ARTICLE

Expression pattern of the microRNA miR-1 in ovarian cancer cell lines and tumor tissue samples implies a loss of miR-1's tumor suppressor properties

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Despite ovarian cancer (OC) represents the leading cause of death in gynecology, however, current understanding of the molecular machinery governing initiation, development and recurrence of the tumor is still limited. Particularly, lack of specific biomarkers defining the complex states of OC limits effective diagnosis and prognosis and, subsequently, hinders appropriate therapy strategies for OC patients. MicroRNA (miR) are small non-coding, regulatory RNA molecules which have critical functions in tumor biology, and which have been functionally grouped into tumor suppressive and tumor promoting miR. miR-1 has been described operating as a tumor suppressor which attenuates proliferation and progression in divers solid cancer entities. Currently, there are no data available concerning miR-1 functionality in progression of OC, particularly, in regard to its potential as a biomarker for OC diagnosis and treatment. Therefore, the present study examined miR-1 expression levels in established OC cell lines as well as in tissue samples from primary and relapsed OC patients to get a first understanding of putative miR-1 properties in OC progression. Notably, we found miR-1 in OC cell lines was linked to higher cell growth rates. Moreover, analysis of patient samples revealed miR-1 levels in relapsed tumors appeared being up-regulated compared to primary tumors. The findings of our preliminary study (1) may suggest a novel role of miR-1 in OC as a promotor of cancer progression or (2) exhibit a dysregulation of miR-1 functions by a so far unknown disruption of miR-1 regulatory cascades.

Keywords: ovarian cancer; microRNA-1; tumor suppressor; progression

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Introduction

Ovarian cancer (OC) is the leading cause of death among all gynecological malignancies [1]. The advanced stage at the time of diagnosis is defined by a lack of specific symptoms and explained by the absence of appropriate screening tests. Despite achievements in both surgical treatment and the development of new drugs the 5-year survival rate remains very low [2]. For these reasons, there is an urgent need to find new predictive and prognostic factors for OC diagnosis and prognosis as well as new targets for novel treatment options.

Even though OC represents the most lethal cancer in gynecological oncology, unfortunately, very little is known about the molecular machinery governing tumor development and progression. MicroRNA (miR) are small

non-coding RNA molecules that play a pivotal role in control of gene expression at the post-transcriptional level [3]. The molecular mode of action is characterized by the specific binding to mRNA followed by (1) an increased degradation of the target mRNA or (2) an enhanced repression of translation [4]. Current studies have identified dysregulated miR expression pattern in various malignancies and, thus, pointed to miR being exceedingly involved in tumor initiation and progression [5, 6]. Numerous miR have been shown to display tumor suppressor properties while others harbor oncogenic activities (oncomirs) [7]. Up to now, approximately 40 miR species have been identified and partially characterized with regard to their characteristics in OC progression; to our best knowledge, nothing is known about miR-1 functionality in OC. Formerly, miR-1 was specified to be specific for skeletal and heart muscle cells with nearly undetectable levels in other tissues [8]. However, recent studies showed miR-1 linked to central mechanisms in tumor biology, e.g. suppression of tumor growth, epithelial-mesenchymal transition, and cell motility [9–11], and from these properties it follows that miR-1 belongs to the miR group of tumor suppressors.

Therefore, the present work investigated for the first time expression levels of miR-1 in OC to get a first understanding of putative miR-1 properties in OC cells. Particularly, this preliminary study focused on miR-1 expression rates in five established OC cell lines and 21 tumor tissue samples to obtain first hints on the regulatory role of miR-1 in OC progression.

