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# Analysis of miRNAs miR-125a-5p, -27a-5p, -193a-5p, -135b-5p, -451a, -495-3p and -136-5p in parental ovarian cancer cells and secreted extracellular vesicles

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**Introduction.** The identification of markers for liquid diagnostics of ovarian cancer is one of the most urgent tasks of gynecologic oncology. Currently, extracellular vesicles (EVs) are of great interest as a source of oncomarkers, including miRNA markers. We have previously shown that the levels of miR-125a-5p, -27a-5p, -193a-5p and 135b-5p are significantly elevated and miR-451a, -495-3p and -136-5p are significantly decreased in the EVs from uterine aspirates of ovarian cancer patients.
**Aim.** Analysis of miR-125a-5p, -27a-5p, -193a-5p, 135b-5p, 451a, 495-3p and -136-5p levels in ovarian cancer cell cultures and secreted EVs.
**Material and methods.** Cultivation of ovarian cancer cell lines: OVCAR-3, OVCAR-4, OVCAR-8 and SKOV3; EVs isolation from conditioned medium by ultracentrifugation; EVs validation by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), western blot analysis of exosomal markers; isolation of miRNAs from cells and EVs; analysis of miRNAs by Stem-Loop – reverse transcription-quantitative polymerase chain reaction.
**Results.** In all cell lines studied, the expression of miR-125a-5p, -27a-5p, -193a-5p and -135b-5p significantly exceeds the expression of -451a, -495-3p and -136-5p. All ovarian cancer cell lines are featured by a "cells >EVs" ratio for highly expressed miRNAs and "EVs >cells" ratio for poorly expressed miRNAs.
**Conclusion.** The results of the study support the relation between the differential expression of studied miRNAs and the pathogenesis of ovarian cancer and confirm the high diagnostic potential of these molecules.

**Keywords:** ovarian cancer, extracellular vesicles, exosomes, miRNA, miR-125a-5p, miR-27a-5p, miR-193a-5p, miR-135b-5p, miR-451a, miR-495-3p, miR-136-5p

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## Анализ микроРНК miR-125a-5p, -27a-5p, -193a-5p, -135b-5p, -451a, -495-3p и -136-5p в клетках рака яичника и секретируемых ими экстраклеточных везикулах

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**Введение.** Поиск маркеров для жидкостной диагностики рака яичника (РЯ) является одной из наиболее актуальных задач онкогинекологии. В настоящее время большой интерес в качестве источника онкомаркеров, в том числе микроРНК, вызывают экстраклеточные везикулы (ЭКВ). Ранее мы показали, что уровень miR-125a-5p, -27a-5p, -193a-5p и -135b-5p достоверно повышен, а miR-451a, -495-3p и -136-5p значимо снижен в ЭКВ маточных аспиратов больных РЯ.
**Цель исследования** – анализ уровней miR-125a-5p, -27a-5p, -193a-5p, -135b-5p, -451a, -495-3p и -136-5p в клеточных линиях РЯ и секретируемых ими ЭКВ.
**Материалы и методы.** Проведены культивирование клеточных линий РЯ (OVCAR-3, OVCAR-4, OVCAR-8 и SKOV3), выделение ЭКВ из кондиционированной среды методом ультрацентрифугирования, валидация ЭКВ с помощью анализа траекторий наночастиц (NTA), трансмиссионной электронной микроскопии и вестерн-блот-анализа экзосомальных маркеров. Также выполнены выделение микроРНК из клеток и ЭКВ, анализ микроРНК методом полимеразной цепной реакции с обратной транскрипцией в реальном времени в модификации Stem-loop.
**Результаты.** В клетках исследуемых линий РЯ экспрессия молекул miR-125a-5p, -27a-5p, -193a-5p и -135b-5p значительно превышала экспрессию miR-451a, -495-3p и -136-5p. Все линии клеток РЯ характеризуются соотношением «клетки >ЭКВ» для высоко экспрессируемых микроРНК и «ЭКВ >клетки» для низко экспрессируемых микроРНК.
**Заключение.** Результаты исследования свидетельствуют о связи дифференциальной экспрессии исследуемых микроРНК с патогенезом РЯ и подтверждают высокий диагностический потенциал данных молекул.

**Ключевые слова:** рак яичника, экстраклеточные везикулы, экзосомы, микроРНК, miR-125a-5p, miR-27a-5p, miR-193a-5p, miR-135b-5p, miR-451a, miR-495-3p, miR-136-5p

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## INTRODUCTION

Extracellular vesicles (EVs) are a heterogeneous class of secreted particles enclosed by a bilipid membrane and containing various bioactive molecules. They play a vital role in intercellular communication by transporting regulatory molecules over both short and long distances. Numerous data support the involvement of EVs in the malignant transformation of cells and the pathogenesis of tumors [1, 2]. Small EVs (30–150 nm), primarily exosomes and exosome-like vesicles, are secreted by most cells of the organism and are found in virtually all biological fluids. Signaling molecules, including proteins, lipids and various classes of nucleic acids (exosomal cargo), are incorporated into EVs through tightly controlled selection and loading mechanisms [3]. Due to the fact that the composition of secreted vesicles reflects the molecular profile of the parent cells, as well as the increasingly evident role of these structures at all stages of tumor progression, EVs are considered a promising source of markers for liquid non-invasive diagnosis of malignant neoplasms [4]. Furthermore, exosomal markers, including regulatory RNAs, have a number of advantages over both tissue and serological tumor markers, such as free circulating nucleic acids. These advantages include high information content, stability in biological fluids, including the bloodstream, and high concentration of molecules [5]. To date, several potential marker panels based on exosomal miRNAs have been proposed, including diagnostic markers for ovarian cancer (OC) [6–8]. However, the proposed sets of miRNAs vary greatly among different studies. This variation can be attributed to the natural heterogeneity of the vesicles and by the great variability in the methods used to isolate EVs from biological fluids and to analyze their molecular composition [9, 10]. Previously, we showed for the first time that EVs corresponding to exosomes can be isolated from uterine aspirates and verified their compliance with the International Society for Extracellular Vesicles (ISEV) guidelines [9]. Analysis of the transcriptome of small exosomal RNAs in a pilot sample revealed significant differences in miRNA profiles between EVs from uterine aspirates of epithelial OC patients and healthy donors [11]. The differential expression (DE) of miRNAs in EVs from OC patients and controls was confirmed through subsequent analysis of an expanded sampling (data in press). The DE miRNAs included molecules that were significantly upregulated (UA-UP) and significantly downregulated (UA-DOWN) in EVs of uterine aspirates of OC patients.

To investigate the relationship between the identified changes (DE) and the pathogenesis of OC, we examined the levels of individual DE miRNAs in both OC cells and their secreted EVs. For this task, we selected four miRNAs from the up-regulated group and three miRNAs from the down-regulated group based on our data on their expression in EVs from uterine aspirates of OC patients (UA-OC EVs). The obtained results indicate that the levels of miRNAs from the UA-UP group were significantly higher than those from the UA-DOWN group in all examined OC cells. Comparison of the expression of the same miRNAs in parental cells and secreted EVs revealed that the levels of miRNAs from the UA-UP and UA-DOWN groups had opposite cell-to-EVs ratio (cell/EV ratio). Specifically, the levels of miRNAs from the UA-UP group were significantly higher in the cells of all OC lines while the levels of miRNA from the UA-DOWN group were significantly higher in the secreted EVs in all cases.

