The Relationship of *FOXR2* Gene Expression Profile with Epithelial-Mesenchymal Transition Related Markers in Epithelial Ovarian Cancer

Vztah profilu exprese genu *FOXR2* a markerů epitelo-mezenchymální tranzice u epiteliálního karcinomu vaječníků

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Summary

Background: Several factors have been evaluated for their competency as applied biomarkers regarding diagnosis and therapy of ovarian cancer as one of the most cause of death due to the gynecologic malignancies. However, some Fox-factors have been shown to modulate cancer progression primarily by their impacts on the proliferation of the cells, the expression and potential function of *FOXR2* (Forkhead Box R2), newly identified as a probable oncogene in a few human cancers, remains undecided in ovarian cancer. The aim of this study was to evaluate the *FOXR2* and some epithelial-mesenchymal transition (EMT)-related gene expression profiles in epithelial ovarian cancer (EOC) tissues and their healthy samples as well as an ovarian cancer cell line (SKOV-3). ***Methods:*** In this observational study, 20 epithelial ovarian adenocarcinoma and their marginal samples, obtained from 20 women with EOC, as well as SKOV-3, were investigated for the relative gene expression levels of *FOXR2*, *CDH1* (encoding E-cadherin) and *FN1* (encoding fibronectin-1) in 2 groups using qualitative real-time polymerase chain reaction technique (qRT-PCR). ***Results:*** The findings demonstrated a significant up-regulation of *FOXR2* and *FN1* despite the *CDH1* down-regulation in case samples compared to controls ($P < 0.05$). There was a significant correlation between *FOXR2* gene expression profile and EMT-related markers in high-grade tumors. Furthermore, the biomarker index of 0.772 was obtained for *FOXR2* gene expression levels. ***Conclusions:*** The findings indicated that the expression levels of *FOXR2* have a significant association with ovarian cancer as far as it can be used as a diagnostic and therapeutic molecular biomarker in ovarian cancer.

Key words

ovarian cancer – *FOXR2* – gene expression – epithelial-mesenchymal transition

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Souhrn

Východiska: Bylo hodnoceno několik faktorů kvůli jejich využití jako aplikovaných biomarkerů pro diagnostiku a léčbu karcinomu vaječníků, jednoho z nejčastějších příčin úmrtí v důsledku gynekologických malignit. U některých faktorů skupiny FOX se sice ukázalo, že modulují progresi karcinomu primárně ovlivněním proliferace buněk a exprese, ale potenciální funkce *FOXR2* (Forkhead Box R2), který je u několika lidských nádorů nově identifikovaným jako pravděpodobný onkogen, nebyla u karcinomu vaječníků zatím potvrzena. Cílem této studie bylo zhodnotit profil exprese genu *FOXR2* a profily exprese některých genů souvisejících s epiteliálně-mezenchymálním přechodem (EMT) v tkáních epiteliálního karcinomu vaječníků (EOC), ve vzorcích zdravé tkáně a v linii buněk karcinomu vaječníků (SKOV-3). ***Metody:*** V této pozorovací studii bylo vyšetřeno 20 vzorků epiteliálního adenokarcinomu vaječníků a jejich marginální vzorky, získané od 20 žen s EOC a SKOV-3 pomocí relativní genové exprese *FOXR2*, *CDH1* (kódující E-kadherin) a *FN1* (kódující fibronektin-1) ve dvou skupinách za použití kvalitativní techniky polymerázové řetězové reakce v reálném čase (qRT-PCR). ***Výsledky:*** Nálezy svědčily o významném zvýšení *FOXR2* a *FN1* i přes sníženou hladinu exprese *CDH1* ve vzorcích v porovnání s kontrolami ($p < 0,05$). U pokročilých karcinomů byla zaznamenána významná korelace mezi expresí genu *FOXR2* a markery souvisejícími s EMT. Pro úrovně exprese genu *FOXR2* byl dále získán index biomarkerů 0,772. ***Závěry:*** Zjištění ukázala, že hladina exprese *FOXR2* má významnou souvislost s karcinomem vaječníků do té míry, že při tomto onemocnění může být použita jako diagnostický a terapeutický molekulární biomarker.

