Three-Dimensional Cellular Arrangement in Epithelial Ovarian Cancer Cell Lines TOV-21G and SKOV-3 is Associated with Apoptosis-Related miRNA Expression Modulation

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Abstract

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy, and the lack of chemoresistance biomarkers contributes to the poor prognosis. Cancer stem cells (CSC) have been investigated in EOC to understand its relationship with chemoresistance and recurrence. In this context, in vitro cultivation-models are important tools for CSC studies. MicroRNAs (miRNAs) play key roles in cancer, CSC regulation and apoptosis. Thus, this study aims to evaluate the tumorsphere model as CSC-enrichment method in EOC studies and investigate apoptosis-related miRNAs in tumorspheres-derived EOC cell lines. TOV-21G and SKOV-3 were cultured in monolayer and tumorspheres. Genetic profiles of cell lines were obtained using COSMIC database. CD24/CD44/CD146/CD177 and ALDH1 markers were evaluated in cell lines and tumorspheres-derived by flow cytometry. Eleven miRNAs were selected by in silico analysis for qPCR analysis. According to COSMIC, TOV-21G and SKOV-3 have eight and nine cancer-related mutations, respectively. TOV-21G showed a CD44^{+/high}/CD24^{-/low}/CD117^{-/low}/CD146^{-/low}/ALDH1^{low} profile in both culture models; thus, no significant difference between cultivation models was identified. SKOV-3 showed a CD44^{+/high}/CD24^{+/high}/CD117^{-/low}/CD146^{-/low}/ALDH1^{low} profile in both culture models, although the tumorsphere model showed a significant increase in CD24^{+/high} subpopulation (ovarian CSC-like). Among eleven miRNAs, we observed differences in miRNA expression between culture models. MiR-26a was overexpressed in TOV-21G tumorspheres, albeit downregulated in SKOV-3 tumorspheres. MiR-125b-5p, miR-17-5p and miR-221 was downregulated in tumorsphere model in both cell lines. Given that tumorsphere-derived SKOV-3 had a higher ratio of CD24^{+/high} cells, we suggest that miR-26a, miR-125b-5p, miR-17-5p and miR-221 downregulation could be related to poor EOC prognosis.

Keywords Epithelial ovarian cancer · Cancer stem cells · microRNAs · Apoptosis · In vitro cultivation-models

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy, and its mortality rate is higher than 60% [1].

Late-stage diagnosis, associated with the lack of effective molecular markers to detect chemotherapy resistance contributes to high mortality rates for EOC [2].

Accordingly, cancer stem cells (CSC) have been increasingly investigated to understand the biology of cancer, and several studies have shown that CSC may be responsible for the high rate of chemoresistance and EOC recurrence [3]. CSC are found in specialized niches or microenvironments and consist of several cell types that interact with each other, including fibroblasts, endothelial and perivascular cells, adipose cells, immune cells, and macrophages [4]. Furthermore, this microenvironment provide the requirements for the maintenance of CSC characteristics such as self-renewal, chemoresistance, anchorage-independent growth, apoptosis resistance, invasion and metastasis [4]. Ponti et al. [5] showed

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that cultivating cells onto an ultra-low attachment surface and into a serum-free medium enriched in growth factors was efficient in maintaining breast CSC. In such conditions, cells grew as multicellular three-dimensional clones termed “tumorspheres” [6]. This cell culture model is widely used to analyze the self-renewal capability of CSC to enrich these cells from bulk cancer and established cancer cell lines [6].

Mechanisms of CSC regulation, drug resistance, progression and EOC recurrence have been associated with MicroRNAs (miRNAs) signatures [7, 8]. miRNAs are small non-coding RNAs that regulate posttranscriptional gene expression and play a key role in cancer progression and expression [8]. Therefore, several studies have investigated the potential use of miRNA to provide information for EOC identification, classification, treatment and/or prognosis [9, 10].