The **aim of this work** – to investigate the expression of the miRNAs miR-125a-5p, -27a-5p, miR-193a-5p, -135b-5p, -451a, -495-3p and -136-5p in OC cell lines and in EVs secreted by OC cells in culture.

## MATERIALS AND METHODS

**Cell cultures.** Ovarian cancer cell lines OVCAR-3, OVCAR-4, OVCAR-8 and SKOV3 were cultured in RPMI-1640 medium (PanEco, Russia) supplemented with 10 % fetal calf serum (FBS) (HyClone, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (PanEco) at 37 °C and 5 % $CO_2$. To obtain exosome-free medium, FBS pre-cleared of native vesicles by overnight ultracentrifugation at 110,000g was used. To collect conditioned medium, cells were seeded into six 175 $cm^2$ culture flasks. The following day, the medium was changed to exosome-free medium. Once the cells reached 90 % confluence, the medium was selected, pooled, and used to isolate EVs.

**Isolation of small extracellular vesicles.** The small EVs were isolated using the differential centrifugation method. The conditioned medium underwent serial centrifugation at 800g for 15 min, 2000g for 15 min, and 10,000g for 30 min, all at 4 °C. The supernatant obtained was then ultracentrifuged for 2 hours at 110,000g, 4 °C. The resulting pellet was dissolved in 5 ml of cold PBS and then precipitated again for 1 hour at 110,000g and 4 °C. The purified pellet, which mainly consists of small EVs, was dissolved in 120 μl of ice-cold PBS, frozen in liquid nitrogen, and stored at –80 °C until further analysis.

**Nanoparticle tracking analysis.** Particle size distribution and concentration were determined by nanoparticle tracking analysis (NTA) using a NanoSight LM14 instrument equipped with an integrated temperature sensor (Malvern Panalytical Ltd., UK), LM 14C laser unit (405 nm, 65 mW) and a high-sensitivity camera with CMOS sensor (C11440-50B, Hamamatsu Photonics, Japan). The measurements were conducted following the methodology previously described [11] and in accordance with ASTM E2834-12(2018).

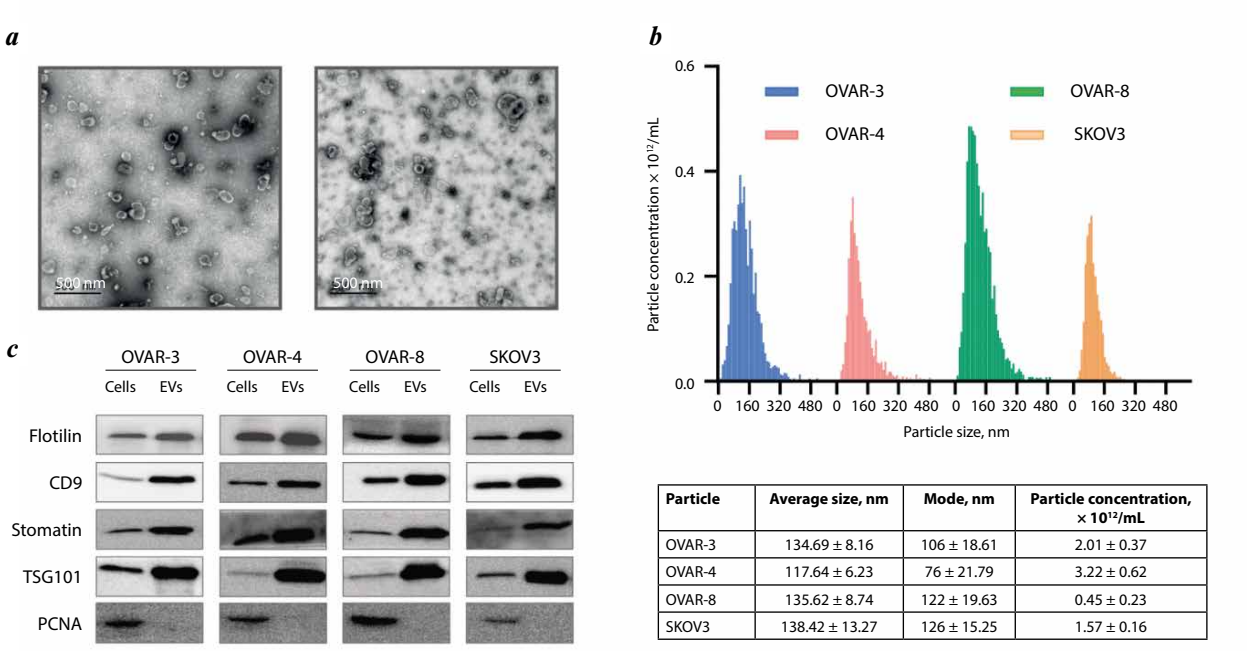
**Immunoblotting and antibodies.** Protein concentration in EVs samples and cells lysed in RIPA buffer was determined using the NanoOrange™ Kit (N6666, ThermoFisher Scientific, USA) according to the manufacturer's recommendations. Immunoblotting was performed as previously described [11], except that 5 μg of protein was applied to SDS-PAGE and visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (34095, ThermoFisher Scientific, USA). The antibodies used in this work were anti-Flotillin-2 (#3436S, 1 : 1000; Cell Signaling Technology, USA), anti-CD9 (#13174, 1 : 2000; Cell Signaling Technology, USA), anti-TSG-101 (ab125011, 1 : 5000; Abcam, UK), anti-PCNA (#sc-7907, 1 : 500; Santa Cruz Biotechnology, USA), anti-mouse goat polyclonal antibody (#ab5887, 1 : 8000; Abcam, UK) and anti-rabbit goat polyclonal antibody (#29902, 1 : 80 000; Cell Signaling Technology, USA).

**MiRNA isolation and analysis.** Isolation of miRNA from cells and EVs was carried out using a kit for isolation of total RNA and miRNA (LRU-100-50, Biolabmix, Russia) according to the manufacturer's recommendations. The concentration of miRNA in the obtained samples was measured using the Qubit™ miRNA assay kit (Q32881, Invitrogen, USA). MiRNA detection was performed by Stem-Loop reverse transcription-quantitative polymerase chain reaction (RT-qPCR) [12]. A total of 10 ng (for cell lines) and 2 ng (for EVs) miRNA was used in the reverse transcription reaction with 1 pmol Stem-Loop primer and 2 U MMuLV H Reverse Transcriptase (RT-10, Dialat Ltd., Russia). Primers for miRNAs were designed using miRBase v22.1 and synthesized by DNA-Synthesis LLC (Moscow, Russia) (the sequences of all oligonucleotides used are given in supplement). The amplification efficiency was assessed by testing serial dilutions of cDNA derived from the reverse transcription reaction of synthetic miRNAs. The PCR parameters were adjusted to ensure that all primer sets had efficiencies between 1.95 and 2.05. The RT products were diluted two-fold with nuclease-free water prior to PCR, which was performed on a CFX96 amplifier (Bio-Rad Laboratories Inc.) in a 20 μL reaction with 20 pmol forward primers, 10 pmol reverse primers and 4 pmol TaqMan™ probe in 5x Mas$^{CFE}$ MIX-2025 buffer (MCFE-100, Dialat) under the following conditions 94 °C 3', followed by 44 cycles of 94 °C 30', Tm 30', 72 °C 30' (Tm for each miRNA detection system are listed in supplement). The reactions were performed in triplicate, and only results with a standard deviation of less than 0.3 were considered acceptable. Bio-Rad CFX Maestro 1.1 v.4.1 software was used to analyze the data and calculate cycle thresholds (Ct). The relative expression level was determined using the $-\Delta Ct$ index, where $\Delta Ct = Ct$ (miRNA) – Ct (reference sequence); in cells, the normalizer used was small nuclear RNA U6, while in the case of EVs, the geometric mean Ct of miR-191-5p and miR-151a-3p was used. The negative value of $\Delta Ct$ was employed to simplify the data presentation on the graph.

