MicroRNA-92gene expression in epithelial ovarian cancer using a novel Real-Time Polymerase change reaction

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Abstract

MiR–92 is one of six miRs encoded by the miR-17-92 cluster and its one of the best-characterized oncogenic miR clusters. A role for miR-92 in the pathogenesis of human cancers has been implicated by the high incidence of amplification in multiple neoplasm. The objective of this study is to estimation the miR-92 gene expression levels in both fresh tissues and serum of same ovarian cancer patients by using stem-loop follow by Taq-Man Real Time PCR (RT-PCR) technique and correlate the miR-92 gene expression with stages and lymph node involvement of ovarian cancer patients. Stem-Loop RT-PCR was performed to identify the level of miR-92 gene expression in both fresh tissues and serum of same ovarian cancer patients. The expression levels of miR-92 relative to mRNA of GAPDH were determined by using the livak method. Mean fold change of miR-92 was statistical significantly higher in ovarian cancer from paracancerous tissues. Mean fold change of serum miR-92 gene expression was higher statistical significantly difference from healthy control, mean fold change of miR-92 in advance stage (III, IV) was higher statistical significantly difference from that early stage (I, II) and mean fold change of miR-92 in patient with positive lymph node was higher statistical significantly difference from that of negative lymph node. There was no significant association between gene expression of miR-92 and age of patients. Together we concluded that the over-expression of miR-92 were observed in serum patients with epithelial ovarian cancer compared with healthy controls, and miR-92 gene expression levels increased with lymph node involvement and advance stage of epithelial ovarian cancer.

Keywords: Epithelial ovarian cancer, miR-92, stem-loop RT-qPCR

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Introduction

Ovarian cancer, particularly epithelial ovarian cancer, which accounts for 90% of all ovarian cancers, continues to be the leading cause of death among gynaecological malignancies [1]. Due to the asymptomatic nature of early stages of epithelial ovarian cancer, over two-thirds of cases are not diagnosed until the disease has spread beyond the ovaries [2]. Thus, early detection of the disease is essential to improve the survival rate in patients with epithelial ovarian cancer. Carbohydrate antigen-125 (CA-125), and Human Epididymis Protein 4 (HE4), are the most widely used biomarkers for epithelial ovarian cancer diagnosis, of which sensitivity and specificity are not quite satisfied. About 70% of patients are diagnosed in advanced clinical stages of the disease due to lack of effective screening methods [3].

During the last few decades, several attempts to develop serum based biomarkers for early detection of ovarian cancer have gained limited success because of their low sensitivities and specificities. Therefore, identifying and developing an alternative, non-invasive, sensitive, and more specific biomarker to detect ovarian cancer at the initial stages is necessary for the improved management of ovarian cancer patients. Fortunately, despite the limitations, recently discovered small RNAs, termed microRNAs (4). MicroRNAs (miRs) belong to a class of endogenously expressed non-coding small RNAs of approximately 18-22 nucleotides, were initially discovered in 1993. These small RNAs influence gene regulation by pairing with mRNAs of protein-encoding genes to repress their expression via decreased translational efficiency and or mRNA levels [5]. A growing body of evidence suggests that dysregulation of miR expression contributes to a wide variety of human diseases, including cancer. Almost 50% of known miRs are located within chromosomal regions frequently amplified or deleted in human cancers [6].

MiR–92 is one of six miRs encoded by the miR-17-92 cluster and its one of the best-characterized oncogenic miR clusters, which comprises members with an effect on cell proliferation. However, the role of miR-92 is unknown. The miR-17–92 cluster, located at 13q, which encodes six miRNAs processed from a common precursor transcript [7]. A role for miR-17–92 in the pathogenesis of human cancers has been implicated by the high incidence of amplification in multiple neoplasms, including diffuse large B cell lymphoma [8]. To the best knowledge, there are few reports determining whether circulating miRs exist in the serum/plasma of patients with epithelial ovarian cancer. Thus, the present study investigated tissue and serum levels of miR-92 in patients with epithelial ovarian cancer and in healthy controls, and evaluated the relationship between serum miR-92 status and clinical characteristics of epithelial ovarian cancer.

