RESEARCH COMMUNICATION

MicroRNAs and Metastasis-related Gene Expression in Egyptian Breast Cancer Patients

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Abstract

   Aim and background: MicroRNAs (miRNAs) are a class of naturally occurring small noncoding RNAs that regulate gene expression, cell growth, differentiation and apoptosis by targeting mRNAs for translational repression or cleavage. The present study was conducted to study miRNAs in Egyptian breast cancer (BC) and their relation to metastasis, tumor invasion and apoptosis in addition to their association with the ER and PR statuses. **Methods:** Real Time RT-PCR was performed to identify the miRNA expression level of eight miRNAs and eight metastatic-related genes in 40 breast cancer samples and their adjacent non-neoplastic tissues. The expression levels of each miRNA relative to U6 RNA were determined using the $^{2-\Delta CT}$ method. Also, miRNA expression profiles of the BC and their corresponding ANT were evaluated. **Results:** The BC patients showed an up-regulation in miRNAs (mir-155, mir-10, mir-21 and mir-373) with an upregulation in MMP2, MMp9 and VEGF genes. We found down regulation in mir-17p, mir-126, mir-335, mir-30b and also TIMP3, TMP1 and PDCD4 genes in the cancer tissue compared to the adjacent non-neoplastic tissues. Mir -10b, mir -21, mir-155 and mir373 and the metastatic genes MMP2, MMP9 and VEGF were significantly associated with an increase in tumor size (P < 0.05). No significant difference was observed between any of the studied miRNAs regarding lymph node metastasis. Mir-21 was significantly over-expressed in ER-/PR- cases. **Conclusion:** Specific miRNAs (mir-10, mir-21, mir-155, mir-373, mir-30b, mir-126, mir-17p, mir-335) are associated with tumor metastasis and other clinical characteristics for BC, facilitating identification of individuals who are at risk.

   Keywords: Breast cancer - MiRNA - gene expression - metastasis risk - Egypt

Introduction

   Breast cancer (BC) is the leading cause of cancer related death in women (Jemal et al., 2009). Over the past years, the worldwide incidence of BC has nearly doubled. Globally, more than one million cases are reported annually, with nearly half million cases in developed countries (Parkin et al., 2005). Accordingly, BC has become a serious threat to the health of women, with noticeably increased mortality. In Egypt, it is the most common malignancy among Egyptian females accounting to about 37.6% of all malignancies (Parkin DM, 2002; Parkin et al., 2005). Recently, the importance of low-molecular-weight (LMW) RNAs, such as small interfering RNAs (siRNAs) and small nuclear RNAs (snRNAs) has been outlined in several studies. In particular, the importance of a new class of small RNA, miRNAs, has been highlighted (Liu et al., 2010). Each miRNA is thought to regulate multiple genes (Chen et al., 2010) and as hundreds of miRNA genes are predicted to be present in higher eukaryotes, the potential regulatory

circuitry afforded by them is enormous.

   MiRNAs are 20~25 nucleotide, the smallest, functional non-coding RNA, plays important roles in post-transcriptional regulation (Borchert et al., 2006). There may be thousands of miRNA genes in the human genome, transcribed by RNA polymerase as long primary-miRNA molecules, then processed in the nucleus forming pre-miRNAs (Lee et al., 2004; Borchert et al., 2006). These pre-miRNAs get transported from the nucleus to the cytoplasm for further processing (Lee et al., 2003; Lund et al., 2004).

   During oncogenesis, deregulated or dysfunctional miRNA can result in increased translation of oncoprotein and/or decreased translation of tumor suppressor protein (Asangani et al., 2008). By binding to the 3'UTR region of targeted genes, miRNA can rapidly inhibit the translation of the mRNA transcript and subsequently, through formation of RNA-induced silencing complex, cause degradation of the transcript (Gregory et al., 2005). MiRNAs can promote the degradation of the targeted mRNA (Valencia-Sanchez et al., 2006). The quantitative

