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RESEARCH ARTICLE

Application of stem-loop RT-PCR procedure for evaluation of microRNA-21 in fresh tissues of invasive ductal carcinoma.

Shoroq Mohammed Al-temimi[1] , Salim Rashid Al-aubaidy[2], Ali Hausin Al-khafaj[3] .

1. Department of pathology/College of medicine/Qaddissia University
2. Department of pathology/College of medicine/Baghdad University
3. pathology unit/Central Public Health lab.Ministryof Health, Baghdad/ Iraq.

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***Corresponding Author**

Shoroq Mohammed Al-temimi

Abstract

Background:- MiR-21 was one of the first oncogenic miRs and as an anti-apoptotic factor, to be characterized, being up-regulated in numerous tumors including BC . A novel miR quantification method has been established using stem–loop RT followed by TaqMan PCR analysis in tissues or culture cells.
Aim of study:-Estimation of miR-21 expression level in fresh tissues of BC / NATs by using stem-loop follow by TagMan real time PCR (RT-PCR) technique and correlate miR-21 gene expression with clinico-pathological parameter of breast cancer .
Material and methods:- Stem-loop RT-PCR was performed to identify the expression level of miR-21 in 50 IDC samples and their NATs. The expression levels of miR-21 relative to mRNA of GAPDH were determined using the livak method.
Results:- Mean cancer tissue fold change of miR-21 was significantly higher than that of NATs. Majority of cases showed up regulation of miR-21, 96%. Up-regulation of miR-21 expression has been statistical significant associated with unfavorable pathological features of the disease, including positive lymph node involvement and higher tumor stage(III,VI)
Conclusion:- Up-regulation of miR-21 expression has been associated with positive lymph node involvement and higher tumor stage(III,VI)

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Introduction

MicroRNAs(miRs) are small non protein coding RNAs involved in gene regulation through binding to the 3' un-translated region of target messenger RNAs (mRNAs) and down-regulate their translation to protein or degrade the mRNAs . So , miRs play critical biological roles in many different cellular processes including metabolism, development , differentiation ,proliferation and apoptosis. They are also linked to human diseases, including cancer [(1)] . miR-21 was one of the first oncogenic miRs and as an anti-apoptotic factor, to be characterized, being up-regulated in numerous tumors including BC . miR-21 expression in BC correlates with advanced stage and metastasis [(1,2)]. Real-time RT- PCR is the gold standard for gene expression quantification . Standard procedures for conducting and publishing RT- PCR experiments have been recently codified in "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments," [(3)]. A novel miR quantification method has been established using stem–loop RT followed by TaqMan PCR analysis in tissue or cultured cells. MiRs are 17 – 24 nucleotides (nt) in length. Standard and quantitative PCR methods require a template that is at least twice the length of either of the specific forward or reverse primers, each typically ∼ 20 nt in length. Thus, the target minimum length is ≥ 40 nt, making miRs too short for standard RT-qPCR methods. The method for quantitative

amplification of specific miRs whereby the target cDNA is lengthened, and design specifics of the PCR forward primer and the hydrolysis probe combine to ensure specificity at great sensitivity[4,5] .

Advantages of Stem-loop protocol :

1-Stem–loop RT primers are better than conventional ones (linear RT primer) in terms of RT efficiency , specificity and stem-loop might enhance the thermal stability of the RNA-DNA hetroduplex .

2-TaqMan miR assays are specific for mature miRs better than SYBR green assay ,and discriminate among related miRs that differ by as little as one nucleotide , they are not affected by genomic DNA contamination

3-The high sensitivity, specificity and precision of this method allows for direct analysis of a single cell without nucleic acid purification [6] .

Materials and methods

The study was conducted during the period from January 2013 through January 2015 . This is a prospective study, where by patients were recruited at the surgical department/ AL-diawaniaTeaching Hospital in diawania city . Fifty-pairs of fresh tissues from both IDC and NATs (which conceder as internal control) , for total RNA extraction and for RT-qPCR. Another 50 pairs specimens of both IDC and NATs for histopathological examination .

