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Circulating MicroRNA 181a As Biomarker In Breast Cancer, Its Impact On Survival and Epithelial Mesenchymal Transformation

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Abstract:
Background: Breast cancer (BC) is the world's most common cancer among women; recent studies have reported that micro RNA 181a may play a significant role in oncogenesis and metastasis through epigenetic post-transcriptional gene regulation. Aim of this study: the detection of miRNA-181a circulating level in females with different stages of breast cancer and to search its role in metastasis through enhancing epithelial mesenchymal transformation (EMT) process as well as its impact on survival. Subjects and Methods: The current research involved 70 women with various stages of breast cancer. Thirty sufferers with metastatic disease and forty sufferers with non-metastatic disease were enrolled in the study. A control group of 20 healthy people was selected. We used qRT PCR to detect miRNA-181a expression in peripheral blood, as well as quantitative PCR to detect TGF-beta, SMAD-4, SNAIL-1, and Bim expression. Results: In metastatic breast cancer, miRNA-181a, TGF-beta, SNAIL-1 and SMAD-4 has been substantially up-regulated relative to patients with non-metastatic disease; whereas in the metastatic versus non-metastatic group, Bim is substantially down-regulated. Up-regulated miRNA181a expression was an important prognosis of progression of the disease and total survival in both metastatic and non-metastatic groups. Conclusion: TGFβ-SMAD signaling pathway regulates miRNA-181a that, in turn, plays a role in promoting tumor aggressiveness and metastasis through epithelial mesenchymal transition, also circulating miRNA 181a could be used as a breast cancer biomarker and to predict survival outcomes.

Keyword: Breast cancer – metastasis – miRNA 181a – TGF-β – SNAIL/SMAD

INTRODUCTION

Breast cancer (BC) is the world's most common cancer and the second main cause of mortality among women, with >40,000 deaths annually [1]. Breast cancer is a heterogeneous tumor with a wide variety of clinical subtypes and molecular aberrations. These aberrations occur both at the genetic and epigenetic levels (regulators of gene expression, such as long non-coding RNAs and microRNAs [miR]) and can disturb the balance between oncogenes and tumor suppressors in human tissues [2]. MicroRNAs are small, non-coding, single-stranded RNAs, involved in regulating basic biological

functions, including development, differentiation, proliferation, and apoptosis [3]. Therefore, altered miRNA levels are supposed to contribute towards stemness, genomic instability, and metastatic potential [4, 5].

MicroRNA-181a (miR-181a) is a member of the miRNA181 family. According to recent research, MiR-181a is involved in a variety of cellular functions, including growth, proliferation, survival, death, and maintenance, as well as carcinogenesis and tumor suppression [6, 7]. While some research have demonstrated that miR-181a expression is down-regulated in various human solid tumors, others have shown that miR-181a up-regulation can enhance metastasis and resistance to chemotherapy [7, 8]. Several research groups reported correlations between altered miRNA levels and the prognosis, as well as several characteristics, including phase/stage, vascular/lymph node invasion, and proliferative index of malignant tumors [9-11]. The underlying mechanistic pathways were linked to its role in the EMT process [7, 11]**.**

Distant spread (metastasis) of BC is the most common reason of mortality in these patients. To achieve it, tumors resort to a developmental embryonic mechanism, known as "Epithelial mesenchymal transition: EMT", in which cells change to a mesenchymal type that can differentiate into several cell types [12]. Tumor cells exploit this mechanism interchangeably (EMT for MET) to execute both tumorigenesis and metastasis. This process is regulated by different signaling pathways which include: TGF-β/SMAD, Wnt, NOTCH, and TNFα/ NF-kB pathways [13]. A recent study exploited this ability in breast cancer cells to turn malignant cells into adipocytes and halt their spread [14].

SNAIL is a protein that represents to a family of transcription factors which help regulate the epithelial to mesenchymal transition (EMT) during embryogenesis by enhancing the suppression of the E-cadherin adhesion molecule. SNAIL is highly expressed in both epithelial and endothelial cells of invasive BC and has been linked to tumor grade, metastasis, and recurrence [15]. Another pro-oncogenic pathway occurs through TGF-β-mediated activation of receptor-regulated SMAD2 and SMAD3, that forms complexes with SMAD-4 [16]. These complexes are regulated by several oncogenic miRNAs as miR-181a [17].

The aim of this work was to detect the level of circulating miRNA-181a in BC patients at various stages and study the possible association between miRNA 181a and SNAIL, TGF-β and pro-apoptotic protein Bim.

