

Downregulation of tumor suppressor gene ribonuclease T2 and gametogenetin binding protein 2 is associated with drug resistance in ovarian cancer

FUQIANG YIN^{1,2*}, LING LIU^{1*}, XIA LIU³, GANG LI¹, LI ZHENG¹, DANRONG LI¹, QI WANG⁴, WEI ZHANG⁴ and LI LI^{2,4}

¹Medical Scientific Research Centre, Guangxi Medical University, Nanning, Guangxi 530021; ²Key Laboratory for High-Incidence Tumor Prevention and Treatment, Ministry of Education; ³Center for Translational Medicine, Guangxi Medical University, Nanning; ⁴Department of Gynecologic Oncology, Affiliated Tumor Hospital of Guangxi Medical University, Nanning, Guangxi 530021, P.R. China

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Abstract. Ribonuclease T2 (*RNASET2*) and gametogenetin binding protein 2 (*GGNBP2*) are tumor suppressor genes whose expression is downregulated in ovarian and other types of cancer. However, whether the proteins encoded by these genes are associated with drug resistance has rarely been studied. Using real-time quantitative polymerase chain reaction, in the present study we showed that *RNASET2* and *GGNBP2* mRNA levels were significantly lower in A2780-CBP (carboplatin-resistant) and A2780-DDP (cisplatin-resistant) ovarian cancer cells than in the parental A2780 cells and were downregulated in drug-resistant ovarian cancer tissues compared with their drug-sensitive counterparts. These findings were consistent with the expression profiles determined from microarray data retrieved from the Gene Expression Omnibus Profiles database. Accordingly, we hypothesized that the downregulation of *RNASET2* and *GGNBP2* is involved in the development of drug resistance in ovarian cancer. A comprehensive bioinformatics analysis of the two genes was therefore conducted, including gene/protein-gene/protein interactions, biological process annotation, pathway enrichment of co-expressed genes and microRNA-mRNA analyses. The integrated results suggested that *RNASET2* and *GGNBP2* contributed to drug resistance in ovarian cancer, via direct or indirect interactions with a number of microRNAs, genes and proteins involved in a wide range of biological processes and pathways. The information provided in the present study provides insight for

further investigations of the drug resistance-related functions of *RNASET2* and *GGNBP2*.

Introduction

Ovarian cancer is the most lethal gynecologic cancer, causing annually a large number of deaths throughout the world. Early stages of ovarian cancer are often asymptomatic such that the disease is usually diagnosed after it has disseminated beyond the ovaries (1). In fact, ~70% of ovarian cancer patients present with advanced-stage disease (2). Although patients usually respond to initial therapy and cytoreductive surgery followed by adjuvant chemotherapy with platinum and paclitaxel (3), ~70% of those with advanced-stage ovarian cancer experience recurrence. In many of these cases, the disease is incurable mainly due to the development of drug resistance (4).

Drug resistance can be intrinsic or acquired and arises from a variety of factors, including genetic differences in somatic cell tumors and individual patient-specific variations (5). In culture, cancer cells have been shown to become resistant to cytotoxic anticancer drugs through several different mechanisms, such as efflux pumps, cell cycle defects, apoptosis, DNA damage, metabolic alterations, changes in drug targets and compartmentalization (5). Some of the molecular mechanisms underlying the development of resistance have been characterized in cellular models of ovarian cancer. Johnson *et al* (6) described three general pathways; increased DNA damage tolerance/repair, decreased cell-drug associations and altered drug inactivation. All of these are thought to be involved in platinum-based resistance mechanisms in ovarian cancer. According to Sorrentino *et al* (7), drug resistance in ovarian cancer reflects the deregulation of growth factor receptor expression, defective DNA damage response, increased DNA repair activity and the increased activity of anti-apoptotic regulators. A more recent study suggested that targeting glycolytic, mitochondrial apoptotic and necrotic pathways offers promising therapeutic strategies in ovarian cancer (3). However, despite the large number of studies and increasing insight into the mechanisms of drug resistance in this disease,

Correspondence to: Professor Li Li, Department of Gynecologic Oncology, Affiliated Tumor Hospital of Guangxi Medical University, 71 Hedi Road, Nanning, Guangxi 530021, P.R. China
E-mail: lili@gxmu.edu.cn

*Contributed equally

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the 5-year survival rate of patients is only ~40%, and only modest improvement has been made over the past 40 years (2).

Tumor suppressor genes (TSGs) play regulatory roles in diverse cellular activities, including cell cycle checkpoint responses, mitogenic signaling, DNA damage repair, cell proliferation, differentiation, migration, protein ubiquitination and tumor angiogenesis (8,9). TSGs have also been implicated in the drug resistance of ovarian cancer and their loss of function contributes to the development of cancer in general (9,10). For example, expression of tumor suppressor PTEN is lower in drug-resistant than in drug-sensitive ovarian cancer cells. The overexpression of PTEN by transfection was shown to reverse chemoresistance to cisplatin, by inactivating the PI3K/AKT cell survival pathway (11). The TSGs BRCA1 and BRCA2 have been convincingly linked to ovarian cancer, and tumors with BRCA1/2 mutations frequently develop platinum resistance (12). In a literature review, Yin *et al* (10) identified 15 TSGs (such as BRCA1, PTEN and p53) that play critical roles in drug resistance and that, consequently, may serve as molecular targets for the treatment of chemoresistant ovarian cancer. Therefore, the targeting of drug resistance-related TSGs would provide the first steps in developing reasonable and feasible methods to meet the challenges posed by drug resistance in ovarian cancer.