Isolation of total RNA :RNA was extracted from fresh tissues using the Trizol reagent (Bioneer ,Korea) according to the manufacture's instructions.The dissolved RNA was stored at -70°C before use .RNA quality was assessed with a NanoDrop 1000 spectrophotometer .

Real-time RT-PCR for miR-21 quantification: The Primers and probes for miR-21 were design in this study by using **(The Sanger Center miR database Registry**) to selected miR-21 sequence **and using miR Primer Design Tool** .as shown in table (1).

Table (1) : The primers and probes for microRNA-21

Primer		Sequence
hsa-miR-21 RT primer		**GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGC CAACTCAACA**
hsa-miR-21primer	**F**	**GTTTGGTAGCTTATCAGACTGA**
	R	**GTGCAGGGTCCGAGGT**
hsa-miR-21 probe		**FAM- CCAGAGCCAACTCAACA-MGB**

GAPDH gene Primers and probes : The mRNA of GAPDH gene Primer and probe were designed by using NCBI-Gene Bank data base and Primer 3 plus design online .These primers were provided by (Bioneer company, Korea) as shown in table (2).

Table (2) : The primers and probes for mGAPDH.

Gene		Sequence
mGAPDH	**F**	**TCAGCCGCATCTTCTTTTGC**
	R	**TTAAAAGCAGCCCTGGTGAC**
mGAPDH probe		**FAM- CCAGCCGAGCCACATCGCTC-TAMRA**

Reverse Transcription and real-time PCR was subsequently performed in duplicate using the M-MLV Reverse Transcriptase kit and AccuPower ® Plus DualStar™ qPCR (Bioneer ,Korea) as described detail in previous study (6) .All miR-21 quantification data were normalized to GAPDH. The data results of RT-qPCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) by using the Livak method that described by (Livak and Schmittgen, 2001) [7] .

Statistical analysis :SPSS version 16 and Microsoft Office Excel 2007 were using in analysis of these data,Chi-square test and Fisher exact test were used to study association between any two nominal variables. P-value of less than or equal to 0.05 was considered significant.

Results

1-MicroRNA-21 gene expression (fold change)

Mean cancer tissue fold change of miR-21 was significantly higher than that of NATs,5.400±0.545 versus 0.768±0.093, (P<0.001) .as shown in table (3) .

Table (3) : Comparison of mean gene fold change between BC tissues and NATs

Parameter	Group	Median	Mean	SE	Minimum	Maximum	P
miR-21	Normal	0.586	0.768	0.093	0.091	2.473	<0.001
	Cancer	4.167	5.400	0.545	0.893	21.120	

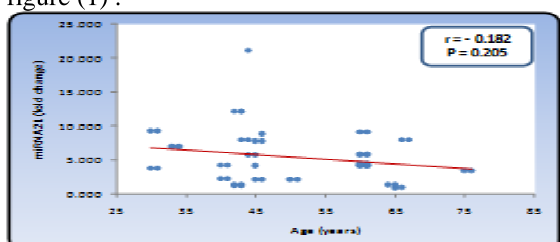
Majority of cases showed up regulation of miR-21, 96% .as shown in table (4) .

Table (4) Down regulation and up regulation of miR-21.

	Up-regulation	Down-regulation	Total
MiR-21	48(96%)	2(4%)	50(100)

2-Correlation between fold change of microRNA-21 gene expression, with age

Despite a negative correlation with age, exhibited by miR-21, there was no statistical significance ,as shown in figure (1) .



Figure(1): Correlation between fold change, of miR-21

Mean miR-21 fold change in patients <50 years was not significantly different from that of patients ≥50 years, (6.259±0.855) versus (4.307±0.526) respectively . These comparisons are outlined in figure(2) .

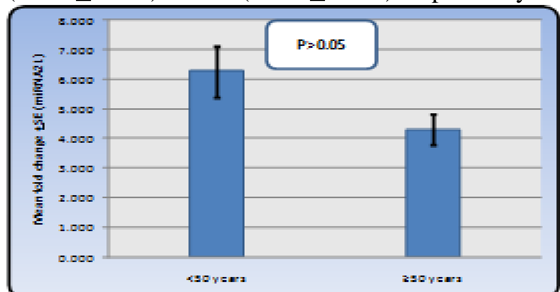


Figure (2): Comparison of mean fold change between patients <50 years and ≥ 50years .

