

MicroRNA expression profiles in non-epithelial ovarian tumors

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Abstract. Ovarian germ cell tumors (OGCTs) and sex cord stromal tumors (SCSTs) are rare gynecologic tumors that are derived from germ and stromal cells, respectively. Unlike their epithelial counterparts, molecular pathogenesis of these tumor types is still poorly understood. Here, we characterized microRNA (miRNA) expression profiles of 9 OGCTs (2 malignant and 7 benign) and 3 SCSTs using small RNA sequencing. We observed significant miRNA expression variations among the three tumor groups. To further demonstrate the biological relevance of our findings, we selected 12 miRNAs for validation in an extended cohort of 16 OGCTs (9 benign and 7 malignant) and 7 SCSTs by reverse transcription-quantitative polymerase chain reaction. Higher expression of *miR-373-3p*, *miR-372-3p* and *miR-302c-3p* and lower expression of *miR-199a-5p*, *miR-214-5p* and *miR-202-3p* were reproducibly observed in malignant OGCTs as compared to benign OGCTs or SCSTs. Comparing with benign OGCTs, *miR-202c-3p* and *miR-513c-5p* were more abundant in SCSTs. Additionally, we examined Beclin 1 (BECN1), a target of *miR-199a-5p*, in the clinical samples using western blot analysis. Our results show that BECN1 expression was higher in malignant OGCTs than benign OGCTs, which is concordant with their lower *miR-199a-5p* expression. This study suggests that these miRNAs may have potential value as tumor markers and implications for further understanding the molecular basis of these tumor types.

Introduction

Ovarian tumors are common with >200,000 women estimated to be diagnosed with ovarian cancer annually worldwide (1).

Three main types are recognized according to the type of cell origin. Epithelial cancer is the most common form seen in the vast majority of cases. Germ cell tumors (GCTs) develop in the egg cells (oocytes) of ovary. Stromal cell tumors, arise from cells involved in ovarian steroid production (e.g., granulosa, theca and Leydig cells) (2).

Although non-epithelial ovarian tumors are uncommon, they are histologically, genetically and clinically heterogeneous causing major challenges for treatment and clinical workup. Ovarian GCTs (OGCTs) typically affect young women (<30 years) (3). It is further subclassified based on histology and clinical behavior as: mature teratoma which is benign, and the malignant form immature teratomas and malignant OGCTs (including dysgerminoma, yolk sac tumor, embryonal carcinoma, choriocarcinoma and mixed GCT). Mature teratoma, which is the most common among all OGCTs, may contain a variety of well-differentiated tissues, such as hair, fat, teeth and bone. In rare cases, complex organs such as brain matter, eyes, torso, hands, feet and other limbs are formed in teratomas (4-7). Immature teratomas, on the other hand, contain incompletely differentiated tissues in addition to mature elements, and have worse prognosis (8). Malignant OGCTs are thought to arise from the pluripotent primordial germ cells, which can differentiate into various histologies. While dysgerminoma is composed of primitive undifferentiated germ cells, the other malignant OGCTs (non-dysgerminomas) are categorized based on the degree of differentiation from their precursor cells that mimic embryonic and extra-embryonic tissues (3).

Patients with sex cord stromal tumor (SCST) can display various hormone-mediated syndromes and a variety of clinical features depending on their cellular origin. Tumors derived from ovarian cells (such as granulosa and theca cells) usually exhibit clinical features associated with excessive production of estrogen, while those derived from Sertoli and Leydig cells exhibit phenotypes resulting from elevated levels of androgens. The prognosis of SCST is generally good, although a minority of patients develop metastasis (9).

The molecular basis underlying the non-epithelial ovarian tumors is still poorly understood. However, accumulating evidence indicate that both OGCTs and SCSTs may reflect the developmental changes of the germ and stromal cells in the ovary. For OGCTs, it is clear that most of the malignant tumors express pluripotency markers. They generally express high level

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of POU class 5 homeobox 1 (POU5F1), v-KIT proto-oncogene receptor tyrosine kinase (KIT), Lin-28 homolog A (LIN28A) and Nanog homeobox (NANOG) (10,11). On the other hand, the benign tumors (mature teratomas) have complex expression patterns due to the cell composition of multiple cell types in different tumors (2).

For SCSTs, they express transcription factors that play important roles in sex determination, e.g., granulosa cell tumors express Forkhead box L2 (FOXL2) and Sertoli-Leydig cell tumors express sex determining region Y box 9 (SOX9), which controls ovary and testis determination, respectively. The majority (97%) of ovarian adult granulosa cell tumors harbor somatic *FOXL2* mutations (12), which promote granulosa cell survival (13) and tumorigenesis (14). On the other hand, somatic missense mutations of Dicer 1 ribonuclease III (*DICER1*) are found in 60% of the ovarian Sertoli-Leydig cell tumors (15). *DICER1* plays an important role in microRNA (miRNA) processing. Its mutations in SCSTs cause selective processing of 3'-strand miRNAs (15). However, how these mutations result in sex cell fate decision or tumorigenesis remain unknown.

miRNAs are single-stranded RNAs of ~22 nucleotides in length, which regulate gene expression in many biological processes, including cell growth, differentiation and tumorigenesis (16). Distinct miRNA expression profiles have been identified in a variety of tumor types associated with histological subtypes and patient outcome, suggesting their potential use as biomarkers for diagnosis and prognosis (17). miRNA expression profiles have been investigated in some ovarian germ cell tumors. However, in all studies, the OGCTs were combined with their testicular counterparts for analysis (18-21). One of the reasons may be due to the rarity of these tumor types and very few samples were analyzed. Although *DICER1* is frequently mutated in SCSTs (15), miRNA expression profiles of this tumor type and how their expression profiles relate to OGCTs are yet to be investigated.

In this study, we characterized miRNA profiles of OGCTs and SCSTs using small RNA (sRNA) sequencing, and compared the expression differences between malignant and benign OGCTs, as well as between SCSTs and OGCTs. We identified miRNA expression patterns associated with different tumor types, suggesting their potential use as a tool for defining their histological and biological differences.

Materials and methods

Clinical samples. This study included 23 frozen non-epithelial ovarian tumors, consisting of 16 ovarian germ cell tumors (OGCT1-16) and 7 sex cord stromal tumors (SCST1-7). The age at diagnosis and the histological type of each tumor are given in Table I. All tumors were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute, USA. Other investigators may have received specimens from the same subjects. The study was approved by the Stanford Human Subjects Review Committee. All clinical samples were de-identified, therefore no written informed consent was required.

