

# Development of Monoclonal antibody against Human Epidermal Growth Factor Receptor 2 a marker of belligerent Breast Cancer

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## Abstract

Human Epidermal Growth Factor Receptor 2 (HER-2) proteins are receptors on breast cells which help to control how a healthy breast cell grows, divides, and repairs itself. In breast cancers, the HER2 gene doesn't work correctly and makes too many copies of itself and signals breast cells to make too many HER2 receptors. Over-expression of this oncogene plays an important role in the development and progression of certain aggressive types of breast cancer by making breast cells to grow and divide in an uncontrolled way. HER2 has become an important biomarker and target of therapy for breast cancer patients. Its epitope is localized in the extracellular domain and consists of an extracellular ligand-binding domain that is connected to a large intracellular domain by a single transmembrane sequence. In this study we have developed a monoclonal antibody against HER-2 and validated it by ELISA, Western Blotting and Immunohistochemistry. It recognizes a protein of 185kDa, which is identified as c-erbB-2/HER-2/neu. C-erbB-2/HER-2 is a member of the EGFR family. Receptors of this family are. c-erbB-2/HER-2 protein is over-expressed in a variety of carcinomas especially those of breast and ovary. The developed monoclonal antibody was used to observe the expression level of HER2 in human breast cancer tissues by Immunohistochemistry. The antibody staining was seen on the membranes of the breast cancer cells which is specific and shows minimal cross-reaction with other members of the EGFR-family. This antibody can be used in the diagnosis of HER-2 positive breast cancers.

Key words: Human Epidermal Growth Factor Receptor 2 (HER-2); Breast cancer marker; Immunohistochemistry.

## 1.Introduction

Despite the major advances in prevention, diagnosis and treatment cancer is still the common cause of death in the world. Among all types of cancer seen in human, breast cancer is one of the most common invasive cancer in women followed by cervical cancer and the highest leading cause of death in women due to cancer [1,2]. Breast Cancer comprises a heterogeneous genomic disease in terms of molecular markers, prognosis and treatment [3]. Female mammary gland growth, development and functions are generally regulated by various hormones and growth factors. Increase level of these factors such as estrogen, progesterone

hormones and growth factors such as epidermal, fibroblasts and insulin like growth factor induces proliferation and differentiation of mammary stem cells, which leads to cancer development [4].

The most prominent research in cancer biology is to find out the cancer specific antigen on the surface of cancer cells, which is serve as target site for antibody therapy. Among all the biomarker which are specific for certain types of cancer, Human epidermal growth factor receptor 2 (HER2) is an important biomarker which is more prominent for breast cancer development and can be seen in 20-25% of breast cancer patients all over the world [5,10]. Human Epidermal Growth Factor Receptor 2 (HER2) belongs to Epidermal Growth Factor Receptor (EGFR) family having tyrosine kinase activity, consists of four members HER1, HER2, HER3 and HER4. HER2 is a transmembrane protein encoded by HER2 gene located on the long arm of chromosome 17. It comprises a cystine rich extracellular ligand binding site, a transmembrane lipophilic segment and intracellular domain with tyrosine kinase catalytic activity [6,7]. HER2 undergoes heterodimerization, therefore the binding of ligands to the external domain of HER2 results activation of major signaling pathways. It causes the autophosphorylation of tyrosine residues in it's intracellular domain and further phosphorylation of downstream intracellular substrates which is associated with cell proliferation, apoptosis, angiogenesis and metastasis [7]. Overexpression of HER2 play an important role in the development and progression of certain aggressive types of cancer such as breast cancer, ovarian cancer, prostate cancer, bladder cancer by making cells to grow and divide in an uncontrolled way. This high level expression of this receptor result in resistance to chemotherapy, radiotherapy and hormonal therapy [8].

As HER2 is a cell membrane receptor it can be easily accessible due to their cell surface location, therefore the anti-HER2 monoclonal antibodies exert their inhibitory effects on cellular signaling or by targeting biological effectors to the target cells. The antitumor activities of this monoclonal antibodies which is produced against HER2 is mediated by several mechanism which includes downstream regulation of HER2 from the cell surface, promotion of apoptosis, inhibition of angiogenesis and immune destruction by the activity of antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) [9].

The prim aim of this present study was to produce monoclonal antibody by using hybridoma technology against human epidermal growth factor receptor 2 (HER2), which is a marker for breast cancer development and validated it by ELISA, Western Blotting and Immunohistochemistry. The developed monoclonal antibody was used to observe the expression level of HER2 in human breast cancer tissues which is specific and shows minimal cross reaction with other members of the EGFR family. This antibody can be used in the diagnosis and treatment of HER2 positive breast cancers.