3-Correlation between size of tumor and fold change

Although miR-21 fold change exhibits a positive correlation with size of tumor, it did not reach a statistical significance.as shown in figure(3) .

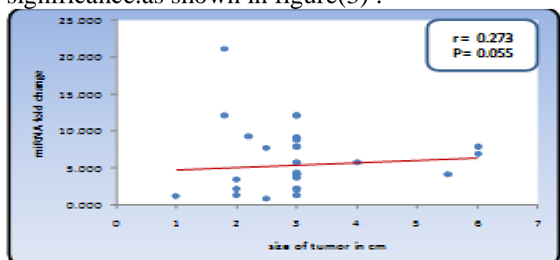


Figure (3): Correlation between fold change and size of tumor

4-Correlation between fold change and lymph node metastasis

Mean miR-21 fold change of patients with positive lymph node was significantly higher from that of patients with negative lymph node involvement, 7.499±0.655 versus 2.252±0.247, and P-value was <0.001 ,as shown in figure(4) .

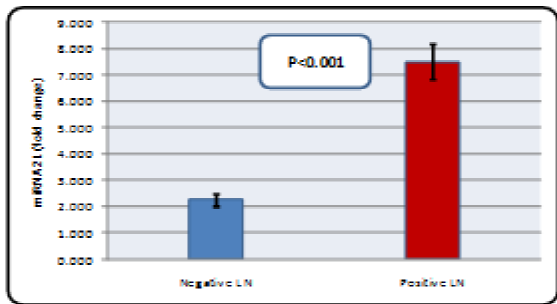


Figure (4): Comparison of mean fold change between patients with positive lymph nodes and patients with negative lymph nodes.

On the other hand there was a significant positive correlation between miR-21 fold change and number of involved lymph nodes.as shown in figure (5) .

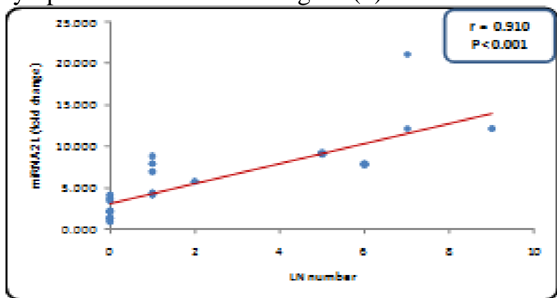


Figure (5): Correlation between number of involved lymph nodes and fold change.

5-Correlation between grade of tumor and fold change

When grade was plotted against miR-21 fold change, a non- significant negative correlation was obtained, r = -0.001 and P=0.999 as shown in (6)

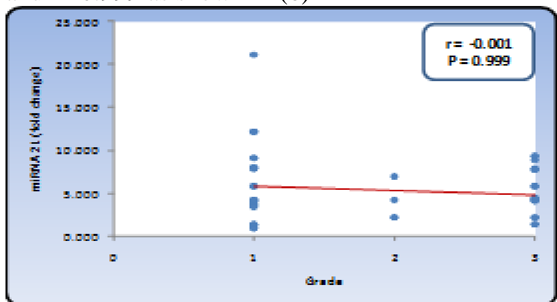


Figure (6): Correlation between fold change and grade of tumor.

6-Correlation between stage and fold change

A significant positive correlation was found between stage and miR-21 fold change. As shown in figure (7) .

