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Effect of Dasatinib and Herceptin on HER-1, HER-2 and ER/PR (Triple Negative) Breast Cancer Cells

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Abstract: Epidermal growth factor receptor (EGFR) levels predict a poor outcome in breast cancer patients and are most commonly associated with proliferative effects of epidermal growth factor (EGF) and drug resistance to anticancer drugs. The study investigated the effect of EGFR expression in Triple negative (ER, PR, Her-2) and EGFR negative MDA-MB-231 human breast cancer cells on sensitivity towards anticancer drugs such as MDA-MB 231 (monoclonal antibody) targeting EGFR-1 and Dasatinib. Clinically the understanding of the role of EGFR-1 function in chemoresistance/ chemosensitivity in breast cancer biology is important to treat women with advanced and non-responsive breast cancers. The modified chemosensitivity of MDA-MB 231 cancer cells were cultured in *in vitro* by transfecting MDA-MB 231 cells with EGGFR-1 (Her-1) gene and showed that cancer cells were differentially sensitive to anticancer therapy (Erbitux + Dasatinib), compared to the untransfected cells. The wound healing assay that measures the invasion potential of cancer cells showed that transfection of Her-1 into MDA-MB 231 cell appreciably increased its invasion property. The result confirmed that Her-1 expression in breast cancer cells drives the metastasis of cancer cells outside its primary site. In future by studying the role of Her-1 genes in overall chemoresistance in breast cancer cells, one can target these genes with specific molecular drugs and improve the chemo sensitivity so that the disease is brought under control. This research is important to understand the mechanism of chemoresistance in breast cancer cells that often develop resistance to chemotherapy in a clinical set up.

Key words: Dasatinib · Hercept · Her1 · Her2 · Er/Pr Breast Cancer Cells

INTRODUCTION

Triple-negative breast cancer (TNBC) is characterized by a lack of expression of estrogen receptor (ER), progesterone receptor (PgR) and the absence of human epidermal growth factor receptor 2 (HER2) over expression [1]. It has been demonstrated that TNBC also typically expresses epidermal growth factor receptor (EGFR) and basal cytokeratins (particularly cytokeratin 5, 14 and 17). Despite TNBC incidence rate of less than 20% of all BC, it remains challenging owing to their resistance to the endocrine and other therapies [2]. In contrast to patients with ER/PgR+ and/or HER2-overexpressing disease [3, 4], systemic treatment options for TNBC are limited to cytotoxic chemotherapy [5, 6].

The EGFR is one of the receptors most commonly associated with human tumors and has been shown to correlate with the progression of many tumor types including breast tumors [7]. Most often associated with aspects of tumor growth (i.e., proliferation, apoptosis and cell survival). However, little work has been done on the effects of EGFR-1 (Her-1) expression on breast cancer cell sensitivity or resistance to anti-cancer drugs. The complex process of drug resistance is a critical component of

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treatment process to arrest the progression of tumors from a noninvasive to an invasive and metastatic phenotype [8].

In the current study, Her-1 transfected into triple negative MDA-MB 231 cells is studied to find the response to MDA-MB 231 and Dasatinib by MTT assay. Besides that the migration/ invasion of Untransfected and transfected (Her-1) MDA-MB 231 cells by wound healing assay is also studied.

MATERIALS AND METHODS

Cell Lines: Established cell lines metastatic breast cancer (MDA-MB-231*, ER/PR, HER-2 and HER-1* negative), 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), MDA-MB 231 and Dasatinib were gifts from Dr. Khaitan's lab associates in USA.

Transfection of *HER-1* in the MDA-MB231 Cell Line: Stable transfection and selection were used to produce HER-1 expressing, MDA-MB-231cells. MDA-MB-231 cells were transfected pcDNA 3.1 vector carrying *HER-1* gene. After hygromycin selection, clones were tested for *HER-1* expression by RT PCR.

Drugs Tested in Untransfected and *HER-1* Transfected MDA-MB231 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 how cells make a number of protein kinases and is called a multi kinase inhibitor. It works by blocking the signals that tell the cells to grow. Dasatinib is a treatment for chronic myeloid leukaemia (CML) – for people who have already had other treatments including imatinib. Acute myeloid leukemia which is Philadelphia chromosome positive, when other treatments are no longer working Acute lymphoblastic leukemia, which is Philadelphia chromosome positive, when other treatments are no longer working (http://www.cancerresearchuk.org/cancer-help/ about-cancer/treatment/cancer-drugs/dasatinib).