Patients and methods

Study population

The study was conducted during the period from March 2013 to May 2015. This is a prospective study, where by (n=40) patients with newly diagnosed ovarian cancer and apparently healthy controls (n=20) were diagnosed without any tumor or physical illness. Aged range (40-60) years, were recruited at the Department of Obstetrics and Gynaecology/Obstetrics and

Gynaecology Teaching Hospital in Diawania City. The patients had not received neoadjuvant therapy, all of whom underwent primary debunking surgery. Serum sample were collected from ovarian cancer patients and healthy controls. Forty - pairs of fresh tissues from same cases of ovarian cancer and paracancerous tissues (which consider as internal control), for total RNA extraction and for RT-qPCR. Another 40 pairs specimens of both ovarian cancer and paracancerous tissues for histopathological examination and tumors were staged according to International Federation of Gynecology and Obstetrics (FIGO) Ovarian Cancer Staging, (2014).

MiRNA isolation from serum and tissue

Serum samples were collected between 8:00 and 9:00 a.m. from patients and healthy controls. A 5-ml sample of peripheral venous blood was drawn from all study participants after an overnight fast and placed at room temperature for 60 min. Then the blood samples were centrifuged at 1000 g for 10 min at 4oC to spin down the blood cells. The supernatants (serum) was then transferred into fresh tubes and stored at 80oC until use. Tissue samples were homogenized in denaturing lysis solution and dissolved RNA was stored at -20°C before use. 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-92 quantification

The Primers and probes for miR-92 were design in this study by using (The Sanger Center miR database Registry) to selected miR-92 sequence and using miR Primer Design Tool. The cDNAs were synthesized by stem-loop primer, UUGCACU UGUCCCGGCCUG. MiR-92 was then analyzed by qPCR and the primer used was: forward, GGGGT-TGGGGGATATAAA and reverse, CCTTTTTCAATTCCTTTT CCCTTTAC. The qPCR analysis was performed using Taq-Man probe, FAM-GGGGGATATA-AAGGAG-MGB. Initial denaturation at 95˚C for 5 min, followed by 40 cycles of 95˚C denaturation for 20 secs, 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-92 quantification data were normalized to housekeeping gene like Glyceraldehyde 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 as design as Random Hexamer primer and the primer used in qPCR was: forward, CAGCCGCATCT-TCTTTTGC and reverse, TTAAAAGC-AGCCCTG GTGAC. Taq-Man probe for mGAPDH was: FAM-CCAGCCGAG-CCACATCGC TC-TAMRA. These primers and probe of miR-92 and GAPDH were provided by (Bioneer company, Korea). The data results of RT-qPCR for miR-92 and GAPDH were analyzed by the relative quantification gene expression levels (fold change) were based on the Ct values by using the Livak method (Fold change = $2^{-\Delta\Delta CT}$) that described by (Livak and Schmittgen, 2001] 9].

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.

Result

Clinicopathological characteristics of patients

In present study a total of 40 ovarian cancer patients, all cases with epithelial ovarian cancer ,10(25%) stage I: 15(37.5%) stage II: 10(25%) stage III: and 5(12.5%) stage IV. Mean age of patients was (47 ±5) years old range (40-60) years and mean age of control group was (50±3.2) years old range (45-56) years, no statistical significant difference between mean age of patients and control groups, (P >0.05), table (1).

Table 1.
Mean age in patients and control groups

Group	N	Mean	SD	Minimum	Maximum	P
Control	20	50	3.2	45	56	
Patients	40	47	5	40	60	0.057
Total	60	48.3	5.1	40	60	

Comparison between microRNA-92 gene expression of epithelial ovarian cancer and paracancerous tissues

Mean cancer tissue fold change of miR-92 was significantly higher than that paracancerous tissue, (4.5±0.002) versus (2.3±0.03), respectively, (P<0.05), figure (1).

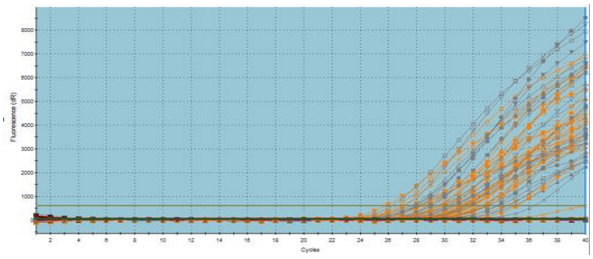

Figure 1.