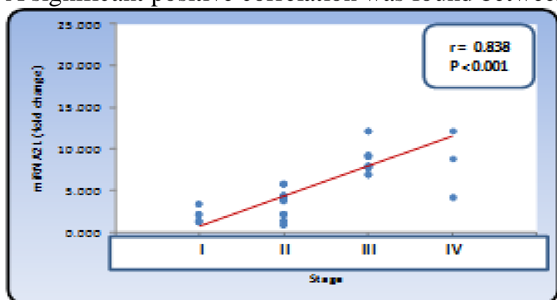


Figure (7): Correlation between stage and fold change

Validity of microRNA-21 fold changes as alteration of gene expression

To find the cutoff value for miR-21 fold change that predict expression gene aberration in BC by using the stem-loop RT-qPCR technique , an ROC curve analysis was done that showed the best cutoff value for miR-21 was 2.940 with a specificity of 100%, sensitivity of 72% and an accuracy of 95.6% .As shown in table (5) and figure (8) .

Table (5): Validity of miR-21 fold change in predicting expression gene aberration in BC tissues in comparison to NATs with p value was <0.001.

Cutoff value	Accuracy	P-value	Sensitivity	Specificity	Interpretation
2.940	0.956	<0.001	72 %	100%	Excellent

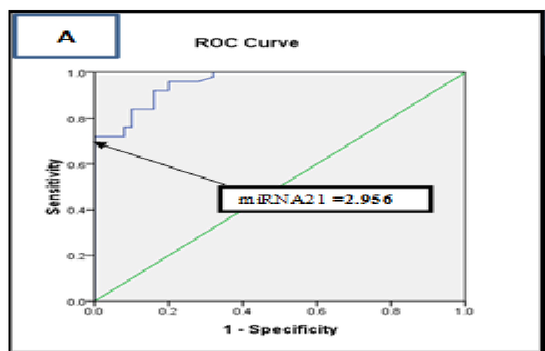


Figure (8) : ROC curve showing the cutoff value for miR-21 fold change that predicts gene expression aberration in breast cancer tissues in comparison to NATs with p value was (<0.05) .

Validity of microRNA-21 fold changes as prognostic markers.

It was found that miR-21 fold change can predict positive lymph node and higher stage (III ,IV) . The ROC results demonstrated that the AUC was (100% ,excellent),(98.5%, excellent) ,when the cutoff value was set to the optimal point, ≥ 4.156 , ≥6.340; specificity was 100% , 97.1%; sensitivity was 100% ,100% respectively. As shown in table (6) and figure(9)

Table(6): Validity of miR-21 gene expression fold change as a prognostic marker

Prognostic parameter	miR-21 Cutoff value	AUC (accuracy)	Specificity	Sensitivity	P-value	Interpretation
Positive LN	≥4.156	1.000 (100%)	100	100	<0.05	Excellent
Size ≥ 2 cm	≥1.290	0.486 (4.86%)	50%	95.7%	>0.05	Poor
Stage (III &IV)	≥6.340	0.985 (98.5%)	97.1%	100%	<0.05	Excellent
Grade (II & III)	≥4.166	0.470 (47%)	53.8%	50%	>0.05	Poor

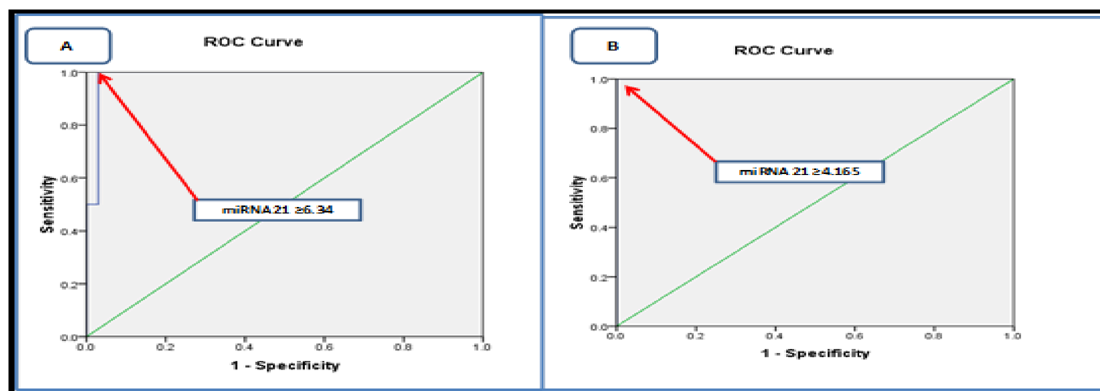


Figure (9): ROC curve showing the cutoff value for miR-21 fold change that predicts higher stage (A); miR-21 fold change that predicts L.N metastasis (B) ; with p value was (<0.05) .

