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EVALUATION OF MICRORNA-21 IN INVASIVE DUCTAL CARCINOMA IN COMPARISON TO CLINICOPATHOLOGICAL STATUS BY STEM-LOOP RT-PCR PROCEDURE.

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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 age of patients and protein expression of ER,PR and HER-2 . **Material and methods:-** Stem-loop RT-PCR was performed to identify the expression level of miR-21 in 50 IDC samples and their NATs. IHC for ER,PR and HER-2 . **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%. No statistical significant associated with age of patients ,protein expression of ER,PR and HER-2 and fold change of miR-21 expression . **Conclusion:-** Up-regulation of miR-21 expression has been no statistical significant associated with age of patients and protein expression of ER,PR and HER-2.

KEYWORDS:- breast cancer,miR-21gene expression ,stem-loop RT-PCR.

Abbreviation: BC: breast cancer ;IDC: invasive ductal carcinoma ;NATs :normal adjacent tissues ;miR: microRNA; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase ; ER: Estrogen receptor ; PR: Progesterone receptor ; HER-2: Human epidermal growth factor receptor-2 ; ROC: Receiver Operating Characteristics.

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 and for protein expression level for ER,PR and HER-2 by IHC.

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	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCAC CAGAGCCAACTCAACA	
hsa-miR-21 primer	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	

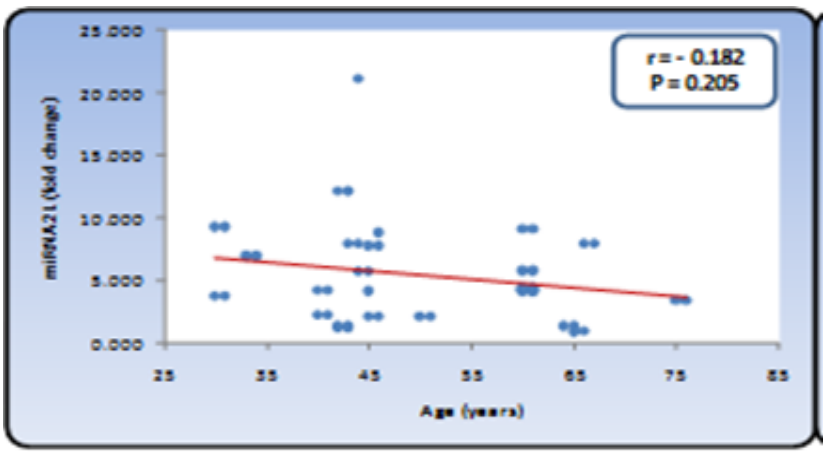
Majority of cases showed up regulation of miR-21, 96% .as shown in table (4).

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).

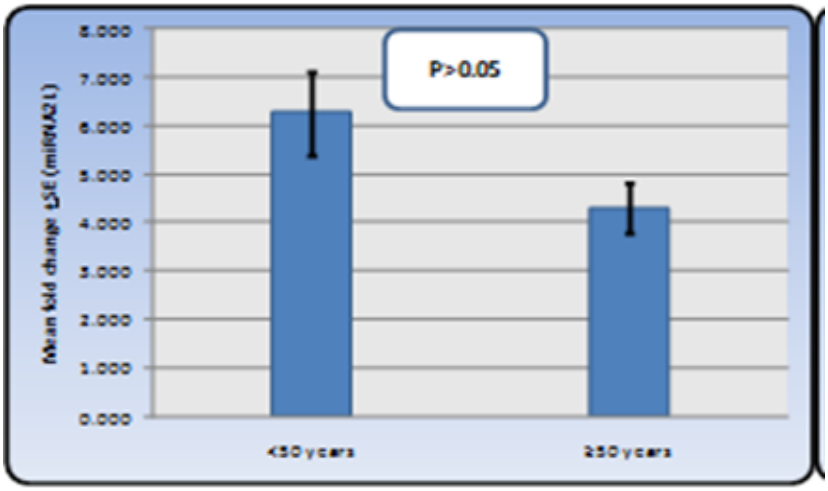


Figure (2): Comparison of mean fold change between patients <50 years and ≥ 50years.