**Statistical analysis.** Statistical processing of the obtained data was performed in GraphPad Prism 9.4.0. The Mann–Whitney U test was used to compare groups of highly and poorly represented miRNAs in cells and EVs, and to compare the level of miRNA representation in cells and EVs of the corresponding cell line. Analysis of variance (one-factor ANOVA followed by Dunnett's post-hoc test) was used for multiple comparisons of individual miRNAs in cells and EVs. Correlation analysis was performed using Pearson's correlation coefficient, $p < 0.05$.

## RESULTS

Characterization of EVs isolated from the conditioned medium of OC cells. To confirm the nature of the samples obtained, EVs from all four OC cell lines were characterized using three different methods in accordance with the MISEV2018 guidelines [9]: NTA to evaluate particle size distribution and concentration, transmission electron microscopy (TEM) to analyze vesicle size and morphology, and immunoblotting to analyze exosomal markers. Based on TEM data, the isolated particles were found to have a membrane and a size of less




| Particle | Average size, nm | Mode, nm | Particle concentration, × 10¹²/mL |
|---|---|---|---|
| OVAR-3 | 134.69 ± 8.16 | 106 ± 18.61 | 2.01 ± 0.37 |
| OVAR-4 | 117.64 ± 6.23 | 76 ± 21.79 | 3.22 ± 0.62 |
| OVAR-8 | 135.62 ± 8.74 | 122 ± 19.63 | 0.45 ± 0.23 |
| SKOV3 | 138.42 ± 13.27 | 126 ± 15.25 | 1.57 ± 0.16 |

**Fig. 1.** *Verification of extracellular vesicles (EVs) isolated from conditioned medium of ovarian cancer cells: a – transmission electron microscopy analysis of the EV morphology, scale bar 500 nm. Examples of EVs isolated from OVCAR-8 and SKOV3 cell lines; b – nanoparticle tracking analysis data for evaluation of the EV size distribution and concentration and mean values for the EV size, median, mode and concentration; c – Western blot analysis of exosomal markers in cells and secreted EVs. The PCNA protein was used to confirm the absence of cellular proteins of non-vesicular origin in EV preparations*

than 200 nm. The particles exhibited a cup-shaped morphology common to this type of analysis (fig. 1, *a*).

The NTA analysis showed that all samples displayed a size distribution typical of small vesicles (fig. 1, *b*). To validate the nature of the obtained particles, exosomal markers were analyzed. For this task, we utilized various proteins from different intracellular compartments, including TSG101, a component of the ESCRT-I complex involved in exosome biogenesis, as well as CD9 tetraspanin and components of lipid rafts such as flotillin-2 and stomatin, in accordance with ISEV recommendations. We previously proposed the latter protein as a highly selective exosome marker [13]. The PCNA protein served as a negative control to confirm the absence of non-vesicular cellular particles in the EV samples. As depicted in fig. 1, *c*, all EV preparations demonstrated a pronounced enrichment of all exosomal markers and the absence of PCNA, which was solely detected in cell lysates of the corresponding OC cell lines.

**Selection of reference molecules for reverse transcription-quantitative polymerase chain reaction data normalization.** A challenge in analyzing miRNAs in EVs using RT-qPCR is the absence of universally accepted reference sequences for data normalization. Although the small nuclear RNA U6 is the most frequently used normalizer for cellular miRNA analysis, numerous different sequences are employed as reference molecules in the study of EV molecular composition. Most studies do not provide justification for the selection of specific molecules or confirm their compliance with normalization criteria. To address this issue, we first validated U6 as a reference molecule for RT-PCR analysis of cellular miRNAs and selected reference sequences for the analysis of EV miRNAs. Based on the data we obtained from the transcriptome analysis of small EV RNAs, we selected miR-342-3p and miR-191-5p, as well as miR-151a-3p, which was suggested as a normalizer [14], as potential references.

The screening of potential reference sequences by Stem-Loop RT-qPCR (fig. 2, *a*) and subsequent analysis using the RefFinder algorithm (https://blooge.cn/RefFinder/?type=reference [15]), which combines four methods to search for normalizers, resulted in the identification of an optimal combination of two reference sequences, miR-151a-3p and miR-191-5p (fig. 2, *b*).

**MiRNAs upregulated in extracellular vesicles of ovarian cancer patients are characterized by higher expression in ovarian cancer cells.** We analyzed the expression of seven miRNAs in OVCAR-3, OVCAR-4, OVCAR-8, and SKOV3 cell lines: miR-125a-5p, -27a-5p, -193a-5p, -135b-5p, -451a, -495-3p, and -136-5p. These miRNAs were previously shown to have significant DE in EVs from uterine aspirates of OC patients (UA-OC EVs) compared to healthy donors. The results of the Stem-Loop RT-qPCR were normalized relative to U6 for cells and to the selected reference molecules, miR-151a-3p and miR-191-5p, for EVs.

Based on the level of expression in cells, the studied miRNAs could be clearly divided into two distinct groups. These groups correspond precisely to the miRNAs that were significantly upregulated (UA-UP – miR-125a-5p, -27a-5p, -193a-5p, and -135b-5p) and downregulated (UA-DOWN – miR-451a, -495-3p, -136-5p) in UA-OC EVs, according to our data.