Stem- Loop qRT-PCR amplification plots for miR-92 cDNA in epithelial ovarian cancer tissue patients by using Taq-Man probe.

Comparison the serum of microRNA-92 gene expression between the epithelial ovarian cancer patients and control groups.

The mean fold change of miR-92 serum levels in ovarian cancer patients were higher statistical significantly from apparently healthy controls, (3.8-fold change) versus (1.4-fold change), respectively, (P<0.001), table (2) and figure (2).

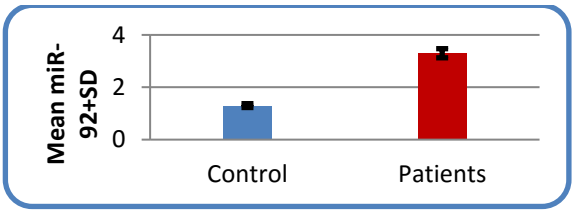

Figure 2.

Mean Serum microRNA-92 in patients and control groups.

Table 2.

Mean and median serum of microRNA-92 in patients and control groups

Group	N	Median	Mean	Minimum	Maximum	P
Control	20	1.3	1.4	1.1	2.0	<0.001
Patients	40	3.3	3.8	2.6	6.2	
Total	60	3.0	3.0	1.1	6.2	

The correlation of microRNA-92gene expression pattern between ovarian cancer tissues and paired serums

The results showed a statistical significant correlation of miR-92 gene expression in the tissues with those in the serums, with r = 0.51 (P<0.001)

Association between serum level of microRNA-92 and age of patients

The result of present study showed that there was no statistical significance correlation between serum level of miR-92 and age of patients (P>0.05), table (1). When divided in two group (<45 and ≥45), no statistical significant between mean fold change of miR-92 serum levels in ovarian cancer patients and age of patients, (P>0.05), as table (3) and figure (3).

Table 3.

Mean fold change of serum microRNA-92 according to age

Age group	N	Median	Mean	SD	Minimum	Maximum	P
<45 years	**15**	**3**	**3.27**	**0.84**	**2.7**	**6.2**	**0.056**
≥45 years	**25**	**4**	**4.11**	**1.14**	**2.6**	**6.1**	
Total	**40**	**3.3**	**3.80**	**1.10**	**2.6**	**6.2**	

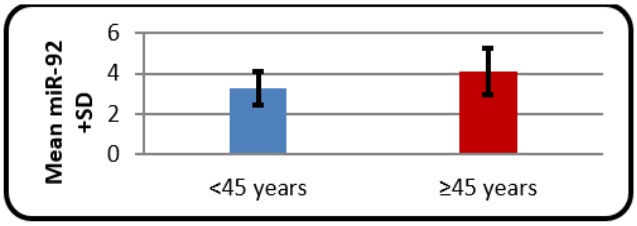

Figure 3.

Patients serum microRNA-92 according to age.

Association between serum level of microRNA-92 and L.N involvement

The mean miR-92-fold change of patients with positive L.N was statistical significantly higher from that of patients with negative L.N involvement, (5.0067 ±0.88355) versus (3.068±0.23402), respectively (P < 0.001), as table (4).

Table 4.

Mean fold change of serum microRNA-92 in patients according to L.N involvement

LN	N	Median	Mean	SD	Minimum	Maximum	P
Positive	15	4.9	5.0067	0.88355	3.5	6.2	<0.001
Negative	25	3	3.068	0.23402	2.6	3.5	
Total	40	3.3	3.795	1.10336	2.6	6.2	

Association between serum level of microRNA-92 and stage of tumor

The mean fold change of miR-92 serum levels in epithelial ovarian cancer patients in stage (III, IV) were statistical significantly higher than that stage (I, II), (5.0067±0.88355) versus (3.068±0.23402), (P <0.001), as table (5).

Table 5.

Mean fold change of serum microRNA-92 in patients according to stage

Stage	N	Median	Mean	SD	Minimum	Maximum	P
I and II	25	3	3.068	0.23402	2.6	3.5	<0.001
III and IV	15	4.9	5.0067	0.88355	3.5	6.2	
Total	40	3.3	3.795	1.10336	2.6	6.2	

Validity of serum microRNA-92 expression as gene aberration

The cutoff value for serum miR-92 gene expression fold change that predict gene expression aberration in ovarian carcinoma by using the RT-qPCR technique, a Receiver Operator Characteristic (ROC) curve analysis was done:

The best cutoff value for serum miR-92 in patients with epithelial ovarian cancer was (2.3-fold change) with a specificity of 100%, sensitivity of 100% and accuracy excellent, table (6) and figure (4).

