ABERRANT METHYLATION GENES MIR-663A AND MIR-663B AND OVARIAN CANCER PATHOGENESIS

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Abstract. Ovarian cancer remains one of the most common causes of death from gynecological cancer in women worldwide. As is known, the course of ovarian cancer depends on many factors, including genetic and epigenetic disorders. MicroRNAs are currently considered one of the most promising prognostic and diagnostic markers for solid tumors. The purpose of this work was to investigate the DNA methylation level of miR-663a and miR-663b in 25 paired tissue samples from patients with an established diagnosis of ovarian cancer and various histological and clinical characteristics by MS-HRM method. Our results indicate a lower frequency of miR-663a methylation in ovarian tumor tissues (0.09% ± 0.01) compared to histologically normal tissues 0.16% ± 0.01 ($p = 0.01$). However, an analysis of the miR-663a and miR-663b microRNA gene methylation level in patients with different clinical parameters, including the stage of disease development, the degree of cell differentiation, the occurrence of distant and regional metastases, as well as therapeutic pathomorphosis, not identify statistically significant differences in the methylation levels of these microRNA genes with any of the clinical characteristics, $p > 0.05$. Thus, our results indicate a potential role of aberrant methylation of the miR-663a microRNA gene in ovarian cancer carcinogenesis. However, additional researches on larger sample sizes are needed to confirm the results obtained.

Keywords: ovarian cancer, microRNA, methylation, miR-663a, miR-663b, metastasis.

List of Abbreviations

OC – ovarian cancer
DNA – deoxyribonucleic acid
PCR – polymerase chain reaction
MS-HRM- Methylation-Sensitive High Resolution Melting

Introduction

Ovarian cancer (OC) is the eighth most common cancer in women, accounting for more than 324,000 new cases and 206,000 deaths annually (Ferlay *et al.*, 2024). Despite significant improvements in ovarian cancer treatment over the past few years, 5-year survival for this disease remains low, mainly due to late diagnosis, recurrence, and metastasis (Xie *et al.*, 2019).

Epigenetic regulation plays an important role in the emergence, development, and formation of sensitivity to cancer therapy. One of the important mechanisms of such regulation is aberrant expression of microRNA (miRNA) in tumor cells, which, in turn, can lead to activation or inhibition of oncogenes and tumor suppressor genes. Current research suggests that miRNA expression can be regulated by methylation modification, which leads to changes in key cellular processes associated with cancer development or progression, such as apoptosis, proliferation, or sensitivity/resistance to chemotherapy. According to current data, the proportion of miRNA genes subject to methylation is significantly higher than that of protein-coding genes. To date, a large body of knowledge has been accumulated about the role of miRNAs and their target genes in the pathogenesis of ovarian cancer (Asl *et al.*, 2023). However, there are not many experimental studies devoted to the study of miRNA gene methylation

in ovarian cancer (Kushlinsky *et al.*, 2020, Pernar Kovač *et al.*, 2023).

One of the microRNAs that can suppress or promote malignant transformation of cells depending on the cellular and tissue environment is miR-663. An important role of this microRNA has been shown in bladder cancer (Yin *et al*., 2020), colorectal cancer (Hong *et al*., 2020), gallbladder cancer (Ma *et al*., 2023), breast cancer (Wang G. *et al.*, 2021) and glioma (Wang L. *et al.*, 2021). However, the role of miR-663 in ovarian carcinoma has not been sufficiently studied.

Based on the above, the aim of this work was to analyze the methylation status of the miR-663a and miR-663b microRNA genes in 25 paired tissue samples of patients with an established diagnosis of ovarian cancer and various histological and clinical characteristics.

Materials and Methods

For methylation analysis, we used DNA obtained from paired paraffin blocks from 25 patients with an ovarian cancer established diagnosis. The average age of disease manifestation is 55.7 years (39-80 years old). The cases clinical and histologically characteristics are presented in Table 1.

