Serum MicroRNA-21 High Level Expression in Breast Cancer in Relation to Advance Stage

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ABSTRACT:

BACKGROUND:

MiR-21 plays an important role in tumor formation and development , and it has been shown to be a key regulator of oncogenic processes and it may be a useful biomarker for breast cancer .

OBJECTIVE:

Estimation of miR-21 gene expression levels in both fresh tissues and serum of same breast cancer patients before and after operation by using stem-loop followed by Taq-Man Real Time PCR (RT-PCR) technique and correlate the miR-21 gene expression with stages of breast cancer and lymph node involvement.

MATERIAL AND METHODS:

Stem-loop RT-PCR was performed to identify the level of miR-21 gene expression in both fresh tissues and serum of same breast cancer patients before and after operation . The expression levels of miR-21 relative to mRNA of GAPDH were determined by using the livak method.

RESULTS:

Mean fold change of miR-21 was significantly higher in breast cancer than that of paracancerous tissues . Before operation ,the mean fold change of serum miR-21gene expression significantly statistical difference from healthy control ,the mean fold change of miR-21 in advance stage(III,IV) was significantly higher than that early stage (I,II) and mean fold change of miR‑21 in positive lymph node was higher than that of negative lymph node . After operation the mean fold change of serum miR-21gene expression was significantly higher than healthy control .A statistically significant difference was noted between advance stage with lymph node involvement and early stage with negative lymph node involvement .In contrast , the mean fold change of serum miR-21 was significantly lower from that before operation .

CONCLUSION:

Circulating miR-21 before and after operation can serve as a good biomarker for breast cancer detection and progression .

KEY WORD: breast cancer, serum miR-21 , stem-loop RT-qPCR .

INTRODUCTION:

Breast cancer is the most common cancer among women. Stage at diagnosis is an important predictor of breast cancer survival and quality of life. Thus, there is an urgent need for diagnostic indicators, with simpleness, rapidness and non-invasiveness, Tumor markers are playing an increasingly important role in breast cancer diagnosis and treatment [(1)] . MicroRNAs (miRs) are a class of noncoding RNAs of (19-25) nucleotides that have been implicated in regulating diverse cellular processes. They have a close relationship with tumor formation and development. MiR-21 is significantly elevated in the majority of human tumors. It plays an important role in tumor formation and development, and it has been shown to be a key regulator of oncogenic processes [(2)] . Iorio *et al*. had reported that miR-21 is overexpressed in breast cancer tissue, and it may be a useful biomarker for breast cancer . But it is invasive to get tissue. The serum sample has the advantages of simple collection, less invasiveness, and easy monitoring [(3)] . The mechanism underlying miR-21 stability is still being investigated for breast cancer detection [(4)] . Expression profiles of circulating miRs may yield

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promising biomarkers for diagnosis and assessment of the prognosis of cancer patients. Sensitive techniques allow the expression levels of many miRs to be determined, and this information can be used for diagnostic purposes [5] . The utility of miR profiles as potential diagnostic or prognostic markers for breast cancer has been gaining interest [6] .

MATERIALS AND METHODS:

Subject :-The study was conducted during the period from April 2014 to May 2015. This is a prospective study, where by (n=45) patients with newly diagnosed breast cancer (all cases underwent modify radical mastectomy and axillary clearance) and apparently healthy controls (n=10) were diagnosed without any tumor or physical illness . The age range between 30-65 years, were recruited at the surgical department/ AL-Diawania Teaching Hospital in Diawania City .The patients had not received neoadjuvent therapy . Serum sample were collected from healthy controls and breast cancer patients (preoperative serum collection at theater Room and serum collection of same patients after 3weaks of operation and before chemotherapy therapy). Forty five-pairs of fresh tissues from same cases of breast cancer and paracancerous tissues (which consider as internal control) were submitted for total RNA extraction and for RT-qPCR . Another 45 pairs specimens of both breast cancer and paracancerous tissues were submitted for histopathological examination . The histopathological classification was performed according to the WHO classification (2013) , tumor staging was carried out according to American join committee cancer (AJCC, 2010) .

