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Expression Analysis of Platinum Sensitive and Resistant Epithelial Ovarian Cancer Patient Samples Reveals New Candidates for Targeted Therapies[1]

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Abstract

Ovarian cancer has the highest mortality rate of all gynecologic malignancies. Identification of new biomarkers is highly needed due to its late diagnosis and high recurrence rate. The objective of this study was to identify mechanisms of therapy resistance and potential biomarkers by analyzing mRNA and protein expression from samples derived from patients with platinum-sensitive and -resistant ovarian cancer (total cohort n = 53). The data revealed new candidates for targeted therapies, such as GREB1 and ROR2. We showed that the development of platinum resistance correlated with upregulation of ROR2, whereas GREB1 was downregulated. Moreover, we demonstrated that high levels of ROR2 in platinum-resistant samples were associated with upregulation of Wnt5a, STAT3 and NF-kB levels, suggesting that a crosstalk between the non-canonical Wnt5a-ROR2 and STAT3/NF-kB signaling pathways. Upregulation of ROR2, Wnt5a, STAT3 and NF-kB was further detected in a platinum-resistant cell-line model. The results of the present study provided insight into molecular mechanisms associated with platinum resistance that could be further investigated to improve treatment strategies in this clinically challenging gynecological cancer.

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Introduction

Epithelial ovarian cancer (EOC) accounts for the majority of mortality from gynecological cancers, with diagnosis often at a late stage. Currently, the golden standard of treatment is primary debulking surgery (PDS) followed by platinum-based chemotherapy [1]. Although most patients initially respond to chemotherapy, cancer cells will eventually develop resistance leading to relapse [2]. Despite intensive efforts to improve targeted therapy in EOC, the five-year survival rate is still only 30% for advanced disease [3]. Therefore, increased knowledge about mechanisms of platinum resistance in EOC treatment is needed in search for cure.

Genetically complex and unstable high-grade serous ovarian cancer subtype (HGSC), accounting for approximately 50–70% of EOC, represents the most aggressive histological subtype [4]. A large-scale integrated genomic data analysis for HGSC identified TP53 mutations in almost 96% of tumors. Recurrent somatic mutations were found in nine other genes including NF1, BRCA1, BRCA2,

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RB1 and CDK12, as well as DNA copy number aberrations and promoter methylation events, indicating biological and molecular heterogeneity that should be considered when developing novel therapeutic strategies [5].

While only 10%–15% of ovarian cancer patients carry BRCA1 or BRCA2 mutations in their germline, ~50% of ovarian cancers exhibit a defect in the homologous recombination (HR) repair of DNA [6]. PARP-1 enzyme became an attractive target for chemotherapeutics for its crucial function in single-strand breaks (SSBs) DNA repair mechanism through base excision repair (BER) pathway [7]. The concept of synthetic lethality has been used in genetic studies to determine functional interactions and compensation among genes for decades and has also been exploited in the development of PARP (Poly (ADP-ribose) polymerase) inhibitors [8]. The responsiveness to platinum and PARP inhibitors associates with so called *BRCAness* profile showing independent prognostic value [9,10].

The chemotherapy resistance can arise due to multiple mechanisms, such as drug target alteration, re-activation or amplification of the oncogenic pathway, activation of parallel pathways, increased DNA damage tolerance/repair, and deregulation of growth factor receptors among others [11]. Deregulation of apoptosis and altered phosphorylation (intracellular signaling), as well as metabolic pathways represent the two main biological processes responsible for oncogene-mediated drug resistance in ovarian cancer [12]. In this context, activation of PI3K/AKT cell survival pathway plays a pivotal role with NF-kB and STAT3 as the main mediations of these intracellular events. On the other hand, tumor suppressor genes such as BRCA1, BRCA2, MLH1 and p21 contribute to ovarian cancer drug resistance via alterations in the DNA damage and repair mechanisms, whereas RASSF1, TP53 and TP73 impair the apoptotic machinery for the same outcome [13]. Epithelial-to-mesenchymal transition (EMT) has also been implicated in HGSC invasiveness and chemoresistance, and in vitro studies using ovarian cancer cell lines have shown that more aggressive, mesenchymal-type cells are more resistant to cisplatin treatment [14]. An important signaling cascade involved in EMT is the Wnt signaling, with increasing evidence suggesting that β-catenin-independent pathway via Wnt5a/ROR1/ROR2 has a critical role in EMT and chemoresistance [15–18]. Consequently, therapies targeting these pathways may offer means to overcome drug resistance.

Active and also productive research in the field of cancer therapy has led to an improved understanding of the molecular mechanisms, providing insight into the development of cancer. This new data has led to the development of new treatment options for cancer patients, including targeted therapies and associated biomarker tests that can select which patients are most likely to respond [19]. The aim of this study was to identify candidate genes and their molecular pathways involved in the pathogenesis of ovarian cancer associating with platinum resistance. Overcoming the paucity of obtaining large collection of tumor samples available for molecular profiling, we investigated differences in mRNA and protein expression between ovarian cancer samples derived from two clinically and molecularly distinct patient cohorts namely high PARP/platinum-sensitive and low PARP/platinum-resistant HGSC cohorts. This comparison aimed at distinction of two cohorts with extremely different clinical behavior. Finally, this analysis led to identification of GREB1 and ROR2 that showed significant differential expression profile between the two groups. Our data suggest new predictive biomarkers for ovarian cancer drug resistance development warranting further investigations.

Materials and Methods

Study Cohort and Tissue Samples

The study was carried out at the University of Tampere and Tampere University Hospital (TAUH), Tampere, Finland. The study protocol was approved by the Ethics Committee of TAUH (identification code ETL-R11137).

The microarray study cohort consisted of 12 HGSC patients who participated in a prospective study addressing PARP enzyme activity in fresh ovarian cancer tumor samples [20]. The selection of this patient subcohort was based on PARP values (high PARP/low PARP, cut off 203 pg/ml, which corresponded to the median value of PARP) and platinum sensitivity/resistance (treatment response), with no differences in respect of age and FIGO (International Federation of Gynecology). Platinum sensitivity was defined as no recurrence within 12 months after the completion of first-line platinum-based chemotherapy. Patient characteristics are presented in Table 1.

The validation cohort of the microarray data consisted of all the patients (n = 53) that participated in the previous prospective study [20]. The median follow-up time of patients was 31 months. The validation cohort is described in Table 2.

For further investigation of ROR2 in EOC, a retrospective subcohort was chosen from the study cohorts described above consisting of a subgroup of patients who had not received NACT (neoadjuvant chemotherapy) and were divided in two categories based on treatment response, i.e. either platinum-sensitive or platinum-resistant (Table 3).

The tumor tissue samples were collected at surgery, two samples approximately 0.5 cm were chosen at the operation room from macroscopically visible tumor and were snap-frozen with liquid nitrogen and stored in −70 °C. The findings from the corresponding archival surgical tumor specimens were assessed by experienced pathologists as part of routine diagnostics at the Department of Pathology at TAUH.

