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Article

Evaluation of miR-146 and miR-196 as potential biomarkers in a sample of Iraqi breast cancer patients

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ABSTRACT
Breast cancer is a heterogeneous disease defined by molecular types and subtypes. It constitutes the most commonly-diagnosed cancer and the leading cause of cancer death in women worldwide, according to the International Agency for Research on Cancer (IARC) World Cancer Reports in 2020. The study aimed to evaluate the miR-146 and miR-196 expression level and their association with the ca15-3 serum level of the participants diagnosed with breast cancer. There were 105 samples, three groups of 35 fresh blood samples and FFPE Tissue samples, which were collected as malignant, benign and healthy control. CA15-3 concentration was elevated in a malignant group with a mean equal to (36.14 Units/ml) in comparison to (27.07 Units/ml) for the benign group and (14.34 Units/ml) for the healthy control group ($p<0.01$). The results revealed that the expression of miR-146 in Malignant breast tumor tissue was (2.378 ±0.76) times more, while in benign breast tissue, with the fold of expression (1.197 ±0.38) in comparison with apparently healthy tissue. At the same time, the expression of miR-196 in Malignant breast tumor tissue was (8.11 ±2.15) times more, while in benign breast tissue, with a fold of expression (2.584 ±0.84) compared with apparently healthy tissue with highly significant differences.

Keyword: Breast Cancer, miR-146, miR-196, ca15-3, FFPE

INTRODUCTION

Breast cancer is the most detectable female malignancy, the second cause of death worldwide. The risk of developing breast cancer is modified by various factors, including age, reproductive and gynecological factors, physical activity, consumption of alcohol and tobacco, as well as family history [1,2,3].

MicroRNAs are a class of small non-coding RNAs (22–23 nucleotides) which regulate gene expression at the posttranscriptional level and play key roles in tumorigenesis and other diseases. They can function as either oncogenes or tumor suppressor genes [4,5,6]. In humans, MIR146A is found within a larger long non-coding RNA host gene, MIR3142HG (chromosome 5q33.3), while MIR146B is

found in an intergenic region of human chromosome 10 (10q24.32). Dysregulation of miR-146a and miR-146b has been observed in many types of malignant tumors, and several studies implicate these miRNAs as metastasis suppressors [7,8].

MiR-196 manifests different functions in different cancers, i.e., oncogenic or tumor suppressor function. Numerous works have reported that dysregulation of the miR-196 family in several types of malignancies is associated with carcinogenesis; this event is involved in tumor development, progression, and clinicopathological status. The miR-196 has an oncogenic role and is upregulated in breast cancer (21-25).

CA15-3 tumor marker is a mucin belonging to a big family of glycoproteins encoded by the MUC 1 gene expressed on the surface of normal epithelial cell types, including those of the breast epithelial cells. It has been shown that most patients with metastatic breast cancer have high levels of CA15-3, while its level rarely increases in patients with early-stage or localized cancer. The measurement of CA15-3 level before surgery is significantly related to the outcome in patients with early breast cancer. In addition, patients with high levels of CA15-3 have a significantly worse prognosis than those with low levels in terms of overall survival (OS) and disease-free survival [9,10].

MATERIAL AND METHODS

105 freshly prepared paraffin-embedded tissue samples were randomly selected from Oncology Teaching Hospital/Medical City /Baghdad archives. The samples were categorized into three groups. Malignant group (IDC, ILC), benign group (FA), and control group were collected from the negatively tested samples. Each group contains 35 samples.

Total RNA was extracted from Formalin Fixed Paraffin Embedded Tissue using RNeasy FFPE Kit (QIAGEN). The concentration of each sample was measured by Qubit 4 Fluorometric Quantification and RNA Quantification, broad range Kit (Thermo Fisher Scientific Australia Pty Ltd, VIC, Australia). cDNA synthesis was performed (ProtoScript® II First Strand cDNA Synthesis Kit) according to the manufacturer's protocol with 18µl of total RNA, 2µl oligo primer in a tube at 37˚C for 10 min, 42˚C for 50 min and then 95 ˚C for 5 min Table 1. The qPCR analysis for the miRNA gene (miRNA146, miRNA196 and U6, which were used as a housekeeping control) was performed using SYBR-Green Reagents (Invitrogen by Thermo Fisher Scientific, USA) and specific primers as shown in Table 2. Amplification conditions were as follows: Taq activation at 94˚C for 2 min; 40 cycles of 94˚C for 20 sec, 60˚C for 1 min and elongation at 72˚C for 30 sec. The result was calculated and analyzed using a Livak formula (20).