Klíčová slova

karcinom vaječníků – *FOXR2* – exprese genů – epiteliálně-mezenchymální tranzice

Introduction

Epithelial ovarian cancer (EOC) is 5th and 7th major cause of deaths among females in the United States and worldwide, respectively [1,2]. There are many clinical complications about this cancer since almost all of the patients are diagnosed late, usually at stages III and IV. As a result, EOC is the most lethal cancer among all gynecological malignancies and the five-year survival rate is < 5% in these patients [3,4]. Therefore, despite vigorous attempts to upgrade the diagnosis and treatment, most women affected by EOC eventually pass away from their cancer. The disease is more prevalent with increasing of women's age and most frequently diagnosed women are 55–65 years old [5,6]. For each woman, the risk of ovarian cancer in her lifetime is estimated 1 in 78 and the probability of death from this disease is about 1 in 108 [6]. The EOC symptoms comprise frequent urination, difficulty in eating, abdominal mass, tiredness and pelvic pain [7]. The most usual treatments for this disease are surgery, chemotherapy and radiotherapy. The most functional chemotherapy agents as the standard treatment for patients with EOC are the platinum-based drugs, namely cisplatin and carboplatin. These drugs are commonly applied in combination with another cytotoxic agent in order to improve the disease [8]. The relapse and development of resistance to first-line chemotherapy routinely occurs among the majority of patients with EOC [9]. Hence, new strategies are required to identify novel prognostic and therapeutic markers for improving patients' long-term survival rate.

The epithelial to mesenchymal transition (EMT) is a key approach in embryogenesis that polarized epithelial cells lose their intercellular adhesion, then transformed to mesenchymal phenotype and gain migratory and invasive characteristics [10]. This process is identified by changes in cell markers like reducing in epithelial markers and increasing in mesenchymal markers [11]. EMT is crucial for numerous developmental processes, and accumulating findings suggest that EMT participates in the initiation of cancer metastasis [12].

The Forkhead box (Fox) proteins organize an extensive family of transcriptional modulators which are characterized by an evolutionary conserved „fork-head" DNA-binding domain [13,14]. The Fox superfamily consists of multifunctional transcription factors, responsible for regulation of a broad range of transcriptional events, which are involved in various cellular events such as cell cycle progression, proliferation, differentiation, metabolism, aging, vitality and apoptosis. In addition to serving as transcription activators, Fox also act as leading factors with the capability of unwinding the compacted chromatin and make it accessible to other factors to bind [15]. Given the great importance of Fox proteins, it is noticeable that the remodelings of their transcriptional regulation and function will result in pathological conditions including cancer. One of the key members of these subfamilies, *FOXR2*, has been discussed here with an emphasis on its carcinogenesis role.

FOXR2 as one of the Fox family members, also known as *FOXN6*, was located on the human chromosome Xp11.21 within genome sequence RP11-167P23 [13]. FOXR protein contains the forkhead binding domain at its C-terminal that followed by a common domain with FOXN5 in N-terminal that is named FN56 [16]. *FOXR2* has been found to be upregulated in breast cancer and is associated with its poor prognosis [17]. Consequently, *FOXR2* has been reported to be overexpressed in prostate and colorectal cell lines and tissues; it was shown that its knockdown by siRNA could suppress cell migration and invasion [18,19]. However, the role of *FOXR2* gene remains undecided in EOC until now. The main goal of the current prospective study was to characterize the expression profile of *FOXR2* in EOC tissue samples as well as SKOV-3 and to investigate if *FOXR2* expression levels were correlated with EMT indicators.

Materials and methods

Patients and tissue specimens

Human EOC tissues and their matching adjacent noncancerous tissues as the control samples were collected from 20 ovarian cancer patients at Shahid Sadoughi Hospital, University of

Medical Sciences, Yazd. Not all patients have received any treatment or chemotherapy before tissue sample biopsies for this study. At first, each sample was evaluated by a pathologist to ensure that it contained more than 70 % tumor cells.

