RESEARCH ARTICLE

Circulating Cell-free miRNA Expression and its Association with Clinicopathologic Features in Inflammatory and Non-Inflammatory Breast Cancer

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Abstract

Recent discovery showing the presence of microRNAs (miRNAs) in the circulation sparked interest in their use as potential biomarkers. Our previous studies showed the diagnostic potential of miR-451 as a serological marker for inflammatory breast cancer (IBC), miR-337- 5p and miR-30b for non-inflammatory breast cancer (non-IBC). The aim of this study is to investigate the prognostic values of circulating miRNAs by comparing the amounts of 12 circulating miRNAs in the serum of IBC and non-IBC from Tunisian breast cancer patients, and by determinating whether correlated pairs of miRNAs could provide useful information in the diagnosis of IBC and non-IBC patients. TaqMan qPCR was performed to detect circulating expression of miRNAs in serum of 20 IBC, 20 non-IBC and 20 healthy controls. Nonparametric rank Spearman rho correlation coefficient was used to examine the prognostic value of miRNAs and to assess the correlation profile between miRNAs expression. Further, a large number of miRNAs were highly correlated (rho>0.5) in both patients groups and controls. Also, the correlations profiles were different between IBC, non-IBC and healthy controls indicating important changes in molecular pathways in cancer cells. Our results showed that miR-335 was significantly overexpressed in premenopausal non-IBC patients; miR-24 was significantly overexpressed in non-IBC postmenopausal patients. Patients with previous parity had higher serum of miR-342-5p levels than those without. Furthermore, patients with HER2+ IBC present lower serum levels of miR-15a than patients with HER2- disease. Together, these results underline the potential of miRNAs to function as diagnostic and prognostic markers for IBC and non-IBC, with links to the menopausal state, Her2 status and parity.

Keywords: Breast cancer - inflammatory breast cancer - circulating miRNAs - biomarker. prognostic - diagnostic

Introduction

MicroRNAs (miRNAs) are small noncoding RNA oligonucleotides (22-25 nucleotides) that suppress gene expression post-transcriptionally via sequence-specific interactions with cognate mRNA targets, they suppress translation and induce degradation of mRNAs. Because of their broad targeting range, miRNAs are expected to be involved in most cellular processes. Therefore, dysregulation of the expression of miRNAs results in diverse disease states (Sayed and Abdellatif, 2011). Aberrations in miRNAs expression levels influence the occurrence and development of tumor and have been linked to the onset and progression of various types of cancer (Palmero et al., 2011). In this particular context, the roles of miRNAs in carcinogenesis and tumor progression are mainly associated with miRNA-regulated expression of oncogenes and tumor suppressor genes (Croce, 2009).

In fact, the overexpression of certain miRNAs could lead to the repression of tumor suppressor genes. Willingly, miRNAs down-regulation could result in increased oncogene expression (Li et al., 2013). Importantly, in previous work it has been demonstrated that extracellular miRNAs levels are surprisingly stable in body fluids ,retrievable and measurable from fresh or archival serum and plasma samples (Chen et al., 2008; Mitchell et al., 2008; Schwarzenbach et al., 2014).

Since then, accumulated studies have convincingly demonstrated that the extracellular miRNAs are released into the blood circulation by apoptotic and necrotic cells (Schwarzenbach et al., 2011) or high density lipoproteine (HDL) particles, and exist either cell-freely, complexed with AGO proteins, or in exosomes in the blood circulation (Simpson et al., 2009). In the case of cancer disease, the use of a less invasive method than tissue biopsy is required and it may represent a major difference in clinical

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outcome. Hence, a large number of studies have assessed the potential use of circulating miRNAs as biomarkers in various types of cancer, including breast cancer, gastric, hepatocellular, non-small cell lung cancer (Konishi et al., 2012; Tomimaru et al., 2012; Hagrass et al., 2015), Table 1.

Breast cancer is a malignant breast neoplasm originating from breast tissues, and it's the cause of cancer related mortality among women worldwide. Breast cancer is a highly heterogeneous disease that has multiple subtypes with distinct clinical outcomes. Inflammatory breast carcinoma (IBC) is the most aggressive manifestation of primary breast carcinoma (Yamauchi et al., 2012). Simultaneously, IBC patients have worse survival outcomes compared with non-inflammatory breast cancer patients even with multiple improved treatments, such as polychemotherapy and endocrine regimens (Dawood and Cristofanilli, 2011; Dawood et al., 2011). Our previous miRNAs expression analysis revealed that miR-451, miR-342-3p, miR-30b, miR-24 and miR-337-5p were dysregulated in inflammatory and non-inflammatory mammary tumors when compared to healthy controls as well as among the two groups of breast cancer. Serum levels of miR-451 were significantly downregulated in IBC compared with healthy controls. While serum levels of miR-337-5p and miR-30 were significantly over-expressed in non-IBC samples compared with healthy controls, suggests that these miRNAs may be used as a biomarker for diagnosis of IBC and non-IBC (Hamdi et al., 2014). These results promoted us to investigate the possible role of these miRNAs in breast cancer carcinogenesis.

