RESEARCH ARTICLE

Circulating miR-195 as a Therapeutic Biomarker in Turkish Breast Cancer Patients

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Abstract

Background: Dysregulation of miRNA expression may be used as a biomarker for specific tumours because it may contribute to development of cancer. Circulating miRNA profiles have been highlighted for their potential as predictive markers in heterogeneous diseases such as breast cancer. In the literature, there is evidence that miR-195 levels are differentially expressed pre- and post-operative periods in breast cancer patients. At the same time, miRNA expression levels may vary because of ethnic origins. This study aimed to determine expression levels and potential roles of miR-195 in Turkish breast cancer patients. Materials and Methods: The expression patterns of miR-195 were initially examined in breast cancer tissues (luminal A and B type) (n=96). Subsequently, blood samples were prospectively collected from preoperative and postoperative Turkish breast cancer patients and disease free controls. Total RNA was isolated, and the expression level of miR-195 was quantified by real-time PCR. Results: We found that miR-195 level was altered in Turkish breast cancer patients, with down-regulation evident in breast cancer tissues compared to normal adjacent specimens. Furthermore, circulating levels of miR-195 was significantly decreased in post-operative blood samples compared with pre-operative levels (p=0.01 and <0.05). However, miR-195 was significantly increased in pre-operative blood samples of the luminal B type (p= 0.04 and <0.05). Conclusions: This study represents the first report of a miR-195 expression profile in Turkish breast cancer patients. Our data suggests that miR-195 levels might be a clinically useful biomarker in the earliest stage of Turkish breast cancer patients.

Keywords: Circulating miRNA - miR-195 - breast cancer patients - Turkey

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Introduction

MicroRNAs are small non-coding RNAs have been regulate gene expression in development, cell differentiation, cell cycle and apoptosis. Aberrant expression of miRNAs are involved in the initiation, migration, invasiveness and metastasis of cancer and therefore unique microRNA expression profiles are very important to determine different types of cancer and early diagnosis (He et al., 2004; Calin et al., 2006; Vasudean et al., 2007; Filipowicz et al., 2008; Allegra et al., 2012).

Breast cancer is a type of cancer which is the most common type among women. The incidence varies widely from country to country, however approximately 39.620 women are expected to die from this disease among US women in 2013 (Desantis et al., 2014). Dysregulated miRNA expression plays an important roles in breast tumor progressions, metastases and response to treatments (Iorio et al., 2008; Mirnezami et al., 2009; Erturk et al., 2014; Demirdogen Sevinc et al., 2015). Several miRNAs such as miR-21, miR-200 family and miR-155 were upregulated, whereas miR-145, miR-10b and especially miR-195 were downregulated in human breast cancer (Iorio et al., 2005; Lu et al. 2005; Corcoran et al., 2011; Kayani et al., 2011).

Circulating biomarkers play an important role in clinical applications including prognosis, diagnosis, monitoring their effects and predicting recurrence in cancer patient. Although the roles of circulating miRNAs in disease therapy remain unclear, recent studies have proved that circulating miRNAs might be a novel strategy for breast cancer therapy due to their stability and predictive properties (Zhu et al., 2009; Roth et al., 2010; Heneghan et al., 2010; Madhavan et al., 2012; EK et al., 2013). Recent studies focus on miR-195 as a potentially ideal breast tumor marker because of its unique circulating levels in patient blood. The levels of miR-195 in the blood is upregulated in before surgery and then downregulated to

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basal levels after surgery particularly 2 weeks post-tumour resection. Furthermore, although blood-based level alters before and after surgery in breast cancer patients, miR-195 expression level is significantly downregulated in breast tumor tissue. Therefore, blood-based miRNA analysis has an important clinical utility for breast cancer (Heneghan et al., 2010; Heneghan et al., 2011; Schrauder et al., 2012).

miRNAs differentially expressed among different ethnic populations because of the effect of socioeconomic, environmental, genetic and epigenetic factors (Shavers et al., 2002; Jack et al., 2013) and also previous studies reported that there was a potential relationship between circulating miR-195 level pre- and post-operative periods of breast cancer bloods compared with tissue levels.