TSGs may be of the class I or class II type. Class I TSGs lose function by deletion, rearrangement or mutation (13); class II TSGs are structurally intact in their sequence, yet are underexpressed or unexpressed due to downregulation or silencing in transcription or translation (14). Ribonuclease T2 (*RNASET2*) is a class II TSG associated with the suppression of ovarian cancer (15); it belongs to a group of genes encoding RNases and is highly conserved among species (16). RNases, including *RNASET2*, have many different functions, with a particularly important one being the regulation of cell growth (17). Consistent with these properties is the observation that in some human malignancies, such as lymphomas and glioma, *RNASET2* expression is downregulated at the transcriptional level (18,19). Monti *et al* (20) showed an apparent decrease in the antitumorigenic activity of *RNASET2* in melanoma cancer cells. In ovarian cancer, *RNASET2* expression was shown to be significantly reduced in 30% of primary ovarian tumors and in 75% of ovarian tumor cell lines (15). Conversely, the transfection of *RNASET2* into ovarian tumor cell lines suppressed their tumorigenicity in nude mice and induced marked cell senescence during *in vitro* growth (15). Besides its antitumorigenic activity, *RNASET2* significantly reduces the metastatic potential of ovarian cancer (21), in addition to controlling ovarian tumorigenesis by modifying the cellular microenvironment and by inducing immunocompetent cells of the monocyte/macrophage lineage (22). Collectively, these studies indicate *RNASET2* is a poorly explored member of the growing family of TSGs (22).

Gametogenetin binding protein 2 (*GGNBP2*), a potential TSG, is a highly conserved gene localized to the tumor-suppressor locus D17S800-D17S930 but also known to be involved in spermatogenesis (23). This gene is also known as *ZNF403* and as laryngeal carcinoma related gene 1 (*LCRGI*), in the latter case since it was cloned from a laryngeal carcinoma (24). The expression of *GGNBP2* is significantly reduced in 40% (12/30) of primary laryngeal carcinomas, where its

primary tumor suppressive functions include control of the cell proliferation rate and of soft agar growth, tumor formation and tumor cell migration (24,25). *GGNBP2* knockdown alters the expression profiles of certain genes, such as upregulating p21 and downregulating MCM2. This participation in cell cycle regulatory pathways suggests that *GGNBP2* contributes to the regulation of the G2/M transition of the cell cycle (25). In fact, cell cycle analysis demonstrated that the downregulation of *GGNBP2* promotes G2/M cell cycle arrest in a dose-dependent manner. Regarding the involvement of *GGNBP2* in ovarian cancer, one study reported downregulation of the gene in six of ten different ovarian cancer cell lines (24).

Although *RNASET2* and *GGNBP2* are important TSGs and disturbances in their expression have been implicated in the development of ovarian and other types of cancer, the literature contains no reports of their specific association with drug resistance. A microarray analysis of carboplatin-sensitive and -resistant ovarian cancer cells carried out in our laboratory showed that the expression of *RNASET2* and *GGNBP2* is significantly decreased in resistant cells (26), indicating the involvement of these two genes in drug resistance. In the present study, on the basis of the results of real-time quantitative polymerase chain reaction (RT-qPCR) and comprehensive bioinformatics analysis, we were able to show, for the first time, that the downregulation of *RNASET2* and *GGNBP2* in drug-resistant ovarian cancer tissues/cells contributes to the regulation of drug resistance in ovarian cancer.

Materials and methods

Cell culture and tissue samples. The human epithelial ovarian cancer cell line A2780 was maintained in our laboratory and propagated *in vitro* by serial passage in RPMI-1640 supplemented with 10% fetal bovine serum. The A2780-CBP and A2780-DDP cell lines were developed by sequential exposure of A2780 cells to increasing concentrations of carboplatin and cisplatin, respectively. The resistance index of A2780-CBP and -DDP ovarian cancer cells was 2.0 and 1.8, respectively.

Ovarian cancer tissues were collected from patients surgically treated for primary epithelial ovarian cancer at the Department of Gynecologic Oncology, Affiliated Tumor Hospital of Guangxi Medical University. The tissue samples were evaluated by pathologists. All patients underwent conventional chemotherapy with 6-8 postoperative cycles of paclitaxel plus cisplatin (TP) or paclitaxel plus carboplatin (TC) after optimal cytoreductive surgery. They were followed up for a minimum of 1 year after the completion of chemotherapy. In addition, the patients were assigned to either the chemotherapy-resistant group (n=10) or the chemotherapy-sensitive group (n=10) in accordance with the 2013 guidelines National Comprehensive Cancer Network (NCCN). The median age of the patients was 50.8 years (range, 35-73 years). All of the malignant tissues were from stage II-III tumors, according to the International Federation of Gynecology and Obstetrics (FIGO) classification. The study was endorsed by the Ethics Committee of Guangxi Medical University. All patients received an explanation concerning the aims of the study and provided signed informed consent. Samples were collected from primary lesions during surgery and stored in a liquid nitrogen tank until they were used for mRNA isolation.

RT-qPCR analysis. Total RNA from ovarian cancer cells/tissues was isolated using TRIzol reagent and digested with amplification-grade DNase I (both from Invitrogen, Carlsbad, CA, USA) prior to cDNA synthesis. The first-strand cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis kit (Roche, Germany). RT-qPCR was performed with the FastStart Universal SYBR-Green Master kit (Roche). Data were collected with the StepOnePlus Real-Time PCR System (ABI, Foster City, CA, USA) in accordance with the manufacturer's instructions. The RT-qPCR gene-specific primers for *RNASET2* were: (forward primer) 5'-ACCCTCCGGATTACTGGACA-3' and (reverse primer) 5'-TGCGATTGGGAAACGAGTGA-3'; for *GGNBP2*, the forward primer was 5'-ATCACGCGAAGTCCTGAGTG-3' and the reverse primer was 5'-GGGAAAAGAGACGCTCCACA-3'. For *GAPDH*, used as the control, the forward primer was 5'-GAAGGTGAAGGTCGGAGT-3' and the reverse primer was 5'-GAAGATGGTGATGGGATTT-3'.