Samples of epithelial ovarian cancer were collected and morphologically characterized at the Republican Clinical Oncology Dispensary, Ufa. This study received permission from the ethics committee of the Institute of Biochemistry and Genetics of the Ufa Federal Research Center of the Russian Academy of Sciences. Informed consent was obtained from all research participants. All ovarian tumors were classified according to the TNM classification of the International Union Against Cancer and histologically verified based on the World Health Organization (WHO) criteria (Kurman *et al*., 2016). To select samples with a high content of tumor cells (at least 70–80%), an additional histological analysis of microsections (3–5 μm) stained with hematoxylin-eosin was performed.

DNA extraction from individual sections from blocks containing formalin-fixed, paraffin-embedded (FFPE) tissue fragments was carried out using the ExtractDNA FFPE kit (Evrogen, Russia). All DNA samples (500 ng) analyzed in this work were subjected to bisulfite conversion. Bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, USA), according to the manufacturer's instructions. The methylation level was assessed using the MS-HRM (Methylation-Sensitive High Resolution Melting) method using a LightCycler® 96 Roche device (Roche, Germany). Primers for the analysis of the methylation status of the miR-663a and miR-663b microRNA genes by the MS-HRM method were selected using the Methyl Primer Express Software v1.0 (Applied Biosystems, USA). Primer sequences miR-663a F: 5' GGGTAAGGGTATTTGGGAG 3', R: 5' AAACCRCCCTCRTATCTATA 3', the length of the PCR product is 117 bp, the primer annealing temperature is 60°C. Primer sequences miR-663b F: 5' GGGGAGAAATTTTAGGTAYGG 3', R: 5' AAAACCRTCCRACATCCTA 3' the length of the PCR product is 113 bp, the primer annealing temperature is 60°C.

MS-HRM was performed using a PCR mixture containing MeltDoctor HRM Dye, MeltDoctor HRM Master mix (Applied Biosystems, USA). Bisulfite-converted samples of 100% methylated and 0% unmethylated DNA (Zymo Research, USA) were used to obtain melting curves of standard controls. Control standards of methylated and unmethylated DNA (100%/0%) were mixed in a percentage ratio, as a result of which the methylation level of the control samples was 0%, 12.5%, 25%, 50%, 75% and 100%, respectively. Methylated standards were used to create a standard methylation curve and quantify the methylation status of the samples. All reactions were performed in duplicate. MS-HRM results were confirmed by sequencing of bisulfite-converted DNA on an ABI PRISM 3500 instrument (Applied Biosystems, USA).

Statistical analysis was performed using SPSS v.23 (SPSS Inc.). The standard curve used to determine the methylation status of unknown samples was constructed using regression analysis. The normality of distribution of quantitative features was tested. Comparison of quantitative features was performed using the Mann-Whitney criterion. Differences were accepted as statistically significant at $p < 0.05$.

Table 1

Clinical and histologically characteristics of ovarian cancer patients (N = 25)

Parameters	Clinical characteristics of patients	
Histology	*Epithelial*	%
	Adenocarcinoma	32
	Cystadenocarcinoma	32
	Serous adenocarcinoma	12
	Malignant Brenner tumor	4
	Mucinous adenocarcinoma	4
	Serous cystadenocarcinoma	4
	Endometrioid adenocarcinoma	4
	Endometrioid cystadenocarcinoma	4
	Non-epithelial	
	Granulosa cell tumor of the ovary	4
Stage	I	32
	II	8
	III	60
	IV	–
Grade	G1	–
	G2	24
	G3	20
	G4	12
	Gx	44
Nodal status	No	82
	Yes	18
Metastasis	No	64
	Yes	36
Menopause status	Premenopause	23
	Postmenopause	77
Bilateral	No	54
	Yes	46
Pathomorphosis	Complete Response - CR	5.6
	Partial Response - PR	55.6
	Stable Disease - SD	27.7
	Progressive Disease - PD	11.1
Recurrence	< 3 months	–
	3-6 months	–
	7-12 months	–
	1-3 years	4
	3-5 years	4
	> 5 years	4
Alive	No	28
	Yes	72

Results

Quantitative assessment of methylation based on HRM analysis is based on the fact that during bisulfite modification, all unmethylated cytosine is converted to uracil, which leads to a decrease in the content of GC nucleotides and is reflected in a decrease in the melting temperature of the samples (Tm). We plotted the differential fluorescence levels (RFU) of methyl-

ated standards using the 0% control as the baseline. Using regression analysis, a standard curve was constructed, which was used to determine the methylation status of unknown samples when studying the methylation level of the miR-663a and miR-663b (Fig. 1, 2).