PATIENTS AND METHODS

2.1. Subjects:

This is a prospective study that included 70 female patients with BC (thirty patients with metastatic disease and forty newly-diagnosed patients with non-metastatic disease) based on clinical, pathological and radiological criteria. As a control group, we enrolled 20 healthy people who were age and sex matched. All patients presented to the Oncology Department at Al-Kasr Al-Ainy Hospital (NEMROK) between July-2014 and July 2015. Then, patients were followed for 3 years for treatment outcomes and survival analysis.

Women were included if they were >18 years old, had pathologically proven BC with a recent onset disease (in the non-metastatic group). Patients were excluded if they received previous treatment, had other oncologic or immune disorders that may have impact on the studied markers, had incomplete data, or withdraw their consent at any stage.

2.2. Data collection:

Data was retrieved from Al-Kasr Al-Ainy Oncology center (NEMROK) patient files. The retrieved data included age at presentation, date of first diagnosis, family history, pathological data (histological subtype, molecular subtype, tumor grade, hormonal status, HER2 neu status), clinical data including TNM stage and management options (type of surgery, chemotherapy, radio therapy and hormonal treatment) and if there was any other comorbidities. The molecular subtypes of breast cancer were classified according to immunohistochemical markers (ER, PR, Her2 status) and ki67 expression according to St- Gallen classification. All patients were followed up for 3 years to assess response to treatment and their survival, including disease free survival (DFS) and overall survival (OS)**.**

2.3. Sample collection and storage:

From each patient 3ml of peripheral blood were collected in sterile EDTA vacutainers and divided into two parts, first part was used for separation of buffy coat for gene expression analysis while that second part was centrifuged at 1000×g. for 20 minutes, plasma was separated and was kept frozen at -80^0 C for later use for detection of MiRNA 181a, TGF-β, SNAIL-1, SMAD4 and Bim expression.

2.4. Detection of MiRNA- 181a expression by qRt-PCR:

The miRNA was extracted by the miRNA extraction kit (mirVanaTM PARISTM Kit, ambion, USA) according to manufacture instructions. -70 °C was used for the storage of purified miRNA. A Nanodrop® spectrophotometer has been used to measure the absorption of isolating RNA at 260 nm and 280 nm in order to assess the efficiency and concentration of isolated RNA.

The purified miRNA was quantified using real-time PCR. Using specific miRNA primers from the TaqMan®MicroRNA Assays and reagents from the TaqMan®MicroRNA Reverse Transcription Kit, cDNA was reverse transcribed from total RNA specimens in the reverse transcription step. The TaqMan®MicroRNA Assay and the TaqMan ®Universal PCR Master Mix were used to amplify PCR products from cDNA specimens during the PCR step. To measure relative shifts in miRNA expression determined from real-time qPCR experiments, the $2^{-\Delta\Delta ct}$ Approach was used**.**

2.5. Detection of TGF-β, SNAIL-1, SMAD-4 and Bim gene expression by RT-PCR:

Ficoll-Paque Premium was used to isolate mononuclear cells from whole blood (Biochrom, Berlin, Germany). After sample centrifugation at 2000 rpm for 10 to 15 min at 18º to 20°C, the cell pellet was removed for RNA extraction SV Total RNA Isolation System kit, Promega, MadisonVVI, USA (Cat # Z3100).The extracted RNA was purified using a HiBind® RNA Spin Column, subjected to repeated buffering and centrifugation.

A spectrophotometer was used to measure the absorbance at 260 nm and 280 nm to estimate the concentration and purity of RNA. For cDNA synthesis, the extracted RNA was reverse transcribed into O.D. using AMV Reverse Transcriptase, Promega, and Madison. VVI, USA (Catalog No.: M5101). For the qPCR step, normalization was performed on the gene-specific forward and reverse primer pair. Each primer (forward and reverse), shown in **Table (1)**, the mixture's concentration was 5 pmol/ µl. Step-one Applied Biosystem software was used to analyze the RT-PCR results.

Table 1: The primer sequence used for real-time PCR

	Primer sequence
miRNA-181a	Forward: 5'-CTAGCCTGCAGGCCTGCTTCTTTTCTTCTGTA-3' Reversed: 5'- ATCCGGCCGGCCCTTTGGTTCTTCCTCCCACC-3'
RU6*	Forward: 5'-CTCGCTTCGGCAGCACA-3' Reversed: 5'- AACGCTTCACGAATTTGCGT-3'
TGF-β	Forward: 5'- CAAGGGCTACCATGCCAACT -3' Reversed: 5'- GTACTGTGTGTCCAGGCTCCAA -3'
Bim	Forward: 5'-GACAAGAATCCGACCAAATGGCAAA-3' Reverse: 5'-AAAAGGATCCATGAGAAATCCTTGTGG-3'
SNAIL-1	Forward: 5'-TCGGAAGCCTAACTACAGCGA-3' Reverse: 5'-AGATGAGCATTGGCAGCGAG-3'
SMAD-4	Forward: 5'-AAGCATTTCAACGCCTCCAAA-3' Reverse: 5'-GGATCTCTGGTTGTGGTATGACA-3'
β- Actin**	forward primer: 5'-GCA CCA CAC CTT CTA CAA TG-3' Reverse primer: 5'-TGC TTG CTG ATC CAC ATC TG-3'.