RNA isolation and quantification. mirVana miRNA isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract sRNA and total RNA for cloning and reverse

transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, respectively. RNA concentrations were determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

sRNA library construction and sequencing. sRNA cloning was performed on 9 OGCTs (7 benign and 2 malignant) and 3 SCSTs, using previously described methodologies (22). Briefly, sRNAs were ligated with adenylated 3'-adaptor and purified on 12% denaturing polyacrylamide gel. Second ligation reaction was performed with 5'-adaptor, followed by gel purification. Complementary DNA (cDNA) synthesis was performed using the reverse transcription enzyme SuperScript II (Thermo Fisher Scientific) together with the reverse transcription primer. cDNA was then PCR amplified for 16-20 cycles with forward and reverse primers. sRNA-sequencing libraries were sequenced by Solexa/Illumina sequencing platform (Illumina 1G Genome Analyzer; Illumina Inc., San Diego, CA, USA). The sequence of the adaptors and primers has been described previously (22). The sequencing data are available at Gene Expression Omnibus (accession no. GSE98536).

miRNA analysis. sRNA sequencing reads from each of the pooled libraries were separated based on their barcode sequence. Adaptor sequences were trimmed and filtered using the package Fastx-toolkit. The resulting dataset was aligned to miRBase database version 21 (<http://www.mirbase.org/>) for mature miRNA using Bowtie short read aligner version 1.1.1 (23) with settings `-f -n 0 -l 15 -k 200 -S -best -chunkmbs 128`. All aligned reads were sorted and indexed by SAMtools version 1.1 (24). For miRNA quantification, the script HTSeq-count of the HTSeq version 0.6.1 was used (25). For clustering analysis, the miRNA counts were normalized by TMM (trimmed mean method) (26) and the normalized expression values were clustered based on Euclidean distance. For identification of differentially expressed miRNAs between two groups, the miRNA counts were normalized and analyzed by DESeq2 using default settings (27).

TaqMan RT-qPCR. RT-qPCR was performed to quantify the expression levels of *miR-373-3p*, *miR-372-3p*, *miR-302c-3p*, *miR-199a-5p*, *miR-125a-5p*, *miR-21*, *miR-34a*, *miR-202-5p*, *miR-513c-5p*, *miR-193a-3p*, *miR-214-5p* and *let-7f*, using TaqMan miRNA assays (Thermo Fisher Scientific). Approximately 120 ng of total RNA was reversed transcribed using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). The synthesized cDNA was then diluted 2- to 10-fold prior to RT-qPCR with TaqMan Universal PCR Master Mix without AmpErase UNG (Thermo Fisher Scientific). The amplification was performed using the Applied Biosystems 7500 Fast Real-time PCR system (Thermo Fisher Scientific) with the following conditions: an initial denaturation at 95°C for 10 min, 40 step cycles of denaturing at 95°C for 15 sec and annealing at 60°C for 60 sec. Each reaction was performed in triplicate. The average Ct-value of each analyzed miRNA was normalized to *RNU6B*, and reported as $2^{-\Delta Ct}$. *RNU6B* was chosen as an endogenous control due to its stability in the samples analyzed (standard deviation of average Ct = 1.8; data not shown).

Table I. Non-epithelial ovarian tumors included in the study.

Sample no.	Age at diagnosis (year)	Diagnosis	Tumor classification		Analysed by	
			Subclassification	Type	sRNA seq	RT-qPCR
OGCT1	19	OGCT	Dysgerminoma	Malignant	-	RT-qPCR
OGCT2	21	OGCT	Dysgerminoma	Malignant	-	RT-qPCR
OGCT3	41	OGCT	Dysgerminoma	Malignant	-	RT-qPCR
OGCT4	73	OGCT	Dysgerminoma	Malignant	sRNA seq	RT-qPCR
OGCT5	24	OGCT	Yolk sac tumor	Malignant	-	RT-qPCR
OGCT6	17	OGCT	Yolk sac tumor	Malignant	-	RT-qPCR
OGCT7	28	OGCT	Primitive germ cell tumor	Malignant	sRNA seq	RT-qPCR
OGCT8	26	OGCT	Mature teratoma	Benign	sRNA seq	RT-qPCR
OGCT9	64	OGCT	Mature teratoma	Benign	sRNA seq	RT-qPCR
OGCT10	47	OGCT	Mature teratoma	Benign	sRNA seq	RT-qPCR
OGCT11	58	OGCT	Mature teratoma	Benign	-	RT-qPCR
OGCT12	77	OGCT	Mature teratoma	Benign	sRNA seq	RT-qPCR
OGCT13	47	OGCT	Mature teratoma	Benign	sRNA seq	RT-qPCR
OGCT14	51	OGCT	Mature teratoma	Benign	sRNA seq	RT-qPCR
OGCT15	54	OGCT	Mature teratoma	Benign	sRNA seq	RT-qPCR
OGCT16	18	OGCT	Mature teratoma	Benign	-	RT-qPCR
SCST1	84	SCST	Signet ring-stromal tumor	Benign	sRNA seq	RT-qPCR
SCST2	45	SCST	Fibrothecoma	Benign	sRNA seq	RT-qPCR
SCST3	46	SCST	Sex cord stromal tumor	Malignant	sRNA seq	RT-qPCR
SCST4	15	SCST	Granulosa cell tumor	Malignant	-	RT-qPCR
SCST5	48	SCST	Granulosa cell tumor	Malignant	-	RT-qPCR
SCST6	15	SCST	Sertoli leydig cell tumor	Malignant	-	RT-qPCR
SCST7	20	SCST	Granulosa cell tumor	Malignant	-	RT-qPCR

No., number; OGCT, ovarian germ cell tumor; SCST, sex cord stromal tumor; sRNA seq, small RNA sequencing; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Western blotting. Western blotting was performed on 7 SCSTs, 9 benign and 7 malignant OGCTs. Total protein lysates were prepared using NP-40 lysis buffer (Thermo Fisher Scientific) with addition of 1 mM phenylmethanesulfonyl (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor (complete protease inhibitor cocktail; Sigma-Aldrich). The concentrations were measured using the Pierce BCA Protein assay kit (Thermo Fisher Scientific). Protein lysates of 30 μ g were separated in NuPAGE Novex 4-12% Bis-Tris gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk diluted in Tris-buffered saline/0.05% Tween-20 prior to incubation with anti-BECN1 rabbit polyclonal (Novus Biologicals; dilution 1:1,000) or anti-GAPDH rabbit monoclonal (Cell Signaling Technology; dilution at 1:1,000) antibodies overnight at 4°C. Anti-rabbit IgG-HRP (Cell Signaling Technology; dilution at 1:2,000) was used as a secondary antibody. Signals were detected using the Novex ECL HRP chemiluminescent substrate reagent (Thermo Fisher Scientific) and LI-COR Odyssey Fc Imaging system (LI-COR Biosciences, Lincoln, NE, USA) and quantified using Image Studio Lite version 5.2 (LI-COR Biosciences).

Statistical analysis. All statistical tests were performed using Statistical Package for the Social Sciences (SPSS) version 24 (IBM, Armonk, NY, USA), unless specified otherwise. Kruskal-Wallis test was used for comparisons among three sample groups, and Mann-Whitney U test was used for two groups. A difference of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miRNA expression profiles in non-epithelial ovarian tumors by sRNA sequencing. To assess the miRNA profiles of non-epithelial ovarian tumors, we performed sRNA sequencing of 9 OGCTs and 3 SCSTs. In total, we obtained 2,949,836 reads for OGCTs (benign, 919,886; malignant, 2,029,950) and 1,198,120 reads for SCSTs. On average, 213,678 reads from the malignant OGCT libraries, 259,915 reads from the benign OGCT libraries and 262,386 reads from the SCST libraries were mapped to mature miRNAs from miRBase release 21.

