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Downregulation of miR-4443 and miR-5195-3p in ovarian cancer tissue contributes to metastasis and tumorigenesis

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Abstract

Background and purpose Ovarian cancer (OC) is one of the most fatal malignancies in women. High mortality rate may be due to problems with diagnosis in the early stages. The use of new biomarkers for faster diagnosis and selection of more efficient therapies is one of the main concerns in this area. miRNAs are non-coding and conserved molecules that are involved in regulating gene expression throughout different cell processes. Few studies have been conducted on the effects of miR-4443 and miR-5195-3p in cancer. Therefore, to determine the role of these miRNAs in OC, this study was directed to investigate the expression rate in OC tissue samples and its relationship with clinical factors.

Methods Expression levels of miR-4443 and miR-5195 were evaluated in 45 ovarian tumor and 45 ovarian non-tumor tissue samples paraffin embedded using qPCR. Expression was investigated by miRNA-specific primers and then statistical analysis was performed to determine the significance. In the next step, the relationship between clinopathologic factors and miRNA expression was investigated.

Results The results showed that miR-4443 decreased in OC in metastatic and serous OC samples (0.154-fold, $P < 0.0001$). As well as, significant reduction in miR-5195-3p was observed in cancer samples (0.373-fold, $P < 0.0001$) and its reduction was associated with metastasis.

Conclusion As a result, the two studied miRNAs may contribute to suppressing tumor, so that decrease in their expression is associated with increased cell proliferation and invasion. Further investigation can help to suggest these miRNAs as diagnostic biomarkers or therapeutic targets in OC.

Keywords miR-4443 · miR-5195-3p · Ovarian cancer · Metastasis

Introduction

Ovarian cancer (OC) is one of the main causes of death which is due to female malignancies and the fifth leading cause of death among women worldwide [1]. Epithelial OC accounts for over 90% of OCs and is often referred to as "silent killer" due to the lack of early signs and symptoms for the disease. More than 70% of OC patients have been diagnosed in advanced stages of this cancer. In this condition, the 5-year survival rate in humans decreases to less than 30% [2, 3]. High mortality rate from OC can be attributed to the lack of primary diagnostic methods for OC. Although pelvic tests, vaginal ultrasonography, and serum CA125 factors are routine diagnostic methods, these methods are not practical in the early stages of this malignancy and for its early diagnosis [4]. Therefore, it seems that new approaches should be developed for diagnosis of the OC at early stages. At the molecular level, a number of genes and signaling pathways play important roles in the pathogenesis of OC. Many of them may be used as molecular targets for treatment, but an effective treatment that can extend the patient's overall survival has not yet been suggested. Meanwhile, certain non-coding molecules such as microRNA have been identified that contribute effectively to regulating gene expression and OC etiology. As molecular biomarkers, these molecules have been studied in different cancer types, including OC [5].

miRNAs are non-coding RNA sequences with an approximate length of 22 nucleotides that have been conserved

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among a wide range of species. These molecules down-regulate genes by binding to the 3′UTR region of the target genes. miRNAs do not require complete binding to the target sequence, and identify the target sequence by binding about 2–8 nucleotides in the seed region. Therefore, an miRNA can handle hundreds of mRNAs, and several miRNAs can regulate one target [6]. Currently, it is well known that miRNAs can be up-regulated or down-regulated in different cancer types. By increasing the expression of miRNAs, they may act by inhibiting tumor suppressor genes as oncogenes, while decreasing the expression of miRNAs can have a negative effect on oncogenes as tumor suppressants [7]. Various studies have shown that miRNAs may be abnormally expressed in OC. The expression level of many of miRNAs has been detected only in a few cancer types, and the tumor suppressor activity in OC remains unknown. Meanwhile, the miR-4443 and miR-5195-3p are among the molecules whose expression levels and functions have not yet been determined in OC.

