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Research Article

Role of miRNA-155 And -10B as Biomarkers of Breast Cancer in Egyptian Women

Amr K S[1*], Ali O S M[2], Afify M[3], Abd-Allah R M[2]

[1]*Medical Molecular Genetics Department, National Research Center, Cairo, Egypt*
[2]*Biochemistry Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt*
[3]*Biochemistry Department, Division of Genetic Engineering and Biotechnology, National Research Center, Cairo, Egypt*

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ABSTRACT

Breast cancer is a common form of cancer among women globally. The potential role of circulating miRNAs as cancer biomarkers relies mainly on their high stability and their capacity to reflect tumor status and predict therapy response. The aim of the study was to find the role of miRNA-155 and -10b in breast cancer Egyptian women. The study enrolled 30 pathologically confirmed breast cancer women beside, 30 apparently healthy age-matched women, comprised the control group. Relative expression of miRNA-155 was (37.2 ± 7.8) and for 10b (28.6 ± 6.8) in BC patients compared to the control subjects (p value < 0.0001). MiRNA-155 was more sensitive (sensitivity: 100%) and specific (specificity: 90.9%) compared to miRNA-10b which exhibits sensitivity and specificity (95.8%) and (86.36%) respectively. Serum PDCD4 and cyt c were significantly increased in BC group ((p value < 0.0001). Plasma miRNA-155 and -10b may be used as early diagnostic markers of BC in Egyptian women.

Keywords: Breast cancer; MiRNA-155; MiRNA-10b; Quantitative RT-PCR; ROC Curve.

INTRODUCTION

Breast cancer is the most common female cancer worldwide representing nearly a quarter (23%) of all cancers in women. The global burden of BC is expected to cross 2 million by the year 2030, with growing proportions from developing countries[1]. It is a leading cancer in the majority of countries in Africa, according to data from 26 African countries for 2012. In Africa, breast cancer is responsible for one in four diagnosed cancers and one in five cancer deaths in women[2]. In Egypt, the rate of breast cancer is higher than the worldwide records representing 32.04% of female cancers diagnosed during 2008–2011. More importantly; it has been reported that 49.7% of the Egyptian patients have regional spread at the time of diagnosis and 11.9% of them have distant metastasis[3]. MicroRNAs (miRNAs) are small single-stranded noncoding RNA of about 20–25 nucleotides in length, these small molecules have an important role in the regulation of a wide range of biological processes, as regulation of gene expression and control many pathways as cell growth, differentiation, and apoptosis by controlling their target gene expression[4]. Circulating miRNAs represent ideal candidates for non-invasive biomarkers to diagnose disease, predict prognosis, and measure therapeutic response[5]. MiRNA-155 is one of the most commonly up-regulated miRNAs in solid and hematological malignancies, and has been linked to the development of leukemia, breast, lung, and stomach tumors[6]. In humans, miRNA-155 is encoded by gene MIR155HG (also termed the B cell integration cluster, or BIC locus), which is located on chromosome 21. The primary miRNA molecule is processed from the BIC gene RNA transcript[7]. One of the first miRNAs implicated as playing a role in metastasis was miRNA-10b. MiRNA-10b is subtype of the miRNA-10 family and plays a role in metastasis and development[8]. The miRNA-10 family consists of miRNA-10a and miRNA-10b. Both genes are situated within the homeobox (Hox) clusters of genes that are essential for normal body patterning in developing vertebrates. They are co-regulated with Hox genes; target multiple Hox mRNAs in mammalian cells[9]. Apoptosis is the cell's natural mechanism for programmed cell death. It is particularly critical in long-lived mammals as it plays a critical role in development as well as homeostasis[10]. The balance between proliferation, differentiation, and death of the cells throughout the mammary gland is critical for normal development and homoeostasis. Situations that can upregulate cell proliferation or downregulate apoptosis may allow accumulation of mutations that result in breast cancer[11]. Programmed cell death protein 4 (PDCD4) is a novel tumor suppressor protein involved in programmed cell death. Up regulation of PDCD4 is observed after the initiation of apoptosis, suggesting that loss of, or reduced, PDCD4 expression could contribute to the anti-apoptotic property of cancer cells[12]. It is expressed in small duct epithelial cells of the normal mammary gland, normal human lung tissue and senescent human fibroblasts. PDCD4 expression is attenuated with progression in human tumors of the colon, prostate and breast[13]. The mitochondrial protein cytochrome c (cyt c) is an important

*Author for Correspondence: khalda_nrc@yahoo.com.