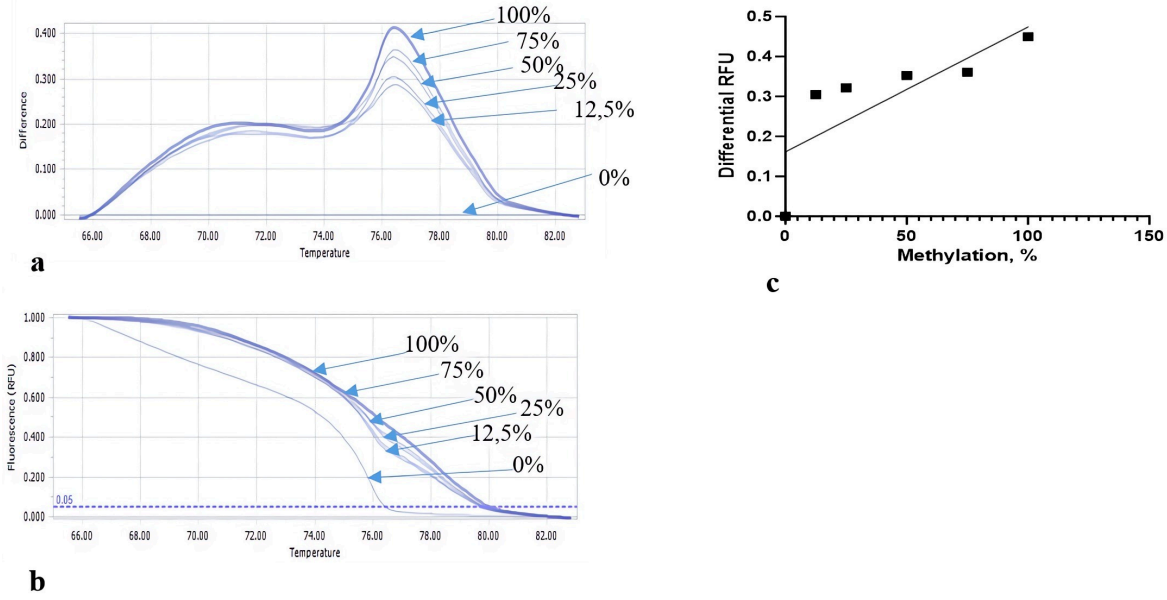

Fig. 1. Melting curves of the miR-663a microRNA gene. a – graph of differential fluorescence level (RFU) values of melting curves of methylated standards with 0% standard used as a baseline; b – normalized melting curves of methylated standards; c – regression plot of differential fluorescence depending on the methylation level of standards (%)

