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RESEARCH ARTICLE

MicroRNA sequencing detects miR-424-5p up-regulation in ovarian cancer stem cells

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Abstract Cancer stem cells (CSCs) are cancer cells that possess the ability to undergo continuous proliferation and self-renewal. It has been postulated that CSCs are responsible for tumor growth, heterogeneity, invasion, metastasis, and recurrence. MicroRNAs (miRNAs), small non-coding RNAs of approximately 22 nucleotides, are known to be involved in the maintenance of CSCs. To gain insight into the role of miRNAs in CSCs, we investigated the differential expression of miRNAs in ovarian CSCs compared to non-CSCs. Ovarian CSCs were isolated from the human ovarian cancer cell line SK-OV-3 using two ovarian CSC-specific surface markers, CD44 and CD117. The expression levels of miRNAs in CSCs and non-CSCs were estimated by miRNA sequencing. We detected four up-regulated miRNAs (miR-29a-5p, miR-34c-5p, miR-106a-5p, and miR-424-5p) in ovarian CSCs, and miR-424-5p was validated by real-time qPCR. MiR-424-5p target genes were predicted using several validated target databases and computational algorithms. Pathway analysis indicated that most miR-424-5p target genes are involved in cancer-related biological pathways. Overall, these results suggest that miR-424-5p is a potential regulator of CSCs that endows human ovarian tissue with tumorigenic potential and thus represents a potential therapeutic target for human ovarian cancer.

Keywords Ovarian cancer stem cells · SK-OV-3 cells · microRNA · microRNA sequencing

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Introduction

Cancer stem cells (CSCs), also known as tumor-initiating cells (TICs), are a small subset of the malignant tumor cell population. Indeed, CSCs that can generate the heterogeneous lineages of cancer cells retain stem cell-like characteristics, such as self-renewal (Kreso and Dick 2014; Reya et al. 2001). Furthermore, increasing evidence from diverse tumor types suggests the functional relevance of CSCs to tumor initiation, progression, invasion, metastasis, and recurrence as well as to therapeutic resistance (Li et al. 2007).

Ovarian cancer is common among women and is one of the leading causes of death from gynecologic malignancies. Ovarian cancer is usually treated by some combination of surgery, radiation therapy, and chemotherapy. Despite advances in ovarian cancer treatment, the majority of patients still fail therapy, resulting in disease recurrence and eventual disease-related death. As expected, CSCs are suspected to be involved in therapeutic failure and the recurrence of ovarian cancer (Bapat 2010). Thus, the strategy of targeting CSCs to inhibit their molecular mechanisms of action is emerging as a promising option for eliminating cancer or improving cancer treatment.

Human ovarian CSCs have been isolated from SK-OV-3 ovarian carcinoma cells using the specific cell surface antigen markers CD44 and CD117 (Chen et al. 2013a, b). It has been demonstrated that $CD44^+CD117^+$ CSCs are highly tumorigenic, and these cells have the ability to re-establish the original tumor hierarchy when injected into nude mice. Zhang and colleagues further identified and characterized ovarian CSCs from primary human tumor samples (Zhang et al. 2008). Additional studies revealed that the surface markers CD117 (Luo et al. 2011) and CD44 (Alvero et al. 2009) were each related to tumorigenesis and chemotherapy outcome. However, the underlying molecular mechanisms of action of ovarian $CD44^{+-}$ $CD117^+$ CSCs have not been clearly elucidated.

MicroRNAs (miRNAs), small non-coding RNA molecules of approximately 22 nucleotides, are a class of post-transcriptional/translational regulators that modulate gene expression by degrading target mRNA or blocking target gene translation (Deiters 2010; He and Hannon 2004). Thus, miRNAs are believed to be involved in the regulation of numerous biological processes, including cancer development (Papagiannakopoulos and Kosik 2008). A number of studies have indeed showed that miRNAs are associated with CSC development (Liu et al. Liu and Tang 2011; Papagiannakopoulos and Kosik 2008) and ovarian cancer (Zaman et al. 2012).

With recent advances in sequencing technology, next generation sequencing (NGS) has emerged as a promising massively parallel high-throughput sequencing method for discovering genetic variation in the whole genome as well as quantifying cellular expression levels. MiRNA sequencing via NGS has been utilized to examine tissue-specific expression patterns, disease associations and isoforms of miRNAs and to discover previously uncharacterized miRNAs. In this study, we isolated CSCs from SK-OV-3 human ovarian cancer cells, conducted miRNA sequencing, and analyzed differentially expressed miRNAs between CSCs and non-CSCs to unravel the underlying molecular basis of CSCs.

