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

Shahnaz Kheirandish
Shahid Sadoughi University of Medical Science
Zeynab Zarezade
Shahid Sadoughi University of Medical Science
Mahdie Yavari
University of Isfahan
Mohammad Hasan Sheikhha
Shahid Sadoughi University of Medical Science
Seyyed Mahdi Kalantar
Yazd Medical Sciences
Mohammad Hossein Sahami-Fard
Shahid Sadoughi University of Medical Science
Shahrzad Sheikh Hasani
Tehran University of Medical Sciences
Nasrin Ghasemi
Shahid Saoughi University of Medical Science
Hamid Reza Jahantigh (hamidreza.jahantigh@uniba.it)
University of Bari

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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 common types of cancer among females worldwide (Lengyel 2010, Jayson, Kohn et al. 2014). The incidence of the disease has been estimated at 9·4 per 100,000 in developed countries and 3.9 per 100,000 in the Iranian population (Sharifian, Pourhoseingholi et al. 2015, Rezaianzadeh, Mokhtari et al. 2018). Early diagnosis of ovarian cancer because lack of specific

symptoms and reliable screening methods is still challenging (Lheureux, Gourley et al. 2019, Stewart, Ralyea et al. 2019).

MicroRNAs (miRNAs) are small, 19–25 nucleotide non-coding RNA molecules that negatively regulate gene expression by translation inhibition or targeting the 3'UTR (Aghaei Zarch, Vahidi Mehrjardi et al. 2019, Aghaei, Khodadadian et al. 2020, Babakhanzadeh, Khodadadian et al. 2020, Babakhanzadeh, Khodadadian et al. 2020, Dehghani, Zarch et al. 2020, Khodadadian, Darzi et al. 2020, Babakhanzadeh, Danaei et al. 2021). Several studies have demonstrated that miRNA expression profiles could be used as prognostic biomarkers in different types of cancer; also, serum and/or plasma are relatively easy to access to measuring circulating miRNAs(Kai, Dittmar et al. 2018, Zhang, Pian et al. 2018, Sohel 2020). Therefore, identifying a unique serum miRNA expression profile could help early diagnose and treat patients with ovarian cancer.

Low transcript levels of miRNA-34a have been associated with tumor progression in ovarian cancer, and reduced miRNA-34a expression in a variety of cell lines and mouse models have been reported. It has been shown that the downregulation of miRNA-34a has been associated with many proteins such as BCL-2 that are involved in the cell cycle and cell survival pathways (Cao, Fan et al. 2013, Liu, Jiang et al. 2014). In addition, some investigations showed that miRNA-143 and miRNA-212 deregulated in many types of cancer. In vitro studies have demonstrated that miRNA-143 targets 3'-UTR of BCL-2 mRNA and causes BCL-2 protein down-regulation. These findings suggest that miR-143 acts as a tumor suppressor and could potentially target cancer therapy. In addition, miRNA-143 has been found to directly interfere with multiple mRNAs such as KRAS, ELK1, MYO6, ERK5, and hexokinase 2 (HK2), which are involved in the pathogenesis of cancers (Zhai, Sun et al. 2017, Xu, Liu et al. 2018). Although increased miRNA-212, by its targeting SRY-box 4 (SOX4) expression, has been reported in colorectal carcinoma, its down-regulation has been associated with many types of cancer. SOX4 usually correlates with cell apoptosis and tumor angiogenesis (Wang, Zhang et al. 2013, Wang, Li et al. 2016). 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 measured in the serum of patients with ovarian cancer. This study investigates the expression levels of circulating miRNA-34a, miRNA-143, miRNA-212, and their target, including Sox4, BCL-2, and E2f5, in the serum of women with ovarian cancer to find new mRNA marker for early diagnosis of ovarian cancer.

Materials And Methods

Samples: After **s**igned consent was obtained from patients undergoing surgery for ovarian cancer at Shahid Sadoughi Hospital (Yazd, Iran), Sample collection was done in patient with histopathological diagnosis of ovarian cancer, and those who received treatment (i.e., chemotherapy and radiotherapy) were excluded from this study. Control subjects were healthy women admitted to the hospital for check-ups, and none of them had been diagnosed with a malignancy nor had any viral infection. Five milliliters of whole blood were collected from each patient (n = 30) and control (n = 30), and serum was immediately separated and stored at -80 ◦C.

