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Effect of dasatinib and herceptin on Her-2 gene transfection in ER/PR positive breast cancer cells

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ABSTRACT

Major nuclear receptors, such as Estrogen (ER) and progesterone (PR) play a significant role in breast cancer biology. However, their role in developing chemoresistance in breast cancer has received less attention. The development of reliable gene expression arrays, real-time quantitative RT-PCR, and immunohistochemical techniques for studying ER/PR have revealed their potential role in developing chemoresistance. Advances in our understanding of both ER/PR functional networks and epidermal growth factor receptor (EGFR) signaling pathways have revealed a frequent interplay between ER/PR and EGFR in cell growth, which is clinically relevant to breast cancer. Understanding how EGFR and their downstream kinases are activated by ER/PR (and vice-versa) is a central goal for maximizing treatment opportunities in breast cancer. In order to understand the connection between nuclear receptor ER/PR function and EGFR-2 (Her-2) in chemoresistance/ chemosensitivity found in breast cancer biology, this study has modified the chemosensitivity of MCF-7 cells by transfecting MCF-7 cells with Her-2 gene in vitro. It showed that cancer cells were differentially sensitive to anticancer therapy (Herceptin with Dasatinib), compared to the untransfected cells. The wound healing assay that measures the invasion potential of cancer cells showed that transfection of Her-2 into MCF-7 cell appreciably increased its invasion property. This result confirms that Her-2 expression in breast cancer cells drives the metastasis of cancer cells outside its primary site.

Keywords: Breast Cancer, Dasatinib, Estrogen (ER), Her-2, Herceptin, Progesterone (PR), Reverse Transcriptase-Polymerase Chain Reaction.

INTRODUCTION

Breast cancer (BC) is the most common cancer in women worldwide, encompassing 16% of all female cancers [1, 2]. It is estimated that BC accounts for an increased number of death in women and thus exists as a persistent health burden. It also varies depending on histopathological features, molecular alterations and clinical symptoms. In India, currently the annual incidence rate of BC that has reached to 23/100,000 women implicates the alarming need for medical concerns. As a consequence, in the management of BC, numerous changes have undergone over the past two decades with the hormone receptor as the main target for chemotherapy[3, 4].

The hormones that play a vital role in the development of normal breast and breast tumor are estrogen and progesterone. Their action is based upon binding to estrogen receptor (ER)[5] and progesterone receptor (PR) [6]. BCs that are initiated inherently from ER and PR positive luminal cells are referred as ER and PR positive carcinomas, respectively [7]. While the human epidermal growth factor receptor-2 (HER-2/neu)[8], a transmembrane cell surface glycoprotein, that originates from normal non-neoplastic epithelia and breast duct epithelium are expressed at low levels.

Conversely, in primary BC, over expression of HER-2/neu is usually evident [9]. In India, an increased mortality rate as compared to the developed countries might be due to the fact that the HER-2 is not included as a marker. As a result, the main focus of our study is to evaluate the effect of HER-2 gene transfection in ER/PR positive BC cells to establish their role in the management of BC with the targeted chemotherapy [10].

MATERIALS AND METHODS

Cell lines:
Established cell lines representing Her 2 negative, non-metastatic breast cancer (MCF-7, ER/PR Positive) obtained from the American Type Culture Collection (Manassas, VA) and maintained as per ATCC guidelines will be used.

Reagents and Antibodies
FuGENE 6 transfection reagent was obtained from Roche Applied Science (Indianapolis, IN), pTet-On and ptTS plasmids from Clontech (Palo Alto, CA), dual-luciferase reporter assay system from Promega (Madison, CA), Herceptin and Dasatinib were gifts from Dr. Khaitan's lab associates in USA.

Transfection of HER-2 in the MCF-7 cell line
Stable transfection and selection were used to produce HER-2 expressing, MCF-7 cells. MCF-7 cells were transfected pcDNA 3.1 vector carrying HER2 gene. After hygromycin selection, clones were tested for HER2 expression by RT PCR.

