Preliminary Study: Prominent miRNAs of Breast Malignant Tissues Compared to Normal Tissues in Turkish Patients with Breast Cancer

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Abstract. *miRNA involvement has been observed in almost every type of cancer, including breast cancer. The etiology of abnormal expression of miRNAs in cancer is still not clearly understood. In order to obtain insight into miRNA de-regulation in breast cancer, we analyzed expression levels of five breast cancer-related miRNAs, miRNA21, miRNA155, miRNA19a, miRNA17-5p and let7a miRNA, in both malignant and neighboring non-tumoral paraffin-embedded tissues of 47 patients with invasive ductal breast cancer. The targeted miRNAs, and a reference snRNA, U6, were analyzed by real-time polymerase chain reaction. let7a Levels were significantly lower in patients with lymphatic invasion than in those without (p=0.047). miR21 was down-regulated in 93.3% of patients with necrosis [p=0.017 (Fisher's exact test (FE))], while at least one oncogenic miRNA was up-regulated in 87.3% of the patients with invasive ductal carcinoma [p=0.009 (FE)]. In addition, tumor-suppressor miRNA was down-regulated or unaltered in 65.8% of the patients with tumor grade 2 or 3 and in all with grade 1 [p=0.047 (FE)]. Based on this preliminary study, we suggest that these miRNAs, especially let7a and miRNA21, might be useful markers in follow-up of breast cancer and in prognosis.*

Breast cancer is a very complex disease resulting from the interaction of numerous genetic, hormonal and environmental factors. Therefore, molecular mechanisms underlying the development and progression of breast cancer are still not fully resolved.

MicroRNAs (miRNAs) are a class of small endogenous RNA molecules that function as post-transcriptional gene regulators by performing gene silencing, with degradation of the mRNA of the target gene or by blocking translation in a sequence-specific manner. Recently, it was discovered that almost one-third of the protein-coding genes are under the control of miRNAs. miRNAs regulate various biological processes, including cellular proliferation, development, differentiation, stress response and apoptosis. Consequently, their contribution to several diseases, and cancer in particular, was not surprisingly recognized (1-3).

miRNAs play important roles in carcinogenesis and tumor metastasis. miRNA profiling studies have shown that the expression levels of all three sub-classes of miRNAs, namely tumor-suppressor, oncogenic and metastatic, are altered in breast cancer. While the reduction or deletion of a tumor-suppressor miRNA or amplification or overexpression of an oncogenic miRNA can cause tumor formation, enhanced expression of a pro-metastatic miRNA or downregulation of an anti-metastatic miRNA may promote tumor metastasis (1-3).

Several miRNAs have been associated with distinct phases of breast cancer, as well as clinicopathological features of breast tumors such as the proliferative index, steroid hormone receptor status, nodal status and tumor stage (4-6). Oncogenic *miRNA21* was found to be the most abundant in breast tumor tissue compared to matched normal tissue (5) and affects tumor invasion and metastasis by targeting tumor suppressors tropomyosin 1 (7), programmed cell death 4 (8, 9), maspin (10), and an inhibitor of matrix metalloproteinases and tissue inhibitor of metalloproteinases (11). It was also shown that altered *miRNA21* expression correlated with the loss of

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phosphatase and tensin homolog expression, and the aggressiveness of the disease related to high tumor grade, advanced clinical stage, shortened patient survival and negative hormone receptor status in breast cancer (12-14). Like *miRNA21*, *miRNA155* has been found also up-regulated, implying that it may potentially act as an oncogene (5, 13, 15). In addition, the expression pattern of *miRNA155* was also altered by molecular subtypes of breast cancer and specific breast cancer clinicopathological factors, including estrogen (ER) and progesterone receptor (PR) status, tumor stage, vascular invasion, and proliferative index (16), with the most altered expression pattern being found in ER^- *versus* ER^+ tumors (5, 17). The miRNA17-92 cluster, which contains *miRNA19a*, was reported as being up-regulated in breast and lung cancers (15). However, it was found that this cluster tends to be deleted in some breast tumors (18). It was suggested that if not deleted *miRNA19* was oncogenic, especially in ER^- tumors, where receptor-associated coactivator would be expected to be inactive. The cluster miRNA17/20, which miRNA17-5p belongs to, regulates cell-cycle progression by targeting multiple cell-cycle regulators (Retinoblastoma (Rb), retinoblastoma-Binding Protein (E2F), avian myelo-cytomatosis viral oncogene homolog (c-MYC), and cyclin D1) in breast cancer (19, 20). Another most significantly de-regulated miRNA in breast cancer was *miRNA let7a*, whose expression was lost at an early stage in breast cancer progression (21). It was reported that the continued expression of *let7a* was associated with low-grade, ER^+ luminal A tumors (16). The let7 family of miRNAs regulates the expression of the retrovirus-associated DNA sequences (RAS) proto-oncogene family (22), thus the let7 is commonly found down-regulated in breast cancer samples.