Table 6.

The ROC curve for cutoff value of serum microRNA-92 in patients

Parameter	Value	Interpretation
Cut off value	2.3	
AUC (accuracy)	1.000(100%)	Excellent
Sensitivity	100%	Excellent
Specificity	100%	Excellent

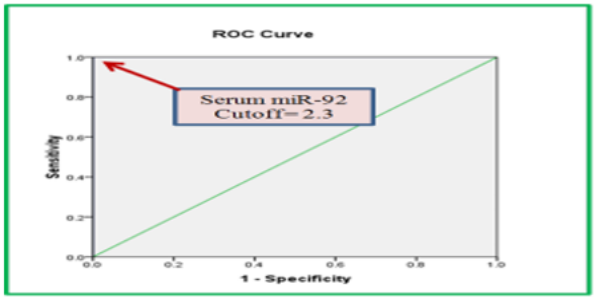

Figure 4.

The ROC curve for cutoff value of microRNA-92 in patients as gene aberration

Validity of serum microRNA-92 expression as prognostic marker

A-The cutoff value of fold change for serum miR-92 gene expression, that predict ovarian cancer patient with positive L.N metastasis, an ROC curve analysis was performed. The best cutoff value of fold change for serum miR-92 in patient with positive L.N was (3.75-fold change with a specificity of 93.3%, sensitivity of 100% and accuracy excellent, as table (7) and figure (5).

Table 7.

The ROC curve for cutoff value of microRNA-92 in patients with positive L.N involvement

Parameter	Value	Interpretation
Cut off value	3.75	
AUC (accuracy)	0.997(99.7%)	Excellent
Sensitivity	100%	Excellent
Specificity	93.3%	Excellent
P	<0.001	Highly significant

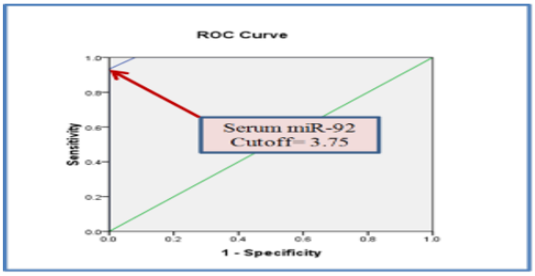

Figure 5.

ROC curve for cutoff value of microRNA-92 in patients with positive L.N involvement.

B-The cutoff value of fold change for serum miR-92 gene expression, that predict epithelia ovarian cancer patient with stage III, IV (advance stage) ovarian cancer from patients with earlier stages (I, II) of ovarian cancer, an ROC curve analysis was performed. The best cutoff value of fold change for serum miR-92 in patient with advance stage was (3.75-fold change) with a specificity of 92%, sensitivity of 100% and accuracy excellent, as table (8) and figure (6).

Table 8.

The ROC curve for cutoff value of microRNA-92 in patients with advance stage

Parameter	Value	Interpretation
Cut off value	3.75	
AUC (accuracy)	0.997(99.7%)	Excellent
Sensitivity	100%	Excellent
Specificity	92%	Excellent
P	<0.001	Highly significant

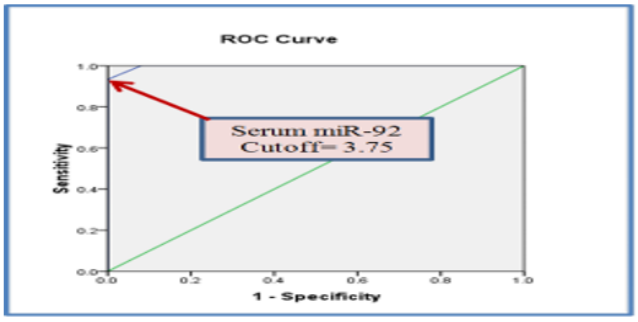

Figure 6.

The ROC curve for cutoff value of microRNA-92 in patients with advance stage.