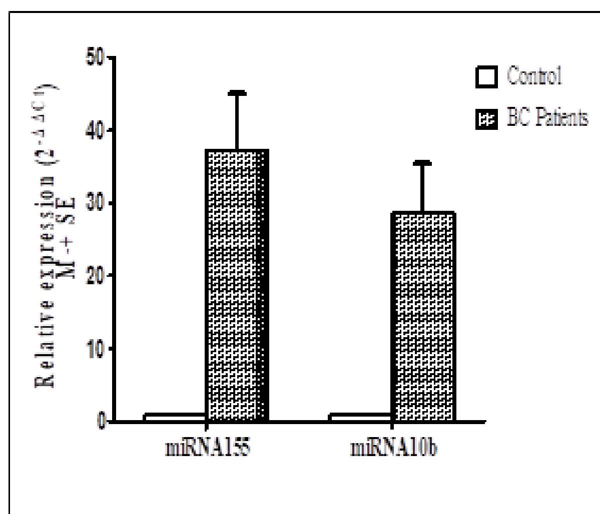

Figure 1: Relative Expression of miR-155 and 10b

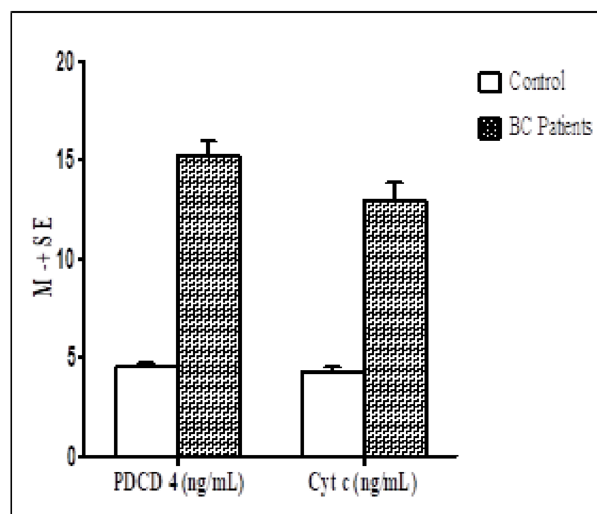

Figure 2: M ± SE of PDCD4 and cyt c.

initiator/ amplifier of programmed cell death or apoptosis. It has been implicated in a number of apoptotic functions and has considerable clinical potential as a serum marker for aberrant apoptosis[14]. The current study aimed to evaluate the potential value of plasma miRNA-155 and -10b in BC Egyptian patients as predictive molecular biomarkers of BC in Egyptian women.

MATERIALS AND METHODS

This study enrolled 60 females: 30 pathologically confirmed BC women have been randomly recruited from BC women who underwent surgical resection for the treatment of breast neoplasm at National Cancer Institute, Cairo, Egypt with the age range from (21-62) years (mean ± S.E. of 46.43 ±1.57). 30 healthy women with matched age ranged from (23-60) years (mean ± S.E. of 45.53 ± 1.44) comprised the control group. Patients were early diagnosed and didn't receive Chemo or radio therapy at the time of diagnosis. Metastatic patients were excluded from the study. All participants in this study gave written informed consent.

Pathological staging

All patients have been diagnosed with primary BC mass and invasive ductal carcinoma stage II. Clinical staging was expressed according to the TNM classification system[15] based on evaluation of findings of physical examination, routine laboratory tests, radiological reports (chest X-ray, liver Echography, bone scan and computed tomography) and pathological records.