The main features of the tumor from patients who participated in the present study including tumor histology, stage and the grades of the tumor as well as the presence of metastasis were evaluated according to the International Federation of Gynecology and Obstetrics global standard [19]. Furthermore, some normal ovarian samples were provided from 3 women who did not have any ovarian disease or endometriosis and were referred to Shahid Sadoughi Hospital for ovarian cyst surgery. The use of all samples was approved by the legal standards in the Ethics Committee of Shahid Sadoughi Hospital. All participants signed the informed consent for this research.

Cell culture

The SKOV-3 cell line (NCBI code: C209, ATCC code: HTB-77) was obtained from the National Cell Bank of Iran (NCBI), Pasteur Institute, and was cultured in RPMI-1640 (Gibco, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS: Gibco, Invitrogen, USA) and 100 IU/ml penicillin-streptomycin (Invitrogen). The cell line was kept at 37 °C in a humidified incubator with 5% CO_2. The culture medium was replaced every 3 days after initial cell culturing and based on cell density. Since reaching 90–95% confluence, the cells were split as a routine passage at the ratio of 1/3. All data represented a duplicate from 2 experiments.

Quantitative real-time PCR

The relative expression of *FOXR2* gene was assessed by real-time polymerase chain reaction technique (RT-PCR). Total RNAs were extracted from the tissue samples and the cell line by using TRIzol reagent (Invitrogen, USA) considering the manufacturer's instructions. After achieving RNA concentration and quality, the measurements of its purity and integrity with a nanodrop spectrophotometer were assessed using agarose gel electrophoresis. The RNAs extracted from 3 normal ovarian samples were pooled together and assessed for the purity and integrity as a unit. Then, the complementary DNA (cDNA) was synthesized in a reverse transcriptase reaction with the Revert Aid cDNA Synthesis kit (Thermo Scientific, USA) according to the manufacturer's recommendations. We used 1µl of total RNA (5 µg) to generate first-stand cDNA as the initial step of RT-PCR protocol. Using specified primers (Tab. 1), relative expressions of target genes (*FOXR2*, *CDH1* and *FN1*) were evaluated by quantitative real-time polymerase chain technique (qRT-PCR). The gene for glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* was applied as a reference gene. Consequently, the qRT-PCR run was carried out by using the QuantiTect SYRB Green dye (TaKaRa, Japan) and Applied Biosystems™ StepOne™ Real-Time PCR System (ABI, Applied Biosystems, USA). All samples were run with the PCR conditions of an initial denaturation step at 95 °C for 2 min, and then 40 cycles at 95 °C for 5 s and 58 °C for 35 s. All reactions were run in duplicates. The relative mRNA expression levels were measured using the comparative CT method $2^{-(\Delta\Delta CT)}$.

Tab. 1. Oligonucleotid primer sequences.

Product size (bp)	Sequence amplified	Primer sequence (5'-3')	Gene
111	NM_198451.3	F:5'-TACAATTTCACCCGACAGCA-3'	*FOXR2*
		R:5'-GTCTGGCACCTTCTCAAAGC-3'	
158	NM_004360	F:5'-TTAGAGGTCAGCGTGTGTG-3'	*CDH1*
		R:5'-CTCCGAAGAAACAGCAAGAG-3'	
169	NM_212482	F:5'-ACAATGTGGGTCCCTCTGTC-3'	*FN1*
		R:5'-ACCTCGGTGTTGTAAGGTGG-3'	
102	NM_002046	F:5'-AGGTGAAGGTCGGAGTCAACG-3'	*GAPDH*
		R:5'-AGGGGTCATTGATGGCAACA-3'	

Statistical analysis

All data were analyzed using the SPSS standard software (Version 23.0, IBM, Armonk, NY, USA) and the results were reported as charts and graphical representation by Excel 2016 software. Based on the one-sample Kolmogorov-Smirnov test, our data did not have a normal distribution. Therefore, the comparisons between the two groups were performed by non-parametric tests. The independent sample t-test, Kruskal-Wallis test and Mann-Whitney test were used to examine the relationship between the quantitative variables and other clinical and pathological parameters. The quantitative data were expressed as the means ± standard deviation (SD). Moreover, a receiver-operating characteristic (ROC) curve was performed as a fundamental tool for evaluating a diagnostic performance and accuracy of the test to discriminate between the cases and controls. Indeed, the biomarker index of *FOXR2* expression was assessed by the ROC curve in MedCalc Statistical Software. The P-values < 0.05 were considered as statistically significant for all data.