Previous studies from our group described different apoptosis-related genes as EOC biomarkers associated with disease prognosis [11–13]. Considering that aberrant miRNAs expression plays a key pathogenic role in different stages of the carcinogenesis process and is involved in the deregulation of several biological processes, including apoptosis, miRNA profile analysis may reveal new biomarkers that can be used for EOC prognosis and chemoresistance [14]. Thus, this study aims to evaluate the tumorsphere model as *in vitro* CSC enrichment method in EOC studies and investigate apoptosis-related miRNAs in tumorspheres-derived EOC cell lines.

Methods

Cell Culture

TOV-21G, (Cat. #CRL-11730™) and SKOV-3 (Cat. #HTB-77™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in monolayer (culture adherent), respectively, in DMEM (Sigma, St. Louis, MO, USA, cat. # M2279) supplemented with 15% fetal bovine serum (FBS) (Sigma, cat. # F6178) and McCoy's 5A Medium (Sigma, cat. # M4892) supplemented with 10% FBS. Tumorspheres were obtained from TOV-21G and SKOV-3. The cell lines were trypsinized, and cell suspensions were plated (2.5×10^4 cells/well) in 96-well plates for non-adherent cells (Corning, Cat. # CLS3474), using 200 μ L per well of DMEM/F-12 medium (Sigma, Cat. # D0547) supplemented with 20 ng/mL epidermal growth factor (EGF) (Sigma, cat. # E1257), 10 ng/mL basic fibroblast growth factor (FGF-b) (ImmunoTools, Friesoythe, Germany, cat. # 11343625) and 10 μ g/mL bovine insulin (Sigma, cat. # I1882) and incubated for seven days at 37 °C in 5% CO₂ to obtain the tumorspheres [15].

Analysis of Genetic Differences between SKOV-3 and TOV-21G Cell Lines

Genetic differences between TOV-21G and SKOV-3 cells were analyzed in the Catalog of Somatic Mutations in Cancer (COSMIC - http://cancer.sanger.ac.uk/cell_lines) to investigate cancer-related mutations in each cell line [16].

Flow Cytometry Analysis

CD44 (Mouse Anti-Human CD44 APC, Immunotools, cat. # 21850446.), CD24 (Human CD24 FITC conjugate, Invitrogen, Carlsbad, CA, USA, cat. #MHCD2401.), CD117 (Mouse Anti-Human CD117 PerCP-Cy™5.5, BD Pharmingen, San Diego, CA, USA, cat. #562094) and CD146 (Mouse Anti-Human CD146 PE, cat. #550315, BD Pharmingen) antibodies and ALDEFLUOR Stem Cell Identification kit (StemCell Technologies, Vancouver, Canada, cat. #01700,) were used for all flow cytometry experiments. SKOV-3 and TOV-21G cells and the tumorspheres derived from them were cultured as previously mentioned and dissociated by trypsinization and/or mechanically into single-cell suspensions. Cells were resuspended in wash buffer (PBS 1X with BSA 0.25% and azide 1 mM). Extracellular staining was performed with fluorochrome-conjugated monoclonal antibodies human or their respective isotype controls at the concentrations recommended by the manufacturer and incubated at 4 °C in the dark for 15 min. ALDEFLUOR Stem Cell Identification kit was used to assess ALDH activity in the EOC cell lines. Cells were suspended in PBS or ALDEFLUOR Stem Cell Identification kit buffer and analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA), and data analysis was performed in FACSDiva 6.1.3 software.

In Silico Selection of Apoptosis-Related miRNA Targets

miRNA targets were selected for qRT-PCR and validated using prediction (TargetScan, miRanda, MirTarget2) and validation (TarBase/DianaTools, miRTarBase) databases. miRNAs indicated in more than one prediction and/or validation databases and those that met the prediction parameters were preferred [17].

Total RNA Extraction and Quality Analysis

SKOV-3 and TOV-21G cells lines and tumorsphere-derived total RNA extraction was performed using mirVana kit (Invitrogen, cat # AM1560), according to the manufacturer's recommendations. Concentrations of total RNA and the 260/280 absorbance ratio were measured using a NanoVue spectrophotometer (GE Healthcare, Little Chalfont, UK). The

quality of the RNA samples was assessed in a 2100 Bioanalyzer using the Pico LabChip Kit (Agilent Technologies, Santa Clara, CA, USA, cat. # 5067–1514), according to manufacturer's instructions.

