MICRORNA MIR-152 METHYLATION IN PATIENTS WITH OVARIAN CANCER: PROGNOSTIC POTENTIAL ANALYSIS

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Abstract. MicroRNAs play a crucial role in the regulation of biological processes variety associated with neoplasm development and progression, such as cell proliferation and differentiation, apoptosis, angiogenesis, inflammation, migration, invasion and metastasis, epithelial-mesenchymal transition and others. The purpose of this work was to investigate the DNA methylation level of miR-152 in 25 paired tissue samples from patients with an established diagnosis of ovarian cancer and various histological and clinical characteristics by the MS-HRM method. Our results indicate a higher frequency of the miR-152 methylation in ovarian tumor tissues (51.5% ± 5.4) compared to normal tissues (43.9% ± 7.2), however, the differences did not reach the statistical level significance, p = 0.5. There was no relationship between the metastatic process in the tumor depending on the level of methylation (46.5% ± 11.9 in patients with metastases vs 45.2% ± 7.8 in cases without metastases). One patient with the highest methylation level of all samples researched – 89.91%, despite a good response to primary therapy, had a relapse of the disease after 7 years. In addition, there is a tendency for a lower level of miR-152 methylation in patients with a complete response to therapy, in contrast to women with a partial response or the tumor process stabilization. Thus, our research provides evidence in favor of the suppressor function of the miR-152 in tumor, and its possible role in sensitivity to polychemotherapy, however, the results did not reach a statistical level of significance and additional studies on larger material are required.

Keywords: ovarian cancer, microRNA, methylation, miR-152, 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 one of the most common malignant neoplasms of the female reproductive system and currently represents a pressing health problem. According to the latest statistics, in 2022, 324,603 new ovarian cancer cases were registered worldwide and 206,956 women died due to this cancer pathology (Singh *et al*., 2023). The high mortality rate from ovarian cancer is primarily explained by the fact that for a long time, it occurs without obvious symptoms and, as a rule, is diagnosed at late stages of development (III-IV), as well as the lack of effective screening methods (Menon *et al*., 2021).

A number of genes and signaling pathways, as well as epigenetic regulatory mechanisms, which include microRNAs, are involved in ovarian cancer pathogenesis and metastasis. MicroRNAs play a crucial role in the regulation of biological processes variety associated with the development and progression of neoplasms, such as cell proliferation and differentiation, apoptosis, angiogenesis, inflammation, migration, invasion and metastasis, epithelial-mesenchymal transition (EMT), etc. Depending on which gene expression is suppressed by mi-

croRNAs, it's can function as tumor suppressors or oncogenes (Braga *et al*., 2017). There are many researchers on the role of microRNAs and their target genes in the ovarian cancer pathogenesis and progression (Nguyen *et al*., 2020; Salem *et al*., 2018; Loginov *et al*., 2018). It has been established that microRNA genes can be suppressed by hypermethylation of the CpG island promoter regions. Moreover, the percentage of genes whose regulation is disrupted by aberrant methylation is significantly higher among microRNA genes than among protein-coding genes (Piletič & Kunej, 2016). However, data on microRNA gene methylation in OC are limited to single experimental researches (Kushlinskii *et al*., 2020; Pernar Kovač *et al*., 2023).

Based on the above, the purpose of this work was to investigate the DNA methylation level of miR-152 in 25 paired tissue samples from 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 cases' clinical and histological 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 & Shih, 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 analyzing the methylation status of miR-152 were selected using the Methyl Primer Express Software v1.0 program (Applied Biosystems, USA). Primer sequences: miR-152 F: 5' TTCGGGTTTAAGTTTTGTTATG 3' and R: 5' ATAACGCAAATCCAACCC 3', PCR product length – 136 bp, primer annealing temperature – 60°C.

