

Generation of CHO Stable Cell Line Overexpressing *HER2*: an *In Vitro* Model for Breast Cancer

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Abstract

Background: Breast cancer is the most common female malignancy and the leading cause of cancer mortality in women worldwide. The human epidermal growth factor receptor2 (*HER2*) is a transmembrane tyrosine kinase receptor that is usually overexpressed in human breast cancers. Stable cell lines heterogeneously overexpressing *HER2* are highly required as *in vitro* models for breast cancer research. The aim of this study was to establish a stable cell line overexpressing *HER2*.

Materials and Methods: CHO Cells were transfected with linearized pCVN/*HER2* plasmid and selected for the recombinant cells with G418 antibiotic. Expression of *HER2* in the transfected cells was analyzed using western blotting and immunofluorescence.

Results: We found that the recombinant cells stably expressed high levels of *HER2* proteins that were mostly concentrated on the cell membrane.

Conclusions: The cell line established here provides a useful *in vitro* model for breast cancer research and any *HER*-related studies.

Keywords: Breast cancer, *HER2*; Stable cell line; Overexpression

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Introduction

HER2 is a proto-oncogene that encodes for a transmembrane tyrosine kinase growth receptor and is involved in several regulatory pathways in breast epithelium such as cell proliferation, survival, and motility. It plays a key role in breast cancer pathogenesis (1). *HER2* (*ErbB2*) amplification is observed in 20 to 50% of breast cancers (2). Women with *HER2*-overexpressing breast cancers have poor prognosis (3). It is the most common cancer in women with a life-time prevalence of about 10-12% (4). Breast cancer is the leading cause of cancer mortality in women worldwide. Approximately 1.38 million new breast cancer cases were diagnosed in 2008 (5). There are wide variations in breast cancer survival rate across the world: approximately 5-year survival of 80% in high-income countries and less than 40% in low-income countries (6). Immunotherapy remains a hot topic in cancer research, and several monoclonal antibodies have been developed to inhibit aberrant activation of the *HER* family members

(7). Generation of these therapeutic agents was originally carried out by first injecting immunogenic linear peptides into mice and later expanded by *in vitro* hybridoma production. However, the more recent methodology of subtractive phage display panning using live cells has allowed production of antibodies that are directed against conformational epitopes (conformation-specific antibodies) or ligand-induced epitopes (activation-specific antibodies), both of which are more desirable in research, diagnosis and treatment (8). This process, however, relies on heterogeneous expression of the target protein in an easy to manipulate cell. Therefore, this study aimed to generate stable cell expressing *HER2* receptor in CHO cell line. The established cell line represents a highly controlled tool for *HER2*-related studies and production of conformation specific antibodies to *HER2* as well.

Materials and methods

Plasmid preparation

HER2 expressing plasmid, pCVN/HER2 (kindly provided by A. Ullrich, Martinsried, Germany) (Figure 1), was eluted from filter paper by incubation in 100 μ L distilled water at 95 $^{\circ}$ C for 10 minutes. Two monograms of the eluted plasmid was used to be chemically transformed into competent *Escherichia coli* TOP10 (NCBI, Pasteur Institute, Tehran, Iran) using the heat-shock method (9). Transformed bacteria were then recovered by plating on LB agar (10g tryptone. 5g yeast extract. 10g NaCl. and 13g agar. All from Merck, Germany) containing ampicillin (100 μ g/mL) as a selective marker.

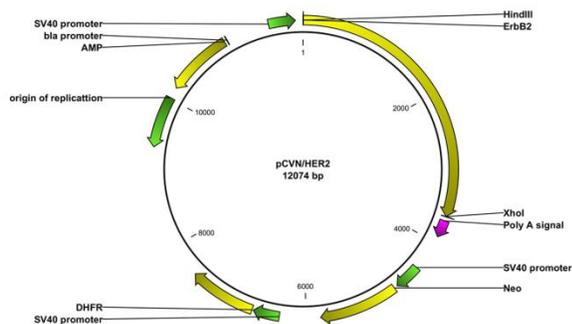


Figure 1. Map of pCVN/HER2 Plasmid.

pCVN/HER2 plasmid was extracted from the *E. coli* TOP10 using the AccuPrep Plasmid MiniPrep DNA Extraction Kit (Bioneer, Korea). The quantity and integrity of the extracted plasmid were checked by spectrophotometry (WPA, UK) and agarose gel electrophoresis, respectively. The extracted plasmid was then linearized using the *Pvu*I restriction enzyme (Fermentas, Vilnius, Lithuania) and separated on a 0.7% agarose gel. To obtain purer plasmids, linearized plasmids were excised from the gel and then purified using the QIAquick Gel Extraction Kit (Qiagen, Germany). The quantity of the purified plasmid was then measured by spectrophotometry (WPA).