The miR-4443 was first identified in 2015 by Xun et al. However, there is little evidence regarding the association between this miRNA and malignant [8]. It has been shown that miR-4443 plays a role in acquiring drug resistance in breast cancer [9], and inhibits cell proliferation and metastasis in colon cancer [10]. Regarding miR-5195-3p in bladder cancer, it has been shown that a decrease in the amount of miRNA is directly correlated with increased proliferation and tumorigenicity [11]. Therefore, the tumor suppressor role of miR-5195-3p in this cancer has been demonstrated. According to current evidence, no study has yet been conducted to investigate OC with respect to these two miRNAs. Therefore, the aim of this study was to evaluate the expression of miR-4443 and miR-5195-3p in OC samples and its association with clinical factors.

Materials and methods

Tissue sections with 30 µm thick from formalin-fixed, paraffin-embedded tissues (FFPE) were provided by the Pathological Laboratory of Al-Zahra Hospital (Isfahan, Iran) in 2017–2018. The samples consisted of 45 ovarian tumors and 45 non-tumor tissues from ovarian. Informed consent was obtained from all available or alive individual participants included in the study. Samples were prepared in accordance with the American Joint Committee on Cancer (AJCC) guidelines. In addition to patients' age, their pathology and clinical information such as tumor size, metastasis, and tumor grade was also provided for each sample.

RNA extraction, cDNA synthesis, and miRNAs expression

RNAs extracted from the paraffin-embedded tissues have many problems due to their various modifications. Especially, there is much less RNA in the solution compared to fresh tissues due to degradation of RNA fragments. Therefore, the steps for extraction were completely accurate and in accordance with the standards of the Kit that included deparaffinization, minimizing the cross links and treatment with DNase. For this purpose, the total RNA was extracted by the MN Kit (Nucleo Spintotal RNA FFPE-Germany) in accordance with the purification Kit instructions. To evaluate the quality and quantity of extracted RNAs used Nanodrop instrument (Nanodrop 2000, Thermo FisherScientific, Wilmington, DE, USA), the RNAs were stored at 70 °C until needed. Synthesis of cDNA was performed on 2 µg of total RNA using MiR-Amp Kit (Pars Genome, Tehran, Iran). In this method, synthesis is performed by adding a poly A tail to small RNAs and using "Syn" primers. The samples were incubated at 37 °C for 10 min to form a poly A tail sample, and then this step was continued with an incubation of 60 min at 43 °C with reverse transcriptase. Finally, to deactivate reverse transcriptase activity, the samples were placed at 85° for 1 min. Real-time PCR was performed using SYBR Green method and Rotor-Gene 6000 machine (Corbett Life Science, Australia). The reaction for internal control (U6) and miRNAs in a volume of 15 µl contained 1.5 µl of a specific miRNA cDNA, 7.5 µl of SYBR Green master mix and 0.5 µl of each the forward and reverse primers. The temperature conditions for miRNA amplification consisted of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 62 °C for 20 s, and 72 °C for 20 s. All reactions were performed in duplicate and then the data were analyzed.

Data analysis

The $2^{-\Delta\Delta Ct}$ method was used to analyze the real-time PCR data. Data were then presented as mean [± standard error of mean (SEM)]. All statistical tests were confirmed by the GraphPad Prism version 7.01 software and the graphs were drawn using this software. The SPSS version 22 was used to conduct data analysis. The *t* test was used to determine the significance of the gene expression in tumor and non-tumor samples, as well as to investigate age groups, size and grade of tumor and metastasis. The ANOVA was used to investigate the relationship between gene expression and the histologic subtypes of ovarian carcinoma. The significance level was considered < 0.05 for all statistical tests.

Results

In this study, 45 tumor tissues and 45 non-tumor tissue samples from ovarian were studied. Table 1 summarizes the clinopathologic data of the samples. For investigating the expression of miRNAs, the qRT-PCR technique was used and the melting curves of internal reference gene (U6) and specific genes (miR-4443 and miR-5195-3p) were plotted as single curve. Then, the gene expression and relative expression of all the samples were evaluated.