Analysis of miRNAs Gene Expression

miRNA expression, selected by in silico analysis, was validated for qRT-PCR. The cDNA was synthesized using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA, cat # 4366597). Inventoried assays (TaqMan® MicroRNA Assays - Applied Biosystems) were used according to manufacturer's recommendation. The small nucleolar SNORD43 and U6 small nuclear 6 (RNU6-6P) were used as endogenous controls. Relative expression levels of miRNAs were calculated using the $2^{(-\Delta\Delta C_q)}$ method as described by LIVAK and SCHMITTGEN, 2001 [18].

Statistical Analysis

Data were analyzed using the REST 2009 software (Qiagen, Hilden, Germany) to evaluate differential gene expression between groups, in addition to the SPSS 18.0 software package (IBM SPSS Inc., Chicago, IL, USA) and Bionumerics 7.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Results

According to information available at the *COSMIC cell line project*, TOV-21G and SKOV-3 have eight and nine cancer-related mutations, respectively (Table 1). The morphology of TOV-21G and SKOV-3 cell lines, cultured in monolayer and tumorsphere models is shown in Fig. 1. TOV-21G-derived tumorspheres grew in loose grape-like clusters, whereas SKOV-3-derived tumorspheres were tightly packed and difficult to separate into single-cell suspensions.

Flow cytometry analysis showed that TOV-21G cell lines have a CD44^{+/high}/CD24^{-/low}/CD117^{-/low}/CD146^{-/low} and ALDH^{low} phenotype profile in both culture models (monolayer and tumorspheres) with no significant differences in subpopulation profile between the cell culture models (Fig. 2a). SKOV-3 cell lines cultured in monolayer and tumorsphere models showed a CD44^{+/high}/CD24^{+/high}/CD117^{-/low}/CD146^{-/low} and ALDH^{low} profile, albeit with a significant

increase in CD24^{+/high} subpopulation and decrease in CD44^{+/high} subpopulation in the tumorsphere model (Fig. 2b).

Eleven miRNAs (miR-125b-5p, miR-128, miR-150-5p, miR-17-5p, miR-181a, miR-181c, miR-20a, miR-221, miR-26a, miR-340 and miR-4478) were selected by in silico analysis of miRNA targets using the relationship with apoptotic pathways as criterion (Table 2) for qPCR gene expression analysis. MiR-125b-5p, miR-340-5p, miR-128, miR-150-5p, hsa-miR-17-5p, miR-20a, miR-340 and miR-4478 was significantly downregulated in the tumorsphere model compared with the monolayer model, in both cell lines (Table 1). Conversely, TOV-21G cells showed miR-26a overexpression in the tumorsphere model compared with the monolayer model, whereas miR-26a was downregulated in SKOV-3-derived tumorspheres. SKOV-3 also showed miR-181c overexpression in the tumorsphere model, with significant differences in miRNAs expression profile between the study cell lines (Fig. 3).

Discussion

EOC is a diverse and genomically complex disease, and the identification of mutated genes is a key step in the diagnosis and treatment of these malignancies [19, 20]. EOC classification as type I and type II tumors, proposed by Kurman and Shih [21], considers the genetic profile of each histological type. Type I tumors have specific mutations in the *KRAS*, *BRAF* and *ERBB2* genes, commonly found in low grade serous carcinomas, whereas *TP53* mutations are rare in this type of tumors [21]. High-grade serous carcinomas, classic type II tumors, are characterized by a high frequency of *TP53* mutations (> 80% of cases) and amplification in *CCNE1* [21]. Type I tumors usually show slow growth, are confined to the ovary at diagnosis and are relatively genetically stable, compared with Type II tumors, comprising low-grade serous, low-grade endometrioid, clear cell, mucinous and transitional (Brenner) carcinomas [21]. Type II tumors are highly aggressive, rapidly progressing carcinomas and are almost always identified at an advanced stage at diagnosis and include conventional high-grade serous carcinoma, undifferentiated carcinoma and malignant mixed mesodermal tumors (carcinosarcoma) [21]. Based on information available at *COSMIC* (Table 1), TOV-21G cells show a typical profile of type I tumors, which frequently have mutations in *KRAS* and *PIK3CA*. SKOV-3, in contrast, shares mutations characteristic