Cell lines and cell culture

CHO and SKBR3 (HER2 expressing cell) cell lines were obtained from Pasteur Institute of Iran (Tehran, Iran). The cells were maintained in RPMI 1640 (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (PAA) and 100 IU/ml penicillin and 100 (μ g/ml) streptomycin (PAA). Cells were grown at 37 $^{\circ}$ C in a humidified incubator (Memmert GmbH & Co., Germany) with an atmosphere of 5% CO₂.

Transfection

To determine the minimum killing concentration of G418 (Santa Cruz Biotechnology) for the CHO cells,

40,000 cells were seeded in 24-well plate (SPL, South Korea) and incubated with a graded (increasing) concentration series of G418 (100, 200, 400, 600, 800 and 1000 μ g/ml) for 10 days. The medium was changed every 3 days with fresh antibiotic added to the medium at the appropriate concentration each time.

CHO cells were transfected with pCVN/HER2 plasmid using Turbofect reagent (Fermentas). Briefly, 40,000 cells were seeded in 24-well plates (PAA) and incubated overnight in the full media. The next day, 1 μ g of linearized pCVN/HER2 plasmid was incubated with 2 μ l of Turbofect and 100 μ l of the serum-free medium for 20 minutes. The mixtures were then added dropwise on cells and incubated for 48 hours before adding G418. Transfected cells were selected by 500 μ g/ml of G418 for three weeks. The cells were then transferred to 6-well plate (PAA) and subsequently to 25 cm² culture flasks.

Cell lysates and Western blotting

Expanded monoclonal cell lines were cultured to subconfluent stage of growth at maintenance concentration of G418 (200 μ g/ml). The cells were washed two times with ice-cold Phosphate-Buffered Saline (PBS) and scraped off from the flask by a cell scraper. Subconfluent cells were scraped off from the flask by a cell scraper. Total cell lysate was prepared from each cell line using radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology, Dallas, TX, USA) containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ l of protease inhibitor cocktail, and 1 mM sodium orthovanadate (Santa Cruz Biotechnology.). Protein content of each cell lysate was measured by the Bradford method (10). Equal amount of each total cell lysate from each clone was separated on an 8% SDS-PAGE under denaturing and reducing conditions in Mini-PROTEAN[®] Tetra Handcast Systems (Bio-Rad, Hercules, CA, USA) at 120 V for 90 minutes. The proteins were then transferred to PVDF membranes (Santa Cruz Biotechnology) in a tank transfer system (Bio-Rad) at 100 V for 60 minutes or at 14 V overnight in the presence of 0.1% SDS. Immunoblotting was performed to detect protein expression of HER2 in the total cell lysate (~30 μ g). All antibodies for immunoblotting were obtained from Santa Cruz Biotechnology: mouse anti-HER2 (clone 3B5) was used as primary antibody; goat anti-mouse IgG-HRP was the secondary antibody; and mouse anti- β -actin antibody (clone C4) was used as the loading control antibody. The immunoblots were developed using reagents from the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL, USA) for 5 minutes and imaged by the G: BOX instrument

(Syngene, England).

Immunofluorescence

Immunofluorescence was used to test HER2 expression on the cell membrane of the transfected CHO cells. Clones with the highest expression level of HER2, as determined by western blotting, were selected and cultured on a sterile coverslip in a 6-well plate (SPL Life Science). The cells were cultured in a medium with a maintenance concentration (200µg/ml) of G418. At 70% confluency, the cells were washed with PBS twice, fixed with 4% paraformaldehyde (Sigma, USA) for 10 minutes, and permeabilized with 0.1 % Triton X-100 (in PBS; Sigma, Germany) for 15 minutes. Subsequently, the cover slips were incubated by anti-HER2 for 2 hours, followed by incubating with a FITC-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, CA) for 1 hour. Finally, the cover slip was mounted using a solution containing antifade and DAPI (Denazist Asia, Iran). Microscopy (Eclipse 50i upright microscope; Nikon, Japan) was used to analyze expression of HER2 on the cells' membranes.

Results

The quantity and integrity of the extracted plasmid were confirmed by spectrophotometry and agarose gel electrophoresis, respectively.

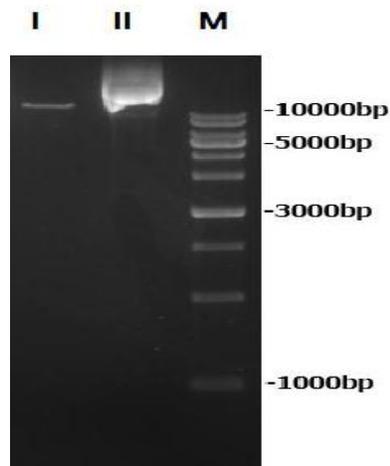


Figure 2. Digestion of pCVN/HER2 plasmid with *PvuI* enzyme (I- linear plasmid, II- native plasmid).