Materials and Methods

Cell Culture

The human OC cell lines OVCAR-3 and SKOV-3 - both received from Cell Lines Service (Eppelheim, Germany) - were propagated in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum (Biochrom), 0.125% gentamicin (Ratiopharm, Ulm, Germany) and 0.1% insulin (Novo Nordisk, Mainz, Germany), and DMEM/F12 (Life Technologies, Darmstadt, Germany) supplemented with 5% fetal calf serum and 0.125% gentamicin, respectively. The OC cell lines UWB1.289, TOV-112D, and TOV-21G - all purchased from the American Type Culture Collection (Manassas, VA, USA) - were propagated in RPMI (Biochrom)/MEGM (Lonza, Basel, Switzerland) 1:1 media mixture containing 3% fetal calf serum and 0.065% gentamicin (UWB 1.289) and MCDB105 (tebu-Bio, Offenbach, Germany)/Medium 199 (Biochrom) mixture containing 15% fetal calf serum and 0.125% gentamicin (TOV-112D, TOV-21G). Cells were passaged twice per week in a humidified atmosphere at 37°C and 5% CO_2.

Proliferation Assay

Cellular growth of OC cells was examined by cell counting utilizing a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany). Therefore, adherent cells were detached by trypsin treatment, suspended in CASYton (Roche Applied Science) as 1:100 dilution and 400 µl of cell suspension were analysed with 3 replicates. Measurement was performed using a capillary of 150 µm in diameter and cell line specific gate settings to discriminate between living , dead cells, and cellular debris: 9.00 µm/15.75 µm (OVCAR-3), 7.00 µm/15.15 µm (SKOV-3), 7.15 µm/15.65 µm (UWB1.289), 5.25 µm/10.15 µm (TOV-21G), and 6.15 µm/11.00 µm (TOV-112D).

Patients Samples

This study was approved by the Ethics Committee of the University Medicine Greifswald (registration no. III SV 05/04) and all patients signed informed consent forms. All samples and related clinical data were obtained from the Department of Gynecology and Obstetrics, Universitiy Medicine Greifswald. The control group consisted of women with no history of malignancy. In order to obtain detailed and complete data about the histo-pathological features of the tumores and the surgical treatment, the IMO (intraoperative mapping of ovarian cancer) documentation system was used [12].

RNA Preparation and cDNA Synthesis

For detection of miR-1 expression in OC cell lines OVCAR-3, SKOV-3, UWB1.289, TOV-112D, and TOV-21G, cells were grown in a 6-well cell culture plate to 80% confluency, and total RNA was prepared using peqGOLD Trifast Reagent (Peqlab Biotechnology, Erlangen, Germany) according to the manufacturer's instructions. For total RNA isolation from OC tumor tissue 50 - 150 g tissue were applied to the NucleoSpin RNA/Protein Kit (Macherey-Nagel, Düren, Germany) according to the supplier's instructions. Subsequently, RNA concentration was determined utilizing a Nanodrop 2000c UV/vis spectrophotometer (Peqlab Biotechnology) and RNA was stored at -80°C.

For reverse transcription 100 ng of total RNA were used with the Superscript II Reverse Transcriptase (Life Technologies) according to the protocol of Chen *et al*. [13]. Stem-loop primers for reverse transcription were designed as follows: miR-1 stem-loop: 5'-GTCGTATCCAGTGCAGGG TCCGAGGTATTCGCACTGGATACGACATACAT-3'; U6 stem-loop: 5'-GTCATCCTTGCGCAGG-3'.

Table 1. Patient characteristics, pathology results, and miR-1 levels

patient				Rel. miR-1 expression	
No.	**age**	**FIGO**	**pathology**	**primary**	**relapse**
1	53	IV	serous papillary	0.29 ± 0.10	-
2	61	IIIc	serous papillary	7.48 ± 3.47	-
3	65	IIIc	serous papillary	0.78 ± 0.94	-
4	71	IIIc	endometroid	0.15 ± 0.06	-
5	72	IV	serous papillary	0.24 ± 0.01	-
6	76	IIIc	serous papillary	0.06 ± 0.05	-
7	70	IIIc	serous papillary	0.37 ± 0.31	-
8	65	IIIb	serous papillary	0.34 ± 0.32	-
9	58	IIIc	serous papillary	0.18 ± 0.01	0.29 ± 0.06
10	55	IV	serous papillary	0.11 ± 0.00	0.36 ± 0.16
11	48	IIIc	serous papillary	0.05 ± 0.02	2.81 ± 0.44
12	35	IIIc	serous papillary	0.03 ± 0.01	0.17 ± 0.03
13	45	III	serous papillary	-	0.03 ± 0.03
14	72	IV	serous papillary	-	1.29 ± 0.03
15	76	IIIc	serous papillary	-	0.98 ± 0.00
16	70	III	serous papillary	-	1.07 ± 1.13
17	65	IIIc	serous papillary	-	0.17 ± 0.15

