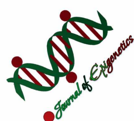

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Assessment of Correlation between Trastuzumab Resistance and miR-885-3p Relative Expression in the BT-474 Human Breast Cancer Cell Line

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A B S T R A C T

Trastuzumab has been applied widely in the treatment of breast cancer. The majority of initial responders display disease progression again within one year. Regardless of the high resistance rate, the molecular mechanisms affected this disease are not well understood. MicroRNAs are small, non-coding RNA molecules involved in gene regulation. There is evidence that promotes miRNAs as potential candidates to mediate therapeutic actions by targeting genes involved in drug response. The purpose of this study is to evaluate miR-885-3p in HER2 positive breast cancer chemoresistance. Trastuzumab-resistant BT-474 cells were generated by in vitro culture of BT-474 cells continuously in the presence of trastuzumab about 9 months. The relative expression of miR-885-3p to U6 RNA was evaluated in trastuzumab-resistant and sensitive cells using Relative Real-Time PCR. The Mann-Whitney test is used to compare the differences between the two groups. The MTT assay showed that BT-474 breast cancer cells were resistant to this drug under long-term culturing with trastuzumab ($p < 0.05$). MiR-885-3p expression was also significantly downregulated in trastuzumab-resistant cells in comparison with the parent cells ($p < 0.05$).: As the relative expression of candidate microRNAs was statistically different in trastuzumab-resistant and sensitive cells, we hypothesize that miR-885-3p downregulation as a possible mechanism of trastuzumab resistance.

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Introduction

Breast cancer is the most prevalent cancer in women worldwide, with around 1.38 million new cases and 458 000 deaths every year. HER2-overexpressing breast cancer displays about 20% of human breast cancers and is related to an increased risk of tumor recurrence and decreased overall survival(Arteaga CL, 2011; Loi et al., 2011). The overexpression of HER2 makes the constitutive activation of growth factor signaling pathways and acts as an oncogenic driver of breast cancer (Rexer and Arteaga, 2012). However, the HER2-targeted therapies are a benefit but the main percentage of patients die during drug resistance (Parra-Palau et al., 2014; Pohlmann et al., 2009). Explaining the molecular pathways involved in trastuzumab resistance has been difficult because of a variety of mechanisms of action of this drug. A number of mechanisms of resistance have been suggested, including reduced HER2 expression or antibody binding affinity, increased pro-survival signaling via alternative receptor tyrosine kinases, which is resulted by activating mutations of PIK3CA or a loss of phosphatase and tensin homolog (PTEN),

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and reduced activity of cell cycle regulator p27kip1, or increased Akt activity, which caused over-proliferation of cells (Nagata et al., 2004; Rexer BN, 2013; Zhang et al., 2011). MicroRNAs (miRNAs) are a class of short, non-coding RNAs that their expression may be regulated by epigenetic mechanisms including DNA methylation and histone modifications. It is well understood that miRNAs play important roles in adjusting multiple pathways for cancer progression. These miRNAs are either pro-oncogenic, by targeting tumor suppressor genes, or tumor suppressive, by silencing the oncogenes (Esquela-Kerscher A, 2006). Over 50% of miRNAs are located in cancer-related genomic regions (Zhu et al., 2015), which shows that miRNAs may play crucial roles in a variety of biological processes (Xie et al., 2016), such as tumorigenesis. It has been exhibited that miR-222 and miR-298 are linked with drug resistance in numerous malignant tumors, such as colorectal cancer, breast cancer, and liver cancer (Liu K, 2014; Robertson NM, 2014; Xu et al., 2012). MiR-542-3p expression was induced by trastuzumab in SKBR3 and MCF7/Her2 cell lines. Also, miRNA-542-3p depletion activated the PI3K-AKT pathway. Additionally, miRNAs are also closely related with drug resistance. Jung et al. showed that miR-210, a hypoxia-induced miRNA in MCF-7 breast cancer cells, was significantly upregulated in trastuzumab-resistant BT474 cells. (Jung et al., 2012). Bai et al. displayed that miR-200c, a well-known chemoresistance-associated miRNA, could induce trastuzumab resistance by adjusting epithelial-mesenchymal transition (EMT) in breast cancer cells (Bai, 2014). Although chemoresistance-related miRNAs have been identified as regulatory molecules in trastuzumab-resistant breast cancers, their underlying mechanisms remain unclear. the role of miR-885-3p in trastuzumab resistance in HER2+ breast cancer cells and its mechanism of action remain unclear. In the present study, we investigated the expression patterns of candidate miRNA in the human breast cancer cell line BT-474 and its trastuzumab-resistant variant BT-474-R.