Heneghan and co-investigators demonstrated that circulating miR-195 was found to be breast cancer specific and it could significantly differentiate breast cancer from other cancers and healthy controls. Furthermore, serum levels of miR-195 were correlated with nodal and estrogen-receptor status (Heneghan et al., 2010) .In addition, miR-155 may be differentially expressed in the serum of women with hormone sensitive compared to women with hormone insensitive breast cancer (Zhu et al., 2009).

Interestingly, another study of breast cancer demonstrated that patients at advanced tumor stages had significantly higher miR-34a than patients at early tumor stages. In the metastatic patients, miR-10b, miR-34a, and miR-155 correlated with the presence of overt metastases circulating microRNAs (Roth et al., 2010).

In the current study, we aimed to investigate the association of 12 serum miRNAs expression levels with clinicopathologic features in IBC and non-IBC serum, and by determining whether correlated pairs of miRNAs could provide useful information in the discrimination between IBC and non-IBC. The findings may increase our understanding of the miRNAs signature in IBC and non-IBC and suggest a potential diagnostic or prognostic strategy for targeting specific miRNAs.

Materials and Methods

Blood samples
Experiments were conducted in accordance with the principles of Declaration of Helsinki. This study was approved by the Medical Ethics Committee of Pasteur Institute of Tunis and the Georgetown University Institutional Review Board. Written informed consents were obtained from all the participants.

Serum samples were collected from 60 women diagnosed and treated at Hannibal International Clinic of Tunis from March 2011 and January 2012 before they underwent any therapeutic procedure : 20 with non-IBC, 20 with IBC, and 20 cancer-free women were enrolled in our study. Control blood samples were collected at the same period from healthy women volunteers with no history of malignant diseases, no blood donations received in the previous 3 years, and no current inflammatory conditions.

5 ml of blood was collected from each woman directly into serum collection tubes. The whole blood was allowed to stand for about 1 h at RT before being centrifuged at 1200 g for 10 minutes at 4°C followed by careful separation of serum. Another centrifugation was performed for 10 min at $10,000 \times g$ at 4°C to remove the cellular debits. The resultant serum was aliquoted into eppendorf tubes and stored at -80°C.

TNM stage was assessed according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging manual. Samples were stained histologically to determine histological grade, which was classified into low, moderate and poor grade according to criteria modified by Elston and Ellis. The status of ER, PR and HER2 were detected by immunohistochemistry (IHC). Tumor characteristics are provided in Table 2.

RNA extraction
Total RNA was extracted from 500 µl serum using mirVana miRNA Isolation Kit (Life Technologies) according to the manufacturer's instructions. The quality and yield of each RNA sample was measured using a NanoDrop ND-1000 Spectrophotometer. The RNA specimens were stored at - 80°C until reverse transcription.

Reverse Transcription and Real-Time PCR
In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using specific miRNA primers from the TaqMan MicroRNA Assays and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies). The amount of RNAs used for RT was 2 ng and the cDNA specimens were stored at - 20°C until PCR. In the PCR step, PCR products are amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan® Universal PCR Master Mix . The PCR reactions were performed on 7900 Real-Time PCR system following the manufacturer's protocol. (Applied Biosystems, United States). For each sample, the miRNAs expression levels were assayed in triplicate and were run with endogenous control RNU-48 (Control miRNAs Assay, Applied Biosystems, USA). also in triplicate, with a single no template negative control reaction. The normalization step was performed according to the 2-((Mean ΔCt Target)-(Mean ΔCt Calibrator)) method (Livak and Schmittgen, 2001).

Statistical analysis was performed using SPSS 20.0 statistical software package for Windows.

Mann-Whitney U nonparametric test was used for comparing two different groups such as premenopausal and postmenopausal. Coefficient correlations were calculated using Spearman's rho test to find a correlation between two variables.. All P values lower than 0.05 were considered statistically significant.

Bioinformatics Tools for Predicting miRNA Targets

The predictive potential target genes of miRNAs were searched in 6 on-line databases, including MIRBD, HSA-MIRBase, TARGETSCAN,TARGETMINER, TARGETSCAN-VERT and KEGG PATHWAY Database.