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and qualitative (mutational) changes in miRNAs and their target binding sites can promote the development and progression of tumors (Calin et al., 2005; Esquela-Kerscher et al., 2006; Cho, 2007; Asangani et al., 2008). MiRNAs profiling studies have revealed their differential expression in various carcinomas compared to that in normal tissue counterparts (Cho, 2007; Zhang et al., 2007) and have further been linked to the repression of tumor suppressor genes or the up regulation of oncogenes at the protein production level (Mayr et al., 2007; Meng et al., 2007; Sampson et al., 2007; Corney et al., 2008). Several miRNAs are associated with BC, miR-155 is up-regulated in BC and may act as an oncogene (Iorio et al., 2005; Ma et al., 2007). Up-regulation of miR-373 and miR-520c promotes metastasis. The increased expression of gene encoding miR-10b can promote tumor invasion (Ma et al., 2007). MiR-21 up-regulated in BC causing down-regulation in programmed cell death 4 and tropomyosin 1 genes (Zhu et al., 2007; Frankel et al., 2008).

Degradation of basement membranes and extracellular matrix is an essential process in invasion and metastasis in malignant tumor. Matrix metalloproteinase (MMP), potent proteolytic enzymes are known to play key roles in this process. Two members of MMP family, MMP-2 and MMP-9 cleave the type IV collagen and gelatin, which are the principal structural components of basement membrane (Toi et al., 1998). Expression of MMP2 and MMP9 has been reported in different cancers (Pyke et al., 1992; Burg-Roderfeld et al., 2007; Wu et al., 2008; Boxler et al., 2010; Chu et al., 2010) and their expression has been correlated with local invasion by the tumor, lymph node metastasis and survival rates (Talvensaari-Mattila et al., 1998). Tissue inhibitors of matrix metallo-proteinases (TIMPs) have the ability to inhibit the catalytic activity of MMPs and can also take part in the activation of MMPs (Gomez et al., 1997). TIMPs seem to have anti-angiogenic activity and they are also able to act as growth factors (Fassina et al., 2000). The expressions of MMPs and TIMPs are associated with the clinical behavior in BC (Turpeenniemi-Hujanen, 2005).

Angiogenesis is known to play an important role in the development of tumor growth and metastasis. Vascular endothelial growth factor (VEGF) an angiogenic factor (Nieves et al., 2009), increases vascular permeability and promotes the formation of new blood vessels by stimulating endothelial cells to migrate and divide (Hicklin et al., 2005). Programmed Cell Death 4 (PDCD4) has been strongly associated with the progression and metastasis of multiple human cancer types. PDCD4 is known to play a role in apoptosis but its specific mechanism has yet to be determined (Vikhreva et al., 2010; Wang et al., 2010). Recent studies indicate that PDCD4 may have important roles in transcription, translation, and signal transduction pathways (Wang et al., 2010). The aim of the present study is to examine the miRNAs expression levels , selected based on previous studies (Iorio et al., 2005; Ma et al., 2007; Vigorito et al., 2007; Zhu et al., 2007; Asangani et al., 2008; Huang et al., 2008; Li et al., 2010), in Egyptian BC patients and their relation to different clinico-pathological features.

Materials and Methods

This study was conducted on 40 BC cases and their matched adjacent non-neoplastic tissues were collected from pathology department, National Cancer Institute, Cairo University between 2007 and 2008. All BC patients were females and had undergone radical mastectomy or modified radical mastectomy. Patients who had received any chemotherapy or radiation therapy before surgery or who had rheumatic disease, acute infection, or other types of cancer were excluded from the current study. Table 1 shows the clinico-pathological features of the participants. The study was conducted in compliance with the Helsinki Declaration and was approved by the Senior Staff Committee. All involved patients gave a written informed consent. All laboratory work was done in the National cancer Institute, Cairo University, and college of pharmacy, pharmacology department, King Saud University.

Nucleic acid extraction and reverse transcription

The total RNA and genomic DNA were isolated from 100 mg of frozen tissue after micro-dissection with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Isolated RNA and DNA were analyzed and quantified on a Nano Drop spectrophotometer. Total RNA

Table 1. Clinicopathologic Characteristics of Breast Cancer in Relation to Genes Expression Level

