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MicroRNA signatures of platinum-resistance in ovarian cancer

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Summary

Objectives: The authors utilized a microRNA (miRNA) array to compare the differentially expressed miRNAs in platinum-resistant associated ovarian cancer cells. *Materials and Methods:* The differential expression of microRNA between COC1 (DDP-sensitive) and platinum-resistant COC1/DDP (DDP-resistant) tumor cell lines was determined using microarray. Expression levels were further validated by real-time quantitive polymerase chain reaction (qRT-PCR). *Results:* The authors identified that several miRNAs are altered in collected 86 samples of human ovarian cancer cell-lines, with four significantly deregulated miRNAs and 13 upregulated miRNAs. Of which, miR-141-3p was the most differentially expressed miRNA between COC1 group (1.7833 ± 0.7213) and COC1/DDP group (14.0433 ± 4.4895) ($p < 0.05$). Additionally, the product curve of PCR amplification indicated that miR-141-3p had a significant higher expression level in chemotherapy resistant group (n=20) rather than in chemotherapy sensitive group (n=20) (9.56 ± 1.04 vs. 1.59 ± 0.91, $p < 0.05$). *Conclusions:* The present results suggest that miR-141-3p might be used as a therapeutic target to modulate platinum-based chemotherapy and as a biomarker to predict chemotherapy response.

Key words: Ovarian cancer; miRNA; Platinum-resistant; Platinum-based drug; Micoarray.

Introduction

Although great advance has been made in the understanding and therapy of ovarian cancer, it still remains the sixth most common leading-death cancer in women in the world [1]. Ovarian carcinoma can be classified into three broad subgroups such as epithelial, stromal, and germ cell tumors, each of them with different etiologies and clinical behavior. Epithelial ovarian cancer is the most common, accounting for more than 85% of all cases of ovarian cancer. Despite progress in platinum-based chemotherapy have resulted in improved survival, patients typically experience disease relapse within two years of initial treatment and develop platinum resistance [2]. Greenlee *et al*. have stated that the diminishing response rate for platinum-based drugs is due to the development of drug resistance, resulting in only 30% of five-year survival rate among patients with advanced ovarian cancer [3].

Currently, extensive studies have illustrated the existence and importance of mechanism of chemotherapeutic resistance mediated by means of short noncoding RNA [4-6]. Miller *et al*. has identified that miR-221/222 expression and HER2/neu overexpression in primary breast tumors that are generally resistant to tamoxifen therapy [7]. Moreover, it has been verified that miR-214 [8] and has-mir-27a [9] can induce platinum-based drug resistance in human ovarian cancer.

Here, the present authors used a cell culture model to determine the miRNA expression profile of a platinum-resistant cell line that was subsequently validated in primary human ovarian cancers.

Materials and Methods

Patients and specimens

Ovarian cancer COC1 and COC1/DDP cell-line were collected from Chinese center for typical culture collection. Specimens for real-time quantitative polymerase chain reaction /qRT-PCR) were collected from 86 patients with ovarian serous carcinoma. All patients treated with paclitaxel and carboplatin were followed up for six months. According to the National Comprehensive Cancer Network (NCCN) ovarian cancer practice guideline [10], chemotherapy resistance was defined if patients were observed tumor recurrence within six months after chemotherapy, whereas sensitive chemotherapy was considered if tumor recurrence was not reported within or more than six months after chemotherapy. In this study, 20 cases were in chemotherapy resistant group and 20 cases were in chemotherapy sensitive group. Characteristics of specimens derived from patients with ovarian cancer are illustrated in Table1.

Cell lines and cell culture

The human ovarian cancer cell line of COC1 and COC1/DDP were cultured in medium RPMI 1640 containing 10% newborn calf serum and 40 μg/ml gentamicin at 37°C in a 5% CO_2 atmosphere. COC1 and COC1/DDP cells at logarithmic growth phase (80%-90%) were harvested and seeded into 250 ml flask at the density of 5 x 10^6/ml for 24 hours.