Results

Correlations between Expression of Different miRNAs.

The expression levels of twelve different miRNAs that were known to be deregulated in various cancers were

quantified by real-time PCR analysis. In total, 20 serum samples from non-inflammatory, 20 from inflammatory breast cancer cases and 20 healthy controls were included in the analysis. Patients were recruited from Hannibal International Clinic of Cancerology in Tunis. In the first step, they were classified on the basis of their clinical and histopathological profiles, according to the tumor size, axillary's lymph node status, and histological grade, ER expression and human epidermal growth factor receptor 2 (HER2) amplification. There were no differences between the groups, the only difference between the groups was related to Oestrogen receptor status (p =0.0018) and metastasis (p=0.001). Tumor characteristics are provided in Table 2.

In complementary to serum miRNAs expression analysis in IBC and non-IBC groups, we assessed not only individual changes in mean miRNAs expression levels among groups, but changes in how miRNAs were correlated with each other across groups. For this analysis, non parametric rank Spearman rho correlation showed that a large number of miRNAs were highly (rho>0.5)

Table 1. A compendium of Circulating miRNAs with Potential as Biomarkers for Breast Cancer and other Human Malignancies

miRNAs	Cancer type	Body fluid source	Clinical correlations	Reference
miR-335	Breast	Serum	Overexpressed miR-335 were associated with breast cancer	(Wang et al., 2010)
			histological tumor grades and hormone receptor expression status	
miR-24	Ovarian	Plasma	lower levels of miR-24 in patients than controls	(Wu et al., 2012; Shapira et al., 2014; Fang et al., 2015)
	Breast	Serum	Increased serum level of miR-24 in Breast Cancer patients.	
	Colorectal	Plasma	Decreased plasma levels of miR-24 serve as novel biomarkers for early stage detection of colorectal	
miR-320	Breast	Serum	Underexpressed in the serum of women who developed lymph node-positive breast tumors.	(Yan et al., 2008)
miR-548d-5p	Bladder	Urine	Expressed predominantly in patients with higher cancer stages	(Brisuda et al., 2014)
miR-342-5p	Ovarian	Whole blood	Decreased levels in ovarian cancer	(Hausler et al., 2010)
miR-15a	Breast	Serum	Increased serum level in older compared to young breast patients	(Kodahl et al., 2014)
miR-342-3p	Breast	Serum	High serum level in older compared to young breast patients	(Hatse et al., 2014)
miR-29a	Colorectal	Plasma	Increased plasma levels in advanced-stage cancer than controls	(Huang et al., 2010)
miR-451	Breast	Plasma	Increased plasma level found in breast cancer patients	(Van der Auwera et al., 2010)
miR-30b	Breast	Serum	high levels in the serum of breast cancer patients than control subjects	(Hamdi et al., 2014)
miR-337-5p	ESCC	Serum	Serum levels were differentially expressed between ESCC patients and controls	(Wu et al., 2014)
miR-486-3p	Prostate	Serum	Serum level were significantly higher in patients compared to controls	(Lodes et al., 2009; Cortez et al., 2011; Hatse et al., 2014)
	Breast	Serum	Increased serum level in older versus young breast cancer patients	

correlated in both patients groups and controls.

In non-inflammatory breast cancer group, all miRNAs analyzed in the current study were highly correlated with each other. Interestingly, absolute correlation was scouted between miR-15a and miR-486-3p (Spearman's rho 1.000; p=0.000). Likewise, miR-320 was highly correlated with miR-24 and miR-548d-5p (Spearman's rho, 0.839 and 0.823 respectively; p=0.000). Moreover, expression of miR-451 were correlated with miR-24 (Spearman's

rho, 0.615; p=0.04), as were miR-30b and miR-337-5p (Spearman's rho, 0.598; p=0.005). Note that miR-342-3p expression did not correlate with other miRNAs except miR-342-5p (Spearman's rho, 0.724; p=0.001). Nonetheless, mir-29a expression profiles did not correlate with other miRNAs (Table 3).

In inflammatory breast cancer, miRNAs correlation profiles were different from non-inflammatory breast cancer. We demonstrated that less correlation was observed in miRNAs expression profiles in IBC compared to that in non-IBC. miR-486-3p expression levels correlate strongly with miR-342-5p (Spearman's rho 1.000; p=0.000) but not with mir-15a as were in non-IBC. Contrary to his correlation profiles in non-IBC, serum miR-451 expression in IBC did not correlate with other miRNAs. Astonishingly, miR-342-5p and miR-342-3p did not significantly correlate, although they belong to the same family and co-localized. There was a slight correlation between miR-320 and mir-15a as compared to that across non-IBC (Spearman's rho 0.590; p=0.016) (Table 3).