Factors	No. Of cases	Mean Fold expression of target gene ± SE							
		MMp2	MMp9	TIMP1	TIMP3	TMP1	PDCD4	VEGF	CyclinD1
Tumor size:									
large >2cm/	28	21.1*± 1.8	11.73±1.05	0.69±0.05	0.69± 0.09	0.70± 0.09	0.61± 0.044	18.60*± 1.68	0.6074±0.032
Small <2cm	12	8.26± 2.07	11.49± 2.06	0.75± 0.17	0.60±0.09	0.54± 0.06	0.8189± 0.13	6.920 ± 1.37	0.8502±0.126
Histological grade:									
I/II	25	8.09± 1.08	12.16± 1.29	0.74±0.08	0.70±0.12	0.63±0.08	0.6593±0.053	6.301±0.75	0.6602±0.46
III	15	7.75±1.18	10.81±1.34	0.66±0.08	0.60±0.07	0.69±0.12	0.6946±0.109	6.572±1.113	0.7136±0.100
Lymph node metastasis:									
Negative	21	6.52±0.91	12.63±1.30	0.60±0.05	0.59±0.07	0.72±0.12	0.7211±0.080	7.291±1.037	0.7005±0.049
Positive	19	9.55±1.28	10.57±1.37	0.83±0.11	0.73±0.12	0.58±0.05	0.6190±0.057	5.421±0.59	0.6578±0.083
ER/PR:									
Positive	32	7.96± 0.98	11.48±1.05	0.71±0.07	0.68±0.08	0.59±0.06	0.6777±0.059	5.993±0.616	0.6929±0.057
Negative	8	7.94± 0.86	12.35±2.25	0.69±0.05	0.61±0.08	0.90±0.20	0.6522±0.094	8.038±1.905	0.6294±0.052

* 'indicated a significant within the same factor

(700ug) was loaded to the micro RNA isolation column (Qiagene, Germany), for isolation of low molecular weight (LMW) RNA following the manufacturer's protocol. One microgram aliquot of DNase-treated total RNA and LMW RNA were reverse transcribed to cDNA using antisense of gene-specific primers, U6 and Thermoscript, thermostable reverse transcriptase (Invitrogen). One microgram RNA was incubated with $1.5\mu l$ of a cocktail containing 10mM each of the antisense gene specific and U6 primers. The reaction was denatured at 800C for 5 min, incubated for 5 min at 600C to anneal the primers, followed by cooling to room temperature and the remaining reagents [5 x buffers, dNTPs, DTT, RNase inhibitor, Thermoscript] were added as specified in the Thermoscript protocol and the reaction proceeded for 45 min at 60°C. Finally, reverse transcriptase was inactivated by incubating the reaction at 85°C for 5 min.

MiRNA expression by Real-Time PCR

The expression of the miRNA precursors was determined by using real-time quantitative PCR (Jiang et al., 2005). miRBase Sequences is the primary online repository for miRNA sequence data and annotation. miRBase Targets is a comprehensive new database of predicted miRNA target genes. miRBase is available at http://microrna.sanger.ac.uk/.Master mix contained 0.5 ul of 10x PCR buffer, 0.7ul of 25mM MgCl2, $0.1\mu l$ of 12.5mM dNTPs, 0.01ul UNG, $0.5\mu l$ of DNA Taq polymerase,SybrGreen, $0.5\mu l$ of dilute cDNA (1:50) and completed with water to $3 \mu l$. Three microliters of the master mix containing all of the reaction components except the primers was dispensed into a 96-well real-time PCR plate (Applied Biosystems, 7500). A $2\mu M$ solution of each pair of primers listed was stored in 12-well PCR strip tubes. Each primer ($2\mu l$) was dispensed into duplicate wells of the 96-well plate. All the reactions were run in triplicate and included no template and no reverse transcription controls for each miRNA. The reactions were amplified for 15s at 95°C and 1 min at 60°C for 40 cycles. The thermal denaturation protocol (dissociation curve) was run at the end of the PCR to determine the number of products in the reaction. Real-time PCR is a sensitive and reproducible gene expression quantitation technique which is now being used to profile miRNA expression in cells and tissues. To correct for systematic variables such as amount of starting template, RNA quality and enzymatic efficiencies, the data is commonly normalized to a universal endogenous control gene, which ideally, is stably-expressed across the test sample set. The expression of each miRNA relative to U6 RNA was determined using the $2^{-\Delta CT}$ where $\Delta CT = (CTmiRNA-CTU6RNA)$ relative gene expression multiplied by 105 in order to simplify the presentation of the data.

