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Minimal HER1 and HER2 Expressions in CHO and HEK-293 Cells cause them Appropriate Negative Cells for HERs-Related Studies

Abasalt Hosseinzadeh Colagar [1], Omolbanin Amjadi [1], Reza Valadan [2], Alireza Rafiei [2]*

[1]Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran.
[2]Molecular and Cell Biology Research Center, Department of Immunology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.

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Corresponding Authors:
Alireza Rafiei
Molecular and Cell Biology Research Center, Department of Immunology, Faculty of Medicine, KM 17 Khazarabad Road, Khazar Sq, Sari, Iran.
Phone: +98-113543614
E-mail: Rafiei1710@gmail.com

Abstract

Background: Understanding of cellular and molecular mechanisms involved in cell/tumor growth and progression has led to molecular-targeted therapy. HER1 and HER2 are two main oncogenic components of HER-receptor family considered as targets for cancer research and therapy. Reliable results of HER-related research and targeted therapies dependent upon cell line with known HER expression status. But there are no published studies focusing on determination of HER1 and HER2 receptors expression in CHO and HEK-293 cell lines.

Materials and Methods: Absolute expressions of HER1 and HER2 in CHO, HEK-293, MDA-MB-468 and SKBR3 cells were evaluated using quantitative real-time PCR. Absolute expression quantification was carried out by serial dilution plasmids harboring HER1 and HER2 to extrapolate standard curve.

Results: Real-time PCR amplification was successfully optimized by HER1 and HER2 plasmids with high efficiency and their copy numbers were calculated. The results showed that HER1 was not expressed in CHO and HEK-293 cells but these cells express HER2 up to 9.4×10^2 and 1.1×10^5 copy/µg, respectively. MDA-MB-468 and SKBR3 cells express HER1 up to 8.4×10^5 and 9.7×10^4, respectively. In addition, the expression of HER2 is higher on the surface of SKBR3 cell (6.5×10^6 copy/µg).

Conclusion: Our study provides a proven insight to HER1 and HER2 expression status in CHO, HEK-293, MDA-MB-468 and SKBR3 cells. Heterologous expression of HER1 and HER2 in cell lines is the first step in structural and functional studies. Since HER1 is not expressed in CHO and HEK-293 endogenously, these cells are ideal for HER receptor related studies.

Keywords: Real-time PCR; HER1; HER2; Expression; Targeted-therapy; CHO and HEK-293

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Introduction

Human epidermal growth factor receptors (HER) play an important role in proliferation, migration, angiogenesis, and resistance to apoptosis in many cancers (1). HER family is a tyrosine kinase family including four homologous members: HER1, HER2, HER3 and HER4. They share common structure consist of: an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (2, 3). The activation of HER receptor depends on ligand binding, dimerization andphosphorylation of downstream adaptor/proteins. *HER1* proto-oncogene encodes a 170 kDa transmembrane glycoprotein related not only to breast carcinogenesis (4) but also in colorectal, gastric, glioblastoma, non- small cell lung, head and neck and ovarian cancers (5). Proto-oncogene *HER2/neu* encodes a 180 kDa tyrosine kinase receptor related to wide variety of human cancers (6, 7). Ligand binding promotes HER receptors homo and hetero dimerization and leads to receptors activation,

subsequently their signaling pathway, Ras/ Raf/ MEK/ ERK and PI3K/ Akt, are triggered. These downstream signaling pathways resulted in cell/tumor growth and metastasis (8). Inhibition of HER1 and HER2 by anti-HER agents can down regulate HER-overexpressing cancers (9). According to the potential of these receptors in pathogenesis of a large number of human cancers, these receptors were considered as promising targets for cancer treatment (10).

Cell lines are indefinite sources of homogenous cell population, manifesting their original properties. Human embryonic kidney cells (HEK)-293 is the most common cell line for rapid production and large scale transient expression of recombinant protein (11). Chinese hamster ovary cells (CHO) is a standard expression system with efficient post translational modification for recombinants therapeutic products (12).

Table 1. Oligonucleotide primer sequences used for RT-PCR amplification.

Name of Gene	Primer Name	Sequences 5' → 3'	Nucleotide position	PCR Product Length (bp)
*Her*1	*HER1F*	GCTTGCATTGATAGAAATGG	3290-3309	204
	HER1R	CAGAGGCTGATTGTGATA	3493-3476	
Her2	*HER2F*	GCTTTGTGGTCATCCAGAA	2883-2901	239
	HER2R	CTCCAGCCCTAGTGTCAG	3121-3104	

Therefore, these cell lines are proper model system for biopharmaceutical products targeting HER family in cancer study.

