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Bone morphogenic protein receptor type 1a (BMPR1A) and Caveolin-1 associated with trastuzumab resistance of breast cancer cells

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ABSTRACT

Trastuzumab is a specific monoclonal antibody used for therapeutic of the human epidermal growth factor receptor 2 (HER-2) -positive metastatic breast cancer. But, resistance to trastuzumab is a major obstacle in clinical efficiency. During the past years, several studies have been done to find the mechanisms contributing to trastuzumab resistance. Previous studies have highlighted that bone morphogenic protein (BMP) signaling can indicate a pathway in cancer for sensitizing cells to chemotherapy. Also, it was suggested that Caveolin-1 is essential for the formation of caveolae and endocytic membrane transport and has a critical role in drug resistance and metastasis in cancer. The purpose of this study was to assess the expression of BMP receptor type1A (BMPR1A) and Caveolin-1 genes in compare with trastuzumab-sensitive and resistance BT-474 cells. Trastuzumab-resistant BT-474 cells were established by continuous subjection to trastuzumab for six months. Then, an MTT assay was done for determining the resistance. After that, the Expression of BMPR1A and Caveolin-1levels were assessed through real-time PCR. Caveolin-1 expression levels increased significantly (2.4 fold, $p<0.05$) whereas BMPR1A levels down-regulated significantly (8.26 fold, $p<0.05$) in BT-474-R compared to the parental cells. Our results proposed that BMPR1A and CAV1 regulation take part in BT-474 trastuzumab resistance breast cancer. Therefore, further experiments are required to confirm the role/s of BMPR1A and CAV1 in trastuzumab resistance breast cancer.

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Keywords
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INTRODUCTION

Amplification or overexpression of HER2 occurs in about 20–30% of metastatic breast cancers. HER2 protein overexpression has come to be identified as important markers for metastatic HER2- overexpressing breast cancer and the target of specific therapies [1]. Trastuzumab is a recombinant humanized monoclonal anti-HER2 antibody (Herceptin) and was the first approved monoclonal antibody by the FDA used for the solid tumor therapy. Most patients with progressive HER-2 positive breast cancer responding initially to trastuzumab acquired resistance over the first year of treatment [2]. several mechanisms have been studied to identify the cause of resistance to trastuzumab in breast cancer, including reduced antibody affinity or HER2 expression, using alternative receptor tyrosine kinases signaling pathways, and changed intracellular signaling including the loss of PTEN expression, increased Akt activity, reduced activity of p27kip1(a cell cycle regulator), leading to the overproliferation of cells [3].

Caveolin-1 (Cav-1), a 21 kDa protein is in “cave-like” invaginations of the cell membrane known as caveolae. Cav-

1 is a main essential component of organelles with a size range of 50–100 nm and is a necessity for their formation. Cav-1/caveolae are associated with several cellular pathways such as endocytosis, signal transduction, and lipid homeostasis [4, 5].

Studies have been displayed that Cav-1 is a molecular hub that integrates the activity of a multitude of signaling molecules including epidermal growth factor receptor (EGFR), HER2, Src and the mitogen-activated protein kinase (MAPK) cascade. Most of these signals are involved in cancer development [6]. Also, recent studies show that Cav-1 can be a factor in mediating stress-associated and drug resistance. caveolin-1 was involved in up-regulation of adriamycin-resistant breast cancer cells [7], Taxol- and gemcitabine-resistant lung cancer cells [8] and multidrug resistant colon cancer cells [9].

BMP is a special extracellular multifunctional cytokine that is a member of the large transforming growth factor-beta (TGF-β) superfamily. For binding to BMP ligands (BMP2, 4, and 7) the BMP type 1 receptor (BMPR1A) that is a transmembrane receptor on the cell surface, forming a heterodimer with the type 2 receptor (BMPR2. Several investigates have revealed that these morphogens play critical roles in proliferation, development, differentiation, and apoptosis [10].

