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Relationship of the expression of circulating *hsa-miR-125a-3p* and *hsa-miR-125b* with breast cancer

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Breast cancer is a major leading cause of cancer death in women in almost all countries, including developing countries. Despite the significant progress that has been made in cancer detection and treatment, around 30% to 70% of breast cancer patients die due to the cancer relapse or metastasis [1]. Therefore, the identification of diagnostically sensitive, specific and non-invasive biomarkers for recurrence and early detection of breast cancer are urgently needed. Circulating microRNAs (miRNAs) are potential blood-based biomarkers for the prognosis and diagnosis of various diseases and malignancies, including breast cancer [2]. MiRNAs are genome-encoded ~22-nucleotide non-protein-coding RNA molecules that act as crucial post-transcriptional regulators of gene expression through degradation of target mRNAs and their translation inhibition [3]. MiRNAs regulate gene expression in numerous biological processes comprising proliferation, development, cellular differentiation and apoptosis [4]. The *miR-125* family is conserved in a wide range of species from nematode to humans. The family has three homologs: *hsa-miR-125a, hsa-miR-125b-1* and *hsa-miR-125b-2*. Controversial properties of the *miR-125* family members have been investigated in diverse cancer types including their potential contribution to the beginning and progression of cancer by acting as either tumour suppressors or tumour promoters. Furthermore, the miR-125 family has a pivotal role in cancer pathogenesis and in response to chemotherapy, so that increased expression levels of *miR-125a* and *miR-125b* are related to cisplatin and paclitaxel resistance in nasopharyngeal carcinoma and colon cancer, respectively [5]. We hypothesized differences in circulating leukocyte *miR-125a-3p* and *miR-125b* in women with breast cancer.

We tested the hypothesis in 90 breast cancer patients recruited from hospitals in Rasht, Iran between April and November 2017, and a control group of 120 age-matched women as healthy controls. Exclusion criteria were previous malignant tumours in other organs; previous chemotherapy or surgery and patients with no pathological data. Clinical data on the patients, including age, menopausal status, breast-feeding history, tumour type and size, staging, LN status, ER, PR and HER2 status were obtained from hospital records. All subjects gave written informed consent in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Peripheral blood samples were collected in EDTA-containing tubes and were immediately processed by ficoll (Biowest, Nuaille, France), then centrifuged at 400 g for 23 min at 4°C to spin down the blood cells, and followed by a second centrifugation of the supernatants at 260 g for 10 min at 4°C. The cell pellet contained white blood cells. MiRNA was isolated using an miRNA isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The miRNA quality and quantity were assessed by agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Fisher Science, Mass, USA), respectively. MiRNAs polyadenylation was performed by *E coli* poly(A) polymerase and a Poly(A) tailing kit, according to the manufacturer's recommendation (New England Biolabs, Mass, USA). Complementary DNA (cDNA) was synthesized using the 2XRT-PCR Pre-mix Kit (BioFact, Kildare, Ireland) followed by RT-PCR. *U48* was used as internal control gene. The anchored oligo-dts (5′GCGTCGACTAGTACAACTCAAGGTTCTTCC AGTCACGACG(T)18V) and *U48* specific primers (F: 5′-TGACCCCAGGTAACTCTGAGTGTGT-3′ and R: 5′-AACT CAAGGTTCTTCCAGTCACG-3′) were designed according to [6]. The *miR-125* specific primers, miR-125a(F: 5′- ACAGGTGAGGTTCTTGGGAG –3′, R: 5′-AACTCAAG GTTCTTCCAGTCACG- 3′) and miR-125b(F: 5′-CTCAGT CCCGACCCTAA-3′, R: 5′-, CGTCGACTACAACTCAA) were designed by Oligo7 software. Analysis of all

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primers was carried out using NCBI primer designing and primer BLAST tools. Primers were synthesized by Macrogen, Seoul, South Korea. Validation of RT-PCR results and evaluation of *miR-125* expression level, real-time PCR was done using an Applied Biosystems (Mass, USA) thermocycler in a 15 µl reaction volume. The PCR reaction included 1 µl of cDNAs, 7.5 µl of 1X SYBR-Green I Mastermix (BioRon, Ludwigshafen, Germany), 5.5µl of RNase-free water, 0.5 µl(5Pm) of each *miRNA* specific forward and reverse primers. The PCR parameters were incubation at 94°C for 5 min, then 40 cycles of 94°C for 30 sec, 53.5°C for 30 sec and 72°C for 30 sec followed by 72°C for 5 min. All reactions were performed in triplicate.

The threshold cycle (Ct) was calculated by one step software (Applied Biosystems) using the automatic threshold setting. The average expression levels of all samples were normalized to *U48* gene and expression fold-changes of *miR-125a* and *miR-125b* in patients' bloods to the healthy people were calculated using $2^{-\Delta\Delta ct}$ method. The Kolmogorov–Smirnov test was applied to test the normality of *miR-125a* and *miR-125b* expressions in both groups, and t-test analysis with P value computations was done at $p < 0.05$. Statistical analysis was performed using SPSS for Windows version 23.0.

