to DII of HER2-ECD (41). These results, once again, corroborate the high specificity of produced antibodies against relevant subdomain.

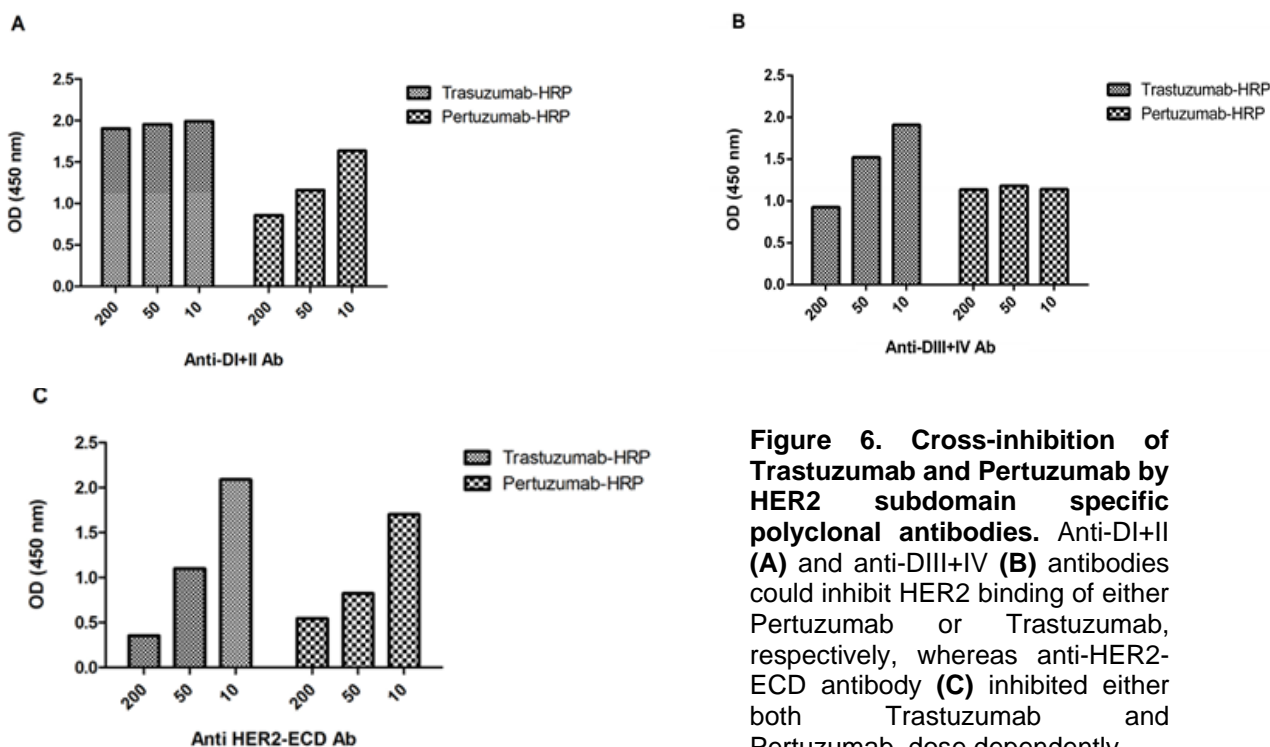


Figure 6. Cross-inhibition of Trastuzumab and Pertuzumab by HER2 subdomain specific polyclonal antibodies. Anti-DI+II (A) and anti-DIII+IV (B) antibodies could inhibit HER2 binding of either Pertuzumab or Trastuzumab, respectively, whereas anti-HER2-ECD antibody (C) inhibited either both Trastuzumab and Pertuzumab, dose dependently.

Tumor growth inhibition by polyclonal anti-HER2 subdomain antibodies.

