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PKC-alpha modulation by *miR-483-3p* in platinum-resistant ovarian carcinoma cells

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ABSTRACT

The occurrence of drug resistance limits the efficacy of platinum compounds in the cure of ovarian carcinoma. Since microRNAs (miRNAs) may contribute to this phenomenon by regulating different aspects of tumor cell response, the aim of this study was to exploit the analysis of expression of miRNAs in platinum sensitive/resistant cells in an attempt to identify potential regulators of drug response. *MiR-483-3p*, which may participate in apoptosis and cell proliferation regulation, was found up-regulated in 4 platinum resistant variants, particularly in the IGROV-1/Pt1 subline, versus parental cells. Transfection of a synthetic precursor of *miR-483-3p* in IGROV-1 parental cells elicited a marked up-regulation of the miRNA levels. Growth-inhibition and colony-forming assays indicated that *miR-483-3p* over-expression reduced cell growth and conferred mild levels of cisplatin resistance in IGROV-1 cells, by interference with their proliferative potential. Predicted targets of *miR-483-3p* included *PRKCA* (encoding PKC-alpha), previously reported to be associated to platinum-resistance in ovarian carcinoma. We found that *miR-483-3p* directly targeted *PRKCA* in IGROV-1 cells. In keeping with this finding, cisplatin sensitivity of IGROV-1 cells decreased upon molecular/pharmacological inhibition of PKC-alpha. Overall, our results suggest that overexpression of *miR-483-3p* by ovarian carcinoma platinum-resistant cells may interfere with their proliferation, thus protecting them from DNA damage induced by platinum compounds and ultimately representing a drug-resistance mechanism. The impairment of cell growth may account for low levels of drug resistance that could be relevant in the clinical setting.

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1. Introduction

Ovarian cancer is the leading cause of cancer deaths among women (Siegel et al., 2013). Drug resistance of tumor cells is a major limitation to the cure of cancer. Although cisplatin represents an effective first-line therapy for ovarian carcinoma, the occurrence of resistance remains a major challenge (Muggia, 2009, Romero and Bast, 2012, Vaughan et al., 2011).

Drug resistance is multi-factorial in nature, with multiple pathways and factors cooperating in reducing the efficacy of the drug. Events occurring at different levels of the action of cisplatin might be responsible for the reduced drug capability to kill tumor cells (Cossa et al., 2009, Beretta et al., 2010, Howell et al., 2010, Cossa et al., 2014). A plethora of factors have been shown to be implicated in the regulation of platinum response, which may result at least in part from transcriptional changes (Shen et al., 2012). Of note, altered expression of specific genes or subset of genes has been implicated in conferring resistance to platinum compounds (Gatti et al., 2004, LaCroix et al., 2014).

MiRNAs are endogenous, non-coding single-stranded RNAs of 19–25 nucleotides, acting as crucial players in the regulation of gene

Abbreviations: API5, apoptosis inhibitor 5; ARF1, ADP ribosylation factor 3; BCA, bicinchoninic acid; *BBC3*/PUMA, BCL2 binding component 3; BIRC5, baculoviral IAP (inhibitor of apoptosis) repeat containing 5; CDC25A, cell division cycle 25A; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; Chk1, checkpoint kinase 1; FACS, Fluorescence-activated cell sorting; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; GO, Gene Ontology; *IGF2*, insulin-like growth factor 2; miRNA, microRNA; MRP2, ABC transporter C family member 2; *pre-483-3p*, *pre-miR-483-3p*; pre-Neg, Pre-miR Negative Control; *PRKCA*, protein kinase C alpha; *PRKCD*, protein kinase C delta; PTEN, phosphatase and tensin homolog; qRT-PCR, quantitative Real Time polymerase chain reaction; RAN, RAN, member RAS oncogene family; Rb, retinoblastoma; RQ, Relative Quantification; RTN3, reticulon 3; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; si*PRKCA*, siRNA specific for *PRKCA*; SLC24A1, solute carrier family 24 member 1; SPTBN4, spectrin beta non erythrocytic 4; STX18, syntaxin 18; SULF2, sulfatase2; *TNFRSF10B*/TRAIL-R2, tumor necrosis factor receptor superfamily, member 10b; Wee1, WEE1 G2 checkpoint kinase; ZZEF1, ZZ type with EF-hand domain 1.