Quantification of miR-1 by Polymerase Chain Reaction

Quantification of miR-1 was performed with the SensiMix SYBR hi-ROX Kit (Bioline, Luckenwalde, Germany) on a CFX96 Real-Time System (Bio-Rad, München, Germany) with the CFX Manager software (Bio-Rad). The sequences of the PCR primers were as follows: miR-1 forward: 5'-GCCCGCTGGAATGTAAAGAAGTATG-3'; miR-1 reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6 forward: 5'-CGCTTCGGCAGCACATATAC-3'; U6 reverse: 5'-AGGGGCCATGCTAATCTTCT-3'. The cycling parameters were one denaturation cycle at 95°C for 5 min and 45 amplification cycles at 95°C for 10 sec, 60°C for 20 sec, and 72°C for 10 sec, followed by a melt curve analysis. For quantification, miR-1 signals were standardized to U6 RNA as reference.

Statistics

Data were evaluated using the graphics and statistics software program Graph Pad Prism (version 5.01) and expressed in column or box plot presentations. Statistical comparisons were performed using the unpaired Student's *t* test with results of $p \leq 0.05$ were given as significant.

Results

Highly variable miR-1 expression rates in OC cell lines

In this study we aimed at analysing expression levels of miR-1 in OC cell lines as well as in OC tissue samples. To proof whether miR-1 is expressed in cells from OC origin, we tempted to compare intracellular miR-1 concentrations in the established OC cell lines SKOV-3, OVCAR-3, UWB1.289, TOV-21G, and TOV-112D. Basal miR-1 signals were detectable in all of these cell lines with relative concentrations of 0.03 ± 0.05 (SKOV-3), 0.08 ± 0.04 (OVCAR-3), 0.24 ± 0.41 (UWB1.289), 0.39 ± 0.48 (TOV-21G:), and 1.10 ± 0.32 (TOV-112D; Fig. 1). Comparison of miR-1 expression levels with SK-OV-3 (set to 1.0) revealed expression values of 2.5-fold (OVCAR-3), 8.1-fold (UWB1.289), 12.8-fold (TOV-21G), and 36.7-fold (TOV-112D), demonstrating highly mutable expression rates of miR-1. However, due to variance only the mean miR-1 expression in TOV-112D was statistically significant compared to the other cell lines.

Cell growth kinetics of OC cell lines correlate with miR-1 expression levels

To determine whether different levels of miR-1 expression define differences in cellular growth of the OC cell lines, we counted living cells of incubated OC cells at indicated time points over a period of 144 h (Figure 2). Notably, cellular proliferation was found being linked to the basal expression of miR-1: TOV-112D (24 h: 2.1 ± 0.7, 48 h: 5.8 ± 0.1, 72 h: 12.2 ± 0.9, 96 h: 20.3 ± 0.8, 120 h: 32.0 ± 6.8, 144 h: 42.2 ± 5.4); TOV-21G (24 h: 2.7 ± 0.6, 48 h: 5.3 ± 0.0, 72 h: 11.9 ± 2.1, 96 h: 24.6 ± 5.4, 120 h: 38.3 ± 7.7, 144 h: 51.3 ± 10.7); UWB1.289 (24 h: 3.0 ± 0.6, 48 h: 4.7 ± 1.1, 72 h: 6.9 ± 1.8, 96 h: 8.6 ± 0.3, 120 h: 13.8 ± 3.4, 144 h: 19.7 ± 4.2); SKOV-3 (24 h: 2.6 ± 1.6, 48 h: 4.3 ± 0.2, 72 h: 7.9 ± 0.8, 96 h: 13.0 ± 1.8, 120 h: 15.7 ± 2.1, 144 h: 20.4 ± 0.5); OVCAR-3 (24 h: 3.0 ± 1.3, 48 h: 4.3 ± 0.1, 72 h: 7.2 ± 0.3, 96 h: 10.9 ± 0.4, 120 h: 13.2 ± 2.2, 144 h: 15.9 ± 3.1). The higher the level of miR-1 in OC cells, the higher the rates of cell growth were detected (TOV-112D, TOV-21G), whereas moderate and low miR-1 expression were related to diminished cell proliferation (UWB1.289, SKOV-3, OVCAR-3).