Drugs tested in Untransfected and HER-2 transfected MCF-7 breast cancer cells:
Dasatinib: It is a protein tyrosine kinase inhibitor (TKI). Tyrosine kinases are proteins that act as chemical messengers to stimulate cancer cells to grow. Dasatinib blocks and interferes with number of protein kinases and is called a multi kinases inhibitor.

Herceptin: (Trastuzumab: monoclonal antibody to HER-2), control the growth of cancer cells that produce too much of a protein called HER 2 (human epidermal growth factor receptor 2).

RNA extraction and PCR
To shed light onto the mechanism responsible for cell death we extracted the RNA from MCF-7 cells after treatment for 24h. Total RNA was extracted from cells which were 75% confluent with TRIzol. The concentration of RNA was determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA). cDNA for RT-PCR was generated by the SuperScript™ First-Strand Synthesis System according to manufacturer's instructions (Invitrogen). PCR was carried out in a total volume of 20µl, containing 0.2mM dNTPs, 1mM MgCl2 and 1 Unit of AmpliTaq Gold DNA Polymerase (Applied Biosystem). The PCR for β-actin cDNA were performed with 30 amplification cycles and the reaction conditions were: denaturation at 94°C for 1 min, annealing at 53°C for 2 min and extension at 72°C for 3 min. Following amplification, 20µl of the samples were separated via electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The PCR primers used were: HER2 Sense primer: 5′-ATATCCAGGAGGTGCAGGG-3′, Anti-sense primer: 5′-CTTCGAAGCTGCAGCTCCC-3′ and β-actin mRNA (243 bp). Sense primer: 5′-CTTCTACAATGAGCTGCGTG-3′, Anti-sense primer: 5′-TCATGAGGTAGTCAGTCAGG-3′.

In vitro wound healing assay
Untransfected and transfected cells were seeded on covers lips until 90% to 95% confluent. Cell monolayer's were then gently scratched with a pipette tip across the diameter of the dish and rinsed with PBS and cell media to remove cellular debris. The surface area of the scratched surface was quantified

after wounding and again after 24 hours on Zeiss microscope. The extent of wound closure was calculated using the ratio of the surface area between the remaining wound edges to the surface area of the initial wound for each time point. These data were then expressed as a percentage of wound closure relative to control conditions for each experiment.

MTT assay for cell viability and growth

Human breast cancer cells (MCF-7) were cultured as monolayer's in complete RPMI 1640 medium. The cells were cultured for 48 hours to allow growth and achieve about 80% confluence in 48-well culture plates, and then exposed to the agents for 24 hours in single and combination treatments (Herceptin and Dasatinib). Post-treatment cell death induction was assessed by the use of MTT.

RESULTS

Expression of HER-2 in MCF7 cell line

HER-2 cDNA was cloned into pcDNA3.1 vector as described above, to produce the plasmid p-HER2. The MCF7 cell line was transfected with p-HER2, and clones were isolated by selection with hygromycin B and screened for expression of HER-2. RNA was extracted from the MCF7 cell and evaluated for ErbB2 expression by RT-PCR (Figure 1).