Rna Extraction And Cdna Synthesis

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

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 performed by Rotor-Gene Q (Qiagen, Hilden, Germany). The expression levels of selected miRNAs were evaluated using Forward specific primers for each miRNA and universal reverse primer that is complementary 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 used in this study are listed in Table 1. The thermal reaction condition was as follows: initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 62 °C for 30 sec, and extension at 72 °C for 30 sec. Real-time PCR was carried out in final reaction volume of 20 µl. Melting curves were plotted at the end of each cycle series to verify the purity of the products The expression levels of miRNA-34a, miRNA-143, and miRNA-212 were normalized to the miRNA-103a as an internal control (β-actin was used as the reference gene and sox4 ,*BCL-2 and e2f5* as the target genes), and ΔCts were calculated by the difference between Ct of the target miRNAs and Ct of miRNA-130a. Relative expression was calculated by $2^{-\Delta\Delta Ct}$ formula.

Statistical analysis

Statistical tests were carried out using SPSS (SPSS, Chicago, IL, USA) and Graphpad Prism version 6.0 (Graphpad Prism Software, Inc., San Diego, CA). Shapiro–Wilk test was performed for analyzing normal distribution of data, and student t-test or Mann-Whitney U-test was used to analyze relative expression of each miRNA between patients and healthy controls. Data were expressed as the mean ± standard error of the mean (SEM), and P-value lower than 0.05 ($P < 0.05$) was considered statistically significant.

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 indicates that the dysregulation of miRNAs might play an essential role in the pathogenesis of ovarian cancer. Besides, circulating miRNAs in serum have emerged as potential biomarkers for detecting various diseases, including cancer. The study results revealed that the expression of miRNA-34a, miRNA-143, miRNA-212 significantly altered in serum of patients compared to healthy subjects.

The results of our study showed that the expression level of circulating miRNA-34a decreased in the patient compared to the control group. Besides, the result of our investigation showed that miRNA-34a could serve as a promising biomarker for the diagnosis of ovarian cancer. MiR34 family members are well known to manage the cell cycle, apoptosis, and invasiveness in cancer (Gou, Dai et al. 2014, Li, Liu et al. 2014)(Li et al., 2014). These miRNAs are verified to have a straight duty in the growth of breast, prostate, bladder, or brain cancer, and miR34a is taken into consideration to be an appealing therapeutic target in cancer therapy (Gou, Dai et al. 2014, Li, Liu et al. 2014)(Li et al., 2014). Besides, the study by Jin et al. has shown that decreased expression of miRNA-34a is associated with a poor prognosis of gallbladder cancer. They indicated that miRNA-34 acts as a tumour suppressor, and overexpression of it resulted in reduced xenograft tumours (Jin, Xiang et al. 2014). In addition, a Raised level of circulating miR34a and miR34b was reported in breast, lung, and prostate cancer (Lodes, Caraballo et al. 2009, Roth, Rack et al. 2010)(Lodes et al., 2009; Roth et al., 2010, 2011). However, restricted info is offered regarding circulating miR34 family members in ovarian cancer, and more in-depth investigation is needed to clarify the exact role of mir34 in ovarian cancer.

Also, the results of our study showed that the level of expression miRNA-143 in patients significantly decreased. Also, Previous reports showed that transfection of HeLa cells with pre-miRNA-143 could significantly decrease their proliferation and increase apoptosis. Also, they found that expression of miRNA-143 was decreased in cancerous cervical tissues compared to non-tumour tissues(Liu, Yu et al. 2012) [34]. Wang et al. also showed that the expression of miRNA-143 was down-regulated in colon cancer but not in rectal cancer(Wang, Zhou et al. 2009) [35]. So that miRNA-143 might be acting as a tumour suppressor, but more investigation is needed to clarify the role of this miRNA in the inhibition of ovarian cancer. In addition, our results showed that it could be an excellent marker for the early diagnosis of ovarian cancer along with miRNA-34a, but to our knowledge, it is the first time to analysis in ovarian cancer and needed more investigations.

Along with miRNA-34a and miRNA-143, we analysed the level of miRNA-212 in ovarian patients and healthy controls. The results showed that along with other miRNAs, it also could predict ovarian cancer. Besides, the literature review showed that the miRNA-212 in different malignancies could change differently. For instance, Incoronato et al. revealed that the miRNA-212 expression decreased in vivo and in vitro in lung cancer. Also, Wei et al. found that the expression of miRNA-212 was significantly down-regulated in both tissue and serum of epithelial ovarian cancer patients(Kenny and Bali 2013). Their results have revealed that overexpression of miRNA-212 in ovarian cancer cells could inhibit cell proliferation, migration, and invasion. In our study, the miRNA-212 level significantly decreased, and it might show possibilities that miRNA-212 can act as a tumour suppressor and a promising biomarker for the detection of ovarian cancer. In addition, the decreasing of miRNA-212 was along with increased expression of SOX4.