A weak miRNAs correlation number was assessed in controls group. With the exception of miR-486-3p, miR-342-3p and miR-342-5p, there was no significant correlation showed in expression profiles of the miRNAs selected (Table 4).

Correlation between Circulating MicroRNA expressions and the clinic–pathologic features of patients with inflammatory and non- inflammatory Breast cancer.

Our first goal was to investigate the potential association of serum miRNA expression with tumor characteristics. We first measured the expression of 12 miRNAs, which have been selected based on previously supported roles in cancer progression and/or diagnostic utility. As shown in Table 5, in non-inflammatory breast cancer group, miR-24 and miR-335 level were correlated with Menopausal status (p=0.01 and p=0.007 respectively), the plasma levels of miR-335 were highly expressed in Pre-menopausal patients than in

Table 2. Tumour Characteristics

Clinicopathological features	IBC (N= 20)	non-IBC (N= 20)	P-value
Patients' ages (years)			
Mean	49	50.2	
Range	34-70	29-78	0.236
Tumour stage			
I	0(0%)	0(0%)	
II	0(0%)	6(30%)	
III	0(0%)	13(65%)	
IV	IVd20(100%)	IVb1(5%)	
Histological tumour gradea			
I	0(0%)	0(0%)	
II	9(45%)	7(35%)	0.264
III	11(55%)	13(65%)	
Node status			
N0	2(10%)	7(35%)	
N1	16(80%)	13(65%)	0.064
N2	2(10%)	0	
N3	0(0%)	0	
Distant metastasis			
M0	14(70%)	4(20%)	
M1	0(0%)	2(10%)	0.001
Unknown	6(30%)	14(70%)	
Oestrogen receptor			
Positive	3(15%)	11(55%)	0.0018
Negative	17(85%)	9(35%)	
HER2 amplification			
Positive	13(65%)	13(65%)	0.2

*HER2: human epidermal growth factor receptor 2; IBC: Inflammatory breast cancer

Table 3. Spearman Rank Correlation Coefficients between miRNA in IBC and non-IBC Samples

	miR-335	miR-24	miR-320	miR-548d-5p	miR-342-5p	miR-15a	miR-342-3p	miR-29a	miR-451	miR-30b	miR-337-5p	miR486-3p
miR-335		-0.16	0.3	0.52	-0.23	-0.11	0.15	0.23	0.04	0.21	0.34	-0.11
miR-24	0.48		0,83**	0.59	0.35	0.59	0.55	0.49	0,61*	0.43	0.23	0.59
miR-320	0.69	0.36		0,82**	0.3	0.53*	0.65	0.74	0.68	0.44	0.39	0.44
miR548d-5p	0.16	0.09	0.45		0.1	0.3	0.66	0.62	0.49	0.53	0.54	0.3
miR-342-5p	0.07	0.39	0.06	-0.23		0.55	0,72**	0.43	0.26	0.35	0.1	0.55
miR-15a	0.62	0.59	0,59*	0.06	0.55		0.27	0.43	0.19	0.35	0.36	1**
miR-342-3p	0.36	0.08	0.25	-0.1	0.57	0.57		0.6	0.59	0.36	0.37	0.27
miR-29a	0.34	0.28	0.21	-0.08	0.33	0.59	0.57		0.69	0.58	0.46	0.43
miR-451	0.57	0.76	0.27	-0.26	0.34	0.69	0.32	0.63		0.51	0.52	0.19
miR-30b	0.48	0.08	0.34	-0.23	-0.1	0.29	0.22	0.21	0.2		0,58*	0.35
miR-337-5p	0.49	0.5	0.12	-0.22	0.56	0.39	0.49	0.06	0.47	0.15		0.36
miR-486-3p	0.07	0.39	-0.03	-0.23	1**	0.55	0.57	0.33	0.34	-0.1	0.6	

non-IBC above the diagonal; IBC below the diagonal

*Correlation coefficients with indicate significant at the 0.05 level; ** Correlation coefficients with indicate significant at the 0.01 level