Material and Methods

Cell culture and generation of trastuzumab-resistant cells:

The human breast cancer BT-474 was taken from the Iranian Biological Research Center. BT-474 cells were cultured in DMEM-f12 media supplemented with 10% fetal bovine serum (FBS). The cell line was incubated in a humidified atmosphere containing 5% CO2 at 37°C. Trastuzumab/Herceptin (Roche, Basel, Switzerland) was dissolved in sterile water. As reported previously, Trastuzumab-resistant cells were generated by continuous culture of BT-474 cells in the presence of 5 µg/ml trastuzumab for 9 months(Gong et al., 2011). Then, trastuzumab-resistant and parental BT-474 cells were cultured with or without trastuzumab, respectively.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated from sensitive and resistant cultured BT-474 cells by treatment with the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The isolated RNA samples were reverse-transcribed into double-stranded (ds) cDNA with the miScript II RT Kit (Qiagen). The samples were subjected to qPCR in the StepOne™ Real-Time PCR System (Applied Biosystems Inc., Hercules, CA, USA). cDNAs were then amplified and detected using SYBR Green PCR Master Mix (Thermo Fisher, England). Mature miR-885-3p was detected by the forward primers miR-885-3pF (Table1), and the MiScript Universal Primer (of the miScript II RT Kit) used as the reverse primer for it. U6 RNA was used as internal loading controls for miRNAs. Relative abundance of detected transcripts was calculated using the $2^{-\Delta\Delta Ct}$ methods.

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

	Primer sequence
Hsa_miR-885-3p Forward	AGGCAGCGGGGTGTAGTGGATA
Universal Reverse	GAATCGAGCACCAGTTACGC
U6RNA Forward	GTGCTCGCTTCGGCAGCACATAT

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Cell survival assay

Cell survival was assessed by a colorimetric MTT assay according to the procedures described previously(21). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Atocel (Graz, Austria). 100 mL of target cell suspension (1 • 104 cells) was added to every well in 96-well plate, and the plate was incubated for 24 h at 37_C in a humidified 5% CO2 atmosphere. Cells were incubated for another 72 h with medium containing trastuzumab (0.21–2100 µg/mL). After drug treatment, 100 µL of 10mM MTT working solution was added in wells of the plate and incubated for 4 h at 37_C. After addition of DMSO, the mixture was agitated for 45 min at RT. The absorbance values were read with a microplate reader at 570 nm. The percentage of survival was estimated by the following formula: survival percentage = (absorbance of drug-treated wells – blank wells) / (absorbance of untreated wells – blank wells) • 100.

Statistical analysis

Statistical analyses were done using SPSS version 16.0 for Windows. The significance of differences between the quantitative PCR results was evaluated by Mann-Whitney test, with the threshold of significance set at P <0.05.

Results

To determine the status of HER2 signaling in sensitive and resistance BT-474 cells, these cells were treated with trastuzumab for 72 h, and cell survival was evaluated by MTT assay. As shown in Figure 1, BT-474-R cells show notably more resistance to trastuzumab than did sensitive cells. (p < 0.01, Fig1). At the highest dose of trastuzumab (2100 mg/mL), around 80% of the resistant cells be viable and just about 44% of the sensitive cells survived. These results showed that trastuzumab-resistant HER2-positive breast cancer cells display proliferation advantages in vitro over non-resistant BT-474 cells.