miR-1 expression in tissue samples from healthy donors and primary OC turmors varies compared to secondary tumor tissues from OC patients

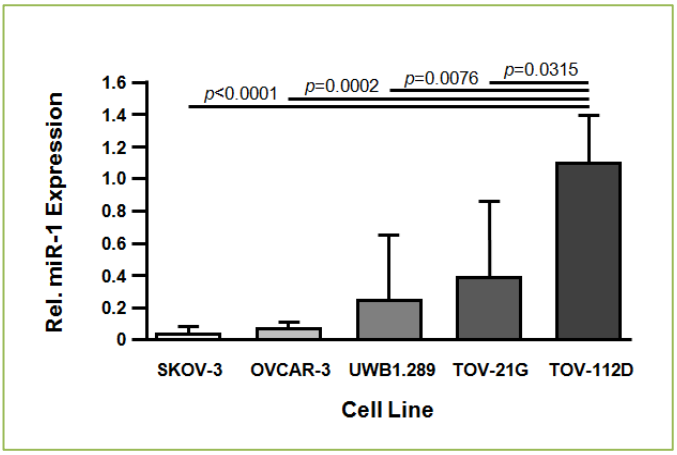

Figure 1. miR-1 analysis in established OC cell lines revealed highly variable expression levels. Relative miR-1 levels normalized to intracellular U6 RNA concentrations ranged from 0.03 ± 0.05 (1.0-fold; SKOV-3) to 1.10 ± 0.32 (36.7-fold; TOV-112D). Columns were calculated as the mean ± SD of relative miR-1 concentrations with given *p* values determined by Student's *t* test.

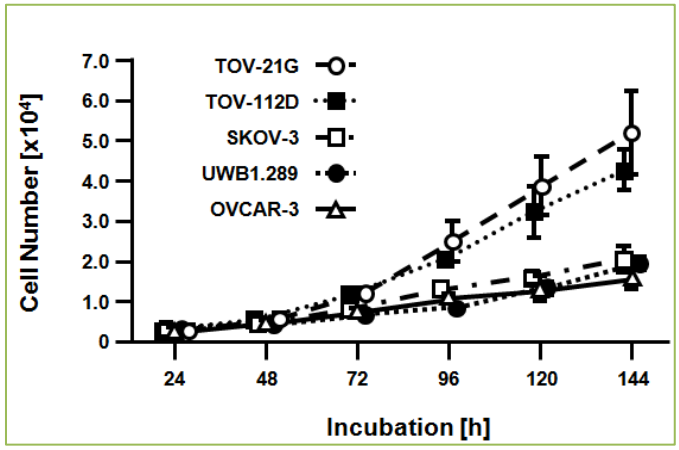

Figure 2. Growth characteristics of OC cell lines correlated with intracellular miR-1 concentrations. Cellular growth was determined at indicated time points utilizing a CASY Cell Counter and Analyzer Model TT. High level miR-1 cell lines (TOV-112D, TOV-21G) demonstrated clearly higher rates of proliferation compared to moderate and low miR-1 expressing cells (UWB1.289, SKOV-3, OVCAR-3). Results are expressed as the mean ± SD of 10^5 cells.