Discussion

Among the various curative and preventive measures considered, early detection has long been the key to successful treatment of multiple life-threatening diseases, including ovarian cancer. Early detection underlies the foundation for better management and intervention of ovarian cancer at its primary stage before it spreads and affects other healthy tissues of the body. The aberrant expression pattern of miRs can be a powerful tool to diagnose ovarian cancer in its earliest stages) [10]. In the present study, focused on tissue and serum levels of miR-92 of same epithelial ovarian cancer patient samples. The mean cancer tissue fold change of miR-92 was statistical significantly higher than that paracancerous tissue. These results were accepted to results of many studies [10-15]. The serum level of miR-92gene expression was higher statistical significantly difference from that apparently healthy controls and the best

cutoff value for gene aberration was (2.3-fold change), from which can detect normal gene expression from gene aberration and also can detect normal cases from malignant one. These results were accepted to results of many studies [10-15]. While these results were disagreement with the result of other study reported by (Iorio et al) [16], whom found that the miR-92a expression was significantly downregulated in ovarian malignant tissues. This difference between the result of present study and that study, could be the sample size, time of collecting the sample, type of sample either serum or tissue or tumor cell culture.

In order to determine the correlation of miR expression in tissue and the matched serum samples, the results of present study shown a significant correlation of miR-92 gene expression in the tissues with those in the serums (Spearman-Rho test $r = 0.51$, $P< 0.01$), which suggests that miR-92 isolated from serums could reflect most of the characteristic expression patterns of their tissue counterparts and further show promise for miR-92 as blood-based biomarkers for detecting and screening ovarian cancers.

Also the serum level of miR-92 gene expression in advance stage (III, IV) and L.N metastasis in present study was higher statistical significantly difference from that stage (I, II) and L.N negative involvement, respectively. And the best cutoff value for gene aberration was (3.75 fold change), from which can detect gene expression in early stage (I, II), in which without L.N involvement according to classification (FIGO, 2014) from gene expression of advance stage (III, IV) ,this suggested the possibility of up-regulated miR-92 levels as a prognostic biomarker .These results were accepted with results of other study reported by (10, 15). While the results of present study were disagreement with other study reported by (Resnick *et al*) [11], whom found that, there was no correlation between miR gene expression and stage or L.N involvement of patients.

In current study, there is no statistical significance between age of patients and fold change of miR-92 gene expression. These findings were accepted with other studies [10, 11]. Best for the present knowledge, this study could be the first study to be conducted in Iraq, evaluating tissue and serum level of miR-92 gene expression in same patients of ovarian cancer by RT-qPCR, in a sample of Iraqi female patients. There was no baseline study regarding tissue and serum level of miR-92 gene expression stratification in apparently healthy control in Iraqi individuals. Although, similar studies were conducted abroad to stratify tissue and serum level of miR-92 in ovarian cancer patients in other countries [10-16].

Conclusion

The extraction of RNA and identification of miR-92 from the serum of individuals diagnosed with epithelial ovarian cancer is feasible and RT- PCR is a novel and practical means for investigation of serum miR samples. MiR-92 could be a useful biomarker for detection of epithelial ovarian cancer, detect early stage from that of advance stage of epithelial ovarian cancer, as well as screening of asymptomatic populations and assessment of disease progression in epithelial ovarian cancer. However, studies with larger number of patients and healthy controls are needed to validate our findings and to investigate whether other miRs are also capable of indicating epithelial ovarian cancer

progression and more importantly whether miR-92 is the best choice among potential epithelial ovarian cancer biomarkers.

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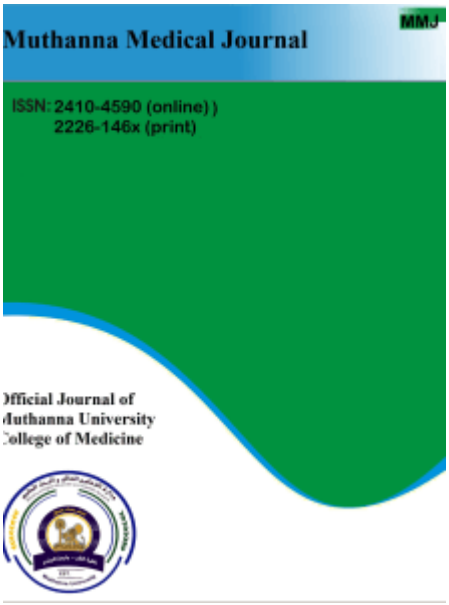

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