MiRNA isolation from serum and tissue: Serum samples were collected between 8:00 and 9:00 a.m. following centrifugation for 30 min at 2,650 g , serum samples were stored at 80o c .Tissue samples were homogenized in adenaturing lysis solution and the dissolved RNA was stored at -20°C before use . Total RNA was extracted from serum and fresh tissues using the Trizol reagent (Bioneer ,Korea) according to the manufactures instructions. .RNA quality was assessed with a Nano Drop 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 .The cDNAs were synthesized by stem-loop primer, TTGGCTCTGGTGCAGGGTCC-GAGGTATTCGCACCAGAGCCAACTCAACA . MiR‑21 was then analyzed by qPCR and the primer used was: forward, GTTTGGTAGCTTATCAGACTGA and reverse , GTGCAGGGTCCGAGGT . PCR analysis was performed using Taq-Man probe, FAM-CCAGAGCCAACTCAACA-MGB . The PCR master mix preparation for miR-21 , as shown in table(1):

Table 1: PCR master mix for miR-21 .

PCR master mix	volume
21 miRNA cDNA template (100ng)	5µL
Forward primer (10pmol)	2.5 µL
Reverse primer (10pmol)	2.5 µL
TaqMan probe (20pmol)	2.5 µL
DEPC water	25 µL
Total	50 µL

Thermal profile included denaturation at 95˚C for 5 min, followed by 40 cycles of 95˚C denaturation for 20 sec, 52˚C annealing for 20 sec and extension at 72˚C for 30 sec. Reverse Transcription and real-time PCR was subsequently performed in duplicate. All miR-21quantification data were normalized to housekeeping gene , Glycer aldehyde 3-phosphate dehydrogenase (GAPDH) . The mRNA of GAPDH gene primers and probe were designed by using NCBI- Gene Bank data base and Primer 3 plus design online. The cDNAs primer of GAPDH was designed as Random Hexamer primer and the primer used in qPCR was: forward, CAGCCGCATCT-TCTTTTGC and reverse, TTAAAAGCAGCCCTGGTGAC .Taq-Man probe for mGAPDH was: FAM-CCAGCCGAGCCACATCGCTC-TAMRA.

These primers and probe of miR-21 and GAPDH were provided by (Bioneer company, Korea). The data results of RT-qPCR for miR-21 and GAPDH were analyzed by the relative quantification gene expression levels (fold change) which were based

on the Ct values by using the Livak method that described by (Livak and Schmittgen) [7] . As following:-
Reference gene CT was normalized to target gene CT as:
ΔCT (calibrator) = CT (ref, calibrator) – CT (target, calibrator)
Second, reference gen CT belonging to target gene for test specimen, as following:
ΔCT (Test) = CT (ref, test) – CT (target, test)
ΔΔCT= ΔCT (test) – ΔCT (calibrator)
Fold change = 2-ΔΔCT

Ratio (reference/target) = 2CT (reference) – CT (target)
Statistical analysis :-SPSS version 16 and Microsoft Office Excel 2007 were used 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- Clinicopathological characteristics of patients **:-** In the present study a total of 45 breast cancer cases were studied cases with invasive ductal carcinoma (IDC) included , stage I : 10(22.3%) ,stage II: 15(33.3%),stage III :15 (33.3%) and stage IV :5(11.1%) . The mean age was 47.96 ±8.94 years old (range 30-65y) and the mean age of control group was 50.50± 9.26 (range40-65y) , table (2) .

Table 2: Mean age for patients and control groups .

Group	N	Mean	SD	Minimum	Maximum	P
Control	10	50.50	9.26	40	65	0.422
Patients	45	47.96	8.94	30	65	

2-Comparison between miR-21 gene expression of breast cancer and paracancerous tissues
The mean cancer tissue fold change of miR-21 was statistical significantly higher than paracancerous tissue , 6 ± 0.5 versus 1 ± 0.02 , respectively (P<0.001) .

3-Comparison of the gene expression of serum miR-21 between breast cancer patients (before and after operation) and apparently healthy controls.

Before operation ,the mean fold change of miR-21 serum levels in breast cancer patients were statistical significantly higher than that of apparently healthy controls. After operation, the mean fold change of miR-21 serum levels in breast cancer patients were still significantly higher than health controls and the mean fold change of miR-21 serum levels in patients before operation were significantly higher than that after operation ,as table (3) and figure (1).

Table 3 :-Median and mean fold change in serum control and patients' group (before and after operation).