In the present study, we determined miR-195 expression level in tumor tissues and blood samples which were collected in pre- and post-operative periods from Turkish breast cancer patients.

Materials and Methods

Patient selection

Both tumor tissues and paired normal adjacent tissues which were pathologically confirmed were collected from 96 cases of breast cancer patients. All the samples were recruited at Uludag University, Medical Faculty, Breast Surgery Department, Bursa, Turkey. The histologic tumor profile of breast cancer cases were consisted of luminal A (53.1%), luminal B type (46.9%) and analyzed in this study. Additionally, 48 of 96 breast cancer patients with tumor and normal tissue were selected for the purpose of detecting circulating miR-195 level in pre- and post-operative periods and this level compared with 12 healthy people blood samples. The pre-operative serum samples of 48 breast cancer patients were collected before surgery and the post-operative samples were obtained after 14 days. All samples were obtained after written informed consent and collected using protocols approved by the local ethics committee (2012/4) and confirmed to the ethical standards of the Helsinki Declaration.

RNA Isolation

TRIZOL LS reagent (Ambion, Thermo Fisher Scientific, ABD) was used to isolate the total RNA from tissues and blood sample according to the manufacture's protocols. The obtained RNA was eluted with 50 μL of RNAse-free water. The concentration of all RNA samples were measured by NanoDrop 2000 Spectrophotometer and was stored at –80°C until cDNA synthesis.

miRNA expression analysis

Reverse transcription was performed by the RT2 miRNA First Strand Kit (QIAGEN, Germantown, Maryland, USA). In brief, 4 µl of total RNA was supplemented with Hispec buffer (X5), nucleics mix (10X), Miscript Reverse Transcriptase mix (RT mix) and nuclease-free water in a total reaction volume of 10µl. Thermal-cycling conditions were as follows: 37°C for 60 min, 95°C for 5 min and 4°C for 5 min followed by melting.

miRNA expression levels were quantified by Roche Light Cycler480 II (Roche, Switzerland) with RT2 miRNA PCR arrays (RT2 Profiler; SABiosciences, Frederick Md, USA). To determine miR-195 expression level, 2,5 µL of RT reaction was mixed with 6,75 µL of Fermentas SYBR green, 1,25 µL of Qiagen primer mix and 2,5 µL water to get a final volume of 13 µL (n=3). To profile RNA integrity in samples, we quantified the expression level of the human SNORD 44 housekeeping gene. As shown in Table 1, the reaction was performed at 95°C for 10 min., followed by 35 cycles at 95°C for 15 s and 60°C for 30 s and then ramped from 65 to 95°C to obtain the melting curve. Each fold change of tissue sample compared with normal tissue in miR-195 expression was calculated and then make average by the comperative cycle threshold (^{2}-ΔΔCT) method. (Livak et al., 2001).

Statistical analysis

RT2 Profiler PCR Array Data Analyse (http://www.sabiosciences.com/pcr/arrayanalysis.php) was used to compare the determined results of RT-PCR array analysis obtained from breast cancer patients. The statistical