Table 1. Characteristics of the Study Patients in the Microarray Cohort (n = 12)

Characteristic	Platinum Sensitive * n (%)	Platinum Resistant * n (%)
All	6	6
PDS †	5	2
NACT ‡	1	4
PARP § low	0	6
PARP § high	6	0
Age		
mean (SD)	65	62
median (range)	63 (46–78)	62 (55–79)
Grade 3 ¶	6 (100%)	6 (100%)
Stage #		
FIGO st I	0	0
FIGO st II	0	0
FIGO st III and IV	6	6
Histology		
serous	6 (100%)	6 (100%)
PFS ** (months)	28	3.5

* Sensitivity defined as relapse or event-free interval> 12 months after completion of platinum based 1st line therapy.

† PDS - primary debulking surgery.

‡ NACT - neoadjuvant therapy.

§ PARP - PARP activity in fresh frozen tumor tissue was assessed by an enzymatic chemiluminescense assay in a previous study [20]; the cut-off level for high PARP activity was set to 203 pg/ml corresponding to median value.

¶ Grade – Grade 3 represents high grade tumors.

FIGO - International Federation of Gynecology.

** PFS - progression free survival.

Table 2. Characteristics of the Study Patients in the Validation Cohort (n = 53)

Characteristic	
Patients in study, n	53
Age at surgery, yrs. (SD)	66 (9.3)
BMI, mean (SD)	26.8 (6.2)
Median follow-up, months (range)	31 (2–50)
Ca 125 level (kU/L) before treatment, median (range)	523 (30–4728)
FIGO * Stage, n (%)	
Stage 1	2 (3,7%)
Stage 2	3 (5,6%)
Stage 3	34 (64,3%)
Stage 4	14 (26,4%)
Histology, n (%)	
Serous	46 (86,8)
Endometroid	4 (7,6%)
Papillar	2 (3,7%)
Mucinous	0 (0%)
Carcinosarcoma	0 (0%)
Transitional cell	1 (1,9%)
Grade †, n (%)	
Grade 1 and 2	10 (18%)
Grade 3	43 (82%)
Sensitivity to platinum therapy ‡, n (%)	
Sensitive	25 (47,2%)
Resistant	15 (28,3%)
Partial sensitive	13 (24,5%)
Neoadjuvant therapy, n (%)	19 (36%)
Recurrence	36 (68%)
Death	22 (42%)

* FIGO = International Federation of Gynecology.
† Grade – Grade 3 represents high grade tumors, Grade 1 and 2 low grade tumors.
‡ Sensitive: Recurrence >12 months after completion of platinum-based 1st line therapy; Resistant: Recurrence ≤6 months after completion of platinum-based 1st line therapy; Partially sensitive: Recurrence 6–12 months after completion of platinum-based 1st line therapy.

mRNA Microarray

Total RNA from ovarian cancer fresh frozen samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The quality and integrity of the RNA was assessed using Fragment Analyzer parallel capillary electrophoresis (Advanced Analytical Technologies, Ankeny, IA, USA). The RNA was subsequently labeled and hybridized using the Agilent gene expression microarray kit (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instruction. Briefly, the RNA from the clinical samples was labeled with Cy3 fluorochrome and subsequently co-hybridized with Cy5 labeled Xpress Ref TM Human Universal Reference Total RNA (SuperArray Bio-science Corporation) as a control. The hybridization was carried out for 21 hours on Agilent 4X44K human gene expression array slides. The slides were subsequently scanned on an Agilent C scanner and raw data were extracted using the Agilent Feature Extraction software ver. 11.0.1.1 and quantile normalized. A fold-change cutoff of 2 was used to determine differential mRNA expression as well as q-value and signal intensity.

Table 3. Characteristics of the study patients in the ROR investigation subcohort (n = 30)

Characteristic	
Patients in study, n	30
Age at surgery, yrs mean (SD)	66 (9.3)
Median follow-up, months (range)	31 (2–50)
FIGO * Stage, n (%)	
Stage 1	2 (6,7%)
Stage 2	4 (14%)
Stage 3	17 (56%)
Stage 4	7(23,3%)
Histology, n (%)	
Serous	22(73,3%)
Endometroid	2 (6,7%)
Papillar	2 (6,7%)
Mucinous	2 (6,7%)
Carcinosarcoma	1 (3,3%)
Transitional cell	1 (3,3%)
Grade †, n (%)	
Grade 1 and 2	8 (26,7%)
Grade 3	22 (73,3%)
Response to platinum therapy ‡, n (%)	
Sensitive	20 (66,7%)
Resistant	10 (33,3%)
Recurrence	20 (66,7%)
Death	9 (30%)

* FIGO = International Federation of Gynecology.
† Grade – Grade 3 represents high grade tumors, Grade 1 and 2 low grade tumors.
‡ Sensitivity defined as relapse or event-free follow up time> 12 months after completion of platinum based 1st line therapy.

qRT-PCR

Putatively differentially expressed genes (*ROR2, CAST, ATP6V1D, GUCY1A3, TMOD1, MYCN, DLK1, PLEKHG4B, GREB1, B4GALNT4, SLC35F3, PTCH2, TNNC1, BNC1*) were selected from the array results based on fold change, q-value, signal intensity and literature data and were validated by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA from ovarian cancer fresh frozen tumor samples were extracted with TRIzol as described above and were reverse transcribed using random hexamere primers and MultiScribe reverse transcriptase (Thermo Fischer Scientific, Waltham, MA, USA). Quantitative Real Time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fischer Scientific) on a BioRad CFX96 ™ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA). Each sample was run in duplicate and expression values were normalized against the TATA-binding protein (TBP). The primer sequences for the two validated genes are as follows:

GREB1 fw	5'ATGGGAAATTCTTACGCTGGAC
GREB1 rev	5'CACTCGGCTACCACCTTCT
ROR2 fw	5'GTGCGGTGGCTAAAGAATGAT
ROR2 rev	5'ATTCGCAGTCGTGAACCATATT
TBP fw	5'GAATATAATCCCAAGCGGTTTG
TBP rev	5'ACTTCACATCACAGCTCCCC

Western blotting

Frozen tumor pieces were thawed, washed 2X with cold PBS and pestered to lyse in lysis buffer (50 mM Tris–HCl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton-x-100, 50 mM NaF) supplemented with protease and phosphatase inhibitor cocktails (Bimake, Houston, TX, USA). Lysates were mixed with 4X Laemmli loading buffer and subjected to SDS-PAGE gel electrophoresis and Western blotting. The primary antibodies used were as following: pAkt S473 (#4060), pMEK1/2 S217/221 (#9121), NF-κB p65 (#6956), pPI3K p85 Y458/p55 Y199 (#4228), PI3K p85α (#13666), Rac-1 (#4651), pSTAT3 Y705 (#9145), STAT3 (#9139), Wnt5a/b (#2530) (Cell Signaling Technology, Danvers, MA, USA); Akt (#sc-5298), Bcl-2 (#sc-7382), MEK1/2 (#sc-6250), β-tubulin (#sc-166,729) (Santa Cruz, Dallas, TX, USA); ROR1 6D4 (Dr. Riddel lab, ref. Balakrishana et al. 2016); ROR2 (#565550, BD Biosciences, San Jose, CA, USA). Secondary antibodies: IRDye® 800CW Donkey anti-Mouse IgG, or IRDye® 680RD Donkey anti-Rabbit IgG (LI-COR, Lincoln, NE, USA). Blots were scanned and quantified using Odyssey CLx and Image Studio

software (LI-COR). Protein quantification was normalized to β-tubulin expression level for each sample.