Table 4. Spearman Rank Correlation Coefficients between miRNA in healthy controls

	miR-335	miR-24	miR-320	miR-548d-5p	miR-342-5p	miR-15a	miR-342-3p	miR-29a	miR-451	miR-30b	miR-337-5p	miR486-3p
					Healty Controls							
miR-335	-	0.41	0.37	0.15	0.47	-0.26	0.25	0.13	0.41	0.18	0.28	0.25
miR-24	-	-	0.11	0.18	0.39	0.56	0	-0.28	-0.07	0.45	0.2	-0.06
miR-320	-	-	-	0.65	0.61	0.21	0.25	0.12	0.54	0.5	0.31	0.25
miR548d-5p	-	-	-	-	0.63	0.25	-0.04	0.1	0.66	0.39	0.51	-0.41
miR-342-5p	-	-	-	-	-	-0.24	0,82**	0.32	0.31	0.56	0.48	0.2
miR-15a	-	-	-	-	-	-	-0.09	0.25	-0.1	-0.31	-0.06	-0.09
miR-342-3p	-	-	-	-	-	-	-	0.3	-0.18	0.18	0.31	1**
miR-29a	-	-	-	-	-	-	-	-	0.13	0.14	0.16	0.3
miR-451	-	-	-	-	-	-	-	-	-	0.12	0.52	0.18
miR-30b	-	-	-	-	-	-	-	-	-	-	0.49	0.18
miR-337-5p	-	-	-	-	-	-	-	-	-	-	-	0.15
miR-486-3p	-	-	-	-	-	-	-	-	-	-	-	-

*Correlation coefficients with indicate significant at the 0.05 level; ** Correlation coefficients with indicate significant at the 0.01 level
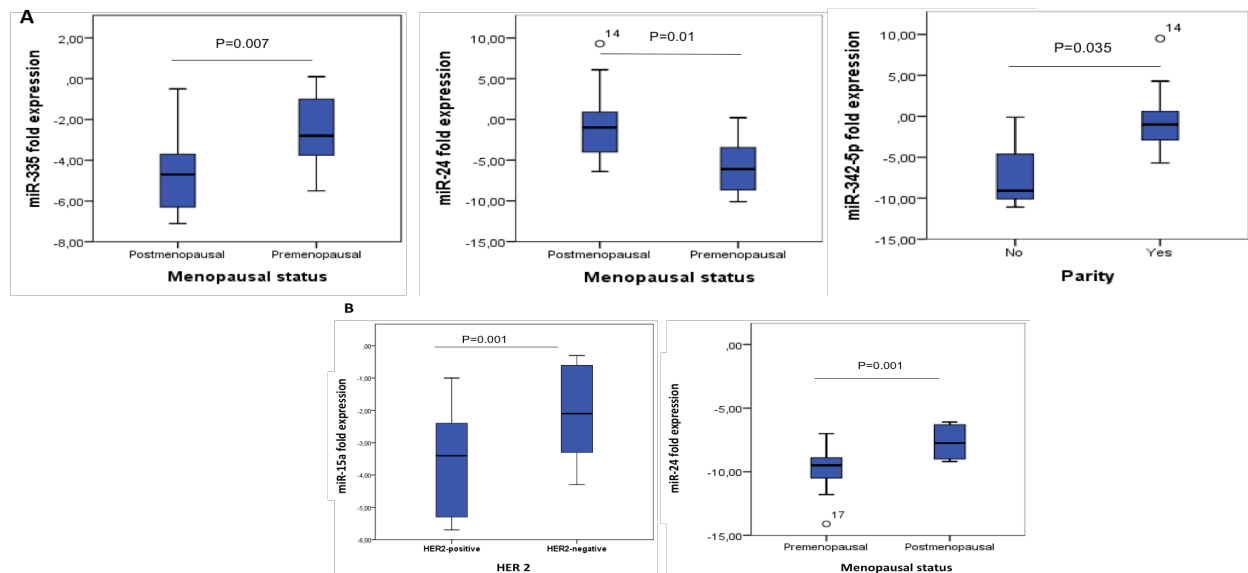

Figure 1. Serum miRNAs Levels in Patients with IBC and non-IBC. The box plots show: A) the fold change of the significantly differentially expressed miR-335 and miR-24 upon non-IBC groups based on menopausal status and miR-342-5p based on parity profile; B) the fold change of the significantly differentially expressed miR-15a and miR-24 upon IBC groups according to HER-2 and menopausal status respectively. The differences in serum levels were evaluated using the Mann-Whitney U test, and p values are indicated above the plots

Post-menopausal. However, Post-menopausal breast cancer patients had a significantly higher plasma levels of miR-24 than Pre-menopausal breast cancer patients. Similarly, miR-342-5p level were significantly associated with parity (p=0.035). We noticed that miR-342-5p had a higher expression level in patients with previous parity than those without (Table 5; Figure 1).