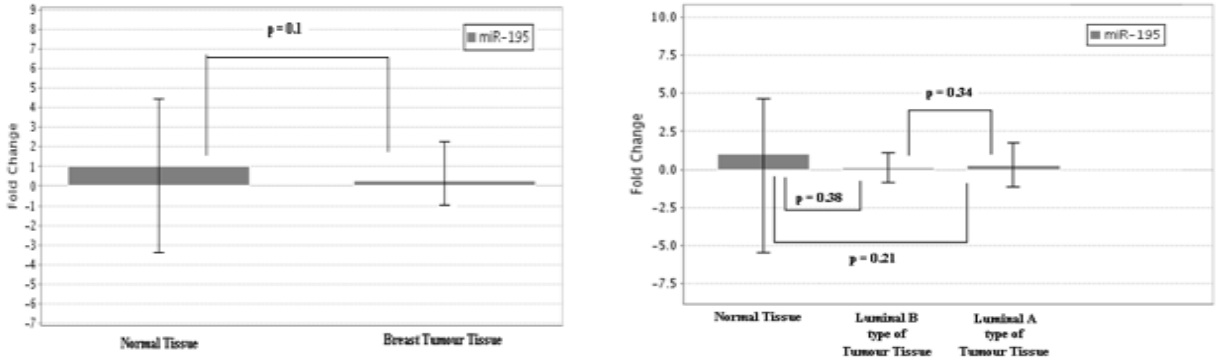

Figure 1. (A) The miR-195 expression level in breast cancer tissues compared with normal tissues. (B) The miR-195 expression level in luminal A and B epithelial subtype compared with normal tissues (n=3)

Table 1. miR-195 Specific forward Primer Sequences and qPCR Conditions

Gene name	Primer sequence	Cycling condition
miR-195	5'UAGCAGCACAGAAAUAUUGGC	95°C/10 min; 35 cycles 95°C/15 s; 60°C/30 s

Table 2. Summary of Demographic, Clinic nd Pathological Details of Breast Cancer Patients

	Breast Cancer Blood Cohort n = 48 n(%)	Breast Cancer Tissues Cohort n = 96 n(%)
Mean age, yr	54.2	55.4
Range	19-78	19-78
Family History		
Positive	27 (56.2)	59 (61.4)
Negative	21 (43.7)	37 (38.5)
Stage		
I	10 (20.8)	11 (11.4)
II	23 (47.9)	42 (43.7)
III	15 (31.2)	43 (44.7)
IV	-	-
Invasive Tumor Type		
Ductal	48 (100)	96 (100)
Lobular	-	-
Inflammatory	-	-
Other	-	-
Tumor size		
<5	32 (66.6)	67 (69.8)
>5	16 (33.3)	29 (30.2)
Lymph Node Metastasis		
Positive	39 (81.2)	71 (73.5)
Negative	9 (18.7)	25 (26.1)
In situ component		
<%25	26 (54.1)	59 (61.4)
>%25	22 (45.8)	37 (38.5)
Epithelial subtype		
Luminal A	26 (54.1)	51 (53.1)
Luminal B	22 (45.8)	45 (46.8)
Basal	-	-
HER2/neu	-	-
Estrogen receptor status		
Positive	48 (100)	96 (100)
Negative	-	-

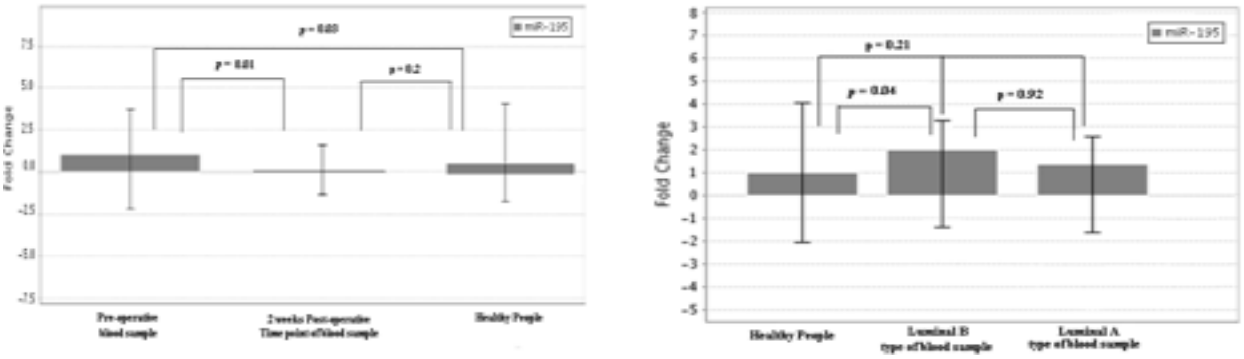

Figure 2. miR-195 Expression. (A) Circulating miR-195 expression levels in pre- and post-operative blood samples from breast cancer patients and controls. (B) Results of miR-195 plasma levels in pre-operative samples according to histologic tumor profile of breast cancer cases (n=3)

analysis was performed by the SPSS software package, version 21.0 (SPSS Inc. USA).The 2-sample t test was used for all 2 sample comparisons to compare the mean response between the levels of the between subject factors of interest. $p<0.05$ was considered statistically significant.