Bioinformatics analysis. Microarray data on gene expression were retrieved from Gene Expression Omnibus (GEO) Profiles (<http://www.ncbi.nlm.nih.gov/geo/profiles/>). The gene/protein-gene/protein interaction network was generated with GeneMANIA (<http://www.genemania.org/>) (25); the annotation of biological processes was analyzed with the Coremine Medical online tool (<http://www.coremine.com/medical/>). The miRWalk online tool (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>) was used for miRNA target-gene prediction (27). Data on genes co-expressed with *RNASET2* and *GGNBP2* according to the TCGA Ovarian 2 database were obtained from the Oncomine online database (<https://www.oncomine.org/resource/login.html>). The pathway enrichment was performed using the DAVID online tool (<http://david.abcc.ncifcrf.gov/>) (28,29). The pathways in which the genes were involved were analyzed using the online database Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>).

Results

RNASET2 and *GGNBP2* mRNA expression is significantly downregulated in drug-resistant ovarian cancer cells/tissues. Based on the microarray data obtained in our laboratory (26) and the data retrieved from the GEO database (Fig. 1A), the expression of *RNASET2* and *GGNBP2* was significantly lower (at least 1.6-fold) in platinum- and vincristine-resistant ovarian cancer cells than in their drug-sensitive counterparts. This result was confirmed by measuring the expression of the two genes in all three ovarian cancer cell lines. Specifically, the RT-qPCR results showed large decreases in *RNASET2* and *GGNBP2* mRNA levels in A2780-CBP and -DDP cancer cells when compared with A2780 cells. Indeed, *GGNBP2* expression was reduced by at least 5-fold in resistant cells (Fig. 1B). Further study of these genes in ovarian cancer tissues revealed that *RNASET2* and *GGNBP2* mRNA levels were significantly lower in drug-resistant than in drug-sensitive tissues (Fig. 1C and D). Compared with the average expression levels in ten sensitive cancers, those of *RNASET2* and *GGNBP2* in ten resistant cancers were decreased 3.2- and 7.4-fold, respectively. Collectively, the data on drug-resistant cells as

provided in the GEO database, the results of our experiments in cultured drug-resistant-A2780 cells, and our analyses of drug resistance in ovarian cancer tissues consistently showed that the expression of *RNASET2* and *GGNBP2* mRNA is down-regulated in ovarian cancer, indicating a role for these genes in the development of drug-resistant variants of these tumors.

Prediction and analysis of function based on gene/protein-gene/protein interactions. The drug resistance-related functions of *RNASET2* and *GGNBP2* were analyzed using GeneMANIA (Fig. 2). *RNASET2* was co-expressed, co-localized, physically interacted and shared protein domains and pathways directly/indirectly with a number of proteins. Specifically, direct genetic interactions were established between *RNASET2* and checkpoint kinase 2 (CHEK2), programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN), split hand/foot malformation (ectrodactyly) type 1 (SHFM1) and Yes-associated protein 1 (YAP1). In addition, *RNASET2* was found to be co-expressed directly with ring finger and WD repeat domain 2 (RFWD2). These results suggested that these genes/proteins and *RNASET2* are functionally related.

CHEK2 is one of the critical kinases governing cell apoptosis, DNA damage repair and cell cycle checkpoint control in response to DNA damage signals. It is degraded at the protein level in response to cisplatin, through a ubiquitin-proteasome pathway. In ovarian cancer, the expression of CHEK2 is decreased in cisplatin-resistant ovarian cancer cell lines, suggesting a role for this kinase in chemoresistance (30).

PDCD4 is another TSG and its expression was shown to enhance chemosensitivity in renal and prostate cancer cell lines (31,32). In ovarian cancer, PDCD4 promotes cisplatin-induced apoptosis, mainly by activating the death receptor pathway and PDCD4 gene transfer in combination with cisplatin therapy may break the chemotherapeutic resistance of ovarian cancer cells (33).

PTEN is a well-known TSG associated with cancer development through the ERK1/2 signaling (34) and PI3K/Akt/mTOR (35,36) pathways. It interacts with TSGs (such as p53 and BRCA1) that contribute to the development of drug resistance in several types of cancer (37). In ovarian cancer, PTEN contributes to multidrug resistance through cell cycle regulation, apoptosis and the PI3K/Akt pathway (38,39).

Yuan *et al* (40) demonstrated that YAP1 is a TSG. Knock-down of the gene in breast cell lines resulted in several abnormal cell behaviors, including inhibition of the response of these cells to taxol. Accordingly, the authors concluded that YAP1 plays a role in drug resistance.

SHFM1 is a TSG required for the stability of BRCA2 (41), yet it has also been implicated in acquired drug resistance in ovarian cancer (42).

Finally, RFWD2, also known as constitutive photomorphogenic 1 (COP1), is a newly discovered TSG (43,44). As reported by Migliorini *et al* (44), it exerts its tumor suppressor function in part by antagonizing c-Jun oncogenic activity. A recent study suggested RFWD2 as a target for gene therapy in the treatment of gastric cancer, suggesting the involvement of this protein in drug resistance in cancer (45).

GGNBP2 is associated with fewer genes/proteins than *RNASET2*. As shown in Fig. 2, *GGNBP2* is co-expressed only