Evaluation of miR-4443 expression in OC and its relationship with clinical factors

Relative expression of miR-4443 was determined in tumor and non-tumor samples. Relative expression of miRNA was normalized by the internal control gene (U6). Data showed that expression of miR-4443 was significantly lower in tumor samples compared to non-tumor samples ($P < 0.0001$) (Fig. 1a). The receiver operating characteristic (ROC) curve was analyzed to determine the specificity and sensitivity of miR-4443 expression to differentiate between tumor and non-tumor samples. The area under curve (AUC) for miR-4443 was 81% ($P < 0.0001$), and it is suggested that this miRNA can be a good tumor marker for OC (Fig. 1b). The relationship between gene expression and metastasis of the ovarian tumors to other regions (uterus, abdominal region, peritoneum and intestines) was also studied (Fig. 1c). It was also observed that gene expression decreased significantly in metastasis than in non-metastasis ($P < 0.05$). The association between miR-4443 expression and the histologic subtypes of ovarian carcinoma (endometrial, mucinous, and serous tissues) was also investigated (Fig. 1d) to determine the association between gene expression and the type of OC, and the effect of the histologic subtypes on gene expression. According to our study, reduction of miRNA expression in serous and mucinous tumor tissues was more obvious, but nevertheless, this change was significant only for serous tumor tissue ($P < 0.05$). However, no significant association was observed between the three groups of tissue (without non-tumor samples). According to the results, a no statistically significant reduction of miR-4443 expression was observed in high-grade (II and III) tumors compared to low-grade tumors (Fig. 1e). In the study of the association between gene expression and tumor size, since the average size of the tumors was estimated 5 cm, the samples were divided into two groups, the tumors smaller and equal to 5 cm and those larger than 5 cm. The comparison of gene expression was performed in both groups (Fig. 1f), and no significant difference was observed in expression of miR-4443 between these two groups of tumors ($P > 0.05$).

Table 1 Clinicopathological characteristics of ovarian cancer patients

Characteristics	
Age (mean)	48.55 ± 0.25
Tumor size (mean)	5.5 ± 0.15
Histological subtype	
Serous (%)	31 (68.89)
Endometrioid (%)	8 (17.78)
Mucinous (%)	6 (13.34)
Tumor grade (%)	
1	11 (24.45)
2	13 (28.89)
3	21 (46.67)
Metastasis (%)	
Negative	26 (57.78)
Positive	19 (42.22)

Evaluation of miR-5195-3p expression in OC and its relationship with clinical factors

The relative expression of miR-5195-3p was also calculated in tumor and non-tumor samples (Fig. 2a). A very significant decrease in miR-5195-3p expression was observed in tumor samples ($P < 0.0001$). ROC curve analysis was performed to assess the sensitivity and specificity of expression of this miRNA. The AUC of 82% ($P < 0.0001$) suggested that miR-5195-3p is more potent as a tumor marker for OC (Fig. 2b). In examining the association of miR-5195-3p expression with metastasis in tumor samples, we observed that in the case of metastasis to other tissues, the level of miRNA expression significantly decreased (Fig. 2c) ($P < 0.001$). According to Fig. 2d, the relationship between gene expression and the type of OC and the effect of histologic subtypes of cancer on the rate of gene expression were also studied. The association between miR-5195-3p expression and the histologic subtypes of ovarian carcinoma (endometrial, mucinous, and serous tissues) and decreased expression in all three groups compared to the control group showed significant changes only in serous and endometrial tissues. Besides that no significant change was observed in gene expression of the three groups of tissues (without non-tumor tissues). The expression level comparison between different grade was not statistically significant ($P > 0.05$) (Fig. 2e). In addition, in tumors larger than 5 cm, there was a decrease of approximately two times when compared to the tumors smaller than 5 cm (Fig. 2f) ($P < 0.05$).