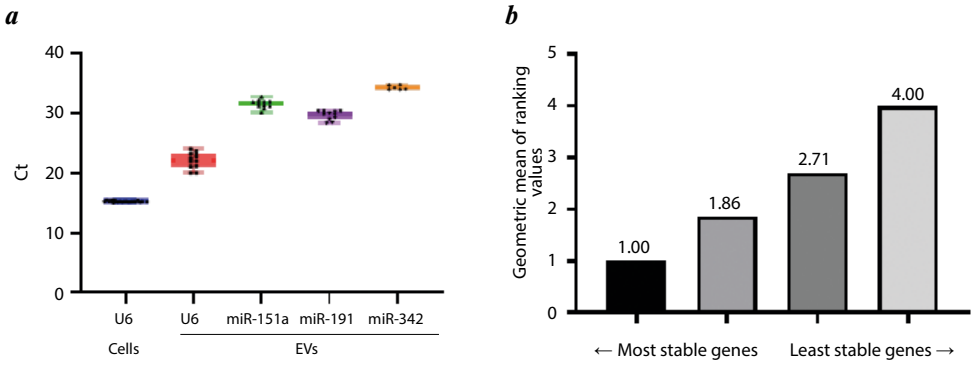



**Fig. 2.** *Analysis of potential reference miRNAs for normalization of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data in cells and secreted extracellular vesicles (EVs): a – amplification cycles of endogenous U6, miR-151a, miR-191 and miR-342 measured by Stem-Loop RT-qPCR in cells and secreted EVs of four ovarian cancer cell lines; b – comprehensive gene stability in secreted EVs evaluated by RefFinder tool*

Figure 3, *a* shows significantly higher expression levels of miRNAs belonging to the UA-UP group compared to miRNAs from the UA-DOWN group in all investigated cell lines (Mann–Whitney U test $p < 0.05$). Additionally, multiple pairwise comparisons using analysis of variance revealed that the level of each miRNA from the first group was significantly higher than that of each miRNA from the second group (ANOVA followed by Dunnett's post-hoc test). On average, the difference in expression was 1500-fold, with miR-125a-5p exhibiting the highest expression level, 40,000-fold higher than the average mean for miRNAs from the UA-DOWN group. The least expressed miRNA was miR-136-5p, which was expressed about 7-fold lower than other miRNAs in the UA-DOWN group.

The miRNA expression pattern in EVs varied (fig. 3, *b*), but no statistically significant differences were found between the two groups. Among all the examined miRNAs miR-125a showed the highest level of expression in both EVs and cells. However, miR-451a from the UA-DOWN group exhibited the second-highest level of expression. It's level in EVs was only one order of magnitude lower than that of miR-125a. In cells, this difference reached four orders of magnitude. Meanwhile, miR-136-5p remained the least expressed of all the examined miRNAs.

**MiRNAs from the UA-UP and UA-DOWN groups exhibit an inverse expression ratio between ovarian cancer cells and secreted vesicles.** In the next step, we analyzed the ratio of each investigated miRNA in OC cells and the vesicles they

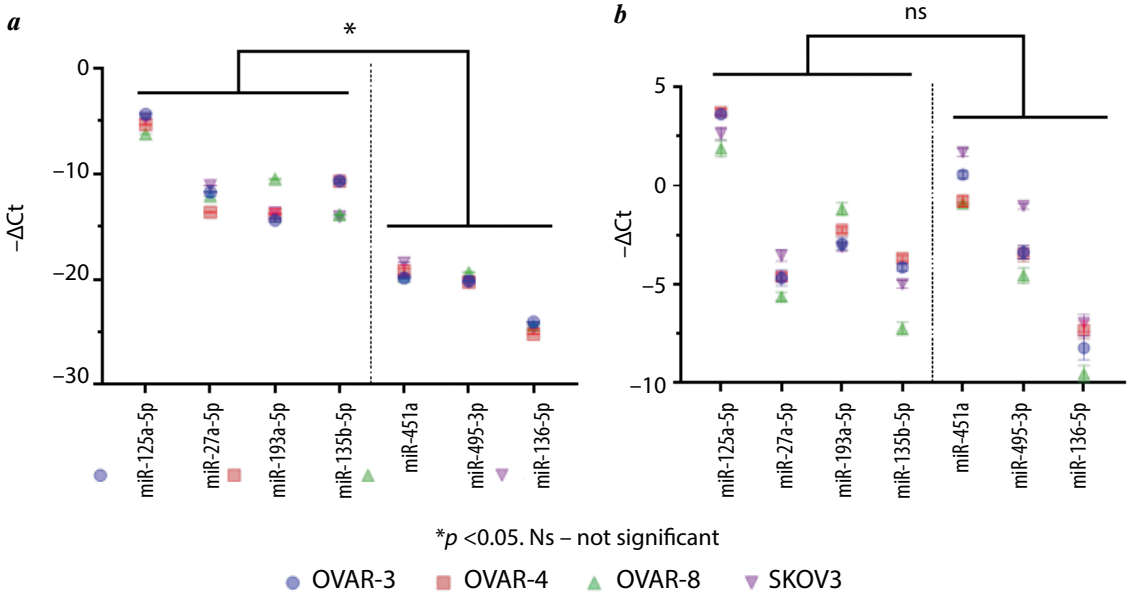



**Fig. 3.** *MiRNA levels in parental cells of four ovarian cancer cell lines (a) and in the small extracellular vesicles (EVs) they secrete (b). The vertical dashed line separates miRNAs from the UA-UP and UA-DOWN groups. The normalized −ΔCt value (negative value is used for convenience of data presentation on the graph) was calculated relative to the corresponding reference gene: U6 in case of cells (a) and geometric mean Ct of miR-191-5p and miR-151a-3p for EVs (b). ΔCt = Ct (miR) – Ct (reference)*

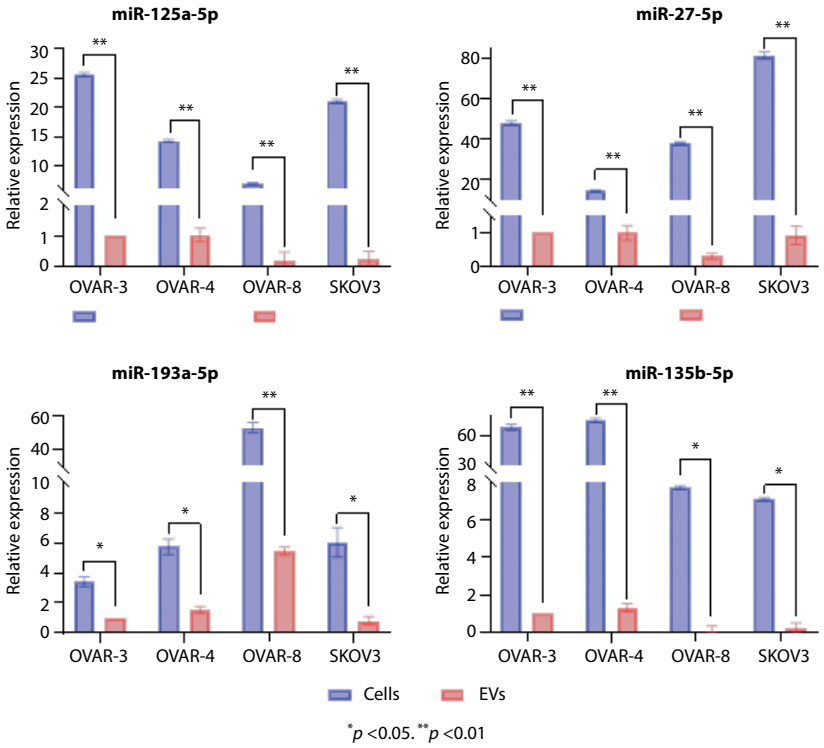



**Fig. 4.** *Comparison of the level of miRNA of the UA-UP group in cells and secreted extracellular vesicles (EVs). Expression values are presented as relative to the level of the corresponding miRNA in EVs of OVCAR-3 line*

produce. As there is currently no universal reference sequence for the simultaneous comparative analysis of miRNAs in cells and EVs, we determined the absolute expression level for each specific miRNA by normalizing it to the mass of total small RNA introduced into the reaction.