Table 1 Cancer-related genes mutations in TOV-21G and SKOV-3 cell lines

Cell line	Cancer-related genes mutations
TOV-21G	<i>ATR</i> , <i>DDX3X</i> , <i>HIF1A</i> , <i>HOOK3</i> , <i>KRAS</i> , <i>NOTCH1</i> , <i>PIK3CA</i> , <i>RNF43</i>
SKOV-3	<i>ARID1A</i> , <i>BCL11B</i> , <i>EP300</i> , <i>ERC1</i> , <i>FBXW7</i> , <i>MYH11</i> , <i>NF1</i> , <i>PIK3CA</i> , <i>TP53</i>

COSMIC cell line project (http://cancer.sanger.ac.uk/cell_lines)

Fig. 1 TOV-21G and SKOV-3 cell lines and tumorsphere-derived cells

of type I (*PIK3CA* mutation) and type II (*TP53* mutation) tumors. Thus, TOV-21G and SKOV-3 are selected as two different and representative types of EOC in both histological and molecular characteristics.

Tumorspheres are a model of in vitro cancer stem cell expansion, established in a serum-free medium supplemented with growth factors widely used for enriching and maintaining CSC from several cell lines [6, 22]. In recent years, the CSC model has been highlighted for further research towards developing therapeutic strategies for cancer treatment [23]. Cellular and molecular heterogeneity among tumors complicate the identification of CSC markers unique to different tumor types [24]. However, some surface markers, such as CD44, CD24, CD133, CD146, EpCAM and the expression

of aldehyde dehydrogenase (ALDH) enzyme are described in the literature as CSC markers in different tumors, including breast, lung, pancreas, prostate, colorectal, renal and ovarian tumors [24–26]. TOV-21G cultured in the tumorsphere model formed loosely adhered grape-like cell clusters that can be easily dispersed by pipetting. A loose morphology can be related to the mesenchymal phenotype, a property that is crucial for tumor cells to become metastatic, and affects their ability to grow as spheroids [27]. Considering the markers analyzed in this study, no significant increase of any cell subpopulation was detected in TOV-21G cultures, regardless of culture model, suggesting that the tumorsphere-enrichment model used was unable to promote the CSC-like growth and proliferation of this cell line. However, the SKOV-3 cell line

Fig. 2 TOV-21G (a) and SKOV-3 (b) Cell population characterization in monolayer and in tumorsphere models Software: SPSS 18.0; Two-way ANOVA, post hoc, DMS method, $n = 3$. *Significantly different ($\alpha = 5\%$), comparing the monolayer cultures with the tumorsphere model