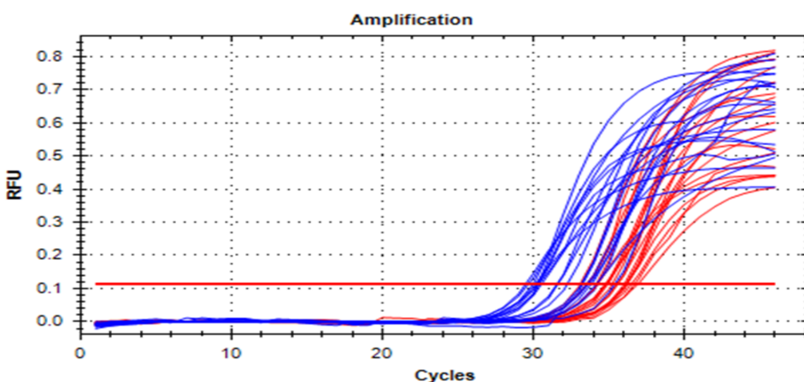


Figure (10): Stem- loop qRT-PCR amplification plots for miR-21 cDNA in breast cancer tissue patients by using TaqMan probe . (FAM), where (blue amplification plot as breast cancer tissue samples) and (Red amplification plot as normal adjacent tissue samples).

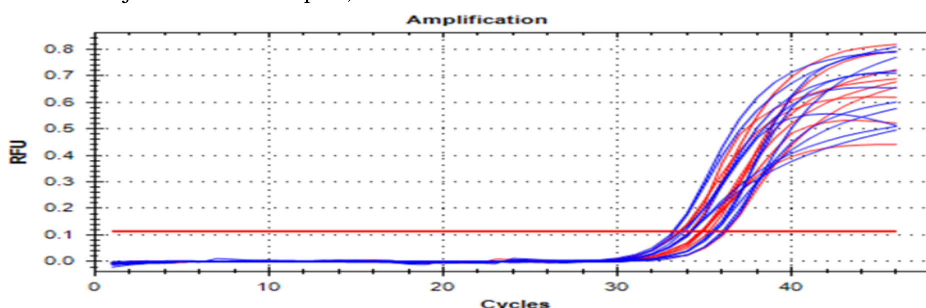


Figure (11): Real-Time PCR amplification plots for GAPDH cDNA gene in breast cancer tissue patients by using TaqMan probe . (FAM). Where (blue amplification plot as breast cancer tissue samples) and (Red amplification plot as normal adjacent tissue samples).

Discussion

An important strength of present study, this is the first study in the literature evaluating miR-21 gene expression by stem-loop RT-qPCR , in the same series of fresh BC/apparently NAT samples to be conducted in Iraq i.e, there was no baseline study regarding miR-21 gene expression stratification in Iraq. Although, similar studies were conducted abroad to stratify miR-21 . Fresh BC tissues were chosen as a sample for the extraction of RNA since it is enriched with many types of miR and mRNA .The fresh tissue is preferable for RNA extraction and make it easier and earlier for molecular diagnosis than (FFPET) , due to the cross linking of RNA with proteins, enzyme degradation occurring during the fixation process reduces the yield, quality and integrity of RNA . So, mRNA detection from archival material is limited due to the labile nature of mRNA and the deleterious effects of enzymatic fragmentation during long periods of storage and RNA modification induced by formalin fixation **(Lwis et al)** [8]. Although miRs are less prone to degradation and modification due to their small size ,stable in tissue by time-course and freeze-thaw cycle analyses and can escape from RNAs degradation **(Liu et al)** [9] .