## 2. Materials and Methods

### 2.1. Immunization of Mice

Six healthy female BALB/C white mice obtained from the animal facility care of Imgenex India Pvt. Ltd were immunized with partial length recombinant protein of HER2 to raise the antiserum level 50µg of immunogen per mouse and mixed with PBS (Phosphate buffer saline) and emulsified with Complete Freund's Adjuvant (CFA) (Sigma) at 1:2 ratio to form emulsion which are injected to these animals through intraperitoneal. These mice were boosted subsequently on 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> day of immunization with 25µg of immunogen emulsified with Incomplete Freund's Adjuvants (IFA) (Sigma).

### 2.2. Bleeding and Serum Collection

After immunization the animals were bled individually on 42<sup>nd</sup> days by ocular vein puncture. The collected blood sample were centrifuged at 4500rpm for 15 min to collect individual serum separately. The collected sera sample were aliquoted and preserved at -20° C for future use.

### 2.3. Measurement of antibody titer by Indirect ELISA

To determine the antibody titer developed against HER2 protein after immunization indirect ELISA was performed. In this process in polystyrene micro titer plates (Nunc.Denmark) each well was coated with 100 ng of immunogen (recombinant protein) in 100µl of coating buffer and incubated at 4°C overnight. Next day wells were washed thrice with TBST (Tris Buffer Saline Tween 20) buffer and after that saturation of each well was done by blocking with 200µl of 5% skimmed milk (prepared in TBST) for 1hr at room temperature. Non adherent proteins were washed off by washing buffer. First immune sera was used as primary antibody whereas pre immune sera was used as control. Primary antibody was applied at a dilution of 1:5000 and incubated for 2 hr at room temperature. After incubation plate was washed thrice with TBST and then the plate was incubated with secondary antibody (HRP conjugated goat anti-mice IgG, Jackson, USA) at 1:5000 dilutions for 1hr at room temperature which is followed by similar washing procedure. Antigen-antibody reaction was visualized by adding TMB (3,3',5,5'-Tetramethylbenzidine) substrate with 100µl/well which is prepared by diluting with distilled water. Addition of TMB substrate to well produce a blue color solution which is followed by addition 50µl of 1N H2SO4 to stop substrate reaction. The absorbance was measured at 450 nm using Multi Scan ELISA Reader (Thermo, USA)

### 2.4. Fusion

After serum ELISA screening the splenocytes of the best mouse was isolated and crushed in 70 µm Nylon cell strainer. 50 µl of splenocytes along with RBC were taken in 45µl RBC lysis buffer for 5 min. to count only B cells. Splenocytes and myeloma cells were added in 4:1 ratio for fusion which is done by addition of

a supplement called PEG (Polyethylene glycol). All fused cells were incubated in 10% HAT IMDM media for 4-5 days in which only fused cells were survived. Then the screening for 96 plates was done and only high titer clones were selected for antibody validation process. After the confirmation of endogenous expression of antibody, hybrid clones were further sub-cloned and the best antibody secreting clones were finalized.

## 2.5. Cell culture and Cell lysates preparation

Frozen vials of MCF-7, Hela and HTC-116 cell lines were thawed and maintained with 10% DMEM media in CO<sub>2</sub> incubator for cell lysates preparation for western blotting. During the cell lysates preparation approximately 10 million cells were harvested and homogenized in addition with 200 µl of RIPA buffer, 2 µl of PMSF and 2 µl of protease inhibitor cocktail solution in which again 2x sample buffer was added in equal proportion. After that the homogenates was centrifuged at 7000rpm for 15 minutes and the supernatant was collected.

## 2.6. Western Blotting

Anti-HER2 antibodies were validated by Western blot method in which pre transferred PVDF membrane with cell lysates proteins of different clots were taken. The strips were soaked in methanol for 1 minute and then washed with TBST to remove extra methanol remaining on the blot. Blot were then incubated with primary antibody at 1:5000 dilutions (i.e. Mice anti-HER2, anti-sera) overnight at 4°C for maximum binding which is followed by washing thoroughly with TBST. Strips were again incubated with secondary antibody (HRP conjugated goat anti-mice IgG, Jackson, USA) at 1:5000 dilution for 1 hour and washed again with TBST for removal of unbound secondary antibodies. Then the strips were soaked in Super Signal West Pico Chemiluminescent Substrate (Pierce USA) from 3-4 minutes and then exposed to hyper film in a cassette. The specific bands were observed and aligned with known molecular weight markers.