RU6 * was used as housekeeping gene for detection of miRNA 181-a expression
β- Actin** was used as housekeeping gene for detection of TGF-β, SNAIL-1, SMAD-4 and Bim expression

2.6. Statistical methods:

The statistical package SPSS version 25 was used to code and enter data. For quantitative variables, mean and standard deviations were used to summarize the data, while categorical variables were summarized using the frequency (%).In normally distributed variables, comparisons among groups were made using an ANOVA with multiple comparisons post-hoc test or an unpaired t-test, whereas non-normally distributed variables were compared using the Kruskal-Wallis and Mann-Whitney tests. The $\chi 2$ test was used to compare categorical results. When the predicted frequency was less than 5, an exact test could be used instead. The Spearman correlation coefficient was used to determine associations among quantitative variables. P values < 0.05 were deemed statistically significant.

RESULTS

3.1. Baseline characteristics:

The age ranged from 23 to 76 years for patient groups. A control group of 20 healthy people of similar ages and sexes was included. Six cases (15%) have positive family history of BC in the non-metastatic group and 10 cases (33.3%) in the metastatic group.

In the non-metastatic group, 14 patients (35%) had HER-2 enriched breast cancer and 9 patients (22.5%) had triple negative disease**.** While in the metastatic group, 17 patients (42.5%) had HER-2 enriched breast cancer and 3 patients (7.5%) had triple negative disease**.** According to site of metastasis: the most common site of metastasis was bone 22 patients (73.3%), followed by liver 11 patients (34%) then lung 8 patients (26.6%) while only one patient had brain metastasis (Table 2). The age of control subjects (28 to 66 years) was not significantly different from patient groups and these subjects were completely free on history taking and on clinical examination.

Table 2: clinical characteristics of both metastatic and non-metastatic patients

	Non metastatic group (n=40)	**Metastatic group (n=30)**
Age (years) Mean ± SD	48.4 ± 10.2	45 ± 11.4
Menopausal status **Pre** **Post**	 23 (57.5%) 17 (42.5%)	 25 (83.3%) 5 (16.7 %)
Pathology **IDC II** **ILC**	 37 (92.5%) 3 (7.5%)	 27 (75%) 3(10.7%)
Grade **2** **3**	 36 (90%) 4 (10%)	 21 (67.9%) 9 (32.1%)
Hormonal status **Positive** **Negative**	 27 (67.5%) 13 (32.5%)	 26 (90%) 4 (10%)
HER2 **Positive** **Negative**	 15 (37.5%) 25 (62.5%)	 17 (53.6%) 13 (46.4%)
Molecular diagnosis **HER 2 enriched** **Luminal A** **Luminal B** **Triple Negative**	 14 (35%) 2 (5%) 15 (37.5%) 9 (22.5%)	 17 (53.6%) 1 (3.6%) 9 (32.1%) 3 (10.7%)
Stage **IIA** **IIB** **IIIA** **IIIB** **IIIC** **IV**	 9 (22.5%) 9 (22.5%) 10 (25%) 5 (12.5%) 7 (17.5%) 0 (0%)	 0 (0%) 0 (0%) 0 (0%) 0 (0%) 0 (0%) 30 (100%)
Site of metastasis **Bone** **Liver** **Lung**	 -- 	 22 (73.3%) 11 (34%) 8 (26.6%)

Data are frequency (percentage) unless stated otherwise. IDC: Infiltrating duct carcinoma- ILC: infiltrating lobular carcinoma – HER2: Human epidermal growth factor receptor.

In the non-metastatic group, all cases underwent surgery; 21 (52.5%) breast conservative surgery and 19 (47.5%) modified radical mastectomy. Thirty-six cases (90%) underwent breast radiotherapy. In the metastatic group, Different chemotherapy regimens were used for treatment according to stage of the disease and molecular diagnosis and organ of metastasis (Supplementary Table 1). Hormonal treatment was used for the 9 hormonal positive patients with bone only metastasis according to their menopausal status.