To determine whether miRNA expression profiles were distinct among the three non-epithelial ovarian tumor groups, we performed unsupervised clustering of the samples and

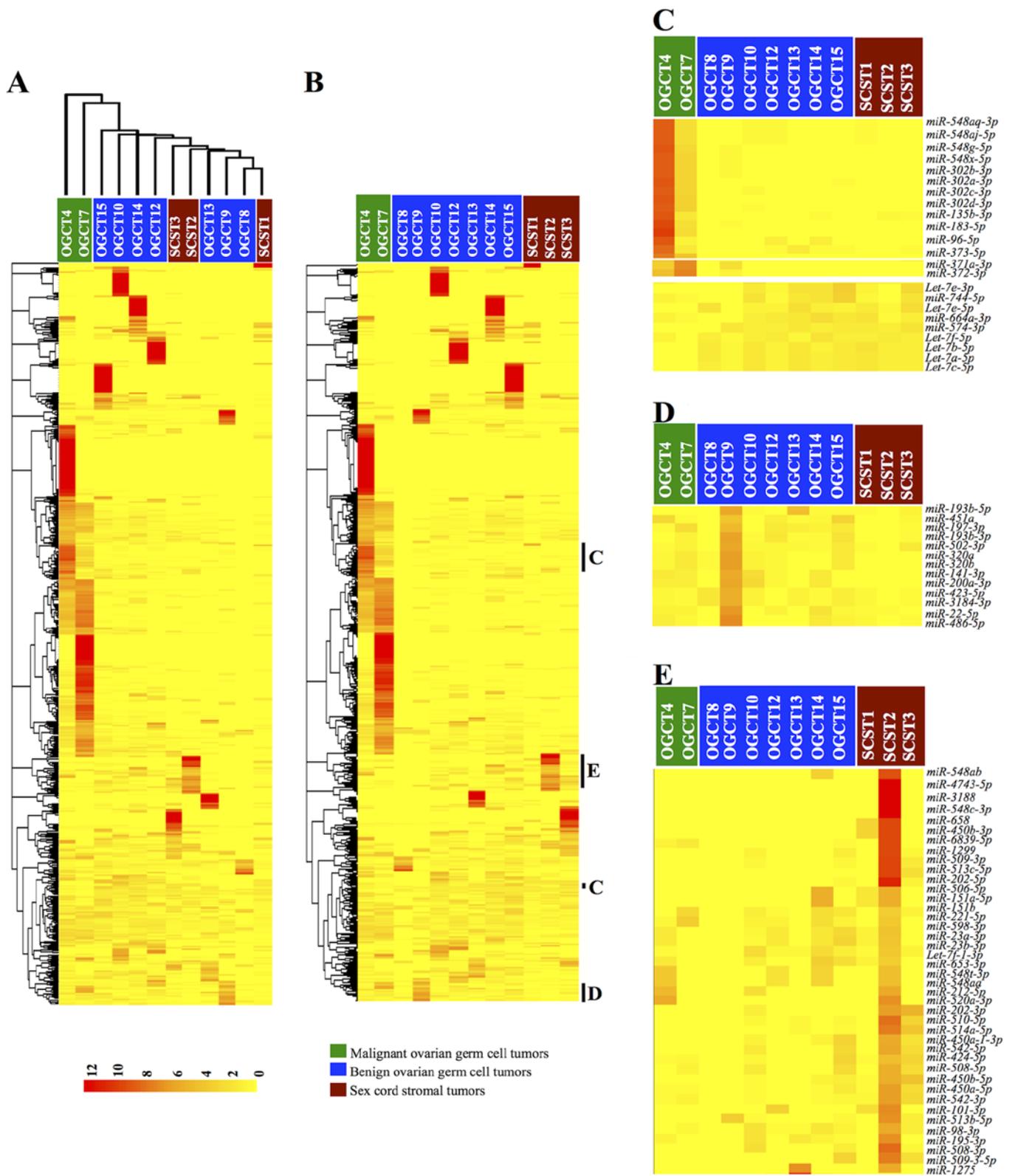


Figure 1. Clustering analysis of miRNA expression in 9 OGCTs (7 benign and 2 malignant) and 3 SCSTs. (A) Unsupervised clustering of the samples based on miRNA expression identified two major groups: one group with both malignant OGCTs and the other group consisted of both benign OGCTs and SCSTs. (B) Clustering of miRNA expression illustrates miRNA expression signatures associated with tumor types, which are further highlighted in (C-E). Clustered miRNA expression patterns associated with malignant OGCTs (C), benign OGCTs (D) and SCSTs (E). Each row represents the relative expression for a single miRNA and each column shows the expressions for a single sample. The red or yellow color indicates relatively high or low expression, respectively. OGCTs, ovarian germ cell tumors; SCSTs, sex cord stromal tumors.

miRNA expression based on Euclidean distance. As shown in the dendrogram in Fig. 1A, the benign OGCTs and SCSTs were

closely related but separated from the two malignant OGCTs, suggesting distinct miRNA expression patterns in malignant

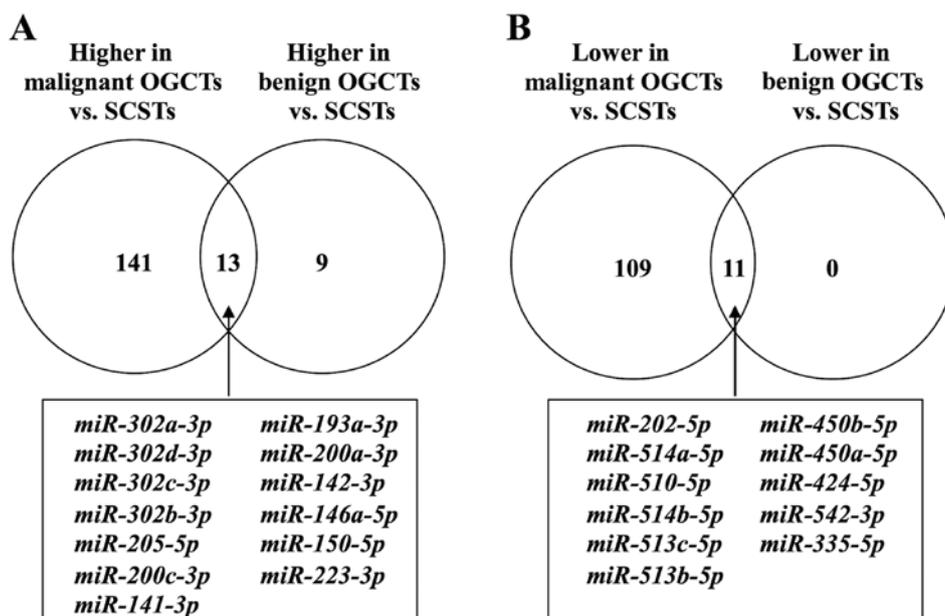


Figure 2. Venn diagrams illustrating the number of differentially expressed miRNAs between malignant and benign OGCTs as compared to SCSTs. (A) Number of miRNAs with higher expression. (B) Number of miRNAs with lower expression in the malignant or benign OGCTs. The common differentially expressed miRNAs are shown in the box, while the unique differentially expressed miRNAs for malignant or benign OGCTs are given in Table II. OGCTs, ovarian germ cell tumors; SCSTs, sex cord stromal tumors.

OGCTs and more similar miRNA expression profiles between benign OGCTs and SCSTs.