MDA-MB 231® (cetuximab): Is a recombinant, human/mouse chimeric monoclonal antibody that binds specifically to the extracellular domain of the human epidermal growth factor receptor (EGFR). Cetuximab is composed of the Fv regions of a murine anti-EGFR antibody with human IgG1 heavy and kappa light chain constant regions and has an approximate molecular weight of 152 KDa. Cetuximab is produced in mammalian cell (murine myeloma).

RNA Extraction and PCR: To shed light onto the mechanism responsible for cell death we extracted the RNA from MDA-MB 231 cells treated with ss\3\p at 10ug/ml 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 20ìl, containing 0.2 mM dNTPs, 1 mM MgCl2 and 1 Unit of AmpliTaq Gold DNA Polymerase (Applied Biosystem). The PCR for â-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, 20ìl of the samples were separated via electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The PCR primers used were: HER1: Sense primer: 5'-AGAGGAGAACTGCCAGAA-3'; Anti-sense primer: 5'-GTAGCATTTATGGAGA GTG-3' and â-actin: Sense primer: 5'-CTTCTACAATGAGCTGCGTG-3'; Anti-sense primer: 5'-TCATGAGGTAGTCAGTCAGG-3'.

***In vitro* Wound Healing Assay:** Untransfected and transfected MDA-MB231 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 (MDA-MB 231) 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 (Erbitux and Dasatinib). Post-treatment cell death induction was assessed by the use of MTT.

Molecular Biology Assays: Ss cDNA was generated using the mRNA extracted from wild type and transfected (with Her-1) MDA-MB 231 cells and then her-1 expression was checked by RT-PCR and Agarose gel electrophoresis.

RESULTS

The *HER-1* cDNA was cloned into pcDNA3.1 vector as described above, to produce the plasmid p-HER2. The MDA-MB 231cells were transfected with p-HER-1 and clones were isolated by selection with hygromycin B and screened for expression of HER-2.

RNA was extracted from the MDA-MB 231cells and evaluated for Her-1 expression by RT-PCR. Over-expression of HER-1 (Figure 1) was shown in MDA-MB 231 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 STAT 3. It has been reported that the regulation of survivin expression in HER-1 induced MDA-MB 231 cells may inhibit specific signal transduction pathways.

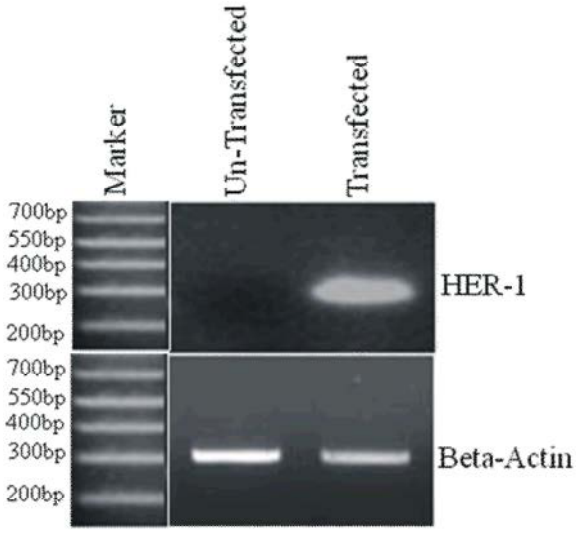

Fig. 1: The MDA-MB 231cells were transfected with p-HER1 and clones were isolated by selection with hygromycin B and screened for HER-1.

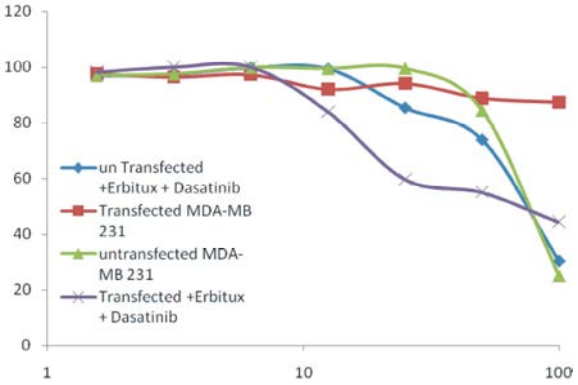

Fig. 2: Effect of HER-1 expression in the MDA-MB 231 cell line compared to untransfected MDA-MB 231 Cells.