Cell Culture

A2780 and A2780cis cells were purchased from Merck & Company Inc. (Kenilworth, NJ, USA) and cultured according to manufacturer's recommendations. The A2780 line was maintained in medium containing 1 μM cisplatin (Selleckchem, Munich, Germany). The cisplatin EC50 response of A2780 and A2780cis cells was validated by incubating cells with increasing concentration of cisplatin for 3 days and cell viability was determined using CellTiterGlo (CTG) Assay (Promega, USA) according to manufacturer's instructions.

qRT-PCR of the Ovarian Cancer Cell Lines

RNA was collected from A2780 and A2780cis using TRI Reagent® (Molecular Research Center Inc. Cincinnati, OH, USA) according to the manufacturer's protocol. qRT-PCR was performed as described for the ovarian cancer clinical samples. Each cell line was run in 4 replicates and the expression of ROR2 and GREB1 was normalized against TBP.

Statistical Analysis

The statistical analysis of mRNA microarray data was implemented in R using packages limma and preprocessCore of Bioconductor project [21–23]. Data was quantile normalized and probe sets were summarized by choosing the probes with the highest average expression [22]. Using limma approach, differential expression was identified between patients who had low PARP value and were platinum resistant, and patients who had high PARP value and were platinum sensitive [23]. P-values were obtained by the empirical Bayes moderated t-test. A fold-change cutoff of 2 was used to determine differential mRNA expression. The results were

Table 4. The 50 Most Upregulated mRNAs in High PARP and Platinum Sensitive OC Patient Samples in Comparison to low PARP and Platinum Resistant Samples.

Gene name	Gene description	log2-fold change	p-value	q-value
COLEC11	collectin subfamily member 11	3.68E+ 00	1.29E-02	0.2588936
MYCN	**MYCN proto-oncogene. bHLH transcription factor**	**3.61E+ 00**	**2.74E-04**	**0.1050305**
ESM1	endothelial cell specific molecule 1	3.18E+ 00	1.64E-04	0.1028588
IGF1R	insulin like growth factor 1 receptor	3.08E+ 00	5.29E-03	0.211962
TNNC1	**troponin C1. slow skeletal and cardiac type**	**3.04E+ 00**	**1.25E-05**	**0.0472319**
LGSN	lengsin. Lens protein with glutamine synthetase domain	3.02E+ 00	1.25E-02	0.2588936
LOC100134423	uncharacterized LOC100134423	2.97E+ 00	2.71E-04	0.1050305
DLK1	**delta like non-canonical Notch ligand 1**	**2.89E+ 00**	**4.17E-03**	**0.2013983**
CRYGC	crystallin gamma C	2.86E+ 00	2.37E-03	0.1822358
A_33_P3286709	NA	2.82E+ 00	9.15E-05	0.0864965
PLEKHG4B	**pleckstrin homology and RhoGEF domain containing G4B**	**2.82E+ 00**	**1.17E-03**	**0.1525447**
TSPAN8	tetraspanin 8	2.78E+ 00	2.99E-02	0.3064189
ENPP6	ectonucleotide pyrophosphatase/phosphodiesterase 6	2.73E+ 00	1.20E-02	0.2563874
FLJ30901	uncharacterized protein FLJ30901	2.60E+ 00	3.26E-04	0.1050305
PROM1	prominin 1	2.59E+ 00	3.02E-02	0.3079072
COL22A1	collagen type XXII alpha 1 chain	2.58E+ 00	2.64E-03	0.1844765
KIAA1324	KIAA1324	2.58E+ 00	5.48E-04	0.1261184
PTCH2	**patched 2**	**2.57E+ 00**	**1.93E-05**	**0.0472319**
SLC35F3	**solute carrier family 35 member F3**	**2.54E+ 00**	**3.87E-05**	**0.0596903**
GABRG3	gamma-aminobutyric acid type A receptor gamma3 subunit	2.51E+ 00	4.50E-05	0.0628517
LOC613266	uncharacterized LOC613266	2.50E+ 00	4.89E-02	0.3485897
LCE1E	late cornified envelope 1E	2.49E+ 00	2.28E-04	0.1050305
B4GALNT4	**beta-1.4-N-acetyl-galactosaminyltransferase 4**	**2.49E+ 00**	**1.24E-05**	**0.0472319**
STC2	stanniocalcin 2	2.48E+ 00	2.86E-04	0.1050305
FOXL2NB	FOXL2 neighbor	2.48E+ 00	1.04E-01	0.4447586
AK124496	NA	2.47E+ 00	2.78E-02	0.3020977
CU677518	NA	2.47E+ 00	2.82E-06	0.0275612
NM_130777	NA	2.45E+ 00	1.10E-02	0.2525629
NR_102701	NA	2.43E+ 00	2.11E-03	0.1762016
NSG1	neuronal vesicle trafficking associated 1	2.43E+ 00	1.05E-03	0.1525447
STAR	steroidogenic acute regulatory protein	2.41E+ 00	5.08E-03	0.2110634
MCTS2P	malignant T-cell amplified sequence 2. pseudogene	2.40E+ 00	5.43E-03	0.2134259
TRABD2A	TraB domain containing 2A	2.36E+ 00	4.89E-03	0.2078187
DEPTOR	DEP domain containing MTOR interacting protein	2.35E+ 00	4.70E-04	0.125416
CLEC4GP1	C-type lectin domain family 4 member G pseudogene 1	2.35E+ 00	4.56E-03	0.2021535
LINC01405	long intergenic non-protein coding RNA 1405	2.33E+ 00	6.95E-04	0.1391319
COL2A1	collagen type II alpha 1 chain	2.32E+ 00	1.82E-02	0.2816697
HS6ST2	heparan sulfate 6-O-sulfotransferase 2	2.31E+ 00	2.15E-02	0.2871745
PRAME	preferentially expressed antigen in melanoma	2.30E+ 00	4.32E-05	0.0628517
LINC02398	long intergenic non-protein coding RNA 2398	2.29E+ 00	1.08E-05	0.0472319
GJB7	gap junction protein beta 7	2.29E+ 00	3.18E-02	0.3122712
PLCXD3	phosphatidylinositol specific phospholipase C X domain containing 3	2.28E+ 00	6.03E-02	0.3707384
ERVI-1	endogenous retrovirus group I member 1	2.27E+ 00	1.28E-02	0.2588936
ZNF556	zinc finger protein 556	2.27E+ 00	1.46E-06	0.0213888
NTS	neurotensin	2.26E+ 00	6.56E-02	0.3828556
BU963192	NA	2.25E+ 00	7.17E-05	0.0751488
GMNC	geminin coiled-coil domain containing	2.24E+ 00	1.52E-02	0.2689731
A_33_P3274001	NA	2.24E+ 00	9.36E-05	0.0864965
CRYGD	crystallin gamma D	2.24E+ 00	2.58E-02	0.2964626
GREB1	**growth regulation by estrogen in breast cancer 1**	**2.22E+ 00**	**1.22E-03**	**0.1542519**

mRNAs selected for further validation are shown in bold.

interpreted by principal component analysis [24] and hierarchical clustering.

qRT-PCR data were analyzed using the Student's t-test and Mann–Whitney U test where appropriate. The Kaplan–Meier regression analyses were used to estimate the survival rates from the date of surgery (primary debulked patients) or from the date of the first dose of neoadjuvant therapy until the date of the event of interest. For progression-free survival (PFS), the event of interest was a recurrence or death, whichever occurred first. Patients alive at the last follow-up without a recurrence were censored at the last follow-up date. Statistical analysis was performed using GraphPad Prism 6 software for Windows (GraphPad Software Inc., La Jolla, CA, USA). A p-value less than 0.05 was considered significant.