To determinate whether miRNAs were linked to the clinicopathological parameters in IBC, Spearman correlation test analysis demonstrated significant correlations between the expression of miR-15a and tumors bearing hormone HER-2 (p=0.001). Results showed that HER-2 tumors had a higher expression level

of miR-15a than HER-2 positive tumors implicating that HER-2 signalisation may have a role in the regulation of miR-15a in inflammatory breast cancer. Lower plasma level of miR-342-3p was observed in Post-menopausal inflammatory breast cancer patients (p=0.012) indicating that the discriminatory potential of miR-342-3p seems to be better for Pre-menopausal patients (Table 6; Figure 1).

Discussion

Considering circulating miRNAs as disease marker has the following advantages: Related to the diseases closely, especially in tumors; Blood samples are available non-

Table 5. Relationship between miRs expression and clinical and histopathology presentations in non-inflammatory breast cancer patients

Patients presentations	N 0	miR-335	miR-24	miR-320	miR-548d-5p	miR-342-5p	miR-15a	miR-342-3p	miR-29a	miR-451	miR-30b	miR-337-5p	miR-486-3p
Age													
≥40 years	7	0.598	0.257	0.064	0.548	0.072	0.442	0.187	0.249	0.193	0.094	0.297	0.774
<40 years	13												
Menopausal State													
Premenopausal	10	0.007	0.01	0.297	0.087	0.71	0.971	0.063	0.087	0.81	0.73	0.118	0.301
Postmenopausal	10												
Parity													
yes	12	0.574	0.591	0.112	0.397	0.035	0.91	0.054	0.314	0.831	0.79	0.071	0.068
No	8												
ER status													
Negative	9	0.75	0.074	0.221	0.319	0.345	0.208	0.614	0.068	0.324	0.217	0.007	0.864
Positive	11												
PR status													
Negative	8	0.904	0.253	0.812	0.627	0.121	0.876	0.531	0.064	0.053	0.072	0.065	0.907
Positive	12												
Her2 overexpression													
Negative	13	0.523	0.739	0.513	0.201	0.257	0.384	0.201	0.087	0.987	0.201	0.214	0.259
Positive	7												
Lymph Node Involvement													
No	7	0.084	0.315	0.07	0.411	0.487	0.214	0.187	0.458	0.573	0.72	0.571	0.201
Yes	13												
Tumor Size													
T1-T2	6	0.091	0.082	0.741	0.841	0.385	0.871	0.397	0.097	0.067	0.854	0.197	0.154
T3-T4	14												
Tumor Grade													
II	7	0.372	0.218	0.397	0.871	0.222	0.287	0.179	0.214	0.91	0.418	0.227	0.128
III	13												
Metastasis													
No	16	0.09	0.55	0.433	0.287	0.454	0.321	0.074	0.27	0.852	0.082	0.812	0.875
Yes	4												

Bold letters represent p<0.05, significant

invasively; Subtle individual differences in the population; Stably present in serum/plasma; Mature methology of miRNA detection technology (Zeng et al., 2012). These advantages indicate that measuring circulating miRNA levels can be beneficial for early IBC and non-IBC screening and detection. Several major profiling platforms are used today in miRNAs detection. A powerful method for the analysis of serum miRNAs involves relative quantification by stem-loop RT-PCR. This method has been widely used for the sensitive detection of low abundance circulating miRNAs (Chen et al., 2005).

The importance of circulating miRNA in the prognostic value in breast carcinoma has been gained much attention. Wu and colleagues found that miR-21and miR-29 were significantly upregulated in the serum of breast cancer patients and may be useful biomarkers for breast cancer detection (Wu et al., 2011). Roth et al, who also analyzed serum samples, found that miR-155 can discriminate between non metastatic breast cancer patients and healthy individuals (Roth et al., 2010). Moreover, Anfossi et al, who investigated plasma samples from IBC patients, non-IBC and healthy women, found that high levels of serum miR-19a may represent a biomarker for IBC that is predictive for favorable clinical outcome in patients with metastatic HER2+ IBC (Anfossi et al., 2014).

In the current study, we analyzed the level of miRNAs to serve as an early detection marker of non-IBC and IBC by analyzing 12 circulating miRNAs (miR-342--3p, miR-320, miR-30b, miR-342-5p, miR-24, miR-15a, miR-548d-5p, miR-486-3p, miR-29a, miR-451, miR-337-5p, miR-335) in serum of IBC patients (N=20), non-IBC patients (N=20) and healthy controls (N=20).