To our knowledge, there is no study on the association of breast cancer with miRNA expression levels in the Turkish population. Therefore, the first aim of the present study was to determine the expression levels of the most significant de-regulated miRNAs, *miRNA21, miRNA155, miRNA19a, miRNA17-5p* and *let7a* in Turkish patients with breast cancer. Subsequently, by determining the expression levels in both normal and malignant tissues of these patients, the identification of their impact on the pathogenesis of the disease may be accomplished. According to their potential role in breast cancer, their development as diagnostic and prognostic biomarkers was also investigated.

Materials and Methods

Patient selection. Paraffin-embedded normal and malignant breast tissues were collected from 47 women with breast cancer diagnosed by the Cerrahpasa Faculty of Medicine Hospital (Istanbul, Turkey) between 2001-2005. Their median age was 58 years (range=37-85 years). All patients were divided into groups according to conventional clinical features: ER^+/ER^-, PR^+/PR^-, positive and negative lymph node status, presence and absence of vascular invasion, high and low proliferative index, and lobular and ductal histopathological subtype. The Scientific Research Projects Coordination Unit of Istanbul University approved the study protocol (no: 11123).

Immunohistochemical analysis of breast cancer samples. Only tumor cells with distinct nuclear immunostaining for ER and PR were recorded as positive. The ER and PR status of the patients was defined by immunohistochemistry on formalin-fixed, paraffin-embedded sections of clinical specimens as part of routine pathological interpretation. Immunohistochemistry was performed using a rabbit monoclonal antihuman ER antibody (clone SP1; Thermo-Scientific, MA, USA) and a polyclonal rabbit antihuman PR antibody (clone 16, Novocastra, Leica Microsystem, Wetzlar, Germany). Nuclear staining of >10% of cells were accepted as positive for ER or PR status.

RNA extraction and cDNA preparation. Isolation of total RNA from paraffin-embedded tumor and normal tissues of patients was carried out with Absolutely RNA FFPE Kit (Agilient Technologies, Strategene Products Division, San Diego, California, USA) according to the instructions of the manufacturer. According to previous studies, no difference was detected between miR levels from analyzing paraffin-embedded tissue samples and those from frozen samples since, due to their small size compared to mRNAs, miRNAs are much more resistant to enzymatic and mechanic degradation (13).

cDNA synthesis was performed using AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilient Technologies, Strategene Products Division). The targeted *miRNAs, miRNA21, miRNA155, miRNA19a, miRNA17-5p* and *let7a*, and a reference small noncoding (sn) RNA, *U6*, were analyzed by real-time polymerase chain reaction (PCR) using a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany).

miRNA21, miRNA155, miRNA19a, miRNA17-5p and let7a expression by quantitative real-time PCR (qPCR). Eva Green-based qPCR was performed using High-Specificity miRNA QPCR Core Reagent Kit (Agilent Technologies, Strategene Products Division) and a LightCycler® 480 system. All primers were supplied by Agilent Technologies (Strategene Products Division).

To calculate the relative concentration, *miRNA21, miRNA155, miRNA19a, miRNA17-5p, let7a* and snRNA *U6* C_T values for all samples were obtained. The normalized expression for each sample was obtained by subtracting the C_T of snRNA U6 from the same sample's miRNA C_T and designating this as ΔC_T. This value was then transformed using $2^{-(\Delta CT)}$. Furthermore, miRNA expressions in tumor tissues and normal breast tissues were compared as previously described (23, 24).

Statistical analysis. All calculations were performed using SPSS Statistical Program Version 21.0 (SPSS Inc. Chicago, USA). The significance of differences in miRNA levels was determined by the Student's *t*-test or Chi-square test where appropriate. All reported *p*-values are from two-sided tests and a value less than 0.05 was considered statistically significant.