Real time PCR for gene expression

Real-time PCR was performed on an Applied Biosystems 7500 to detect the expression of TIMP1, TIMP3, MMp2, MMp9, TPM1, PDCD4, VEGF, and cyclin-D1 genes. All reactions were run in triplicate and included no template and no reverse transcription controls for each gene. The reactions were amplified for 15 s at

95°C and 1 min at 60°C for 40 cycles. The expression of each gene relative to GAPDH was determined using the $2^{-\Delta\Delta}CT$.

Melting curve and agarose gel electrophoresis analysis

Following amplification, melting curve analysis was performed to verify the specific product according to its specific melting temperature (Tm). The results were analyzed by the melting curve analysis software of applied biosystem. Amplification plots and Tm values were analyzed to confirm the specificities of the amplicons for SybrGreen -based PCR amplification.

Validation of miRNA precursor primers by SybrGreen PCR

Each pair of primers included in this study was validated on extracted genomic DNA, mouse genomic DNA and no template control reaction. All of the primers worked successfully on genomic DNA by the presence of single peak on the thermal melting curve but not on mouse genomic DNA.

Statistical analysis

Statistical analysis was performed using the SPSS version 9.0 software program and a statistical difference was considered significant when the p value was 0.05 or less.

Results

To generate a comprehensive set of miRNA expression profiles for Egyptian BC patients, 40 primary BC samples and their normal adjacent tumor were investigated for miRNA expression profiling. In BC tissues, mir-155, mir-10, mir-21 and mir-373 were significantly over-expressed by 14.46, 5.7, 10.69 and 5.87 folds respectively compared to normal adjacent tumor tissues Figure 1. In contrast, mir-17p, mir-126, mir-335 and mir-30b were significantly decreased by 0.31, 0.297, 0.17 and 0.55 folds expression respectively compared to normal adjacent tumor tissues Figure 2.

In the BC tissues, MMP2, MMP9 and VEGF genes were significantly over expression by 7.1, 15.3 and 7.6 folds respectively expression compared to non-neoplastic tissues. Conversely, in cancer tissue TIMP3, TMP1 and PDCD4 genes were significantly decreased by 0.16, 0.23 and 0.18 folds respectively compared to non-neoplastic
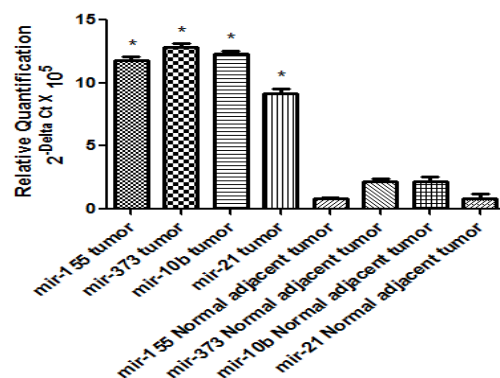
Figure 1. MiR Expression in Breast Cancer Tissue in Relation to Normal Adjust Tumor Tissue
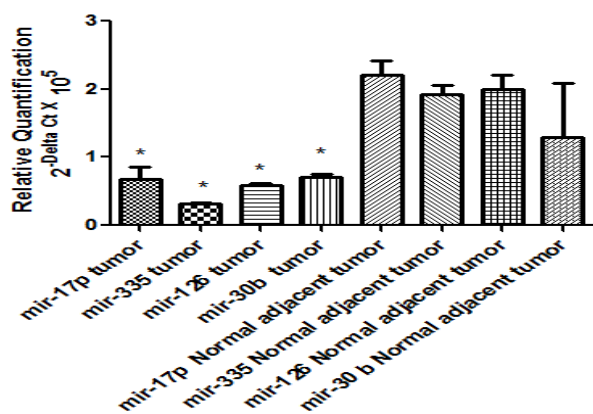
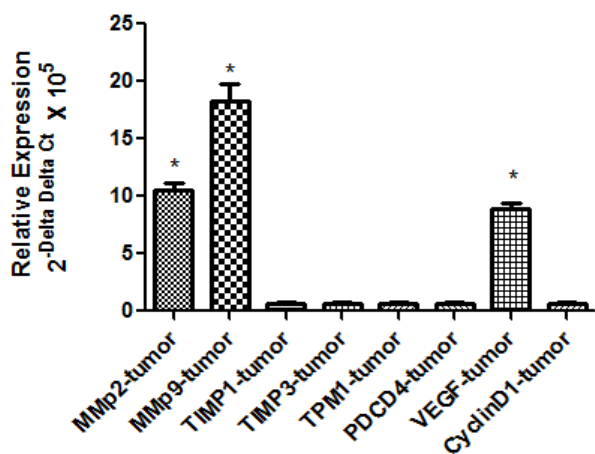**Figure 2. MiRs Expression in Breast Cancer Tissue in Relation to Normal Adjust Tumor Tissue**