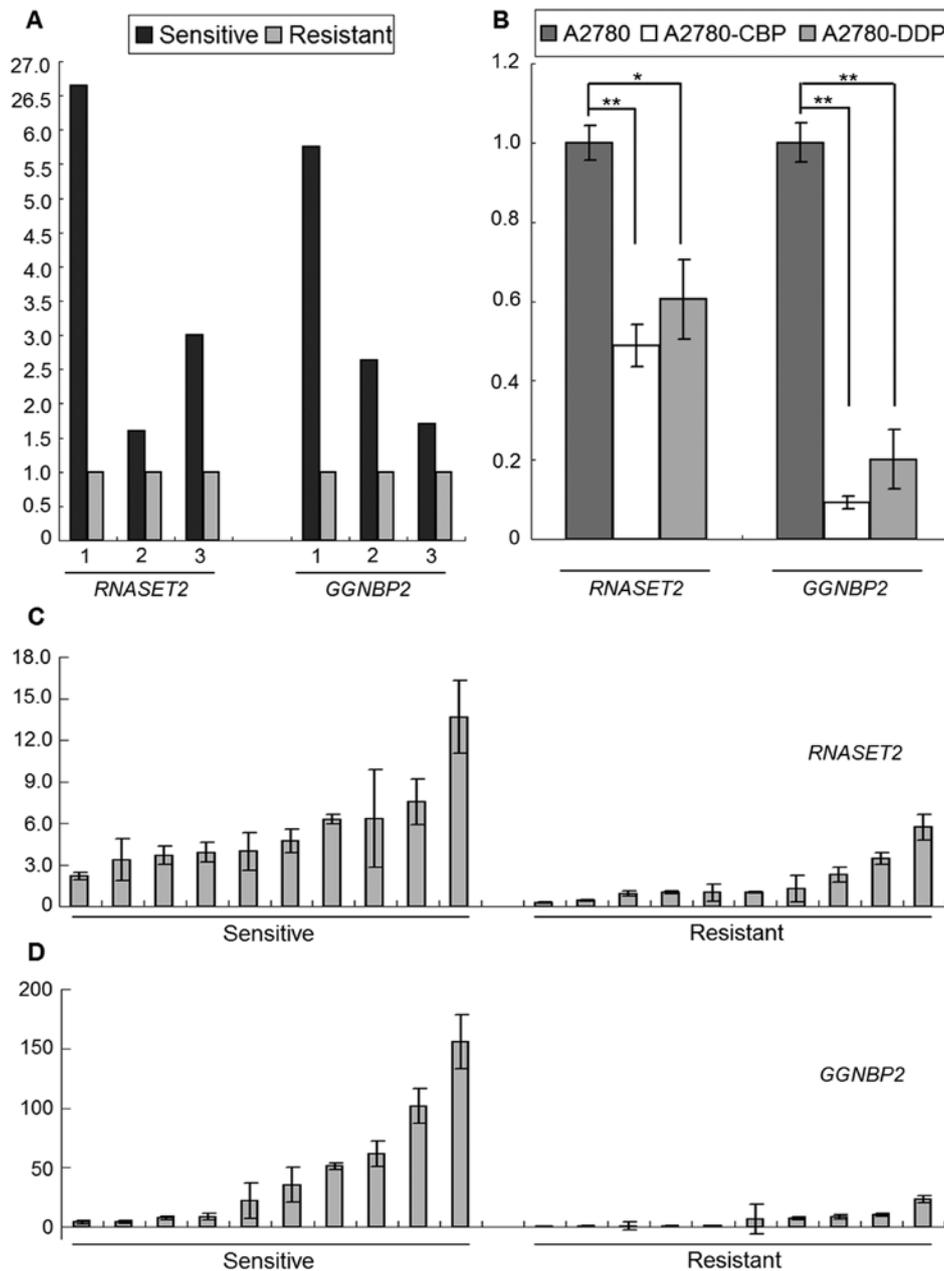


Figure 1. RNASET2 and GGNBP2 expression at the transcriptional level is downregulated in drug-resistant ovarian cancer cells/tissues. (A) RNASET2 and GGNBP2 mRNA expression in drug-resistant ovarian cancer cells, as determined by microarray analysis. Lane 1, carboplatin-sensitive and -resistant SKOV3 ovarian cancer cells (4 biological replicates each) from our laboratory; Lane 2, vincristine-sensitive and -resistant SKOV3 ovarian cancer cells (4 biological replicates each) according to the GEO profiles database (GDS2963); Lane 3, platinum-sensitive and -resistant A2780 ovarian cancer cells (10 biological replicates each) according to the GEO profiles database (GDS3754). Gene expression in the drug-resistant cancer cells was normalized to 1.0. (B) RNASET2 and GGNBP2 mRNA expression in A2780, A2780-CBP and -DDP ovarian cancer cells. * $p < 0.05$, ** $p < 0.01$. (C) RNASET2 mRNA expression in drug-sensitive and -resistant ovarian cancers. (D) GGNBP2 mRNA expression in drug-sensitive and -resistant ovarian cancers. RNASET2, ribonuclease T2; GGNBP2, gametogenin binding protein 2.

with zinc finger CCCH-type containing 11A (ZC3H11A) and genetically interacts with WW domain containing oxidoreductase (WWOX). ZC3H11A is a poorly characterized protein that belongs to a large family of zinc finger proteins, with 58 known members (46). Zinc finger proteins are known to mediate the epithelial-mesenchymal transition, which in lung cancer is related to drug resistance (47). Thus, the function of ZC3H11A, as a member of the zinc finger protein family, may also be drug resistance-related. As a TSG, WWOX plays important roles in ovarian and many other types of cancers (48). For

example, the overexpression of WWOX preferentially inhibits the viability of human glioblastoma cells expressing mutant p53 and induces apoptosis via a mechanism independent of the intrinsic apoptotic pathway (49). Conversely, WWOX suppression by RNA interference reverses platinum resistance in DDP-resistant SKOV3 ovarian cancer cells (50).

In the present study, we determined direct interactions of six genes/proteins with RNASET2 and two with GGNBP2, and in all cases these were linked to drug resistance in ovarian and other types of cancers. In addition, indirect interactions

