The circulating miRNA-34a, miRNA -143 and miRNA -212 are promising biomarkers in ovarian cancer

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Abstract

Objective: Ovarian cancer is one of the most common gynaecological malignancies among females worldwide. Early diagnostic of ovarian cancer is challenging, and miRNAs could serve as a potential biomarker for early detection of ovarian cancer regarding easy to detect. The present study investigated the expression profile of miRNA-34a, miRNA-143, miRNA-212, Sox4, BCL-2, and E2f5 in the serum of participants to find new biomarkers for early diagnosis of ovarian cancer.

Materials and Methods: Five milliliters of whole blood were collected from each patient (n=30) and control (n=30), and total RNA was extracted using Ambion™ TRIzol™ Reagent. Real-time PCR methods evaluated the expression of targeted miRNAs and their targeted genes.

Result: The Disease status of all ovarian cancer patients were classified as FIGO stage III (21) and (IV 9) according to imaging studies and surgical pathological findings. Our results showed that expression levels of miRNA-34a ($p<0.0001$), miRNA-143 ($p=0.028$), and miRNA-212 ($p<0.0001$) were significantly decreased in patients with ovarian cancer compared to controls and expression levels of Sox4($p<0.0001$), BCL-2($p<0.0001$) and E2f5($p<0.0001$) were significantly increased in patients with ovarian cancer compared to controls. In addition, Receiver Operating Characteristic (ROC) curve revealed that the expression profile of miRNA-34a (AUC= 0.82; P <0.0001), miRNA-143 (AUC= 0.66; P = 0.026) and miRNA-212 (AUC= 0.78; P = 0.0001) could be used as potential biomarker for discriminating patients with ovarian cancer.

Conclusion: In conclusion, our data showed that the expression profile of miRNA-34a, miRNA-143, and miRNA-212 could act as a potential biomarker for diagnosing ovarian cancer patients.

Introduction

Ovarian cancer is one of the most frequent kinds of cancer amongst women globally [1, 2]. The occurrence of the disease has been approximated at 9 · 4 per 100,000 in industrialized nations and 3.9 per 100,000 in the Iranian population [3, 4]. Early identification of ovarian cancer because absence of certain signs and reliable testing techniques is still difficult [5, 6].

MicroRNAs (miRNAs) are tiny, 19– 25 nucleotide non-coding RNA molecules that negatively manage gene expression by translation inhibition or targeting the 3 ' UTR [7–13]. Numerous reports have shown that miRNA expression profiles could be utilized as prognostic biomarkers in various kinds of cancer; additionally, serum and/or plasma are relatively convenient to access to determining circulating miRNAs [14–16]. As a result, determining a special serum miRNA expression profile can aid early identify and treat people with ovarian cancer.

Reduced transcript levels of miRNA-34a have been related to tumor development in ovarian cancer, and decreased miRNA-34a expression in a variety of cell lines and mouse models have been reported. It has been revealed that the downregulation of miRNA-34a has been connected with several proteins such as

BCL-2 that are associated with the cell cycle and cell survival paths [17, 18]. Furthermore, some examinations revealed that miRNA-143 and miRNA-212 decontrolled in numerous sorts of cancer. Artificial insemination reports have shown that miRNA-143 targets 3'-UTR of BCL-2 mRNA and triggers BCL-2 protein down-regulation. These findings recommend that miR-143 functions as a tumor suppressor and can possibly target cancer treatment. On top of that, miRNA-143 has been located to straight interfere with several mRNAs such as KRAS, ELK1, MYO6, ERK5, and hexokinase 2 (HK2), which are associated with the pathogenesis of cancers [19, 20]. Although boosted miRNA-212, by its targeting SRY-box 4 (SOX4) expression, has been reported in colorectal cancer, its down-regulation has been related to several sorts of cancer. SOX4 typically associates with cell apoptosis and tumor angiogenesis [21, 22]. To our knowledge, the expression levels of miRNA-34a, miRNA-143, and miRNA-212 and their targeted genes Sox4, BCL-2, and E2f5 have not been determined in the serum of people with ovarian cancer. This report explores the expression levels of circulating miRNA-34a, miRNA-143, miRNA-212, and their target, consisting of Sox4, BCL-2, and E2f5, in the serum of ladies with ovarian cancer to locate brand-new mRNA pen for very early identification of ovarian cancer.

