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Micro-RNAs associated with the evolution of ovarian cancer cisplatin resistance

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Abstract

Objectives—Ovarian cancer (OVCA) is the leading cause of mortality among women with gynecologic malignancy, in part due to the development of chemoresistance. We sought to identify micro-RNAs (miRNAs) associated with in vitro development of OVCA chemoresistance that may also represent potential targets for therapy.

Methods—In this study, four OVCA cell lines (A2780CP, A2780S, IGROV1, and OVCAR5) were serially treated with cisplatin in parallel with measurements of miRNA expression changes.

Results—Nine miRNAs were found to be associated with increasing cisplatin resistance (IC_{50}) ($p<0.01$); however, only 5 of these miRNAs have publically available information. Pathway analysis identified 15 molecular signaling pathways that were represented by genes predicted to be

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targets of the 5 miRNAs (false discovery rate <0.05), 11 of which are associated with the epithelial-mesenchymal transition (EMT). Further analysis identified 2 of those pathways as being associated with overall survival in 218 patients with OVCA.

Conclusions—Collectively, this panel of miRNAs associated with in vitro evolution of OVCA cisplatin resistance and the pathways identified to be associated with EMT and overall patient survival provide a framework for further investigations into EMT as a therapeutic target in patients with OVCA.

Keywords

ovarian cancer; micro-RNA; chemoresistance; epithelial-mesenchymal transition

Introduction

Although the rate of diagnosis has been declining for the past 20 years, ovarian cancer (OVCA) remains the leading cause of mortality from gynecologic cancer and the fifth overall for cancer in women [1-4]. It is estimated that 21,290 new cases will be diagnosed in the United States in 2015 and that 14,180 women will die of the disease [5]. The high numbers are in part due to the absence of reliable screening tests for asymptomatic early-stage diagnosis and the fact that many patients will ultimately develop disease that is unresponsive to therapy [1-3, 6].

The primary therapy for OVCA is cytoreductive surgery followed by platinum-based chemotherapy. The initial response rate to this primary therapy is nearly 70% [4]. Since 1978, cisplatin, *cis*-[Pt(II)(NH(3))(2)Cl)]($PtCl_2(NH_3)_2$] or CDDP, has been used as a cancer therapeutic, binding to DNA, forming adducts, and activating apoptosis [7]. The development of cisplatin resistance has long been a focus of OVCA research because, despite response to initial platinum-based therapy, the majority of patients with OVCA will ultimately experience chemoresistant relapse and succumb to disease [1-3, 6-8]. A multitude of mechanisms result in the development of cisplatin resistance including, increased DNA repair, decreased accumulation of the drug within the cells, and post-translational modification [3, 8].

Recently, it has been recognized that micro-RNAs (miRNAs) may influence the development of cisplatin resistance [8]. miRNAs are small (~22 bp) endogenous, non-protein-coding nucleotides that regulate gene expression by base-pairing to the 3’ untranslated region of the target mRNA [3, 9-12]. miRNA expression levels vary between normal cells and cancer cell lines and between chemoresistant versus chemosensitive in follicular lymphoma [13], breast cancer [11, 14], pancreatic cancer [11], and OVCA [15] cells.

In this study, we evaluated changes in miRNA expression associated with the experimental induction of cisplatin resistance in OVCA cells. Furthermore, in an effort to determine the mechanisms underlying the development of cisplatin resistance, we investigated the molecular signaling pathways represented by miRNA target genes. In doing so, our goal was

to find potential targets for treatment or biomarkers for diagnosis and chemotherapy response.

Methods

Cell lines

OVCA cell lines A2780CP, A2780S, IGROV1, and OVCAR5 were kind gifts provided by Dr. Patricia Kruk, Department of Pathology, College of Medicine, University of South Florida (Tampa, FL). All cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Fisher Scientific, Pittsburgh, PA), 1% sodium pyruvate, 1% penicillin/streptomycin (Cellgro, Manassas, VA), and 1% nonessential amino acids (HyClone, Hudson, NH). Mycoplasma testing was performed every 6 months in accordance with the manufacturer's protocol (Lonza, Rockland, ME). The cell lines used in this study have been genotyped by STR profiling to ensure tissue of origin.