No significant differences were observed between the age, menopausal status and breastfeeding history status of cases and controls (Table 1). Mean expression level of *miR-125a-3p* in breast cancer patients was significantly decreased by almost half compared to healthy controls, while *miR-125b* was elevated nearly twofold. *miR-125a-3p* and *miR-125b* expression levels had a negative correlation ($r = -1.00$, $p = 0.001$) in breast cancer patients. *miR-125a-3p* and *miR-125b* expression levels in different clinico-pathological conditions are shown in Table 2.

We report a significant down-regulation in *miR-125a-3p* and upregulation of *miR-125b* in breast cancer subjects compared to the control group, in agreement with many other findings. Hsieh *et al*. showed serum *miR-125a* decreased in breast cancer blood [7]. He et al. showed *miR-125a-5p* and *miR-125b* significantly decreased in breast cancer tissues while *miR-125a-3p* was not linked with breast cancer [8]. Likewise, down-regulation of *miR-125a* has been observed in breast cancer tissue, and other types of cancers show *miR-125a* inhibits the proliferation, invasion and migration of cancer cells [9–11]. Also, up-regulation of *miR-125a-5p* suppresses proliferation and induces apoptosis through augmentation of p53 signalling in lung cancer [12]. *hsa-miR125a-3p* expression is significantly decreased in chemoresistant breast cancer cells, suggesting that *hsa-miR125a-3p* may act as a tumour suppressor through modulation of BRCA1 signalling [10].

Overall, previous studies and our results demonstrated that *miR-125a* is negatively correlated with human breast cancer progression and could function to inhibit tumour growth. Results of this work clearly show statistically significant high expression of leukocyte *miR-125b* in breast cancer patients. Wang et al. [13] indicated that chemotherapeutic resistance in advanced ductal carcinoma breast cancer was correlated to high expression levels of circulating *miR-125b*. Also, *miR-125b* expression significantly elevated in the breast cancer tissues and correlated with Tumour-Node-Metastasis stage and tumour size in HER2-positive breast cancer patients [14]. Moreover, *miR-125b* directly targets the tumour suppressor gene p53 and other genes belonging to the p53 network [15]. The results indicate that circulating *miR-125* family members could function as potential prognostic biomarkers and molecular targets in breast cancer treatment. However, further functional analyses

Table 1. Clinical characteristics of breast cancer patients and healthy controls.

Variable	Cases n (%)	Controls n (%)	P-value
Age (years)	58.4 [7.6]	57.9 [8.2]	0.774
Menopausal status			
Premenopausal	33 (36.7)	48 (40)	0.62
Postmenopausal	57 (63.3)	72 (60)	
Breastfeeding history			
Positive	69 (76.7)	104 (86.7)	0.06
Negative	21 (23.3)	16 (13.3)	
miR-125a-3p	0.62 [0.1]	1.34 [0.13]	<0.001
miR-125b	0.61 [0.04]	0.31 [0.01]	<0.001

Data mean [SD] or n (%)

Table 2. *miR-125a-3p* and *miR-125b* expression levels in each clinico-pathological status.

	miR-125a-3p expression		*miR-125b* expression	
Characteristic	Mean[SD]	P	Mean[SD]	P
Oestrogen receptor				
Positive (n = 53)	0.32 [0.09]	<0.001	0.76 [0.06]	<0.001
Negative (n = 37)	0.64 [0.09]		0.53 [0.08]	
Progesterone receptor				
Positive (n = 65)	0.57 [0.06]	<0.001	0.73 [0.06]	<0.001
Negative (n = 25)	0.68 [0.07]		0.57 [0.07]	
HER2 status				
Positive (n = 21)	0.64 [0.06]	<0.001	0.68 [0.05]	<0.01
Negative (n = 69)	0.75 [0.04]		0.61 [0.06]	
Nodal status				
Positive (n = 66)	0.57 [0.06]	0.87	0.59 [0.02]	0.011
Negative (n = 24)	0.57 [0.04]		0.55 [0.03]	
Tumour size				
≤5 cm (n = 29)	0.62 [0.04]	<0.001	0.67 [0.07]	0.033
>5 cm (n = 61)	0.68 [0.02]		0.60 [0.04]	
Tumour site				
Ductal (n = 77)	0.54 [0.05]	0.81	0.59 [0.05]	0.88
Lobular (n = 13)	0.53 [0.06]		0.58 [0.04]	
Stage				
0-Ⅱ (n = 32)	0.71 [0.06]	<0.01	0.58 [0.04]	0.79
Ⅲ-Ⅳ (n = 58)	0.81 [0.07]		0.57 [0.04]	

Data mean [SD]

determining the precise role of circulating *miR-125* family in the pathogenesis of human breast cancer are suggested.

This work represents an advance in biomedical science because it shows a link between leukocyte *miR-125a* and *miR-125b* expression levels and breast cancer.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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