Materials and methods

Cell culture

SK-OV-3 human ovarian cancer cells were obtained from American Type Culture Collection (ATCC) and were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. All the supplements were purchased from HyClone (USA). The cells were cultured in a humidified atmosphere of 5 % CO_2 at 37 °C.

Isolation of ovarian CSCs

The ovarian CSCs from SK-OV-3 cells were isolated by fluorescence-activated cell sorting (FACS) according to a previously described method (Chen et al. 2013a, b) with slight modifications. Briefly, the cells were dissociated with trypsin and then washed with PBS. The cells were suspended in sorting buffer (1X PBS containing 1 mM EDTA, 1 % heat inactivated-FBS, and 25 mM HEPES, pH 7.0). Prior to cell sorting, cell surface markers were labeled with direct fluorochrome-conjugated antibodies under sterile conditions. Two antibodies, the FITC-conjugated CD44 antibody (BD Biosciences) and the PE-conjugated CD117 antibody (Miltenyi Biotec., Germany), were used at the concentrations recommended by the manufacturers. Labeled cells were sorted using an S3e™ cell sorter (BIO-RAD, USA). A sorting purity of greater than 89 % was ensured for our experiments.

Total RNA extraction and miRNA isolation

Total RNA was extracted using TRIzol® reagent according to the manufacturer's protocol (Invitrogen, USA). Extracted RNA was purified using RNeasy Mini Kit Columns (Qiagen, USA). MiRNA was isolated using a High Pure miRNA Isolation Kit (Roche, USA).

MiRNA sequencing

Six total RNA samples (triplicate CSC and non-CSC samples) were prepared as described above for sequencing. The 5′ and 3′ ends of the total RNAs were ligated with adapters. Subsequently, the cDNAs synthesized from the ligated RNAs were amplified by PCR. The enriched PCR products were separated by PAGE, and the small fragments (approximately 120–140 bp) were isolated from the gel. These fragments were used for cluster generation and sequencing on an Illumina HiSeq 2000 instrument (Illumina, Inc., USA).

Sequencing data analysis

The quality of the sequence reads (from the FASTQ files) generated by the Illumina sequencer was checked using SolexaQA, a Perl-based software package, to remove the low quality reads. Adapter sequences in the reads were trimmed using an in-house script. All the trimmed reads were clustered according to the unique base sequence of the reads using CD-HIT (http://weizhong-lab.ucsd.edu/cd-hit/). To identify miRNA sequences, unique sequences identified after clustering were mapped to known miRNAs in the miRBase database (http://www.mirbase.org/) by blast search. Trimmed reads were considered to be miRNAs if

they possessed 100 % identity at the sequence level and covered the whole length of the miRNA based on the sequences in miRBase.

Identification of differentially expressed miRNAs

The read counts of detected miRNAs were considered to represent the level of miRNA expression. The reads counts from triplicate CSC and non-CSC samples were normalized by the quantile method (Bolstad et al. 2003). MiRNAs with a read count less than ten were removed from subsequent analyses. The average read count in triplicate samples for each miRNA was compared between CSCs and non-CSCs to identify differentially expressed miRNAs according to the criteria of a t test P value < 0.05 and a fold change ≥ 2. Statistical analyses were performed using R (version 3.1.2).

Prediction of miRNA target genes

Eight programs were utilized to predict miRNA target genes in this study. The rule-based algorithms included DIANA-microT Web server 5.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php? r = microT_CDS/index), TargetScan 6.2 (http://www.targetscan.org/), miRDB (http://mirdb.org/miRDB/) and PITA (http://genie.weizmann.ac.il/index.html). The data-driven algorithms for experimentally validated target prediction included miRecords (http://mirecords.biolead.org/), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), miR-TarBase (http://mirtarbase.mbc.nctu.edu.tw/), and TarBase 7.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index). To reduce the false negative rate from using these programs, we integrated the results obtained from each target prediction analysis.

Pathway analysis of the predicted miRNA target genes

The predicted miRNA target genes were submitted to the DAVID online tool (http://david.abcc.ncifcrf.gov/home.jsp) (Dennis et al. 2003; Huang et al. 2009) for gene ontology (GO) annotation (Ashburner et al. 2000) and pathway enrichment analysis. Functional classification of the targets was performed by the KEGG pathway database (Kanehisa and Goto 2000; Kanehisa et al. 2006).