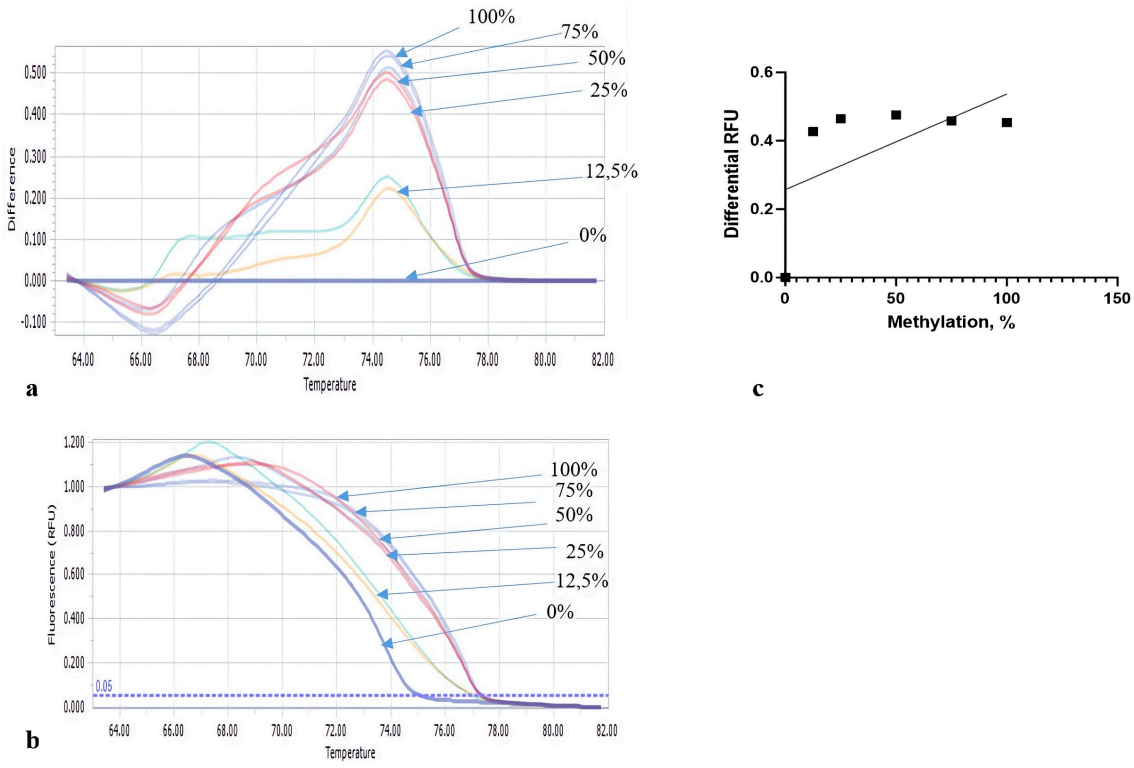

Fig. 2. Melting curves of the miR-663b microRNA gene. a – plot of differential fluorescence units (RFU) of melting curves of methylated standards with 0% standard used as a baseline; b – normalized melting curves of methylated standards; c – plot of regression of differential fluorescence depending on the methylation level of standards (%)

The profiles of normalized curves and melting peaks of MS-HRM analysis for the miR-663a microRNA gene in OC patients (tumor and normal) are shown in Figure 3 (a, b). The average methylation level in tumor samples according to differential RFU values was statistically lower and amounted to 0.09% ± 0.01 than in normal tissue samples – 0.16% ± 0.01 ($p = = 0.01$) (Fig. 3c). The methylation level was confirmed by sequencing of bisulfite-converted DNA. Figure 3 d, e shows the sequencing results of patient samples with the methylation level of the miR-663a microRNA gene in normal tissue and tumor tissue.

The normalized curve and melting peak profiles of MS-HRM analysis for the miR-663b microRNA gene in OC patients are shown in Figure 4 (a, b). The differential fluorescence values at the maximum peak temperature of the HRM curves were used in the regression formula derived from the diluted methylated standards and the percentage of methylation among the unknown samples was calculated. The average methylation level in tumor samples according to differential RFU values did not differ from the level in normal tissue samples ($p > > 0.05$). The methylation level was confirmed by bisulfite-converted DNA sequencing. Figure 4 d, e shows the sequencing results of patient samples in normal tissue and tumor tissue.