The results of the analysis indicated that the miRNAs from the UA-UP and UA-DOWN groups had an opposite expression ratio between the parental cells and their secreted vesicles (EV/cell ratio). In all lines studied, the levels of each miRNA from the UA-UP group (miR-125a-5p, -27a-5p, -193a-5p, and -135b-5p) were significantly higher in cells than in their secreted vesicles ("cells >EVs"; $p < 0.05$), with an average difference of 47-fold (fig. 4). The difference was minimal for miR-193a, which showed a 3-10-fold higher level in cells than in EVs. This indicates a more balanced distribution of this miRNA between intracellular and vesicular compartments. The remaining miRNAs displayed more significant differences in expression ratios.

Interestingly, two miRNAs, specifically miR-193a and miR-135b, showed a strong positive correlation between expression levels in cells and EVs, with Pearson correlation coefficients of 0.99 and 0.98, respectively ($p < 0.05$). This suggests a stable distribution pattern of these miRNAs between these compartments.

For all miRNAs in the UA-DOWN group (miR-451a, -495-3p, -136-5p), the correlation was the opposite. In all cell lines, the level of each miRNA from this group was significantly higher in vesicles compared to the intracellular level ("EVs >cells") ($p < 0.05$) (fig. 5).

In particular, miR-451a expression in EVs exceeded its intracellular level by 27-118-fold, depending on the cell line. For miR-495-3p, a 3-fold increase in EVs was detected in the OVCAR-8 line and an even more pronounced 36-fold increase in the SKOV-3 line compared to the cellular level. Similarly, the level of miR-136-5p in EVs was 3 times higher than the intracellular level in the OVCAR-8 line and 32 times higher in the OVCAR-4 line.

Therefore, we found that miRNAs differentially expressed in EVs from uterine aspirates of OC patients exhibit significant DE in OC cells, with a significant predominance of molecules upregulated in the EVs of OC patients. Additionally, these miRNAs display an inverse ratio between their levels in the parental cells and the secreted vesicles. The miRNAs that were found to be upregulated in the EVs of OC patients are characterized by "cells >EVs" ratio, while the downregulated miRNAs are characterized by the ratio "EVs >cells".

The observed enrichment of OC cells with miRNA molecules that are upregulated in UA-OC EVs indicates their tumor-promoting activity. Conversely, the decreased levels of miRNAs that are downregulated in UA-OC EVs suggest their tumor-suppressive activity. Meanwhile, each miRNA from the latter group was significantly more represented in vesicles than in parental cells ("EVs >cells") ($p < 0.05$) (fig. 5).

In summary, the analysis of miRNAs in parental OC cells and secreted EVs revealed the controlled and context-dependent nature of miRNA distribution between ve-

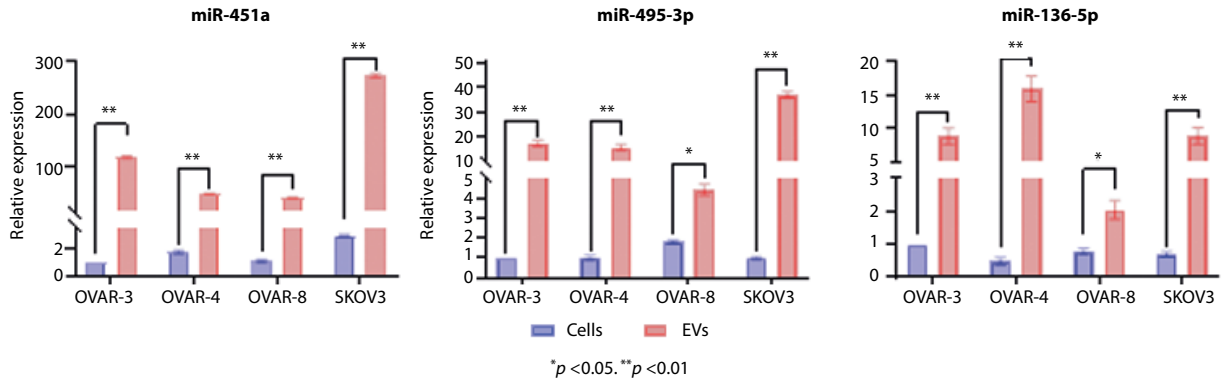



**Fig. 5.** *Comparison of the level of miRNA of the UA-DOWN group in cells and secreted extracellular vesicles (EVs). Expression values are presented as relative to the level of the corresponding miRNA in cells of OVCAR-3 line*

sicular and cellular compartments. MiRNAs with potential tumor-promoting activity accumulate to a greater extent in cells, while molecules with potential tumor-suppressing activity are eradicated as part of EVs.

## DISCUSSION

In recent decades, secreted EVs, and exosomes in particular, have gained significant scientific attention in experimental oncology due to their potential in both the diagnosis and therapy of malignant neoplasms. Initially described as cellular waste disposal units in the 1980s, these nanoscale vesicles have since been recognized as playing a crucial role in intercellular communication under both normal and pathological conditions [3]. Numerous studies have demonstrated their fundamental role in carcinogenesis. EVs play a crucial role in various processes, including tumor cell proliferation, epithelial-mesenchymal transition, migration, invasion, angiogenesis, and metastasis [16]. They provide intercellular transport of bioactive molecules, which can affect antitumor immunity, alter the microenvironment, and promote tumor development and resistance to therapy. Exosomes selectively and controllably load miRNAs, which are crucial regulators of expression, through various mechanisms [17]. Exosomal miRNAs can be transported locally or to distant organs and tissues, where they participate in epigenetic regulation and alter intracellular signaling in recipient cells.

Therefore, exosomal miRNAs have gained increasing attention as potential diagnostic and prognostic markers of malignant neoplasms [18]. Previously, we showed for the first time that vesicles corresponding to EVs according to ISEV criteria can be isolated by ultracentrifugation from individual uterine aspirate samples in sufficient concentration for subsequent transcriptome analysis [11]. Additionally, we optimized techniques for small RNA isolation from EVs and their subsequent analysis [19]. According to the results of next-generation deep sequencing (NGS-small RNA-seq) performed on a pilot sample of EVs from uterine aspirates, significant differences were observed in the miRNA profiles of patients diagnosed with OC and healthy donors [11]. A follow-up study performed on an expanded sampling, using stringent selection criteria (false discovery rate <0.001, FC >2), revealed 35 differentially expressed miRNAs (data in press). Notably, the miRNAs identified as up- or downregulated in UA-OC EVs include molecules with known tumor-promoting or tumor-suppressing activities. For instance, miR-205-5p and the members of the miR-200 family, which have been repeatedly shown to be upregulated in EVs from OC patients [20], as well as several other known cancer promoters. The second group included known miRNAs with tumor suppressor activity such as miR-152-3p [21], -424-5p [22, 23], -199a and -199b [24, 25], among others. Meanwhile, we detected molecules whose role in carcinogenesis in general and in OC progression remains unclear in the literature, as well as miRNAs that are little known in this context.

To assess the extent to which the identified changes are related to the pathogenesis of OC, in this study we evaluated the expression of certain miRNAs from both groups (upregulated and downregulated in UA-OC EVs) in OC cells cultured in vivo and in EVs from conditioned medium.