Table 2 Apoptosis-related miRNA target selection and expression analysis

miRNA selected	Predicted targets related to apoptosis	Prediction Database	Validation Database	Taqman Assay ID	Cell line		
					TOV-21G		SKOV-3
					Log ₂ (FC)**	Interpretation	Log ₂ (FC)
hsa-miR-150-5p	TP53, AIFM2	TargetScan, miRanda	miRTarBase	000473	-0.003	*Sub expressed ($p < 0.01$)	-4.094
hsa-miR-181a	DDX3X, BCL2	TargetScan, miRanda	TARBASE (DianaTools)	002317	-2.932	*Sub expressed $p = 0.498$	-3.580
hsa-miR-340-5p	DDX5, BCL2L2	TargetScan	TARBASE (DianaTools)	002258	-1.797	*Sub expressed ($p < 0.01$)	-4.645
hsa-miR-128	ACIN1, AFF1	TargetScan,	miRTarBase	002216	-3.777	*Sub expressed ($p < 0.01$)	-5.090
hsa-miR-125b-5p	BAK1, BCL2	TargetScan	TARBASE (DianaTools)	000449	-1.999	*Sub expressed ($p < 0.01$)	-7.722
hsa-miR-26a	BID, THAP5	TargetScan	TARBASE (DianaTools)	002443	0.558	*Sub expressed $p = 0.502$	-2.866
hsa-miR-181c	BCL2, BCL2L1	TargetScan, miRanda	miRTarBase	000482	1.426	$p = 0.502$	0.445
hsa-miR-20a	BCL2, BCL2L1, THAP1	TargetScan	TARBASE (DianaTools)	000580	-2.056	*Sub expressed ($p < 0.01$)	-4.792
hsa-miR-221	BMF, APAF1	TargetScan	miRTarBase	000524	-0.746	$p = 0.498$	-9.155
hsa-miR-17-5p	CAAP1, CASP8, AREL1	MirTarget2	–	002308	-0.795	*Sub expressed ($p < 0.01$)	-14.744
hsa-miR-4478	APAF1, TNFRSF10C, TNFRSF10B	MirTarget2	–	465302_mat	-1.636	*Sub expressed ($p < 0.01$)	-5.974

Software: REST 2009 (Qiagen). *Significantly different ($\alpha = 5\%$), comparing tumorspheres to monolayer model, $n = 2$ **Fold Change (FC) = $(2^{-\Delta\Delta C_q})$

Fig. 3 miRNAs expression profile between EOC cell lines (TOV-21G and SKOV-3) comparing tumorspheres with the monolayer model

had a compact spherical morphology that required disaggregation by enzymatic and mechanical treatment and showed a significant increase in the CD24^{+/high} subpopulation when cultured in the tumorsphere model. Experiments *in vitro* showed that the CD24^{+/high} fraction has higher chemoresistance, quiescence and self-renewal capacity than the CD24^{-/low} fraction [28]. Furthermore, CD24 can be related to epithelial-mesenchymal-transition (EMT) induction in ovarian cancer, a process involved in cell invasion, resistance to chemotherapy and the formation of CSC side populations [29]. Ishiguro et al. [30] have cataloged antineoplastic agents according to their effectiveness against platinum-resistant and platinum-sensitive ovarian carcinoma cell lines and concluded that SKOV-3 is the most chemoresistant cell line for platinum derivatives. Thus, the CD24^{+/high} subpopulation increase observed in SKOV-3 tumorspheres suggests CSC-like enrichment and may explain the higher chemoresistance observed in this cell line.

Several miRNAs involved in various biological processes, such as developmental timing, differentiation and apoptosis, are aberrantly expressed in various cancer types and some act as tumor suppressors, whereas others act as oncogenes (oncomiRs), depending on which genes or pathways they regulate [31]. Each tumor has a characteristic miRNAs combination; this key information can be used, with the advent of personalized medicine, to direct therapeutic decisions by analyzing patient-specific molecular biomarkers [32]. Among the miRNAs analyzed, miR-125b-5p, miR-340-5p, miR-128, miR-150-5p, hsa-miR-17-5p, miR-20a, miR-340 and miR-4478 were significantly underexpressed in TOV-21G-derived tumorspheres compared with their levels in TOV-21G monolayers (Table 2). The difference in expression of these miRNAs between the culture systems used is apparently more

related to the three-dimensional profile (Fig. 1) than to the CSC phenotype (Fig. 2), which was shown to be the same in the TOV-21G line in both culture models. In contrast, SKOV-3 showed changes in the three-dimensional cell profile (Fig. 1), in the ratio of cell subpopulations (Fig. 2), and in the differential expression of the miRNAs evaluated between the two culture models (Table 2).