MS-HRM was carried out using a PCR mixture containing MeltDoctor HRM Dye, MeltDoctor HRM Master mix (Applied Biosystems, USA). Bisulfite-converted 100% methylated and 0% unmethylated DNA samples (Zymo Research, USA) were used to generate melting curves of standard controls (Hussmann & Hansen, 2018). Methylated and unmethylated DNA control standards (100%/0%) were mixed as a percentage, resulting in the methylation level of the control samples being 0%, 5%, 25%, 50%, 75% and 100%, respectively. Methylated standards were used to generate a methylation standard curve and quantify the methylation status of samples. All reactions were performed in duplicate. The MS-HRM results were confirmed by sequencing 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 that was used to determine the methylation status of unknown samples was generated using regression analysis. The normality of the distribution of quantitative characteristics was checked. The comparison of quantitative characteristics was carried out using the Mann-Whitney test. Differences were accepted as statistically significant at $p < 0.05$.

Table 1

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

Parameters	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
Age, years	55.7 (39-80)	
Menopause status	premenopause	23
	postmenopause	77
Bilateral	No	54
	Yes	46
Pathomorphosis	Complete Response - CR	25
	Partial Response - PR	25
	Stable Disease - SD	50
	Progressive Disease - PD	-
Recurrence	< 3 months	-
	3-6 months	-
	7-12 months	-
	1-3 years	12,5
	3-5 years	12,5
	> 5 years	12,5
Alive	No	33
	Yes	67

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 methylated 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-152 (Fig. 1).

The profiles of normalized curves and melting peaks of MS-HRM analysis for the miRNA - miR-152 in OC patients are shown in Figure 2 (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 was 51.5% ± 5.4, which was higher than in normal tissue samples – 43.9% ± 7.2, but the differences did not reach the level of statistical significance ($p = 0.5$) (Fig. 2c). The level of methylation was confirmed by sequencing of bisulfite-converted DNA. Figure 2 (d, e) shows the results of sequencing patient samples with a methylation level of 5.89% of the miR-152 in normal tissue and 50.61% in tumor tissue.

When comparing the methylation level between patients with metastases (46.5% ± 11.9) and those without metastases (45.2% ± 7.8), no significant differences were found between the compared groups ($p = 0.9$).

Discussion

Ovarian cancer is the deadliest among all gynecological malignancies and its 5-year survival rate does not exceed 45% (Chan *et al*., 2008). The most common histological type of ovarian cancer is epithelial tumors, which account for 85% of all cases of the disease (Meinhold-Heerlein *et al*., 2016). Traditionally, epithelial ovarian cancer is treated by surgical debulking of the original tumor followed by a combination of platinum and taxane treatments-primarily carboplatin and paclitaxel. Platinum therapy promotes the appearance of DNA strand breaks, which trigger miss-match repair (MMR) and apoptosis signaling pathways (Dijt *et al*., 1988). Taxanes bind β-tubulin, preventing microtubule depolymerization, which in turn promotes to cell cycle arrest and apoptosis (Abal *et al*., 2003). Although 70% of patients with ovarian cancer initially respond to chemotherapy, subsequent chemoresistance and disease relapse remain a serious problem and account for 90% of eventual deaths (Agarwal & Kaye, 2003).

Determining the methylation status of CpG island of genes in malignant neoplasms is an important step for understanding the function of a gene in the oncogenesis (Braga *et al.*, 2020) and the development of tumor chemoresistance, in particular.

In this work, we analyzed the aberrant methylation level of miR-152, associated with carcinogenesis, in 25 paired (tumor/normal) OC samples. This microRNA is a member of the miR-148/152 family, members of which act as tumor suppressors and their activity is reduced in various types of cancer, including ovarian cancer (Chen *et al*., 2016; Lopes *et al*., 2021). It has been established that some of miR-152 oncogenic targets are *MET*, *TGFα*, *FGF2*, *CD151* and *MMP3* genes, acting on which miR-152 leads to inhibition of cell proliferation and tumor metastasis (Liu *et al*., 2016).