Results

Clinical and histopathological features

In this study, 20 patients with ovarian cancer with a mean age of 45.25 ± 2.56, ranged 19–64 years, were included. The general characteristics of the ovarian adenocarcinoma patients were presented in Tab. 2.

Assessment of RNAs quality

Fig. 1 showed both 18S and 28S rRNA appeared as typical bands after electrophoresis of total RNA, represented to be intact. The 28S rRNA band was of more

Tab. 2. Characteristics of pataints with epithelial ovarian cancer.

Variables	Frequency (%)
Age (year)	
Mean	45.25 ± 2.56
< 40	4 (20)
40–49	8 (40)
50–59	7 (35)
> 60	1 (5)
Tumor Differentiation	
Grade 1	8 (40)
Grade 2	6 (30)
Grade 3	6 (30)
Histology	
Mucinous	3 (15)
Serous	13 (65)
Teratoma	2 (10)
Endometrioid	2 (10)
Metastasis	
Positive	11 (55)
Negative	9 (45)

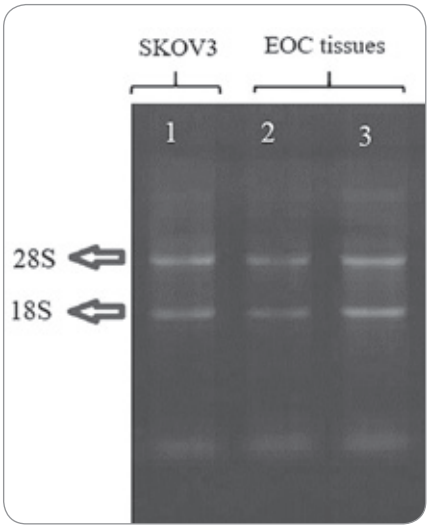

Fig. 1. Denaturing gel electrophoresis stained with ethidium bromide showing total RNA extracted from cell line (SKOV-3) and ovarian tissue samples of patients with EOC.
Total RNA (2 µl) was separated on agarose (1% w/v) gel.

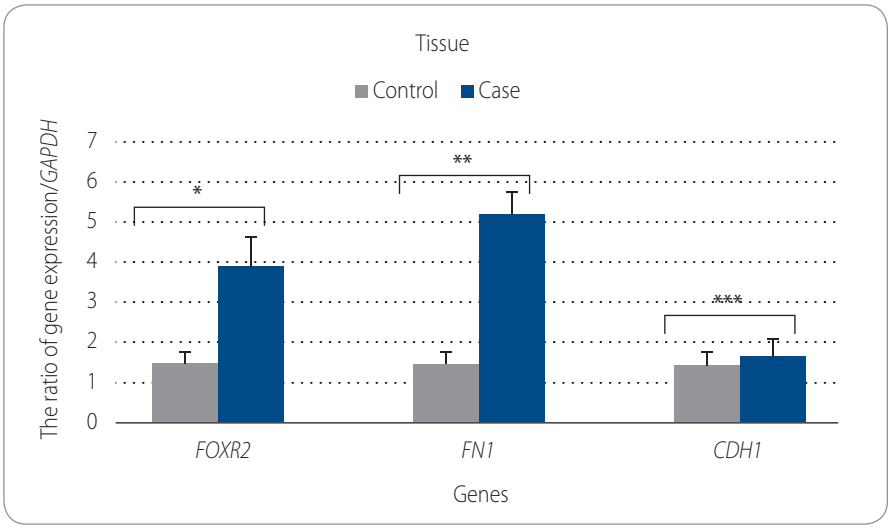

Fig. 2A. Comparison of expression of target genes to *GAPDH* in epithelial ovarian tissues (EOC) obtained from EOC patients and SKOV-3 cell line by Mann-Whitney test. The ratio of genes expression of *FOXR2*, *FN1*, *CDH1* and *GAPDH*.
There were significant differences between tumor (case) and control groups regarding mRNA levels of *FOXR2* (*P = 0.015), *FN1* (**P = 0.003) and *CDH1* (***P = 0.025).