## 2.7. Immunohistochemistry

Breast cancer tissues was stored in 4% paraformaldehyde for 24-48 hours after which it removed from fixatives and washed properly in cold 1XPBS (Phosphate buffer saline) for 1 minutes and then treated with upgraded series of ethanol (30, 50, 70, 90 and 100) followed by xylene and embedded in paraffin. The tissue block were sectioned into 4 µm thick in microtome (Leica) and the sections were coated on albumin coated slides. During the following day the slides were deparaffinized using xylene and tissues were rehydrated with down graded series of ethanol for 2-3 minutes in each conc.. Next the antigen retrieval of the sample was carried out by autoclave method in which slides were immersed in an autoclave tray containing 1mm Tris Sodium Citrate buffer having 0.05% Tween 20 and pH 6 and then the slides were boiled at high temperature for 5 minutes. After washing for 3 times in Tris buffered saline having 0.1% Triton X, the slides

then incubated in 3% hydrogen peroxide from methanol (10ml methanol and 300 µl hydrogen peroxide) for 30 min followed by washing in TBST. Appropriate dilution of antibody was prepared (15µl/ml) corresponding to its concentration (4.5mg/ml) in blocking solution. Individual sections was enriched using PAN pen and incubated with primary antibody in different concentrations with control in blocking solutions. Then the sections were incubated overnight at 4°C in humidity chamber box and next day sections was removed from the box and kept at room temperature. After that the slides were washed in 1X TBST for 3 times. Biotin conjugated secondary antibody was diluted in blocking solution in the ratio 1:2000, in which section were incubated in a humidity chamber for 30 min. after removing unbound antibodies by washing buffer. To prepare the ANC (Avidin-Biotin Complex) two 2.5 ml of 1XPNS, 2 drop of reagent A and 1 drop of reagent B (VECTASTAIN ABC KIT, Vector Laboratories, USA) was added to each section. The section were then incubated for 30 minutes at room temperature followed by washing in washed buffer 1XTBST. The Ag-Ab reaction was determined by color development with the addition of VECTOR NOVA RED substrate (VECTOR LABS, USA) to the section. When appropriate color was developed the slides were then counter stained with hematoxylin for 10-15 sec to intensify the nucleus after that it was then washed in running tap water for 2 mins. To clear extra color. Next sections were dehydrated in ascending conc. of alcohol and then mounted with DPX carefully and not allowing any air bubbles to enter. Sections were observed in microscope and the photographs were taken. Antigen exposure was done by boiling in EDTA buffer. HRP was applied to the section followed by DAB substrate. The sections were then counterstained with Hematoxylin and coverslip were mounted with DPX mounting media.

### **3. Results and Discussion**

#### **3.1. Result**

##### **3.1.1. ELISA**

The affinity and specificity of antibody developed against HER2 in the sera of all mice before and after immunization were detected by indirect-ELISA technique and the absorbance value indicative of titer were represented in the graph (Fig.1).

##### **3.1.2. Western Blotting Analysis**

The expression of HER2 was probed by Western blot analysis using Anti-HER2 as primary antibody. Upon immunoprobng of proteins obtained from cell lysates of SKBR3 (Breast Cancer) of stage specificity and subsequent electro blotting on to PVDF membrane, specific endogenous bands of 137kDa right size band was observed on SKBR3 (Breast Cancer) cell lysates (Fig. 2).

### 3.1.3. Immunohistochemistry Analysis

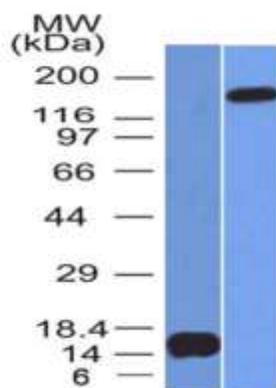
We further investigated to check the expression of HER2 on human breast cancer tissues by immunohistochemistry analysis. The resulting microscopic images showed that anti-HER2 antibody is binding to HER2 at membrane bound region (Fig. 3).