Figure 3. Gene Expression in Breast Cancer Tissue in Relation to Normal Adjacent Tumor Tissue

tissues. There were insignificant decrease in TIMP1 and cyclin D1 genes by 0.36 and 0.5 compared with non-neoplastic tissues (Figure 3).

Tumor characteristics and their associations with the expression levels of the miRNAs are summarized in Table 2. We tested miRNAs for statistically significant associations with tumor characteristics such as tumor size, histological grade, lymph node metastasis and ER/PR status. According to the tumor size, mir -10b, mir -21, mir-155 and mir373 were significantly associated with increasing in tumor size (P < 0.05). Wherein, in large tumor size (>2cm) mir -10b and mir -21, mir-155 and

mir373 expression levels were 10.5, 13, 13.14 and 14 folds compared to 6.25, 9, 10.2 and 10.4 folds respectively in cancer samples having smaller tumor size (<2cm). Whereas, mir-30b, mir-126 and mir-335 were equally expressed in tumor tissues having large or small tumor size.

In relation to pathological grade, mir-10b and mir-373, mir-155 and mir-21 were significantly associated with increasing in the pathological grade (P < 0.05). Wherein, in BC samples having grade III, the mir-10b and mir-373, mir-155 and mir-21 expression were 13.27, 14, 13 and 10.43 compared to 11.75, 12.16, 11 and 8.4 folds respectively in cancer samples having grade I or grade II. Whereas, mir30b, mir-126 and mir-335 were equally expressed in cancer samples having high or low tumor grades.

There were no significant differences observed between any of the studied miRNAs in the BC patients having positive lymph node metastasis as compared to their counterparts (P>0.05).

In relation to estrogen/ progesterone receptors, there were non-significant high expression level of mir-10b, mir-155, mir-17b and mir-373 (16.25, 15.87, 1.45 and 13.45 respectively) in the patients having ER–/PR– compared to 12.11, 11.48, 0.5 and 12.56 folds respectively in BC patients having ER+/PR+. In contrast, mir-21 was significantly over-expressed (18.7 fold) in BC cases having both ER–/PR– compared to 8.83 folds in cases having ER+/PR+. Furthermore, there were no significant differences in the down-regulation of mir-30b, mir-126 and mir-335 in patients with positive or negative ER/PR. The expression levels of the selected studied genes for the metastasis and invasion and their relations to clinicopathological features of BC patients are summarized in Table 2. According to the tumor size there was significant difference observed in MMP2, MMP9 and VEGF in comparison to non-neoplastic tissue P<0.05. Whereas, in large tumor size (>2cm) the gene expression level of MMP2, MMP9 and VEGF were significantly high 12.5, 21.1 and 10.26 as compared to 5.9, 11.5 and 5.6 folds in the patients having small tumor size. There were an equal expression levels in PCDC4, TMP1, TIMP1, TIMP3, and cyclin D1genes in patients having large or small tumor size. In relation to histological grade, lymph node metastasis and ER/PR, there were no statistical

Table 2. Tumor Characteristics and Their Associations with the Levels of the miRNAs