Supplementary Table (1): chemotherapeutic regimens used for treatment of both metastatic and non-metastatic groups.

		Metastatic		non metastatic	
		Count	%	Count	%
CTH	AC X4	*0*	*.0%*	*7*	*17.5%*
	AC X4 then T X4	*0*	*0%*	*6*	*15%*
	AC X4 then TH X4	0	*0%*	*7*	*17.5%*
	EC X4 then T X4	*0*	*.0%*	*2*	*5%*
	FAC X3 then TC X3	*1*	*3%*	*0*	*.0%*
	FAC X4 then TH X4	*0*	*.0%*	*2*	*5%*
	FAC X6	*6*	*20%*	*0*	*0%*
	FEC X 6	*8*	*27%*	*1*	*2.5%*
	FEC X3 then TH X3	*0*	*0%*	*3*	*7.5%*
	FEC X3 then TX3	*0*	*0%*	*11*	*27.5%*
	Hormonal ttt	9	*30%*	*0*	*.0%*
	TCH X6	*3*	*10%*	*1*	*2.3%*
	TH X6	*3*	10%	*0*	*.0%*

3.2. Survival curves for patients:

The median DFS for the whole non-metastatic group was 28 (CI 95%: 22.2-33.7) months, while most patients have survived the three-year follow up period with 82.5% 3 year OS (Figs. 1A and 1B). The median DFS for the whole metastatic group was 8 (CI 95%: 6.3-9.7) months, while 3 year OS reached 22.5% (Figs. 1C and 1D).

3.3. Estimation of miRNA-181a (by qRT PCR), TGF-β, SMAD, SNAIL and Bim (by real time PCR) among different groups:

Table 3 show significant increase in miRNA-181a, TGF-β, SMAD-4 and SNAIL-1 expression in non-metastatic patients and metastatic patients compared with the normal control ($p < 0.001$). However there was significant decrease in Bim expression non-metastatic patients and metastatic patients compared with the normal control ($p < 0.001$). Also there was significant increase in miRNA-181a SMAD-4 and SNAIL-1 expression in metastatic patients compared with the non-metastatic patients ($p < 0.001$). However also

there was significant decrease in Bim expression metastatic patients compared with the non-metastatic patients (p <0.001).

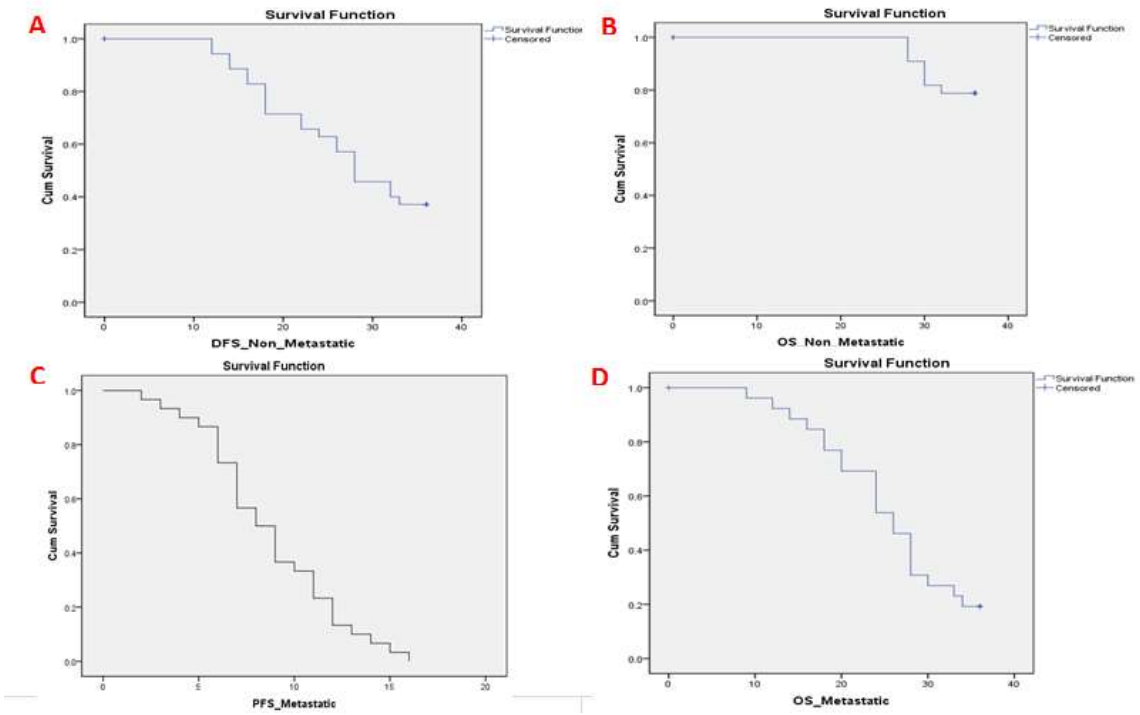

Fig 1: A) 3 year disease-free survival of patients with non-metastatic group, B) 3 year overall survival of patients with non-metastatic group, C) 3 year disease-free survival of patients with metastatic group, and D) 3 year overall survival of patients with metastatic group.

