

Production and Characterization of Monoclonal Antibodies Against the Dimerization Domain of Human *HER2*

Mohammad Nadri¹; Mohammad Soukhtanloo^{1,2}; Amir Hosein Jafarian²; Mahboobeh Alamdari¹; Mohsen Sisakhti¹; Tayebbeh Kianoush¹; Farnaz Zahedi Avval^{1,3,*}

¹Department of Biochemistry, School of Medicine, Mashhad University of Medical Sciences, Mashhad, IR Iran

²Cancer Molecular Pathology Research Center, Ghaem Hospital, School of Medicine, Mashhad University of Medical Sciences, Mashhad, IR Iran

³Biochemistry & Nutrition Research Center, Mashhad University of Medical Sciences, Mashhad, IR Iran

*Corresponding author: Farnaz Zahedi Avval, Department of Biochemistry, School of Medicine, Mashhad University of Medical Sciences, P. O. Box: 917794-8564, Mashhad, IR Iran. Tel: +98-5138002365, Fax: +98-5138828574, E-mail: zahediaf@mums.ac.ir

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Background: Human Epidermal Growth factor Receptor 2 (*HER2*), also known as ErbB2 is a 185 kDa protein belonging to the Human Epidermal Receptor (HER) family of tyrosine kinase receptors overexpressed in 20% - 30% of patients with breast cancer. Similar to other members of the HER family, *HER2* glycoprotein comprises of multiple domains including an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain with tyrosine kinase activity. The extracellular domain of *HER2* with 632 amino acids is composed of four subdomains (I - IV); subdomains I and III form a ligand binding site, and cysteine-rich subdomains II and IV play an important role in dimerization of the receptor.

Objectives: In this study we aimed to produce murine Monoclonal Antibodies (MAbs) with the ability of specific recognition of the *HER2* dimerization arm.

Materials and Methods: Primarily, BALB/c mice were immunized with a 30-aminoacid peptide as a part of the human *HER2* subdomain II. Splenocytes from hyperimmunized mice were fused with myeloma cells (SP2/0), selected in hypoxanthine-aminopterin-thymidine (HAT) medium, and screened by indirect Enzyme-Linked Immunosorbent Assay (ELISA). Secreted MAbs were characterized according to isotypes, reactions with the native *HER2* in SKBR3 cells by western blotting, and in tissue sections from *HER2* positive breast cancer specimens by Immunohistochemistry (IHC).

Results: Isotype of 1F1 clone was determined to be IgG1, which reacted with native protein in the western blot experiment and stained 20% of the membrane of neoplastic cells overexpressing *HER2* with 3+ grade. However, 3L5 clone showed a low reaction (10%) with native *HER2* in immunohistochemistry.

Conclusions: The results of both western blotting and Immunohistochemistry showed that native *HER2* can be detected with 1F1 monoclonal antibody.

Keywords: Monoclonal antibody; Immunohistochemistry; Western blotting; *HER2*

1. Background

Human epidermal growth factor receptor 2 (*HER2*) known as ErbB2 was identified in the early 1980s. This receptor belongs to the human epidermal receptor (HER) family of tyrosine kinase receptors consisting of four members, *HER1* through *HER4* (1, 2). Similar to other members of the HER family, *HER2* (185 kDa) glycoprotein has an extracellular ligand-binding domain, a single hydrophobic transmembrane domain and a cytoplasmic domain with tyrosine kinase activity. The extracellular domain (ECD) of *HER2* with 632 amino acids is composed of four subdomains (I - IV), where subdomains I and III form a ligand-binding site, and cysteine-rich subdomains II and IV play an important role in dimerization of the receptor (3, 4). Overexpression of *HER2* has been shown in 20% - 30% of patients with

breast cancer (5), 20% of gastric cancers (6), and has also been associated with poor prognosis and progression of other multiple neoplastic conditions (7). Besides overexpression, the dimerization arm of *HER2* is always available for dimerization with other members of HER family receptors; *HER2* is probably the preferred dimerization partner for other receptors of the HER family (8). Heterodimerization of *HER2* induces tyrosine kinase activity of the receptor and finally activates major signaling pathways including mitogen-activated protein kinase (MAPK), phosphoinositide phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K), which are all involved in cellular oncogenic processes such as proliferation, survival, motility and angiogenesis (2, 9). In addition to the important role of *HER2* in develop-

ment of breast cancer, it is considered as a diagnostic and therapeutic target for breast cancer. Monoclonal antibodies directed against HER2 extracellular domain inhibit its dimerization with other HER family receptors. Fendly and colleagues produced the 4D5 monoclonal antibody against HER2 extracellular domain (10) that was later developed to trastuzumab; a humanized monoclonal antibody, approved by the food and drug administration (FDA) in 1998, for the treatment of patients with metastatic HER2 overexpressing breast cancer (11, 12). Pertuzumab (2C4), the second humanized monoclonal antibody against different epitopes of HER2 ECD (13), in combination with trastuzumab and docetaxel, was approved by the FDA for the neoadjuvant treatment of HER2-positive patients (14, 15).