Oncomine (https://www.oncomine.org/resource/login.html) and Kaplan–Meier plotter (http://kmplot.com/analysis/index.php?p=service&cancer=ovar) databases were searched for gene expression and survival data.

Results

Microarray Analysis of HGSC Patient Samples

In order to identify differentially expressed genes between ovarian cancer samples with platinum-sensitivity with high PARP levels and samples with platinum resistance with low PARP levels (n = 12), gene expression microarray was performed on total RNA isolated from the freshly frozen tumor samples. The analysis of gene expression showed a total of 3001 differentially expressed genes between the two comparison groups when a log fold change cutoff 2 was implemented. In this comparison, 1463 genes were downregulated and 1538 genes were upregulated. 50 most upregulated and 50

Table 5. The 50 Most Downregulated mRNAs in High PARP and Platinum Sensitive OC Patient Samples in Comparison to Low PARP and Platinum Resistant Samples

Gene Name	Gene Description	Log2-Fold Change	p-Value	q-Value
EFEMP1	EGF containing fibulin extracellular matrix protein 1	−3.85E+ 00	1.50E-03	0.1587368
FAP	fibroblast activation protein alpha	−3.66E+ 00	9.07E-04	0.1525447
TMOD1	**tropomodulin 1**	**−3.41E+ 00**	**2.92E-08**	**0.0008577**
BNC1	**basonuclin 1**	**−3.36E+ 00**	**2.74E-04**	**0.1050305**
ENST00000453673	NA	−3.22E+ 00	2.22E-02	0.2877726
ENST00000411475	NA	−3.18E+ 00	2.30E-02	0.2898232
SCRG1	stimulator of chondrogenesis 1	−3.18E+ 00	1.64E-03	0.1627023
PTGFR	prostaglandin F receptor	−3.04E+ 00	1.05E-03	0.1525447
POSTN	periostin	−3.01E+ 00	1.10E-02	0.2525629
ENST00000390237	NA	−2.97E+ 00	1.54E-02	0.2695379
BF175071	NA	−2.92E+ 00	5.12E-02	0.3538495
GUCY1A3	**guanylate cyclase 1 soluble subunit alpha**	**−2.89E+ 00**	**5.68E-05**	**0.0723946**
ENST00000390628	NA	−2.87E+ 00	4.44E-03	0.2021325
CXCL13	C-X-C motif chemokine ligand 13	−2.87E+ 00	1.02E-02	0.2487313
ACKR4	atypical chemokine receptor 4	−2.86E+ 00	9.73E-05	0.0864965
IGLL5	immunoglobulin lambda like polypeptide 5	−2.86E+ 00	3.46E-02	0.318167
AREG	amphiregulin	−2.83E+ 00	1.43E-02	0.2670241
A_33_P3251412	NA	−2.81E+ 00	1.46E-02	0.2682963
FYB1	FYN binding protein 1	−2.79E+ 00	2.51E-03	0.1843078
CD38	CD38 molecule	−2.78E+ 00	1.54E-03	0.1587368
ENST00000468879	NA	−2.77E+ 00	4.36E-02	0.3380632
ENST00000390252	NA	−2.73E+ 00	1.49E-02	0.2682963
JCHAIN	joining chain of multimeric IgA and IgM	−2.70E+ 00	6.61E-03	0.2195342
ENST00000390547	NA	−2.69E+ 00	5.85E-03	0.2163421
MEDAG	mesenteric estrogen dependent adipogenesis	−2.69E+ 00	4.49E-02	0.3403785
CNR1	cannabinoid receptor 1	−2.68E+ 00	9.85E-03	0.2471112
PRRX1	paired related homeobox 1	−2.68E+ 00	7.45E-04	0.1427284
NR4A3	nuclear receptor subfamily 4 group A member 3	−2.67E+ 00	2.00E-02	0.2850432
CH25H	cholesterol 25-hydroxylase	−2.65E+ 00	2.21E-03	0.1762175
AB363267	NA	−2.65E+ 00	4.23E-02	0.3347815
ENST00000426099	NA	−2.63E+ 00	4.11E-02	0.331398
CCL18	C-C motif chemokine ligand 18	−2.57E+ 00	1.16E-04	0.0898927
THBS2	thrombospondin 2	−2.57E+ 00	3.91E-02	0.327707
ENST00000410078	NA	−2.56E+ 00	5.30E-02	0.3580372
BCHE	butyrylcholinesterase	−2.55E+ 00	1.00E-02	0.2471753
ATP6V1D	**ATPase H+ transporting V1 subunit D**	**−2.55E+ 00**	**1.54E-04**	**0.1028588**
ENST00000390323	NA	−2.55E+ 00	2.42E-02	0.2937808
CCR2	C-C motif chemokine receptor 2	−2.54E+ 00	9.37E-03	0.24523
ENST00000479981	NA	−2.54E+ 00	9.43E-03	0.24523
CAST	**calpastatin**	**−2.54E+ 00**	**1.02E-03**	**0.1525447**
ASPN	asporin	−2.52E+ 00	2.94E-02	0.3049408
HBB	hemoglobin subunit beta	−2.52E+ 00	2.08E-02	0.2862875
ENST00000390247	NA	−2.51E+ 00	2.16E-02	0.2871745
SULF1	sulfatase 1	−2.50E+ 00	1.80E-02	0.2805948
EPYC	epiphycan	−2.49E+ 00	1.28E-02	0.2588936
FGFBP1	fibroblast growth factor binding protein 1	−2.49E+ 00	1.03E-02	0.2499163
LRRC15	leucine rich repeat containing 15	−2.48E+ 00	4.14E-02	0.3321649
DOCK11	dedicator of cytokinesis 11	−2.45E+ 00	5.13E-04	0.1261184
OOEP	oocyte expressed protein	−2.45E+ 00	4.23E-03	0.2013983
ROR2	**receptor tyrosine kinase like orphan receptor 2**	**−1.84E+ 00**	**2.83E-05**	**0.0506967**

mRNAs selected for further validation are shown in bold.

most downregulated genes are summarized in Tables 4 and 5, respectively. The comparison of patient groups according to PARP levels and treatment responses are shown in Figure 1, a-b.