Factors	No. Of cases	Mean Fold expression of target gene ± SE							
		Mir10b	Mir21	Mir17p	Mir30b	MiR-126	MiR-155	MiR-373	MiR-335
Tumor size:									
large >2cm	28	53.7*# ±4.2	62.2*# ±3.7	0.4±0.0	0.7 ± 0.1	0.6 ± 0.0	71.5* ± 3	27.53* ± 5.0	0.336 ±0.015
Small <2cm	12	35.3± 4.99	34.6± 4.6	0.3± 0.0	0.8±0.1	0.6± 0.0	46.3± 7.4	29.51± 9.98	0.3178±0.017
Histological grade:									
I/II	25	29.1*± 2.2	62.3* ± 4.8	0.7±0.2	0.7±0.0	0.5723±0.023	53.4*± 5	6.156*±0.35	0.3239±0.013
III	15	67.3±4.01	60.9±5.4	0.6±0.3	0.8±0.1	0.6379±0.065	54.7 ±6.5	61.30±3.4	0.3293±0.023
Lymph node metastasis:									
Negative	21	53.8±4.9	64.2±5.2	0.9±0.35	0.7±0.1	0.5733±0.034	33.9*±3	29.77±6.3	0.3197±0.018
Positive	19	54.4±4.7	59.2±5.0	0.3±0.031	0.7±0.1	0.6231±0.043	71.0±3.9	23.59±6.57	0.3329±0.016
ER/PR:									
Positive	32	43.3±3.9	58.5±4.1	0.5±0.1	0.7±0.1	0.6099±0.034	52.9±4.4	26.37±4.9	0.3202±0.0144
Negative	8	43.9±9.1	75.0±6.1	1.5±0.8	0.7±0.1	0.5450±0.037	58.0±8.1	28.70±11.5	0.3491±0.017

* 'indicated a significant within the same factor

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differences observed in the expression levels of the studied genes (P> 0.05). In the tumor tissues having increase in the mir-21, the expression level of PCDC4, TMP1, TIMP1, TIMP3, and cyclin D1genes were down-regulated and the expression levels of MMP2, MMP9 and VEGF genes that promote cell migration and invasion were up regulated. Breast cancer patients with highly overexpression for miR-10b and miR-373 showed an over expression in the level of the MMP2 and MMp9 genes and a decrease in the TIMp1 and TIMP3.

In the cancer tissues having up-regulation for the mir-155, the expression levels of TIMP1, TIMP3, PDCD4, and TMP1 genes were down regulated, and have up-regulation for MMP2 and MMP9 genes. In the BC tumor tissue, high expression level for MMP2, MMP9 and VEGF genes were observed in tissues having low expression level mir-17p, mir-126, mir-335 and mir-30b,and have low gene expression level of PCDC4, TMP1, TIMP1, TIMP3, and cyclin D1.

Discussion

MicroRNAs (miRNA) are family of short non-protein-coding RNAs that negatively regulate gene expression and are required for cell viability. MiRNAs control normal rates of cellular growth, proliferation, differentiation and apoptosis, hence, down-regulation of them may play a role in the development or progression of cancer since they inhibit cell cycle progression and drive terminal differentiation (Croce, 2009). By targeting and controlling the expression of mRNA, miRNAs can control highly complex signal transduction pathways and other biological pathways. The biologic roles of miRNAs in cancer may correlate with diagnosis, prognosis and therapeutic outcome. In this study, 40 BC and their adjacent non-neoplastic tissues were studied for detecting the expression levels of miRNAs and some of their target genes.

In mammalian cells, several miRNAs were involved in cell death and regulated a variety of cellular pathways through regulation of expression of multiple target genes (Schmid et al., 2008). In the present study, BC miRNAs expressions were quantified by using real-time PCR in which miR-21, mir-373, mir-10b and mir-155 were up-regulated, whereas, mir-17p, mir-335 and mir-126 were down-regulated in tumor tissue compared to ANT. Their expressions in tumor tissues were accompanied by over-expression in MMP-2, MMP-9 and VEGF and suppression in TIMP-1, TIMP-3, TMP-1, PDCD-4 and cyclin-D1 genes.

The mir-21 gene is located on chromosome 17q23.2, having anti-apoptotic function and its inhibition led to a profound increase in cell growth (Cheng et al., 2005). Up-regulation of mir-21 is associated with solid cancers including breast and pancreatic cancer (Schmittgen et al., 2004; Chan et al., 2005; Chang et al., 2007). In the present study, mir-21 was significantly associated with increasing tumor size, in large tumor size its expression levels was 13 fold compared to 9 folds in smaller tumor size. Mir-21 was significantly associated with increasing in the pathological grade, in samples having grade III its expression was two folds more than the samples having grade I or grade II. In

a similar previous study on the expression of miRNAs, mir-21 was significantly up-regulated in BC (Iorio et al., 2005). Our data are consistent with other reports indicating that mir-21 expression increased with advanced clinical stage (Li et al., 2010; Reis et al., 2010) .