Fold change	Control	Patients (Before)	Patients (After)	P1*	P2*	P3!
Median (Mean ±SD)	1.1 (1.05± 0.17)	10.00 (9.33±3.83)	5.00 (4.78±2.01)	<0.001	<0.001	<0.001

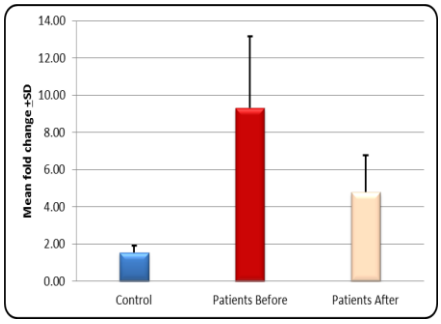

Figure 1: Mean fold change in control and patients' group (before and after operation).

4- The correlation of miR-21gene expression pattern between breast cancer tissues and paired serums

The results showed a statistical significant correlation of miR-21 gene expression in the tissues with those in the serums before and after operation, with r = 0.51 and r = 0.61 ($p<0.001$) , respectively .As shown in figure (2) and (3) .

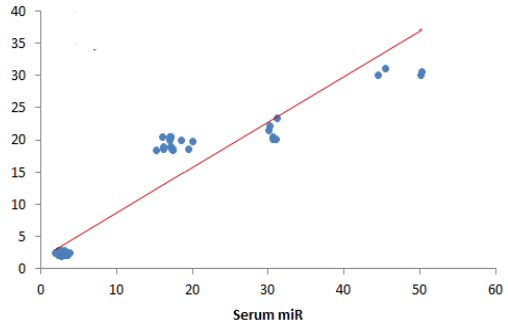

Figure 2: Before operation.

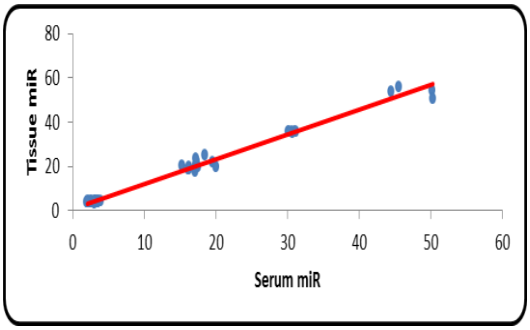

Figure 3 : After operation

5-Assiosiaion between serum level of miR-21 and age of patients

The result of present study showed that there was no significant statistical correlation between serum level of miR-21 and age of patients ($P>0.05$) , table (2) . When patients were divided in two group (<50 and ≥50), before operation, no statistical significant association between mean fold change of serum miR-21 in breast cancer patients and age of patients was noticed. Similar finding were noticed after operation, as shown in table (4)

Table 4 : Median and mean fold change in patients' group according to age.

Age group	Fold change before	Fold change after
<50 years (n=23)	10.00 (9.26±3.47)	5.00 (4.74±1.71)
≥ 50 years (n= 22)	9.00 (9.41±4.25)	5.00 (4.82±2.32)
P!	0.819	0.854

6-Assiosiaion between serum miR-21 and positive L.N involvement .

Before operation , the mean serum of miR-21 of patients with positive L.N was significantly higher than that of patients with negative L.N involvement, 10.66±3.22 versus 4.70±1.34 , respectively (P <0.001) . After operation the mean serum of miR-21 of patients with positive L.N was significantly higher than that of patients with negative L.N involvement , 5.43±1.79 versus 2.50±0.53 , (P <0.001) as well .

In contrast , in negative L.N involvement there was significantly higher difference between before and after operation ,again same results in positive L.N involvement ,as shown in table (5).

Table 5: Median and mean fold change in patients with and without L.N (before and after operation).

LN	Fold change before	Fold change after	P*
Negative (n= 10)	4.50 (4.70±1.34)	2.50 (2.50±0.53)	<0.001
Positive (n = 35)	10.00 (10.66±3.22)	6.00 (5.43±1.79)	<0.001
P!	<0.001	<0.001	

7- Association between serum miR-21 and stage of tumor.

Before operation ,the mean fold change of miR-21 serum levels in breast cancer patients with stage III,IV were significantly higher than those of stage I,II (P <0.001) . After operation , the mean fold change of miR-21 serum levels in patients with stage III,IV were also significantly higher than that of stage I,II (P <0.001). In each stage the mean fold change of miR-21 serum levels in patients before operation were significantly higher than that of the same stage after operation (P <0.05) as table (6) .