The increased level of methylation detected in tumor tissues found in our study may also indicate the tumor suppressor properties of this microRNA. These results obtained on clinical samples are consistent with the data of functional studies by other authors, which also indicate the suppressor properties of miR-152. Thus, in a study conducted by Lian-Wei et al., it was found that the miR-152 suppresses the proliferation, migration and invasion of ovarian cancer cells and promotes apoptosis through inhibition of the ERBB3 protein *in vitro* (Li *et al*., 2018). Recent work by Wu et al. (2023) also showed that the miR-152 suppresses the proliferation, migration and infiltration of cervical cancer cells and reduces their resistance to cisplatin chemotherapy by inhibiting protein expression in the ERBB3/Akt/c-myc and ERBB3/Akt pathways/Snail (Wu *et al*., 2023). A study by Liu et al. (2019) showed that miR-152 has an inhibitory effect on the growth

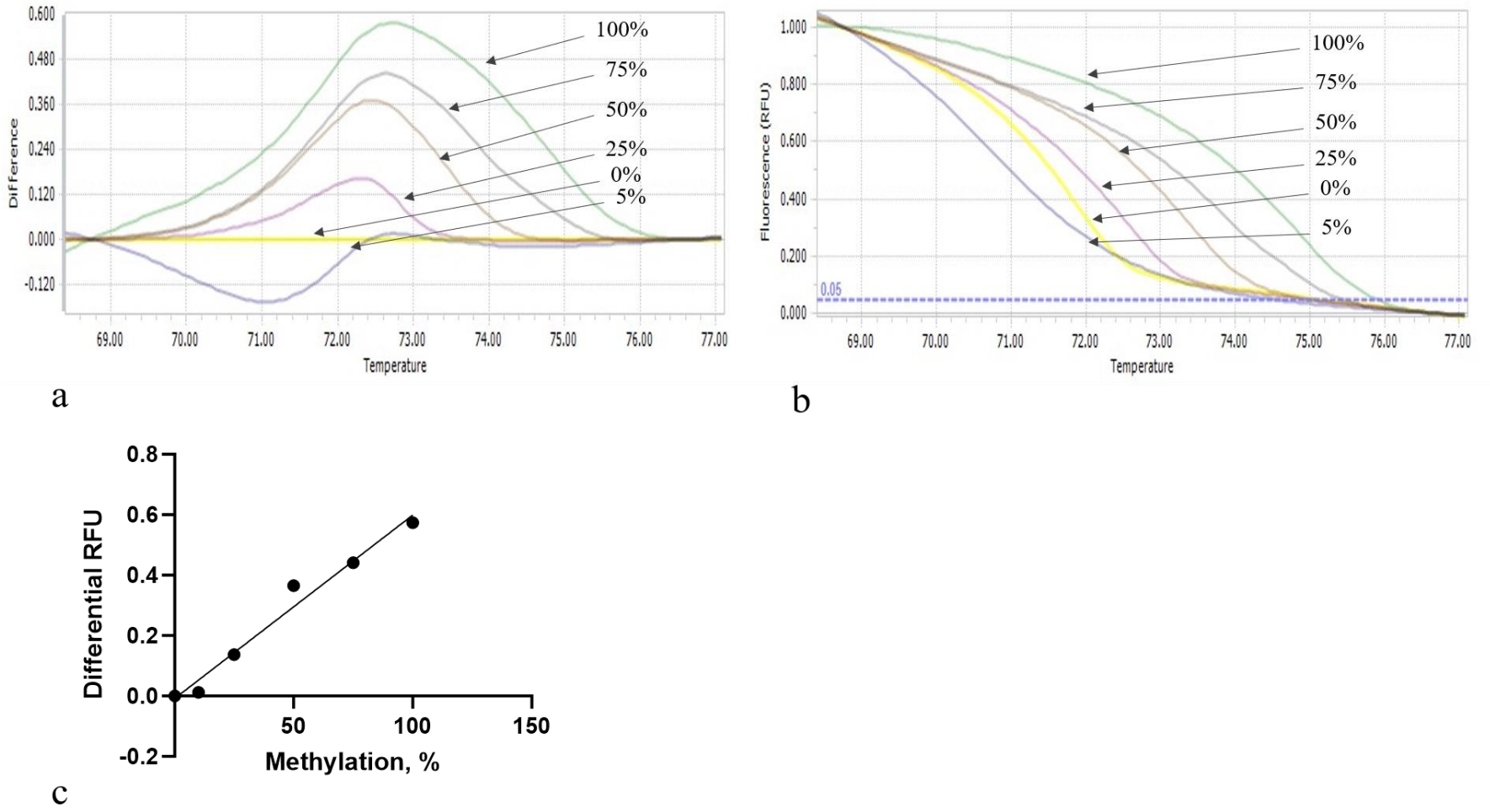

Fig. 1. Melting curves of miR-152. a – graph of differential fluorescence level (RFU) values of melting curves of methylated standards with 0% standard used as a baseline; b – normalized melting curves of methylated standards; c – regression plot of differential fluorescence depending on the methylation level of standards (%)