2. Objectives

In this study, we reported anti-HER2 monoclonal antibodies that specifically recognize the dimerization arm of HER2 subdomain II.

3. Materials and Methods

3.1. Immunization of Mice and Monoclonal Antibodies (MAbs) Production

The mice were immunized with a 30-amino acid (266 - 296) peptide from subdomain II of HER2 coupled with bovine serum albumin (BSA) as a carrier protein. Synthesis and high performance liquid chromatography (HPLC) analysis of HER2 peptide was carried out by China Peptide Co. Six to eight-week-old male BALB/c mice were immunized with intra-peritoneal injections of 0.2 mL phosphate-buffered saline (PBS) containing 30 µg of BSA conjugated-HER2 peptide emulsified with an equal volume of Freund's Complete Adjuvant in the first injection; the next five infusions were performed by half concentrations of the same peptide in combination with Freund's Incomplete Adjuvant every two weeks. Mice were screened for anti-HER2 serum titer by indirect enzyme-linked immunosorbent assay (ELISA) using the HER2 peptide. Splenocytes from hyperimmunized mice were fused at a ratio of 4:1 with SP2/O-Ag14 myeloma cells in the presence of polyethylene glycol (PEG) 1500 (Sigma-Aldrich) (16). Established hybridomas were cultivated in HAT media (Sigma-Aldrich) supplement with serum, 1 µg/mL Plasmocin™ Treatment (Sigma-Aldrich) and 100 mg/mL penicillin/streptomycin (Gibco Invitrogen). After two weeks of fusion, to screen the hybridoma cells, supernatants were tested using indirect ELISA with the HER2 (266 - 296) peptide. The hybridomas with high reactivity to HER2 (266 - 296) peptide were sub-cloned twice by the limiting dilution technique. Clones with higher optical density (OD) were assessed using cell ELISA by HER2/*neu*-overexpressing human breast cancer SKBR3 cell line (ATCC), and isotypes of positive clone were determined

by IsoStrip mouse monoclonal antibody isotyping kit (Roche, Germany), according to the manufacturer's instructions.

3.2. Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

For screening of clones and serum titer of mice we developed an indirect ELISA. Ninety-six-well plates were coated with 3 µg/mL HER2 peptide in bicarbonate buffer (50 mM, pH 9.6) and left overnight at 4°C; the remaining protein-binding sites were blocked by adding 120 µL of 3% skimmed milk in PBS for one hour at 37°C. Furthermore, 50 µL of supernatant of clones or serum sample dilutions was incubated for 90 minutes on a shaker at room temperature. After three washes, plates were incubated with 50 µL of 1:4000 dilution of anti-mouse IgG antibody produced in rabbits (sigma Aldrich) containing 0.1% BSA for one hour on a shaker at room temperature. After four washes, plates were developed by adding and incubating 50 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate for 10 - 15 minutes. The Optical Density (OD) was read at 450 nm by the ELISA reader after stopping the reaction using 2 M HCl. In this study, normal mouse serum was used as the negative control.

3.3. Cell Enzyme-Linked Immunosorbent Assay

The HER2 overexpressing human breast cancer cell line (SKBR3) was cultured in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich) containing 10% Fetal Bovine Serum (FBS) and 100 mg/mL penicillin/streptomycin. The cells were seeded at a density of 1.5×10^4 cells per well on 96-well plates and incubated for 24 hours at 37°C and 5% CO₂ to allow adherence. After reaching 70% confluence, the culture medium of cells was changed and immediately fixed by 3.7% paraformaldehyde in PBS for 20 minutes at room temperature. Non-binding sites were blocked by incubating in blocking buffer for 1.5 hours with moderate shaking, followed by five washes with PBS. Next, the cells were incubated with supernatant of clones overnight at 4°C. The 1:4000 dilution of rabbit anti-mouse IgG antibody containing 0.1% BSA was added and the solution was shaken for one hour at room temperature. Finally the reactions were developed by adding the 3, 3', 5, 5'-Tetramethylbenzidine (TMB) as a substrate for 10 - 15 minutes, and the Optical Density (OD) was measured at 450 nm by the ELISA Reader.