Serological study: For the serological study, 105 fresh serum samples were prepared in gel tubes (Biopro Company). The sample was categorized into three groups. Malignant, benign, and control groups were collected from the negatively tested samples. Each group contains 35 samples.

CA15-3 Measurement by Enzyme-Linked Immunosorbent Assay (ELISA): The serum CA15-3 tumor marker level was determined using an automated immunoassay system (Enzyme-Linked Immunosorbent Assay). The procedure was carried out according to the manufacturer's instructions. The cutoff value of the CA15-3 level was the increment (or decrement) of 25 U/ml Table 3.

Material	Volume (µL)
RNA	18
Oligoprimer	2
cDNA	5
Total	25

Table 1. cDNA reaction

The reaction was performed according to the manufacturer's protocol of a OneScript® Plus cDNA Synthesis Kit with 18µl of total RNA, 2µl oligoprimer and 5 µL cDNA in a tube at 37˚C for 10 min, 42˚C for 50 min and then 95 ˚C for 5 min.

Gene	Specific Primers
RNU6 RT-primer	5`-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAG AGCCAACAATCAG-3`
RNU6	F 5`- (CTCGCTTCGGCAGCACA) 3` R 5`- (AACGCTTCACGAATTTGCGT) 3`
miR-146 RT-primer	miR-146 RT 5`-(GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGAC AACCCA) 3`
miR-146	F 5` ( GGGTGAGAACTGAATTCCA) 3` R 5` ( CAGTGCGTGTCGTGGAGT ) 3`
miR-196 RT-primer	miR-196 RT 5` ( GTCAGAAGGAATGATGCACAGCCAACAACA ) 3`
miR-196	F 5` ( ACCTGCGTAGGTAGTTTCATGT ) 3` R 5` ( CGTCAGAAGGAATGATGCACAG ) 3`

Table 2. Primer Sequence for miRNA Gene Expression

Components
12Assay plate (12 x 8 coated Microwells)
Standard (Freeze-dried)
Biotin-antibody (100 x concentrate)
HRP-conjugate (100 x concentrate)
antibody Diluent
HRP-avidin Diluent
Sample Diluent
Wash Buffer (25 x concentrate)
Substrate A(1 x 7)ml
Substrate B(1 x 7)ml
TMB Substrate
Stop Solution
Adhesive Strip (For 96 wells)

Table 3. Components of human CA15-3 ELISA kit.

RESULTS

These results revealed that CA15-3 concentration was elevated in a malignant group with a mean equal to (36.14 Units/ml) in comparison to (27.07 Units/ml) for the benign group and (14.34 Units/ml) for the healthy control group ($p<0.01$) as displayed in Table 4.

MicroRNA 146a functions as a tumor suppressor in various types of cancer. The molecular experiment of miR-146 expression was performed to detect the amplification plots of the target miR-146 and RNU6 (reference gene) to find the threshold cycle (Ct) value for each. The results revealed that the expression of miR-146 in Malignant breast tumor tissue was (2.378 ±0.76) times more, while in benign breast tissue, with a fold of expression (1.197 ±0.38) compared with apparently healthy tissue Table 5.

The results revealed that the expression of miR-196 in Malignant breast tumor tissue was (8.11 ±2.15) times more, while in benign breast tissue, with a fold of expression (2.584 ±0.84) compared with apparently healthy tissue Table 6.

It had been noticed that the lack of promoter methylation in miR-196b may explain its overexpression in the majority of cancers and that miR-196b overexpression may be a tumor marker. This group later reported that miR-196b expression is significantly repressed by the transcription factor E26 transformation-specific sequence-2 (ETS2), whose expression is implicated in a reduced incidence of solid tumors. A knockdown of ETS2 significantly promotes migration and invasiveness of cancer cells.

Group	No.	Mean ± SD of CA15-3
Control	35	**14.34 ±4.13 c**
Benign	35	**27.07 ±9.02 b**
Malignant	35	**36.14 ±11.13 a**
LSD Value	--	**4.083 ****
P-Value	--	**0.0001**

** ($P \leq 0.01$). **Table 4. The mean of CA15-3 concentrations in patients and control groups.**

Groups	Means of MiR146 CT	Means of RNU6 CT	ΔCT	2^{-} ΔCT	Experi-mental group/Control group	Fold of Expres-sion
Control	19.66	30.3	10.64	1260.69	1595.7/1595.7	**1.00 ±0.00 b**
Benign	20.36	31.26	10.9	1910.85	1910.85/1595.7	**1.197 ±0.38 b**
Malignant	21.05	32.94	11.89	3795.3	3795.3/1595.7	**2.378 ±0.76 a**
P-value	--	--	--	--	--	**0.0371 ***
This means having different letters in the same column differed significantly. * (P≤0.05).						