GREB1 and ROR2 are Significantly Differentially Expressed in Ovarian Cancer Tumor Samples Based on Platinum Sensitivity and PARP Levels

Fourteen differentially expressed mRNAs (namely: *ROR2, CAST, ATP6V1D, GUCY1A3, TMOD1, MYCN, DLK1, PLEKHG4B, GREB1, B4GALNT4, SLC35F3, PTCH2, TNNC1, BNC1*) from the microarray analysis were selected for validation by qRT-PCR in the cohort of 53 ovarian cancer patients, based on fold change, q-value, signal intensity and previous literature (Table 2). Two genes, namely *ROR2* and *GREB1*, were significantly differentially expressed in high PARP/platinum-sensitive vs. low PARP/platinum-resistant groups (P = .02 and 0.002, respectively) (Figure 2, a-b). *ROR2* was downregulated in microarray data in high PARP/platinum-sensitive tumor samples and the same result was obtained by qRT-PCR analysis in the validation cohort (n = 53). A trend towards statistically significant difference between the platinum sensitive and resistant groups regardless of PARP levels was observed (P = .058) (Figure 2a). The comparisons described above were based on treatment response as a significant clinical feature and issue of interest. In addition, previously determined PARP levels were taken into account to further investigate tumors from the BRCAness profile point of view. The platinum sensitive and high PARP level cohort is considered a group of better prognosis befitting with the BRCAness profile, whereas the platinum resistant and low PARP level a group with poorer prognosis.

On the other hand, *GREB1* was upregulated in microarray data in high PARP/platinum-sensitive tumor samples, and this result was validated by qRT-PCR (Figure 2b). Furthermore, GREB1 also showed a trend of overexpression in platinum sensitive samples, regardless of PARP level (P = .26).

GREB1 Expression Defines Platinum Sensitivity and Correlates with Longer PFS in Ovarian Cancer

Furthermore, we investigated whether *GREB1* expression affects PFS in ovarian cancer patients (n = 53). High *GREB1* expression was significantly associated with longer PFS as shown in Figure 2c (P = .019 in log-rank test). In Kaplan–Meier database search, which included 1465 ovarian cancer patients, a similar result was shown associating high GREB1 expression with better PFS (P = 0,014 in log-rank test), as shown in Figure 2d. In addition to Kaplan–Meier plotter database and Oncomine (website links are reported in statistical analysis section) were searched for the *ROR2* and *GREB1* genes. No similar results in expression correlation were found, however, no similar study settings were found (HGSC, treatment response comparison).

High ROR2 Expression in LowPARP/Platinum Resistant Ovarian Cancer Samples Correlated with Higher Wnt5a, STAT3 and NF-kB levels

Previous data have shown no association with ROR2 expression and relapse-free survival [25] in ovarian cancer. However, ROR2 and ROR1 expression is increased in cisplatin resistant A2780 cell line compared to parental cells ([18]), and silencing their ligand Wnt5a in serous adenocarcinoma OVCAR3 cell line had greater effect in inhibiting cell migration and invasion than silencing either ROR alone [25]. Since *ROR2* was significantly upregulated in low PARP/platinum-resistant patient samples in our microarray data, we decided

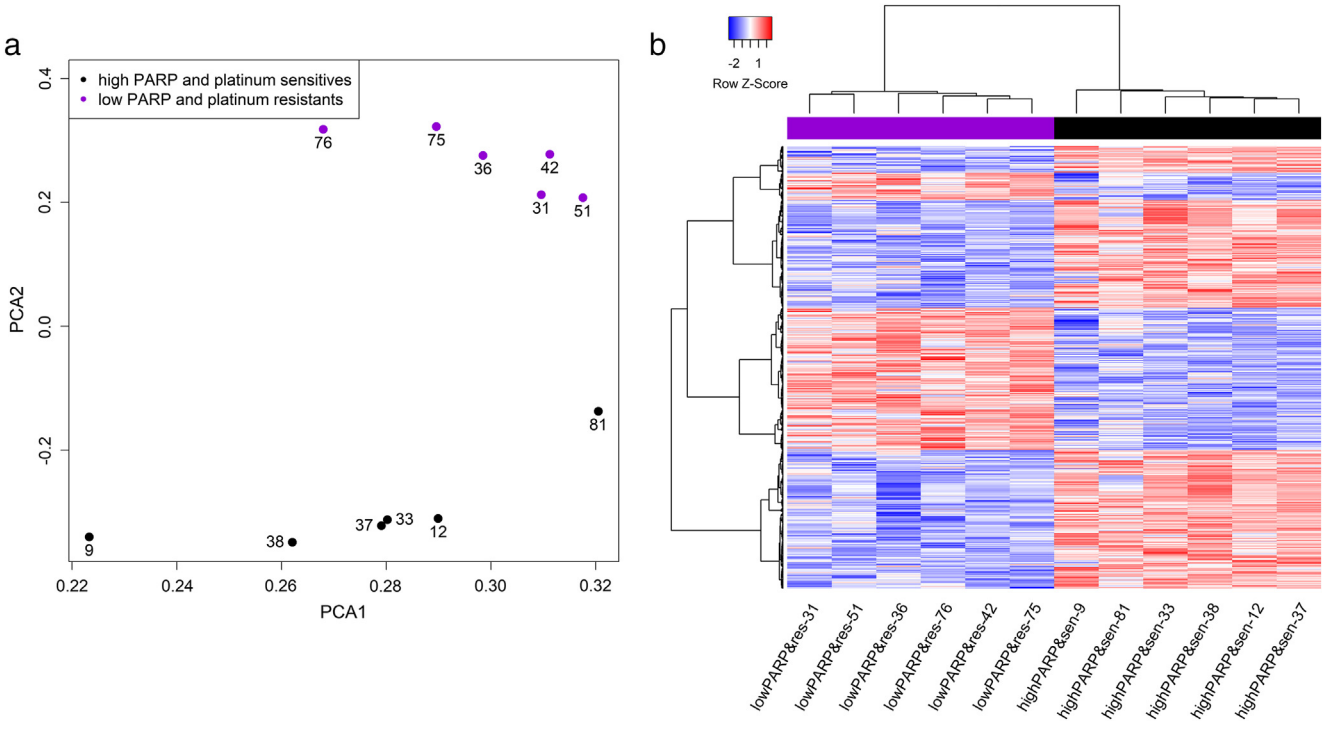

Figure 1. a. Scatterplot showing location of samples of high PARP and platinum sensitive and low PARP and platinum resistant in patients along the first two principal components (PC1 and PC2). Platinum sensitive and high PARP level samples are represented by black and low PARP and platinum resistant samples by purple dots.b. Unsupervised clustering of the clinical samples shows differential expression patterns in patients with high PARP levels and platinum sensitivity compared to patients with low PARP levels and platinum resistant.