While differential expression of miR-1 was detectable in OC cell lines, tumor samples were included in this preliminary study to perform a miR-1 expression profiling. A total of 21 tumor samples from OC patients were compared to ovarian tissue samples from 4 non-malignant donors obtained from the Department of Gynecology and Obstetrics at the University Medicine Greifswald. OC patients were caucasians with an average age at the time of surgery of 59.7 and FIGO stages from III to IV (Table 1). OC samples were sub-classified in primary tumor tissue (n=12; patient no. 1 to 12) and samples from relapsed tumors (n=8; patient no. 9 to 17). Noteworthy, four samples of both groups were obtained from the same four individuals (patient no. 9 to 12).

Box plot analysis of intracellular miR-1 concentration graphically depicted no significant differences in miR-1 expression in healthy tissue and primary tumor samples (Figure 3A). In contrast, miR-1 expression in relapsed tumors appeared being upregulated compared to primary tumors. Notably, a subset analysis of patients 9 to 12 demonstrated that there was a distinct rise in miR-1 expression comparing primary and relapsed tumor tissues in individual patients (Figure 3B).

Discussion

OC is a highly heterogenous and severe malignant disease, for which no satisfactory treatment options are yet available. Due to the fact that molecular research should provide much-needed information about novel predictive and prognistic factors, we analyzed the expression of miR-1 in OC cell lines and tumor samples. The panel of probed OC cell lines demonstrated highly variable miR-1 concentrations reaching a basal level up to 36.7-fold higher in TOV-112D cells than in the low-expression cell line SKOV-3. miR-1 was strongly expressed in TOV-112D and TOV-21G cells, and moderate expressed in UWB1.289 cells, whereas SKOV-3 and OVCAR-3 cells weakly expressed miR-1. Recent studies stated miR-1 functionality in solid malignancies as tumor-suppressive. Molecular analysis in bladder cancer [14, 15], prostate cancer [9, 10, 16], colon cancer [11, 17], and renal cell carcinoma [18] strongly interrelated cellular miR-1 properties with mechanisms of growth inhibition and general anti-oncogenic cell response. On the contrary and rather surprisingly, miR-1 levels in OC cell lines as determined in this study were linked to *in vitro* growth characteristics of the cells: high level miR-1 cell lines (TOV-21G, TOV-112D), however, demonstrated clearly higher proliferation rates than OC cells bearing lower levels of miR-1 (UWB1.289, SKOV-3, OVCAR-3).

In order to shed further light on the putative role of miR-1 in OC, we assessed miR-1 expression levels in primary and relapsed states of OC. A comparison of ovarian miR-1 status of healthy women with primary OC tumors disclosed none or even low differences in expression. Interestingly, miR-1 levels analyzed in the subsets of primary and relapsed tumors, however, revealed elevated values and a higher median of miR-1 concentration in relapsed tumors. Admittedly, variations as well as few numbers of samples in this preliminary study prohibited statistical analysis of the box plot presentation. Further analysis of primary and relapsed tumors from individuals underscored the previous findings: all samples of the four patients showed an increase of miR-1 expression from primary to relapsed tumor samples.