Table 3: miRNA-181a, TGF beta, SMAD-4, SNAIL-1 and Bim expression among the studied groups

	Control Group	Non-Metastatic Group	Metastatic Group	P1 value*	P2 value#
miR-181a	1±0.01	4.2±2.7*	11.7±1.5*#	<0.001	<0.001
TGF-β	1.1±0.14	5.7±2.8*	12.8±2.3*#	<0.001	<0.001
SMAD-4	1.01±0.03	4.7±2.6*	12.04±4.07*#	<0.001	<0.001
SNAlL-1	1.01±0.028	4.3±2.8*	11.8±2.3*#	<0.001	<0.001
Bim	1.01±0.03	0.77±0.21*	0.21±0.07*#	<0.001	<0.001

Data were expressed as Mean ± SD, p value <0.05 was significant
(*) Denotes significant difference between each group versus control subjects
(#) Denotes significant difference between metastatic versus non metastatic patient

3.4. Association between miRNA-181a and TGF-β pathway as well as Bim:

Positive correlation was observed between miRNA-181a, TGF beta, SNAIL-1 and SMAD-4, (r=0.81), (r=0.81), (r=0.77) respectively while there was negative correlation between miRNA-181a and Bim (r=-0.87) and these results were statistically significant as shown in Supplementary Table 2.

Supplementary Table (2): Correlation analysis between the different laboratory parameters among studied groups

		miRNA181a	TGF beta	SNAIL-1	SMAD-4	Bim
miRNA-181a	**R**	1	.817**	.818**	.774**	-.871-**
	P value		.000	.000	.000	.000
TGF beta	**R**	.817**	1	.848**	.781**	-.811-**
	P value	.000		.000	.000	.000
SNAIL-1	**R**	.818**	.848**	1	.728**	-.807-**
	P value	.000	.000		.000	.000
SMAD	**R**	.774**	.781**	.728**	1	-.789-**
	P value	.000	.000	.000		.000
Bim	**R**	-.871-**	-.811-**	-.807-**	-.789-**	1
	P value	.000	.000	.000	.000	

r = correlation coefficient, r < 0.3: no correlation, r = 0.3 - < 0.5: weak correlation, r = 0.5: fair correlation, r = > 0.5 – 0.75: good correlation, r > 0.75: very good correlation

3.5. Association between miRNA 181a and clinicopathological characteristics:

The correlation between miRNA 181a and age as well as its association with tumor stage, molecular subtypes, histopathological subtypes and tumor grade was investigated. As shown in Fig.2, the only significant association was found between miRNA 181a expression and clinical stage with higher expression in stage IV compared to stage (II and III) (p=0.001). There was no significant correlation among age and miRNA 181a (p=0.19). No significant association was found between miRNA 181a expression and molecular subtype (p=0.77), histopathological subtype (p=0.96) and tumor grade (p=0.79).

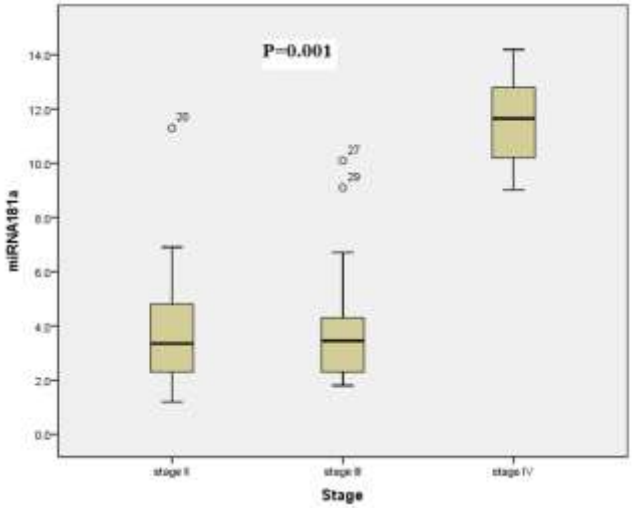

Fig 2: Association between miRNA-181a and tumor stage among BC patients

3.6. Prognostic value of measured molecular parameters:

Supplementary Table 3A shows the results of Cox regression analyses for all variables; it reveals that up-regulated miRNA181a expression is a significant prognostic factors for 3 year disease free survival in patients with non-metastatic breast cancer (p=0.000).