Induction of chemoresistance

We previously reported the in vitro induction of platinum resistance in the OVCA cell lines used for this study [16]. Briefly, cell lines were serially treated with increasing doses of cisplatin with intermittent cell recovery/expansion phases to induce resistance. Three dosing schedules were followed: *schedule A* (3 treatments with 1 μg/mL followed by an additional 3 treatments with 3 μg/mL), *schedule B* (3 treatments with 2 μg/mL followed by an additional 3 treatments with 4 μg/mL), and *schedule C* (3 treatments with 5 μg/mL followed by an additional 3 treatments with 3 μg/mL). After each recovery phase, OVCA cell cisplatin sensitivity was quantified using CellTiter-96 MTS cell viability assays (Fisher Scientific) and in parallel, microRNA (miRNA) expression profiles were evaluated. In total each OVCA cell line underwent 6 treatment-recovery cycles with corresponding miRNA profiling and cisplatin sensitivity quantification.

MicroRNA isolation and labeling

This process was completed as described by Boren *et al*. In brief, before the initial treatment and following each recovery phase, Ambion *mir*Vana microRNA isolation kit (Ambion, Austin TX) was used to extract miRNA. The Agilent Bioanalyzer (Agilent Technologies, Santa Clara CA) was used to assess the quality of total RNA. Using the miRCURY Labeling Kit (Exiqon, Vedbaek Denmark), 10 μg of Ambion ovarian total RNA and total RNA from each cell line was labeled. Ambion ovarian samples and cell line samples were co-hybridized to printed arrays containing 622 of Invitrogen's NCode multispecies miRNA probes (Invitrogen, Carlsbad CA), which had 335 unique human miRNAs, and 562 Ambion *mir*Vana miRNA probe set (Ambion, Austin TX). A GenePix 4000B scanner was used to scan the hybridized arrays and the GenePix Pro software (Molecular Devices, Sunnydale CA) generated the miRNA expression data [4].

Statistical analysis

Expression data from the OVCA cell lines were subjected to background correction and normalization using the robust multichip average algorithm [17] and then implemented to the Bioconductor (http://www.bioconductor.org) extensions to the R-statistical programming

environment as described previously [18]. Probe sets with expression ranges less than 2-fold (maximum/minimum) and control probes (i.e. AFEX_*probe sets) were excluded from the analysis. miRNAs associated with acquired cisplatin resistance (IC_{50}) were determined using Pearson correlation. A Pearson correlation coefficient, r, less than −0.50 or greater than 0.50 were considered statistically significant. The target genes of significant miRNAs ($p<0.01$) were determined using the MiRanda database [19].The genes were then analyzed using GeneGo software in order to identify the significantly (false discovery rate <0.05) represented pathways. Pathways identified in this manner were evaluated for associations with overall survival from OVCA using principal component analysis (PCA) modeling. The first component of the PCA model, PC1, which contains the largest variance, was used to define high versus low pathway score. The median PC1 was used as a threshold when testing pathways for an association with overall survival, using log-rank test, within a publically available clinico-genomic OVCA dataset (n=218 patients; GSE9891) [16].

Results

Nine miRNAs are associated with the evolution of platinum resistance

Correlation analysis identified 9 miRNAs that were significantly ($p<0.01$ and Pearson correlation coefficient $-0.50>r>0.50$) associated with the IC_{50} of the 4 OVCA cell lines with acquired resistance to cisplatin (Table 1). From the 9 miRNAs, 4 demonstrated a positive correlation (miR-496, miR-485-5p, let-7g, and miR-152) and 5 were negatively correlated (miR-422b, miR-17-3p, miR-520h, miR-27b, and miR-432*).

miRNAs associated with CDDP resistance dominantly regulate EMT-related genes and pathways

Target genes were identified for 5 of the miRNAs that had been previously determined and made publically available on the MiRanda database (Supplemental Table 1). With these target genes, GeneGo was used to perform pathway analysis, revealing 15 significant (false discovery rate <0.05) pathways common to 3 or more of the miRNAs (Table 2).