Real-time quantitative PCR to validate differentially expressed miRNAs

MiRNA cDNA was synthesized using a miRCURY LNA™ cDNA Synthesis Kit II (EXIQON, Denmark) according to the manufacturer's recommendations. Mature miRNA expression was detected using LightCycler® 480 SYBR Green I Master (Roche) with a miRCURY LNA™ UniRT primer set. All the PCR primer sets were purchased from EXIQON (Supplementary Table S1). U6 snRNA was used as the internal control to normalize the amplification data. The amplifications were performed in an LC480 Detection System (Roche) in 384-well plates. The real-time qPCR data were analyzed by one-way ANOVA with Dunnett's multiple comparison test using GraphPad Prism 5 (GraphPad Software, Inc., USA), and differences were considered to be statistically significant at $P < 0.05$.

Results

To isolate CSCs, SK-OV-3 cells were stained for the CD44 and CD117 surface marker antibodies and sorted by FACS. A large population of SK-OV-3 cells were CD44$^+$/CD117$^-$ (Fig. 1), as previously reported (Chen et al. 2013a, b). Two subpopulations of cells, CD44$^+$/CD117$^+$ and CD44$^+$/CD117$^-$, were grouped as the CSCs and non-CSCs, respectively. CSCs were gated at a maximum of 14 % in the double-positive extremity. The sorting purities were estimated to be 89 and 98 % for CSCs and non-CSCs, respectively. In total, 6×10^5 cells from each subpopulation were isolated from SK-OV-3 cells and used for subsequent experiments.

The expression levels of a total of 1199 different miR-NAs were estimated by miRNA sequencing. Of these, 474 miRNAs passed our filtering criterion (read count ≥ 10) and were subsequently analyzed to detect differentially expressed miRNAs between CSCs and non-CSCs. We detected four up-regulated miRNAs (miR-29a-5p, miR-34c-5p, miR-106a-5p, and miR-424-5p) in CSCs by statistical analysis (t test P value < 0.05) and fold change (≥ 2) (Table 1; Fig. 2). We further confirmed the up-regulation of these miRNAs in CSCs by real-time qPCR. Of the four miRNAs, only miR-424-5p showed evidence of significant up-regulation (1.8-fold increase) in SK-OV-3 CSCs (Fig. 3).

To gain insight into the functional role of miR-424-5p in SK-OV-3 CSCs, we predicted target genes using experimentally validated miRNA target databases (for example, miRTarBase and TarBase) and computational algorithms (for example, DIANA-microT and miRDB). The validated and computational targets, which included 2330 genes, were united to reduce false negative results (Supplementary Table S2). The predicted target genes for miR-424-5p were largely involved in cancer-related biological pathways based on KEGG pathway analysis (Table 2). In addition, gene ontology analysis indicated that miR-424-5p target genes were highly related to protein phosphorylation (Supplementary Table S3).

Fig. 1 CD44/CD117 analysis and the isolation of SK-OV-3 cells by FACS. The cells were stained with CD44-FITC and CD117-PE. Approximately 6×10^5 CSCs (CD44$^+$/CD117$^+$) and non-CSCs (CD44$^+$/CD117$^-$) were isolated from SK-OV-3 cells. CSCs or non-CSCs were gated at a maximum of 14 % of the entire viable cell population
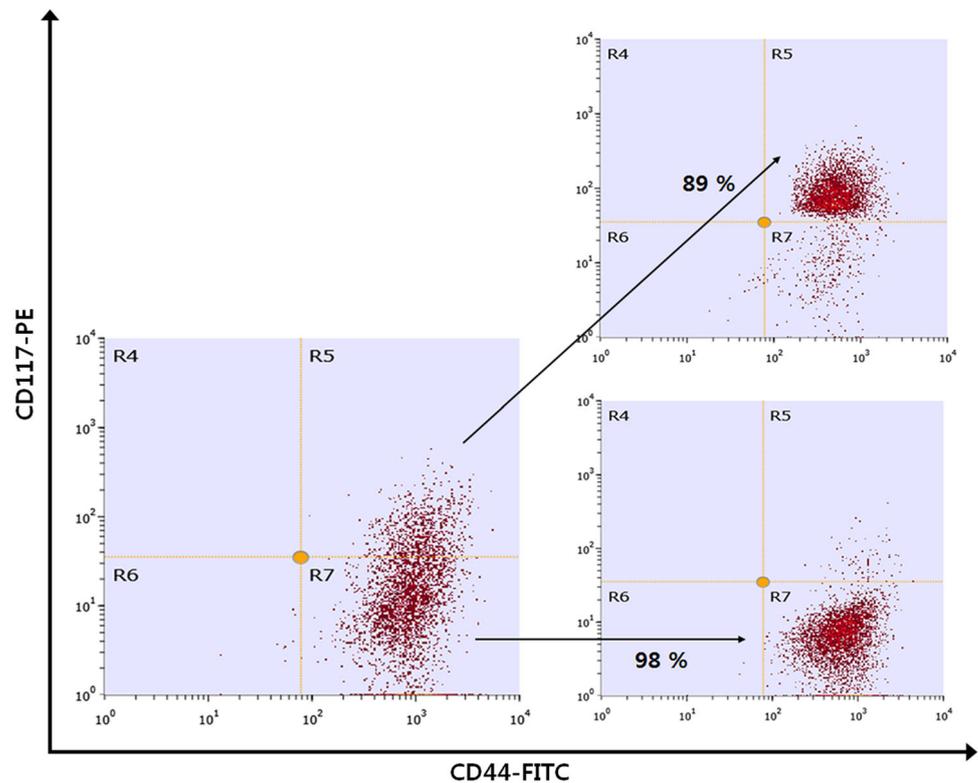