Table 6 : Median and mean fold change in patients' group according to stage.

Stage	Fold change before	Fold change after	P*
I (n=10)	4.50 (4.70±1.34)	2.50 (2.50±0.53)	0.005
II (n=15)		4.00 (4.00±1.36)	<0.001
III (n=15)	12.00 (12.00±1.51)	6.00 (6.00±0.76)	0.001
IV (n=5)	15.00 (15.00±3.61)	8.00 (8.00±1.22)	0.042
P!	<0.001	<0.001	

8-Validity of serum microRNA-21 gene expression as gene aberration

The cutoff value for serum miR-21 gene expression fold change that predict gene expression aberration in breast carcinoma by using the RT-qPCR technique , was done by an Receiver Operator Characteristic (ROC) curve analysis: The best cutoff value for serum miR-21 was 2.5 with a specificity of 100%, sensitivity of 100% and accuracy excellent ,table (7) and figure (4).

Table 7 :- The ROC curve for cutoff value of serum miR-21 in patients.

Parameter	Value	Interpretation
Cut off value	2.5	
AUC (accuracy)	1.000(100%)	Excellent
Sensitivity	100%	Excellent
Specificity	100%	Excellent

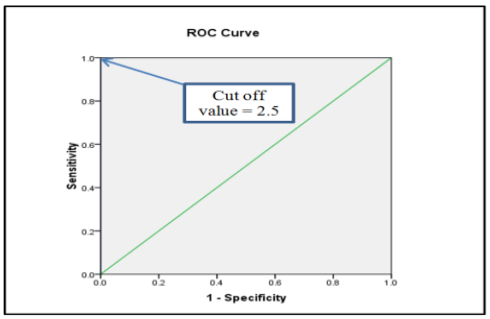

Figure 4: ROC curve for cut off value fold change of serum miR-21 in breast cancer patients

9-Validity of serum microR**NA-21 gene expression as prognostic marker.**

A-The cutoff value of fold change for serum miR-21 gene expression, that predict breast cancer patient with positive L.N metastasis , an ROC curve analysis was performed .The best cutoff value of fold change for serum miR-21 with positive L.N was 7.5 with a specificity of 100%, sensitivity of 80% and excellent accuracy , as shown in table (8) and figure (5).

Table 8:-ROC curve for serum miR-21 in patients with positive L.N metastasis.

Parameter	Value	Interpretation
Cut off value	7.5	
AUC (accuracy)	1.000(100%)	Excellent
Sensitivity	8 0%	Very good
Specificity	100%	Excellent

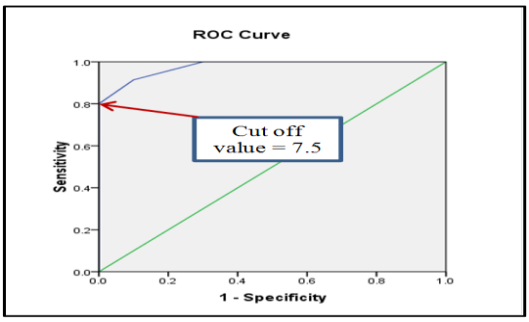

Figure 5: ROC curve for cut off value fold change for positive LN in patients .

B-The cutoff value of fold change for serum miR-21 gene expression , that predict breast cancer patient with AJCC stage III, IV (higher stage) breast cancer from patients with earlier stages of breast cancer , an ROC curve analysis was performed. The best cutoff value of fold change for serum miR-21with stage III,IV was 11.00 with a specificity of 100%, sensitivity of 75% and accuracy excellent, as shown in table (9) and figure (6).

Table 9: The ROC curve for cut off value for higher stage (III and IV) in patients.

Parameter	Value	Interpretation
Cut off value	11.00	
AUC (accuracy)	0.985 (98.5%)	Excellent
Sensitivity	75%	Good
Specificity	100%	Excellent

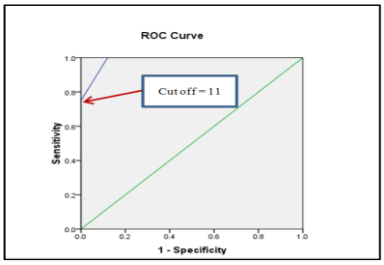

Figure 6: The ROC curve for cut off value for higher stage (III and IV) in patients .