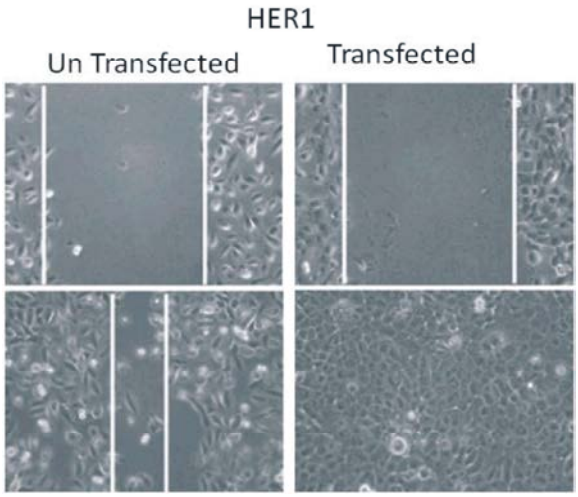

Fig. 3: Wound healing assay of MDA-MB 231 cell lines at 24hrs of culturing. The result of growth rate in wound healing assay increased in the MDA-MB 231. Magnification 40X

Activated HER-1 in MDA-MB 231 Cells Increases the Sensitivity to Drugs: Due to a significant overlap of biological features attributable to the over expression of HER-1 (i.e., enhanced proliferation, improved cell survival, resistance to chemotherapy, correlation with poor prognosis, etc). Her-1 expression was investigated on triple negative MDA-MB 231 cells. The MTT experiments were repeated twice with separately.

Figure 2 d emonstrated pronounced increase in MDA-MB 231 cell death when exposed to Erbitux and Dasatinib. The modulation of cell death after by 48 hrs treatment with Dasatinib and Erbitux combination with or without *Her-1* transfection in the MDA-MB 231 breast cancer cell line was studied (Table 1). Expression of Her-1 was verified by RT-PCR and Agarose gel electrophoresis.

Table 1: Determination of cell death after by 48 hrs with Dasatinib and Erbitux in presence and absence of Her-1 transfection in the MDA-MB 231

Drug Concentration	un Transfected + Erbitux + Dastanib	Transfected MDA-MB 231+Dasatinib	untransfected MDA-MB 231+ Dasatinib	Transfected + Erbitux + Dastanib
100	30.4556962	87.33333333	25.12236287	44.32067511
50	74	88.79324895	84.4978903	55.05485232
25	85.52320675	94.09704641	99.52320675	59.70886076
12.5	99.50632911	92.00421941	99.50632911	83.92827004
6.25	99.86075949	97.3164557	99.86075949	100
3.125	97.51476793	96.48945148	97.51476793	100
1.5625	96.99156118	97.69620253	96.99156118	98.22362869

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 (%) = $[(\text{Initial area}_{t0} - \text{Final area}_{tf})/\text{Initial area}_{t0}] \times 100$ (Figure 3).

DISCUSSION

TNBC which occurs more frequently in younger patients (<50 years old) usually harbour a more aggressive behavior [9]. In a study by Dent and colleagues, it has been observed an increased risk of distant recurrence following diagnosis among patients with TNBC tumors compared with other subtypes (hazard ratio [HR] = 2.6; 95% confidence interval [CI], 2.0–3.5; $p < 0.0001$) in a cohort of 1061 patients with BC. The median overall survival (OS) among patients with TNBC was also shorter than that with other subtypes (4.2 versus 6.0 years; $p < 0.0001$). As a consequence, the above data strongly suggested that TNBC has a particularly poor prognosis, usually presenting with high-grade tumors. The overall poor prognosis of patients with TNBC and their tendency to relapse with distant metastases make the need for effective systemic therapies an absolute clinical imperative, especially in the early setting [10].

Her-1 plays an important role in human malignancies. Her-1 gene is amplified or over expressed in significant number of human breast cancer patients. Patients with Her-1 -over expressing breast cancer have substantially lower overall survival rates and shorter disease-free intervals than patients whose cancer does not over express Her-1. Moreover, over expression of Her-1 leads to increased breast cancer metastasis. The important roles of Her-1 in cancer progression render it a highly attractive target for therapeutic interventions of breast cancer. The humanized Her-1 -targeting antibody Trastuzumab (MDA-MB 231) was approved for the treatment of Her-1 over expressing colon cancers.