Results

Clinical investigation. The baseline characteristics of the study population are given in Table I. The mean age (±SD) of the study group (n=47) was 58.68±11.75 years. No age-

related expressional differences were found (data not shown). The highest mean expression was observed for *miRNA21*, while the lowest was that for *miRNA155*.

Most patients had disease of histological grade II or more (38/47, 80.9%).

Patients with ER^+PR^+ disease formed 78.7% of the group, 87.2% had invasive ductal carcinoma, while the rest had sub-types of invasive ductal carcinoma. Moreover, 66.0%, 40.0%, 93.6% and 38.3% of the patients had lymphatic vascular, axilla, blood vessel and perineural invasion, respectively. An *in situ* component and necrosis were present in 76.6% and 31.9% of patients, furthermore 44.7% of the tumors exhibited moderate mitosis (8-14 mitoses per 10 high power fields).

Patients with grade III tumors were alike regarding ER and PR expression (ER^+PR^+ and ER^-PR^-), on the other hand 86.5% (n=32) of patients with grade I or II tumors expressed neither receptor (ER^+ or PR^+) (Relative risk (RR)=1.73, 95% Confidential interval (CI)=0.92-3.26, p=0.012; Data not shown).

miRNA Alterations according to clinicopathological characteristics. Only *let7a* alteration and lymphatic invasion were statistically significant associated (p=0.047) (data shown in Table II). As lympatic invasion being a poor prognostic factor and let7 being a tumor-suppressor miRNA, without the proctective regulation of *let7* lymphatic invasion seems sensible. Expressional alterations were defined according to a 1.5-fold difference by comparing malignant tissues to normal surrounding tissues.

Distribution of up- and down-regulations of miRNAs according to the thresholdare shown in Table III.

The present of tumor necrosis and expression of *miRNA21* were found to be associated in statistical analysis (Table IV): *miRNA21* expression was down regulated in 93.3% of patients with necrosis [p=0.017, Fisher's exact test (FE)]. Yet when other oncogenic miRNAs were also taken into account, this influence was no longer apparent (Table VI).

According to the common findings in literature of how these miRNAs act (oncogenic or tumor suppressor), here we defined two combined groups of miRNAs conferring their basic roles and oppositions and investigated what they would display individually or in combination. Therefore, unaltered or down-regulated tumor-suppressor miRNAs (*let7a* and *miRNA17-5p*) and/or up-regulated oncogenetic miRNAs (*miRNA155, miRNA19a* and *miRNA21*), which all are shown to act positively on cancerogenesis individually, are defined as tendency to poor prognosis or cancerogenesis (PPC). Correspondingly, up-regulated tumor-suppressor miRNAs and/or down-regulated or unaltered oncogenetic miRNAs, which all are shown to act as a suppressive of cancerogenesis, are defined as protective or suppressive against cancerogenesis (PSC).

Table I. *Clinicopathological characteristics*.

Patient characteristics	Mean±SD	Range
Age (years)	58.68±11.75	37-85
	Status	n (%)
Histological grade	I	9 (19.1%)
	II	28 (59.6%)
	III	10 (21.3%)
Receptor status	ER^+ PR^+	37 (78.7%)
	ER^- PR^-	10 (21.3%)
Lymphatic vascular invasion	Yes	31 (66.0%)
	No	16 (34%)
Axilla invasion	Yes	19 (40.4%)
	No	28 (59.6%)
In situ component	Yes	36 (76.6%)
	No	11 (23.4%)
Blood vessel invasion	Yes	44 (93.6%)
	No	3 (6.4%)
Necrosis	Yes	15 (31.9%)
	No	32 (68.1%)
Perineural invasion	Yes	18 (38.3%)
	No	29 (61.7%)
Cancer type	Invasive ductal	41 (87.2%)
	Other	6 (12.8%)
Mitosis	I	19 (40.4%)
	II	21 (44.7%)
	III	7 (14.9%)
miRNA expression*		
let7a	0.59±1.69	–2.63-5.53
miRNA17	–0.82±2.41	–8.05-3.86
miRNA155	–0.98±3.23	–13.54-5.21
miRNA21	0.83±2.13	-4.61-6.29
miRNA19a	–0.53±3.09	–7.35-7.22

*Tumor tissue compared to surrounding normal tissue. ER: Estrogen receptor; PR: Progesteron receptor.

miRNA combinations. In addition, we grouped the oncogenic and tumor-suppressor miRNAs and searched for a possible association in between. Combinations of at least 2 to less PPC miRNAs are shown in Table V; no statistical significance was found.