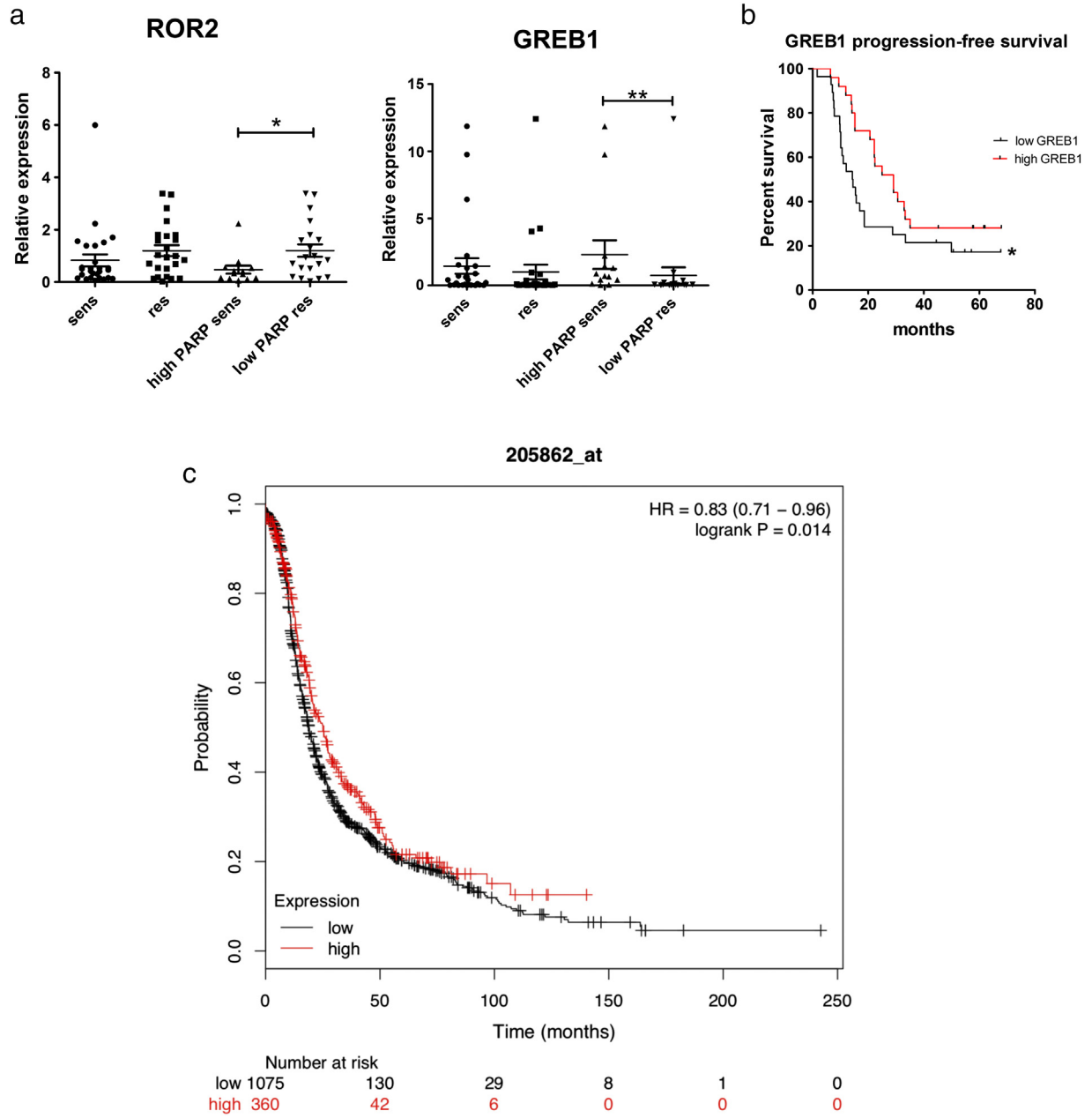

Figure 2. a. Expression of ROR2 and GREB1 in platinum sensitive (n = 38) vs resistant (n = 15) samples as well as high PARP level and platinum sensitive (n = 17) vs low PARP level and platinum resistant (n = 14) samples according to qRT-PCR (P = .02 and 0.002 respectively). The graphs show mean ± SEM.b. Kaplan–Meier analysis of progression free survival (PFS) according to median level of GREB 1 concentration (log rank P = .019). Vertical lines represent censored patients.c. Kaplan–Meier analysis of progression free survival (PFS) according to median level of GREB 1 concentration (log-rank P = .014) from Kaplan–Meier plotter database.

to investigate the expression levels of Wnt5a, ROR1 and ROR2 proteins by Western blot in a subcohort of samples described in Table 3. As shown in Figure 3a-b, higher expression levels of Wnt5a, ROR1 and ROR2 proteins were found in low PARP/platinum-resistant group compared to high PARP/platinum-sensitive group. Moreover, upregulation of downstream signaling mediators such as pSTAT3 (Y705) and NF-kB was also observed in tumor lysates from low PARP/platinum resistant group. Noteworthy, patient samples with high levels of ROR1 and ROR2 from low PARP/platinum-resistant group showed higher pSTAT3 (Y705) levels (Figure 3a), indicating that STAT3 could mediate signaling downstream ROR1 and ROR2. Database search (Oncomine, Kaplan–Meier plotter; website links are reported in statistical analysis section) revealed no additional information regarding ROR in therapy response and ovarian cancer setting.

ROR2 and GREB1 Show Differential Expression in Cisplatin Resistant Ovarian Cancer Cell Line Model

The human epithelial ovarian cancer cell line A2780 and its cisplatin resistant model A2780cis were selected to investigate the expression levels of ROR2 and GREB1. We confirmed the chemoresistant phenotype of A2780cis cells compared to A2780 parental cells by CTG assay and found significant increase of EC50 to cisplatin treatment for A2780cis cells (Figure 4a). The expression of