3-Association between estrogen and progesterone receptors for protein expression by IHC and fold change

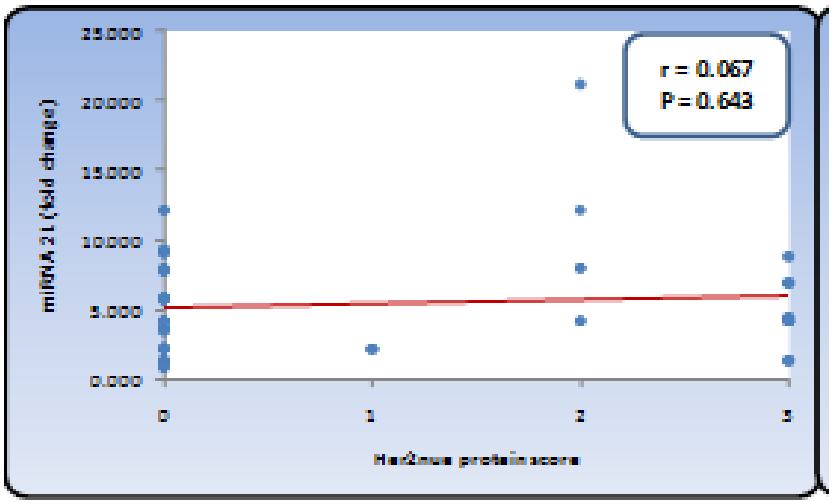
No significant association was found between miR-21 fold change and ER expression. Similarly, no significant association was found between miR-21 fold change and PR expression . These results are shown in table(5).

Table (5): Association between IHC expression, of ER and PR, and fold change .

	Negative ER		Positive ER		Negative PR		Positive PR		Total	
miR-21	No.	%	No.	%	No.	%	No.	%	No.	%
down regulation	0	0.00	2	5.88	0	0.00	2	5.26	2	4.00
up regulation	16	100.00	32	94.12	12	100.00	36	94.74	48	96.00
Total	16	100.00	34	100.00	12	100.00	38	100.00	50	100.00
P-value	1.000				1.000					

4-Correlation between HER-2/neu expression and fold change

miR-21 fold change showed a positive non-significant correlation with IHC HER-2 expression . These results are illustrated in figure(3).



Figure(3):Correlation between HER-2 protein expression and fold change.

Table (6)solidified the above mentioned absence of a significant association between HER-2 protein and fold change.

Table(6) : Association between HER-2 by IHC and miR-21 fold change.

	HER-2/neu protein					
	Negative		Positive		Total	
miR-21	**No.**	**%**	**No.**	**%**	**No.**	**%**
down regulation	2	5.88	0	0	2	4
up regulation	32	94.12	16	100	48	96
Total	34	100.00	16	100	50	100
P=1.000						

5-Validity of microRNA-21 fold changes as gene expression aberration

To find the cutoff value for miR-21 and PTEN gene expression fold change that predict gene expression aberration in BC by using the stem-loop RT-qPCR technique , an ROC curve analysis was done that showed the following results:

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(7) and figure(4) .

Table (7): Validity of miR-21 fold change in predicting expression gene aberration in BC tissues.

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

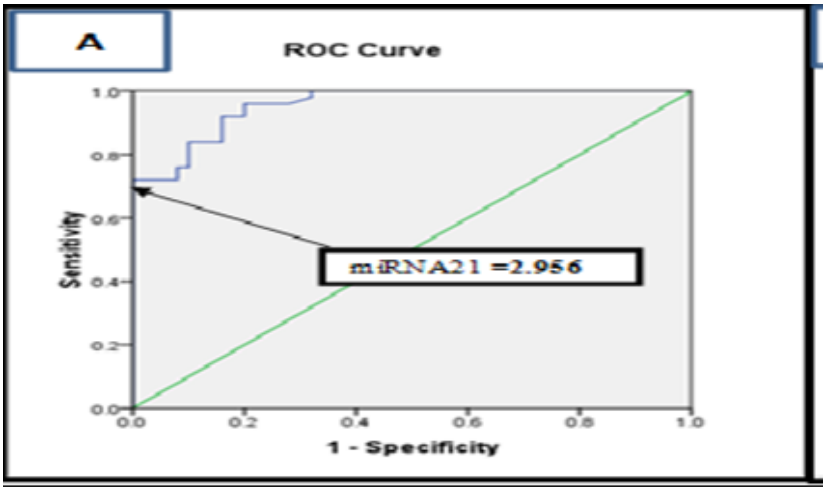


Figure (4): ROC curve showing the cutoff value for miR-21 fold change that predicts gene expression aberration in BC tissues in comparison to NATs with p value was (<0.05).

5-Validity of microRNA-21 gene expression fold changes as prognostic markers.

To find the cutoff value of fold change for miR-21 gene expression , that predict BC patient with positive ER,PR and HER-2 ,an ROC curve analysis was performed.