Table 1 Differentially expressed miRNAs with *t*-test *P* values less than 0.05 and a fold change greater than 2 between CSCs and non-CSCs. The expression levels of a total of 1199 different miRNAs were estimated by miRNA sequencing

miRNA	*t* test *P* value	Fold change (CSC/non-CSC)
hsa-miR-34c-5p	0.0091	2.69
hsa-miR-424-5p	0.0023	2.26
hsa-miR-106a-5p	0.0094	2.82
hsa-miR-29a-5p	0.0349	2.02

Real-time qPCR result of miR-424-5p showed evidence of significant (*P* value < 0.05) up-regulation (1.8-fold increase) in CSCs, while those of three miRNAs (miR-29a-5p, miR-34c-5p, and miR-106a-5p) were not available

Discussion

Dysregulation of miRNAs is commonly observed in many cancers, resulting in the up-regulation of oncogenes or the down-regulation of tumor suppressor genes. Accumulating evidence has suggested that miRNAs are also involved in the regulation of CSCs and are aberrantly expressed in ovarian cancer (Di Leva and Croce 2013; Iorio et al. 2007; Liu and Tang 2011). Based on these previous findings, the identification of differentially expressed miRNAs in ovarian CSCs and their molecular targets may enable the development of therapeutics for the treatment of ovarian
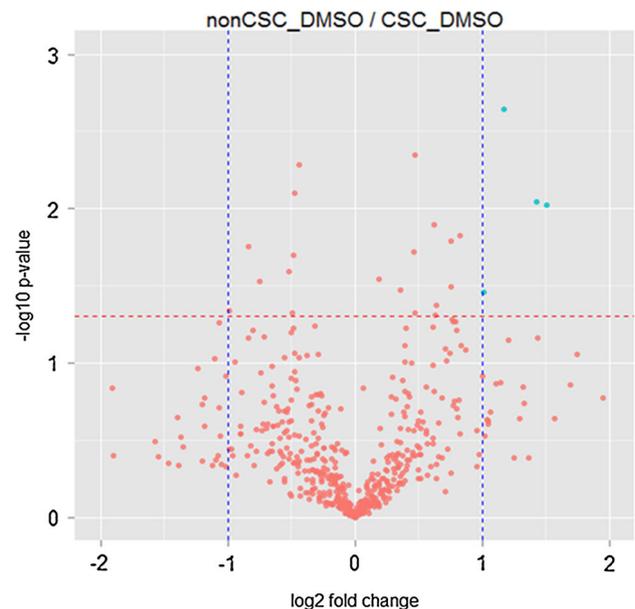