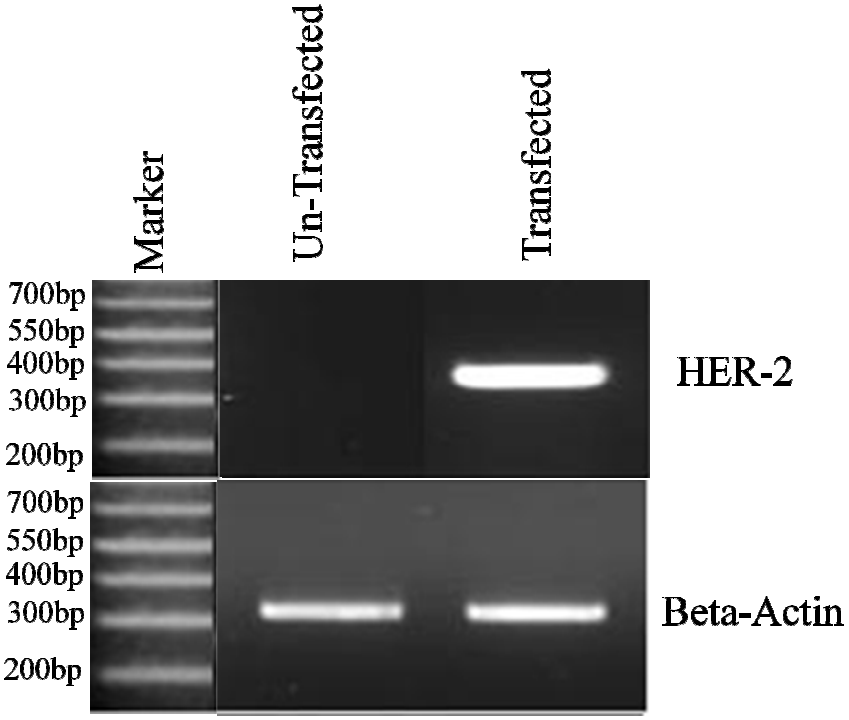

Figure 1: The MCF7 cell line was transfected with p-HER2 and clones were isolated by selection with hygromycin B and screened for HER-2

Activated HER-2 in MCF-7 cells increases the sensitivity to drugs

Due to a significant overlap of biological features attributable to the overexpression of HER-2 (i.e., enhanced proliferation, improved cell survival, resistance to chemotherapy, correlation with poor prognosis, etc), Her-2 expression was investigated along with ER/PR expressions in HER-2-overexpressing MCF7 cells. The MTT experiments were repeated twice with separately. Figure 2 demonstrates pronounced increase in MCF-7 cell death when exposed to Herceptin and Dasatinib.

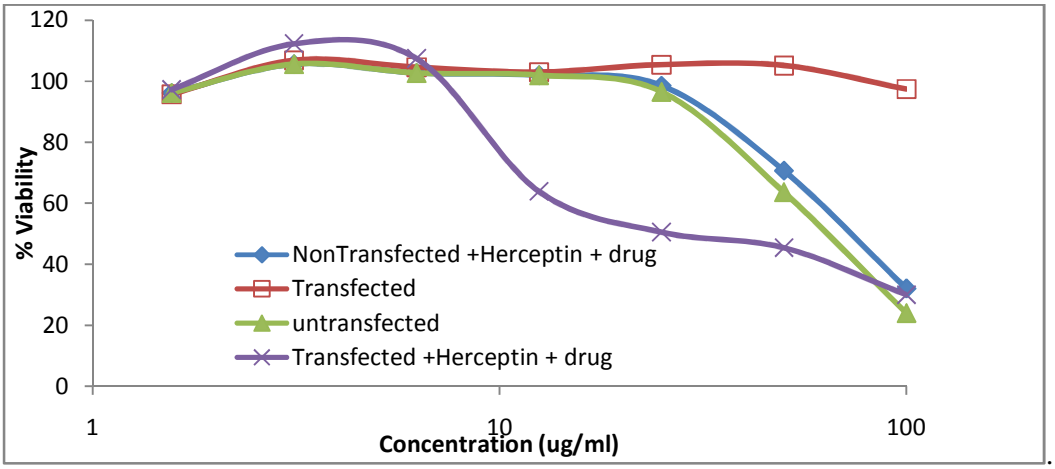

Figure 2: Effect of HER-2 expression in the MCF7 cell line compared to untransfected MCF-7 Cells. Her-2 transfection has increased the sensitivity of HER-2 expressing MCF7 cells to Dasatinib and Herceptin

Over-expression of HER-2 (Figure 2) was shown in MCF-7 cells by RT-PCR method. It has been reported to activate multiple signal transduction pathways including Ras-MAPK, the PI3-K-Akt-NF-kappaB cascade and STAT3 [11]. It has been reported that the regulation of survivin expression in HER-2 induced MCF7 cells may inhibit specific signal transduction pathways. The modulation of cell death after by 48 hrs treatment with Dasatinib and Herceptin in combination with or without Her-2 transfection in the MCF7 breast cancer cell line was studied (Table 1). Expression of Her-2 was verified by RT-PCR and Agarose gel electrophoresis.