Results

Patient characteristics

The present study included 96 unrelated breast cancer patients and 48 pre- and post-operative blood samples were selected among them. Details of the clinical profiles and pathological features of breast cancer patients characteristics, including age range of patients, family history, tumor types and sizes, lymph node metastasis, in situ companent, epithelial subtype, estrogen receptor and HER2/neu status were shown in Table 2.

Dysregulated Expression of miR-195 in the Breast Cancer Tissues

We analyzed the mRNA expression levels of miR-195 in 96 tumor samples (Luminal A and B type) and compare the adjacent normal tissues by real time PCR. As shown in Figure1A, miR-195 expression was decreased in

breast cancer tissues compared with those in the normal adjacent tissues. Additionally, we found that miR-195 expression was reduced in Luminal B type compared to Luminal A type (p=0.34). Although miR-195 level could also be identified in each type (Figure 1B), we found no significant data.

Detection of Circulating miR-195 Plasma Level in the Breast Cancer Patients

To investigate whether the levels of the miR-195 in blood of breast cancer patients depends on breast cancer type, we measured the blood levels of the miR-195 in 48 breast cancer patients. In general, miR-195 was observed to be significantly overexpressed in the blood of breast cancer patients before operation in comparison to (p=0.01) post-operative samples (Figure 2A). Additionally, miR-195 level was significantly increased in pre-operative period compared to disease-free control subjects (p=0.03). Our data showed miR-195 levels in Turkish breast cancer patients before surgery were 4.5-fold (unlogged fold change) higher than after surgery. Furthermore, circulating miR-195 level was differentially expressed in pre-operative period in luminal A (p=0.21) and B types (p=0.04) compared to control in Figure 2B, respectively.

Discussion

Alterations in miRNA expression signatures in tumor tissues could be used as biomarkers in diagnosis and prognosis of breast cancer due to the fact that they have correlated with tumorigenesis, metastasis, poor prognosis and response to therapy in human breast cancer. Thus, a promising approach is to detect cancer at an early stage by developing miRNA-based therapeutics (Lowery et al., 2008; Shen et al., 2008; Wu et al., 2008; Asaga et al., 2011; Kavitha et al., 2014). Additionally, recent research studies have focused on the exact mechanism of cancer-associated circulating miRNAs as biomarkers because of their potential for detection of disease, diagnosis and prognosis, and prediction of response to therapy and recurrence (Kosaka et al., 2010; Ma et al., 2012; Cortez et al., 2012; Chen et al., 2013; Aquilar et al., 2013).

In breast cancer, some studies have assessed the use of circulating miRNAs as biomarkers (Roth et al., 2010; Zhao et al., 2010; Qu et al., 2011). Sun et al. found that serum miR-155 expression is a potential biomarker to distinguish breast cancer patients from healthy people and declined after surgery and chemotherapy. So it may be used as an indicator for treatment response (Sun et al., 2012). Zhu et al. demonstrated that PR-positive tumors had higher miR-155 expression levels in serum from 21 women with and without breast cancer (Zhu et al., 2009). Wang et al. evaluated that circulating miR-125b expression is associated with chemotherapeutic resistance of breast cancer (Wang et al., 2012). Si et al. determined that circulating miR-92a may be used to discriminate the breast cancer patients from healthy controls correlates with tumor size and lymph node metastases (Si et al., 2013). Chan et al. found that mir-145, and mir-21 the most consistently detected and attractive candidate for clinical applications for breast cancer patients (Chan et al., 2013). Cuk et al. identified that four miRNAs (miR-148b, miR-376c, miR-409-3p and miR-801) were shown to be significantly upregulated in the plasma of breast cancer patients (n=127) compared healthy controls (n=80) and they might be of potential use to improve early detection of breast cancer (Cuk et al., 2013). Madhavan et al. detected nine miRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801) which were able to discriminate between metastatic breast cancer patients (Madhavan et al., 2013). Wu et al. found that miR-122 level might be use in the circulation predicts breast cancer metastasis in early-stage patients (Wu et al., 2012). As a result, circulating microRNAs differentially expressed serum and cancer tissues compared with control groups and so they can be used as diagnostic and prognostic biomarker and they also can be used effectively for response to therapy.