It was found that miR-21gene expression fold change cannot predict positive ER,PR and HER-2 . The ROC results demonstrated that the AUC was (67%),(56.8%) , (60.5%) when the cutoff value was set to the optimal point, ≥6.341 , ≥4.272, ≥4.389 ; specificity was 73.5%, 50%,58.8%; sensitivity was 50% , 33.3%,50% respectively. Results obtained were summarized and presented in table(8).

Table (8): Validity of miR-21 fold change as a prognostic marker.

Prognostic parameter	miR-21 Cutoff value	AUC (accuracy)	Specificity	Sensitivity	P-value	Interpretation
Positive HER-2/neu	≥4.389	0.605 (60.5%)	58.8%	50%	>0.05	Poor
Negative ER	≥6.341	0.670 (67%)	73.5%	50%	>0.05	Poor
Negative PR	≥4.272	0.568 (56.8%)	50%	33.3%	>0.05	Poor

Stem- loop qRT-PCR amplification plots for miR-21 cDNA in BC tissue patients by using TaqMan probe as shown in figure(5) and qRT-PCR amplification plots for GAPDH cDNA in BC tissue patients by using TaqMan probe as shown in figure(6).

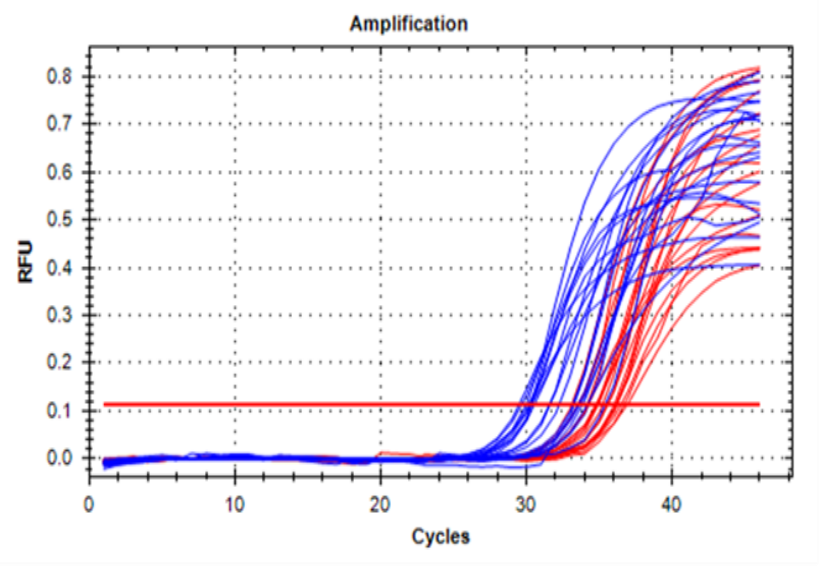


Figure (5): Stem- loop qRT-PCR amplification plots for miR-21 cDNA in BC tissue patients by using TaqMan probe. (FAM), where (blue amplification plot as breast cancer tissue samples) and (Red amplification plot as normal adjacent tissue samples).

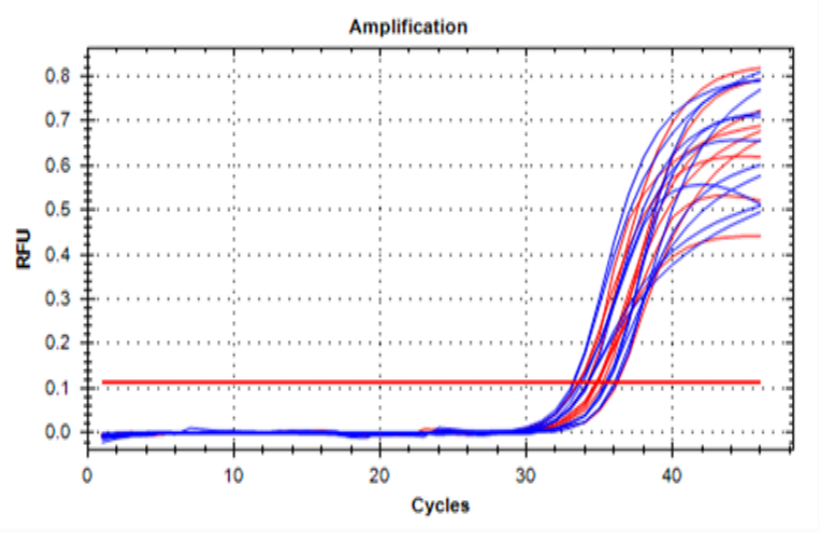


Figure (6): Real-Time PCR amplification plots for GAPDH cDNA gene in BC tissue patients by using TaqMan probe . (FAM). Where (blue amplification plot as BC tissue samples) and (Red amplification plot as NATs samples).