Reliable results of HER- targeted therapies dependent upon cell line with known HER expression status. But, there are no published studies focusing on determination of HER1 and HER2 receptors expression in CHO and HEK-293 cell lines. Immunobloting analysis done by Shi *et al* (13) implied no EGFR or HER1 expression, while others (14) mentioned low or undetectable expression level of ErbB receptor in CHO cells. In the same way, HER1 and HER2 expression in HEK-293 cells are controversial in different analysis such as Lanteri *et al* (15) and Xu *et al* (16). The present study investigated the precise endogenous expression levels of HER1 and HER2 receptors in CHO and HEK-293 cells.

Materials and Methods

Cell culture

CHO, HEK-293, SKBR3 (HER2 positive control), and MDA-MB-468 (HER1 positive control) cell lines were purchased from Pasteur Institute, Iran. The cells were grown in suspension culture at 37 °C under 5% humidified CO_2 in bioactive medium RPMI 1640 (PAA, Pasching/ Austria) containing inactivated fetal bovine serum (PAA, Pasching/ Austria), L-glutamine (300mg/l), penicillin (100 U/ mL), and streptomycin (100µg/ml). The experiments were carried out at cell densities of 3×10^6 cells per milliliter.

RNA isolation and cDNA synthesis

The total RNA was isolated from 80% confluent cultured cells by RNeasy Mini Kit (Qiagen, Germany) according to manufactures' instructions. Finally, RNA pellet dissolved in DEPC-treated water.

Concentration and quality of RNA was checked by the absorbance values at 260 nm, $A_{260/280}$ and $A_{260/230}$ ratio and confirmed by electrophoresis in 1% agarose gel. Total RNA (1µg) was subjected to single strand cDNA synthesis using a cDNA synthesis kit (Parstous, Mashhad/ Iran). Briefly, cDNA synthesis was carried out using 200 U MMLV RTase, 2mM dNTP mixture and 200 ng/µl random hexamer in a total volume 20 µl reaction containing: 10 µl 2X RT Premix, 2 µl random hexamer, 1 µg RNA template and 7 µl DEPC-treated water.

Quantitative real time PCR

*Her*1 or *Her*2 gene expression was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using DNA Mastermix SYBR Premix Ex Taq II (Takara, Japan) on an iCycler iQ5 real- time PCR machine (BioRAD, USA). The gene sequences of *Her*1 and *Her*2 were derived from the NCBI National Center for Biotechnology Information (NCBI) and primers were designed using Allele ID 7.5 software (Table1).

The final reaction volume for *Her*1 was 20 µl which contains: 10 µl Mastermix SYBR Green II (Takara, Japan), 333 pmol forward primer, 285 pmol reverse primer, 2 µl cDNA and 6.7 µl distilled water. *Her*2 amplification was carried out in 20 µl reaction including: 10 µl Mastermix SYBR Green II (Takara, Japan), 285 pmol forward primer, 333 pmol reverse primer, 2 µl cDNA and 6.7 µl distilled water. *Her*1 gene amplified in 40 cycles including; 95 °C for 3 min, 95 °C for 10 sec, 62.7 °C for 30 sec and 72 °C for 30 sec and a final extension for 5 min at 72 ˚C, followed by a standard melting curve analysis. Amplification of *Her*2 is similar to *Her*1 except annealing temperature that is 62 ˚C in 30 sec. Absolute

quantification was performed by comparing the threshold cycle values (C_T) for the samples, with standard curves generated with HER1 and HER2 plasmid DNA.

Plasmid preparation

To determine the absolute copy number of the receptors; HER1 and HER2 plasmids and Escherichia coli Top 10 competent cells were used. HER1 and HER2 plasmids were transformed to CaCl2 treated E.Coli Top 10 by heat shock method. Then transformed bacteria plated on LB agar containing 100 µg/ ml Ampicillin.

Positive colonies were picked and used for plasmid purification. Plasmids were extracted by AccuPrep Plasmid MiniPrep DNA Extraction Kit (Bioneer, South Korea). Purified plasmids were then linearized with Pvu I restriction enzyme (Fermentas, Germany) and run on a 1% agarose gel. The linear plasmid band was excised from the gel and the DNA extracted to obtain pure linear plasmid DNA by QIAquick Gel Extraction Kit (Qiagen, Germany). This DNA was then quantified by Nanodrop spectrophotometer (WPA, UK).