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expression (Calin and Croce, 2006; Garzon and Croce, 2011). MiRNA microarray profiling of human tumors has shown that some groups of miRNAs may offer new opportunities in the definition of novel biomarkers and therapeutic targets (Mulrane et al., 2014, Yang et al., 2012). Epithelial ovarian cancers are remarkably heterogeneous and this heterogeneity is reflected in deregulation of multiple miRNAs (Zhang et al., 2015). For example, the Cancer Genome Atlas project has published an integrated analysis of nearly 500 high-grade serous ovarian cancers that clearly documents multiple changes of miRNA levels (Network: TCGAR, 2011).

Several miRNAs have been reported as being associated to platinum drug-resistance or response in ovarian carcinoma, but the role of miRNAs and their target mRNAs in determining ovarian carcinoma chemosensitivity remains to be elucidated (Boren et al., 2009). For example, resistance to platinum compounds in ovarian carcinoma cells has been proposed to involve *miR-130a*, whose overexpression resulted in down-regulation of the PTEN phosphatase (Yang et al., 2012). Recently, *miR-141-3p* was found to be the most differentially expressed miRNA between platinum-sensitive and –resistant ovarian cancer cells (Ying et al., 2015). Interestingly, the passenger strand *miR-21-3p* has been reported to play a role in mediating cisplatin resistance in ovarian carcinoma cells, whereas *miR-21-5p* seems to have the opposite effect (Pink et al., 2015). Several clinical studies were aimed at elucidating how the deregulation of miRNAs impacts on the prognosis of patients treated with platinum-based chemotherapy. Specifically, three miRNAs (*miR-146a*, *miR-148a* and *miR-545*) targeting BRCA1/2 and associated with a good prognosis in ovarian cancer patients were recently identified (Gu et al., 2015). The expression of miR-136 was significantly reduced in ovarian carcinoma patients who were refractory to cisplatin treatment (Zhao et al., 2015a). Additionally, the expression of miR-9 was found associated with increased cisplatin sensitivity in ovarian cancer patients (Zhao et al., 2015b). Thus, given the increasing number of miRNAs which are proposed to regulate the aggressiveness of tumor cells, there is an urgent need to better define how deregulated miRNAs can modulate drug response of ovarian carcinoma and possibly interfere with the multiple pathways and/or factors implicated in the phenomenon. Based on this background, in the present study we comparatively assessed miRNA/mRNA profiles of drug-sensitive and -resistant ovarian carcinoma cells with the final aim of clarifying the biological determinants of ovarian carcinoma drug response.

2. Materials and methods

2.1. Cell culture

IGROV-1, a human ovarian carcinoma cell line established from a patient with a mixed (endometrioid/clear cell) ovarian carcinoma was kindly provided by Dr. Bénard (Institut Gustave Roussy, Villejuif, France) (Perego et al., 1998). The resistant cell lines IGROV-1/OHP and IGROV-1/Pt1 generated by exposure to oxaliplatin and cisplatin, respectively, maintained resistance for at least six months in the absence of drugs (Benedetti et al., 2008, Perego et al., 1996). Two additional ovarian carcinoma pairs of cisplatin sensitive and -resistant cell lines (A2780-A2780/CP, and OVCAR-5-OVCAR-5/Pt) were employed (Cossa et al., 2014). The resistant sublines were grown in the absence of selecting agent and routinely tested. Cell lines were all grown in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Gibco) and were routinely checked for possible mycoplasma contamination (MycoAlert® Mycoplasma Detection Kit, Lonza).