The selection of miRNAs for the study was made in such a way that each of the miRNA groups (up- and downregulated in UA-OC EVs) included both known tumor promoter/suppressor molecules and miRNAs with contradictory or poorly represented activity data. Accordingly, from UA-UP group we selected miR-135b, which has been shown to promote OC tumor cell growth and resistance to chemotherapy [26–28]; miR-125a, which is considered to be more of a suppressor miRNA, inhibiting proliferation, epithelial-mesenchymal transition, migration and invasion of OC cells [29–31], and miR-193a-5p, which also display distinct and predominantly tumor suppressor activity [32–34], although the latter two miRNAs have been shown to be overexpressed in stage III OC compared to stage I OC [35]. Also, miR-27a-5p shows conflicting data in the context of OC progression (increased sensitivity of OC cells to cisplatin [36], stimulation of migration and

invasion [37], decreased representation of the $CX_3CR1$ receptor in natural killer cells [38]).

Among the miRNAs downregulated in UA-OC EVs, we selected the known suppressor molecules, miR-495-3p [39–41] and miR-136-5p [42, 43], as well as miR-451a. The data on miR-451a in the context of OC are contradictory: while in one study miR-451 expression was reduced in OC compared to normal tissues and its low level was associated with late the International Federation of Gynecology and Obstetrics FIGO) stage, high serum CA-125 levels, metastasis to lymph nodes and poor prognosis for patients [44], in another study, miR-451a was overexpressed in stage III OC compared to stage I [35]. In support of this claim, a third study showed that the expression of miR-27a-3p and miR-451a was upregulated in multidrug-resistant OC and cervical cancer cell lines. This upregulation led to the activation of P-glycoprotein, a product of the *MDR1* gene, which confers resistance of tumor cells to a wide range of chemotherapeutic drugs [45].

We found that cells from all four OC lines examined were enriched in miRNAs that were upregulated in UA-OC EVs. Conversely, the cellular expression of all miRNAs that were downregulated in UA-OC EVs was significantly lower, with a difference of several orders of magnitude. These results were quite expected and supported our assumption of a tumor-promoting or tumor-suppressive role of these miRNAs in OC. It should be noted that although, as mentioned above, miR-125a-5p and miR-193a-5p are more likely to be classified as suppressor molecules according to the literature data, both miRNAs have been shown to be associated with OC progression, particularly with overexpression in stage III compared to stage I [35]. Furthermore, according to the results of a large cohort study published in Lancet Oncol in 2016, increased miR-193a-5p expression was associated with negative prognosis and recurrence [46]. These findings were later confirmed by the authors in a different cohort [47] and may explain the high levels of these miRNAs in OC cells, as well as their increase in UA-OC EVs, especially considering that the majority of EV preparations from uterine aspirates were obtained from patients with high-grade adenocarcinoma, the most aggressive form of OC, and predominantly represented late stages of the disease (mainly stage III).

In contrast, the analysis of the ratio of miRNAs in parental OC cells and secreted EVs yielded unexpected results. Given the putative role of these miRNAs as promoters/suppressors of tumor progression, we expected to see either a similar miRNA levels in EVs and parental cells, or even enrichment of EVs with potential tumor-promoting miRNAs, and vice versa. However, the study revealed the contrary result – the "cells >EVs" ratio characterized potential tumor-promoting molecules, while the "EVs >cells" ratio characterized suppressor molecules. These ratios were observed for all studied miRNAs and all cell lines. It should be noted that despite significant progress in understanding the mechanisms of loading molecules, including miRNAs, into EVs [17, 48], the question of what determines the selection of such molecules for inclusion in the EV cargo is still discussed in the literature and remains unanswered. In this context, the results of several studies demonstrating selective loading of certain miRNAs into EVs are of particular interest. It has been suggested that miRNAs can be divided into those that are secreted to a greater extent and those that are preferentially retained in the cell and incorporated into EVs to a lesser extent [49, 50]. However, there is currently little data on which miRNAs belong to each group, and it is clear that their composition depends on cell growth conditions. Notably, miR-451a is reported to be an example of miRNAs preferentially loaded into EVs, which is consistent with our findings [49].

Another possible explanation for the results obtained is the peculiarities of EVs secretion by cells growing in two-dimensional culture. It is assumed that during the growth of clonal culture on the substrate, tumor cells are primarily guided by "their own needs". They overexpress and accumulate tumor-promoting miRNAs for their own growth, while limiting the expression and releasing from suppressor molecules, including through their secretion as part of EVs.

In contrast to growth in two-dimensional culture, tumor development *in vivo* involves competitive growth of cells in a heterogeneous population and remodeling of the microenvironment. This includes reprogramming of stromal fibroblasts, endothelial cells, immune cells, and stimulation of neoangiogenesis, as well as activation of other survival mechanisms. Under these conditions, tumor cells produce more vesicles for delivery to cells in the microenvironment and distant tissues. This is likely to be accompanied by changes in the spectrum of molecules, including miRNAs, that are preferentially secreted as part of EVs, which may lead to a shift in the "cell/EV" ratio. Results comparing EVs secreted by cells in 3D and 2D models suggest that such changes occur [51, 52]. Furthermore, the molecular composition of EVs secreted by cells in 3D models appears to be closer to the composition of EVs from the corresponding biological fluids [53, 54], which confirms our hypothesis and is consistent with the data obtained on the enrichment/reduction of tumor-promoting/suppressor miRNAs in EVs from uterine aspirates.

## CONCLUSION

Here, we showed for the first time that the levels of miR-125a-5p, -27a-5p, -193a-5p and -135b-5p in OC cells are significantly higher than those of miR-451a, -495-3p and -136-5p, indicating the tumor-promoting and tumor-suppressive activities of these molecules, respectively, and confirming their potential diagnostic value in OC. The cell-to-EV ratio for studied miRNAs is characterized by a decrease in EV levels for highly expressed miRNAs and an increase for low-expressed miRNAs.

**Supplement**

*Sequences of primers and TaqMan probes used. Annealing temperatures (Tm) of primers*