Methods

Venous blood samples (4 mL) have been withdrawn from patients and controls under complete aseptic conditions then divided as the following: 2 mL was collected in EDTA and centrifuged for plasma separation and stored at - 80 ◦ C until used for MiRNA extraction. 2 mL was left to clot for 30 min at room temperature and then centrifuged at 3000 rpm for serum separation. Extraction of MiRNA-155 and -10b was carried out from the stored plasma at -80◦C using miRNA isolation kit (Qiagen), NanoDrop® (Thermo Scientific) spectrophotometer was used to measure the absorbance of isolated RNA at 260 nm, 280 nm and 230 nm. TaqMan® miRNA assays used a stem-looped primer for reverse transcription and a sequence-specific TaqMan assay to accurately detect mature miRNAs. Then, amplification of cDNA using Taq Man master mix (Applied Biosystem) and primer assay of each miRNA were quantified by Real Time PCR. Analysis of relative miRNA expression data from qRT- PCR was accomplished using the change in expression of the target miRNA relative to the reference control. Using the $2^{-\Delta\Delta CT}$ method, the data is presented as the fold change in miRNA expression normalized to an endogenous control and relative to the healthy controls. For the healthy control sample, -ΔΔCT equals zero and 2^0 equals one, so that the fold change in miRNA expression relative to the healthy control equals one. For the samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in miRNA expression relative to the healthy control[16].

The kits (Glory Science Co., Ltd, USA) used double – antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human PDCD4 and Human cyt c in serum. Antibody specific for PDCD4 or cyt c has been pre-coated onto a microplate. When standards and samples are pipetted into the wells, any PDCD4 or cyt c present will be bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for PDCD4 or cyt c must be added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution must be added to the wells and the developed color will be proportion to the amount of PDCD4 or cyt c bound in the initial step. The color development will be stopped and the intensity of the color will be measured.

Statistical analysis

GraphPad Prism® 5 software was used for analysis of data. Data were presented as mean ± standard error (SE). Comparison between two groups was carried out using unpaired t test. Correlation analysis was done by Spearman's method. Receiver Operating Characteristic (ROC) curve was used to determine the cut-off values, sensitivity and specificity using MedCalc version 13.2.2.

Table 1: Output data for receiver operating characteristic (ROC) curve for each of PDCD4, cytochrom c, miRNA-155 and -10b:

Correlation variables	Spearman's coefficient (r)	p value
miRNA-10b and -155	0.43	0.02*
miRNA-10b and PDCD4	0.38	0.03*
PDCD 4 and cyt c	0.54	0.002**

0. *P*-value was considered statistically significant when $p < 0.05$ vs. corresponding control.

RESULTS

Figure 1 shows the relative expression ($2^{-\Delta\Delta ct}$) of miRNA-155 and -10b in the plasma expressed as M±SE. The difference between the two groups was statistically significant. M ± SE of miRNA-155 relative expression (37.2 ± 7.8) for BC relative to healthy women, *p* value was < 0.0001. With regard to plasma miRNA-10b; M ± SE of fold change was (28.6 ± 6.8)
for BC patients; *p* value was < 0.0001. Serum PDCD4 and cyt c were significantly high in BC women with respect to control patients. M ± SE for BC patients was (15.2 ± 0.72) and (12.9 ± 0.92) respectively. In control group the M ± SE was (4.5 ± 0.23) and (4.2 ± 0.18) for PDCD4 and cyt c respectively (Figure 2).

The output data for receiver operating characteristic (ROC) curve revealed that miRNA-155 exhibited the highest sensitivity followed by PDCD4 then miRNA-10b (95.8%). Both serum PDCD4 and cyt c as apoptotic markers were more specific than miRNA-155 and -10b (Table 1). Spearman's correlation method showed that there was a significant positive correlation between miRNA-10b and each of miRNA-155 and PDCD4 in BC group. In addition, PDCD 4 showed a significant positive correlation with cyt c in BC group (Table 2).

DISCUSSION

Breast cancer is a worldwide health problem affecting women with growing incidence over the past decades. Fortunately, with the development of treatment protocols including chemotherapy, surgery and irradiation, BC is potentially curable if the detection was done in less advanced stages[17]. With the rapid development of sequencing and molecular biology techniques, amplified or decreased miRNA can easily be detected[18].