Pathways associated with platinum resistance may influence overall survival from OVCA

In an effort to explore the clinical relevance of the identified target pathways, these pathways were further explored using PCA to find associations with survival. Using a publically available dataset, 218 patients with OVCA were divided into high and low groups using the PC1 as the median and then log-rank tests were performed to compare the survival probability between the two groups of patients (Supplemental Table 2). This analysis revealed that 2 pathways (*TGF, WNT and cystoskeletal remodeling* and *Regulation of EMT*) were associated with overall patient survival from OVCA ($p<0.05$) (Figure 1).

Discussion

It is believed that a single miRNA may target hundreds of mRNAs and more than 18,000 miRNA-mRNA interactions have been reported [20]. The central role of miRNAs in biologic processes makes them appealing candidates as biomarkers for diagnosis and prognosis, therapeutic selection, or as therapeutic targets [3, 21].

We identified 9 miRNAs to be significantly associated with the evolution of OVCA cisplatin resistance ($p<0.01$). Four of the miRNAs demonstrated a positive correlation (miR-496, miR-485-5p, let-7g, and miR-152) and 5 a negative correlation (miR-422b, miR-17-3p, miR-520h, miR-27b, and miR-432*). Previous studies support our findings that miR-485-5p, let-7g, miR-27b, and miR-152 may be associated with chemoresistance in human malignancy [2, 22-24]. Let-7g, miR-422b, miR-152, and miR-27b have all been found to be associated with OVCA in addition to gastric, cholangiocarcinoma, endometrial, hepatitis B and C-related hepatocellular carcinoma, breast, colorectal, prostate, and head and neck squamous cell [23-29]. Although miR-496, miR-485-5p, and miR-520h have not been reported to be associated with OVCA, they have been found to be associated with breast cancer [22, 30-32]. Little is known about the roles of miR-17-3p and miR-432* in cancer.

We also explored how miRNAs may influence OVCA biologic processes associated with cisplatin resistance by seeking to identify genes and represented signaling pathways predicted to be targets of cisplatin resistance-related miRNAs. We found that 11 pathways regulated by cisplatin resistance-associated miRNAs were involved in epithelial-to-mesenchymal transition (EMT). Furthermore, two of these pathways (*TGF/WNT* and *Development Regulation of EMT*) were also shown to be associated with overall survival in patients with OVCA.

EMT is a process by which epithelial cells undergo morphologic and phenotypic changes and assume the features of a mesenchymal cell, including loss of cell polarity and cell-cell adhesion and gain of migratory and invasive properties. As such, EMT is recognized to be a fundamental process in embryogenesis (type I), fibrosis and wound healing (type II), and cancer (type III) [33]. Cells that undergo changes to become more phenotypically mesenchymal have been associated with more aggressive, metastatic, and chemo-resistant cancers [34-37]. Our findings are consistent with previous reports that highlight the importance of EMT in cancer chemoresistance and thus may serve as a possible therapeutic target [35, 38, 39]. In a similar study, after inducing resistance to paclitaxel in the NOS-2 cell line, it was found that the NOS-PR cells showed EMT-like changes. There was an upregulation in Snail and Twist transcription factors, which play a part in the development of EMT, along with vimentin, smooth muscle actin, and fibronectin. In addition, the NOS-PR cells showed an increase in migration and invasiveness in wound assays [35]. A study published by Marchini *et al* compared tumor samples from the primary surgery versus those taken during a secondary cytoreductive surgery following resistance to platinum chemotherapy and found that 70% of the samples expressed genes involved in pathways that are a part of EMT. A possible obstacle in future studies would be determining the exact target location within the EMT pathway because the mechanism is still unknown, with a recent study suggesting that there are varying states of EMT that are dependent on cell models and inducer combinations [37].