A total of 87.3% of patients with invasive ductal carcinoma had at least one oncogenic miRNA up-regulated [p=0.009 (FE)], while 65.8% patients with tumor grade II+III and all those with grade I had down-regulation or unchanged expression of tumor-suppressor miRNAs [p=0.047 (FE)].

Discussion

In recent years, extensive studies have been conducted on genes that coordinate cancer development and progression. Furthermore, small non-coding RNAs that are the regulators of gene translation, so-called miRNAs, have particularly come to the fore in molecular cancer research and it has recently become clear that the alterations of miRNA expression contribute to cancer pathogenesis (25-27).

Table II. *Distributions of miRNAs according to patient characteristics.*

Patient characteristic		n	let7a miRNA	miRNA17-5p	miRNA155	miRNA21	miRNA19a
Receptor status	ER^+ PR^+	37	0.74±1.80	−0.97±2.17	−1.35±3.48	0.76±2.14	−0.19±3.28
	ER^- PR^-	10	0.03±1.05	−0.20±2.17	0.41±1.47	1.10±2.16	−0.62±3.08
	p-Value		0.246	0.375	0.127	0.660	0.700
Axillary invasion	Yes	19	0.17±1.51	−0.51±2.07	−1.75±3.36	0.45±2.28	−0.32±2.99
	No	28	0.87±1.76	−1.02±2.63	−0.45±2.67	1.09±2.02	−0.67±3.21
	p-Value		0.161	0.479	0.178	0.320	0.707
Prognosis	At least one invasion*	33	0.39±1.54	−0.87±2.22	−1.23±3.55	0.79±2.22	−0.63±3.03
	No Invasion	14	1.06±1.97	−0.69±2.88	−0.38±2.30	0.92±1.96	−0.30±3.35
	p-Value		0.220	0.815	0.415	0.847	0.735
Necrosis	Yes	15	0.81±1.78	−0.82±2.82	−1.27±2.62	0.12±1.68	−0.98±3.93
	No	32	0.48±1.66	−0.82±2.24	−0.84±3.51	1.16±2.25	−0.32±2.66
	p-Value		0.544	0.949	0.677	0.117	0.500
Lymphatic vascular invasion	Yes	31	0.24±1.33	−0.69±2.15	−1.39±3.61	0.68±2.20	−0.57±3.03
	No	16	1.26±2.11	−1.06±2.91	−0.17±2.22	1.12±2.00	−0.46±3.31
	p-Value		0.047	0.626	0.225	0.505	0.914
Perineural invasion	Yes	18	0.43±1.80	−1.54±2.48	−0.45±3.04	0.62±2.39	−0.44±3.55
	No	29	0.69±1.64	−0.37±2.30	−1.30±3.36	0.96±1.98	−0.59±2.84
	p-Value		0.621	0.107	0.385	0.609	0.881
Tumor grade	1+2	37	0.63±1.77	−0.66±2.27	−0.96±3.38	1.03±2.25	−0.32±2.93
	3	10	0.45±1.42	−1.39±2.92	−1.02±2.76	0.09±1.44	−1.31±3.70
	p-Value		0.772	0.399	0.962	0.223	0.373

ER: Estrogen receptor; PR: progesteron receptor. *There are three main invasion types; Lymphatic, vascular and neural invasion. Here having at least one of three is shown

Because of their regulatory function in cell differentiation and renewal under physiological and malignant conditions, miRNAs are emerging as a new class of effective biomarkers for cancer research. miRNAs have crucial regulatory roles in oncogenic and tumor-suppressor pathways (28, 29), which may be a consequence of 50% of miRNA genes being located at fragile, deletion or amplification regions of chromosomes. Since the genomics of cells are dramatically altered in cancer, miRNAs can be induced or suppressed, and alterations can culminate in cancer initiation (5, 30).

The present study is the first to be conducted on a Turkish population and shows the association between the expression levels of the most significant deregulated *miRNAs, miRNA21, miRNA155, miRNA19a, miRNA17-5p* and *let7a* miRNA in both normal and malignant tissues of patients with breast cancer and their impacts on pathogenesis of the disease.

Cancer development starts with the accumulation of multiple mutations, which makes the molecular basis of every person's cancer unique. Different miRNA expressions originate from these mutations, thus each individual' s miRNA pattern might have dissimilarities. miRNA binding sites may also harbor polymorphisms that inhibit binding, and therefore certain miRNAs would be impotent. These personal diversities may create numerous patterns that will subsequently be reflected in cancer characteristics at a more specific level. The more miRNAs that are taken into account, the greater the specificity.