It has been shown that patients with HER-2 amplification and HER-1 expression had lower ER levels and were modestly less responsive to tamoxifen, suggesting that molecular events in addition to those involving the ErbB receptors are important in determining the endocrine-resistant phenotype [11, 12]. The majority of patients with breast carcinoma still receive chemotherapy as a critical component of multimodality treatment. Thus, understanding the effect and mechanisms of Her-1 on chemo sensitivity is important for anticancer agent selection and individualization of patient treatment [13], which is critical to the success of treatment of breast cancers.

Until recently, the study of EGFR function in breast cancer biology has been largely limited to HER-2 receptor [14]. By adding *HER-1* in MDA-MB 231 cancer cells I showed how the transfected cancer cells respond to anticancer drugs. Understanding how growth factor receptors and their downstream kinases are activated by HER-1 (and vice-versa) is a central goal for maximizing treatment opportunities in breast cancer. In addition to other EGFRs, it is predicted that modulating the activity of Her-1 is expected to provide novel prevention and treatment approaches for breast cancer patients.

Chemotherapy, radiation and immunotherapy all rely heavily on apoptosis to kill breast cancer cells [15]. Her-1 (EGFR-1) over expression is associated with poor overall survival and drug resistance in breast and ovarian cancer patients. Although the molecular mechanisms by which Her-1 induces drug resistance are not well established, there is increasing evidence that this resistance is a consequence of deregulation of apoptotic pathways in cells. Therefore, investigated the effect of Her-1 expression in triple negative MDA-MB 231 cells. The results showed that acute over expression of HER-1 in MCF7 cells increases the sensitivity of cancer cells to Erbitux and Dasatinib.

The cell death was appreciable in Her-1 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 [16]. 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 -MB 231 –HER-1 cells, suggesting up-regulation of oncogenes that promote invasion and migration.

Wound healing assay showed lower growth rate of the untransfected MDA-MB 231 cells compared to Her-1 transfected MDA-MB 231 cells. Growth and survival signals elicited by activated HER-1 are largely mediated via PI3K-Akt and Ras-MAPK signaling pathways. Down-regulation of survivin may occur independent of p-Akt expression. PI3K-dependent, but Akt-independent, mechanisms by which HER-1 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-1 regulates survivin in part through PI3K-dependent effects on SGK and/or phospholipase Cγ.

CONCLUSION

After a short term (48 hours) induction of HER1 in MDA-MB 231 breast cancer cells increased sensitivity to Erbitux and Dastininb. In addition the Her-1 expression advanced the wound healing property of MDA-MB 231-HER1 cells, suggesting up-regulation of oncogenes that promote invasion and migration. Understanding the regulation of HER-1 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-1 induced signaling pathways for their survival.

Until recently, the study of EGFRs function in breast cancer biology has been largely limited to EGFR-2 (Her-2) receptors. By adding Her-1 gene in MDA-MB 231 cancer cells will show how the transfected cancer cells respond to drug treatments. By understanding how growth factor receptors and their downstream kinases are activated by EGFR-1oncologists may maximize treatment opportunities in breast cancer. In addition to other EGFR receptors, it is predicted that modulating the activity of Her-1 is expected to provide novel prevention and treatment approaches for breast cancer patients. In this study that the combination drug treatment of untransfected MDA-MB 231 Cells with Erbitux and Dasatinib increased cell death. However, when MDA-MB 231 cells were transfected with her-1, the cells developed chemo resistance to Erbitux and Dasatinib. It is concluded that Her-1 expressing cancer cells are much more sensitive to chemotherapy but when the cells express other oncogenes such as Her-2, their chemo resistance increase and such cancers are hard to treat. Therefore molecular profiling of the tumour is important to understand their response to chemotherapy before and during the treatment regimen in breast cancer patients.

List of Abbrivations:

HER 1 - Human Epidermal Receptor 1
SGK - Serum- and Glucocorticoid-induced Kinases
TNBC - Triple-Negative Breast Cancer
EGFR-2 - Epidermal Growth Factor Receptor-2
PI3K – Phosphatidylinositide 3-kinases
RT-PCR - Reverse transcription polymerase chain reaction

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