Discussion

The present study was conducted to investigate the expression of miR-4443 and miR-5195-3p in OC tumor samples compared to non-tumor samples. In addition, the association

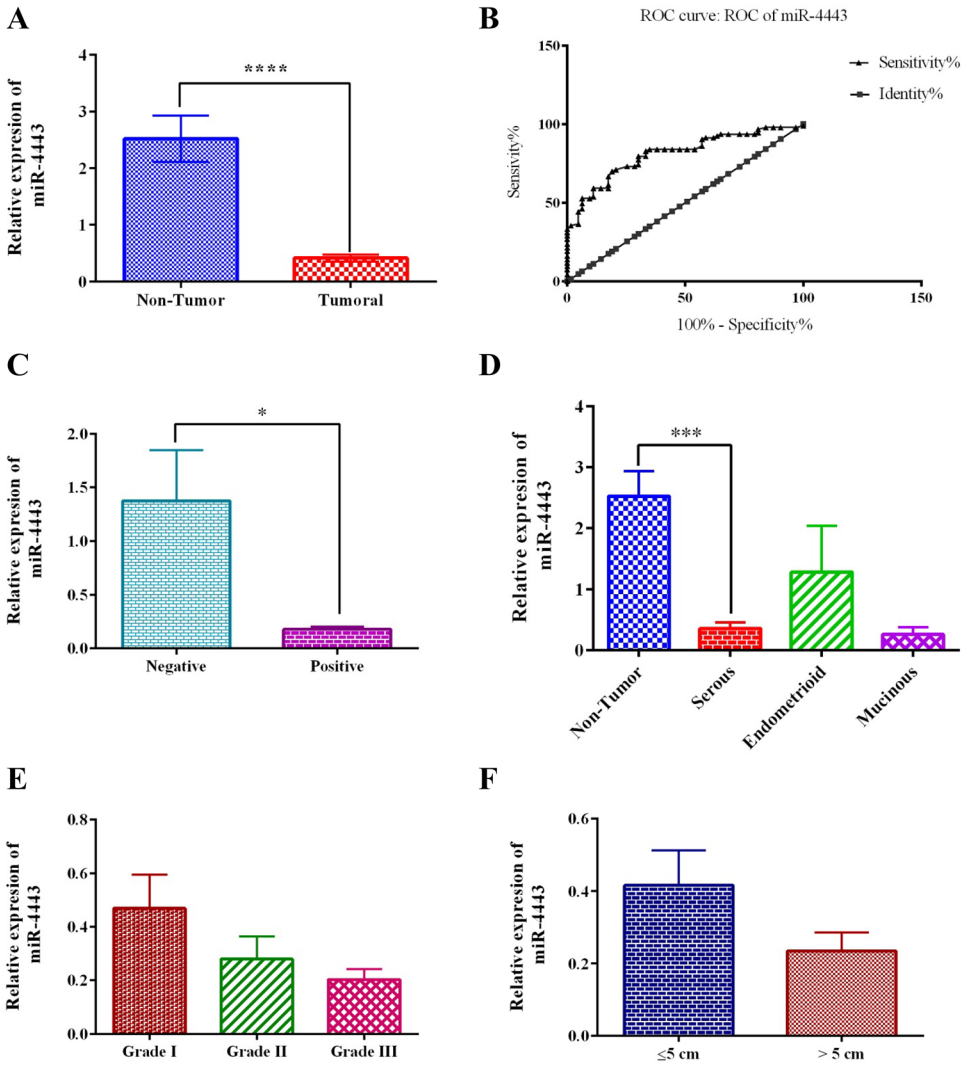

Fig. 1 miR-4443 expression in tumor compares non-tumor ovarian tissue cancer. **a** Histogram displays the mean values of relative miR-4443 expression in tumor and non-tumor samples. As shown, the expression of miR-4443 is significantly down-regulated in tumor vs. non-tumor samples ($P = 0.0001$). **b** Receiver operating characteristic (ROC) curve analysis shown an area under the curve of 81% for miR-4443. Comparative expression levels of miR-4443 are presented for **c** metastasis, **d** histologic subtypes of ovarian carcinoma, **e** low and high grades (**f**) and tumor size. *$P < 0.05$

of miRNAs expression with clinical characteristics was investigated in the samples to determine if these miRNAs could be used as diagnostic biomarkers in the people with OC. Many cases of OC have poor prognosis and, when diagnosed, the disease has progressed to an advanced stage. Only 20% of cases are diagnosed in the early stages of OC [12, 13]. On the other hand, the use of diagnostic markers such as CA-125 has not been satisfactory so that the level of this marker increases under certain conditions such as inflammation, diabetes mellitus and other types of cancers [14]. Therefore, finding a therapeutic or diagnostic biomarker for OC is very valuable. A group of these ideal biomarkers include DNA, RNA, and protein markers that are found in tissues as well as in body fluids. In addition, recently, abundant evidence has suggested the role of miRNAs as biomarkers such that in some cases, cancer types can be distinguished from one another [15]. For instance, miR-30e and miR-223 in hepatocellular cancer, or miR-200c in gastric cancer that can serve as a biomarker [16, 17].