Table III. *Distribution of miRNAs by threshold value (≥1.5-fold change) for up and down-regulation.*

miRNA	Up-regulated, n (%)	Down-regulated, n (%)	Unaltered, n (%)
let7a	10 (21.3%)	19 (40.4%)	18 (38.3%)
17-5p	5 (10.6%)	16 (34.0%)	26 (55.3%)
155	11 (23.4%)	5 (10.6%)	31 (66.0%)
21	15 (31.9%)	6 (12.8%)	26 (55.3)
19a	10 (21.3%)	19 (40.4%)	18 (38.3)

Some miRNAs were found to be down-regulated in malignant tissues compared to normal tissues in this study, which as mentioned before, might be a result of deleted regions of tumor-suppressor miRNAs and may be involved in cancer development. For instance, *miRNA19a* was found to be up-regulated in breast cancer, yet also tended to be deleted (18). In this study, it was up-regulated in 21.3% and down-regulated in 40.4%. Down-regulated patients must have deleted miR-19a regions, which can be seen in some breast tumors, thus in these patients miR-19a did not participate in the carcinogenesis process, yet some other carcinogenesis related miRNA alteration must have taken place, as the ones studied or else. Once more, cancer development may include numerous different deregulated pathways.

Table IV. *Comparison of alterations of miRNA threshold values by patient characteristics.*

	miRNAlet7a		*miRNA155*		*miRNA21*		*miRNA17-5*		*miRNA19a*	
	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated
Axillary invasion										
No	20 (71.4)	8 (28.6)	7 (25.0)	21 (75.0)	10 (35.7)	18 (64.3)	26 (92.9)	2 (7.1)	6 (21.4)	22 (78.6)
Yes	17 (89.5)	2 (10.5)	4 (21.1)	15 (78.9)	5 (26.3)	14 (73.7)	16 (84.2)	3 (15.8)	4 (21.2)	15 (78.9)
	p=0.168 (FE)		p=1.000 (FE)		p=0.498 (FE)		p=0.381 (FE)		p=1.000 (FE)	
At least one invasion*										
No	26 (78.8)	7 (21.2)	5 (35.7)	9 (64.3)	4 (28.6)	10 (71.4)	29 (87.9)	4 (12.1)	4 (28.6)	10 (71.4)
Yes	11 (78.6)	3 (21.4)	6 (18.2)	27 (81.8)	11 (33.3)	22 (66.7)	13 (92.9)	1 (7.1)	6 (18.2)	27 (81.8)
	p=1.000 (FE)		p=0.194		p=1.000 (FE)		p=1.000 (FE)		p=0.456 (FE)	
In situ component										
No	8 (72.7)	3 (27.3)	3 (27.3)	8 (72.7)	2 (18.2)	9 (81.8)	10 (90.9)	1 (9.1)	1 (9.1)	10 (90.9)
Yes	29 (80.6)	7 (19.4)	8 (22.2)	28 (77.8)	13 (36.1)	23 (63.9)	32 (88.9)	4 (11.1)	9 (25.0)	27 (75.0)
	p=0.679 (FE)		p=0.703 (FE)		p=0.461 (FE)		p=1.000 (FE)		p=0.413 (FE)	
Necrosis										
No	25 (78.1)	7 (21.9)	7 (21.9)	25 (56.2)	14 (43.8)	18 (78.1)	30 (93.8)	2 (6.2)	7 (21.9)	25 (78.1)
Yes	12 (80.0)	3 (30.0)	4 (26.7)	11 (73.3)	1 (6.7)	14 (93.3)	12 (80.0)	3 (20.0)	3 (20.0)	12 (80.0)
	p=1.000 (FE)		p=0.725 (FE)		p=0.017 (FE)		p=0.309 (FE)		p=1.000 (FE)	
Lymphatic invasion										
No	12 (75.0)	4 (25.0)	6 (37.5)	10 (62.5)	5 (31.2)	11 (68.8)	15 (93.8)	1 (6.2)	4 (25.0)	12 (75.0)
Yes	25 (80.6)	6 (19.4)	5 (16.1)	26 (83.9)	10 (32.3)	21 (67.7)	27 (87.1)	4 (12.9)	6 (19.4)	25 (80.6)
	p=0.209 (FE)		p=0.101		p=0.944		p=0.648 (FE)		p=0.716 (FE)	
Perineural invasion										
No	24 (82.8)	5 (17.2)	7 (24.1)	22 (75.9)	10 (34.5)	19 (65.5)	26 (89.7)	3 (10.3)	6 (20.7)	23 (79.3)
Yes	13 (72.2)	5 (27.8)	4 (22.2)	14 (77.8)	5 (27.8)	13 (72.2)	16 (88.9)	2 (11.1)	4 (22.2)	14 (77.8)
	p=0.391		p=1.000 (FE)		p=0.632		p=1.000		p=1.000 (FE)	
Histological grade										
Comparison A										
I+II	30 (81.1)	7 (18.9)	10 (27.0)	27 (73.0)	13 (35.1)	24 (64.9)	33 (89.2)	4 (10.8)	8 (21.6)	29 (78.4)
III	7 (70.0)	3 (30.0)	1 (10.0)	9 (90.0)	2 (20.0)	8 (80.0)	9 (90.0)	1 (10.0)	2 (20.0)	8 (80.0)
	p=1.000 (FE)		p=0.413 (FE)		p=0.465 (FE)		p=1.000 (FE)		p=1.000 (FE)	
Comparison B										
I	9 (100.0)	-	4 (44.4)	5 (55.6)	7 (77.8)	2 (22.2)	9 (100.0)	-	1 (11.1)	8 (88.9)
II+III	28 (73.7)	10 (26.3)	7 (18.4)	31 (81.6)	13 (34.2)	25 (65.8)	33 (86.8)	5 (13.2)	9 (23.7)	29 (76.3)
	p=0.453 (FE)		p=0.183 (FE)		p=0.697 (FE)		p=0.567 (FE)		p=0.660 (FE)	