Tumor growth inhibition was assessed by XTT assay. The results indicated that, similar to HER2-ECD specific polyclonal antibody, anti-DI+II and anti-DIII+IV antibodies obstructed HER2-overexpressing tumor cell growth *in vitro* (Figure 7). Moreover, 100 µg/ml of SPG purified anti-DI+II or anti-DIII+IV antibodies (equivalent to 50 µg of Trastuzumab mAb) inhibited tumor growth. Different concentrations of induced polyclonal antibodies showed dose-dependent tumor growth inhibition. Anti-DI+II and anti-DIII+IV antibodies, similar to anti HER2-ECD antibody and Trastuzumab mAb, had significant tumor growth inhibition compared to cells treated with 100 µg/ml irrelevant control antibody ($P < 0.001$). Furthermore, treatment with a combination of anti-DI+II and anti-DIII+IV antibodies induced a slightly more growth inhibition compared to when each antibody was separately utilized.

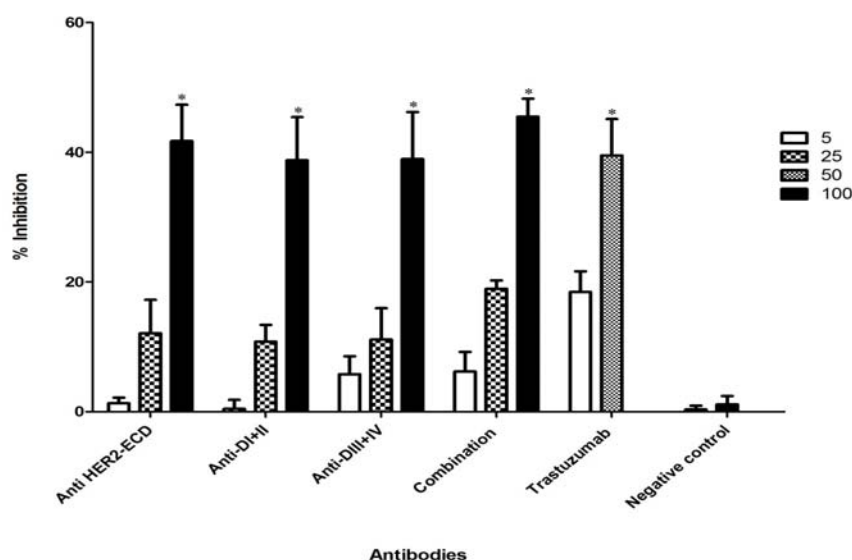


Figure 7. Growth inhibition of HER2 overexpressing tumor cells by polyclonal HER2 subdomain specific antibodies. BT-474 tumor cells were treated with 5, 25 and 100 µg/ml of HER2 specific polyclonal antibodies, 5 and 50 µg/ml of Trastuzumab or 25 and 100 µg/ml of irrelevant non-immune rabbit IgG (negative control). The results represent mean and standard deviation of percent of inhibition obtained by XTT assay. * shows significant growth inhibition ($p < 0.001$) of different treatments compared with negative control. Combination treatment refers to combination of anti-DI+DII and anti-DIII+DIV polyclonal antibodies.

DISCUSSION

Several studies have shown that passive therapy with anti-HER2-ECD mAbs hinders tumor growth and proliferation through different mechanisms (19,20,38,42-44). Despite promising clinical outcomes, antibody therapy encounters a number of limitations including resistance to mAbs, disease recurrence and the need for multiple high dose administrations of mAbs (21,45-47). Active immunization, on the other hand, induces