Mean cancer tissue fold change of miR-21 was significantly higher than that of NAT , and majority of cases showed up regulation of miR-21, 48(96%) . Similar result was in agreement with **(Iorio et al)** [10], who conceder as the first one ,since 2005, reported that miR-21 was up-regulated in BC in comparison to NATs .Also Similar to that result of **(Li-Xu et al)** [11] **; (Bao et al)** [12] **; (Mohamed et al)** [13]**; (Haiyan et al)** [14] **; (Yang et al)** [15] **.**

Our result showed no statistical significance between age of patients and fold change of miR-21. Similar result were in agreement with **(Haiyan et al)** [14] .

Our result showed that there was no statistical significance correlation between miR-21 fold change and tumor size ,and there was a positive correlation and statistical significance with positive lymph node and higher stage (III,IV) of tumor. **(Li-Xu et al)** [11] who was the first one which described the significance of miR-21 to clinical stage, lymph node metastasis, and prognosis of BC patients, who found that a statistically significant association between miR-21 fold change expression level and clinical stage and metastasis of breast cancer . Similar result was seen with **(Shahram et al)** [16]**.** While disagreement with **(Mohamed et al)** [13],who found that the fold change of miR-21 was significantly associated with tumor size and higher stage and no significantly association with positive lymph node.

Previous studies have demonstrated that the miR-21 promotes growth of the BC cell line MCF-7 both in vitro and in vivo. Which may be due to the ability of miR-21 to suppress the expression of the tumor suppressor PTEN and

matrix metalloproteinases inhibitors ,such as tropomyosin 1 (TPM1) . As tumor suppressor genes, PTEN and TPM1 are also implicated in cell migration and invasion **(Si et al)** [17]. Many studies have demonstrated that the PI3K/AKT/PTEN pathway is important for tumorigenesis. The PI3K signaling pathway is associated with almost all aspects of tumor biology, including cell transformation, growth, proliferation, migration and apoptosis evasion and genomic instability, angiogenesis, metastasis and cancer stem cell maintenance. PTEN degrades the product PI3K by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate at the 3' position . The loss of function or reduced expression of PTEN leads to the accumulation of critical messenger lipids, which in turn increases AKT phosphorylation and activity, leading to decreased apoptosis and/or increased mitogen signaling . Suggesting that miR-21 may also have a role in invasion and metastasis**(Menget al)** [18] . However, the PI3K/AKT/PTEN pathway contains a number of attractive therapeutic targets **(Shaw et al)** [19]**; (Janzen et al)** [20] . The future holds great potential for the rapid development of selective novel anticancer agents specifically targeting components of this pathway.
The result of present study showed no statistical significant correlation with tumor grade and miR-21.Our result was agreement with **(Li-Xu et al)** [11] , while disagreement with those reported by **(Yan et al)** [2] **;(Mohamed et al)** [13], who found that the mean fold change of miR-21 is significantly up-regulated in high grade tumor.
It was found that miR-21 fold change can predict for gene expression aberration. The best cutoff value was, (2.940) with best accuracy. It suggests that miR-21 gene expression quantification by stem-loop RT-qPCR may be used to discriminate the aberration in gene expression in BC tissues from the NATs .
(Chang ,et al) [21], it was found that miR-21 fold change can predict for gene aberration by using RT-qPCR technique and the best cutoff value was > 2-fold change than normal expression .
Also it was found that miR-21 fold change can predict positive lymph node . The best cutoff value was (≥ 4.156) with best accuracy . Also it was found that miR-21 fold change can predict higher stage (III ,IV) . The best cutoff value was (≥6.340) with best accuracy . In the other hand ,the fold change of miR-21 gene expression was poor with age ,size and grade of invasive BC .

Conclusion:

MiR-21 is significantly up-regulated in BC tissues, implicating miR-21 as oncogene in breast cancer. Up-regulation of miR-21 expression has been associated with unfavorable pathological features of the disease, including positive lymph node involvement and higher tumor stage(III,VI) .

Recommendation:

Evaluation of miR-21 , other miRs and related genes in serum and other body fluid of breast cancer patients which conceder as non- invasive technique before and after operation ,and before and after gene therapy and for follow up patient with BC . For miR-21 up-regulation gene expression there was possibly a potential target for gene therapy.

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