Absolute quantitative assay

To evaluate absolute quantitative gene expression, HER1 and HER2 plasmids (Sino biological, China) were used. Serially diluted standards of HER1 and HER2 plasmids with known concentration were used to produce a standard curve. The standard curve revealed a linear relationship between C_T and initial amount of total RNA or cDNA, so the concentrations were interpolated from standard curves on the basis of C_T values (17). Six 10-fold dilutions of HER1 plasmid and eight 10-fold dilutions of HER2 plasmid were prepared for plotting standard curve (Figure 1). The real-time PCR procedures included appropriate positive and negative controls.

Results

RNA quality of cell lines was evaluated on a 1% agarose gel electrophoresis. The concentration and purity of the RNA was described by the mean $A_{260/280}$ and $A_{260/230}$ ratio and were on average 1.83 and 1.89, respectively.

RNA isolation was performed from trypsinized cells and 1 µg RNA was reverse transcribed to cDNA. Gene copy numbers were calculated by absolute real-time PCR using serially diluted plasmids as standards. For quality control all RNA isolation and cDNA synthesis carried out two times.

The standard curves were analyzed for two properties, the coefficient of correlation (R^2) and amplification efficiency (E) derived from the slope of standard curve. The standard curves forHER1 ranging from 2.88×10^9 to 2.88×10^4 copies and HER2 ranging from 2.5×10^9 to 2.5×10^2 copies are shown in Figure 1. The copy numbers of plasmid DNA were calculated using the online calculator provided by the URI Genomics&Sequencing Center(18).The formula used is: (amount$\times6.022\times10^{23}$) / (length$\times$ 1×10^9 $\times650$), in which amount, is concentration of plasmid in nanogram/microliter, 6.022×10^{23} (molecule/mole) is Avogadro's number, length, is full length of plasmid in base pair, 650 (gram/mole) is the average molecular weight of a base pair and 1×10^9 is used for conversion gram to nanogram.

Copy numbers were calculated for 30 nanogram (ng) of HER1 and HER2 plasmid DNA. Both curves were linear in the range tested (R^2> 0.99) by the duplicate reactions. The slopes of the standard curves for HER1 and HER2 were -3.5 and -3.4, respectively. From the slopes, high amplification efficiencies of 0.91 and 0.96 were determined for HER1 and HER2 in the tested range.

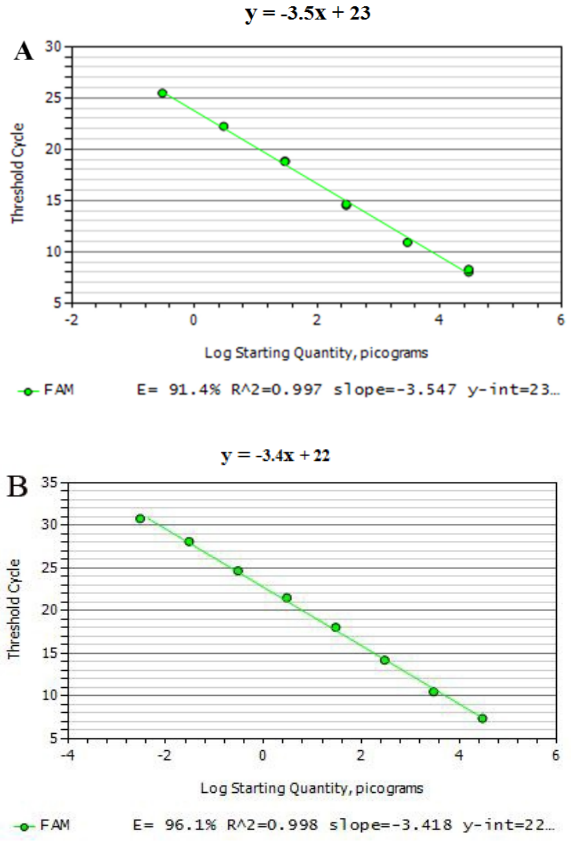

Figure 1. Standard curve for evaluating qPCR optimal assay: Standard curve was plotted with C_T value against the log of the starting quantity of standard template for each dilution. R^2 value and calculated amplification efficiency were shown below the graph. **A.** represent HER1 standard curve. **B.** represent HER2 standard curve.