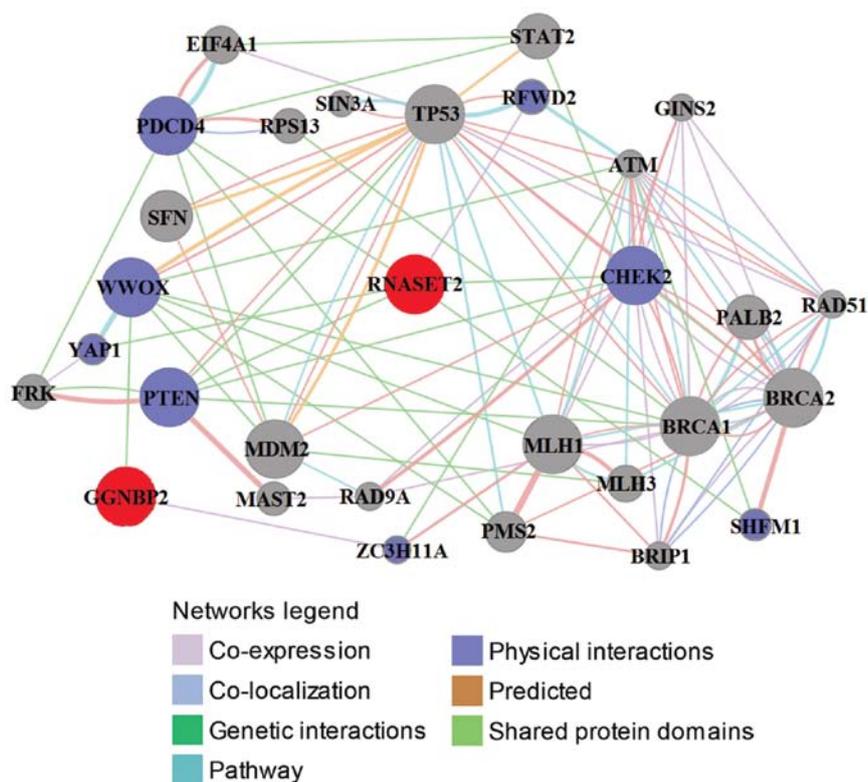


Figure 2. Gene/protein interaction networks of RNASET2 and GGNBP2, generated using the GeneMANIA online tool. Queries in red refer to target genes/proteins; those in blue refer to the genes/proteins that interact directly with the target. RNASET2, ribonuclease T2; GGNBP2, gametogenetin binding protein 2.

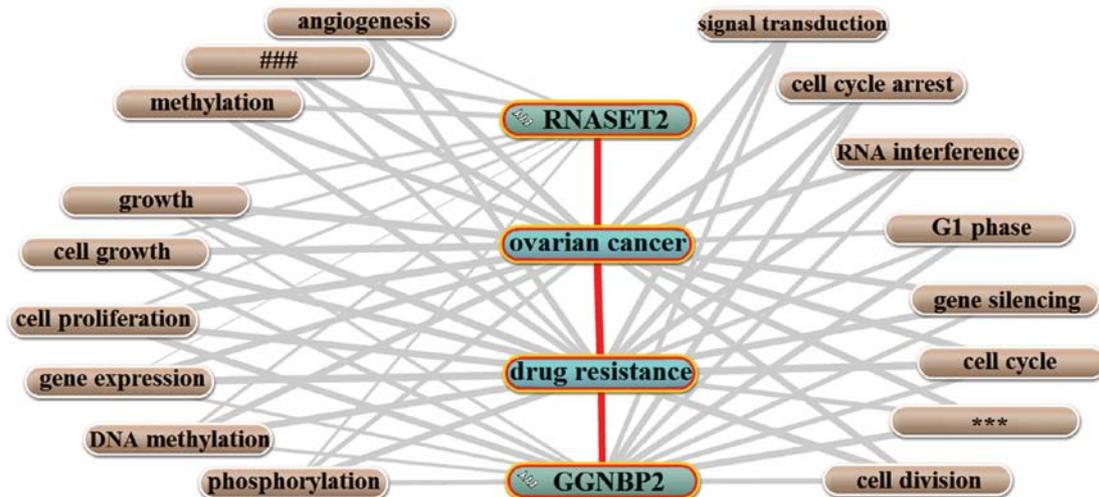


Figure 3. Annotated biological processes involving RNASET2 and GGNBP2 and their relationships with both drug resistance and ovarian cancer, as determined using the Coremine online tool. ###, cell cycle regulation; ***, respond to DNA damage stimulus. RNASET2, ribonuclease T2; GGNBP2, gametogenetin binding protein 2.

between several TSGs, such as TP53, MLH1, MLH3, BRCA1, and BRCA2, and RNASET2, and GGNBP2 were demonstrated. All of these TSGs are linked to drug resistance in ovarian cancer (10), a relationship that supports our conclusion that RNASET2 and GGNBP2 are associated with drug resistance in ovarian cancer.

Functional prediction based on the annotated biological process. In Coremine Medical (51), gene and protein names

are cross-referenced to terms relevant to an understanding of their biological function and importance in disease. We therefore used the Coremine online tool and probabilistic scoring ($p < 0.009$) to annotate RNASET2 and GGNBP2 with respect to biological processes. As shown in Fig. 3, 17 biological processes closely associated with ovarian cancer, drug resistance, and the two genes were annotated, suggesting their mutual involvement in drug resistance in ovarian cancer. Among the nine processes annotated to RNASET2, three were related to

Table I. The 8 miRNAs most strongly targeting GGNBP2 and RNASET2, and their functions in cancer.

Gene name	MicroRNA	miRNA-gene interaction prediction tools									Drug resistance and related functions in cancer (ref.)
		A	B	C	D	E	F	G	H	I	
<i>GGNBP2</i>	hsa-miR-135a	1	1	1	1	1	1	1	1	1	Drug resistance in breast cancer (52)
	hsa-miR-23b	1	1	1	1	1	1	1	1	1	Drug resistance in ovarian cancer (53) and breast cancer (52)
	hsa-miR-135b	1	1	1	1	1	0	1	1	1	Drug resistance in esophageal cancer (54)
	hsa-miR-23a	1	1	1	1	1	0	1	0	1	Drug resistance in ovarian cancer (55)
	hsa-miR-150	1	1	0	1	1	0	1	1	1	Drug resistance in breast cancer (52)
	hsa-miR-323-3p	1	1	1	1	1	0	1	0	1	-
	hsa-miR-210	1	1	1	1	0	0	1	0	1	Drug resistance in breast cancer (56)
	hsa-miR-224	1	1	1	1	0	0	1	0	1	Drug resistance in lung cancer cells (57) and gastric cancer cells (58)
<i>RNASET2</i>	hsa-miR-758	0	1	0	1	0	0	1	0	1	Drug resistance in breast cancer (52)
	hsa-miR-320c	0	1	0	1	0	0	1	0	1	-
	hsa-miR-320a	0	1	0	1	0	0	1	0	1	Cell proliferation in colon cancer cell (59) and tumor invasion in colorectal cancer (60)
	hsa-miR-320d	0	1	0	1	0	0	1	0	1	Regulation of stem cell differentiation in colon cancer stem cells (61)
	hsa-miR-320b	0	1	0	1	0	0	1	0	1	-
	hsa-miR-635	0	1	0	1	0	0	0	0	1	-
	hsa-miR-22	0	1	0	1	0	0	1	0	0	Drug resistance in ovarian cancer (62)
	hsa-miR-1257	0	1	0	0	0	0	1	0	1	Tumor dedifferentiation and/or progression in liposarcoma (63)