potent humoral and cellular immune responses against multiple epitopes of HER2, leading to tumor eradication without the subsequent risk of tumor resistance (37,48,49). Anti-HER2 has been reported to be spontaneously produced with intrinsic anti-proliferative effects in myriad HER2 positive patients with breast cancer (50). However, due to the low immunogenicity and insufficient levels of endogenously-induced antibodies, there is no considerable tumor growth inhibition. Parallel with these findings, endogenous anti-HER2 antibodies induced by peptide vaccination have been shown to improve survival in patients with breast cancer (51). However, the anti-tumor immune response induced by short peptides may not be as efficient as intact HER2-ECD protein. When combined with other HER2 specific therapeutics, HER2-ECD immunization induces a potent anti-tumor response and significant tumor growth inhibition in patients with HER2-overexpressing breast cancer (24,52). Furthermore, the fusion proteins of HER2-ECD with costimulatory molecules expressed on antigen presenting cells have been demonstrated to induce highly powerful anti-tumor activity (53-55). Such promising results together with the fact that different subdomains of HER2 might induce either inhibitory or stimulatory mAbs (38) and Pertuzumab and Trastuzumab are respectively directed against subdomains II and IV of HER2-ECD, prompted us to investigate antibody response to isolated human HER2 subdomains in Balb/C mice. DNA immunization failed to induce an efficient antibody response, yet coupled with a booster protein immunization, a modest antibody response was observed in immunized mice (37). In the next step, we prepared recombinant prokaryotic proteins from each HER2-ECD subdomains and evaluated their immunogenicity. All subdomains were able to generate a strong antibody response; the antibodies, however, reacted neither with the native HER2 protein expressed on tumor cells nor with recombinant eukaryotic HER2-ECD protein. Similar to the present study, the fusion subdomains of HER2 with anti-CD19 scFv produced in prokaryotic host, induced a high titer of polyclonal antibodies against each subdomain; however, the anti-DI and DII polyclonal antibodies did not inhibit the growth of the tumor cells (53), implying that the lack of reactivity is attributed to either the distortion in the conformation of isolated subdomains or the lack of post-translational modifications in these prokaryotic proteins. To circumvent these limitations, we produced and employed eukaryotic recombinant subdomains I+II, III+IV and full length HER2-ECD. Immunization of rabbits with the purified subdomain proteins induced high titers of specific antibody which could bind to native HER2 protein on tumor cells similar to Trastuzumab and anti-full HER2-ECD specific antibody. Interestingly, the purified polyclonal antibodies produced against DI+II and DIII+IV proteins, in a dose dependent manner, inhibited the binding of Pertuzumab and Trastuzumab to HER2-ECD, respectively (Figure 6), indicating the specificity of these antibodies and their binding activity to native HER2. In order to assess the functional activity of these antibodies, their effect on growth and proliferation of HER2 over-expressing BT-474 (human breast cancer tumor cells) was determined *in vitro* by XTT assay. Similar to Trastuzumab and anti-full HER2-ECD specific antibody, both subdomain specific antibodies inhibited tumor cell growth, dose dependently (Figure 7). Interestingly, although the HER2-ECD and subdomains specific antibodies represented a small fraction (5-10%) of the total IgG purified by Streptococcus protein G, they could still inhibit the growth of tumor cells as potently as Trastuzumab ($p < 0.001$).

Since our antibodies are polyclonal, they may also bind to other members of the HER family, influencing their *in vitro* inhibitory activity. It has been demonstrated that

combining mAbs specific for HER1, HER2 and HER3 may synergistically inhibit growth of different cell lines expressing these molecules (56,57). As a matter of fact, BT-474 cells overexpress HER2, HER3 and, to a lesser extent, HER1 proteins (58,59). Accordingly, we checked the reactivity of our polyclonal antibodies with other members of the human HER family, as well as Cynomolgus monkey HER2 which is 99% homologous to human HER2 (41,60). Unexpectedly, the antibodies did not display any cross-reaction to other HER family members which exhibit more than 45% sequence homology to HER2 (41); these antibodies, on the other hand, strongly reacted with Cynomolgus monkey HER2. Despite the substantial structural similarity, the homologous sequences might be embedded in regions of the molecule not accessible to the polyclonal antibody, suggesting that the inhibitory effect of our HER2 subdomain specific antibodies is restricted to their binding to HER2 molecule.

In conclusion, altogether, the present findings suggest that the paired eukaryotic subdomains of HER2-ECD are highly immunogenic and, if formulated with appropriate adjuvants, are capable of inducing efficient humoral and cellular immune responses capable of eradicating HER2 positive tumors. These subdomains are potentially useful tools for active immunotherapy.

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