RNA extraction

The small and total RNA fractions were isolated using the miRNeasy mini kit and TRI reagent, respectively. Both proce-

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Table 1. — *Characteristics of ovarian cancer specimens.*

Ovarian cancer specimens	Number
Chemotherapy sensitive group	20
Chemotherapy resistant group	20
Age	36-76 (mean 51.25)
Postmenopausal women	31
Premenopausal women	9
Stage I	5
Stage II	7
Stage III	28
Stage IV	0
G1	21
G2	22
G3	18

dures were followed according to the manufacturer's recommendations. Quantity and purity of the RNA were tested using a nanodrop spectrophotometer. RNA integrity was determined by running agarose gel electrophoresis.

miRNA microarray hybridization

One µg total RNA from each sample were labeled with Hy3 and Hy5 fluorescent label, respectively, with the help of the miRCURY array power labeling kit following the instructions. The Hy3 and Hy5 labeled reference RNA sample were mixed pairwise and hybridized to the miRCURY LAN array, which contained 1,891 capture probes targeting all of the miRNAs for all the species registered in the miRBase (Version16.0) at the Sanger Institute. Hybridization signals were detected by biotin binding of a streptavidin alexa 647 conjugate using a scanner 4000B. The images were quantified by GenePix 6.0 software.

miRNA microarray data analysis

Microarray data were analyzed in R using the linear models for microarray data package. Poor quality (flagged) spots were excluded from the analysis. The array results were background-corrected using the Normexp method [11]. The intensities were then log2-transformed and normalized, using the LIMMA implementation in quantile normalization. Subsequently, the normalized microarray data were managed and analyzed by scatter plot, volcano plot, and MEV software (version 4.6).

qRT-PCR

The expression level of miR-141-3p in COC1 vs. COC1/DDP group as well as chemotherapy resistant group (n=20) vs. chemotherapy sensitive group (n=20) were detected by qRT-PCR. Here, the authors only validated the most significant miRNA namely miR-141-3p. To quantify miRNA, the expression of the miR-141-3p included in the TaqMan MicroRNA assays human panel kit was examined according to the manufacturer's protocol. To validate the results, the TaqMan kit specified that the quantification of miR-141-3p be used, with normalization to the U6 small nuclear RNA (U6 snRNA). RNA (20 ng) from each sample was reverse transcribed using a TaqMan human microRNA assays kit. To quantify the mRNA, 20 *ul* of RNA from each sample was reverse transcribed into complementary DNA (cDNA) using a PrimeScript RT reagent kit. The resulting cDNA was amplified by PCR using TaqMan microRNA assay primers with the TaqMan universal PCR master mix and analyzed with a 7500 ABI sequence detector system according to the manufacturer's instructions. The non-coding RNU6B (U6 control) was used as housekeeping control: F: 5 'GCTTCGGCAGCACATATACTAAAAT3'; R: 5'CGCTTCACGAATTTGCGTGTCAT3'. In addition, gene-specific primer (GSP) of hsa-miR-141-3p was F: 5'GGGGTAACACTGTCTGGTAA3': R: 'TGCGTG TCGTGGAGTC3'. Reaction substrate was dissolved in diethyl-pyrocarbonate (DEPC)-treated water in the ratio of 1:10, 1:100, 1:1000 and 1:10000.