Table 5. Fold of MiR146 Expression

Groups	Means of MiR196 CT	Means of RNU6 CT	ΔCT	2^{-} ΔCT	Experimental group/Control group	Fold of Expres-sion
Control	31.3	30.3	1	2	2/2	**1.00 ±0.00 c**
Benign	33.63	31.26	2.37	5.169	5.169/2	**2.584 ±0.84 b**
Malig-nant	34.96	30.94	4.02	16.22	16.22/2	**8.11 ±2.15 a**
P-value	--	--	--	--	--	**0.0001 ****
This means having different letters in the same column differed significantly. ** (P≤0.01).						

Table 6. Fold of MiR196 Expression

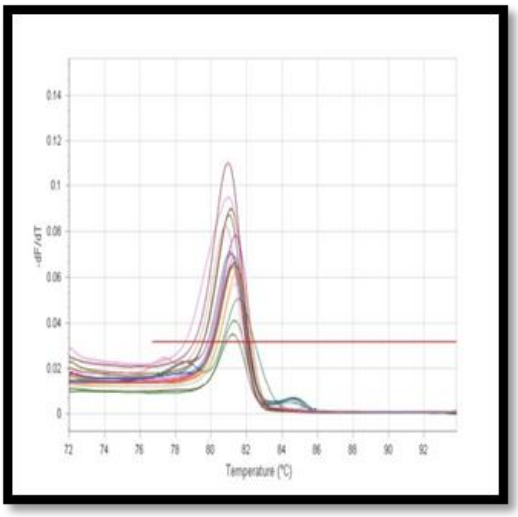