In this study, we defined a panel of miRNAs associated with in vitro evolution of OVCA cisplatin resistance. Pathways predicted to be modulated by these miRNAs are associated with EMT processes and may influence overall patient survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EMT	epithelial-mesenchymal transition
miRNA	micro-RNA
OVCA	ovarian cancer

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Highlights

- Cisplatin resistance in OVCA cell lines is associated with 9 miRNAs.
- Phenotypically EMT cells are associated with more chemo-resistant cancers.
- *TGF/WNT* and *Development Regulation of EMT* are associated with overall survival.

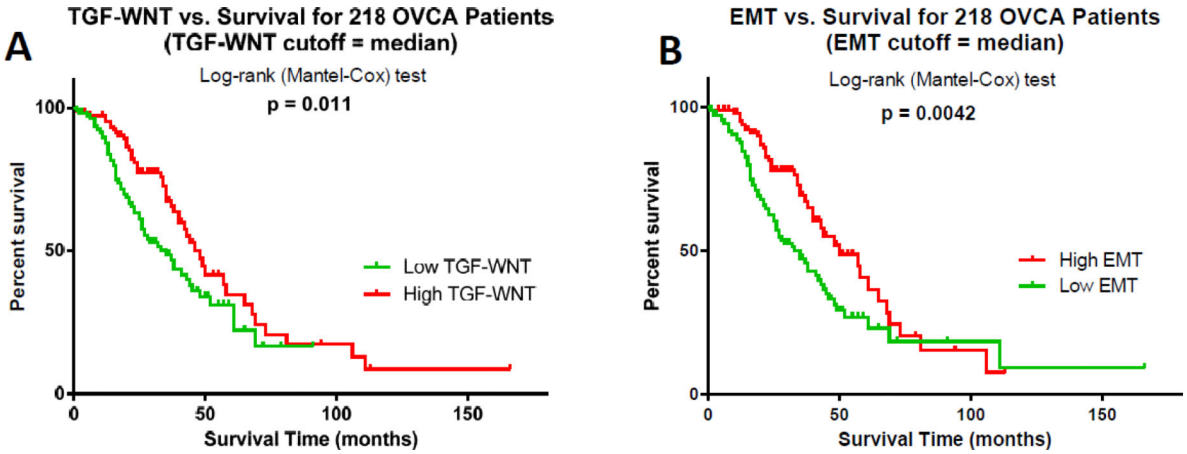

Figure 1.

Pathways regulated by platinum resistance-associated miRNAs influence clinical outcome. Pathway genes for the (A) TGF/WNT and (B) Regulation of EMT (EMT) pathways were modeled using principal component analysis and tested for associations with overall survival using a publically available OVCA clinico-genomic dataset (n=218 patients). Kaplan-Meier curves depict the association with survival using median principal component analysis score as a threshold. Log-rank test *p* values indicate significance.

Table 1

Micro-RNA expression correlation with IC_{50} of cisplatin-resistant cell lines

miRNA	Pearson correlation coefficient, r	*P* value	Correlation
hsa-miR-496	0.68	0.00026	Positive
hsa-miR-485-5p	0.65	0.00060	Positive
hsa-let-7g	0.56	0.00430	Positive
hsa-miR-422b	−0.55	0.00522	Negative
hsa-miR-152	0.55	0.00587	Positive
hsa-miR-17-3p	−0.54	0.00660	Negative
hsa-miR-520h	−0.54	0.00697	Negative
hsa-miR-27b	0.53	0.00797	Negative
hsa-miR-432*	−0.52	0.00940	Negative