The previous investigation showed that miRNA-212 acts as a tumour suppressor in colorectal carcinoma by targeting SOX4(Mou, Zhang et al. 2019) [23]. SOX4 is a vital developmental transcription factor in invertebrates and is essential for accurate differentiation and proliferation in various tissues.

Furthermore, SOX4 is overexpressed in many human malignancies; however, the exact role of SOX4 in cancer progression is not well understood. Besides, SOX4 has been reported to be overexpressed in renal cell carcinoma, which promoted cell migration and invasion inducing EMT. In addition, SOX4 has been discovered to participate in metastasis and EMT in renal cell carcinoma, lung adenocarcinoma, and non-small cell lung cancer(Ruan, Yang et al. 2017, Tong, Meng et al. 2017) [29, 45]. Another experiment revealed that the Aryl hydrocarbon receptor-microRNA-212/132 axis in human breast cancer suppresses metastasis by targeting SOX4(Hanieh 2015). In addition, Dysregulation of the SOX4 correlates with the outcome of colorectal cancer (Andersen, Christensen et al. 2009).

Besides, our results showed that the level of E2F5 was significantly higher than the control group. E2F5 is an essential member of cell growth and proliferation by regulating the genes involved in cell cycle progression(Ren, Cam et al. 2002, Chen, Tsai et al. 2009) [27, 37]. In addition, it has been shown that some miRNA can inhibit cancer development via repressing E2F5 (Ren, Cam et al. 2002)[40]. Also, one investigation revealed that knockdown of E2F5 induces cell death via the TP53-dependent pathway in breast cancer cells carrying wild-type TP53 (Inagaki, Wu et al. 2020)[41].

Also, the results showed that in cancer patients, the BCL-2 expression was higher than in the control group. Bcl-2-family proteins regulate all significant types of cell death, including apoptosis, necrosis, and autophagy, thus operating as nodal points at the convergence of multiple pathways with broad relevance to oncology(Chipuk, Moldoveanu et al. 2010) [50]. Also, therapies targeting Bcl-2-family are currently in clinical testing, raising hopes that a new class of anticancer drugs may soon be available.

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. In addition, miRNA-34a, miRNA-143 circulating levels significantly decreased in patients and could serve as a tumour suppressor, but more investigation is needed to further show different targets to light on their role in the pathogenesis of ovarian cancer.

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

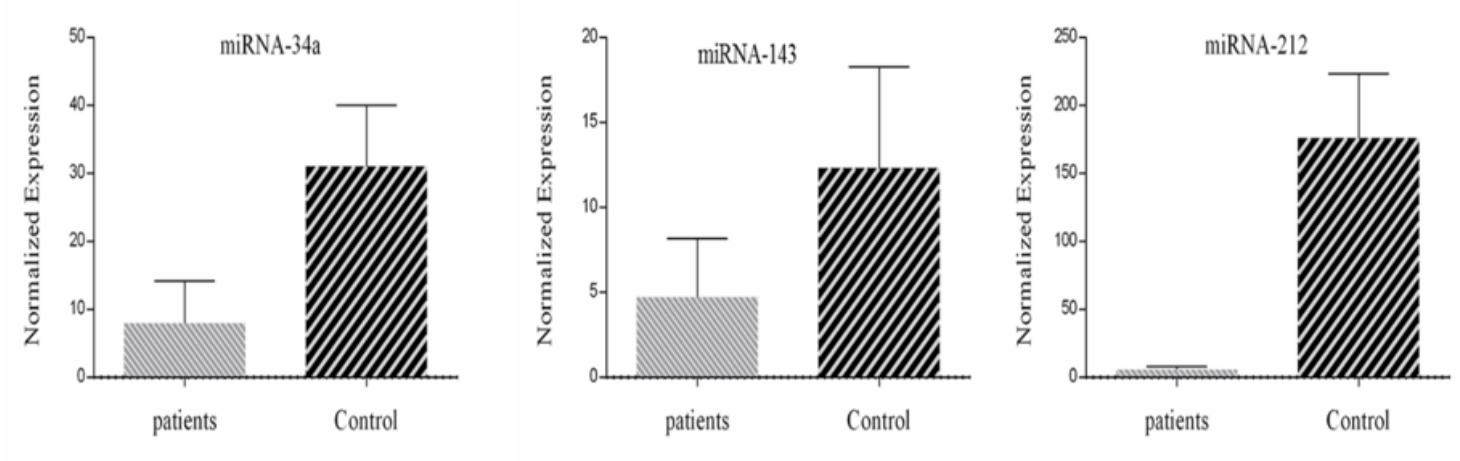

Figure 1

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.