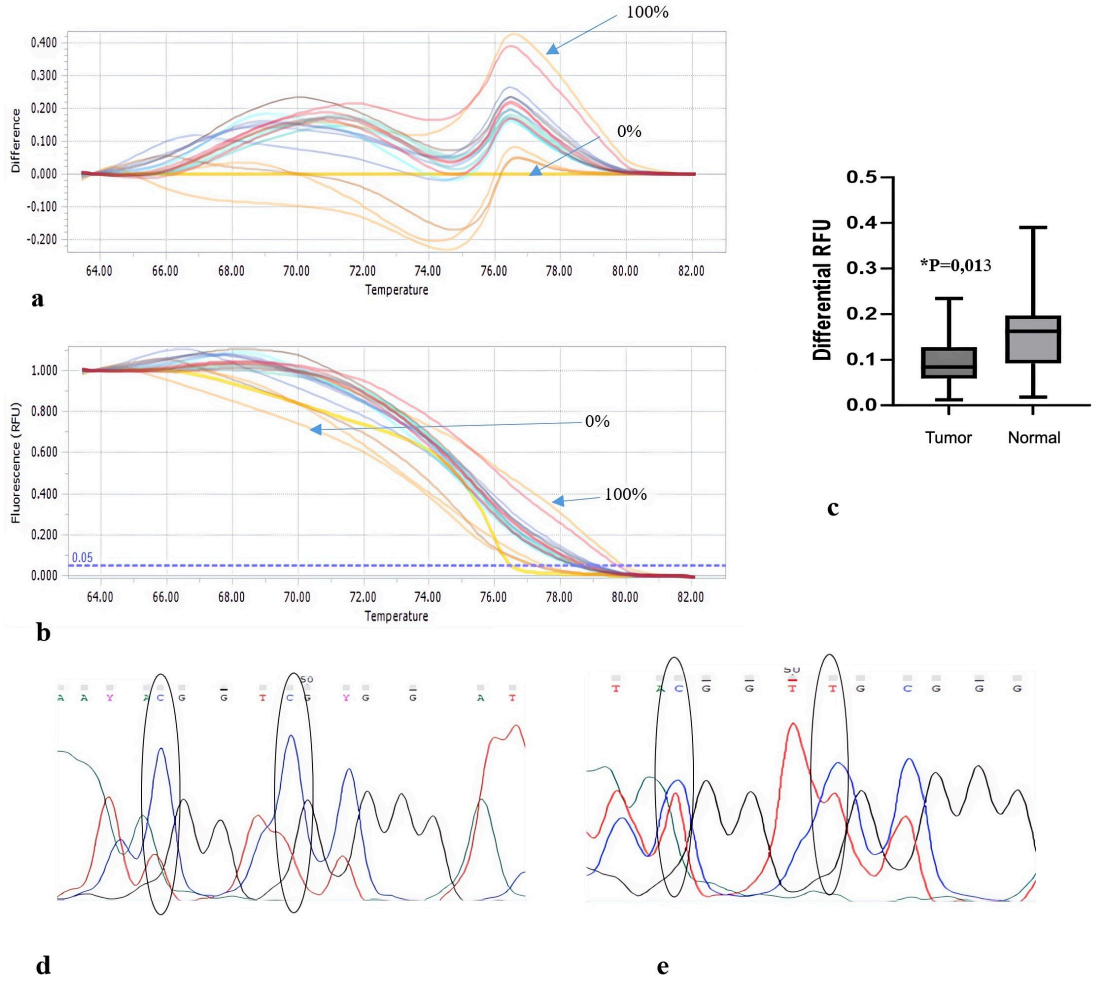

Fig. 3. MS-HRM analysis of the miR-663a microRNA gene in OC patients (normal and tumor). a – Melting curves of the MS-HRM analysis transformed into a difference plot with 0% control standard used as a baseline. b – Normalized melting curves of the miR-663a microRNA gene in OC patients (normal and tumor). c – Relative methylation level of the miR-663a microRNA gene in OC patients (tumor, normal). d, e – Results of sequencing of the miR-663a microRNA gene region in a normal tissue sample from an OC patient (d) and in a tumor tissue sample (e)