FE: Fisher's exact test. *There are three main invasion types; Lymphatic, vascular and neural invasion. Above at least one of three is shown.

let7a is a tumor-suppressor miRNA, expected to defend the cells from cancerogenesis, regulating numerous cell cycle-related genes (31). A total of 65.9% of the patients with lymphatic invasion had altered expression of *let7a* (p=0.047) and in 80.6% of these patients, let-7a expression was down-regulated. Down-regulation of *let7a* was found in highly metastatic human breast cancer tissues (5). Lymphatic invasion is a poor prognostic factor and some research has been conducted on its significance as a prognostic marker and target in breast cancer treatment (32, 33). Moreover, an inverse correlation between levels of *let7a* and C-C chemokine receptor type 7 (CCR7), known to play an important role in cancer metastasis, was found in both human breast cancer tissues and cancer cell lines (34). Thus, our finding is compatible with previous miRNA target studies.

In tumor cells, induced overexpression of *miRNA21* resulted in increased tumor growth, on the other hand *miRNA21* knock-down paved the way for cell-cycle arrest, increased apoptosis, increased chemosensitivity and reduced invasion (35). Presence of necrosis is related to rapid growth of the tumor, which is a factor of poor prognosis. *miRNA21* was down-regulated in 93.3% of patients with necrosis (p=0.017 (FE)), yet this effect was found to be specific to *miRNA21* among all oncogenic miRNAs since no association was found with other miRNA alterations when investigated individually or were combined. Thus, our finding on this matter is contrary to other findings (12-14). A high level of *miRNA21* was proposed as an indicator of disease progression (36), yet our findings were compatible with another group's findings (37). From this point of view

Table V. *Combination of having at least two poor prognosis related or cancerogenic (PPC) miRNAs to less.*

	Two or more PPC miRNAs n (%)	
	<2	≥2
Receptor status		
ER⁻ PR⁻	3 (30.0)	7 (70.0)
ER⁺ PR⁺	3 (8.1)	34 (91.9)
		p=0.101 (FE)
Axillary invasion		
No	4 (14.3)	24 (85.7)
Yes	2 (10.5)	17 (89.5)
		p=0.865
In situ component		
No	2 (18.2)	9 (81.8)
Yes	4 (11.1)	32 (88.9)
		p=0.614 (FE)
Necrosis		
No	3 (9.4)	39 (90.6)
Yes	3 (20.0)	12 (80.0)
		p=0.367 (FE)
Lymphatic invasion		
No	2 (12.5)	14 (87.5)
Yes	4 (12.9)	27 (87.1)
		p=1.000 (FE)
Perineural invasion		
No	3 (10.3)	26 (89.7)
Yes	3 (16.7)	15 (83.3)
		p=0.662 (FE)
Histological grade		
I-	9 (100.0)	
II + III	16 (5.8)	32 (84.2)
		p=0.579 (FE)

FE: Fisher's exact test; ER: estrogen receptor; PR: progesteron receptor.