Materials And Methods

Sample

After signed permission was acquired from patients undertaking surgery for ovarian cancer at Shahid Sadoughi Hospital (Yazd, Iran), Sample collection was carried out in patient with histopathological diagnosis of ovarian cancer, and those who obtained treatment (i.e., radiation treatment and radiotherapy) were left out from this research. Control subjects were healthy women admitted to the hospital for exams, and none of them had been identified with a malignancy neither had any viral infection. Five milliliters of whole blood were gathered from each patient (n = 30) and control (n = 30), and serum was instantly divided and kept at -80 ∘ C.

Rna Extraction And Cdna Synthesis

Acid guanidinium-phenol-chloroform methods by Ambion ™ TRIzol ™ Reagent (Invitrogen, Waltham, Massachusetts, USA) was utilized to extract total RNA from serum samples. Complementary DNA (cDNA) of Sox4, BCL-2 and E2f5 was manufactured utilizing Revert Aid first Strand cDNA Synthesis Kit (Thermo Scientific, C.N: 4368813,4368814, 4374966, and 4374967) according to the producer's guidelines. After treatment with DNase I (Thermoscientific, Canada) to remove DNA contamination, 3 micrograms of complete RNA from each sample were subjected to reverse transcription utilizing the NG dART RT kit (EURX, Poland) and specific stem-loop primers (Table 1) for each of the picked miRNAs, according to the producer's guidelines.

Table 1
List and sequence of all Primers used in this study

Targets	cDNA synthesis primer (stem loop)
miRNA34a-3p	5'GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGGGCA3'
miRNA143-3p	5'GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGAGCTA3'
miRNA212-3p	5'GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGGCCGT3'
miRNA103a-3p	5'GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCATAG3'
-	**Real time PCR primers (Forward)**
miRNA34a-3p	5'CACGCACAATCAGCAAGTATAC3'
miRNA143-3p	5'CACGCATGAGATGAAGCACTG3'
miRNA212-3p	5'CACGCATAACAGTCTCCAGTC3'
miRNA103a-3p	5'CACGCAAGCAGCATTGTACAGGG3'
-	**Real time PCR primer (Reverse)**
Universal	5'-CCAGTGCAGGGTCCGAGGTA-3'
BCL-2	F: ACAACATCGCCCTGTGGATGAC
	R: ATAGCTGATTCGACGTTTTGC C
Sox4	F: GTGAGCGAGATGATCTCGGG
	R: CAGGTTGGAGATGCTGGACTC
E2f5	F: TCAGGACCTATCCATGTGCTGCTT
	R: TCAGAGACATGTTGCTCAGGCAGA
ACTB	F: CCAACCGCGAGAAGATGA
	R: AGGGCATACCCCTCGTAGAT

Real-time Pcr

Quantitative real-time PCR was done by Rotor-Gene Q (Qiagen, Hilden, Germany). The expression degrees of chosen miRNAs were assessed utilizing Forward specific primers for each miRNA and universal reverse primer that is corresponding to a sequence within the RT stem-loop primers, 1 µl of RT product, and RealQ PCR 2x Master Mix Green (AMPLIQON, Odense, Denmark) and for genes 1.0 µL of produced cDNA, 10 µL of the SYBR Green master mix (AB Applied Biosystems), 1 µL of each primer and 7.0 µL of DNase/RNase-free water for the gene expression profile. The sequences of all primers utilized in this research are provided in Table 1. The thermal reaction condition was as follows: initial denaturation at 95 ◦ C for 15 minutes, followed by 40 cycles of denaturation at 95 ◦ C for 30 sec, annealing at 62 ◦ C for 30

sec, and expansion at 72 ◦ C for 30 sec. Real-time PCR was performed in last reaction volume of 20 µl. Melting curves were plotted at the end of each cycle series to confirm the purity of the products The expression degrees of miRNA-34a, miRNA-143, and miRNA-212 were normalized to the miRNA-103a as an inner control (β-actin was utilized as the reference gene and sox4,BCL-2 and e2f5 as the target genes), and Δ Cts were computed by the difference between Ct of the target miRNAs and Ct of miRNA-130a. Relative expression was computed by 2-ΔΔCt formula.