A, DIANAmt; B, miRanda; C, miRDB; D, miRWalk; E, RNAhybrid; F, PICTAR4; G, PICTAR5; H, RNA22; I, TargetsCan; 1, predicted by the software; 0, not predicted; GGNBP2, gametogenetin binding protein 2; RNASET2, ribonuclease T2.

growth, cell growth and cell proliferation, consistent with the theory that the drug resistance-related functions of RNASET2 mainly proceed through the regulation of cell growth and the RNase activity of RNASET2 (17). GGNBP2 annotated with 14 of the 17 processes: three (growth, cell growth and cell proliferation) were cell-growth related, four (gene expression, DNA methylation, RNA interference and gene silencing) were gene-expression related, and three (cell cycle arrest, G1 phase and cell cycle) were cell cycle related. Of these, the relationship between the cell cycle-related function of GGNBP2 and drug resistance is in agreement with a previous study in which GGNBP2 was shown to be involved in cell cycle regulation during cancer development (25).

Functional prediction based on the functionality of the miRNAs that target them. Among the transcriptional targets of GGNBP2 and RNASET2 were 228 and 179 miRNAs, respectively, as predicted through miRWalk, which is made up of ten miRNA-mRNA prediction tools. Eight miRNAs, i.e., those yielding the highest score for each gene, were selected for subsequent analysis (Table I). As shown in Table I, except for hsa-miR-323-3p, all the miRNAs that targeted GGNBP2 influenced drug resistance in ovarian and other types of cancers. For RNASET2, although only hsa-miR-758 and hsa-miR-22 were specifically involved in drug resistance in ovarian and breast cancer, three other miRNAs (hsa-miR-320a, hsa-miR-320d

and hsa-miR-1257) contributed to drug resistance-related processes, such as cell proliferation, tumor invasion and cell differentiation. Collectively, among eight miRNAs most strongly targeting RNASET2 and especially GGNBP2, the majority were involved in drug resistance in ovarian and other cancers, suggesting that the two genes also mediate drug resistance.

Potential pathways by which RNASET2 and GGNBP2 contribute to drug resistance. Pathway enrichment of the genes co-expressed with RNASET2 and GGNBP2 in drug-resistant ovarian cancer cells was performed to mine the possible pathways in which these two TSGs may exercise their drug resistance-related functions in ovarian cancer. Genes co-expressed with RNASET2 and GGNBP2 in eight irinotecan-resistant, four paclitaxel-resistant and five topotecan-resistant ovarian cancer cells were retrieved from the Oncomine online database. Of the co-expressed genes, the 50 genes with the highest correlation efficiency with GGNBP2 and RNASET2 were selected for further analysis (Table II). For GGNBP2, two pathways (cytokine-cytokine receptor interaction and chemokine signaling pathway) were found to be enriched, as determined using DAVID (28,29), whereas with RNASET2 no enriched pathway was identified. Thus, a basic search in the KEGG database was performed to retrieve the pathways to which the co-expressed genes belonged. This

Table II. The 50 genes most strongly co-expressed with RNASET2 and GGNBP2 in drug-resistant ovarian cancer cells and their drug resistance-associated pathways.

Genes co-expressed in drug-resistant ovarian cancer cells ^a (correlation efficiency >0.873)	Annotated pathway ^b	Genes belong to certain pathway based on the KEGG database ^c and the pathways were involved in the drug resistance in ovarian and other cancers (ref.)
RNASET2		
<i>FGFR1OP, CCR6, T, PRR18, SFT2D1, BRP44L, RPS6KA2, C6orf118, PDE10A, UNC93A, TTLL2, TCP10, GPR31, TCP10L2, QKI, PACRG, PARK2, SMOC2, DACT2, PSMB1, TBP, PDCD2, FAM120B, DLL1, THBS2, PHF10, WDR27, C6orf120, TCTE3, C6orf70, HGC6.3, KIF25, FRMD1, C6orf124, MLLT4, MAP3K4, AGPAT4, SOD2, WTAP, TCP1, ACAT2, PNLDC1, MRPL18, MAS1, IGF2R, LPA, LPAL2, SLC22A3, SLC22A2, SLC22A1</i>	-	<i>CCR6</i> , I (69) and II (71) <i>PARK2</i> , III (77) <i>THBS2</i> , IV (77) and V (39,78) <i>TCP1</i> , V (39,78) <i>PSMB1</i> , VI (30,79) <i>MAP3K4</i> , VII (80-83)
GGNBP2		
<i>DHRS11, MYO19, PIGW, MRM1, ZNHIT3, AATF, LHX1, SLFN13, SLFN11, SLFN12, CCL3, CCL23, CCL18, MMP28, TAF15, C17orf50, CCL5, C17orf66, GAS2L2, RASL108, LYZL6, RDM1, CCL16, CCL14, AP2B1, SLFN12L, PEX12, SLFN14, SLFN5, AMAC1, C17orf75, ZNF207, RHBDL3, PSMD11, CDK5R1, MYO1D, TMEM98, LRRC37B, RHOT1, UTP6, C17orf79, SUZ12, C17orf102, TMEM132E, CCL7, CCL1, CCL13, CCL8, CCL11, CCL2</i>	I II	<i>PIGW</i> , VIII (3) <i>CCL3, CCL5, CCL7, CCL1, CCL8</i> and <i>CCL11</i> , I (69), II (71) <i>CCL23, CCL18, CCL16</i> and <i>CCL14</i> , I (69) <i>CCL2</i> , I (69) and IX (73,74) <i>CCL13</i> , IX (73,74) <i>AP2B1</i> , X (84) <i>PSMD11</i> , VI (30,79) and XI (85)