In the current study, the relative expression of miRNA-155 was significantly increased in BC Egyptian women compared to healthy group (*p* value < 0.0001). Our results were in accordance with other findings including[19-22] who reported that serum miRNA-155 relative expression could differentiate early-stage BC form healthy subjects.

Liu *et al.*[23] found that the up regulation of miRNA-155 in BC patients was significantly differed in terms of Ki-67 and p53 with the relative expression of Ki-67(+) higher than Ki-67(-) and that of P53 (-) more than P53 (+), indicating that serum miRNA-155 levels were associated with tumor cell proliferation, invasion, apoptosis and prognosis. The same study exhibited that the highly expressed miRNA-155 was clearly detected when tumor cells had high proliferative activity and decreased apoptotic capability where nuclear antigen Ki-67 expression represents the proliferative activity of tumor cells and therefore is often used as a reliable marker of proliferation. Moreover, Wild-type tumor suppressor p53 gene is well known as key gene which can trigger cell cycle arrest, induce apoptosis, and promote differentiation. While, the mutant p53 can induce the inhibition of cell apoptosis leading to uncontrolled cellular proliferation and ultimately cause the cancer development[23].

Cancer is marked by known six hallmarks including proliferative signaling maintenance, evading growth inhibitors, resisting cell death, enabling immortality, inducing angiogenesis and activating invasion and metastasis. As a prominent oncomir, miRNA-155 plays a role in many of the above oncogenic processes where it down-regulates BCL6 (B-cell lymphoma 6) protein, which is an evolutionarily conserved zinc finger transcription factor. Reduction of BCL6 in turn leads to up-regulation of known BCL6 targets such as inhibitor of differentiation (Id2), Interleukin-6 (IL6), cMyc and Cyclin D1, all of which promote cell survival and proliferation[6].

MiRNA-155 was also found to be a potent suppressor of apoptosis, since it acts as an effector on caspase associated with the initiation of the "death cascade" and is therefore an important marker of the cell's entry point into the apoptotic signaling pathway [24-25]. The role for miRNA-155 as an oncomir can be credited to multiple studies which have revealed it to be a prolific inhibitor of tumor suppressor genes, including suppressor of cytokine signaling 1(SOCS1), forkhead box O3A (FOXO3a), Ras homolog family member A (RhoA), and telomeric repeat-binding factor 1 (TRF1). In addition, the consistent over-expression of miRNA 155 makes it a viable prospect for use as a biomarker for cancer detection[26-27].

In the current study, we observed that miRNA10b expression was significantly increased in BC patients in comparison to healthy subjects which support its promising value as new breast cancer biomarker. Our results are supported with different previous studies presented by[22, 28]. Zhao *et al.*[29] indicated that a significant upregulation of serum miRNA-10b in breast cancer patients with bone metastases compared to without bone metastasis or control suggesting that miRNA-10b may be worthy of further evaluation as a prognostic marker in breast cancer. MicroRNAs have been also found to both promote and inhibit metastasis in breast cancer by controlling the expression of numerous mRNA targets which regulate the ability of tumors to metastasize. Pro-metastatic miRNAs include, miRNA10b which targets HOXD10[27]. This findings support that miRNA10b, which was classified as pro-metastatic miRNA can detect BC before the incidence of metastasis because all BC women in the current study were in non metastatic stage. Our results were also supported by Zhang et al.[30] who showed that miRNA-10b expression levels was correlated with breast cancer disease stage, survival status, age, ER/PR/HER2 status, molecular subtypes, tumor size, lymph node status and Ki-67 expression levels and

Table 2: Spearman's correlation of (miRNA-155 and -10b) with each other and with (PDCD 4 and cyt c) and between (PDCD4 and cyt c) with each other.