To illustrate whether specific miRNA expression signatures were distinct among these tumor groups, we performed hierarchical clustering of the miRNA expressions (Fig. 1B). Notably, several miRNAs were apparently unique to specific tumor types (Fig. 1C-E). For malignant OGCTs, several *miR-548* family, *miR-302* and *miR-371~373* clusters were more abundant, while several *let-7* family members were lower than the benign OGCTs and SCSTs (Fig. 1C). For benign OGCTs, the miRNA expression patterns were not homogenous among the same tumor type (Fig. 1D). However, *miR-193b-5p/3p*, *miR-320a/b* and *miR-22-5p* were frequently more abundant in the benign OGCTs as compared to the malignant OGCTs and SCSTs. Similarly, we also observed heterogeneous miRNA expression profiles among the three SCSTs. However, *miR-202* and *miR-506~514* cluster were higher in at least two SCSTs and absent or lower expression in the malignant and benign OGCTs (Fig. 1E).

Differentially expressed miRNAs among the three tumor types. We applied DESeq2 analysis to identify differentially expressed miRNAs between malignant and benign OGCTs. The analysis identified 128 and 59 miRNAs with higher and lower expression respectively, in the malignant OGCTs compared to the benign OGCTs (false discovery rate <0.5; data not shown). Notably, *miR-302~367* and *miR-371~373* clusters were among the differentially expressed miRNAs that had higher expression in the malignant tumors compared to the benign tumors. Additionally, expression of several *let-7* family members was lower in the malignant than the benign OGCTs.

For comparison between malignant OGCTs and SCSTs, we identified 120 miRNAs with higher expression and

154 miRNAs with lower expression in the malignant OGCTs compared to SCSTs (data not shown). Among the differentially expressed miRNAs, lower expression of *miR-202-5p*, *miR-506~514* cluster and *let-7* family, as well as higher expression of *miR-302* and *miR-371~373* cluster in the malignant OGCTs were also noted in the clustering data (Fig. 1C and E).

Strikingly, the number of differentially expressed miRNAs between SCSTs and benign OGCTs was much fewer, i.e., 11 miRNAs had lower expression and 22 had higher expression in the benign OGCTs than the SCSTs (data not shown). Notably, all 11 miRNAs with lower expression and 13 out of the 22 miRNAs with higher expression in the benign OGCTs were overlapped in both comparisons between SCSTs and malignant or benign OGCTs (Fig. 2), suggesting that these miRNAs may be specific for SCSTs. Comparing with SCSTs, 141 miRNAs with higher expression and 109 with lower expression were unique for malignant OGCTs, while only 9 miRNAs with higher expression were unique for benign OGCTs (Fig. 2 and Table II).

Validation of differentially expressed miRNAs by RT-qPCR. To validate the sRNA sequencing findings, we selected 12 miRNAs (i.e., *miR-373-3p*, *miR-372-3p*, *miR-302c-3p*, *miR-199a-5p*, *miR-125a-5p*, *miR-21*, *miR-34a*, *miR-202-5p*, *miR-513c-5p*, *miR-193a-3p*, *miR-214-5p* and *let-7f*) for RT-qPCR in an extended cohort of samples, which consisted of 16 OGCTs (9 benign and 7 malignant) and 7 SCSTs. These miRNAs were selected from different comparisons, and because of their involvement in testicular GCTs (*miR-373-3p*, *miR-372-3p*, *miR-302c-3p*, *miR-21*, *miR-513c-5p*, *miR-199a-5p*, *miR-214-5p* and *let-7f*) (11,19,20,28-32) or other tumor types (*miR-125a-5p*, *miR-34a*, *miR-193a-3p* and *miR-202-5p*) (33-35).

In the comparison between malignant and benign OGCTs, 8 of the 12 miRNAs were concordant between the sRNA

Table II. Unique differentially expressed miRNAs in malignant and benign OGCT as compared to SCST.