| Primer s and TaqMan probes | miRNA | Sequence 5'-3' | Tm, °C |
|---|---|---|---|
| RT-primers | miR-125a-5p | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAG | – |
| | miR-27a-5p | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCTCA | – |
| | miR-193a-5p | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATCT | – |
| | miR-135b-5p | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAT | – |
| | miR-451a | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTC AG | – |
| | miR-495-3p | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGAAG | – |
| | miR-136-5p | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAT | – |
| | miR-151a-3p | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTCAA | – |
| | miR-191-5p | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG | – |
| | U6 | CGCTTCACGAATTTGCGTGTCAT | – |
| F-primers | miR-125a-5p | ACGCATCCCTGAGACCC | 56 |
| | miR-27a-5p | CACGCAAGGGCTTAGCTG | 56 |
| | miR-193a-5p | AACAAGTGGGTCTTTGCG | 56 |
| | miR-135b-5p | CACGCATATGGCTTTTCA | 54 |
| | miR-451a | CACGCATAAACCGTTACCA | 56 |
| | miR-495-3p | CGGCGGAAACAAACATGG | 53 |
| | miR-136-5p | CGGCGGACTCCATTTGTT | 54 |
| | miR-151a-3p | CGGCGGCTAGACTGAAGC | 54 |
| | miR-191-5p | CACGCACAACGGAATCCC | 56 |
| | U6 | CTCGCTTCGGCAGCACATA | 60 |
| Universal R-primer | | GTGCAGGGTCCGAGGT | – |
| R-primer | U6 | CGCTTCACGAATTTGCGTG | – |
| TaqMan-probes | miR-125a-5p | FAM-GCACTGGATACGACTCACAGGTT-BHQ1 | – |
| | miR-27a-5p | FAM-TGGATACGACTGCTCACAAG-BHQ1 | – |
| | miR-193a-5p | FAM-CTGGATACGACTCATCTCGCC-BHQ1 | – |
| | miR-135b-5p | FAM - CTGGATACGACTCACATAGGA - BHQ-1 | – |
| | miR-451a | FAM-TTACTGAGTTGTCGTATCC-BHQ1 | – |
| | miR-495-3p | FAM-TGGATACGACAAGAAGTGCA-BHQ1 | – |
| | miR-136-5p | FAM-TGGATACGACTCCATCATCA-BHQ1 | – |
| | miR-151a-3p | FAM-CTGGATACGACCCTCAAGGA-BHQ1 | – |
| | miR-191-5p | FAM-CTGGATACGACCAGCTGCTT-BHQ1 | – |
| | U6 | FAM-CCTTGCGCAGGGGCCATGC-BHQ-1 | – |

## REFERENCES / ЛИТЕРАТУРА


1. Raposo G., Stahl P.D. Extracellular vesicles – on the cusp of a new language in the biological sciences. Extracell Vesicles Circ Nucleic Acids 2023;4(2):240–54. DOI: 10.20517/evcna.2023.18
2. Kalluri R., LeBleu V.S. The biology, function, and biomedical applications of exosomes. Science 2020;367(6478):eaau6977. DOI: 10.1126/science.aau6977
3. Liu Y.-J., Wang C. A review of the regulatory mechanisms of extracellular vesicles-mediated intercellular communication. Cell Commun Signal 2023;21(1):77. DOI: 10.1186/s12964-023-01103-6
4. Xu R., Rai A., Chen M. et al. Extracellular vesicles in cancer – implications for future improvements in cancer care. Nat Rev Clin Oncol 2018;15(10):617–38. DOI: 10.1038/s41571-018-0036-9
5. Logozzi M., Mizzoni D., Di Raimo R., Fais S. Exosomes: a source for new and old biomarkers in cancer. Cancers 2020;12(9):2566. DOI: 10.3390/cancers12092566
6. Staicu C.E., Predescu D.V., Rusu C.M. et al. Role of microRNAs as clinical cancer biomarkers for ovarian cancer: a short overview. Cells 2020;9(1):169. DOI: 10.3390/cells9010169
7. Meng X., Müller V., Milde-Langosch K. et al. Diagnostic and prognostic relevance of circulating exosomal miR-373, miR-200a, miR-200b and miR-200c in patients with epithelial ovarian cancer. Oncotarget 2016;7(13):16923–35. DOI: 10.18632/oncotarget.7850
8. Pan C., Stevic I., Müller V. et al. Exosomal microRNAs as tumor markers in epithelial ovarian cancer. Mol Oncol 2018;12(11):1935–48. DOI: 10.1002/1878-0261.12371
9. Théry C., Witwer K.W., Aikawa E. et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 2018;7(1):1535750. DOI: 10.1080/20013078.2018.1535750
10. Salmond N., Williams K.C. Isolation and characterization of extracellular vesicles for clinical applications in cancer – time for standardization? Nanoscale Adv 2021;3(7):1830–52. DOI: 10.1039/d0na00676a
11. Skryabin G.O., Komelkov A.V., Zhordania K.I. et al. Extracellular vesicles from uterine aspirates represent a promising source for screening markers of gynecologic cancers. Cells 2022;11(7):1064. DOI: 10.3390/cells11071064
12. Kramer F. Stem-Loop RT-qPCR for miRNAs. Curr Protoc Mol Biol 2011;Chapter 15:Unit15.10. DOI: 10.1002/0471142727.mb1510s95
13. Skryabin G.O., Komelkov A.V., Galetsky S.A. et al. Stomatin is highly expressed in exosomes of different origin and is a promising candi-date as an exosomal marker. J Cell Biochem 2021;122(1):100–15. DOI: 10.1002/jcb.29834
14. Burdiel M., Jiménez J., Rodríguez-Antolín C. et al. MiR-151a: a robust endogenous control for normalizing small extracellular vesicle cargo in human cancer. Biomark Res 2023;11(1): 94. DOI: 10.1186/s40364-023-00526-0
15. Xie F., Wang J., Zhang B. RefFinder: a web-based tool for comprehensively analyzing and identifying reference genes. Funct Integr Genomics 2023;23(2):125. DOI: 10.1007/s10142-023-01055-7
16. Wang X., Huang J., Chen W. et al. The updated role of exosomal proteins in the diagnosis, prognosis, and treatment of cancer. Exp Mol Med 2022;54(9):1390–400. DOI: 10.1038/s12276-022-00855-4
17. Zhang J., Li S., Li L. et al. Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics 2015;13(1):17–24. DOI: 10.1016/j.gpb.2015.02.001
18. Liu Q.-W., He Y., Xu W.W. Molecular functions and therapeutic applications of exosomal noncoding RNAs in cancer. Exp Mol Med 2022;54(3):216–25. DOI: 10.1038/s12276-022-00744-w
19. Skryabin G.O., Vinokurova S.V., Elkina N.V. et al. Comparison of methods for microRNA isolation from extracellular vesicles obtained from ascitic fluids. Biochemistry 2022;87(11):1354–66. DOI: 10.1134/S0006297922110141
20. Koutsaki M., Libra M., Spandidos D.A., Zaravinos A. The miR-200 family in ovarian cancer. Oncotarget 2017;8(39):66629–40. DOI: 10.18632/oncotarget.18343
21. Liu X., Li J., Qin F., Dai S. miR-152 as a tumor suppressor microRNA: target recognition and regulation in cancer. Oncol Lett 2016;11(6):3911–6. DOI: 10.3892/ol.2016.4509
22. Xuan J., Liu Y., Zeng X., Wang H. Sequence requirements for miR-424-5p regulating and function in cancers Int J Mol Sci 2022;23(7):4037. DOI: 10.3390/ijms23074037
23. Timofeeva A.V., Fedorov I.S., Asaturova A.V. et al. Blood plasma small non-coding RNAs as diagnostic molecules for the progesterone-receptor-negative phenotype of serous ovarian tumors. Int J Mol Sci 2023;24(15):12214. DOI: 10.3390/ijms241512214
24. Gadducci A., Sergiampietri C., Lanfredini N., Guiggi I. MicroRNAs and ovarian cancer: the state of art and perspectives of clinical research. Gynecol Endocrinol 2014;30(4):266–71. DOI: 10.3109/09513590.2013.871525
25. Jiang Y., Shi Y., Lyu T. et al. Identification and functional validation of differentially expressed microRNAs in ascites-derived ovarian cancer cells compared with primary tumour tissue. Cancer Manag Res 2021;13:6585–97. DOI: 10.2147/CMAR.S320834
26. Wang J., Zhang R., Zhang B. et al. MiR-135b improves proliferation and regulates chemotherapy resistance in ovarian cancer. J Mol Histol 2022;53(4):699–712. DOI: 10.1007/s10735-022-10080-y
27. Chen H., Mao M., Jiang J.D. et al. Circular RNA CDR1as acts as a sponge of miR-135b-5p to suppress ovarian cancer progression. OncoTargets Ther 2019;12:3869–79. DOI: 10.2147/OTT.S207938
28. Yu S., Yu M., Chen J. et al. Circ_0000471 suppresses the progression of ovarian cancer through mediating mir-135b-5p/dusp5 axis. Am J Reprod Immunol 2023;89(4):e13651. DOI: 10.1111/aji.13651
29. Cao Y., Shen T., Zhang C. et al. MiR-125a-5p inhibits EMT of ovarian cancer cells by regulating TAZ/EGFR signaling pathway. Eur Rev Med Pharmacol Sci 2019;23(19):8249–56. DOI: 10.26355/eurrev_201910_19134
30. Lee M., Kim E.J., Jeon M.J. MicroRNAs 125a and 125b inhibit ovarian cancer cells through post-transcriptional inactivation of EIF4EBP1. Oncotarget 2015;7(8):8726–42. DOI: 10.18632/oncotarget.6474
31. Yang J., Li G., Zhang K. MiR-125a regulates ovarian cancer proliferation and invasion by repressing GALNT14 expression. Biomed Pharmacother 2016;80:381–7. DOI: 10.1016/j.biopha.2015.12.027
32. Wang Y., Li N., Zhao J., Dai C. MiR-193a-5p serves as an inhibitor in ovarian cancer cells through RAB11A. Reprod Toxicol 2022;110:105–12. DOI: 10.1016/j.reprotox.2022.04.003
33. Zhang S., Liu J., He J., Yi N. MicroRNA-193a-5p exerts a tumor suppressive role in epithelial ovarian cancer by modulating RBBP6. Mol Med Rep 2021;24(2):582. DOI: 10.3892/mmr.2021.12221
34. Khordadmehr M., Shahbazi R., Sadreddini S., Baradaran B. miR-193: a new weapon against cancer. J Cell Physiol 2019;234(10): 6861–72. DOI: 10.1002/jcp.28368
35. Eitan R., Kushnir M., Lithwick-Yanai G. et al. Tumor microRNA expression patterns associated with resistance to platinum based chemotherapy and survival in ovarian cancer patients. Gynecol Oncol 2009;114(2):253–9. DOI: 10.1016/j.ygyno.2009.04.024
36. Wambecke A., Ahmad M., Morice P.M. et al. The lncRNA 'UCA1' modulates the response to chemotherapy of ovarian cancer through direct binding to miR-27a-5p and control of UBE2N levels. Mol Oncol 2021;15(12):3659–78. DOI: 10.1002/1878-0261.13045
37. Che X., Jian F., Chen C. et al. PCOS serum-derived exosomal miR-27a-5p stimulates endometrial cancer cells migration and invasion. J Mol Endocrinol 2020;64(1):1–12. DOI: 10.1530/JME-19-0159
38. Regis S., Caliendo F., Dondero A. et al. TGF-β1 downregulates the expression of CX3CR1 by inducing miR-27a-5p in primary human NK cells. Front Immunol 2017;8. Available at: https://www.frontiersin.org/articles/10.3389/fimmu.2017.00868
39. Huldani H., Malviya J., Rodrigues P. et al. miR-495–3p as a promising tumor suppressor in human cancers. Pathol Res Pract 2023;248:154610. DOI: 10.1016/j.prp.2023.154610
40. Chen H., Wang X., Bai J., He A. Expression, regulation and function of miR-495 in healthy and tumor tissues. Oncol Lett 2017;13(4):2021–6. DOI: 10.3892/ol.2017.5727