## 3.2. Discussion

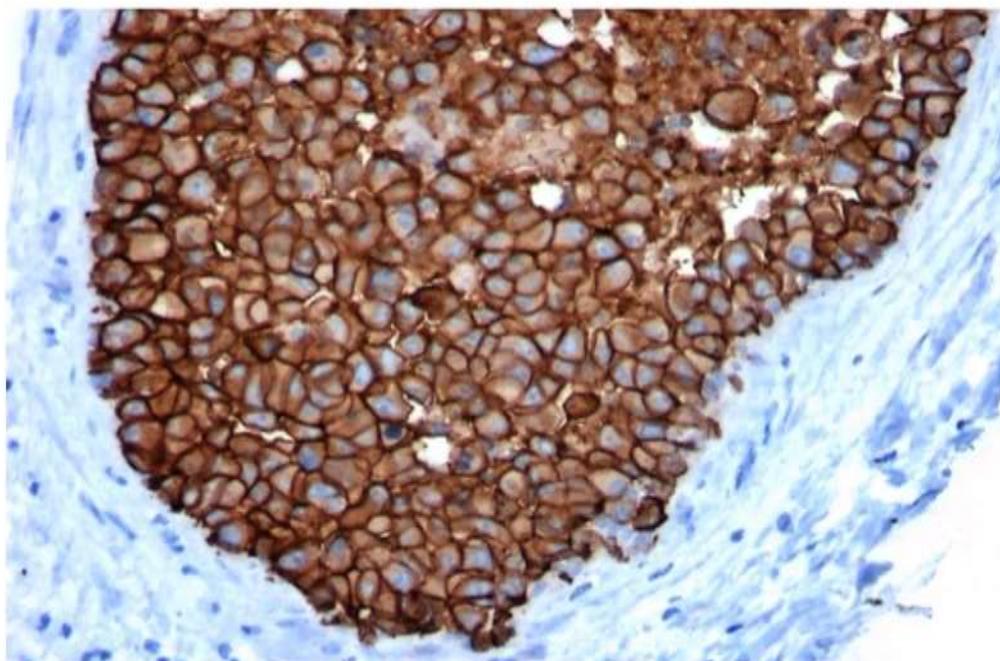
Breast Cancer is one of the most common cancer seen in women with highest global incidence. There are various tumor markers used for the diagnosis of breast cancer: among all HER2 is one of the most important tumor marker, which is overexpressed in 20-25% of patients with invasive breast cancer. This cancer mainly occurs due to overexpression of HE2 receptor protein or HER2 gene amplification. When this cancer occurs in human breast cells the amount of receptor increases its expression in cell membrane as it is a membrane bound receptor. So by detecting the expression of HER2 receptor we can diagnose this kind of cancer. Monoclonal antibody which is developed by using hybridoma technology against HER2 is used as a diagnostic agent, which is used to express the HER2 status on breast cancer specimens using several methods, especially Immunohistochemistry, which is a standard method because it requires very less time in comparison to other and easy to perform. By considering the overexpression and amplification of HER2 receptor due to its dimerization with other EGFR family members, the extracellular domain of HER2 is therefore considered as the major site for the therapeutic targets with overexpressed breast cancer.

In this study by using hybridoma technology the developed hybridoma secreting monoclonal antibody characteristics against HER2 is evaluated by indirect ELISA, Western Blotting and Immunohistochemistry. By conducting Indirect ELISA analysis during and after production of hybrid cells we must know the specificity of developed monoclonal antibody. Then the expression of HER2 protein was detected in cell lysates as we observed 137kDa band on SKBR3 (Breast Cancer) cell lines by using Western blot analysis which is further confirmed by Immunohistochemistry. Immunohistochemistry analysis shows expression of HER2 protein in the tissue sample of SKBR3 breast carcinoma at cell membrane region. As HER2 is a membrane bound protein the developed anti-HER2 monoclonal antibody is bind with HER2 protein at cell membrane region. Our study helps in finding of HER2 as a molecular marker for diagnostic purpose in case of belligerent breast cancer as well as different scientific study in the field of cancer biology.

## 4. Figures



**Figure 2:** Western Blot analysis of HER2: Lane1: HER2 antibody detected 16kDa right size band on partial length HER2 recombinant protein (immunogen) and Lane 2: detected 137kDa right size bands on SKBR3 (Breast Cancer) cell lysates.



**Figure 3:** Immunohistochemical staining of HER2 Mouse monoclonal antibody on Breast Cancer tissue: Formalin-fixed, paraffin-embedded human breast carcinoma stained with HER2 mouse monoclonal antibody. HER2 antibody shows specific membrane staining of the breast cancer cells.

## 5. References

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