Differentially expressed miRNAs compared to SCST					
Higher in malignant OGCT					
<i>miR-373-3p</i>	<i>miR-376b-3p</i>	<i>miR-519e-5p</i>	<i>miR-520c-3p</i>	<i>miR-155-5p</i>	<i>miR-148a-3p</i>
<i>miR-372-3p</i>	<i>miR-9-5p</i>	<i>miR-654-3p</i>	<i>miR-758-3p</i>	<i>miR-544a</i>	<i>miR-501-5p</i>
<i>miR-182-5p</i>	<i>miR-377-3p</i>	<i>miR-18a-3p</i>	<i>miR-1296-5p</i>	<i>miR-190a-5p</i>	<i>miR-185-5p</i>
<i>miR-183-5p</i>	<i>miR-363-3p</i>	<i>miR-381-5p</i>	<i>miR-519c-3p</i>	<i>miR-505-3p</i>	<i>miR-15b-5p</i>
<i>miR-767-3p</i>	<i>miR-629-3p</i>	<i>miR-183-3p</i>	<i>miR-543</i>	<i>miR-93-3p</i>	<i>miR-433-3p</i>
<i>miR-371a-3p</i>	<i>miR-18a-5p</i>	<i>miR-361-3p</i>	<i>miR-494-3p</i>	<i>miR-149-5p</i>	<i>miR-15a-5p</i>
<i>miR-302b-5p</i>	<i>miR-3180-3p</i>	<i>miR-545-3p</i>	<i>miR-146b-5p</i>	<i>miR-769-5p</i>	<i>miR-339-3p</i>
<i>miR-7-5p</i>	<i>miR-655-3p</i>	<i>miR-345-5p</i>	<i>miR-590-5p</i>	<i>miR-20a-5p</i>	<i>miR-340-3p</i>
<i>miR-3529-3p</i>	<i>miR-18b-5p</i>	<i>miR-1277-3p</i>	<i>miR-409-3p</i>	<i>miR-92a-1-5p</i>	<i>miR-93-5p</i>
<i>miR-96-5p</i>	<i>miR-373-5p</i>	<i>miR-1248</i>	<i>miR-154-5p</i>	<i>miR-134-5p</i>	<i>miR-378a-5p</i>
<i>miR-767-5p</i>	<i>miR-136-5p</i>	<i>miR-652-5p</i>	<i>miR-625-3p</i>	<i>miR-487b-3p</i>	<i>miR-181d-5p</i>
<i>miR-105-5p</i>	<i>miR-5585-3p</i>	<i>miR-210-3p</i>	<i>miR-31-3p</i>	<i>miR-30e-5p</i>	<i>miR-505-5p</i>
<i>miR-367-3p</i>	<i>miR-493-3p</i>	<i>miR-3679-5p</i>	<i>miR-512-3p</i>	<i>miR-16-2-3p</i>	<i>miR-493-5p</i>
<i>miR-135b-5p</i>	<i>miR-372-5p</i>	<i>miR-299-3p</i>	<i>miR-377-5p</i>	<i>miR-876-5p</i>	<i>miR-130b-3p</i>
<i>miR-380-3p</i>	<i>miR-20b-5p</i>	<i>miR-339-5p</i>	<i>miR-324-5p</i>	<i>miR-651-5p</i>	<i>miR-92b-3p</i>
<i>miR-154-3p</i>	<i>miR-376a-5p</i>	<i>miR-548b-5p</i>	<i>miR-30d-5p</i>	<i>miR-19a-3p</i>	<i>miR-16-5p</i>
<i>miR-539-3p</i>	<i>miR-515-5p</i>	<i>miR-382-5p</i>	<i>miR-421</i>	<i>miR-127-5p</i>	<i>miR-301a-3p</i>
<i>miR-381-3p</i>	<i>miR-548d-5p</i>	<i>miR-379-3p</i>	<i>miR-519b-3p</i>	<i>miR-342-3p</i>	<i>miR-374a-5p</i>
<i>miR-425-5p</i>	<i>miR-329-3p</i>	<i>miR-517a-3p</i>	<i>miR-92b-5p</i>	<i>miR-485-5p</i>	<i>miR-34a-5p</i>
<i>miR-495-3p</i>	<i>miR-181c-5p</i>	<i>miR-517b-3p</i>	<i>miR-187-3p</i>	<i>miR-423-3p</i>	<i>miR-21-5p</i>
<i>miR-302a-5p</i>	<i>miR-302d-5p</i>	<i>miR-103a-3p</i>	<i>miR-519a-3p</i>	<i>miR-550a-3p</i>	<i>miR-19b-3p</i>
<i>miR-485-3p</i>	<i>miR-548y</i>	<i>miR-103b</i>	<i>miR-337-3p</i>	<i>miR-550b-2-5p</i>	
<i>miR-9-3p</i>	<i>miR-484</i>	<i>miR-107</i>	<i>miR-454-3p</i>	<i>miR-3065-5p</i>	
<i>miR-409-5p</i>	<i>miR-487a-3p</i>	<i>miR-1323</i>	<i>miR-941</i>	<i>miR-127-3p</i>	
Lower in malignant OGCT					
<i>miR-206</i>	<i>let-7b-5p</i>	<i>miR-199a-3p</i>	<i>miR-195-3p</i>	<i>miR-200b-3p</i>	<i>miR-502-3p</i>
<i>miR-509-3p</i>	<i>let-7c-5p</i>	<i>miR-199b-3p</i>	<i>miR-30c-1-3p</i>	<i>miR-615-3p</i>	<i>miR-125a-5p</i>
<i>miR-508-3p</i>	<i>let-7a-5p</i>	<i>miR-1294</i>	<i>let-7f-5p</i>	<i>miR-199b-5p</i>	<i>miR-30a-5p</i>
<i>miR-30c-2-3p</i>	<i>let-7d-3p</i>	<i>miR-98-3p</i>	<i>miR-1299</i>	<i>miR-1270</i>	<i>miR-145-3p</i>
<i>miR-542-5p</i>	<i>miR-574-3p</i>	<i>miR-508-5p</i>	<i>miR-135a-5p</i>	<i>miR-1180-3p</i>	<i>miR-193a-5p</i>
<i>miR-204-5p</i>	<i>let-7f-2-3p</i>	<i>miR-214-5p</i>	<i>miR-664a-3p</i>	<i>miR-744-5p</i>	<i>miR-99b-5p</i>
<i>miR-211-5p</i>	<i>miR-874-5p</i>	<i>miR-125b-1-3p</i>	<i>miR-1271-5p</i>	<i>miR-365a-3p</i>	<i>miR-660-5p</i>
<i>miR-1269a</i>	<i>miR-143-3p</i>	<i>miR-99a-5p</i>	<i>miR-10b-5p</i>	<i>miR-365b-3p</i>	<i>miR-532-5p</i>
<i>miR-450a-2-3p</i>	<i>miR-133a-5p</i>	<i>miR-30b-3p</i>	<i>miR-95-3p</i>	<i>miR-331-5p</i>	<i>miR-29b-2-5p</i>
<i>miR-506-5p</i>	<i>let-7b-3p</i>	<i>miR-196a-5p</i>	<i>miR-196b-5p</i>	<i>miR-140-5p</i>	<i>miR-181a-5p</i>
<i>miR-483-5p</i>	<i>miR-214-3p</i>	<i>miR-30a-3p</i>	<i>miR-574-5p</i>	<i>miR-26a-5p</i>	<i>miR-29a-3p</i>
<i>let-7c-3p</i>	<i>miR-675-5p</i>	<i>miR-100-5p</i>	<i>miR-23a-3p</i>	<i>let-7d-5p</i>	<i>miR-29c-3p</i>
<i>miR-133a-3p</i>	<i>miR-1</i>	<i>miR-509-3-5p</i>	<i>miR-23b-3p</i>	<i>miR-125a-3p</i>	<i>miR-30b-5p</i>
<i>miR-375</i>	<i>miR-193b-5p</i>	<i>miR-30e-3p</i>	<i>miR-122-5p</i>	<i>miR-191-5p</i>	<i>miR-186-5p</i>
<i>miR-133b</i>	<i>miR-125b-5p</i>	<i>miR-98-5p</i>	<i>miR-664a-5p</i>	<i>miR-184</i>	
<i>miR-135a-3p</i>	<i>miR-3609</i>	<i>miR-199a-5p</i>	<i>miR-539-5p</i>	<i>miR-221-5p</i>	
<i>let-7e-5p</i>	<i>miR-99a-3p</i>	<i>miR-532-3p</i>	<i>miR-26a-2-3p</i>	<i>miR-423-5p</i>	
<i>miR-4500</i>	<i>let-7f-1-3p</i>	<i>miR-30d-3p</i>	<i>miR-32-3p</i>	<i>miR-3184-3p</i>	
<i>miR-450a-1-3p</i>	<i>let-7e-3p</i>	<i>miR-708-3p</i>	<i>miR-28-3p</i>	<i>miR-1306-5p</i>	
Higher in benign OGCT					
<i>miR-1247-5p</i>	<i>miR-203a</i>	<i>miR-338-3p</i>	<i>miR-145-5p</i>	<i>miR-10a-5p</i>	<i>miR-22-3p</i>
<i>miR-199b-5p</i>	<i>miR-320a</i>	<i>miR-320b</i>			

miRNA, microRNA; OGCT, ovarian germ cell tumor; SCST, sex cord stromal tumor.

Table III. Evaluation of 12 selected miRNAs using sRNA sequencing and RT-qPCR methods.

miRNA	Malignant vs. benign OGCTs ^a			Malignant OGCTs vs. SCSTs ^b			Benign OGCTs vs. SCSTs ^b		
	sRNA seq	RT-qPCR	Concordance	sRNA seq	RT-qPCR	Concordance	sRNA seq	RT-qPCR	Concordance
<i>miR-372-3p</i>	Higher	Higher	Yes	Higher	Higher	Yes	Equal	Equal	Yes
<i>miR-373-3p</i>	Higher	Higher	Yes	Higher	Higher	Yes	Equal	Equal	Yes
<i>miR-302c-3p</i>	Higher	Higher	Yes	Higher	Higher	Yes	Higher	Equal	No
<i>miR-199a-5p</i>	Lower	Lower	Yes	Lower	Lower	Yes	Equal	Equal	Yes
<i>miR-214-5p</i>	Lower	Lower	Yes	Lower	Lower	Yes	Equal	Equal	Yes
<i>miR-202-3p</i>	Lower	Lower	Yes	Lower	Lower	Yes	Lower	Lower	Yes
<i>miR-513c-5p</i>	Equal	Lower	No	Lower	Lower	Yes	Lower	Lower	Yes
<i>let-7f</i>	Lower	Equal	No	Lower	Equal	No	Equal	Equal	Yes
<i>miR-21</i>	Equal	Equal	Yes	Higher	Equal	No	Equal	Equal	Yes
<i>miR-34a</i>	Higher	Equal	No	Higher	Equal	No	Equal	Equal	Yes
<i>miR-125a-5p</i>	Equal	Equal	Yes	Lower	Equal	No	Equal	Equal	Yes
<i>miR-193a-3p</i>	Higher	Equal	No	Higher	Equal	No	Higher	Equal	No