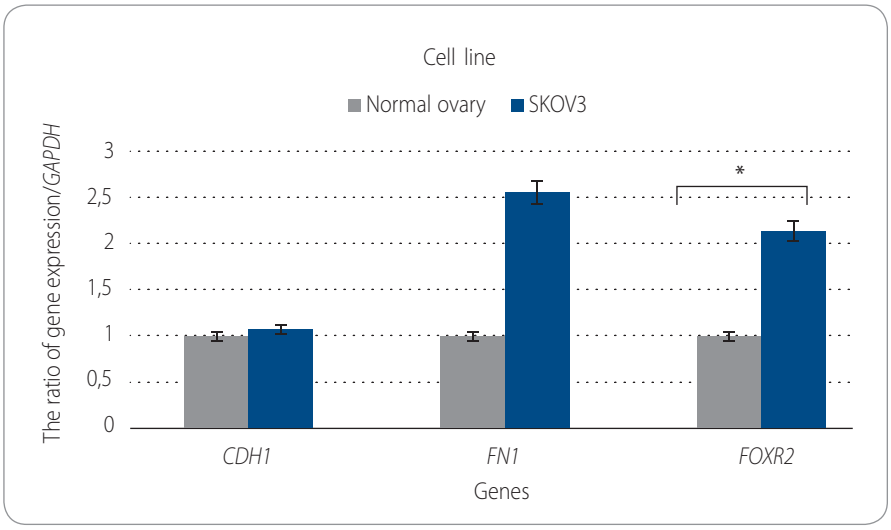

Fig. 2B. Comparison of expression of target genes to *GAPDH* in epithelial ovarian tissues (EOC) obtained from EOC patients and SKOV-3 cell line by Mann-Whitney test. The ratio of genes expression of *FOXR2*, *FN1*, *CDH1* and *GAPDH*.
The mRNA expression levels of *FOXR2* was remarkably upregulated in SKOV-3 cell lines (P = 0.035) when compared to the normal ovarian pool tissues. However, there were no significant higher *FN1* and *CDH1* expression levels between SKOV-3 cell lines and normal controls (P = 0.09 and P = 0.3). P < 0.05 was considered as a significant value.

intensity than the 18S rRNA. The 28S rRNA band was approximately twice as intense as the 18S rRNA. Also, the results from a nanodrop spectrophotometer showed that OD260/280 was 1.8–2 for all samples and the average of tissues RNA density was about 1,000 ng/µl (ranged 655–1,400 ng/µl) and it was 1,178 ng/µl for RNA extracted from the cell line.

Expression of *FOXR2* and EMT markers in EOC tissues and cell line

As we conducted RT-PCR to evaluate the relative expression levels of *FOXR2* in case and control groups, there was a significant up-regulation (about 2-fold) in *FOXR2* expression in EOC cases compared to non-cancerous controls (P = 0.015) (Fig. 2A). It was also found that the remarkable over-

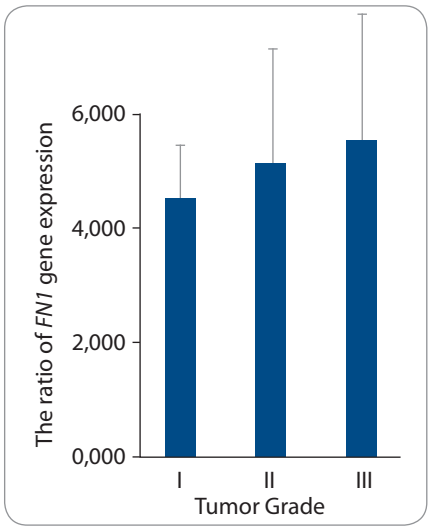

Fig. 3A. Gene expression levels of *FOXR2* in patients with epithelial ovarian cancer based on tumor grades.
The rate of *FN1* expression was no significantly increased among all tumors grades (P = 0.8).