DISCUSSION:

At present, miR have become the rising stars in cancer genetics. MiRs are excellent candidates for novel molecular targeting treatments because of their ability to regulate multiple genes in molecular pathways. Chemotherapy is an important component in the treatment paradigm for cancers. However, the resistance of cancer cells to chemotherapeutic agents frequently results in the subsequent recurrence and metastasis. Recently, a new data suggest that the expression level of miR-21 in tumor tissue and plasma might be used as a biomarker to predict adjuvant platinum-based chemotherapy response and disease free survival in patients with non-small-cell lung cancer. Thus, it may serve as a novel therapeutic target to modulate platinum-based chemotherapy [8,9,10,11] .

The present study , focused on tissue and serum levels of miR-21 before and after operation of same breast cancer patient samples. The mean cancer tissue fold change of miR-21 was significantly higher than the paracancerous tissue . These results were accepted to results of many studies [12,13,14] .

Before operation, the serum level of miR-21gene expression was significantly higher than that of apparently healthy controls and the best cutoff value for gene aberration was (2.5 fold change) ,from which we can detect normal gene expression from gene aberration and also can detect normal cases from malignant ones .These result were in concordance with results of many studies [12,13,14] .The miR-21 gene is located on chromosome 17q23.2, which is inside the common fragile site FRA17B. This region is frequently amplified in breast and lung cancer [15] . The serum level of miR-21 gene expression in advance stage (III,IV) and L.N metastasis in present study was significantly higher than that of stage (I,II) and L.N negative involvement ,respectively. And the best cutoff value for gene in advance stage and positive L.N involvement was (11 and 7.5 fold change) ,in which we can detect gene expression in early stage (I,II), from gene expression of advance stage (III,IV) and positive L.N from negative one ,respectively .These results were also in concordance with other studies [12 , 14]. Previously, Gao *et al.* [11] , reported that serum miR-21 was a more sensitive breast cancer marker than CA15-3 or CEA ; in particular, miR-21 was a potential tumor marker for the diagnosis of early-stage breast cancer. Also, miR-21 levels before and after chemotherapy demonstrated a detectable association with overall survival that was independent of anti-Her-2 therapy [16,17] .

After operation , the result of presented study , on the one hand, the mean serum level of miR-21 gene expression was significantly higher than that of apparently healthy controls and higher in advance stage and in L.N involvement from that of early stage and negative L.N involvement .On the other hand ,the mean serum level of miR-21 was significantly lower than that of before operation in both higher stage and L.N involvement . These results could be due to the bulk of tumor mass removed after operation. No similar studies

concerning the of miR-21in patients before operation in comparison to same patients after operation.

In current study, there is no significant association between age of patients and fold change of miR-21 gene expression before and after operation. These findings are accepted with other studies [12,13,14] . In order to determine the correlation of miR expression in tissue and the matched serum samples, the results of present study showed a significant correlation of miR-21 gene expression in the tissues with those in the sera before and after operation, with r = 0.51 and r = 0.61 ($p<0.001$) respectively, which suggests that miR-21 isolated from sera could reflect most of the characteristic expression patterns of their tissue counterparts and further show promise for miR as blood-based biomarkers for detecting and screening breast tumors .

Best for the present knowledge, this study could be the first study of its type to be conducted in Iraq, evaluating tissue and serum level of miR-21 gene expression in same patients before and after operation by RT-qPCR , in a sample of Iraqi female patients . There was no baseline study regarding serum level of miR-21 gene expression stratification in apparently healthy control in Iraqi individuals. Although, similar studies were conducted abroad to stratify serum level of miR-21 in breast cancer patients in other countries [12 ,13 ,14] .

CONCLUSION:

The RT-PCR is a applied means for investigation of serum miR samples and the extraction of RNA and identification of miR-21 from the serum of individuals diagnosed with breast cancer is possible. Circulating miR-21 before operation can serve as a good biomarker for breast cancer screening , could be used in detection the early stage (I,II) from late stage (III, IV) and can differentiate patient with L.N positive from negative L.N involvement . While serum level of miR-21 after operation can be used for follow- up patient, gene therapy and for management of patients with breast cancer. However, studies with larger number of patients and healthy controls are needed to validate the present findings and to investigate whether other miRs are also capable of indicating breast cancer progression and more importantly whether miR-21 is the best choice among potential breast cancer biomarkers .

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