Mir-21 functions as an oncogene because it is over-expressed in tumor compared with the normal tissues (Vandesompele et al., 2002; Sieuwerts et al., 2006; Kulshreshtha et al., 2007; Smid et al., 2008) and its suppression inhibits cell growth through activation of apoptosis pathways causing activation of effector caspases and increased cell death (Kulshreshtha et al., 2007; Smid et al., 2008). Endogenous angiogenesis inhibitors TIMPs are necessary to block the mitogenic stimuli in the vascular endothelium (Curran et al., 2000). Tropomyosin 1 (TPM1) is a member of the tropomyosin family of proteins, which are associated with actin and serve to stabilize microfilaments and act as tumor suppressor gene (Perry, 2001). In this study, for the up-regulated mir-21, the genes that belonged to the class of tumor suppressor genes TIMP1, TIMP3 and TMP1 were suppressed. Similar to our data, others found suppression in the tumor suppressor genes, TIPM1 and TIPM3 that played a role in the malignant phenotype (Mulder et al., 1994; Yu et al., 2004). The Suppression of TPM1 has been reported in malignant cells, suggesting a role for these proteins in neoplastic transformation(Raval et al., 2003). The down-regulation of TIPM1 and TIPM3 with the up-regulation of mir-21 may confirm the suppression of mir-21 and can inhibit tumor growth (Jones et al., 1999) supporting the concept that mir-21 functions as an oncogene. Our study suggested that the expression of mir-21may play a role in suppression of TIMP3 and TIMP1.

Tumor-suppressor PDCD4 targets translation by inhibiting transformation and invasion in cancers (Schmid et al.,2008). High level of PDCD4 play a role in apoptosis rendered cells resistant to transformation (Cmarik et al., 1999). Down-regulation of PDCD4 at both the mRNA and protein levels significantly associated with regional disease, more advanced tumor stages, and poorer survival .(Reis et al., 2010). In the present study there was down-regulation of PDCD4 in the breast cancer tissues compared to the adjacent non-neoplastic tissues and was associated with the increase in the expression of mir-21. Similarly, the expression of mir-21 and suppression of PDCD4 has been previously reported in BC and colorectal cancer (Leupold et al., 2007; Lu et al., 2008). Other studies had also shown that PDCD4 is the direct targets of mir-21 (Meng et al., 2007; Zhu et al., 2007; Frankel et al., 2008).

The up-regulated of mir-155 in BC, is suggesting that it may act as an oncogene (Vigorito et al., 2007). In this study, mir-155 was over-expressed by 14.5 folds in BC samples compared to the ANT tissues respectively. Similar studies on BC using microarray technique, the mir-155 was up regulated (Iorio et al., 2005; Volinia et al., 2006; Iorio et al., 2007). In our study, the over-expression of mir-155 was associated with the over-expression of MMP2, MMP9 and VEGF genes. The up-regulation of mir-155 has been found to be associated with down-regulation in the gene expression level of TIMP1, TMP1 and TIMP3 in the same cancer tissues. Mir-155 was significantly associated

with increasing in tumor size and pathological grade. Our study suggested that the targeted genes for the mir-155 over-expression may be MMP2, MMP9 and VEGF genes.

Mir-30 family members include mir-30a, -30b, -30c, -30d and -30e. Our present study shows that mir-30b is down-regulated in BC compared to ANT tissues. Mir30b was equally expressed in tumor tissues having large or small tumor size and in samples having high or low tumor grades. Expression of the gene encoding mir-30 seems to correlate with estrogen receptor and progesterone receptor status; down-regulation of this miRNA is found in estrogen receptor– and progesterone receptor–negative tumors (Bartels et al., 2009). The current study showed no difference observed in the expression level of mir-30 and the ER/PR status. The suppression of mir-30b was associated with over-expression in MMP2, MMP9 and VEGF genes and down-regulation of TIMP1, TIMP3, TMP1 and PDCD4 genes. Similarly, other study found that miR-30 family are able to suppress apoptosis (Li et al., 2010). To our knowledge, there has been no publication delineating the relationship between miR-30 and metastasis.