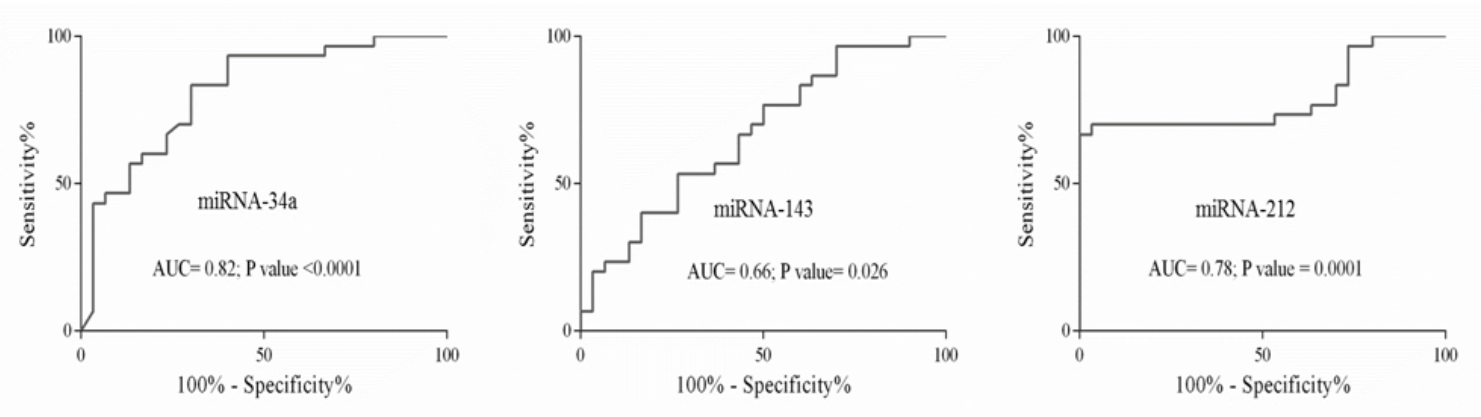

Figure 2

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

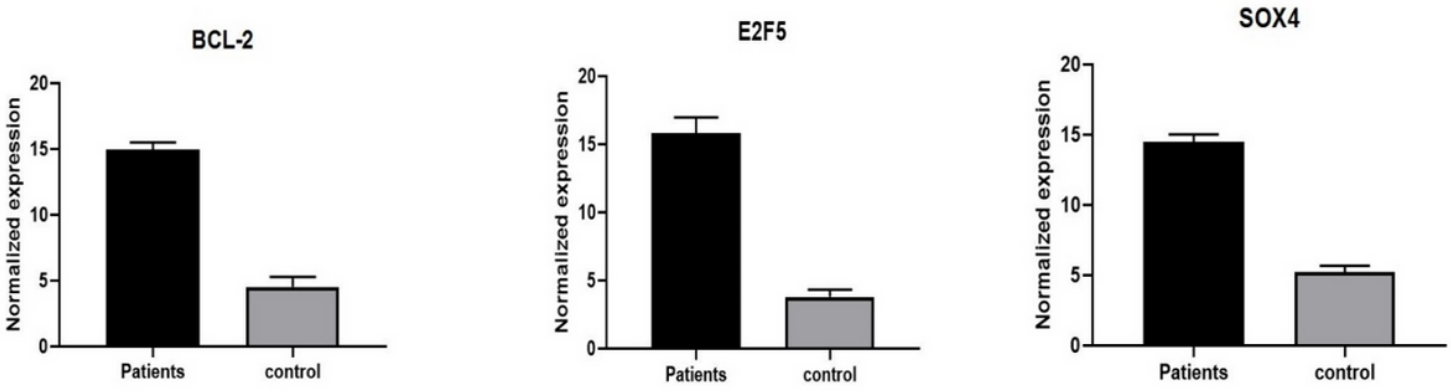

Figure 3

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