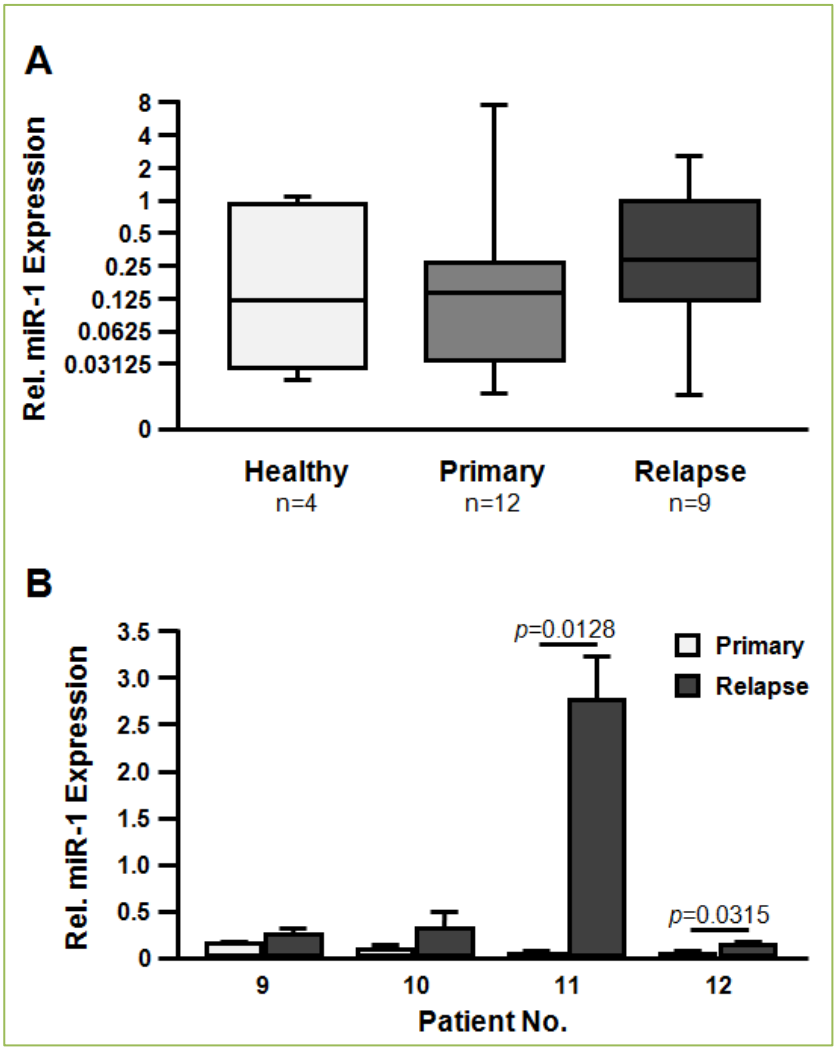

Figure 3. Analysis of primary and relapsed OC tumor samples demonstrated no attenuation of miR-1 compared to healthy tissue and tended to result in increased levels of miR-1 in relapsed tumors. Relative miR-1 levels normalized to intracellular U6 RNA concentrations of healthy (n=4) ovarian samples, and primary (n=12) as well as relapsed (n=9) OC tumor samples showed no reduction in miR-1 expression in malignant tissues. Comparison of primary and relapsed tumor samples indicated a weak increase of miR-1 within the subset of relapsed tissues. Results are expressed as box plot presentation; variations and few numbers of samples prohibited statistical analysis (A). Analysis of four patients from which primary as well as relapsed samples were available indicated elevated levels of miR-1 in all of the relapsed state of OC. Columns were calculated as the mean ± SD of relative miR-1 concentrations with given *p* values determined by Student's *t* test (B).

In conclusion, a clear decrease of miR-1 in malignant compared to healthy tissue as recently described in cancer of bladder [14], colon [17], prostate [19], and lung [20], has not been detected in OC. Furthermore, combining miR-1 in OC cell lines was linked to higher proliferation, together with potentially up-regulated miR-1 levels in relapsed and therefore highly oncogenic OC, makes miR-1 role as tumor suppressor rather implausible. This may suggest that (1) miR-1 may be able to govern OC cell growth contradicting the tumor suppressor properties observed in other cancer types, or (2) miR-1 tumor suppressor functions may be dysregulated in OC cells by the disruption of miR-1-depending signaling cascades and/or effector molecules. Although miR-1 functionality in OC oncogenesis remains unclear, however, elevated levels of miR-1 during tumor progression may serve as a promising marker for OC recurrence.

Abbreviations

Ovarian cancer (OC), microRNA-1 (miR-1), intraoperative mapping of ovarian cancer (IMO)

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Conflicting Interests

No potential conflicts of interest were disclosed.

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