As the levels of circulating miRNAs may reflect the clinicopathologic status of cancer patients, the association between the miRNAs expression levels in the serum and the clinicopathological characteristics of the patients was investigated in the present study to assess the prognosis value of miRNAs in IBC and non-IBC. miR-24 expression in serum of postmenopausal non-IBC patients was significantly higher compared with the premenopausal non-IBC patients. While we are the first to demonstrate the prognostic potential of Circulating miR-24 in breast cancer, its prognostic potential was supported by a study that found high miR-24 expression in HCC cases with

Table 6. Relationship between miRs Expression and Clinical and Histopathology Presentations in Inflammatory Breast Cancer Patients

Patients presentations	N 0	Inflammatory Breast Cancer p-value											
		miR-335	miR-24	miR-320	miR-548d-5p	miR-342-5p	miR-15a	miR-342-3p	miR-29a	miR-451	miR-30b	miR-337-5p	miR-486-3p
Age													
≥40 years	9	0.413	0.698	0.098	0.874	0.074	0.354	0.187	0.249	0.193	0.094	0.297	0.774
<40 years	11												
Menopausal State													
Premenopausal	10	0.224	0.154	0.347	0.056	0.634	0.157	**0.012**	0.192	0.259	0.454	0.576	0.312
Postmenopausal	10												
Parity													
yes	12	0.881	0.844	0.816	0.95	0.359	0.935	0.72	0.457	0.617	0.892	0.524	0.12
No	8												
ER status													
Negative	3	0.76	0.074	0.282	0.199	0.454	0.208	0.108	0.456	0.676	0.35	0.767	0.697
Positive	17												
PR status													
Negative	4	0.97	0.329	0.43	0.302	0.371	0.483	0.79	0.812	0.405	0.413	0.347	0.297
Positive	16												
Her2 overexpression													
Negative	13	0.989	0.178	0.138	0.394	0.304	**0.001**	0.058	0.053	0.074	0.421	0.39	0.297
Positive	7												
Lymph Node Involvement													
No	2	0.09	0.682	0.351	0.671	0.487	0.13	0.102	0.241	0.251	0.935	0.925	0.061
Yes	18												
Tumor Size													
T1-T2	0	0.884	0.091	0.682	0.926	0.35	0.671	0.487	0.13	0.102	0.241	0.215	0.935
T3-T4	20												
Tumor Grade													
II	9	0.273	0.49	0.532	0.153	0.344	0.859	0.83	0.721	0.659	0.799	0.678	0.289
III	11												
Metastasis													
No	14	0.819	**0.028**	**0.005**	0.921	0.68	0.151	0.479	0.712	0.211	0.187	0.405	0.736
Yes	6												

Bold letters represent p<0.05, significant

larger tumor size (Liu et al., 2014). Whereas, a tendency to higher levels of miR-335 was observed in the blood of premenopausal non-IBC patients indicating that the postmenopausal status may affect miR-335 expression levels. This observation is in the same line with other studies showing that menopausal status may deregulate level of miRNAs in breast cancer. In fact, miR-155 was upregulated in postmenopausal samples made it a considerable breast cancer biomarker for postmenopausal patients (Nassar et al., 2014). Thus, miR-24 may be a considerable non-IBC biomarker for postmenopausal patients and miR-335 for premenopausal non-IBC patients which could help the diagnosis of early onset breast cancer in Tunisian non-IBC patients. We also found that miR-342-5p was upregulated in patients with positive parity as compared with those with nulliparity. Further, in the IBC samples, we found that serum miR-342-5p levels was downregulated in postmenopausal IBC samples indicating that the postmenopausal status may affect miR-342-5p expression levels.

Serum miR-15a levels were significantly lower in Her2+ IBC patients than in patients with HER2- IBC patients implying that miR-15a expression levels may be altered by HER2/neu overexpression in Her2+ IBC patients. Thus, it can be hypothesized that tumor cells overexpressed HER2 may contribute to the decreased miR-15a levels in the serum of Her2+ IBC patients. In this regard, it has been shown that HER2Δ16 (a clinically important oncogenic isoform of HER2) can suppresses miR-15a (Cittelly et al., 2010). In addition, miR-15a was shown to mediate cell cycle arrest and promoting cell apoptosis of breast cancer by directly targeting Cyclin E1 suggesting the tumor suppressive activity of miR-15a in a breast cancer cell line (Luo et al., 2013; Shinden et al., 2015). Moreover, the identification of HER2/neu-induced miR-15a repression provides a greater understanding into how HER2/neu overexpression promotes invasion and metastasis in IBC.

miRNAs involvement in the pathogenesis of human cancers has been well documented. However, co-

expression and co-regulation of miRNAs seems to be less pronounced. Through our analysis we observe a difference in miRNAs correlation profiles between IBC, non-IBC and healthy controls indicating important changes in molecular pathways in cancer cells. Many pairs of miRNAs were highly correlated with each other in both breast cancer groups, the largest pairwise miRNAs correlation was observed in non-IBC group suggesting that correlated miRNAs may be under similar transcriptional control, co-expressed together in exosomes.