3.4. Western Blotting

To determine the reactivity of monoclonal antibodies with native HER2, we designed a western blot method using SKBR3, the HER2-overexpressing cell line. Cell lysate of SKBR3 cell line was prepared by addition of 1 mL/10⁷ cell lysis buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris (pH 7.4), 0.1% SDS 1%, Triton X-100) containing

20 mg/mL protease inhibitor phenyl methyl sulfonyl fluoride (PMSF) (Sigma-Aldrich) and incubation on ice for one hour and centrifugation at 10000 grams for ten minutes. The concentration of the cell lysate was determined with the Bradford method (17). Furthermore, 40 µg of the cell lysis proteins and HER2 (266 - 296) peptide were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in non-reduced status and transferred to a nitro-cellulose membrane. The membrane was incubated with non-diluted supernatant of hybridoma clones overnight on a shaker at room temperature, followed by blocking of nonbinding sites. After four washes with washing buffer using tris-buffered saline and tween 20 (TBST), the 1:8000 diluted secondary antibody (Anti mouse IgG antibody produced in rabbit, Sigma-Aldrich) containing 1% BSA was added and the solution was incubated for two hours on a shaker at 37°C. After the final washing step, the detection of specific signal bands was performed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) substrate (Sigma-Aldrich).

3.5. Immunohistochemistry

Sections (4 µm thick) of formalin fixed paraffin-embedded tissue from HER2 positive (3 + / 3 +) invasive breast carcinoma cases were prepared and mounted on silanized glass slides. The slides were fixed at 50 - 60°C for 10 minutes and left overnight at room temperature. During the following day, the sections were deparaffinized using xylene and rehydrated with incubation in descending series of ethanol for two to three minutes in each concentration. Next, the antigen retrieval of the samples was performed by adding Tris-Ethylenediaminetetraacetic acid (EDTA) buffer and incubation in a hot bath at 92°C for 30 minutes. After washing with distilled water, the endogenous peroxidase activity was blocked using 3% H₂O₂ in ethanol for 10 minutes. After two washes with Tris buffer, each five minutes in duration, the sections were incubated with undiluted supernatant of clones, 1:200 dilution of polyclonal rabbit anti-human HER2 (clone A0485, Dako, Glostrup, Denmark) as the positive control, and 1:50 dilution of normal mouse serum as the negative control, for one hour at room temperature. The sections were then incubated with the 1:4000 dilution of the secondary antibody (anti mouse IgG antibody produced in rabbit) containing 1% BSA for 45 minutes at room temperature after removing unbound antibodies by washing buffer (50 mM Tris-base). The sections were visualized by DAB solution and hematoxylin, dehydrated in ascending concentrations of alcohol and mounted for optical analysis by a pathologist.

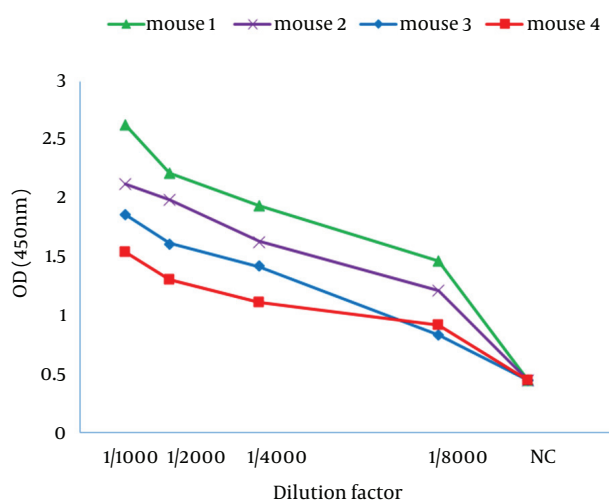
4. Results

4.1. Production of Monoclonal Antibodies

Three days after the last injection, serum antibody ti-

ters of immunized mice were measured by indirect ELISA, using the HER2 (266 - 296) peptide (Figure 1). More than 400 mouse hybridoma were screened in four separate fusions of spleen cells from hyperimmunized mice with SP2/0 by indirect ELISA; three stable hybridoma clones after the first screening, based on reactivity with HER2 (266 - 296) peptide, were selected and subcloned using limiting dilution. To determine the specificity of clones (1F1, 3L5 and 3G7) the reactivity against native HER2, on membrane of SKBR3 cell line, was evaluated by cell ELISA. As results of indirect and cell ELISA indicated, the HER2-peptide and SKBR3 cell line were strongly recognized by the 1F1. The 1F1 clone produced IgG1 isotype with kappa light chain.