Mir-17–5p , also known as mir-91, is encoded by a gene located on chromosome 13q31, which is a region that undergoes loss of heterozygosity in BC. This miRNA normally represses translation of the AIB1 (amplified in breast cancer 1) mRNA. AIB1 is a co-activator of the cell cycle regulator E2F1, and it also enhances estrogen receptor– dependent transcription (Bartels et al., 2009). In the current study mir-17p was found to be down-regulated in cancer compared to adjacent non-neoplastic tissues. Similarly, mir-17–5p has been found to be down-regulated in BC (Bartels et al., 2009).

In vivo ectopic expression of mir-10b conferred invasive properties on otherwise non-invasive BC cells; mir-10b over-expressing tumors exhibited an invasive behavior and were highly vascularized (Negrini et al., 2008). The over expression of mir-10b can promote tumor invasion and is associated with the increase of VEGF that play role in the angiogenesis. In this study, mir-10b was over-expressed by 5.7 fold in BC tissues compared to the ANT tissues. Some studies found the same elevation pattern for the mir-10b , in contrast others found down-regulation in BC in comparison with normal breast tissue (Negrini et al., 2008).. Mir-10b was significantly associated with increasing in tumor size, its expression level in large tumor size was 10.5 fold compared to 6.25 folds in smaller size. It was significantly associated with increasing in the pathological grade, in grade III samples, the mir-10b expression was 13.27 compared to 11.75 in grade I or grade II. Similarly, a recent study showed that mir-10b initiates BC invasion and metastasis (Ma et al., 2007). Mir-10b can promote metastasis in otherwise non-metastatic BC cells (Negrini et al., 2008). In contrast to our study, mir-10b was found to be down-regulated in about 50% of the metastatic breast cancers by using microarray technique in comparison with normal tissue (Iorio et al., 2005; Ma et al., 2007). This difference might be due to the difference in the technical methods used.

Mi-373 is metastasis-promoting micro-RNAs, by inhibiting Cyclin-D1 expression (Tavazoie et al., 2008).

In this study, the BC tissues had over-expression of the mir-373 by 6 folds compared to the adjacent non-neoplastic tissue. The up-regulation of mir-373 was found to be associated with down-regulation of TIMP1, TMP1 and TIMP3 and with over-expression in MMP2, MMP9 and VEGF genes in tumor tissues. Our results confirm that the up-regulation of mir-373 promotes metastasis by suppression of TIMP1 and TIMP3 and expression of MMP2 and MMP9. Similarly, others reported that mir-373 stimulates cancer cell migration and invasion (Huang et al., 2008; Tavazoie et al., 2008). In vitro studies found that over-expressing mir-373 developed metastatic nodules, which were absent in the control cells. Mir-373 was up-regulated in cancer, particularly in tumors exhibiting lymph node metastasis (Negrini et al., 2008).

Down-regulation of mir-335 promotes metastasis by up-regulating the extracellular matrix protein tenascin C and the transcription factor SOX4 . The role of miR-126 seems to be that of a tumor suppressor, and a decreased concentration of this miRNA promotes cell proliferation (Bartels et al., 2009). In this study, we found down-regulation of mir-335 and mir-126 in the BC tissues compared to ANT. The suppression of both mir-335 and mir-126 in cancer tissues were associated with over-expression in MMP2, MMP9 and VEGF genes and down-regulation of TIMP1, TIMP3 and PDCD4 genes. Similar to our study, Tavazoie et al (Tavazoie et al., 2008) found that mir-335 and mir-126 are metastasis-suppressor miRNAs. They found that miR-335 and miR-126 were consistently down-regulated in metastatic foci and restoring the expression of them significantly decreased the number of metastatic foci and significantly associated with poor metastasis-free survival. Our results suggest that the down-regulation of both mir-335 and mir-126 may promote metastasis by inhibiting the expression of TIMP1 and TIMP3 and increase the expression of MMP2, MMP9 and VEGF genes. Thus, these two miRNAs can be taken as biomarkers for developing metastasis.

In conclusion, MiRNAs are associated with clinical characteristics for Egyptian BC, unlike most other biomarkers that are currently available. To the best of our knowledge, this is the first report to demonstrate that miRNA are related to invasion and metastasis of BC in Egyptians. This study suggests that the studied miRNAs (mir-10, mir-21, mir-155, mir-373, mir-30b, mir-126, mir-17p, mir-335) can be used in BC patients prognosis.

Acknowledgements

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