Fig. 2 A volcano plot showing the differentially expressed miRNAs between ovarian CSCs and non-CSCs. Red dotted line indicates a *t*-test *P* value of 0.05, while *blue dotted lines* indicate a fold change of two in miRNA expression between CSCs and non-CSCs. Four miRNAs (represented as *blue dots*) showed significantly increased expression in CSCs (*t* test *P* < 0.05 and fold change >2.0)

cancer. In our study, we detected up-regulated miRNAs, including miR-29a-5p, miR-34c-5p, miR-106a-5p, and miR-424-5p, by miRNA sequencing. Of these four
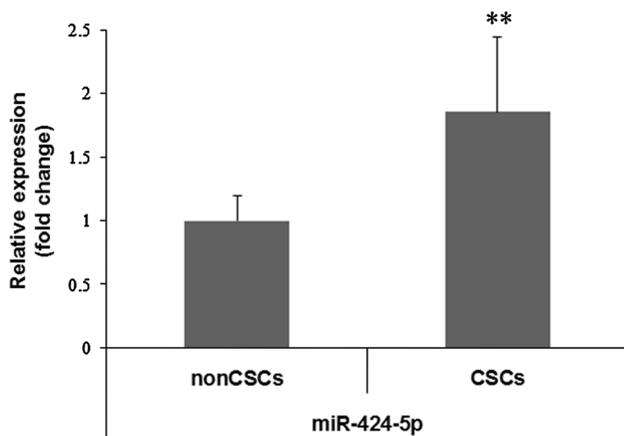
Fig. 3 Real-time qPCR results of miR-424-5p expression in ovarian CSCs and non-CSCs. Increased miR-424-5p expression was detected by real-time qPCR in SK-OV-3 CSCs compared to non-CSCs. The data are presented as the mean $\pm$SD (n = 3) of four independent experiments. The *double asterisk* denotes a significant difference in miRNA expression between CSCs and non-CSCs (**$P < 0.01$)

Table 2 KEGG pathway analysis of the predicted target genes of hsa-miR-424-5p that were up-regulated in CSCs compared to non-CSCs

Pathway of target genes	Count	P value
Pathways in cancer	74	1.19×10^{-7}
Neurotrophin signaling pathway	36	1.09×10^{-6}
Pancreatic cancer	25	2.29×10^{-6}
mTOR signaling pathway	20	5.70×10^{-6}
Colorectal cancer	26	1.39×10^{-5}

Count indicates the number of genes involved in the term (Pathway)

P value means the modified Fisher Exact P value

miRNAs, only the expression of miR-424-5p was validated by real-time qPCR. Significant differential expression of the other three miRNAs was not observed by qPCR.

Generally, RNA sequencing technology enables the whole transcriptome (not just detected transcripts) to be investigated in a high-throughput and highly accurate manner to quantify expression levels (Wang et al. 2009). Usually, qPCR analysis requires nucleotide sequences longer than miRNAs to ensure the successful detection of expression levels. Thus, the failure to validate three miR-NAs (miR-29a-5p, miR-34c-5p, and miR-106a-5p) that were detected by miRNA sequencing likely resulted from the limitations of qPCR to detect miRNA expression.

MiR-424-5p, which was validated by qPCR, is a potential regulator of CD44$^+$/117$^+$ ovarian CSCs isolated from SK-OV-3 cells. Numerous studies have shown that miR-424 has oncogenic effects; it is known as an "on-comir" (having oncogenic activity) and is associated with tumorigenesis. Some studies have demonstrated that high levels of miR-424 are related to the induction of tumorigenesis (Park et al. 2013; Zhang et al. 2014). However, another study showed that high levels of miR-424 repressed tumorigenesis (Oneyama et al. 2013). These contradictory observations suggest different oncogenic effects of miR-424 in different tissue types.

MiR-424-5p target genes were predicted from databases of experimentally validated targets, such as miRTarBase and TarBase, and using computational algorithms, such as DIANA-microT and miRDB (Supplementary Table S2). Pathway analysis demonstrated that the predicted target genes of miR-424-5p are highly related to cancer development (Table 2). Gene ontology analysis further suggested that miR-424-5p may regulate target genes involved in cancer development via particular signaling pathways (Supplementary Table S3). Taken together, these results suggest that miR-424-5p is potential key regulator of SK-OV-3 ovarian cancer cells and CSCs.

We also predicted target genes for three miRNAs (miR-29a-5p, miR-34c-5p and miR-106a-5p) using target gene prediction programs (Supplementary Tables S4–S6). Pathway analyses demonstrated that these target genes are also related to cancer development (Supplementary Table S7), suggesting that further studies might be worthwhile to elucidate the biological roles of these miRNAs in CSCs.

Conflict of interest The authors declare no conflicts of interest.

Ethical statement In this study, human ovarian cancer cells were obtained from the commercially available human ovary adenocarcinoma (ATCC$^®$ HTB-77^{TM}). This study was approved by Hallym University Institutional Review Board (HIRB).

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