Figure 1. Titration of Antibody in Serum of Immunized Mice With Bovine Serum Albumin-Conjugated HER2 (266 - 296) Peptide



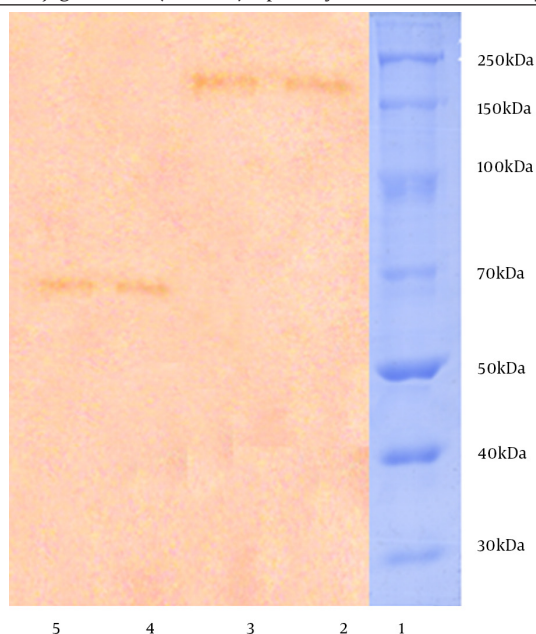
Serum samples were collected after four intraperitoneal injections, and different dilutions were evaluated for presence of anti-HER2 (266 - 296) peptide using indirect ELISA. (Negative Control: NC).

4.2. Specificity for HER2 Receptor

4.2.1. Western Blot Analysis

To confirm that MAb producing clones recognize the native HER2, western blot test was performed with extracted proteins from human SKBR3 breast cancer cell line. Western blot analysis showed that 1F1 monoclonal antibody reacts strongly with 185 kDa HER2 protein from SKBR3 cell line and BSA conjugated HER2 (266 - 296) peptide with molecular weight of around 70 kDa (Figure 2); whereas, 3L5 and 3G7 did not or weakly bounded to native HER2 (not shown).

Figure 2. Western Blotting of SKBR3 Cell Lysate and Bovine Serum Albumin-Conjugated HER2 (266 - 296) Peptide by 1F1 Monoclonal Antibody



Cell lysate of SKBR3 and HER2 peptide after separation on 10% SDS-PAGE gel electrophoresis, were blotted to nitrocellulose membrane and reactivity of 1F1 MAb to native HER2 and HER2 (266 - 296) peptide was evaluated. Lane 1, protein marker (10 - 250 kDa); lane 2 and 3, cell lysate of SKBR3; lane 4 and 5, BSA-conjugated HER2 (266 - 296) peptide.