Statistical analysis

Statistical examinations were executed utilizing SPSS (SPSS, Chicago, IL, USA) and Graphpad Prism version 6.0 (Graphpad Prism Software, Inc., San Diego, CA). Shapiro– Wilk examination was done for assessing normal distribution of data, and student t-test or Mann-Whitney U-test was utilized to evaluate relative expression of each miRNA between patients and healthy controls. Data were shared as the mean ± standard error of the mean (SEM), and P-value less than 0.05 ($P < 0.05$) was considered statistically substantial.

Results

Patient

A total of 60 participants under ethical approval of the Shahid Sadoughi Hospital (Yazd, Iran), were involved in this study, including 25 patients with malignant epithelial ovarian tumor, 5 patients with non-malignant ovarian masses, and 30 age-matched healthy volunteers without any ovarian disorder whose samples were used as controls in the comparisons. Disease status of all ovarian cancer patients were classified as FIGO stage III and IV according to imaging studies and surgical pathological findings.

Expression Level Of Mirna-34a, Mirna-143 And Mirna-212

Statistical analysis of ΔCt values revealed that the expression levels of miRNA-34a and miRNA-212 significantly decreased in the serum of patients with ovarian cancer compared to healthy controls. As shown in Fig. I, the expression level of miRNA-34a (Fold change = 0.059; P value < 0.0001) and miRNA-212 (Fold change = 0.024; P value < 0.0001) has significant differences in patients compared to healthy controls. Also, the expression level of miRNA-143 (Fold change = 0.15; P value = 0.028) has decreased in patients with ovarian cancer compared to healthy controls (Fig. 1).

Receiver Operating Characteristic (Roc) Curve

To examine whether the expression profile of studied miRNAs could act as potential biomarkers, receiver operating characteristic (ROC) curve analysis was performed and the area under the ROC curve (AUC) was calculated. Data from ROC curve analysis revealed that the expression profile of miRNA-34a (AUC =

0.82; P value < 0.0001), miRNA-143 (AUC = 0.66; P value = 0.026) and miRNA-212 (AUC = 0.78; P value = 0.0001) could be used as potential biomarker for discriminating patients with ovarian cancer (Fig. 2).

In addition, spearman correlation test showed that the expression level of miRNA-143 and miRNA-212 has a significant positive correlation (r_s= 0.56; P value = 0.001) with ovarian cancer (Table 2).

Table 2
correlation of the expression levels of studied miRNAs

	Correlation with	Patients		Controls	
		r	P values	r	P values
miRNA-34a	**miRNA-143**	**0.403**	**0.027**	**0.419**	**0.021**
	miRNA-212	**0.424**	**0.02**	**0.427**	**0.019**
miRNA-143	miRNA-212	**0.561****	**0.001**	.287	0.125
** Significant P values (< 0.01)					

Expression Analysis of Sox4, BCL**-2 and** E2f5 **Genes**

Statistical analysis of ΔCt values revealed that the expression levels of Sox4, BCL-2 and E2f5 significantly increased in the serum of patients with ovarian cancer compared to healthy controls (P value < 0.0001) (Fig. 3).

Discussion

Previous evidence shows that the dysregulation of miRNAs could play a vital function in the pathogenesis of ovarian cancer. Besides, circulating miRNAs in serum have become prospective biomarkers for identifying numerous diseases, consisting of cancer. The report results disclosed that the expression of miRNA-34a, miRNA-143, miRNA-212 dramatically changed in serum of patients compared to healthy subjects.