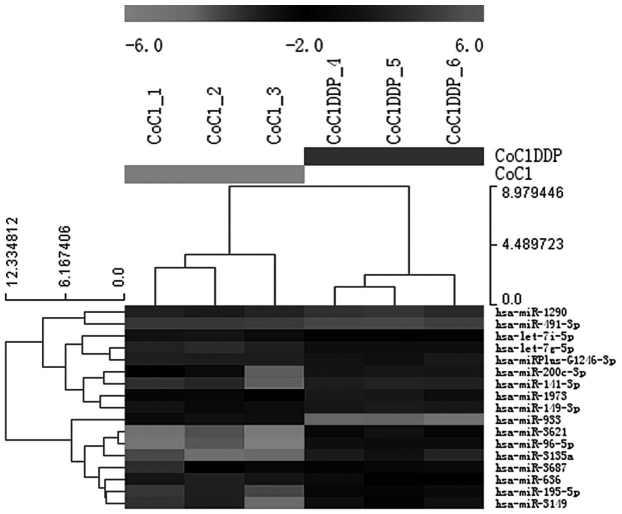

Figure 1. — Results from cluster analysis. Total RNAs isolated from three biological replicates of COC1 and COC1/DDP were subjected to miRNA microarray analysis

Statistical analysis

The relative gene copy number was estimated by real time PCR using the ΔΔCt method [12]. Statistical analysis was performed by using the SPSS statistics software package (SPSS). All results were expressed as mean ± SD were evaluated for statistical significance using Student's t test. A p value < 0.05 was used for significance.

Results

Distinct miRNA signatures in ovarian cancer COC1/DDP, compared with COC1

To identify miRNAs differentially expressed in COC1/DDP compared with the COC1, the authors used a customized miRNA microarray that contained more than 1800 miRNAs (Figure 1). Seventeen miRNAs were differentially expressed between COC1 and COC1/DDP. The up-regulated miRNA (n=13) were has-miR-1290, hsa-miR-636, hsa-miR-1973, hsa-miR-3687, hsa-miR-3621, hsa-miR-200c-3p, hsa-miR-195-5p, hsa-miR-96-5p, hsa-miR-141-3p, hsa-miR-491-3p, hsa-miR-3135a, hsa-miR-149-3p, and hsa-miR-3149 (Table 2). The downregulated miRNAs (n = 4) were hsa-miR-933, hsa-let-7g-5p, hsa-let-7i-5p, hsa-miRPlus-G1246-3p (Table 3). Of these miRNAs, hsa-miR-141-3p had a fold-change of 31.01228 (the highest among all groups), which suggests that hsa-miR-141-3p was the most significantly different ex-

Table 2 — *Upregulated miRNAs in COC1/DDP compared with COC1.*

Name	Fold change COC1/DDP vs CoC1	*p*-valve COC1/DDP vs CoC1
hsa-miR-1290	2.430505	0.035101
hsa-miR-636	1.719201	0.02538
hsa-miR-1973	3.742112	0.025768
hsa-miR-3687	2.837062	0.023905
hsa-miR-3621	5.48781	0.001494
hsa-miR-200c-3p	10.21796	0.000222
hsa-miR-195-5p	4.036994	0.039189
hsa-miR-96-5p	5.937419	0.00032
hsa-miR-141-3p	31.01228	0.00291
hsa-miR-491-3p	1.845917	0.018582
hsa-miR-3135a	2.941407	0.006129
hsa-miR-149-3p	1.961863	0.030071
hsa-miR-3149	2.23344261	0.041212

Table 3. — *Downregulated miRNAs in COC1/DDP compared with COC1.*

Name	Fold change COC1/DDP vs CoC1	*p*-valve COC1/DDP vs CoC1
hsa-miR-933	0.229047	0.00014
hsa-let-7g-5p	0.255458	0.015381
hsa-let-7i-5p	0.251553	0.01992
hsa-miRPlus-G1246-3p	0.574549	0.02653

miRNAs that are up-regulated and down-regulated in OCO1/DDP compared to COC1, $p < 0.05$.

pressed miRNA between COC1/DDP and COC1 group, and may have a potential role in the mechanism of platinum-resistance in ovarian cancer.