^aHigher and lower refer to expression in the malignant OGCTs compared to the benign OGCTs. ^bHigher and lower refer to expression in malignant or benign OGCTs as compared to SCSTs. miRNA, microRNA; OGCT, ovarian germ cell tumor; SCST, sex cord stromal tumor; sRNA seq, small RNA sequencing; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

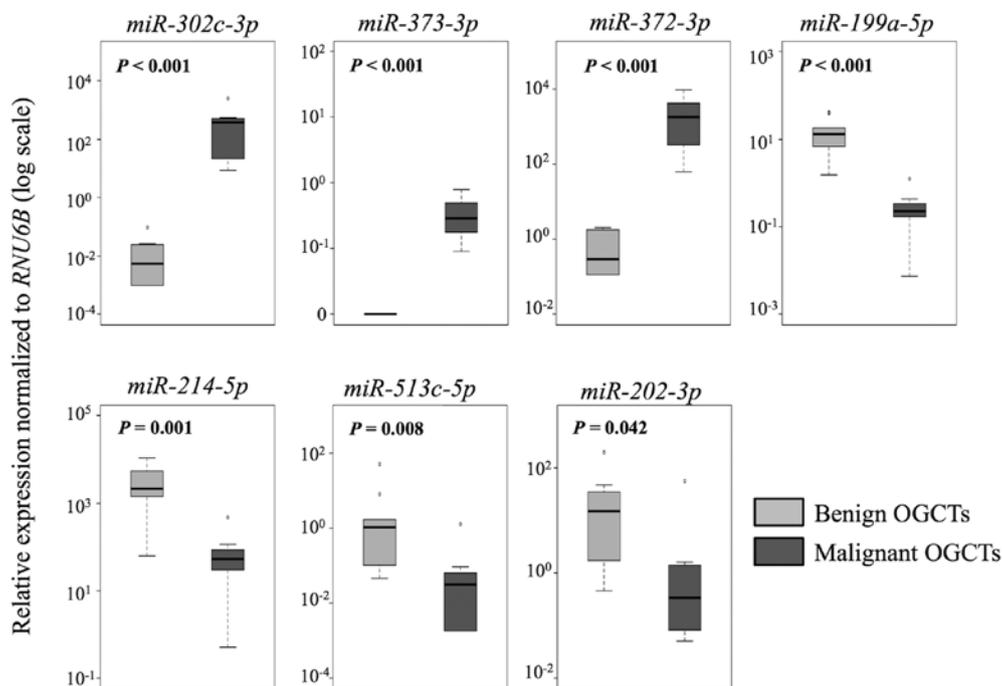


Figure 3. RT-qPCR analysis of miRNA expression in 9 benign and 7 malignant OGCTs. The boxplots show the relative expression of individual miRNAs normalized to *RNU6B*. P-values were calculated using the Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference. OGCTs, ovarian germ cell tumors; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

sequencing and the RT-qPCR results (Table III and Fig. 3). *miR-373-3p*, *miR-372-3p* and *miR-302c-3p* had higher expression, whereas *miR-199a-5p*, *miR-214-5p* and *miR-202-3p* had lower expression in the malignant OGCTs compared to the benign OGCTs (P<0.05; Mann-Whitney U test). *miR-21* and *miR-125a-5p* were not differentially expressed between the two groups using both sRNA sequencing and RT-qPCR.

Similarly, 7 and 10 out of the 12 miRNAs were concordant in both methods for the comparisons between SCSTs and malignant or benign OGCTs, respectively (Table III and Figs. 4 and 5). Three miRNAs (*miR-372-3p*, *miR-373-3p* and *miR-302c-3p*) were higher and four miRNAs (*miR-513c-5p*, *miR-202-3p*, *miR-199a-5p* and *miR-214-5p*) were lower in the malignant OGCTs compared to the SCSTs (Fig. 4A), while