Supplementary Table 3B shows the results of Cox regression analyses for all variables; it reveals that up-regulated miRNA181a and TGF beta expression were a significant prognostic factors for 3 year progression free survival in patients with metastatic breast cancer (p=0.001), (p=0.24) respectively.

Supplementary Table 3A: Analyses for 3 year disease free survival of various prognostic parameters in non-metastatic patients by Cox-regression analysis

Lab. Parameter	P value.	HR	95.0% CI	
			Lower	Upper
miRNA181a	.000	.644	1.434	2.531
TGF-β	.375	.098	.888	1.369
SNAIL-1	.638	-.059-	.737	1.206
SMAD	.616	.058	.844	1.331
Bim	.425	1.240	.164	72.685

Supplementary Table 3B: Analyses for 3 years progression free survival of various prognostic parameters in metastatic Patients by Cox-regression analysis

Lab. Parameter	Sig.	HR	95.0% CI	
			Lower	Upper
miRNA181a	.000	1.664	2.565	10.869
TGF-β	.024	-.266-	.608	.966
SNAIL-1	.776	-.022-	.844	1.135
SMAD	.393	-.050-	.848	1.067
Bim	.361	-2.670-	.000	21.343

Supplementary Table 3C shows the results of Cox regression analyses for all variables; it reveals that up-regulated miRNA181a expression is a significant prognostic factors for 3 year overall survival in metastatic breast cancer patients (p=0.006). Supplementary Table 3D shows the results of Cox regression analyses for all variables; it reveals that up-regulated miRNA181a and TGF beta expression were a significant prognostic factors for 3 year overall survival in metastatic breast cancer patients (p=0.00) and (p=0.019) respectively.

prognostic parameters in non-metastatic breast cancer patients by Cox-regression analysis

	Sig.	HR	95.0% CI for Exp(B)	
			Lower	Upper
miRNA181a	.006	.707	1.227	3.349
TGF-β	.527	.192	.669	2.194
SNAIL	.425	-.395-	.255	1.780
SMAD	.437	-.328-	.315	1.646
Bim	.453	3.382	.004	201543.160

Supplementary Table 3D: Analyses for 3 years overall survival of various prognostic parameters in metastatic breast cancer patients by Cox-regression analysis

	Sig.	HR	95.0% CI for Exp(B)	
			Lower	Upper
miRNA181a	.000	1.728	2.486	12.741
TGF-β	.019	-.345-	.531	.945
SNAIL	.095	.177	.970	1.468
SMAD	.399	-.061-	.817	1.084
Bim	.223	-3.834-	.000	10.343