Validation of the microarray results

The method of qRT-PCR was used to validate the microarray results in COC1/DDP and COC1 cell-lines. The authors selected the most frequently hsa-miR-141-3p for qRT-PCR test. Of note, the expression level of hsa-miR-141-3p was significantly higher in COC1/DDP rather than in COC1 cell-lines (14.0433 ± 4.4895 vs. 1.7833 ± 0.7213, $p < 0.05$, Figure 2). U6 amplification plot and hsa-miR-141-3p amplification plot are clearly illustrated in Figure 3.

The expression level of hsa-miR-141-3p between chemotherapy resistant and chemotherapy sensitive group

In order to detect the different expression level of hsa-miR-141-3p between chemotherapy resistant (n=20) and chemotherapy sensitive group (n=20), the authors also used qRT-PCR. Similar to the result of comparing COC1/DDP and COC1 cell-lines, hsa-miR-141-3p was more frequently upregulated in chemotherapy resistant group rather than in chemotherapy sensitive group (9.56 ± 1.04 vs. 1.5 9 ± 0.91, $p < 0.05$)

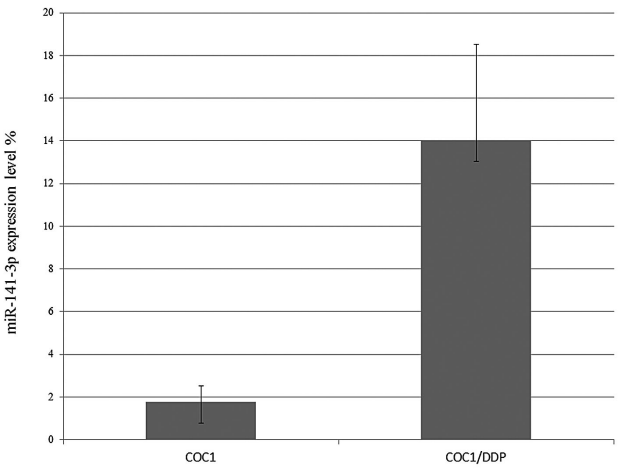

Figure 2. — Total RNAs isolated from three biological replicates of COC1 and COC1/DP were subjected to real-time RT-PCR to validate its differential expression level of miRNA-141-3p

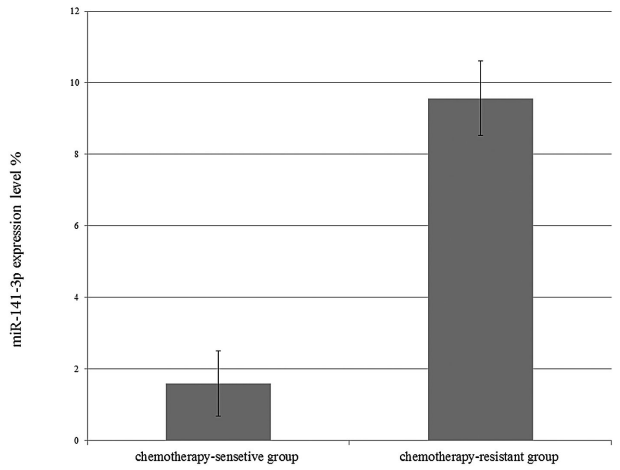

Figure 3. — Total RNAs isolated from three biological replicates of chemotherapy-sensitive group and chemotherapy-resistant group were subjected to RT-PCR to validate its differential expression level of miRNA-141-3p.

Discussion

To date, numerous findings have confirmed a critical role of miRNA as powerful diagnostic and prognostic indicators of human ovarian cancer [10, 13-15], resulting in the development of novel approaches to ovarian cancer management [8]. Despite the well-established role of miRNA in cancer [16, 17] and the understanding of the molecular mechanisms involved in the development of chemotherapy-resistant cancer cells, the role of miRNA in cancer drug resistance is still largely unexplored.