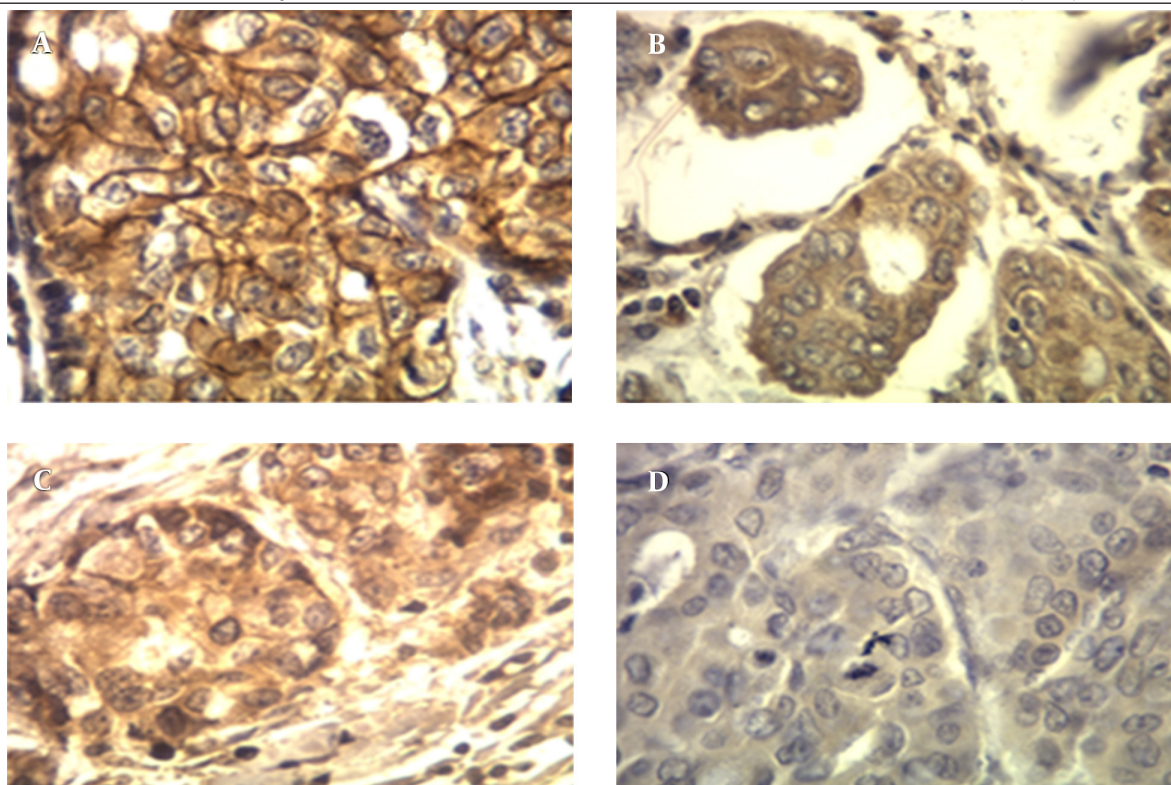
4.2.2. Immunohistochemistry Analysis

We further investigated the specificity of MAbs with an immunohistochemistry test; staining of neoplastic tissues from human invasive breast cancer with HER2 (+ 3 / + 3) by supernatant of clones 1F1, 3L5 and A0485 (as a positive control). The resulting microscopic images showed that 1F1 and 3L5 MAbs stained 20% and 10% of the membranes of neoplastic tissue with HER2 overexpression, respectively (Figure 3). None of the three MAbs stained the membrane of non-HER2 overexpressing tissue (0 / + 3).

5. Discussion

Breast cancer is the most common cancer among women. There are many tumor markers used for diagnosis of breast cancer; HER2 is one of the most important tumor markers, which is overexpressed in 20% - 30% of the patients with invasive breast cancers (5). Anti-HER2 MAbs is used as a diagnostic agent for measuring the HER2 status of breast cancer specimens using several methods especially IHC, which is the standard method considering that is both easy to perform and time efficient (18, 19). As regards to overexpression and preferred partner in dimerization with other HER family receptors, the HER2 extracellular domain, especially dimerization arm is considered as a major therapeutic target for specific immunotherapy in patients with HER2 overexpressing breast cancers (20, 21).

Figure 3. Immunohistochemical Analysis of 1F1 and 3L5 Monoclonal Antibodies on a Tissue Section From Human Breast Cancer (400 ×)



A, Positive control (1:200 rabbit polyclonal anti-human HER2, clone A0485, DAKO®). B, Supernatant of 1F1 clone anti-HER2; C, Supernatant of 3L5 clone anti-HER2; D, Negative control (normal mouse serum 1:50).

Several studies have shown that two-thirds of patients with HER2 overexpressing invasive carcinoma breast cancer are resistant to trastuzumab (22), the humanized 4D5 monoclonal antibody (11). Also in the presence of HER ligands (paracrine or autocrine), trastuzumab cannot inhibit the formation of HER2-containing heterodimers or the activation of mitogenic signaling pathways (23, 24). However, pertuzumab, the 2C4 humanized monoclonal antibody against subdomain II near the dimerization arm (13), can block the formation of ligand-induced HER2-containing heterodimers (24) and prevent the growth and HER2 downstream signaling pathways, even in human breast cancer cell line or tumors that express low levels of HER2 (25).

In this study we generated three hybridomas secreting MAbs using a 30-amino acid peptide from subdomain II of HER2 extracellular region (a part of the dimerization arm), and evaluated their characterization by indirect ELISA, cell ELISA, western blot and immunohistochemistry. The 1F1 clone secreting IgG isotype monoclonal antibody was determined to be the most capable among produced antibodies in detecting native HER2 in SKBR3 cell line by cell ELISA. Also, western blot analysis showed that the 1F1 MAb was capable of binding to 185 kDa HER2 and BSA-conjugated HER2 (266 - 296) peptide bonds.

As described above, the immunohistochemistry method is used as a routine method for detection of the HER2 expression level. Thus, the identification of native HER2 by 1F1 and 3L5 was evaluated with the immunohistochemistry technique, which showed 20% and 10% staining in neoplastic tissue, respectively. However, the poor results of the IHC test could probably be due to the low accessibility of the dimerization arm for detection by MAbs. This might be because HER2 is the preferred partner to dimerize with other receptors of HER family.

In conclusion, 1F1 monoclonal antibody had an adequate reactivity with native HER2 and could be useful for detection of HER2 in research techniques such as IHC and western blot.

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