^aGenes co-expressed with RNASET2 and GGNBP2 in 8 irinotecan-resistant, 4 paclitaxel-resistant and 5 topotecan-resistant ovarian cancer cells in accordance with Oncomine online tool and database. ^bPathway enrichment of the genes was determined using DAVID. ^cPathways of the genes co-expressed with RNASET2 and GGNBP2 were searched in the KEGG online database. I, Cytokine-cytokine receptor interaction; II, chemokine signaling pathway; III, ubiquitin-mediated proteolysis; IV, focal adhesion; V, PI3K-Akt signaling pathway; VI, proteasome pathway; VII, mitogen-activated protein kinase signaling pathway; VIII, metabolic pathways; IX, NF- κ B signaling pathway; X, endocytosis pathway; XI, Epstein-Barr virus infection. RNASET2, ribonuclease T2; GGNBP2, ametogenetin binding protein 2; KEGG, Kyoto Encyclopedia of Genes and Genomes.

resulted in the identification of 12 RNASET2-associated genes and 19 GGNBP2-associated genes as a member of certain pathways, several of which could be related to drug resistance in ovarian and other types of cancers (Table II).

For example, the genes *THBS2* and *TCP1*, co-expressed with RNASET2, are part of the PI3K/Akt signaling pathway, which suggested that it is among the pathways used by this TSG to regulate drug resistance. In fact, a strong association between PI3K/Akt signaling and drug resistance has been shown in ovarian cancer (11,39). Pathway enrichment analysis suggested that the hypomethylation-mediated activation of cell growth-promoting pathways, including PI3K/Akt, contributes to the onset of chemoresistance in ovarian cancer cells (64). Studies on the genes involved in the activation of this pathway identified two mechanisms, PTEN reduction and PIK3CA mRNA amplification, that finally contribute to cisplatin resistance in an ovarian cancer cell line (39). Similarly, the chemoresistance caused by interleukin (IL)-6 and -8 expression is associated with the activation of several pathways, including PI3K/Akt (65,66). Accordingly, altered RNASET2 expression in drug-resistant ovarian cancers may

well contribute to chemoresistance by activating the PI3K/Akt signaling pathway.

The cytokine-cytokine receptor interaction and chemokine signaling pathways were enriched in the 50 genes most highly co-expressed with GGNBP2 in drug-resistant ovarian cancer cells, suggesting that GGNBP2 contributes to drug resistance via these two pathways. In tumorous spleens, downregulated genes that have been linked to transformation are mainly associated with the cytokine-cytokine receptor interaction pathway (67); similarly, most of the genes associated with lung carcinogenesis are annotated to this same pathway (68). Cytokine-cytokine receptor interactions with drug resistance are thought to proceed via tumor-stroma communication, as shown in primary tumors with a high risk of relapse (69). In a non-small cell lung cancer cell line, functional annotation showed that genes with altered expression induced by docetaxel are strongly co-related with several pathways, including cytokine-cytokine receptor interactions (70). These results provide evidence of the interaction between the cytokine-cytokine receptor pathway and the drug resistance of several types of tumor.

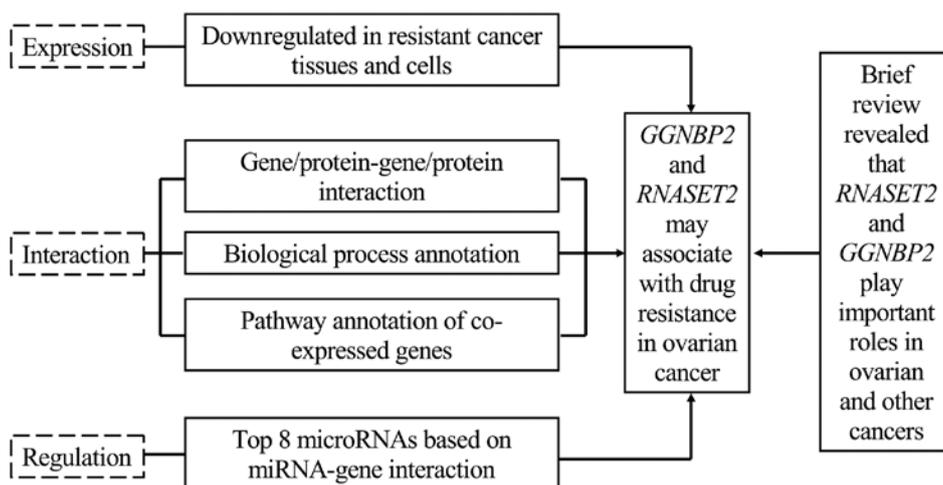


Figure 4. Summary of the bioinformatics analysis of the association of GGNBP2 and RNASET2 with drug resistance in ovarian cancer. RNASET2, ribonuclease T2; GGNBP2, gametogenetin binding protein 2.

Crosstalk networks among pathways associated with prostate cancer (primary and metastatic), by integrating protein-protein interactions and KEGG pathways, point to the regulation of multiple processes, including drug resistance, by the chemokine signaling pathway during cancer progression (71). Oxaliplatin-induced chemokine signaling was shown to reinforce NF- κ B transcriptional activity, thus potentiating the resistance of prostate cancer cells to this platinum-based drug (72). The NF- κ B signaling pathway contains two genes (*CCL2* and *CCL13*) that are co-expressed with GGNBP2 in drug-resistant ovarian cancer cells. Many studies have shown that this pathway plays a critical role in anti-apoptosis and the drug resistance of tumor cells during chemotherapy (73). For example, curcumin downregulates the multidrug-resistance *mdr1b* gene by inhibiting the PI3K/Akt and NF- κ B signaling pathways in multidrug-resistant L1210/Adr cells (74). Pre-clinical models demonstrated that many chemotherapy drugs, such as platinum-based agents, anthracyclines and taxanes, promote NF- κ B pathway activation, which finally leads to chemoresistance through the subsequent activation of a multitude of mediators including anti-apoptotic genes (75). In ovarian cancer, NF- κ B functions as an oncogene in drug-resistant ovarian cancer cells and exerts its anti-apoptotic effects by repressing mitogen-activated protein kinase phosphorylation in aggressive chemoresistant cell lines (76).