The outcomes of our report revealed that the expression level of circulating miRNA-34a reduced in the patient compared to the control group. Besides, the outcome of our examination revealed that miRNA-34a might function as an appealing biomarker for the diagnosis of ovarian cancer. MiR34 family members are popular to manage the cell cycle, apoptosis, and invasiveness in cancer [23, 24]. These miRNAs are validated to have a straight task in the development of breast, prostate, bladder, or brain cancer, and miR34a is considered to be an attractive healing target in cancer treatment [23, 24]. Besides, the report by Jin et al. [25] has revealed that lowered expression of miRNA-34a is related to a poor prognosis of gallbladder cancer. They showed that miRNA-34 functions as a tumor suppressor, and overexpression of it led to decreased xenograft tumors [25]. On top of that, an Increased level of circulating miR34a and miR34b was reported in breast, lung, and prostate cancer [26, 27]. Nevertheless, limited information is

provided concerning circulating miR34 family members in ovarian cancer, and extra comprehensive examination is required to clear up the specific function of mir34 in ovarian cancer.

Additionally, the outcomes of our report revealed that the level of expression miRNA-143 in patients substantially lowered. Additionally, Previous records revealed that transfection of HeLa cells with pre-miRNA-143 might substantially lower their proliferation and boost apoptosis. Additionally, they discovered that expression of miRNA-143 was lowered in malignant cervical tissues compared to non-tumour tissues [28]. Additionally they revealed that the expression of miRNA-143 was down-regulated in colon cancer however not in rectal cancer [29]. To ensure that miRNA-143 could be serving as a tumor suppressor, yet extra examination is required to clear up the function of this miRNA in the restraint of ovarian cancer. On top of that, our outcomes revealed that miRNA-143 might be a superb marker for the early diagnosis of ovarian cancer in addition to miRNA-34a, yet to our understanding, it is the first time to evaluation in ovarian cancer and required additional examinations.

In addition to miRNA-34a and miRNA-143, we evaluated the level of miRNA-212 in ovarian patients and healthy controls. The outcomes revealed that in addition to other miRNAs, it additionally might forecast ovarian cancer. Besides, the literature review revealed that the miRNA-212 in various malignancies can alter differently. As an example, other investigation disclosed that the miRNA-212 expression reduced in vivo and in vitro in lung cancer. Additionally, Wei et al. [30] located that the expression of miRNA-212 was considerably down-regulated in both tissue and serum of epithelial ovarian cancer patients [30]. Their outcomes have disclosed that overexpression of miRNA-212 in ovarian cancer cells can hinder cell proliferation, migration, and invasion. In our report, the miRNA-212 level dramatically reduced, and it could reveal opportunities that miRNA-212 can serve as a tumor suppressor and an encouraging biomarker for the discovery of ovarian cancer. Furthermore, the lowering of miRNA-212 was in addition to raised expression of SOX4.

The previous examination revealed that miRNA-212 works as a tumor suppressor in colon cancer by targeting SOX4 [31]. SOX4 is a crucial developing transcription factor in invertebrates and is important for precise differentiation and proliferation in different tissues. Additionally, SOX4 is overexpressed in numerous human malignancies; nevertheless, the specific function of SOX4 in cancer development is not well recognized. Besides, SOX4 has been reported to be overexpressed in kidney cell cancer, which promoted cell migration and invasion generating EMT. Additionally, SOX4 has been uncovered to take part in metastasis and EMT in kidney cell cancer, lung adenocarcinoma, and non-small cell lung cancer [32, 33]. Another experiment disclosed that the Aryl hydrocarbon receptor-microRNA-212/ 132 axes in human breast cancer reduces metastasis by targeting SOX4 [34] .On top of that, Dysregulation of the SOX4 associates with the end result of colon cancer [35].

Besides, our outcomes revealed that the level of E2F5 was dramatically higher than the control group. E2F5 is a necessary participant of cell development and proliferation by managing the genes associated with cell cycle development [36, 37]. On top of that, it has been revealed that some miRNA can hinder cancer growth using repressing E2F5 [37]. Additionally, one examination disclosed that knockdown of

E2F5 causes cell death by means of the TP53-dependent path in breast cancer cells bring wild-type TP53 [38].