Further experiments showed BMP signaling act as a tumor suppressor. Howe et al. displayed the formation of Juvenile Polyposis Syndrome through the loss of BMPR1a [11]. Also, the loss of BMPR1a form hamartomas that usually are benign tumors. Recent works have offered that low expression of BMPR1A may be related to poor prognosis in tumors [12, 13]. However, the expression levels of cav1 and BMPR1A and their effect on drug resistance of HER2 positive breast cancer have not been thoroughly evaluated.

MATERIALS AND METHODS

Cell culture and establishment of trastuzumab-resistant cells

The human BT-474 breast cancer cells were taken from the Iranian Biological Research Center. The cells were cultured in media (DMEM-f12) with 10% fetal bovine serum (FBS) and incubated under humidified chamber containing 5% CO_2 at 37°C. As reported previously, to obtain Trastuzumab-resistant cells, The BT-474 parental cells were cultured continuously in the presence of low-dose trastuzumab (5 µg/ml) for 6 months [14]. Then, parental and trastuzumab-resistant BT-474 breast cancer cells were cultured in the absence and presence of trastuzumab, respectively.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from each samples (sensitive and resistant cultured BT-474 cells) by using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). MRNAs were reverse transcribed to cDNAs using the miScript II RT Kit (Qiagen). qPCR was done through the StepOne™Real-Time PCR System (Applied Biosystems Inc., Hercules, CA, USA). Then cDNAs were detected and amplified by Thermo Fisher Master Mix of SYBR Green PCR (Thermo Fisher, England). The sequence information used in this quantitative Real-Time PCR was listed in Table 1. GAPDH was as internal controls for mRNAs. Quantitation of gene expression evaluated by ΔΔCt calculation, where Ct is the threshold cycle. QRT-PCR was performed in triplicate.

Cell survival assay

Cell survival was estimated by a colorimetric MTT assay [21]. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Atocel (Graz, Austria). Briefly, BT-474 cells were cultured for 24 h into the 96-well plate at a density of 1 • 104 for each well. Then, cells were incubated with medium containing various concentrations of trastuzumab (0.21–2100 µg/mL) for another 72 hours. After the incubation period, 100 µL of 10mM MTT working solution was added into every well of the plate and incubated for 4 h at 37°C. After addition of DMSO, the plate was placed on an orbital shaker for 45 min at RT for dissolving the formazan crystals. The absorbance intensity of cells was evaluated at 570 nm with the Epoch Microplate Reader. The relative cell viability (%) was calculated through the following formula: survival percentage = $(A_{\text{drug-treated cells}} - A_{\text{blank cells}}) / (A_{\text{untreated cells}} - A_{\text{blank cells}}) \cdot 100$.

Statistical analysis

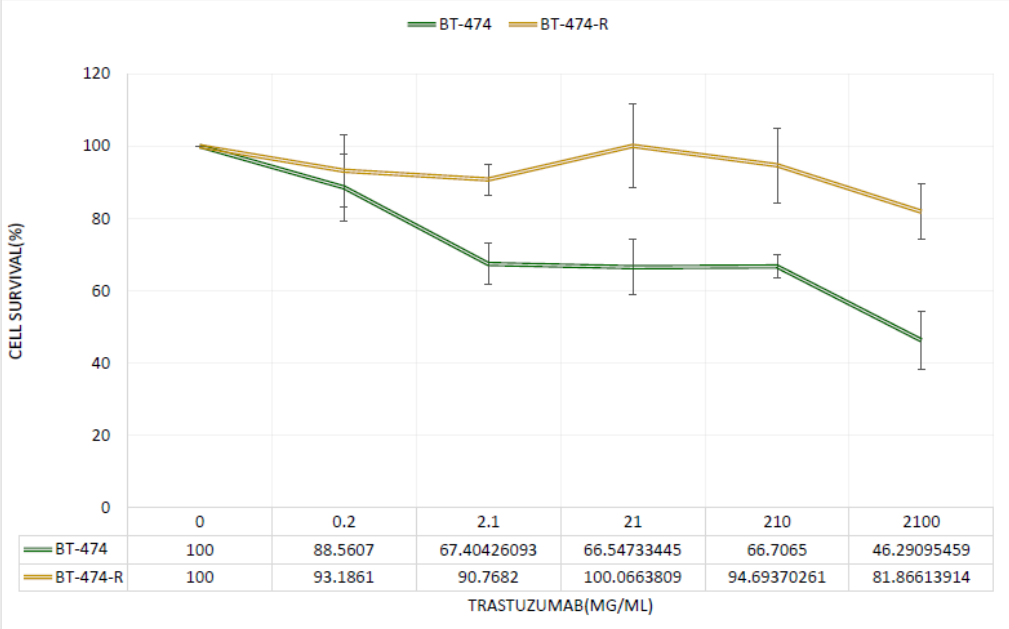
Statistical analyses were done using SPSS version 16.0 for Windows. The significance of differences in the quantitative PCR results was evaluated by Mann-Whitney test. The P-values less than 0.05 were *assumed* as statistically *significant.*