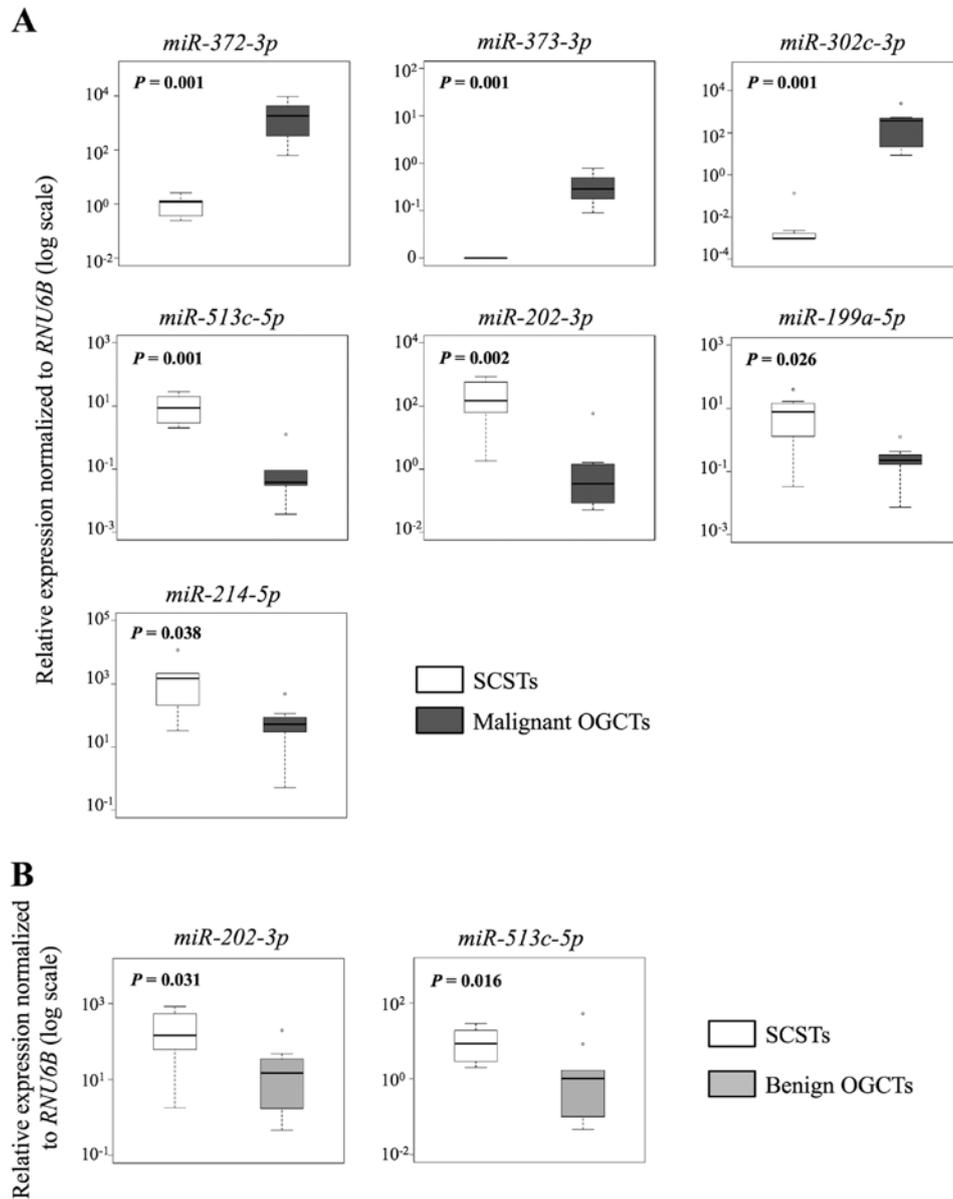


Figure 4. Validation of differentially expressed miRNAs between 7 malignant or 9 benign OGCTs and 7 SCSTs by RT-qPCR. Boxplots illustrating the relative miRNA expression levels (A) between malignant OGCTs and SCSTs, and (B) between benign OGCTs and SCSTs. All miRNA expressions were normalized to RNU6B. $P < 0.05$ was considered to indicate a statistically significant difference by Mann-Whitney U test. OGCTs, ovarian germ cell tumors; SCSTs, sex cord stromal tumors; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

only *miR-202c-3p* and *miR-513c-5p* were lower in the benign OGCTs than the SCSTs (Fig. 4B). Eight miRNAs (*miR-372-3p*, *miR-373-3p*, *miR-199a-5p*, *miR-214-5p*, *let-7f*, *miR-21*, *miR-34a* and *miR-125a-5p*) were not differentially expressed between benign OGCTs and SCSTs using both methods. Comparing the three groups, 7 miRNAs (*miR-302c-3p*, *miR-373-3p*, *miR-372-3p*, *miR-513c-5p*, *miR-199a-5p*, *miR-202-3p* and *miR-214-5p*) were significant using Kruskal-Wallis test ($P < 0.01$; Fig. 5), suggesting their diagnostic potential in ovarian non-epithelial tumors.

Evaluation of BECN1 expression in non-epithelial ovarian tumors. Beclin 1 (*BECN1*) is one of the known direct targets of *miR-199a-5p* (36,37) and it plays an important role in germ cell survival and proliferation (38,39). We therefore examined *BECN1* expression in 16 OGCTs (7 malignant and 9 benign)

and 7 SCSTs using western blot analysis. The results showed that *BECN1* was the most abundant in all malignant OGCTs analyzed, moderate to high levels in SCSTs, and very low or undetectable levels in benign OGCTs (Fig. 6). For quantification, we excluded two samples, OGCT14 and OGCT11, due to low concentrations of the protein lysates. *BECN1* expression was significantly different among the three groups (Kruskal-Wallis test, $P < 0.001$), and the significant difference was observed in the comparison between malignant and benign OGCTs.

Discussion

miRNAs play important roles in gene regulation of many cellular processes that contribute to cancer development and progression. Despite miRNA expression and function have been characterized in a broad range of tumor types, miRNA

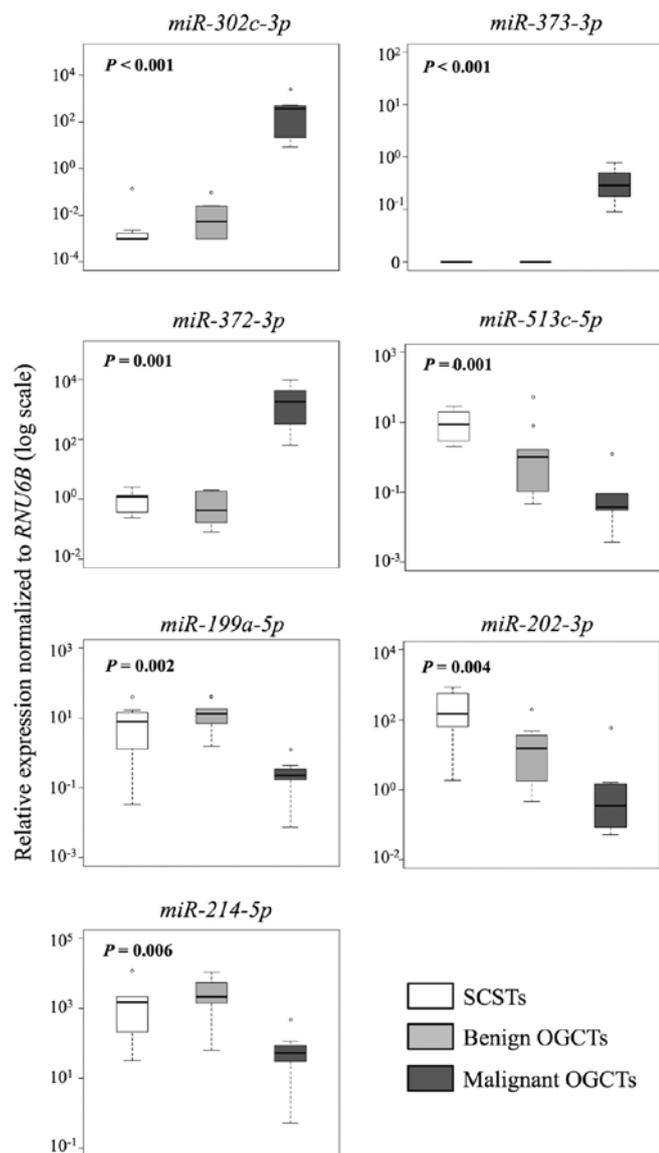


Figure 5. Significant differentially expressed miRNAs among the three non-epithelial ovarian tumor types. The boxplots show relative miRNA expression levels in 7 SCSTs, 9 benign and 7 malignant OGCTs, as determined by RT-qPCR. RNU6B was used for normalization. Statistical significance was calculated by Kruskal-Wallis test. $P < 0.05$ was considered to indicate a statistically significant difference. OGCTs, ovarian germ cell tumors; SCSTs, sex cord stromal tumors; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

profiles of non-epithelial ovarian tumors are still scarce. In this study, we characterized miRNA expression pattern of OGCTs and SCSTs using sRNA sequencing. Our analysis of miRNA profiles indicated similarities and differences of expression level in these tumor types.

We observed higher expression of *miR-302* and *miR-371~373* cluster in malignant OGCTs, which is consistent with previous studies (18-21,40). Although the clinical significance of these miRNAs in OGCT has not been investigated, these miRNAs appear as potential serum biomarkers for diagnosis and follow-up of malignant testicular GCTs (TGCTs) (29,30,41,42). Importantly, *miR-372* and *miR-373* have been demonstrated as oncogenes in TGCTs (28), and *miR-302* can regulate stemness, differentiation and tumorigenesis (43-47).