Additionally, the outcomes revealed that in cancer patients, the BCL-2 expression was higher than in the control group. Bcl-2-family proteins control all substantial sorts of cell death, consisting of apoptosis, necrosis, and autophagy, hence running as nodal factors at the merging of several paths with wide importance to oncology [39]. Additionally, treatments targeting Bcl-2-family are presently in clinical screening, increasing hopes that a new class of anticancer drugs might quickly be readily available.

To conclude, our data revealed that the expression profile of miRNA-34a, miRNA-143, and miRNA-212 can work as a prospective biomarker for detecting ovarian cancer patients. Furthermore, miRNA-34a, miRNA-143 circulating levels dramatically reduced in patients and can act as a tumor suppressor, yet extra examination is required to additional reveal various targets to light on their function in the pathogenesis of ovarian cancer.

Statements & Declarations

Ethics approval

This study has been ethically approved by the Ethics Committee of Yazd University of Medical Sciences with codes number of IR.RSI.REC.1394.11.

Consent to participate

Informed consent was obtained from all individual participants included in the study

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HRJ, Sk, and ZZ did study design and manuscript drafting. SK and MY performed statistical analyses of data. MHS, SMK, and MHSF performed data collection and extraction. SSH and NG helped with the study design, the interpretation of data. HRJ and NG critically revised the manuscript. All authors approved the final version of the manuscript and therefore agreed to be accountable for all aspects of the work.

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Figures

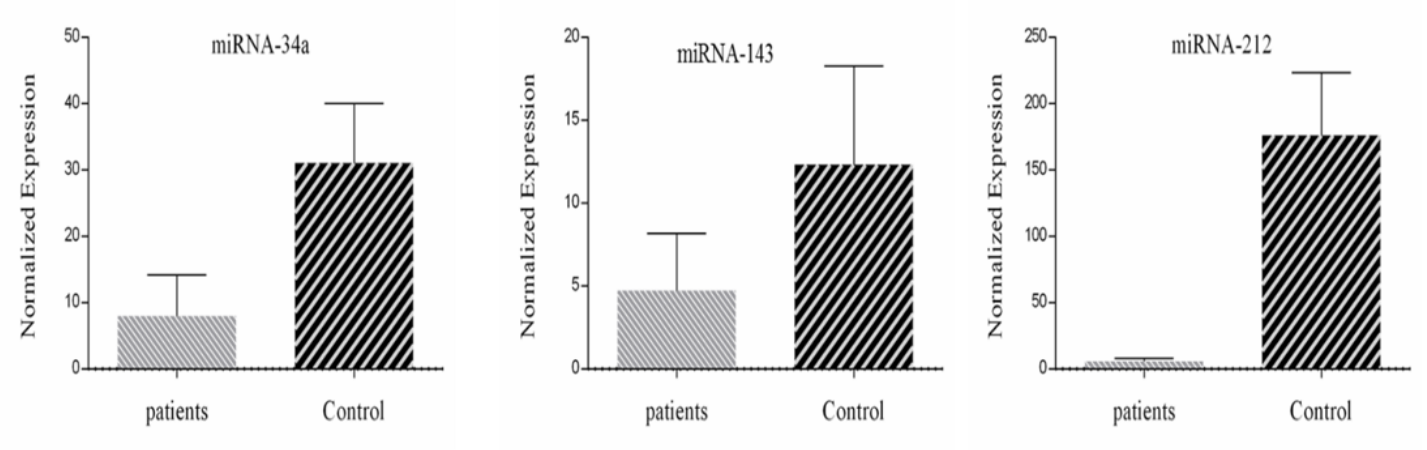

Figure 1

Expression level of miRNA-34a, miRNA-143 and miRNA-212 in the serum of patients with ovarian cancer (n=30) and healthy subjects (n=30). Expression levels of miRNA-34a (p<0.0001), miRNA-143 (p=0.028) and miRNA-212 (p<0.0001) were significantly down-regulated in ovarian cancer patients. miRNA-103 was used as an internal control for normalization. Error bars indicate means ± standard errors of the mean.