Note that, correlation analysis is complementary to expression analysis, since two miRNAs may be highly correlated regardless of whether either one shows any mean change across groups (Lugli et al., 2015).

Interestingly, we found that miR-486-5p is still the most connected miRNA in IBC, non-IBC and healthy controls. In fact, miR-486-5p correlates perfectly with miR-342-3p in controls but not in breast cancer group in which miR-486-5p was correlated with miR-342-5p in IBC cases and miR-15a non-IBC group. This suggests changes in the regulatory network that occur in IBC and non-IBC. Moreover, it was described that miR-486-5p plays a tumor suppressor role in breast cancer by direct targeting PIM-1 oncogene (Zhang et al., 2014).

In addition, miR-30b and miR-337-5p interacts strongly together according to Spearman's rank correlation coefficient in non-IBC group. Moreover, In our previous studies it has been shown that high level of miR-337-5p and miR-30b may represent a new efficient biomarkers for early diagnosis for non-IBC patients. This co-up regulation provides additional evidence that miR-337-5p and miR-30b may contribute together to promote initiation and development of non-IBC. This observation is in agreement with other studies showing that these miRNAs can promote the evolution of malignant behaviors by enhancing cellular invasion and immunosuppression (Gaziel-Sovran et al., 2011). Likewise, miR-320 was highly correlated with miR-24 and miR-548d-5p. A possible explanation is that these miRNAs may be secreted into the circulation by the same mechanisms. Nevertheless, the significant biological effect of correlated miRNAs on clinical pathology of breast cancer need to be further investigated.

In inflammatory breast cancer, miRNAs correlation profiles were different from non-inflammatory breast cancer. Thus, miR-320 revealed significant correlation with miR-15a expression. Interestingly, correlation analyses revealed no significant association of miR-451 with others miRNAs. A possible explanation for this finding is that exosomes containing miR-451, which is the most downregulated miRNAs in IBC, with an average decrease of 57.5-fold according to our previous IBC and non-IBC circulating miRNAs expression profile, are released into the circulation to a lesser extent in IBC subjects and processed independently of other miRNAs in IBC.

Astonishingly, miR-342-5p and miR-342-3p did not significantly correlate in IBC, although they belong to the same family. This might give reason to speculate that these two miRNAs may have the same transcription and maturation process, with different use and intake by IBC

tumor cell, or different target. However, the appearance of significant strong correlation between miR-342-5p and miR-342-3p in both non-IBC and controls group may indicate important changes in molecular pathways in IBC versus non-IBC and controls.

Lastly, a weak miRNAs correlation number was observed in controls group as compared to IBC and non-IBC miRNAs correlation profile. Indeed, except miR-486-3p, miR-342-3p and miR-342-5p which were pairwise correlated as mentioned and discussed above, there was no significant correlation observed among other miRNAs. Thus, the loss of miRNAs correlation in healthy controls could represent an overall change in the regulatory network that occur in breast cancer cells during cancer progression, leading to the preferential co-regulation and co-expression of miRNAs in breast cancer group in opposition to healthy controls. These findings indicated that, as a novel noninvasive biomarker, circulating miRNAs would provide considerable diagnostic values in screening breast cancer.

In conclusion, This study is a continuity of our previous study in which we have shown that miR-451could be considered as a serological marker for IBC. However, miR-337-5p and miR-30b could discriminate non-IBC patients. Our current study showed that, in non-IBC group, the expression of circulating miR-335 and miR-24 was correlated with the menopausal state. Also, miR-342-5p was positively correlated with parity. We showed also that higher levels of miR-15a and miR-24 was positively correlated with Her2 status and menopausal state respectively in IBC patients. Moreover, we noticed that miRNAs interaction were different between IBC, non-IBC and healthy controls indicating important changes in molecular pathways in cancer cells and different mechanisms of carcinogenesis between the two Breast cancer subtype that may contribute to the dynamic of miRNAs profiles. Future large studies are warranted to validate our preliminary results and further allow us to examine whether miRNAs alterations are notably related to risk, onset or progression of the breast cancer and indicate their potential as molecular biomarkers for early cancer diagnosis, prognostic and facilitate the development of novel strategies for cancer therapy.

Acknowledgements

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