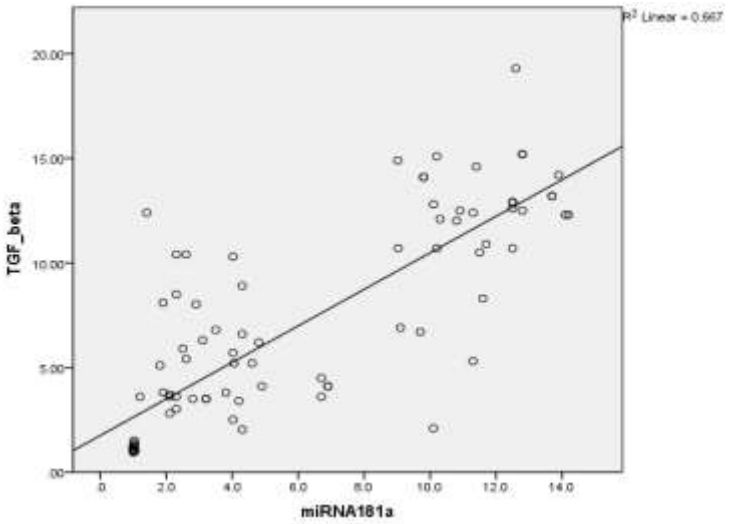

Fig. S1: Correlation between miRNA-181a and TGF beta among studied population

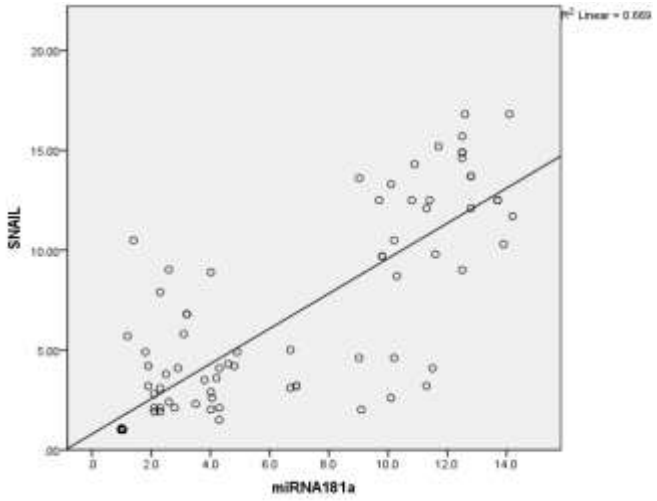

Fig. S2: Correlation between miRNA-181a and SNAIL-1 among studied population

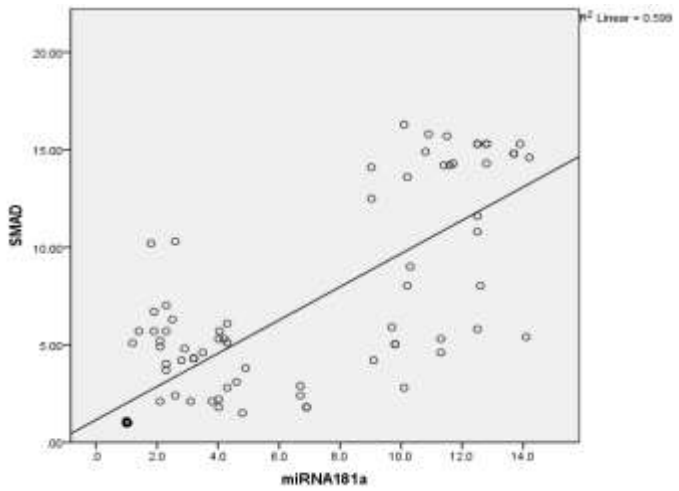

Fig. S3: Correlation between miRNA-181a and SMAD among studied population

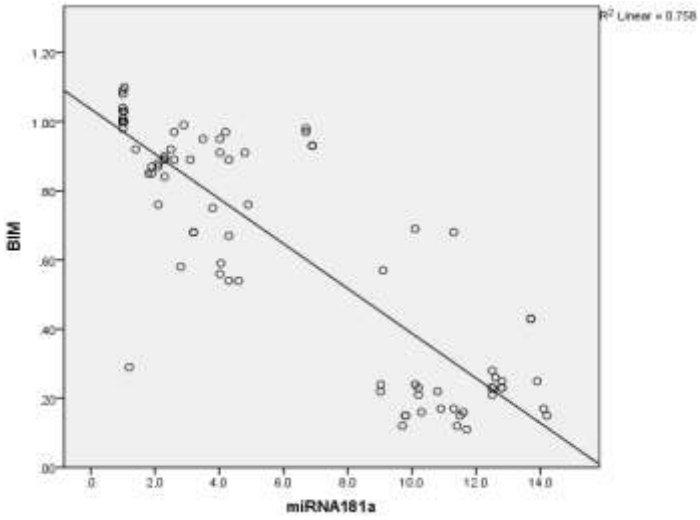

Fig. S4: Correlation between miRNA-181a and Bim among studied population

DISCUSSION

In this study, the expression miRNA-181a was significantly higher in BC patients, compared to normal controls. This is in agreement with Godfrey et al. in their prospective study of over 2005 BC women [18]. In addition, when compared to non-metastatic patients, metastatic patients had a significant increase in miRNA-181a. This finding is in consistent with Lopez et al. and Taylor et al. who found that miR-181a expression was upregulated selectively in metastatic BC, especially triple-negative BC, and was a good predictor of reduced OS in BC sufferers [9, 10]. However, another study by Guo et al. reported no significant differences regarding miR-181a in women with various clinical TNM classification stages and histological grades [19]. In the present work, there were no significant correlations between plasma miRNA 181a and age, tumor grade or histologic subtype. Although miRNA 181a upregulation in triple negative BC was less than other molecular subtypes, this result was not statistically significant. A former study found that miR-181a expression was significantly downregulated in triple negative BC

cell lines MDA-MB-231 and BC stem SK-3rd cells as compared with less aggressive BC cell lines such as MCF-7 and SK-BR3 (ER-/HER2+) [20].