One of the major cell regulatory pathways that is affected in cancer is apoptosis [33]. Although several studies show that one miRNA targets a several mRNAs, plays either anti- or pro-apoptotic roles in different tumors, and is important for apoptosis regulation during cancer development [34, 35]. Therefore, the investigation of miRNAs involved in apoptotic pathways may provide new information about the mechanisms of tumorigenesis and chemoresistance [34]. Then, the *in vitro* expression profile of miR-181c, miR-125b-5p, miR-17-5p, miR-221 and miR-26a evaluated in this study may provide important information for EOC prognosis (Fig. 3). MiR-181c was overexpressed in the tumorsphere model, compared with the monolayer model, in both cell lines, although no significant difference was found in TOV-21G cell lines. MiR-181c inhibited the anti-apoptotic factors BCL2 and MCL1, and is related to apoptosis induction [36]. However, the function of miR-181c depends on tumor type and cellular context because miR-181c is downregulated in glioblastoma multiforme, yet is overexpressed in gastric cancer, skin basal cell carcinoma and in osteosarcomas [37]. MiR-125b-5p is downregulated in both TOV-21G and SKOV-3-derived tumorspheres and is more strongly downregulated in SKOV-3-derived tumorspheres. Yang et al. [38] showed that miR-125b-5p directly suppresses BCL2 expression and increases the

sensitivity of gallbladder cancer cells and mouse models to cisplatin treatment. Furthermore, the combination of miR-125b-5p low expression and BCL2 high expression is highly correlated with poor prognosis in gallbladder cancer patients [38]. Given that SKOV-3 is a platinum-resistant cell line and that miR-125b-5p is strongly downregulated in the tumorsphere model, miR-125b-5p may be a platinum chemoresistance marker. Another strongly miRNA downregulated in SKOV-3-derived tumorspheres is miR-17-5-p. Among the predicted targets of miR-17-5-p (Table 2), CAAP1 was very interesting. CAAP1 is an anti-apoptotic protein and its knockdown expression induces apoptosis in different cancer cell lines that proceeds independently of the caspase-8-dependent death receptor pathway [39]. Li et al. [40] showed that miR-17-5p overexpression plays a regulatory role in the malignant progression of non-small cell lung cancer (NSCLC), promoting significantly inhibited proliferation while inducing the apoptosis of NSCLC H460 cells. Thus, strategies of co-transfection can be elucidating the relationship of miR-17-5p and CAAP1 expression in apoptosis resistance. In addition, miR-17-5p overexpression is also associated to significantly inhibited cell proliferation, migration and invasion in triple-negative breast cancer (TNBC) cells and miR-17-5p down-regulated in TNBC samples were significantly correlated with shorter overall survival, larger tumors size and advanced stages of disease [41]. MiR-221 is also downregulated in both TOV-21G and SKOV-3 tumorspheres-derived, but more strongly downregulated in SKOV-3. However, Li et al. [42] showed, for the first time that APAF1 is a direct target of miR-221 in human EOC cells, and APAF1 overexpression, through miR-221 inhibition, suppressed EOC cell lines proliferation and induced cell apoptosis in vitro. MiR-26a was overexpressed in TOV-21G-derived tumorspheres compared with monolayer TOV-21G cells and downregulated in SKOV-3-derived tumorspheres. MiR-26a expression is frequently abnormal in tumors, indicating that it may play key roles in tumor formation [43]. Deng et al. [44] showed that miR-26a levels were associated with the gastric cancer clinical stage and presence of lymph node metastases and analysis revealed that patients whose primary tumors displayed low expression of miR-26a had shorter survival and relapse-free survival. Moreover, miR-26a overexpression enhanced the sensitivity to doxorubicin, inducing apoptosis in hepatocellular carcinoma cells [45] and significantly inhibit the proliferation, promoting apoptosis in ovarian cancer cells [46].

Conclusion

Considering that miR-26a, miR-125b-5p, miR-17-5p and miR-221 expression was lower in SKOV-3-derived

tumorspheres, which had a higher ratio of CD24^{+/high} cells (ovarian CSC-like), we suggest the need to investigate the relationship of the downregulation of these miRNAs towards understanding apoptosis resistance and poor prognosis in EOC. Moreover, co-transfection strategy can be help to elucidate the relationship of miR-17-5p and CAAP1 expression in apoptosis resistance mechanisms.

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Compliance with Ethical Standards

Conflict of Interest The authors have no conflicts of interest to declare.

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