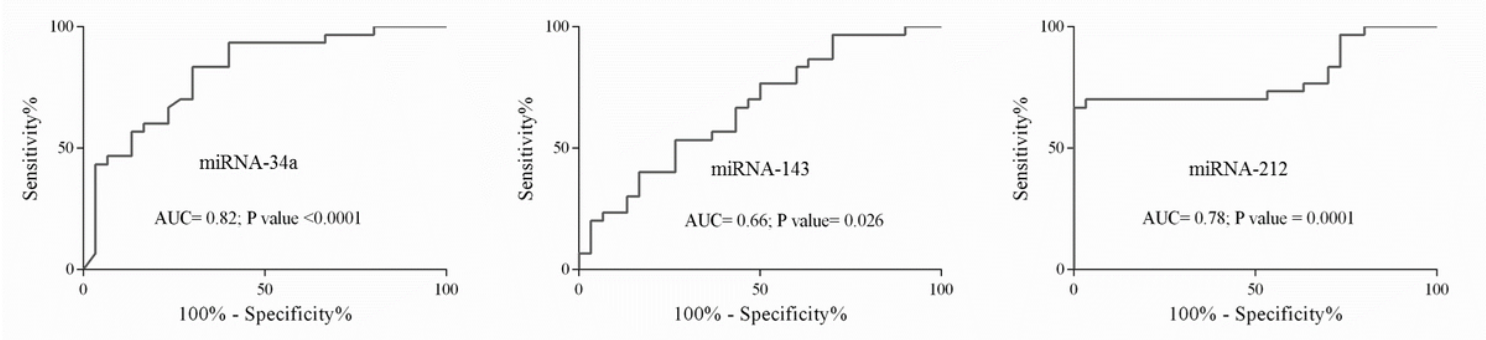

Figure 2

Result of ROC curve analysis for miRNA-34a, miRNA-143 and miRNA-212 expression as a potential biomarker

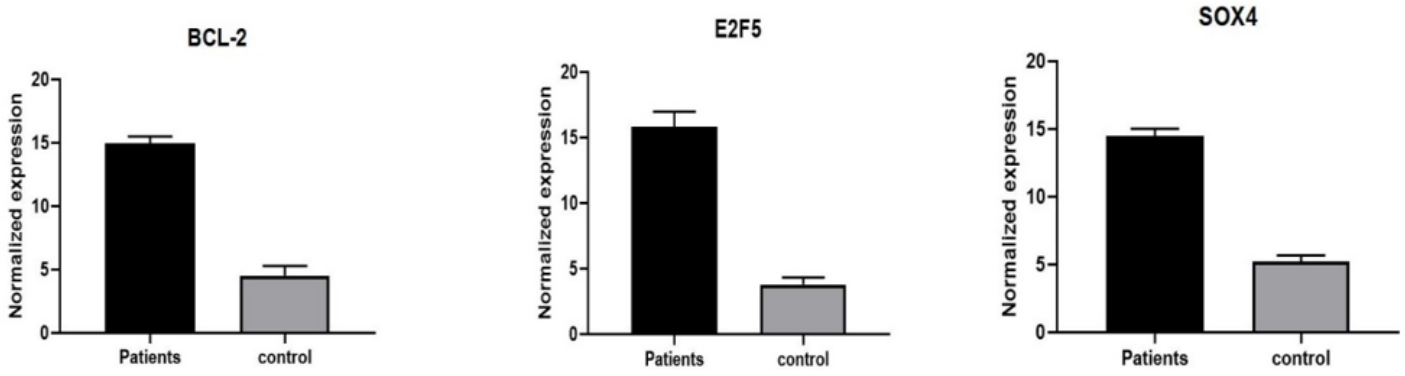

Figure 3

Expression level of Sox4, BCL-2 and E2f5 in the serum of patients with ovarian cancer (n=30) and healthy subjects (n=30). Expression levels of Sox4 ($p<0.0001$), BCL-2 ($p<0.0001$) and E2f5 ($p<0.0001$) were significantly up-regulated in ovarian cancer patients.