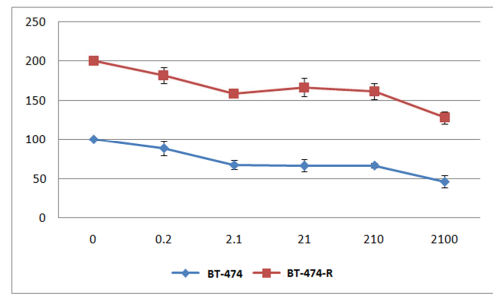

Fig. 1- Analysis of Sensitivity to trastuzumab by MTT cell survival assay. Cells were seeded in 96-well plates and trastuzumab (0.21–2100 µg/mL) containing medium was added after 24 h. MTT assay was done after 72 h incubation. *p < 0.05 or **p < 0.01 statistically significant when compared between two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

Expression status of miR-885-3p detected by qRT-PCR

The expression status of miR-885-3p was then evaluated in the resistant and sensitive BT-474 cells. The results show that expression of miR-885-3p was significantly downregulated in the resistant BT-474 cells. (Table2)(FIG2). The amplification curve of miR-885-3p shown in Fig.3.

Table 2- Relative expression levels of miR-885-3p in BT474 sensitive and resistance cells

	Fold change	p-value
miR-885-3p	-2.4	0.049

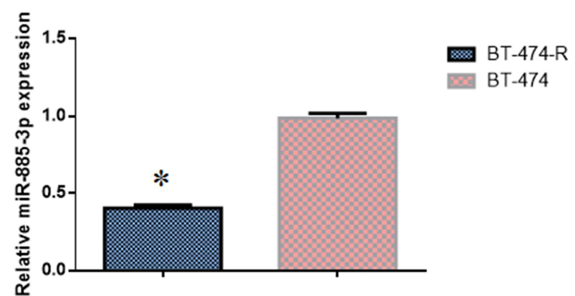

Fig. 2 - The fold change in expression of miR-885-3p in BT-474 and BT-474-R.

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.

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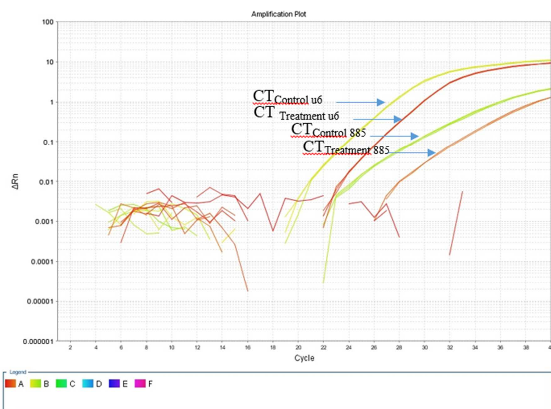

Fig. 3 - The amplification curve of the qRT-PCR of miR-885-3p. *U6* has been *used as an internal* reference gene.