Table VI. *Oncogenic and tumor suppressor miRNAs together*

	miRNAlet7 and miRNA17-5, n (%)		miRNA21/ 155/19a, n (%)	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Receptor status				
ER⁻ PR⁻	4 (40.0)	6 (60.0)	7 (70.0)	3 (30.0)
ER⁺ PR⁺	9 (24.3)	28 (75.7)	16 (43.2)	21 (56.8)
	p=0.429 (FE)		*p*=0.168 (FE)	
Axillary invasion				
No	8 (28.6)	20 (71.4)	13 (46.4)	15 (53.6)
Yes	5 (526.3)	14 (73.7)	10 (52.6)	9 (47.4)
	p=0.865		*p*=0.676	
Cancer subtype				
Invasive ductal	12 (29.3)	29 (70.7)	5 (12.2)	36 (87.8)
Other	1 (16.7)	5 (83.3)	6 (100.0)	-
	p=1.000 (FE)		*p*=0.009 (FE)	
In situ component				
No	3 (27.3)	8 (72.7)	7 (63.6)	4 (36.4)
Yes	10 (27.8)	26 (72.2)	16 (44.4)	20 (55.6)
	p=1.000 (FE)		*p*=0.318 (FE)	
Necrosis				
No	8 (25.0)	24 (75.0)	13 (40.6)	19 (59.4)
Yes	5 (33.3)	10 (66.7)	10 (66.7)	5 (33.3)
	p=0.552		*p*=0.096	
Lymphatic invasion				
No	4 (25.0)	12 (75.0)	8 (50.0)	8 (50.0)
Yes	9 (29.0)	22 (71.0)	15 (48.4)	16 (51.6)
	p=1.000 (FE)		*p*=0.917	
Perineural invasion				
No	4 (25.0)	12 (75.0)	3 (10.3)	26 (89.7)
Yes	9 (29.0)	22 (71.0)	3 (16.7)	15 (83.3)
	p=0.493		*p*=0.908	
Histological grade				
Comparison A				
I+II	10 (27.0)	27 (73.0)	17 (45.9)	20 (54.1)
III	3 (30)	7 (70)	6 (60.0)	4 (40.0)
	p=1.000 (FE)		*p*=0.494 (FE)	
Comparison B				
I	-	9 (100.0)	4 (44.4)	5 (55.6)
II+III	13 (34.2)	25 (65.8)	19 (50.0)	19 (50.0)
	p=0.047 (FE)		*p*=1.000 (FE)	

FE: Fisher's exact test; ER: estrogen receptor; PR: progesteron receptor.

miRNA21 seems to have a possible effect on cancer development but not prognosis, since it was not associated with other prognostic factors such as invasion status and high grade. We also searched for a possible pattern with these miRNAs, not surprisingly; finding statistically significant patterns in this small group was not likely.

Among patients with invasive ductal carcinoma, 87.8% had up-regulation of at least one oncogenic miRNA [*p*=0.009 (FE)]. Invasive ductal carcinoma being the most common breast cancer type, finding a possible pliable alteration profile that can assist all patients may be the key for diagnosing, predicting prognosis and deciding on targeted therapy approaches.

miRNAs are highly associated with tumor grade, which is another point making them great candidates as aids in diagnosis or treatment. In this study, 65.8% of the patients with tumor grade II + III and all those with grade I had down-regulation or unaltered expression of tumor-suppressor miRNA [*p*=0.047 (FE)] where oncogenic miRNA alterations were the same for both groups (*p*=1.000). In other words, as the tumor becomes less differentiated, some other oncogenic miRNAs must be stepping in since the protective tumor-suppressor miRNAs have no influence against progression, and poor differentiation occurs despite this suppression.

This study carries importance as a preliminary study and, as mentioned above, with every carcinoma being unique, in order to find patterns or generally accepted markers, this study will be carried on with a larger group of miRNAs. Finding effective therapy approaches for cancer is crucial, as is early diagnosis. From our findings, we have the impression

that miRNAs will be the glowing star of the next-generation approach to breast cancer, especially since they are known to have a finger everywhere in the pie.

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