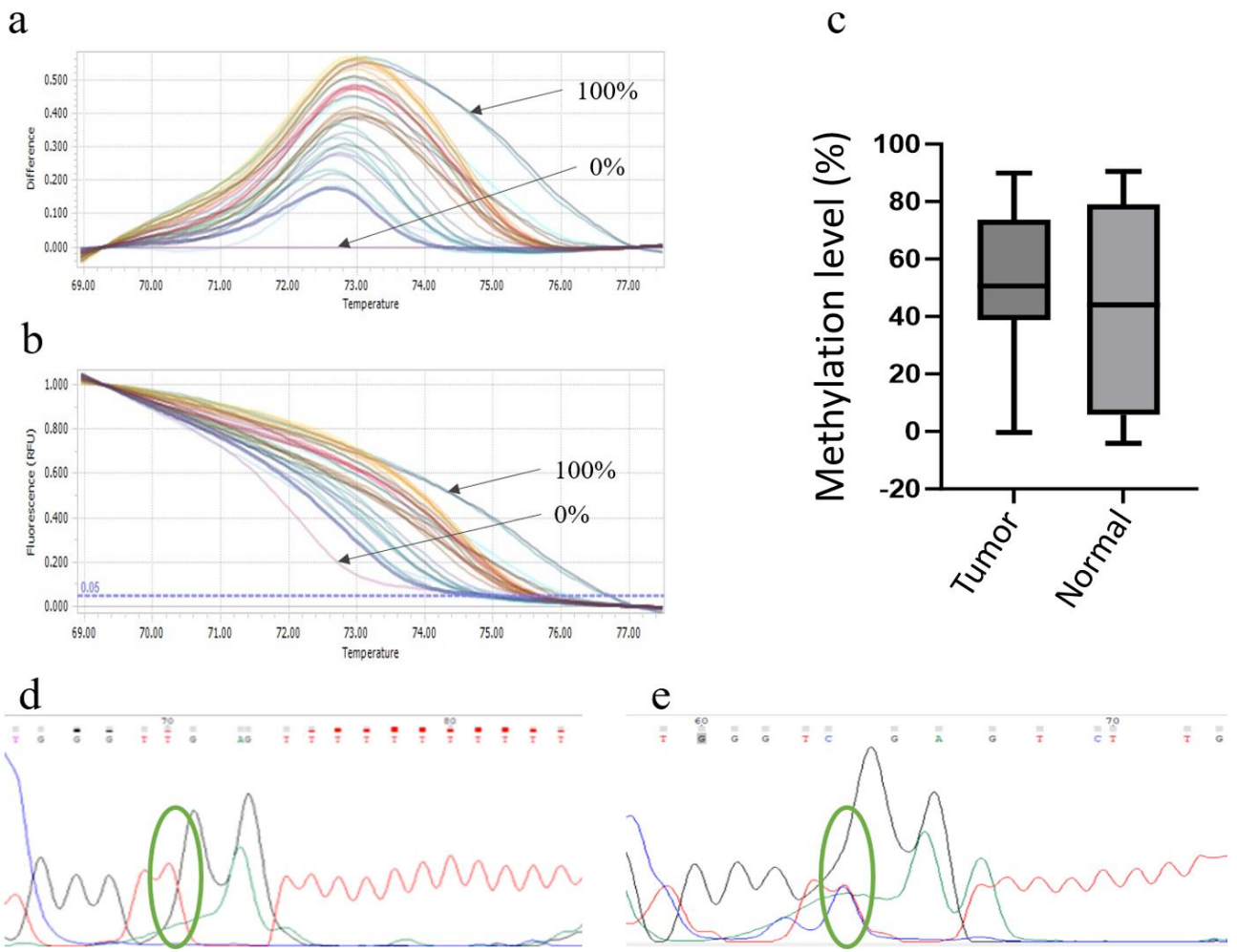

Fig. 2. MS-HRM analysis of miR-152 in patients with ovarian cancer (normal and tumor). a – Melting curves of MS-HRM analysis converted to difference plot with 0% control standard used as a baseline. b – Normalized melting curves of miR-152 in patients with ovarian cancer (normal and tumor). c – Relative level of methylation of miR-152 in patients with ovarian cancer (tumor, normal). d, e – Results of sequencing the region of miR-152 in a sample of normal tissue from a patient with ovarian cancer (d) and in a sample of tumor tissue (e)

of xenografts in mice obtained by subcutaneous injection of the human ovarian cancer cell line SKOV3 (Liu *et al*., 2019).

The main mechanism of miR-152 suppression is hypermethylation of the promoter region (Liu *et al*., 2016). A high degree of methylation of the CpG island of miR-152 is found in mixed lineage leukemia (MLL), rearranged acute lymphoblastic leukemia, endometrial and gastrointestinal cancer (Chen *et al*., 2013). It was also observed that the expression level of miR-152 was significantly reduced in cisplatin-resistant epithelial ovarian cancer cell lines A2780/DPP and SKOV3/DPP. DNA methyltransferase 1 (DNMT1), the main enzyme that maintains DNA methylation patterns, is one of miR-152 targets (Xiang *et al*., 2014). Reduced expression of this miRNA leads to overexpression of DNMT1, promoting DNA methylation, which can lead to the suppression of genes involved in chemotherapy sensitivity (Yan *et al*., 2016). Thus, Khajehnoori et al. (2020) in their work demonstrated an increase in the levels of miR-152 and miR-148 and a decrease in the