Circulating miR-195 is differentially expressed in breast cancer patients identified in the literature and its level changes according to tumor type and size. Therefore circulating miRNAs may be useful in clinical management. Zhang et al. identified that miR-195 was significantly elevated in breast tumor compared with normal breast tissue (Zhang et al., 2009). Luo et al. determined that the expression level of miR-195-5p was significantly decreased in 40 breast cancer compared with adjacent normal breast tissues. Besides, overexpression of miR-195-5p inhibited cell proliferation and caused an accumulation of cells in the G1 phase of the cell cycle (Luo et al., 2014). Hennegan et al. compared mir-195 and let-7 expression level in breast cancer blood and breast tumor tissue. They demonstrated that miR-195 and let-7a were significantly increased in the blood of breast cancer patients in comparison to control subjects. Additionally, they found that miR-195 levels in breast cancer patients were associated with certain clinicopathologic variables, included lymph node status, tumor size, presence of lymphovascular invasion, histological grade and ER status (Heneghan et al., 2010; Heneghan et al., 2010).

The miRNA expression profiles of breast cancer patients differ in ethnic origins because of genetic and environmental factors. The unique features of racial groups derived from alterations in the gene and miRNA expression profiles of the tumors give rise to biologically important differences in the molecular profile of a tumor and so determine the patient's response to cancer therapies (Shavers et al., 2002; Wiencke et al., 2004; Sarfati et al., 2006). In present study, we measured miR-195 expression level in Turkish breast cancer patients. Circulating miR-195 level was significantly decreased breast cancer patients tissues compared to healthy controls.

Additionally, we were also able to identify miR-195 signature that were associated with Luminal A and Luminal B type, estrogen receptor (ER), progesterone receptor (PR), and Her2 status. The luminal type of breast cancer is morphologically well differentiated and exhibits a relatively good prognosis however luminal B subtype is understood to be the more aggressive form of ER+ breast cancer and worse outcomes (Creighton et al., 2012; Yanagawa et al., 2012;Uchida et al., 2013; Zhang et al., 2013). In our study, circulating mir-195 levels that

were indicative of the tumour's type have the potential to be developed as blood biomarker. We think that miRNA expression may be used for classification of molecular tumor subtypes. miR-195 expression level can be able to distinguish breast cancer samples from healthy controls as well as its level changes tumor tissues compared blood samples before and after surgery. We found that circulating miR-195 levels have altered in pre- and post-operative periods of breast cancer patients. It is possible that the decreased levels of circulating miR-195 in post-operative periods provide evidence that certain miRNA signatures might be used as considerable biomarker for follow-up and management of patients with breast cancer. Three possible mechanisms which related to tumor-associated circulating miRNAs with tumors level are predicted: releasing from tumor cell death, secretion by tumor cells and originate from immunocytes in the tumor microenvironment. However, their exact mechanism and functions in cancer are not well understood (Ma et al., 2012; Wang et al. 2014).

In conclusions, Circulating tumor associated miRNAs could indicative to the improvement of new diagnostic and therapeutic biomarkers for breast cancer patients. The present study provides the first report of the possible relationship between circulating and tissue miR-195 level of breast cancer patients in the Turkish population and miR-195 expression level can differentiate in epithelial subtype of tumors. However, circulating microRNA signatures should be validated how to detect breast cancer even in its earliest stages and more studies are necessary to explore the potential role of circulating miRNAs as clinically novel biomarkers for breast cancer.

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