Our qPCR assay is optimized accurately regarding to

R^2>0.99 showing linear standard curve and efficiency equal to 90-105 representing high amplification efficiency. Therefore, absolute copy numbers of HER1 and HER2 in each cell lines were calculated based on its C_T value with its plasmid DNA standard curve. The copy numbers of HER1 and HER2 receptors were shown in Table 2.

Discussion

Precise gene expression profiling by quantitative real-time PCR assay is used to determine HER1 and HER2 expression in CHO and HEK-293 cells. The results demonstrated no endogenous HER1 expression and low level of HER2 expression in these cells. Real-time qPCR provides a fast and reliable technology for gene expression quantification (19).

This sensitive method applies in two convenient ways: absolute and relative. Absolute qPCR assay allows detecting target gene copy numbers based on the standard curve. Plasmid DNA is often used as an external standard for generating standard curve.

As previous studies have confirmed that linear plasmids are more reliable for copy number estimation (20), *Pvu* I- linearized HER1 and HER2 plasmids were used to measure copy numbers. After ampicillin resistance screening and plasmid purification, these plasmids are brought to create standard curves. The copy numbers of samples were calculated following the qPCR optimizing. Our study showed no *Her*1 amplification in CHO and HEK-293 while HER2 levels were 9.4×10^2 copy/µg RNA of CHO and 1.13×10^5 copy/µg RNA of HEK-293, as shown in Table 2.

Table 2. HER1 and HER2 expression in the most common cell lines.

	HER 1			HER 2	
Sample name	**C_T**	**Copy number**	**Sample name**	**C_T**	**Copy number**
CHO	0	-	CHO	34.07	9.4075×10^2
HEK- 293	0	-	HEK- 293	26.82	1.13775×10^5
MDA-MB-468	28.73	8.4×10^5	MDA-MB-468	29.16	1.5×10^4
SKBR-3	36.43	9.7×10^4	SKBR-3	21.85	6.5622×10^6

*The copy number of each cell line was calculated in regard to its C_T and known copy number of HER1 and HER2 plasmids.

According to our results, CHO and HEK-293 don't express HER1 but they express low level of HER2.

Determining HER1 and HER2 expression profiling of CHO and HEK-293 is consequential for many researches. Different results have been reported by Tzahara *et al* (21), Krug *et al* (22), Hatakeyama *et al* (14) and others about HER1 and HER2 expression in CHO. The contradictory results of HER1 and HER2 expression have been reported in HEK-293 (Table 3). The main finding of this study is confirmation and extension previous knowledge about HER1 and HER2 expression by an accurate, sensitive, and fast method.

High levels expression of HER1 (8.4×10^5 copy/µg) in MDA-MB-468 and HER2 (6.5622×10^6 copy/µg) in SKBR3 in our study are concordance to previous studies which reported that MDA-MB-468 and SKBR3 highly expressed HER1 and HER2, respectively (23-28). While HER2 expression has not been detected in different studies (29, 30), our results showed that MDA-MB-468 expressed HER2 as much as 1.5×10^4 copy/µg. In addition, SKBR3 cells expressed HER1 up to 9.7×10^4 copy/µg. In other words, real time PCR is a highly sensitive and accurate method for determining the precise expression of HER1 and HER2 on the cell surface. HER1 and HER2 activation initiate potent signaling pathways resulted in many key cellular functions, including cell growth, division, migration, adhesion, angiogenesis, and apoptosis (31). These receptors are involved in the pathogenesis of variety of human cancers such as breast, ovarian, bladder, glioblastoma, gastric, lung, and pancreas (6, 7).

Since HER1 and HER2 expression profile is a crucial determinant in cancer pathogenesis and cancer related studies, these two main oncogenic components of HER-receptor family considered as targets for biological researches and cancer therapy. Heterologous expression studies are principle part of biological and biomedical researches for analyzing interaction between receptors and their mutations. These studies must be evaluated on cell lines as reliable system model for validation. In addition, many cancer-related studies such as targeted therapy and drug screening require to right control cell lines. Knowing the precise expression levels of these receptors on cell surface is a fundamental need in the development of any HER- related study.

In the present study, CHO and HEK-293 cells were selected as two most applicable cells used in biomedical and biological studies. Quantitative real-time PCR was performed in order to measure HER1 and HER2 expression levels in these cells. HER1 and

HER2 expression analysis in these cell lines revealed no copies of HER1 while low copy numbers of HER2 receptor were measured. This is the agreement with the finding of some previous studies used immune precipitation, blotting, and other methods and imply HER1 and HER2 expression pattern in CHO and HEK-293 cells.