2.2. Cell growth and colony formation inhibition assays

For cell growth inhibition assays, cells were seeded in 12-well flat-bottom plates (1.3×10^5 cells/cm^2) and 24 h later, they were treated for 1 h with the drug. IGROV-1 cells were exposed to cisplatin (Teva, Petah Tikva, Israel) in a range of concentration from 1 to 30 μM, IGROV-1/OHP cells from 3 to 100 μM and IGROV-1/Pt1 cells from 10 to 300 μM. IGROV-1 cells were exposed to GO6983 (Selleck, Houston, TX) in a range of concentrations from 1 to 30 μM. In drug combination studies, IGROV-1 cells were exposed to GO6983 for 24 h before adding cisplatin for 48 h. Seventy-two hours after treatment start, cells were harvested with trypsin-EDTA (Gibco) and counted with a cell counter (Coulter Electronics, Luton, United Kingdom). For colony formation inhibition assays, cells were seeded in 12-well flat-bottom plates, exposed to cisplatin for 1 h as indicated above and then harvested and seeded in 19.6 cm^2 dishes (500 cells/dish). After 14 days, colony were fixed in ethanol 100%, coloured using crystal violet and counted. All experiments were performed at least three times.

2.3. Quantitative Real Time PCR

RNA was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), further purified with RNeasy MinElute Cleanup Kit (Qiagen) and quantified using the Nanodrop 2000c (NanoDrop, Thermo Fisher Scientific, Monza, Italy) spectrophotometer. RNA quality was then evaluated by agarose gel denaturing electrophoresis. One microgram of RNA was reverse transcribed using miSCRIPT II RT kit (Qiagen) which allows retro-transcription of both mRNA and miRNA. Mature miRNA expression was assayed by miScript Primer Assays specific for *miR-483-3p* (MS00009751) and normalized on *SNORD48* (MS00007511) (Qiagen). Quantitative Real Time PCR (qRT-PCR) was conducted using miScript SYBR Green PCR Kit (Qiagen). The reaction was carried out in a 96-well PCR plate at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s, followed by dissociation step to distinguish specific from non-specific amplification products. Each sample was analyzed in triplicate. qRT-PCR for mRNA was performed using TaqMan® Assays (Applied Biosystem). The reactions were incubated in a 96-well PCR plate at 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Each sample was analyzed in triplicate. TaqMan gene expression assays were performed for *BBC3, TNFRSF10B CDK6* and *PRKCA* genes using primers and probes (Hs00248075_m1, and Hs00366278_m1 and Hs00248075_m1, Applied Biosystems and Hs.PT.58.4563689, Integrated DNA Technologies, Inc. Coralville, IA). The expression of *GAPDH* (Hs02758991_g1, Applied Biosystems) was used as an endogenous control. The levels of miRNA and mRNA were measured using the ΔΔCt method. The amount of target, normalized to the endogenous reference and relative to a calibrator, is given by relative quantification value corresponding to $2^{-\Delta\Delta Ct}$.

2.4. Gene and miRNA expression profiling

Gene expression analysis was carried out as previously described (Cossa et al., 2014). Mature miRNA expression profiling was performed using the Illumina human_v2 MicroRNA expression kit based on DASL (cDNA-mediated Annealing, Selection, Extension, and Ligation) assay, according to the manufacturer's instructions. Briefly, 600 ng of total RNA was converted to cDNA and annealed to a miRNA-specific oligonucleotide pool. After PCR amplification and fluorescent labeling, the probes were hybridized on Illumina miRNA BeadChips and fluorescent signals were detected by the Illumina BeadArray Reader. Primary data were collected using BeadStudio v.3 software. All microarray data are MIAME compliant and the raw data were deposited into the NCBI's Gene Expression Omnibus (GEO) database (http://www.ncbi.nmlm.nih.gov/projects/geo/) with accession number GSE58472. Class comparison analyses to identify differentially expressed genes between cell lines was then carried out using BRB-ArrayTools v3.8 developed by Dr. Richard Simon and the BRB-ArrayTools Development Team (National Cancer Institute, Rockville, Maryland). We considered only miRNA with FDR < 5% and P value < 0.005.

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