The present study showed that in OC, miR-4443 and miR-5195-3p can act as tumor suppressor, so that there was a significant reduction in the expression in tumors when compared to the non-tumor tissues. As well as, the role of these two miRNAs in controlling metastasis is questionable. As metastatic samples have less expression than non-metastatic samples. These conditions have also been recognized in other studies, which is consistent with our study. A study of colorectal cancer showed that leptin and insulin treatment of intestinal cancer cells significantly increased miR-4443 expression, which led to reduced cellular invasion. Cell transfection with miR-4443 mimic decreases invasion and proliferation in colorectal cancer cells and inhibits the expression of TRAF4 and NCOA1 genes [10]. In our study, TRAF4 gene expression was measured in OC samples, and linear regression showed a negative gradient for expression level with miR-4443 (data not shown). Increasing the expression of TRAF4 is a common characteristic of most human carcinomas, including the breast, prostate, lung and pancreas

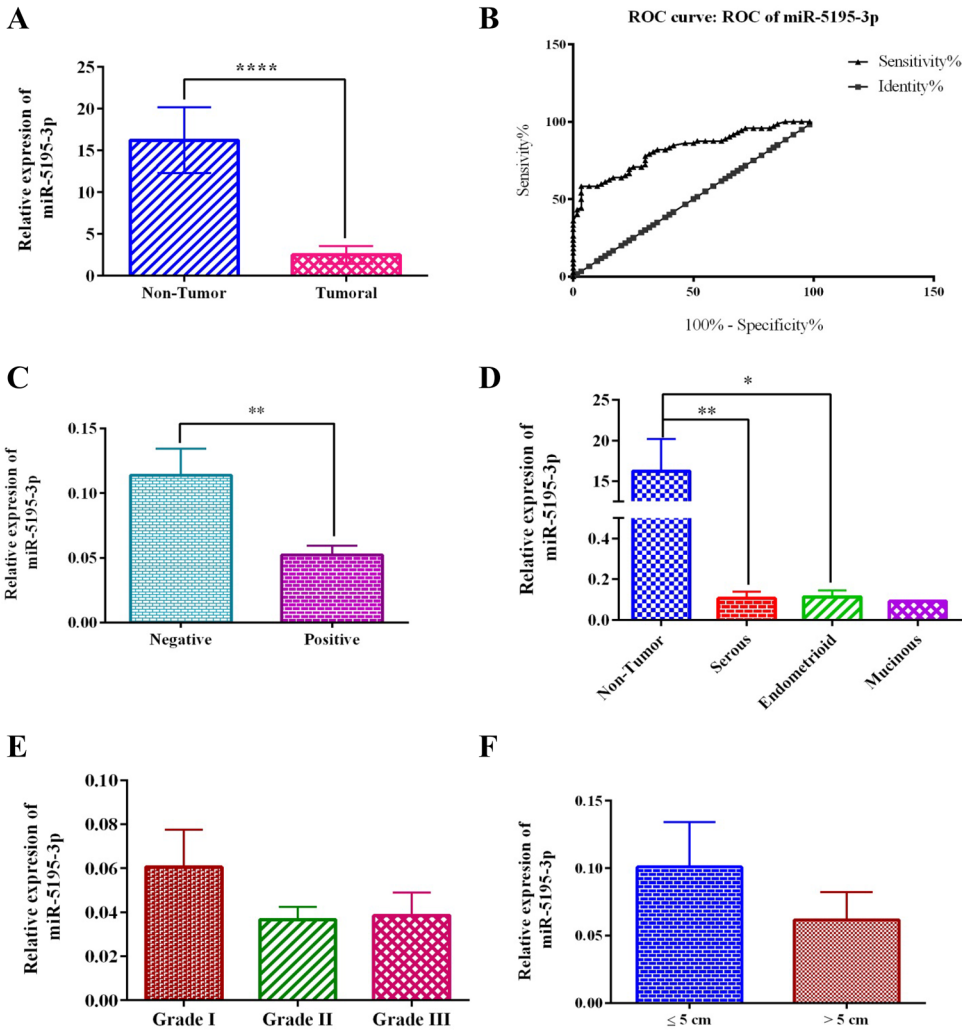

Fig. 2 miR-5195-3p expression in tumor vs. non-tumor ovarian tissue samples. **a** Histograms display the mean values of relative miR-5195-3p expression in tumor and non-tumor samples. As shown, the expression of miR-5195-3p is significantly down-regulated in tumor vs. non-tumor samples ($P=0.0001$). **b** Receiver operating characteristic (ROC) curve analysis shown an area under the curve of 82% for miR-5195-3p. Comparative expression levels of miR-5195-3p are presented for **c** metastasis, **d** histologic subtypes of ovarian carcinoma, **e** low and high grades (**f**) and tumor size. *$P<0.05$

[18, 19]. A remarkable point is that one of the mechanisms for increased expression of TRAF4 protein in human cancers is gene duplication. TRAF4 is located in a region of gene amplification that does not have any known oncogene. In the present study, an approximately 4-time increase in ovarian tumor samples was observed. The study of Zheng et al. to compare breast cancer cell lines with normal breast cells revealed that TRAF4 exhibited higher expression in cancer cells than in normal cells in vitro. The expression of TRAF4 in breast cancer cell lines with positive expression of estrogen receptor, was higher than that of the breast cancer cell lines without expression of estrogen receptor [20]. Therefore, the reduction of inhibitory effect on TRAF4 can be one of the factors involved in the metastasis of ovarian tumor cells. However, study of the expression profile of breast cancer samples and also comparing cell lines resistant to chemotherapy with sensitive