The quantity was 240ng/µl for pCVN/ HER2 and most of the plasmids were circular. Furthermore, the results of DNA sequencing showed a complete open reading frame (ORF) for HER2. The plasmid was then linearized with *PvuI* restriction enzyme and separated on an agarose gel (Figure 2)

The results of killing curve showed that CHO cells were killed in 500 µg/ml concentration of G418 in

less than 10 days. Therefore, we used 500 and 200 µg/ml of G418 for selection and maintenance of transfected cells, respectively.

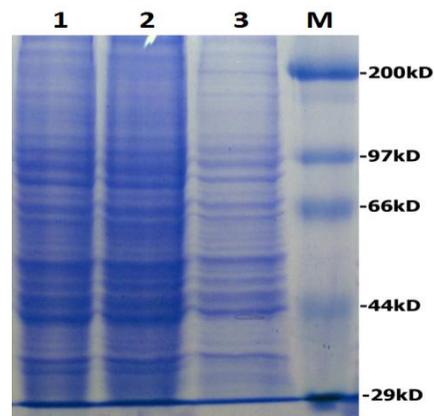


Figure 3. SDS-PAGE of CHO, CHO/HER2 and SKBR3 cells.

CHO cells were maintained in RPMI 1640 before transfection and grown to subconfluent stage. The transfected cells were selected with 500 µg/ml of G418 for 48 hours post-transfection until the single colonies were appeared. The cell lysates were separated on an SDS-PAGE. The results showed that all cell lysates were separated on SDS-PAGE with no evidence of protein lysis (Figure 3).

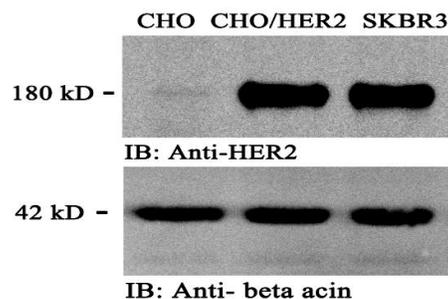


Figure 4. Immunoblotting analysis of HER2 expression in the transfected cells.

The transfected CHO/HER2 showed strong signals on western blots of the polyclonal cells (batch cultures). The SKBR3 positive control cells showed a high level of HER2 expression in the total cell lysate, as expected, since this cell line carries the *HER2/Neu* amplified loci. On the other hand, HER2 was not significantly expressed in the parental CHO cells (Figure 4). Membrane localization of HER2 in the transfected cells were investigated by immunofluorescence assay. The CHO/HER2 cells produced strong immunofluorescent signals that were concentrated on the surface of all cells (Figure 5).

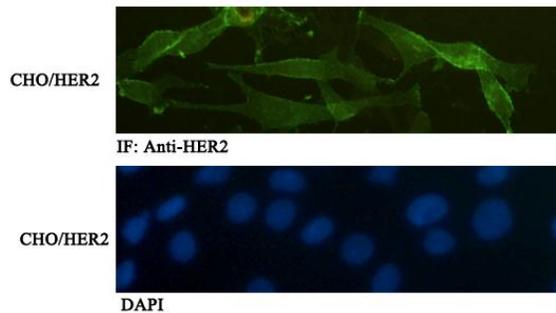


Figure 5. Immunofluorescence analysis of HER2 expression on the cell surface of CHO/HER2 cells.

Discussion

Stable cell lines have been extensively used in molecular and cellular studies. Regarding the application of a gene amplification system for industrial processes, one of the most important factors, is the selection method employed for obtaining highly productive recombinant cell lines that can stably produce the desired recombinant proteins (11). HER2 receptors play an important role in the development and progression of many types of human cancers (12) such as breast cancer, therefore, generation of a HER2-expressing cell line would be highly beneficial. In this study, we have successfully generated a stable cell line for future use for antibody screening, particularly in the cell-based approaches (8). Therapeutic proteins are predominantly manufactured using stable mammalian cell lines in large-scale bio reactors. The variability in protein production among individual cell lines is assumed to be resulted from differences in the transgene copy number and the site of transgene integration (13). HER2 is a transmembranes tyrosine kinase receptor overexpressed in a large percentage of human breast cancers (14). In summary, in this study we generated a stable cell line based on CHO cells that over-expressed HER2 and confirmed that by western blotting and immunofluorescence methods that can be used in the cell-based methodologies.

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Authors' contributions

H-OA and VR performed the experiments and wrote the draft. VR and RA designed the study and supervised study protocol. AA, TM, A-NR and RA analyzed data and reviewed the manuscript.

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Conflict of interest

No competing financial interests exist.

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