Table 1: Assay of cell death after by 48 hrs treatment with Dasatinib and Herceptin in combination with or without Her-2 transfection in the MCF7 breast cancer cell line

Drug Concentration (µg/ml)	Non-Transfected + Herceptin + Dastinib	Transfected + Dasatinib	No-transfected + Dasatinib	Transfected + Herceptin + Dasatinib
100	32.06521739	97.41666667	24.06521739	30.08333333
50	70.69565217	105.1594203	63.69565217	45.48188406
25	98.55797101	105.4275362	96.55797101	50.57608696
12.5	101.9637681	102.9927536	101.9637681	63.91304348
6.25	102.7065217	104.5724638	102.7065217	107.4528986
3.125	105.6014493	106.8369565	105.6014493	112.2826087
1.5625	96.15942029	95.69565217	96.15942029	97.25362319

Wound healing (Cell Invasion) Assay

The cell lines were seeded in 6-well plates until the cells reach confluence. Then, a straight scratch was made using a yellow plastic pipette tip. Next, the plates were rinsed twice with PBS to remove floating cells. The underside of the dish was marked to indicate the wounded area where the initial photos were taken, which allowed the imaging of both wound edges using the 10X objective Wound closure (%) = [(Initial $area_{t0}$ - Final $area_{tf}$)/Initial $area_{t0}$] ×100. This corroborates with the clinical finding that HER 2 over expression leads to metastasis of cancer. Figure 3 shows that HER 2 transfection increases the migration of cells resulting in faster wound healing.

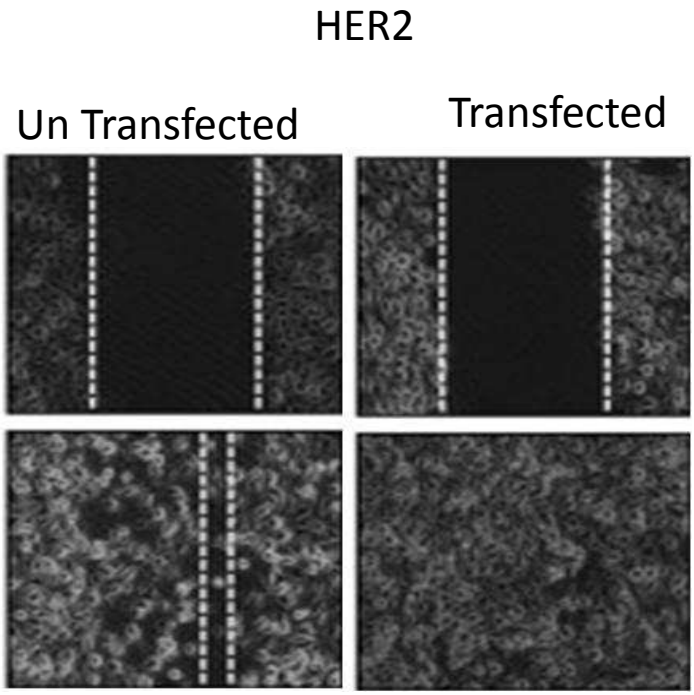

Figure 3: Wound healing assay of MCF-7 cell lines at 24hrs of culturing. The result of growth rates in wound healing assays increased in the MCF-7 cells
Magnification 40X

DISSCUSSION

Estrogen receptor (ER) is the most essential prognostic and predictive marker for BC [12]. A good understanding of the prognostic features of BC potentially assists in the selection of suitable treatment for the individual patient [13]. Consequently, the following features like lymph node involvement, tumor size and grade, status of ER and PgR, status of the cancer biomarker HER-2/neu gene expression profile, and patient's age are considered in the management of BC [14].