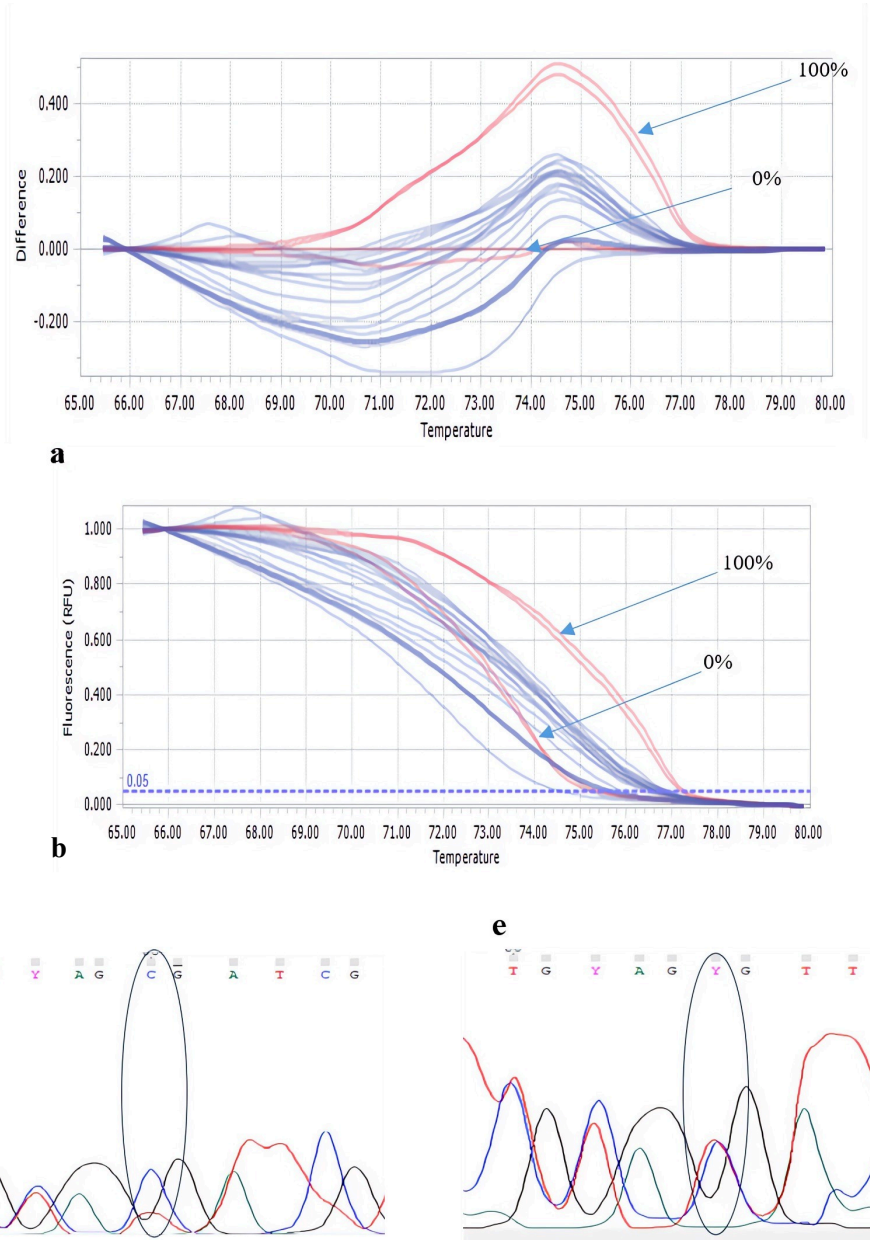

Fig. 4. MS-HRM analysis of the miR-663b microRNA gene in OC patients (normal and tumor). a – Melting curves of the MS-HRM analysis transformed into a difference plot with 0% control standard used as a baseline. b – Normalized melting curves of the miR-663b microRNA gene in OC patients (normal and tumor). d, e – Results of sequencing of the miR-663 b microRNA gene region in a normal tissue sample from an OC patient (d) and in a tumor tissue sample (e).