Similarly, lower expressions of *miR-199a* and *miR-214* were also observed in malignant TGCTs (32,48). *miR-199a-2* (one of the two loci encoding *miR-199a*) and *miR-214* are derived from the same cluster located on chromosome 1q24.3, and their expressions are regulated by the same promoter and transcription factor (49). Previous studies have shown that the promoter of this locus is frequently hypermethylated in malignant testicular tumors leading to decreased expressions of *miR-199a* and *miR-214* (48,50,51). *miR-199a-5p* has been reported to act as a tumor suppressor in TGCTs by suppressing cell proliferation, migration, invasion and metastasis, possibly through its targets podocalyxin-like (*PODXL*) and MAF BZIP transcription factor B (*MAFB*) (48,51). Although *miR-214* has been demonstrated to directly regulate proteasome 26S subunit, non-ATPase 10 (*PSMD10*) in TGCT (32), its functional role remains undetermined. Contrary to their reduced expression in OGCTs, increased expression of *miR-199a* and *miR-214* is found in epithelial ovarian cancer (52). *miR-199a* has been shown to regulate nuclear factor κ B (NF- κ B) activity via targeting inhibitor of nuclear factor κ B kinase subunit beta (*IKK β*) (53), while *miR-214* can induce cell survival and cisplatin resistance by targeting phosphatase and tensin homolog (*PTEN*) in ovarian carcinoma cells (52,54).

Comparing with both malignant and benign OGCTs, two miRNAs (*miR-202-3p* and *miR-513c-5p*) are significantly higher in SCSTs. Expression of *miR-202-3p* is detected specifically in gonads, and predominantly in the granulosa (55) and Sertoli cells (56), supporting that this miRNA is specific for SCSTs. Additionally, *miR-202-3p* is tightly linked to sex hormone secretion and sex differentiation (57-59), suggesting its important role in gonadal development and differentiation. Indeed, *miR-202-3p* prevents spermatogonial stem cell differentiation by suppressing multiple cell cycle regulators and RNA binding proteins (60). *miR-202-3p* expression is also correlated with the expression level of testis-associated (e.g., *SOX9*) and ovary-associated genes (e.g., *FOXL2*) (57), in which both *SOX9* and *FOXL2* are commonly expressed in SCSTs. *FOXL2* is a transcription factor required for granulosa cell differentiation and ovary development (61), and its somatic mutation has been linked to the development of adult granulosa cell tumors of the ovary (12). The mutation can protect granulosa cells from apoptosis (13) and promote tumorigenesis through enhanced glycogen synthase kinase 3 β (*GSK3 β*)-mediated S33 phosphorylation (14). Further studies are yet to be conducted to determine the expression relationship between *miR-202-3p* and *FOXL2* and the functional role of *miR-202-3p* in the development of SCST.

miR-513c-5p is one of the *miR-513* subfamily that belongs to the *miR-506~514* cluster. Although hardly anything is known about the functional role of *miR-513c-5p*, the miRNA cluster has been demonstrated as an oncogene or a tumor suppressor depending on cellular context. In melanoma, this cluster can promote melanocyte transformation and melanoma growth (62). On the other hand, it has been shown to suppress cell growth and induce senescence in ovarian carcinoma (63), and inhibit NF- κ B pathway in TGCT (31). Notably, the *miR-506~514* cluster can be induced by Forkhead box protein O1 (*FOXO1*) (64). *FOXO1* is a transcription factor that plays important roles in regulation of apoptosis, cell cycle progression, insulin signaling and metabolic homeostasis in

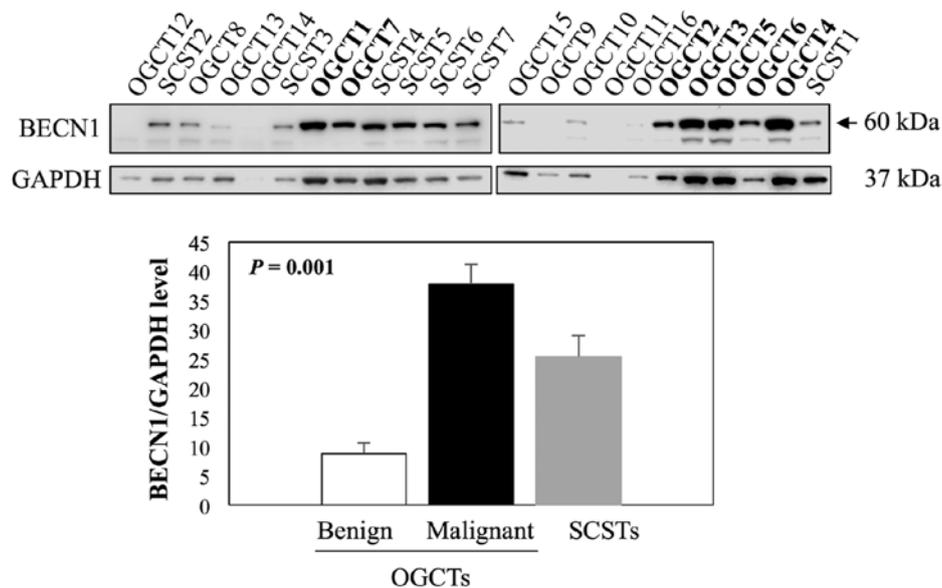


Figure 6. Evaluation of BECN1 protein expression in 7 SCSTs (SCST1-7), 9 benign (OGCT8-16) and 7 malignant OGCTs (OGCT1-7) using western blot analysis. GAPDH was used as a loading control. Bar chart represents relative mean expression with standard error of the mean. P-value was calculated using Kruskal-Wallis test. Malignant OGCTs are indicated in bold. OGCTs, ovarian germ cell tumors; SCSTs, sex cord stromal tumors; BECN1, Beclin 1.

response to oxidative stress (65). FOXO1 is expressed in granulosa cells of growing follicles (66), and is critical in granulosa cell fate decisions and follicle growth (67,68). Importantly, Liu *et al*, showed that inactivation of the two FOX proteins FOXO1 and FOXO3 expression in mouse granulosa cells can promote ovarian granulosa cell tumor development, and coordinate *PTEN* depletion enhances the granulosa cell tumor occurrence in *FOXO1/3* depletion mice (69). Together, it is tempting to speculate that *miR-506~514* cluster may contribute to the development of SCST (in particular the granulosa cell tumor subtype) through FOXO1/*PTEN* pathways.

We also observed increased expression of BECN1, a target of *miR-199a-5p*, in malignant OGCTs, suggesting its importance in ovarian germ cell maintenance and/or tumorigenesis. In line with such speculation, BECN1 is required to control germ cell proliferation/survival (38,39) and cell cycle progression (38,70). BECN1 is one of the key regulators of autophagy, an evolutionally conserved regulatory pathway of cellular degradation and recycling (71). Autophagy has been shown to play important roles in germ cell function in multiple organisms, including mouse (72), fish (73), worm (74,75), moss (76) and sea urchin (77). Although the role of BECN1 or autophagy in human germ cell tumorigenesis remains unclear, Rossi *et al* demonstrated that mitogen-activated protein kinase 15 (MAPK15)-mediated autophagy can promote cell proliferation and prevent DNA damage accumulation in TGCTs (78). On the other hand, BECN1 expression is lower in malignant epithelial ovarian cancers than their non-malignant counterparts (79), and induction of autophagy in ovarian carcinoma cells can lead to tumor suppression (80). Together, autophagy may function as a tumor suppressor in ovarian epithelial tumors and an oncogene in OGCTs.

In conclusion, we showed that different non-epithelial ovarian tumors have distinct miRNA expression pattern, implying their role in tumorigenesis and their potential values as diagnostic markers. Our data also provide the starting

points to elucidate the molecular mechanisms underlying the pathogenesis of these tumor types.

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