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]**

The result of present study showed no statistically significant association was found between ER,PR expression and both miR-21 gene expression.

Regarding to miR-21,the result of present study were in agreement with **(Iorio et al) [10]; (Li-Xu et al) [11] ; (Shahram et al).[16]** While disagreement with those reported by **; (Yan et al) [2] ; (Mohamed et al) [13]** , who found that, miR-21 was significantly up-regulation in breast cancer cases having both ER−/PR− compared to cases having ER+/PR+ .

The result of present study showed no statistical significant correlation between HER-2 status (protein and gene expression) and miR-21 gene expression . Similar result were seen in **(Iorio et al) [10]; (Li-Xu et al) [11] ; (Haiyan et al) [14] ; (Shahram et al) [16]** ,regarding to miR-21.

Despite to miR-21 gene expression does not appear to discriminate between the different receptor statuses in breast tumors (ER,PR,HER-2), miR-21 gene expression might still be a potential prognostic marker for predicting lymph node involvement and advanced stages of breast cancer **(Li-Xu et al).[11]**

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) [17], 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.

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 no statistical significant association with unfavorable pathological features of the disease, including ER,PR and HER-2 status of IDC.

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.

REFERENCES

1. Huang G, Zhang X , Guo G ,et al . Clinical significance of miR-21 expression in breast cancer: SYBR-greenI-based real-time RT-PCR study of invasive ductal carcinoma. Oncol Rep 2009; 21: 673e679.
2. Yan L , Huang X , Shao Q , et al . MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA 2008; 14: 2348-2360.
3. Hasemeier B , Christgen M, Kreipe H,et al . Reliable microRNA profiling in routinely processed formalinfixed paraffin-embedded breast cancer specimens using fluorescence labelled bead technology. BMC Biotechnol 2008; 8: 90.
4. 4-Bustin S, Benes V, Garson J, et al. Minimum information for publication of quantitative real-time PCR experiments . Clinical Chemistry 2009; 55: 1–12.

5. Ridzon D, Chen C, Broomer A, et al. Realtime quantification of microRNAs by stem-loop RT-PCR . Nucleic Acids Res 2005; 33: 179.
6. Martha – Kramer F. Stem-Loop RT-qPCR for miRNAs . Curr Protoc Mol Biol 2011; 10: 1002.
7. Livak and Schmittgen . The $2^{-\Delta\Delta CT}$ (Livak) Method Real-Time PCR. Applications Guide 2001: 41.
8. Lwis F, Maughan N , Smith V, et al . Unlocking the archive-gene expression in paraffin-embedded tissue. J Pathol 2001; 195: 66–71.
9. Liu A , Tetzlaff M, Vanbelle P, et al. MicroRNA expression profiling outperforms mRNA expression profiling in formalin fixed paraffin-embedded tissues. Int J Clin Exp Pathol 2009; 2: 519–27.
10. Iorio M . MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005; 65: 7065-70.
11. Li -XU Y , Xiu F , Qiong S, et al . MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA 2008; 15: 2350–2362.
12. Bao Song , Chuanxi Wang , Jie Liu, et al .MicroRNA-21 regulates breast cancer invasion partly by targeting tissue inhibitor of metalloproteinase 3 expression. Journal of Experimental & Clinical Cancer Research 2010; 29: 29.
13. Mohamed M Hafez, Zeinab K Hassan , Abdel Rahman N Zekri et al. MicroRNAs and Metastasis-related Gene Expression in Egyptian Breast Cancer Patients. Asian Pacific Journal of Cancer Prevention 2012; 13.
14. Haiyan Si, Xiaoming Sun, Yingjian Chen, et al. Circulating microRNA-92a and microRNA-21 as novel minimally invasive biomarkers for primary breast cancer. J Cancer Res Clin Oncol 2013; 139: 223–229.
15. Yang Zhang, Wenhai Zhang, et al .Genetic Heterogeneity of Breast Cancer Metastasis May Be Related to miR-21 Regulation of TIMP-3 in Translation. International Journal of Surgical Oncology 2013.
16. Shahram Savad , Parvin Mehdipour , Mohammad Miryounesi, et al. Expression Analysis of MiR-21, MiR-205, and MiR-342 in Breast Cancer in Iran. Asian Pacific Journal of Cancer Prevention 2012; 13:873-877.
17. Chang G, Yan dan Y, Erwei S, et al. Up-regulation of miR-21 Mediates Resistance to Trastuzumab Therapy for Breast Cancer. J Biol Chem 2011; 286: 19127–19137.