An analysis of the variability of clinical parameters (stages grade, nodal status, metastasis, tumor pathomorphological response) depending on the different methylation level of microRNA genes in patients with ovarian can-cer was performed. We conducted research in the following groups: I and II stages vs III and IV; G1-G2 Low grade (well-differentiated) vs G3-G4 High grade (poorly or un-differenti-ated); regional metastasis to the lymph nodes vs without lymph nodes metastasis; distant metastases to the greater omentum, lungs and liver vs without distant metastases; patients with complete (CR) and partial (PR) response of the tumor to treatment vs patients with progression (PD) and stabilization (SD) of response to chemotherapy (Table 2).

As a result, no statistical differences were established between the compared groups of patients ($p > 0.05$). We suppose this may be related to the size of the sample researched.

Table 2

Variability of clinical parameters in ovarian cancer patients (tumor and normal tissues) with different methylation level of microRNA genes

Methylation level (differential RFU values)	Stage (M ± SE)		Grade (M ± SE)		Nodal status (M ± SE)		Metastasis (M ± SE)		Tumor pathomorphosis (M ± SE)	
	I, II	III, IV	G1, G2	G3, G4	Yes	No	Yes	No	I, II	III, IV
miR-663a	0.12 ± 0.03	0.15 ± 0.04	0.15 ± 0.03	0.14 ± 0.02	0.17 ± 0.03	0.15 ± 0.02	0.17 ± 0.02	0.14 ± 0.03	0.14 ± 0.02	0.16 ± 0.04
miR-663b	0.17 ± 0.03	0.19 ± 0.03	0.17 ± 0.03	0.21 ± 0.08	0.17 ± 0.03	0.15 ± 0.02	0.17 ± 0.02	0.14 ± 0.03	0.14 ± 0.02	0.16 ± 0.04

Discussion

Ovarian cancer remains one of the most common causes of gynecological cancer deaths in women worldwide (Binju *et al.,* 2019). The high mortality rate of ovarian cancer is primarily due to its non-specific symptoms, which usually appear during disease progression, and the lack of effective screening methods for its early detection (Xie *et al.,* 2021). Currently, clinical treatment of OC is based on cytoreductive surgery to reduce the tumor volume and subsequent combination chemotherapy using cisplatin and paclitaxel. However, despite an initial good response to therapy, most patients progress to relapse and eventually develop chemotherapy-resistant disease. In addition, ovarian cancer has a high metastatic and invasive potential, and metastasis increases multidrug resistance and dramatically reduces patient survival (Tian *et al.,* 2022).

MicroRNAs are now recognized to play a central role in the molecular dysfunctions linking inflammation to cancer. Several studies have found that miR-663 plays an important role in many pathological processes, including autoimmune diseases, infection, and inflammatory response. However, its function in tumor progression is controversial. MiR-663 can either suppress or promote cell proliferation and/or migration under various conditions. Thus, Wang et al. showed that miR-663a expression was significantly reduced in breast cancer tissue samples and cell lines. Functional experiments showed that downregulation of miR-663a promoted cell proliferation, migration, and invasion, which correlated with lymph node metastasis, TNM stage, subtypes, and poor survival in breast cancer patients (Wang G. *et al.,* 2021). A similar decrease in miR-663a expression was found in glioblastoma tissues and cells. Overexpression of miR-663a suppressed the proliferation, migration, invasion, and cancer stem cell (CSC) properties of U-251 MG cells and primary human glioblastoma cancer cells (pGBMC1), while miR-663a knockdown had the opposite effects. Overexpression of miR-663a was found to attenuate the KDM2A/TGF-β/Smad signaling pathway and alleviate the CSC properties of THP1 cells-mediated glioma (Wang L. *et al.,* 2021). In gallbladder cancer, on the contrary, increased expression of miR-663a resulted in the suppression of its target epithelial membrane protein 3 (EMP3), indicating a promoter function of this miRNA (Ma *et al.,* 2023).