41. Liu S., Xi X. LINC01133 contribute to epithelial ovarian cancer metastasis by regulating miR-495-3p/TPD52 axis. Biochem Biophys Res Commun 2020;533(4):1088–94. DOI: 10.1016/j.bbrc.2020.09.074
42. Zhu J., Luo J.E., Chen Y., Wu Q. Circ_0061140 knockdown inhibits tumorigenesis and improves PTX sensitivity by regulating miR-136/CBX2 axis in ovarian cancer. J Ovarian Res 2021;14(10):136. DOI: 10.1186/s13048-021-00888-9
43. Zhao H., Liu S., Wang G. et al. Expression of miR-136 is associated with the primary cisplatin resistance of human epithelial ovarian cancer. Oncol Rep 2015;33(2):591–8. DOI: 10.3892/or.2014.3640
44. Ling S., Ruiqin M., Guohong Z., Ying W. Expression and prognostic significance of microRNA-451 in human epithelial ovarian cancer. Eur J Gynaecol Oncol 2015;36(4):463–8.
45. Zhu H., Wu H., Liu X. et al. Role of microRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells. Biochem Pharmacol 2008;76(5):582–8. DOI: 10.1016/j.bcp.2008.06.007
46. Bagnoli M., Canevari S., Califano D. et al. Development and validation of a microRNA-based signature (MiROvaR) to predict early relapse or progression of epithelial ovarian cancer: a cohort study. Lancet Oncol 2016;17(8):1137–46. DOI: 10.1016/S1470-2045(16)30108-5
47. De Cecco L., Bagnoli M., Chiodini P. et al. Prognostic evidence of the miRNA-based ovarian cancer signature MiROvaR in independent datasets. Cancers 2021;13(7):1544. DOI: 10.3390/cancers13071544
48. Pucci M., Reclusa Asiáin P., Duréndez Sáez E. et al. Extracellular vesicles as miRNA nano-shuttles: dual role in tumor progression. Target Oncol 2018;13(2):175–87. DOI: 10.1007/s11523-018-0551-8
49. Guduric-Fuchs J., O'Connor A., Camp B. et al. Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. BMC Genomics 2012;13:357. DOI: 10.1186/1471-2164-13-357
50. Ohshima K., Inoue K., Fujiwara A. et al. Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. PLoS One 2010;5(10): DOI: 10.1371/journal.pone.0013247
51. Bordanaba-Florit G., Madarieta I., Olalde B. et al. 3D cell cultures as prospective models to study extracellular vesicles in cancer. Cancers 2021;13(2):307. DOI: 10.3390/cancers13020307
52. Kusuma G.D., Li A., Zhu D. et al. Effect of 2D and 3D culture microenvironments on mesenchymal stem cell-derived extracellular vesicles potencies. Front Cell Dev Biol 2022;10:819726. DOI: 10.3389/fcell.2022.819726
53. Rocha S., Carvalho J., Oliveira P. et al. 3D cellular architecture affects microRNA and protein cargo of extracellular vesicles. Adv Sci Weinh Baden-Wurtt Ger 2019;6(4):1800948. DOI: 10.1002/advs.201800948
54. Thippabhotla S., Zhong C., He M. 3D cell culture stimulates the secretion of in vivo like extracellular vesicles. Sci Rep 2019;9(1):13012. DOI: 10.1038/s41598-019-49671-3

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