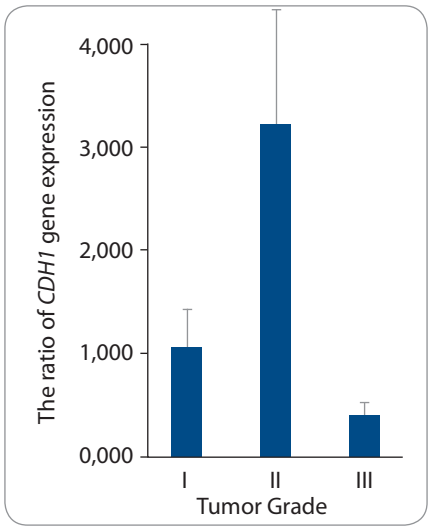

Fig. 3B. Gene expression levels of *FOXR2* in patients with epithelial ovarian cancer based on tumor grades.
There was almost no gene expression regarding *CDH1* in high grade (III) compared to low (I) and moderate grade (II) tumors (P = 0.05).

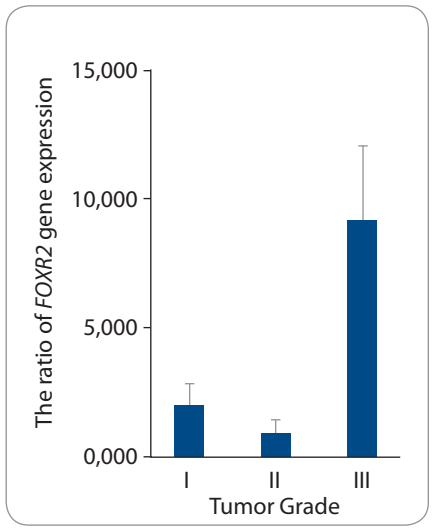

Fig. 3C. Gene expression levels of *FOXR2* in patients with epithelial ovarian cancer based on tumor grades.
There was a positive relationship between *FOXR2* mRNA levels and tumor grades. A progressive increase was seen in *FOXR2* mRNA levels and high tumor grade (III) based on Kruskal-Wallis test despite the insignificancy between the three tumor grades (P = 0.5). P < 0.05 was considered as a significant value.

expression levels of *FOXR2* in SKOV-3 cell line and 3 normal ovarian tissue samples pool (P = 0.035), but it was not significant statistically for *FN1* (P = 0.09) and *CDH1* (P = 0.3) genes (Fig. 2B). To investigate the expression level of *CDH1* and *FN1* genes as EMT-related markers (E-cadherin and fibronectin), no difference was found between case and control groups regarding *CDH1* mRNA levels (P = 0.025). In contrast, the significantly higher expression levels were detected in the *FN1* gene (2.8-fold) (P = 0.003) (Fig. 2A) as well as in SKOV-3 cell line (Fig. 2B).

Association of gene expression and clinical aggressiveness of ovarian cancer

There was a positive relationship between *FOXR2* mRNA levels and tumor grades. Despite insignificancy, the higher *FOXR2* expression levels were associated with a high grade (grade III) of EOC compared to low and moderate grades – I and II) (P = 0.5) (Fig. 3A). We did not observe any significant change in *FOXR2* expression related to the histological type and the metastatic features of tumor samples according to the Kruskal-Wallis test (P = 0.949 and P = 0.656, respectively) (Tab. 3). It was also demonstrated that the mRNA levels of EMT-related markers correlated with clinicopathological parameters so that *FN1* expression increased in high-grade tumors (P = 0.8) despite almost no expression levels of *CDH1* in high-grade tumors, compared to those with a low-grade status (P = 0.05) (Fig. 3B and 3C).

Correlation of *FOXR2* expression and epithelial to mesenchymal transition

In addition, the data demonstrated a significant positive correlation between the rate of *FOXR2* expressions and EMT-related markers (*CDH1* and *FN1*) in EOC (P= 0.038, P = 0.007, respectively). Furthermore, the area under the curve (AUC) ROC demonstrated that the biomarker index for *FOXR2* was about 0.722 with specificity and sensitivity of 80/00 and 55/00, respectively (P = 0.006) (Fig. 4 – available at www.linkos.cz).

Discussion

Ovarian carcinoma is a highly lethal cancer with an average of almost 30% of patients achieving the five-year survival [5]. Most of the women with ovarian cancer appear with late stage with expanding resistance to first-line chemotherapy

Tab. 3. Correlations between FOXR2 expression levels and clinicopathological parameters of epithelial ovarian cancer.