We also found a significant increase in TGF-β in BC patients compared with the normal control. Moreover, TGF-β was found to be significantly higher in metastatic patients compared to non-metastatic patients. These results are in consistent with several published studies [21-23] who found that TGF-β1 could induce and promote EMT. Thus, TGF-β1 is regarded as a metastasis inducer. Several studies also demonstrated that SMAD-dependent pathways are implicated in the tumor-promoting activity of TGF-β1. SMAD-2, -3 and -4 are essential for the metastatic extension of bone, lung, liver, and brain tumors [24, 25]. On the other hand, some studies have shown that TGF-β was involved in inhibition of epithelial cell cycle progression and promoting apoptosis of cancer cells [26, 27]. TGF-β induces G1-arrest by stimulating cdk inhibitors such as p16INK4A, p15INK4B, p21CIP1, and/or p27Kip1 [28]. Increased TGF-1 expression in early development inhibits mammary epithelial outgrowth in vivo, according to Boulanger [29].

The current study showed that Bim expression was significantly decreased in BC patients compared to normal controls with further reduction in metastatic patients. This result is consistent with Merino et al. who found a striking connection among the expression of Bim and the EMT transcription factor SNAIL2 at the proliferative edge of tumors [30]. They suggested that SNAIL2-driven Bim induced apoptosis may temper metastasis. In the four molecular subtypes, the Bim expression was substantially different. Survival analysis has shown that Bim expression has led to shorter OS, particularly in sufferers with luminal A tumors [31].

SNAIL-1 expression was found significantly increased in BC patients compared to normal controls with further increase in metastatic patients. our results are in agreement with former investigations who found that Slug and SNAIL genes were significantly overexpressed in BC tissue compared to normal mammary tissue especially tumors associated with lymph node metastasis [25, 32]. Further, Zhang et al. found that down-regulated SNAIL expression is associated with a substantial inhibition of RhoA GTPase and MMP-2 expression and activity and concluded that SNAIL transcription factor play important role in BC progression [33].

In this work, SMAD-4 expression was found significantly higher in BC patients compared to normal controls with further increase in metastatic patients. This results are in consistent with Deckers et al. [34] who found SMAD4 knockdown inhibited TGF-β-induced EMT of NMuMG cells as assessed by morphologic conversion from epithelial to fibroblast-like cells, inhibition of the expression of E-cadherin and gain of expression of different mesenchymal markers, and they suggested that SMAD-4 may have role in metastasis. SMAD4 knockdown in MDA-MB-231 BC cells significantly hindered bone metastasis at 75 % in nude mice and substantially improved metastasis-free survival. Others have shown that TGF-β triggered invasion of premalignant and highly malignant BC cells by induced SMAD3/SMAD4-dependent expressions of MMP2 and MMP9 [35, 36]. On the other hand, Liu et al. found that SMAD-4 expression was reduced in ductal

breast carcinoma as relative to adjacent normal breast epithelia [37]. Other authors studied expression of SMAD-4 by immunohistochemistry technique on BC tissues and SMAD4 expression has been shown to be decreased in lobular and ductal breast carcinoma as relative to surrounding uninvolved lobular and ductal breast epithelia and it was correlated positively with expression of TGF-β-receptor [38].

In addition, we found significant strong positive correlation was observed between miRNA-181a and TGF-β, SNAIL-1, SMAD-4, while there was negative correlation between miRNA-181a and BIM and these results were statistically significant. Similarly, former articles [39, 40] described a positive correlation between TGF-β and miRNA-181a. Samanta and Datta also found a positive correlation between TGF-β and SMAD-4 [41]. Others studied TGF-β pathway in EMT and tumor progression which was correlated with SMAD3,4, SNAIL1,2 in BC cell lines and suggested they play role in BC progression [16, 23, 42]. Feng et al. also suggested that Bim as a potential target for miRNA-181a for regulation of mitochondrial mediated apoptosis and is downregulated in malignant transformation [43].

Also up-regulated miRNA181a expression was a significant negative prognostic factors for 3 year DFS in patients with non-metastatic BC, while both upregulated miRNA181a and TGF-β expression were a significant prognostic factors for 3 year DFS in patients with metastatic BC. These results are inconsistent with [39, 40]. However, upregulated SNAIL-1, SMAD-4 and downregulated Bim did not have a prognostic value for disease progression or survival which in in contrast to former investigations [31, 33, 37].

In conclusion, this study showed significant overexpression of miR-181a, TGF-β, SNAIL-1, and SMAD-4, as well significant Under-expression of Bim in BC patients in comparison to normal controls. These aberrations were more marked in metastatic patients, compared with non-metastatic patients. Moreover, it highlights the regulatory effects of miR-181a on these molecules and hence the involvement of miR-181a in regulating EMT. Our study also revealed that circulating miRNA 181a can be used as a breast cancer biomarker and to predict survival outcomes in all patients with BC.

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