Table 3. Mini review of previous studies about HER1 and HER2 expression in CHO, HEK-293, MDA-MB-468 and SKBR3 cell lines.

Cell lines	Receptors	Method(s) of Analysis	Results	Reference and Year
CHO	HER2	Affinity labeling and co-immunoprecipitation	Low expression (19)	Tzahara *et al.*, 1996
CHO	EGFR	Affinity labeling and co-immunoprecipitation	No expression (19)	Tzahara *et al.*, 1996
CHO	ErbB receptors	Western blot	Low expression (21)	Hatakeyama *et al.*, 2004
CHO	HER1	Western blot	No expression (22)	Krug *et al.*,2002
CHO	EGFR	Immunobotting	No expression (13)	Shi *et al*., 2000
CHO	HER1	-	No expression (32)	Kim et al., 2002
CHO	HER2	BiFC	Low expression (33)	Tao and Maruyama, 2008
HEK-293	HER1	BRET	Low expression (34)	Schiffer et al., 2007
HEK-293	HER1	-	Expression (35)	Schmidit et al., 2003
HEK-293	HER1	Immunoblotting	No expression (36)	Kancha et al., 2013
HEK-293	HER2	-	Low expression (37)	Vermeer et al., 2012
HEK-293	HER2	-	Not detected (38)	Kang et al., 2011
HEK-293	HER2	-	No expression (16)	Xu et al., 2004
HEK-293 and SKBR3	HER2	Northern blot	Low expression in HEK-293 and high expression in SKBR3 (15)	Lanteri *et al*., 2005
HEK-293	HER2	-	No expression (39)	Nam *et al*., 2013
MDA-MB-468 and SKBR3	EGFR and HER2	Western blot	High HER1 and HER2 expression in MDA-MB-468 and SKBR3, respectively (40)	Xu *et al*., 2005
MDA-MB-468 and SKBR3	HER1 and HER2	Western blot	High HER2 and HER1 in SKBR3 and MDA-MB-468 (24)	Lopez *et al*., 2008
MDA-MB-468 and SKBR3	HER1 and HER2	Western blot	High HER1 expression in MDA-MB-468 and HER1 and 2 expression in SKBR3 (30)	Belsches- Jablonski et al., 2001
MDA-MB-468 and SKBR3	HER1 and HER2	ELISA, CEER method and western blot	2.0×10^6 HER1 receptors/ cell in MDA-MB-468 and 2.5×10^6/ cell HER2 receptors in SKBR3 (28)	Kim *et al*., 2011
MDA-MB-468 and SKBR3	HER1 and HER2	FACS	1,402,832 HER2 and 143,599 HER1 receptors in SKBR3 and 3,389,807 HER1 and 1,209 HER2 receptors in MDA-MB-468 (41)	DeFazio- Eli et al., 2011
SKBR3	HER2	Immunohistochemistry, FISH, Laser- scan cytometry and quantitative real- time PCR	High expression (42)	Xiao *et al*., 2009
MDA-MB-468 and SKBR3	HER1 and HER2	Immunoblotting	High HER1 expression in MDA-MB-468 and High HER2 expression in SKBR3 (43)	Gunawardane *et al*., 2008
MDA-MB-468	HER1 and HER2	Two- color Raman image	High HER1 and low HER2 expression (44)	Liu et al., 2010
SKBR3	HER2	Immunohistochemistry	High expression (45)	Subik *et al*., 2010
MDA-MB-468 and SKBR3	HER2	Northern blot and western blot	High expression in SKBR3 but not in MDA-MB-468 (46)	Faltus *et al*., 2004

HER1 and HER2 are linked to development of many cancers; therefore, they are key targets in functional studies. Such researches need to powerful experimental tools to achieve accurate results. The popularity of cell lines is largely due to their top feathers, making them an ideal model for many biomedical researches especially cancer related-studies. The choice of cell line as an *in vitro* model is the first and important step in cancer research. This indicates the need for more attempts to consider molecular profiles of cell lines.

Taken to gather, this is the first study that quantitatively report HER1 and HER2 expression in CHO and HEK-293 cells. These results provide an important insight to consider HER1 and HER2 expression pattern even in low level for basic and applied studies. Our interest lies in describing right cell lines especially for heterologous expression studies. With respect to data derived from absolute real-time PCR, we suggest that CHO and HEK-293 are proper cell lines for HER1-related heterologous study while low endogenous expression level of HER2 made these cells suitable for HER receptor-related research.

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