Figure 1: The miR-146 expression Melting Curve

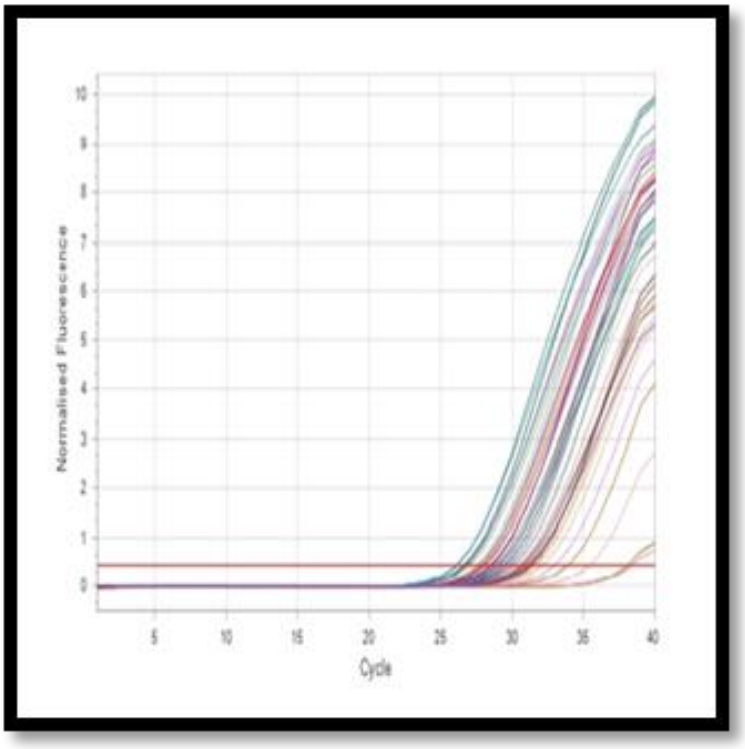

Figure 2: Amplification plots for miR-146 expression Obtained by Real-Time PCR

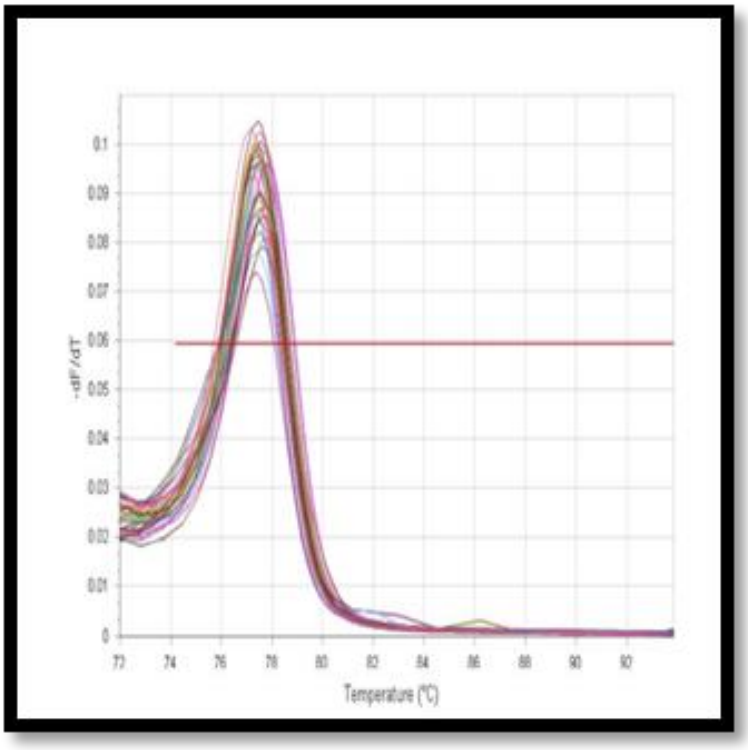

Figure 3: Amplification plot for RNU6 gene expression.

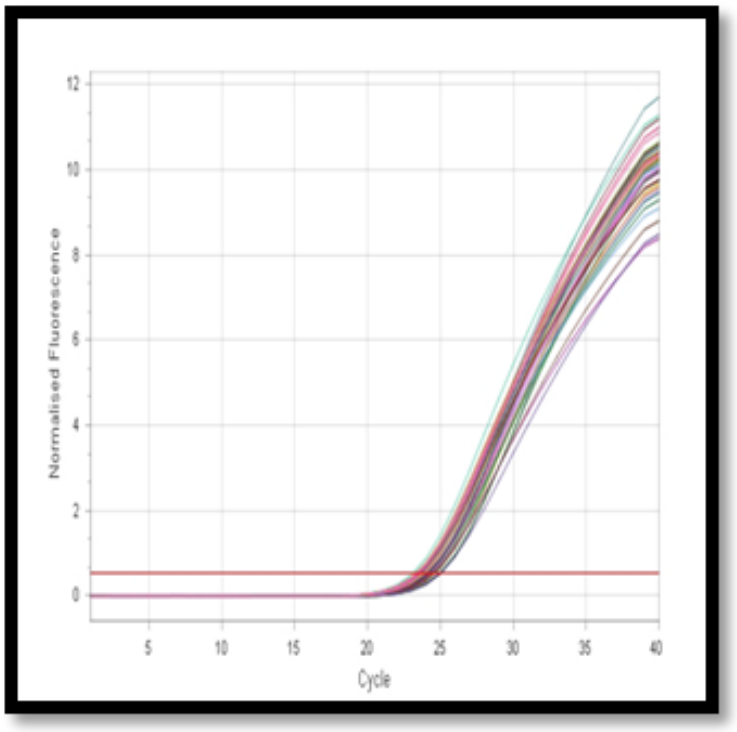

Figure 4: The RNU6 expression Melting Curve

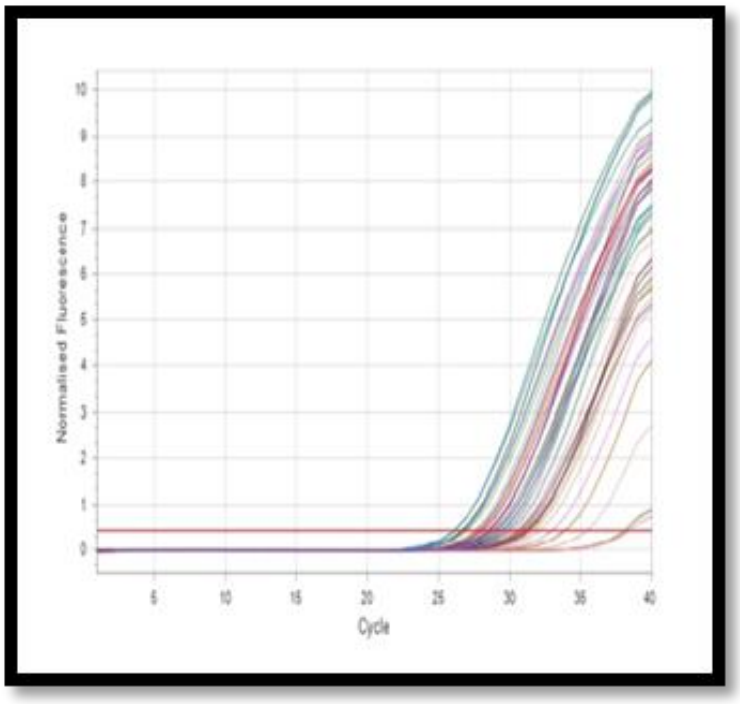

Figure 5: Amplification plots for miR-196 expression Obtained by Real-Time PCR.