cells showed an increase in the expression of miR-4443. An increase in the expression of miR-4443 increases IC50 levels for miRNA-transfected cells against epirubicin, while miR-4443 inhibition leads to the return of sensitivity to chemotherapy in cells. In addition, decreased expression of miR-4443 significantly increases the epirubicin-induced apoptosis. miR-4443 also caused an increase in migration and malignancy in cancer cells [9].

Regarding the expression of miR-5195-3p, a study on glioma malignant cells showed that the level of expression of miRNA decreased and miR-5195-3p played a key role in cell migration and invasion in these cancer cell lines [21]. Another study on bladder cancer indicated that an expression decline was observed at the surface of miR-5195-3p. In the bladder cancer cells, the inhibitory role of miRNA in cell proliferation and invasion has been determined. The inhibitory function of miR-5195-3p in bladder cancer acts by influencing KLF5, which acts as an oncogene in this type of cancer [11]. The results of our study are in complete agreement with two studies so that expression reduction in tumor samples was significant compared to non-tumor samples and positive metastatic samples. Decrease in expression of the two studied miRNAs was more marked in serous tissue than in the other tumor tissues. Therefore, these miRNAs can

play a beneficial and effective role in detecting the invasive effects of cancer.

Conclusion

Taken together, miR-4443 and miR-5195-3p can be considered tumor suppressors, and reductions in their expression can be associated with increased tumorigenicity and cell invasion. However, considering that these miRNAs have been studied in a few number of cancer types, further studies are needed to determine their exact function. On the other hand, the level of expression and function of these miRNAs in OC have not yet been studied, and this study can be an introduction to identifying these factors in early diagnosis or determining the appropriate approach of treatment for OC.

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Author contributions SR coordinated the study, designed the experiments and wrote the manuscript and data analysis. SOE performed molecular experiment and the statistical analyzes and participated in intellectual discussions of the data and manuscript writing.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest with any non-financial or financial organization.

Ethical approval All the procedures performed in studies involving FFPE samples 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 available or alive individual participants included in the study.

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