level of DNMT1 protein after treatment with both 5-azacytidine (5-aza) and trichostatin A (TSA) in cisplatin-resistant OC cell lines A2780. The authors of the study hypothesized that increased expression of miR-152 and miR-148 under the influence of 5-Aza and TSA may reverse cisplatin resistance by suppressing the DNMT1 protein in ovarian cancer, and a decrease in the expression level of these microRNAs may be associated with DNA methylation and histone deacetylation (Khajehnoori *et al.*, 2020).

Our results indicate a higher frequency of miR-152 methylation in ovarian tumor tissues (51.5% ± 5.4) compared to normal tissues (43.9% ± 7.2), however, the differences did not reach the statistical level significance, $p = 0.5$. There was no relationship between the metastatic process in the tumor depending on the level of methylation (46.5% ± 11.9 in patients with metastases vs 45.2% ± 7.8 in women without metastases). One patient with the highest methylation level of 89.91% of all samples studied, despite a good response to primary therapy, had a relapse of the disease after 7 years. In addition, there is a tendency for a lower level of miR-152 methylation in patients with a complete response to therapy, in contrast to women with a partial response or stabilization of the tumor process.

In general, our study provides evidence in favor of the suppressor function of the miR-152 in tumor formation, and its possible role in sensitivity to polychemotherapy, however, the results did not reach a statistical level of significance and additional studies on larger material are required.

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