However, a proportion of patients positive for ERs will not respond to endocrine treatment. In a study by Wright et al, it was demonstrated that the coexpression of HER-2/neu was associated with a reduced response rate of ER–positive patients to first-line hormone therapy of metastatic breast cancer from 48% to 20%. Thus it implicates the role of HER-2 gene in metastasizing the BC [15].

Leitzel K et al studied the serum level of circulating HER-2/neu extracellular domain prior to treatment with second-line hormone therapy for metastatic cancer in 300 patients [16]. The study reported a lower response rate, decreased time to progression and reduced survival in association with elevated level of HER-2/neu. In a study by Yamauchi H et al, it was found that the serum level of higher HER-2/neu expression is also correlated with the reduced response rate to an antiestrogen droloxifene as first-line endocrine therapy [17].

However, a study by Willsher *et al*,. showed no significant correlation between pretreatment serum HER-2/neu levels and response to first-line tamoxifen therapy using a serum enzyme-linked immunosorbent assay (ELISA) in 77 patients with stage III or IV breast cancer [18]. With the prevailing evidence, the current study has been conducted to study the effect of chemotherapy on HER-2/neu gene transfected in ER positive breast cancer cells.

The cell death was appreciable in Her-2 expressing cancer cells. The induction of cell death may be due to up-regulation of apoptotic protein Bax or Bad Oncogenes such as c-Myc, which drive cells to enter the cell

cycle, also engage Bax/Bak-dependent apoptosis. Likewise, reduced expression of pro-apoptotic Bax levels has been associated with poor response to chemotherapy and shorter overall survival for patients with breast cancers, whereas enhanced expression of Bax protein correlate with a good response to chemotherapy in vivo.

Her-2 expression also advanced the wound healing property of MCF7-HER2 cells, suggesting up-regulation of oncogenes that promote invasion and migration. Wound healing assay showed lower growth rate of the Untransfected MCF-7 cells compared to Her-2 transfected MCF-7 cells. Growth and survival signals elicited by activated HER-2 are largely mediated via PI3K-Akt and Ras-MAPK signaling pathways. Down-regulation of survivin may occur independent of p-Akt expression [19]. PI3K-dependent, but Akt-independent, mechanisms by which HER-2 might regulate survivin include effects on serum- and glucocorticoid-induced kinases (SGK), which are serine/threonine kinases that are highly homologous to Akt and, like Akt, are regulated by PI3K. Thus, it is possible that HER-2 regulates survivin in part through PI3K-dependent effects on SGK and/or phospholipase Cγ.

CONCLUSION

After a short term (48 hours) induction of HER2 in MCF7 breast cancer cells increased sensitivity to Herceptin and Dastininb. In addition the Her-2 expression advanced the wound healing property of MCF7-HER2 cells, suggesting up-regulation of oncogenes that promote invasion and migration. Understanding the regulation of HER-2 signaling pathways and genes that induce resistance to drugs will help to identify new targets/strategies for the treatment of patients with tumors that are dependent on HER-2 induced signaling pathways for their survival. Until recently, the study of nuclear receptor (NR) function in breast cancer biology has been largely limited to estrogen and progesterone receptors. By adding Her-2 gene in MCF-7 cancer cells will show how the transfected cancer cells respond to chemotherapeutics. By understanding how growth factor receptors and their downstream kinases are activated by ER/PR (and vice-versa) oncologists may maximize treatment opportunities in breast cancer.

In addition to the ER/PR receptors, it is predicted that modulating the activity of Her-2 is expected to provide novel prevention and treatment approaches for breast cancer patients. In this study that the combination drug treatment of untransfected MCF-7 Cells with Herceptin and Dasatinib increased cell death. However, when MCF-7 Cells were transfected with Her-2, the cells developed chemoresistance to Herceptin and Dasatinib. It is concluded that ER/PR expressing cancer cells are much more sensitive to chemotherapy but when the cells express other oncogenes such as Her-2, their chemoresistance increase and such cancers are hard to treat. Therefore molecular profiling of the tumors is important to understand their response to chemotherapy before and during the treatment regimen in breast cancer patients.

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