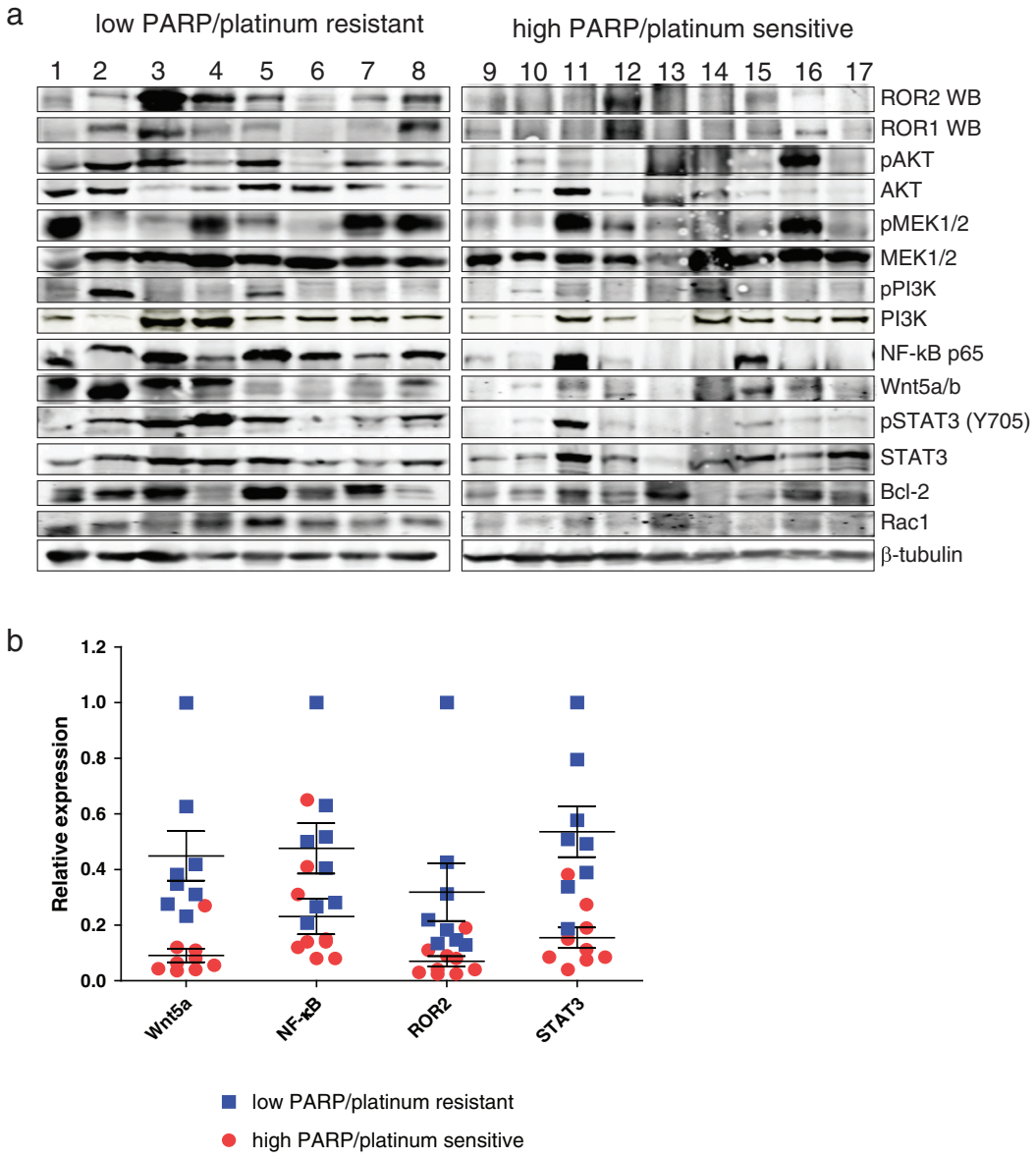

Figure 3. Wnt5a/ROR2 expression is increased in lysates from platinum resistant tumors. A. Western blot analysis of lysates from platinum resistant vs. platinum sensitive patient samples with the indicated primary antibodies. B. Quantification of protein expression for Wnt5a, ROR2, STAT3 and NF-kB based on β-tubulin levels. Horizontal lines represent the average and errors bars are SEM (standard error of mean). After normalization, samples were quantified based on the highest expression level of all samples.

both ROR2 and GREB1 mRNA level was investigated in A2780 and A2780cis cells (Figure 4b and c). We observed ROR2 mRNA upregulation in platinum resistant cell line A2780cis compared to platinum sensitive A2780 cells (P = .0046), as previously shown ([18]), whereas GREB1 mRNA level was downregulated in A2780cis compared to A2780 parental cells (P = .0012). Furthermore, A2780cis cells have increased protein expression levels of ROR2 and Wnt5a compared with the parental A2780 cells as shown by Western blotting of cytoplasmic cell lysates (Figure 4d). In addition, we detected higher nuclear expression levels for STAT3 and NF-kB in chemoresistant A2780cis compared to parental A2780 cells (Figure 4d). Thus, our microarray data from patient samples are validated in chemoresistant ovarian cancer cell line and investigations are ongoing to decipher the molecular mechanisms employed by ROR2 and GREB1 for their differential expression associated with cisplatin resistance.

Discussion

Due to its poor prognosis, identification of new therapeutic approaches is highly needed in ovarian cancer. Although approximately 50% of HGSC tumors with HR defects may benefit from PARP inhibitors, beyond this, identification of other commonly deregulated pathways could provide opportunities for better therapeutic interventions. As such, our goal was to examine gene and protein expression in a HGSC tumor sample cohort defined by response to platinum therapy and the level of PARP expression. Patients were carefully stratified based on their PARP levels and platinum responsiveness as previously indicated [20] and monitored

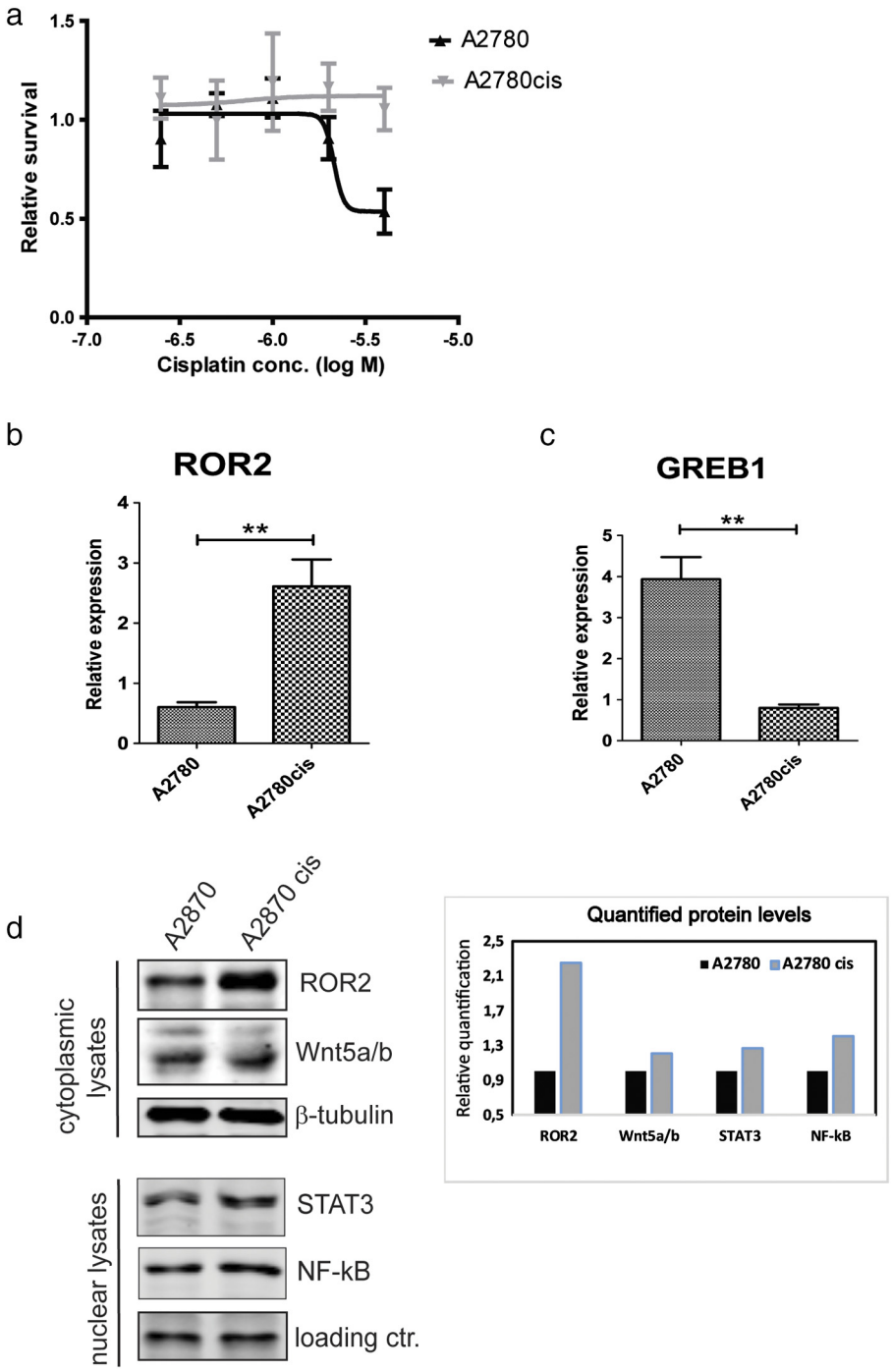

Figure 4. a. Cisplatin sensitivity testing of A2780 and A2780cis cell lines. Cells were incubated as five replicates for 3 days with increased concentrations of cisplatin as indicated and cell-viability was measured by CTG assay. EC50 was calculated using Graph Prism software and shown as approximate values.b. ROR2 mRNA expression in platinum sensitive and resistant cell lines A2780/A2780cis ($P = .0046$). The graphs show mean ± SEM.c. GREB1 mRNA expression in platinum sensitive and resistant cell lines A2780/A2780cis ($P = .0012$. The graphs show mean ± SEM.d. Protein expression levels for ROR2, Wnt5a, NF-kB and STAT3 in the cytoplasmic and nuclear cell lysates of A2780 and A2780cis cell lines. Protein quantification was done using Oddyssey Licor software and normalized against the loading control.

for relatively long time, up to 50 months to ensure a comprehensive analysis of their disease progression.