Test	Sensitivity	Specificity	Cutoff	Accuracy	AUC	P- value
PDCD 4	96.7%	100%	≥ 7.2	96%	0.991	< 0.001
Cytochrom c	93.3%	100%	≥ 5.6	94%	0.98	< 0.001
MiRNA-155	100%	90.9%	≥ 1.4	89%	0.97	< 0.001
MiRNA-10b	95.8%	86.36%	≥ 3.1	88%	0.93	< 0.001

conclude that miRNA-10b may be a biomarker for breast cancer and is considered as a potential treatment target.

The reported downstream targets for miRNA-10b include HOXD10, T-lymphoma invasion and metastasis-1 factor and stress-induced cell surface molecule MICB. Under the control of the TWIST transcription factor, miRNA-10b binds HOXD10 gene, enhancing cell migration and invasion. HOXD10, in turn, inhibits the Ras homolog gene family, member C (RHOC) protein, favouring metastatic diffusion of the tumor. Furthermore, over-expression of miRNA-10b leading to cancer metastasis has been correlated with the metastasis-promoting transcription factor Twist which induces epithelial-to-mesenchymal transition (EMT). MiRNA-10b requires Twist to induce EMT and the resulting cell motility and invasiveness in the breast epithelial cells. E-cadherin, another important determinant of EMT which is an essential component for maintaining epithelial cell polarity and a potent suppressor of breast cancer invasion and metastasis, has also been proposed to be a target of miRNA-10b. Thus, it appears that miRNA-10b influences metastasis through a complex regulation of multiple factors that determine EMT[28].

Both of serum PDCD4 and cyt c in the current study showed a significant increase in BC patients in comparison to the healthy women. In addition they showed a significant positive correlation with each other. Our findings are supported by Afify *et al*.[31] who evaluated the expression of PDCD4 using immunohistochemistry and showed a moderate and strong positive staining in BC patients. Schafer *et al*.[32] found that breast cancer cells display an unusual sensitivity to cyt c-induced apoptosis when compared with their normal counterparts. In addition, serum concentrations of apoptotic markers such as cyt c were increased after administration of the first cycle of chemotherapeutic drugs and suggesting that the measurement of these circulating apoptotic markers may help clinicians in evaluating treatment efficacy in breast cancer[33]. During carcinogenesis, cell death rates are changed according to disease stage and individual conditions. Although proliferation of malignant cells often already starts to increase years before the manifestation of cancer disease, effective immune surveillance leads to the elimination of transformed cells by a simultaneously elevated cell death rate[31]. Up regulation of PDCD4 is observed after the initiation of apoptosis, suggesting that loss of, or reduced, PDCD4 expression could contribute to the anti-apoptotic property of cancer cells. PDCD4 could inhibit neoplastic transformation through inhibition of adaptor protein-1 (AP-1) activation. Structurally, PDCD4 could interact with RNA helicase eukaryotic translation initiation factor 4A (eIF4A), inhibiting its helicase activity and affecting protein translation[12]. ROC curve for miRNA-155 and -10b revealed that both of them may differentiate BC patients from control subjects. MiRNA-155 showed the highest sensitivity (100%) and its specificity was higher than miRNA10b. Furthermore, both miRNAs exhibited a good accuracy which supports the idea that they can be considered as non invasive diagnostic markers for BC. Our results regarding miRNA-155 are supported by other findings who suggested that the overall diagnostic accuracy of miRNA-155 was reliable with sensitivity range (65.0% to 79%) and specificity range (81.8% to 85.5%)[20, 34, 35]. On the other hand Mar-Aguilar *et al*.[36] carried out the ROC curve for miRNA-10b and showed sensitivity and specificity of 83.30% and 100.00% respectively. The current study revealed associations between miRNA-155, miRNA-10b and breast cancer and apoptotic markers (PDCD4 and cyt c) which suggest a potential role for these miRNAs in BC and apoptosis. The ROC curve of miRNA-155 and -10b significantly discriminated between patient group and controls. The limitations of our study were that all participants were selected from one hospital and some level of selection bias could not be avoided, and the small number of sample size and controls. Further studies are recommended to understand the mechanism of action of miRNA-155 and -10b as well as to assess their possible roles as therapeutic targets for such disease.

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