Table 2

Micro-RNAs and the pathways that they control

Pathway	miRNA	p-value	False discovery rate	Object ratio
Cytoskeleton remodeling	miR-152	2.22E-06	1.17E-04	26/102
	miR-27b	3.75E-04	6.35E-03	25/102
	miR-485-5p	9.66E-05	4.33E-03	25/102
	let-7g	8.14E-07	2.12E-04	24/102
	miR-496	1.55E-07	1.21E-04	26/102
TGF, WNT and cytoskeletal remodeling	miR-152	6.16E-09	2.44E-06	32/111
	miR-27b	1.52E-07	4.62E-05	34/111
	miR-485-5p	2.13E-05	2.33E-03	28/111
	let-7g	4.62E-09	3.61E-06	29/111
	miR-496	9.24E-07	1.80E-04	26/111
Chemokines and adhesion	miR-152	5.06E-05	1.07E-03	23/100
	miR-27b	2.70E-04	5.69E-03	25/100
	let-7g	2.58E-04	1.42E-03	21/100
	miR-496	5.90E-05	3.71E-03	21/100
Regulation of actin cytoskeleton by Rho GTPases	miR-152	8.91E-04	6.07E-03	8/23
	miR-27b	1.68E-04	4.19E-03	10/23
	miR-485-5p	8.06E-05	4.07E-03	10/23
	let-7g	3.28E-05	1.61E-03	9/23
Thromboxane A2 pathway signaling	miR-152	3.13E-05	7.72E-04	15/49
	miR-27b	4.62E-04	7.38E-03	15/49
	miR-485-5p	2.30E-06	6.18E-04	18/49
	miR-496	2.91E-05	3.24E-03	14/49
G-Protein alpha-12 signaling pathway	miR-152	1.90E-03	9.58E-03	10/37
	miR-27b	2.42E-04	5.69E-03	13/37
	miR-485-5p	2.08E-05	2.33E-03	14/37
	miR-496	1.41E-04	5.78E-03	11/37
Receptor-mediated axon growth repulsion	miR-152	9.86E-06	3.39E-04	15/45
	miR-27b	4.01E-05	1.78E-03	16/45
	miR-485-5p	6.01E-05	4.04E-03	15/45
	miR-496	2.22E-04	8.26E-03	12/45
PIP3 signaling in cardiac myocytes	miR-152	3.25E-04	3.04E-03	13/47
	miR-27b	8.03E-07	1.28E-04	19/47
	let-7g	2.05E-04	5.95E-03	12/47
Regulation of EMT	miR-152	2.51E-04	2.61E-03	16/64
	miR-27b	1.10E-05	8.80E-04	21/64
	miR-496	5.86E-07	1.80E-04	19/64
Slit-Robo signaling	miR-152	5.62E-05	1.11E-03	11/30

Pathway	miRNA	p-value	False discovery rate	Object ratio
	miR-27b	9.98E-05	3.14E-03	12/30
	let-7g	6.01E-05	2.38E-03	10/30
H-RAS regulation pathway	miR-152	1.90E-03	9.58E-03	10/37
	miR-496	2.66E-05	3.24E-03	12/37
	let-7g	8.47E-05	3.32E-03	11/37
Dynein-dynactin motor complex in axonal transport in neurons	miR-152	1.29E-06	9.23E-05	18/54
	miR-485-5p	1.12E-05	2.26E-03	18/54
	let-7g	2.25E-06	4.41E-04	16/54
Ovarian cancer (main signaling cascades)	miR-152	2.51E-04	2.61E-03	16/64
	miR-496	1.85E-04	7.21E-03	15/64
	let-7g	9.86E-05	3.68E-03	15/64
Calcium signaling	miR-152	1.89E-06	1.06E-04	16/45
	miR-27b	4.01E-05	1.78E-03	16/45
	miR-496	4.92E-05	3.50E-03	13/45
Macropinocytosis by growth factors	miR-152	8.08E-07	6.38E-05	20/63
	miR-27b	3.37E-04	6.25E-03	18/63
	let-7g	3.04E-04	8.50E-03	18/63

Abbreviations: EMT: epithelial-to-mesenchymal transition.