Discussion

Resistance to common anticancer drugs is a hallmark of progressive breast cancers that lead to mortality in the great numbers of patients by facilitating cancer progression and distant metastasis (Hu et al., 2009; Morris PG, 2009). Overexpression of the ERBB2 gene, that encodes the oncoprotein of HER2, occurs in 20 to 25% of human breast cancers and is related to poor prognosis. HER2 overexpressing breast cancers tumor treatment has been greatly improved by targeted cancer therapy of anti-HER2 agents like trastuzumab; but, primary and secondary resistance is an important problem(Rexer and Arteaga, 2012). To examine the possible mechanisms of this resistance we must try to find out the pathways and mechanisms altered by trastuzumab treatment. Modified miRNA profiles have been recognized in several cancers relative to malignancy and prognosis, and in metastatic breast cancer tumors, miR-10b and miR-21 have been found to be upregulated(Sato et al., 2011). Several miRNAs, such as miR-34a or miR-221, are involved in treatment response and are related to response to docetaxel and tamoxifen(Kastl, 2012; Qian B. Katsaros D, 2009). miR-30b and miR-26a are upregulated in HER2 overexpressing breast cancer cells treated with trastuzumab(Ichikawa et al., 2012). In our investigation, we showed that this downregulation occurs in acquired-resistance BT474 cells. Thus, this downregulation of miR-885-3p may be a marker for sensitivity to trastuzumab treatment. It is potential that changes in the expression levels of miRNAs participate in trastuzumab sensitivity by targeting some genes that are necessary for the acquisition of resistance in breast cancer cells in vivo. While the specific roles of miRNA(s) dominates over others in various models of trastuzumab resistance, there might be wide crosstalk among the signaling events acted by miRNAs (Le et al., 2012; Rezaei et al., 2019).

Conclusions:

In conclusion, we identified miR-885-3p that may have important roles in drug resistance of breast cancer. The results suggest that HER2 positive breast cancer chemoresistance might be associated with dysregulation of miRNAs. We think that thoughtful changes in expression status of dysregulated miRNAs might be a potential therapeutic option to improve chemotherapy results of HER2 positive breast cancer.

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ارزیابی ارتباط بین مقاومت به تراستوزوماب و بیان نسبی miR-885-3p در سلول های سرطانی پستان در رده سلولی BT-474

زهره رضائی[1]، کاظم دستجردی[2]*

چکیده

تراستوزوماب به طور وسیعی در درمان سرطان پستان HER2 مثبت استفاده می گردد. اغلب کسانی که در ابتدا به دارو پاسخ می دهند پس از یک دوره یکساله مقاومت به این دارو را نشان می دهند. صرف نظر از میزان مقاومت بالا به این دارو، مکانیسم های ملکولی مقاومت به تراستووزماب به خوبی فهمیده نشده است. miRNA ها ملکول های کوچک غیر کد کننده RNA می باشند که در تنظیم بیان ژن شرکت می کنند. شواهدی وجود دارد که miRNA ها را، به عنوان کاندید های بالقوه، در هدف گذاری ژن های در گیر در مقاومت دارویی معرفی می کند. هدف از این مطالعه، ارزیابی بیان miR-885-3p در مقاومت دارویی به تزاستوزوماب در سرطان پستان HER2 مثبت است. سلول های BT-474 مقاوم به تراستوزوماب، با کشت مداوم ۹ ماهه در حضور تراستووزماب در محیط آزمایشگاه ایجاد گردید. بیان نسبی miR-885-3p نسبت به U6 در سلول های حساس و مقاوم به تراستوزوماب با استفاده از بررسی نسبت به U6 RNA با روش Relative Real Time PCR در سلول‌های مقاوم و حساس به تراستوزوماب و اختلاف بین دو گروه با استفاده از آزمون Whitney-Mann بررسی گردید. تست MTT نشان داد که سلول‌های BT-474 تحت کشت طولانی‌مدت با تراستوزوماب به این دارو مقاوم شدند؛ همچنین میزان بیان miR-885-3p در سلول‌های مقاوم به تراستوزوماب در مقایسه با سلول‌های والدی BT-474 به‌صورت معنی‌داری کاهش یافت (P=۰/۰۴۹). با توجه به اینکه بیان نسبی miRNA ی کاندید به طور معناداری بین سلول های حساس و مقاوم به تراستوزوماب متفاوت است، احتمالا بیان کاهش یافته ی miR-885-3p به عنوان مکانیسم احتمالی مقاومت به تراستوزوماب می تواند در نظر گرفته شود.

واژگان کلیدی: سرطان پستان، فاکتور شماره ۲ اپیدرمال انسان، مقاومت به تراستوزوماب، میکروآران‌ای 885.

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