RESULTS

To define the status of HER2 signaling in sensitive and resistance BT-474 cells, these cells were treated with trastuzumab for 72 hours, and the survival of cells was as-

Table 1. Primer sequences for quantitative real-time polymerase chain reaction

Primer	Sequence
CAV1 Forward	GCGACCCTAAACACCTCAAC
CAV1 Reverse	ATGCCGTCAAAACTGTGTGTC
BMPR1A Forward	TAGTTCGCTGAACCAATAAAGG
BMPR1A Reverse	GTCAGAAAATGGAGTAACCTTA
GAPDH Forward	TGGACTCCACGACGTACTCAG
GAPDH Reverse	CGGGAAGCTTGTCATCAATGGAA



	0	0.2	2.1	21	210	2100
BT-474	100	88.5607	67.40426093	66.54733445	66.7065	46.29095459
BT-474-R	100	93.1861	90.7682	100.0663809	94.69370261	81.86613914

Figure 1. Analysis of Sensitivity to trastuzumab by MTT cell survival assay. Cells were plated in 96-well plates and trastuzumab (0.21–2100 mg/mL) containing medium was added 24 h later. MTT assay was performed after 72 h incubation. *p < 0.05 or **p < 0.01 statistically significant when compared between two groups using t-test.

sessed by MTT assay. As revealed in Figure 1, BT-474-R cells represent significantly higher resistant to trastuzumab in compare with the sensitive cells (P < 0.01, Fig. 1). At the maximum concentration of trastuzumab (2100 mg/mL), the survival cells were about 80% , but just about 44% of the sensitive cells be viable [15]. These results proposed that these resistant cells show in vitro proliferation advantages over non-resistant BT-474 cells.

Upregulation of cav1 and downregulation of BMPR1A contributing to trastuzumab-chemotherapy resistance in bt-474 cells

To assess the function of BMPR1a and CAV1 in trastuzumab resistance of breast cancers, the expression of these genes were surveyed in parental and trastuzumab-resistant BT-474 cells by Quantitative RT-PCR. The expression level of CAV1 was meaningfully upregulated (P < 0.05, fold change 2.4); but BMPR1a was downregulated significantly (P < 0.05, fold change -8.26) (Table 2 and Fig. 2).

DISCUSSION

In the present study, we showed that enhanced level of CAV1 or downregulation of BMPR1A could be independent predictors of drug resistance in HER2 positive breast cancer. Caveolin-1 was shown that interact with different intracellular signaling pathways. Agelaki et al displayed that Cav-1 contribute EGFR signaling and promote proliferation and migration [16]. Cav-1 inhibits EGFR pathway through ERK1/2 and Grb2-Sos-Ras, but activates PI3K pathway [17]. Interaction of Cav-1 with Akt causes EGFR activation which causes an increase in cell survival [18].