Variable	No. of patients	FOXR2 expression levels	P-value
Metastasis	11	11.17	0.656
No metastasis	9	9.95	
Mucinous	3	9.67	0.949
Serous	13	10.35	
Teratoma	2	12.75	
Endometrioid	2	10.50	

caused by late diagnosis due to the obscure symptoms of ovarian cancer [20]. Therefore, identifying the novel prognostic and therapeutic biomarkers is crucial for improving the long-term survival rate of patients. Recently, the researchers suggested the biomolecular agents with important potentials for diagnosing and treating ovarian cancer.

The FOX gene family has frequently been studied and it has been reported to play a vital regulatory role as transcriptional factors in a broad spectrum of biological processes, especially during the development and tumorigenesis [14]. Furthermore, many of these family members can act as oncogenes in EOC. For example, overexpression of *FOXM1* was reported by Zhao et al. in EOC [21]. Li et al. found that *FOXC2* was significantly upregulated in cisplatin-resistant ovarian cancer tissues or ovarian cancer cell lines [22]. Also, in a new study, Li et al. identified that the upregulation of *FOXR2* gene in ovarian cancer cell lines play a significant role in angiogenesis and malignancy in EOC by Sonic Hedgehog pathway [23].

In this study, the expression profile of *FOXR2* gene in EOC samples was examined and it was compared with normal ovarian tissues for the first time. It was demonstrated that *FOXR2* was overexpressed in EOC tissues/SKOV-3 cell line compared to controls. In addition, the elevated expression of *FOXR2* was found to be significantly associated with a high grade (III) of EOC samples. *FOXR2* is a scarcely studied member of the Fox protein superfamily [24]. Recently, the *FOXR2* overexpression has been declared in various human malignancies including breast, prostate, colorectal and medulloblastoma tumors [17–19,25].

One of the most important processes in metastasis is EMT wherever polarized epithelial cells lose their intercellular adhesion, then transformed to mesenchymal phenotype and gain migratory and invasive characteristics [10]. Fibronectin was overexpressed, too; but E-cadherin had no significant difference between case and control samples. Therefore, it is likely that *FOXR2* overexpression and its interaction with the other important transcription factors (TFs) MYC and MAX contribute to tumorigenesis of the ovary and maybe these stable TFs complexes affect the *CDH1* promoter and inhibit in this gene expression. In previous studies, it was demonstrated that Fox proteins acted as the final effectors in various signaling pathways like Wnt/B-catenin, Shh, TGF-B1 and MAPK, leading to the cross-talks between parallel signaling pathways, so they caused more suitable cell responses to extracellular signals. Overall, the post-translational modifications on Fox proteins determined their target genes [14]. It was revealed that TFs are strongly regulated by the association with other proteins and these protein-protein interaction networks are crucial for their transcriptional activities via on and off chromatin [26]. A recent study showed that *FOXR2* has a potential interaction with MYC and MAX in the cell nucleus. Indeed, *FOXR2* promotes the effect of MYC transcriptional activity in tumor growth [27]. Despite the high expression levels of MYC, a recent study by Song et al. showed that E-cadherin expression was down-regulated in ovarian cancer [28,29]. It was established that the inhibition of *FOXR2* reduced the migratory and the invasions abilities of cancer cells as well as their EMT [18,19].

Also, the samples of EOC patients with high-grade tumors showed no expression of *CDH1* despite the *FN1* with significantly up-regulations in all grades, specifically in grade III. It means that *FOXR2* expression was significantly associated with high grades of ovarian cancer. These results indicated that *FOXR2* inhibited the *CDH1* expression rates and stimulated the cells to migration. Therefore, supplementary studies should be done to elucidate the exact mechanism of action of this gene in tumorigenesis of ovarian cancer.

Conclusions

The data declared that *FOXR2* as an oncogene in EOC has a crucial impact on the EMT pathway, ovarian carcinogenesis and progression. The findings suggest that *FOXR2* can serve as an independent prognostic factor as a new biomarker in early diagnosis of ovarian cancer or may be used as a novel drug agent for gene therapy in EOC patients, and therefore earlier treatments that lead to a reduction in mortality due to EOC.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

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

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