In this present study, the authors provided data indicating the importance of altered expression of miRNA in the acquisition of ovarian cancer cells resistance to platinum-based

drugs. Their findings showed that upregulation of has-miR-1290, hsa-miR-636, hsa-miR-1973, hsa-miR-3687, hsa-miR-3621, hsa-miR-200c-3p, hsa-miR-195-5p, hsa-miR-96-5p, hsa-miR-141-3p, hsa-miR-491-3p, hsa-miR-3135a, hsa-miR-149-3p, hsa-miR-3149, and downregulation of hsa-miR-933, hsa-let-7g-5p, hsa-let-7i-5p, hsa-miRPlus-G1246-3p were in COC1/DDP compared to COC1 cell-lines. Of which, let-7 family [18] and miR-200c family [19] and miR-141 [20] have been widely reported in the development of tumorigenesis.

Ectopic expression of miR-200 family (miR-200a, miR-141, miR-200b, miR-200c, miR-429) expression has been detected in human ovarian cancer [21-23] . Of which, miR-200c was found directly target the mRNA of the E-cadherin transcriptional repressors ZEB1 (TCF8/δEF1) and ZEB2 (SMAD-interacting protein 1 [SIP1]/ ZFXH1B). Altered expression of miR-200 resulted in up-regulation of E-cadherin in cancer cell lines [24]. Li *et al*. have demonstrated that the gradually increment of upregulated miR-141 and miR-200c expression was detected in normal ovarian tissue, borderline ovarian tumors, and ovarian cancer, respectively. However, expression of miR-200c was downregulated in ovarian metastatic carcinoma, poorly differentiated ovarian carcinoma and ovarian clear cell adenocarcinoma. In addition, poor prognosis of ovarian cancer was found in miR-200c downregulated group compared to upregulated counterpart, suggesting that miR-200c can be severed as a potential prognostic indicator in the clinical practices [25]. In this study, miR-200c-3p, one of miR-200 membership, was detected to be upregulated in COC1/DDP group, which indicates that miR-200c-3p may be associated with the development of platinum-resistance in ovarian tumors.

Furthermore, PTEN (antioncogene) as one of the target genes of miR-141 was found to inhibit tumor cell growth, migration and invasion through regulating the P13K/PKB signaling pathway. Indeed, PTEN can induce the cell cycle arrest at G1 stage and initiate apoptosis. Lost function of PTEN can result in cell over-proliferation and elicit tumorigenesis [26]. Importantly, in this present study, the authors identified that miR-141 was upregulated in COC1/DDP compared to that in COC1 group, indicating that miR-141 may have a potential role in the mechanism of platinum-resistance in ovarian cancer cell line.

Takamizawa *et al*. have reported that Let-7 can inhibit the growth of lung carcinoma A549 cell *in vitro*. Hence the Let-7 expression level is considered as an independent prognostic factor in lung cancer [27]. The present findings further demonstrate that hsa-let-7g-5p and hsa-let-7i-5p (Let-7 family) were downregulated in ovarian cancer COC1/DDP compared to COC1. Conversely, Van *et al*. have stated that let-7g was upregulated in platinum-resistant ovarian cancer cell line [28]. Looking at this discrepancy of the results, the present authors elucidate that the expression of miRNAs may have a highly association with some factors such as different tumor stage, primary or recurrent cancer and various platinum-based drugs.

The present authors will concentrate on studying the role of miR-141-3p and its target genes in platinum-resistant ovarian cancer because of several reasons: 1) miR-141-3p was found to be most frequently up-regulated in COC1/DDP compared to COC1 (a fold-change of 31.01228); 2) most studies have reported the mechanism of miR-141 in regulating tumor apoptosis. Yet, less evidence has shown its role in platinum-resistance; 3) the molecular function of other two miR-141-3p target genes, BRD3and UBAP1, still remains unclear.

Conclusions

Altogether, the present authors have identified other miRNAs that are differentially expressed in platinum-resistant cell lines and chemotherapy resistant group. Some of these miRNAs could emerge as potential biomarkers of platinum-resistant tumors. Identification of target genes of these miRNAs will further enhance our knowledge of platinum resistance and facilitate design of new therapeutic agents targeting these proteins.

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