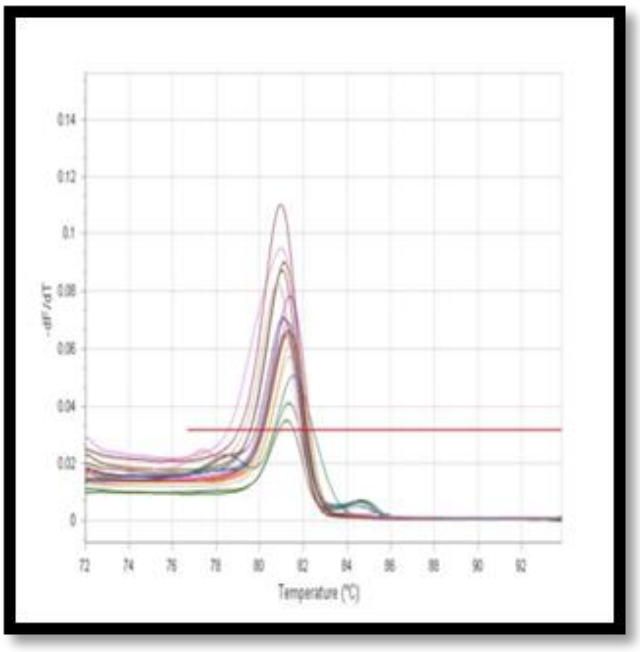

Figure 6: The miR-196 expression Melting Curve.

DISCUSSION

The patients with elevated CA15-3 levels have worse overall survival than those with normal CA15-3 levels. The association between high CA15-3 and CEA and the adverse outcome was found because elevated marker levels are directly tied to tumor burden and the presence of antigens about the tumor at the time of diagnosis of breast cancer reveals vascularization in the tumor cell, with the possibility of micrometastases [12]. However, Ebeling et al. conducted a study including 1046 breast cancer cases and did not find CA15-3 an independent prognostic marker [13], and Clinton's study supports this view [14]. A prior study reported no association between breast cancer outcome and CA15-3 among patients younger than 40 years [15], and another research by [16] did not offer prognostic information for tumor markers. Possible reasons for the inconsistent results may include differences in treatment modalities at the time of measurement, sample size and follow-up time. Serial determination of these markers may be useful in routine therapy monitoring and early detection of recurrence and progression during follow-up.

The results of RT-qPCR [11] demonstrated that the expression of miR-146 in breast cancer tissue was (3.2±0.3) times more compared with paraneoplastic tissue. High expression of miR-146 could significantly promote the proliferation, and low expression of miR-146 could significantly inhibit it. BRCA1 was preliminarily confirmed as the target gene of miR-146 by bioinformatics prediction. BRCA1 is a tumor suppressor gene which frequently mutates in hereditary breast cancer. BRCA1 is important in DNA repair, cell cycle control, transcriptional activation, ubiquitin and other processes; high expression of miR-146 in breast cancer and breast cancer cell lines [11].

A study by Chen et al. found that the expression of miR-146a was significantly increased in breast cancer and was closely related to the invasion of breast cancer. Transfection of miR-146a could significantly promote the proliferation, migration and invasion of breast cancer and directly interact with the 3′-UTR of NM23-H1. NM23-H1 is an important class of tumor suppressor enzymes [17].

Alterations in ETS2 and miR-196b expression in cancer cell lines influence the expression of epithelial-mesenchymal-transition-related genes [18]. A genetic and epigenetic association study suggested that miR-196 might have a potentially oncogenic role in breast tumorigenesis, and the functional genetic variant in its mature region could serve as a novel biomarker for breast cancer susceptibility. Hypermethylation of a CpG island upstream (-700 bp) of the miR-196 precursor was also associated with reduced breast cancer risk [19].

CONCLUSION

For patients with diagnosed breast cancer, the most widely used serum markers are CA 15-3, which, combined with other clinical parameters, could have significance in patient surveillance. Serial determination of these markers may be useful in routine therapy monitoring and for early detection of recurrence and progression during follow-up. The expression of miR-146a was significantly increased in breast cancer. At the same time, the high expression level of miR-196 in BC patients confirms that miR-196 has a strong prognostic role in BC (tumorigenesis) and could be considered a significant predictor (prognostic biomarker) in BC.

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