Two functionally unrelated genes, *GREB1* and *ROR2* were identified in our analysis as significantly differentially expressed between high PARP/platinum-sensitive and low PARP/platinum-resistant groups.

While initially sequenced from brain tissue, human *GREB1* is highly expressed in normal and neoplastic ovarian tissue and in several other hormone-responsive tissues such as breast, uterine and prostate [26–29]. *GREB1*, along with *CCND1* and *MYC*, are common transcription targets for E2 (17β-estradiol)-mediated proliferative responses, via ESR1 (estrogen receptor one) engagement [28].

Estrogen receptor positive ($ESR1^{+}$) breast cancers usually express GREB1, whereas in ovarian cancer, its expression could be detected in both, $ESR1^{+}$ or estrogen receptor negative ($ESR1^{-}$) tumors. ESR1-independent expression of GREB1 may indicate the existence of other signaling pathways for estrogen-promoting growth in the absence of E2 and/or ESR1, underlining differences in E2-responsiveness between breast and ovarian cancer that play an important role in response to antiestrogenic therapies [30]. *GREB1* was upregulated in all EOC tumors and was suggested to have potential biomarker role in ovarian cancer [29]. *GREB1* was upregulated in our microarray data in high PARP/platinum-sensitive patients, and high *GREB1* expression was associated with longer PFS, suggesting a prognostic value for *GREB1* in ovarian cancer.

It has been demonstrated that *GREB*1 knockdown inhibits proliferation of ovarian cancer cell lines and consequently, prolongs survival in an orthotopic mouse model [28]. Also, hypomethylation at specific CpG site associated with *GREB1* has been associated with longer PFS in ovarian cancer in a DNA methylation study designed to investigate epigenetic modifications [31]. Interestingly, loss of GREB1 has been linked to tamoxifen resistance in breast cancer due to loss of sensitivity to endocrine agents in general, underlining its important role in endocrine resistance [32–33]. Although endocrine therapy has overall limited efficacy in ovarian cancer patients, more research is needed to assess whether GREB1 is associated with antiestrogen sensitivity in this cancer. In view of this previous data, our results are in accordance with these findings reaffirming the understanding that *GREB1* is highly expressed in high PARP/platinum-sensitive patient group that has been associated with a positive outcome befitting with the BRCAness profile [10]. Our data show for the first time that *GREB1* could have prognostic value in ovarian cancer and warrants further investigation, especially associated with response to hormone-based therapy.

ROR2 belongs to the ROR receptor family (*ROR1* and *ROR2*) from the non-canonical Wnt pathway [17]. ROR1 and ROR2 form heterodimers in response to Wnt5a, which leads to RhoA/Rac1 activation and increases migration and invasion properties of cancer cells [34]. Recent studies have demonstrated that upregulation of *ROR2* and its ligand Wnt5a in EOC regulates EMT and correlates with worse prognosis [35]. *ROR1* and *ROR2* regulate migration and invasion of ovarian cancer cells and more importantly, their expression was increased in platinum resistant A2780 ovarian cancer cell line compared to parental cells ([18]). TCGA data analysis of over 500 ovarian tumor samples identified high expression of Wnt5a and Wnt5a protein was found prevalent in ascites samples of ovarian cancer patients [36]. In our study, *ROR2* gene expression was significantly upregulated in low PARP/platinum-resistant vs. high PARP/platinum-sensitive patient samples. We also found higher protein levels of ROR2, ROR1 and Wnt5a ligand in lysates of platinum-resistant tumors (Figure 3, a-b), confirming our gene expression analysis. Interestingly, higher expression levels of NF-κB and pSTAT3 (Y705) proteins were also noted in platinum-resistant tumor lysate with high ROR1 and ROR2 levels, indicating that STAT3 could be downstream mediator of ROR signaling in ovarian cancer. These results showing Wnt5a-ROR2 and STAT3/NF-kB signaling pathway in clinical ovarian tumor samples are novel findings. A direct link between ROR1 and STAT3 expression has been demonstrated previously in leukemia, showing that STAT3 promoter harbors two ROR1 binding sites [37]. Moreover, previous studies have shown that Wnt5a is involved in cancer multidrug resistance (MDR)[36]. High Wnt5a expression levels in ovarian cancer cell lines correlated with lower chemosensitivity to paclitaxel, oxaliplatin, 5-fluorouracil, epirubicin and etoposide ([38,39]. Furthermore, upregulation of Wnt5a and ROR2 was detected in colon cancer cells resistant to butyrate, a histone deacetylase inhibitor (HDACi)[40], along with activation of AKT/PKB (protein kinase B) signaling pathway. Wnt5a orchestrates multiple signaling networks involved in chronic inflammation and carcinogenesis, and crosstalk with STAT3 and NF-κB pathways have been previously documented [41]. Thus, upregulation of Wnt5a, ROR1 and ROR2 signaling pathways could be common mechanisms in ovarian cancer chemoresistance and could serve as putative biomarkers. ROR1 targeted therapies have shown promising results in preclinical and clinical models, with anti-ROR1 monoclonal antibody cirmtuzumab being efficient not only in leukemia, but also in ovarian cancer [42], indicative of high therapeutic potential for targeting these receptors.

Our mRNA expression data from patient samples were validated using a cisplatin resistant cell line model A2780cis and we found that upregulation of ROR2 at the mRNA and protein levels is associated with cisplatin resistance. Moreover, GREB1 mRNA level was downregulated in A2780cis compared to A2780 parental cells. Furthermore, we observed higher expression levels for Wnt5a, STAT3 and NF-kB proteins in chemoresistant A2780cis cells compared to parental cells, in support of our results from patient samples (Figure 3).

In conclusion, we found the expression of ROR2 and GREB1 to be associated with treatment response in HGSC. The association between Wnt5a/ROR2 expression and development of platinum resistance reported herein suggests that the Wnt5a/ROR2 pathway is potentially actionable for possible modulation of chemoresistance. Because silencing *ROR1* and *ROR2* restores the chemosensitivity of carboplatin-resistant ovarian cancer cells [18], a combination of ROR antagonists and chemotherapeutic agents may offer a promising treatment option. Also, our findings regarding *GREB1* expression in highPARP/platinum-sensitive patients should reinforce the interest in this gene for future investigations.

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Disclosure

The authors declare no conflicts of interest.

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