Exosomal miR-663b, stimulated by TGF-β1, promotes cervical cancer metastasis and epithelial-mesenchymal transition via targeting MGAT3 (You *et al.,* 2021). Similar results were obtained by Yin et al. when studying the level of exosomal miR-663b in patients with bladder cancer. The authors of the study found an increased level of miR-663b in the plasma of patients compared to healthy individuals and identified the target of this microRNA, the repression factor Ets2 (Yin *et al.,* 2020). Overexpression of miR-663b was also detected in colorectal cancer cells and cell lines. Reduced miR-663b expression suppressed cell proliferation and sphere formation ability, resulting in inactivation of Ras/Raf signaling and decreased expression of YAP1 and CD44. Using TargetScan software, TNK1, a negative regulator of Ras/Raf signaling, was predicted to be the target gene of miR-663b (Hong *et al.,* 2020). In a study by Xie et al., miR-663 acted as a tumor promoter in ovarian cancer, promoting cancer cell growth, migration, and invasion through inhibition of TUSC2 (Xie *et al.,* 2019).

Methylation plays a critical role in silencing gene expression, and hypermethylation of genes has been found to correlate with low or no transcription (Suzuki & Bird, 2008). Furthermore, although overall methylation levels and the methylation completeness of specific promoters are similar among individuals, there are significant differences in overall and specific methylation levels between normal cells and tumor cells from the same tissue (Phillips, 2008). High methylation of the miR-663a promoter region was found in pediatric B-cell precursor acute lymphoblastic leukemia (Chaber *et al.,* 2022), pancreatic cancer (Gu *et al*., 2020), and breast cancer (Hu *et al.,* 2013). Transfection of miR-663a mimic into pancreatic cancer cell lines resulted in hypomethylation of CpG islands and increased expression of this miRNA. Moreover, increased expression of

miR-663a increased the sensitivity of pancreatic cancer cells to gemcitabine and inhibited the EMT process. The authors of the study further investigated the possible molecular mechanisms and demonstrated that miR-663a targets the TGF-β1 gene (Gu *et al*., 2020). Another study showed that miR-663a expression is upregulated in the drug-resistant MDA-MB-231-derived ADM breast cancer cell line and that increased miR-663a expression is associated with downregulation of heparin sulfate proteoglycan 2 (HSPG2) and chemoresistance to cyclophosphamide and docetaxel. Moreover, MDA-MB-231/ADM contained fewer methylated CpG sites than its parental cell line, and 5-aza-29-deoxycytidine treatment of MDA-MB-231 cells significantly increased miR-663a expression, indicating that miR-663a is downregulated in MDA-MB-231 cells through promoter hypermethylation (Hu *et al*., 2013).

Aberrant methylation of miR-663a and miR-663b genes in ovarian cancer is analyzed for the first time in this research. Previously, methylation of the miR-663 gene family, of which only miR-663a (or miR-663) and miR-663b have been identified in humans (Carden *et al*., 2017), has been found in acute lymphoblastic leukemia, pancreatic cancer, breast cancer, and endometrial cancer (Chaber *et al.,* 2022; Gu *et al.*, 2020; Hu *et al.,* 2013; Yanokura *et al*., 2017). In this research, we analyzed aberrant methylation of miR-663a and miR-663b microRNA genes in 25 paired (tumor/normal) OC samples. Our results indicate a lower frequency of miR-663a methylation in ovarian tumor tissues ($0.09\% \pm 0.01$) compared to histologically normal tissues $0.16\% \pm 0.01$ ($p = 0.01$). However, when analyzing the methylation level of the miR-663a microRNA gene in patients with different clinical parameters, including the stage of disease development, the degree of cell differentiation, the occurrence of distant and regional metastases, as well as therapeutic pathomorphosis, we were unable to identify statistically significant differences in the methylation levels of the miR-663a microRNA gene with any of the clinical characteristics studied. The methylation level of the miR-663b gene did not differ between tumor and normal tissue samples ($p > 0.05$).

Thus, our results indicate a potential role of aberrant methylation of the miR-663a microRNA gene in ovarian cancer carcinogenesis. However, additional research on a larger sample are needed to confirm our data.

The authors declare no conflict of interest.

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