The expression of was mainly associated with EGFR positivity, for instance, CAV1 was expressed in 68% of HER2 positive breast cancers, however in EGFR-negative

Table 2. Relative expression levels of CAV1 and BMPR1A in BT474 sensitive and resistance cells

	Fold change
CAV1	2.4
BMPR1A	-8.26

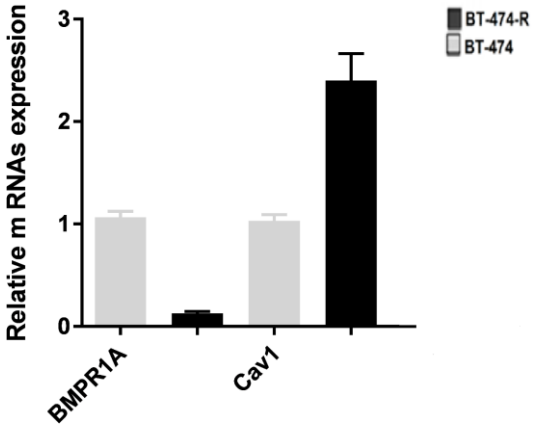


Figure 2. The fold change in expression of BMPR1A and CAV1 in BT-474 and BT-474-R. The RNA was extracted using TRIzol reagent, converted into cDNA by RT-PCR and submitted to qRT-PCR using specific primers, as described in methods. Each bar indicates the mean ± SD of triplicate assays.

breast carcinomas just exhibit 3.6% expression of CAV1 [19]. Primary experiments proposed that EGFR could be localized within caveolae and CAV1 expression would adjust the EGFR signaling pathway by receptor sequestration and controlling receptor trafficking [20].

Gang et al in 2016 showed that Cav-1 overexpression increases HER-2 signaling and enhances proliferation and migration in breast cancer cells [21]. Upregulation of Cav-1 associated with histological differentiation, intrahepatic metastasis, expression of VEGF and venous invasion [22]. In inflammatory breast cancer cells and also tissues expression of Cav-1 was increased [23].

Several studies showed that Cav-1 expression was meaningfully enhanced in some drug-resistant cancer cells. In A549-T12 (taxol-resistant lung cancer cell line) that displayed 9-fold resistance taxol compared with the parental cell line, the expression of Cav-1 upregulated 3.4 fold. Increasing resistance taxol to 17-fold, enhanced Cav-1 expression 9.5-fold [24]. However, upregulation of Cav-1 level has been described in another drug-resistant cancer cells including MCF-7 cells resistant to adriamycin, SKVLB1 cells resistant to vinblastine and HT-29 cells resistant to colchicine [25].

In 1965 BMPs primary presented as an osteogenic factor. BMps are critical extracellular multifunctional signaling cytokine that demonstrates as a member of TGF-b superfamily [26]. BMPRs are transmembrane serine/threonine kinase receptors. when BMPs interacted with type I and II transmembrane serine/threonine kinase receptor, their signals were transduced [27]. There are four different types I receptors and three distinct types II receptors including —Bmpr1a (Alk3), Bmpr1b (Alk6), Acvrl1 (Alk1), Acvr1 (Alk2), Bmpr2 (BMPRII), Acvr2a (ActRIIA) and Acvr2b (ActRIIB) [28]. In this study, we showed that BMPR1A level was lower in BT-474 resistant cells compared with parental BT-474 cells. Due to the metastatic potential of resistance cells, downregulation of this tumor suppressor can have a critical role in the induction of metastasis. Our results in this study showed that downregulation of BMPR1a decreased tumor burden and metastatic potential. Several surveys have displayed that BMPR1A has critical roles in the suppression of tumor progression [29] BMPR1A has been demonstrated to be a tumor suppressor in tumorigenesis of skin .whereas findings of the lower lip squamous cell carcinoma showed that upregulation of BMPR1A displayed a very strong connection with high malignancy score and advanced clinical staging [30].

CONCLUSION

In conclusion, we evaluated the levels of Caveolin-1 and BMPR1A in trastuzumab-resistant and sensitive BT-474 cells. These results show that CAV1 and BMPR1A may be independent tumor markers reflecting the resistance in BT-474 HER2 positive breast cancer. More researches are essential for defining the function of CAV1 and BMPR1A in the HER2 positive breast cancer.

CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article

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