Thus, some of the genes co-expressed with RNASET2 and GGNBP2 in drug-resistant ovarian cancer cells belong to pathways associated with drug resistance, suggesting the similar involvement of these two TSGs. In particular, GGNBP2 may participate in the regulation of drug resistance in ovarian cancer via cytokine-cytokine receptor interactions, chemokine signaling and NF- κ B signaling, whereas for RNASET2, the PI3K/Akt pathway is relevant.

Discussion

Drug resistance is an important obstacle to successful chemotherapeutic treatment in ovarian cancer and causes treatment failure in over 90% of patients with metastatic disease (86). Several mechanisms, including DNA damage repair and disrup-

tions in the cell cycle, apoptosis and metabolism, are associated with the rise of drug resistance in ovarian cancer (3,7), mostly through aberrant gene expression. As key regulators of genes related to drug resistance, the mining and exploration of TSGs can provide insights into meeting the therapeutic challenge of drug resistance in ovarian cancer. The prediction of gene function based on comprehensive bioinformatics analysis offers a valuable tool for gene function mining. For example, on the basis of a comprehensive bioinformatics analysis, Yin *et al.* (87) reported the association of SPARCL1 and CCL21 with drug resistance in ovarian cancer. In another study, 15 TSGs associated with drug resistance in ovarian cancer were analyzed by comprehensive bioinformatics to better understand the relationships of these genes to drug resistance (10).

The Gene Expression Omnibus (GEO), a public repository at the National Center for Biotechnology Information (NCBI), stores tens of millions of expression profiles, including those obtained from microarray-based experiments measuring mRNA abundance (88). The identification of large-scale networks of molecular interactions within the cell has highlighted the need to go beyond one-dimensional approaches to study gene/protein function (89). One such alternative is GeneMANIA, a fast and highly accurate web-based database and tool for the prediction of gene function on the basis of multiple networks, as determined from varied genomic or proteomic data/sources (90). Coremine Medical is a product of the PubGene Company, which itself grew out of the PubGene online tool, a gene/protein database and web-based tool for literature mining. By the automated analysis of titles and abstracts in over 10 million MEDLINE records, Coremine Medical creates a gene-to-gene co-citation network for millions of named human genes. The identified relationship networks provide both an overview of related terms and an intuitive system for exploring the information space (91). In the present study, the GEO database was used to retrieve data on *RNASET2* and *GGNBP2* expression in drug-sensitive and drug-resistant ovarian cancer cells; GeneMANIA and Coremine Medical were then used to predict the functions of *RNASET2* and *GGNBP2* through both their interaction networks and annotated biological processes.

MicroRNAs (miRNAs) are a class of small (22 bp) endogenous non-coding RNAs that regulate gene expression and thus many cellular processes, both pathological and physiological, by binding to the 3'-UTR of the target mRNA, causing mRNA cleavage, destabilization or translational repression (92-95). This method of post-transcriptional gene regulation has been shown to play a crucial role in drug resistance in many types of cancers, including ovarian cancer (7). However, the specificity of miRNAs for their target genes also makes them of great interest in diagnostics, prognosis and therapy (96) and in predicting gene function. miRWalk is a comprehensive database on miRNA-mRNA interactions that gathers predicted and validated miRNA binding sites on all mRNAs, mitochondrial genes, and the 10-kb upstream flanking regions of all known genes in humans, mice and rats. More importantly, it is a real-time database in which the 'Validated Target' module is updated every month and the 'Predicted Target' module every 6 months (27). As a result, the functional prediction of genes as determined by their miRNAs through miRWalk is both feasible and reliable.

In the present study, *RNASET2* and *GGNBP2* were shown to be notably and consistently downregulated in drug-resistant ovarian cancers and ovarian cancer cells, which suggested the involvement of these TSGs in drug resistance. Support for this hypothesis came from a network analysis based on gene/protein-gene/protein interaction networks and biological processes annotation. The former indicated that *RNASET2* and *GGNBP2* directly interact with many genes/proteins (Fig. 2), all of which are associated with drug resistance in ovarian cancer. Similarly, the annotated biological processes analysis related *RNASET2* and *GGNBP2* mainly to processes closely linked to drug resistance (Fig. 3). In addition, determinations of miRNA-mRNA interaction showed that among the eight miRNAs most strongly targeting *RNASET2* and *GGNBP2* (Table I), the majority were involved in the regulation of drug resistance in ovarian and other cancers, suggesting roles for the two target genes in drug resistance. Further support of an association between *RNASET2* and *GGNBP2* and drug resistance came from a bioinformatics analysis of the drug resistance-related pathways in which their co-expressed genes were involved (Table II).

Currently, there are no data on the genetic pathways related to *RNASET2* and *GGNBP2*, either in the literature or in the KEGG database. Instead, in the present study, examination of the pathways enriched in the expression of genes co-expressed with *RNASET2* and *GGNBP2* in drug-resistant ovarian cancer cells suggested that the downregulation of the former resulted in drug resistance through the activation of the PI3K-Akt signaling pathway, whereas for the latter, cytokine-cytokine receptor interactions, chemokine signaling pathways and NF- κ B signaling were implicated.

Collectively, on the basis of our studies of gene expression at the transcriptional level, protein interactions, annotated biological processes, miRNA-gene interactions and pathway analysis (Fig. 4), we were able to attribute the downregulation of *RNASET2* and *GGNBP2* in drug-resistant ovarian cancers and ovarian tumor cells to drug resistance in ovarian cancer. The present study sets the stage for further experimental investigations into the associations between *RNASET2* and *GGNBP2* and drug resistance.

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