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ABCF2, an Nrf2 target gene, contributes to cisplatin resistance in ovarian cancer cells

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Abstract

Previously, we have demonstrated that NRF2 plays a key role in mediating cisplatin resistance in ovarian cancer. To further explore the mechanism underlying NRF2-dependent cisplatin resistance, we stably overexpressed or knocked down NRF2 in parental and cisplatin-resistant human ovarian cancer cells, respectively. These two pairs of stable cell lines were then subjected to microarray analysis, where we identified 18 putative NRF2 target genes. Among these genes, *ABCF2*, a cytosolic member of the ABC superfamily of transporters, has previously been reported to

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contribute to chemoresistance in clear cell ovarian cancer. A detailed analysis on *ABCF2* revealed a functional antioxidant response element (ARE) in its promoter region, establishing *ABCF2* as an NRF2 target gene. Next, we investigated the contribution of *ABCF2* in NRF2-mediated cisplatin resistance using our stable ovarian cancer cell lines. The NRF2-overexpressing cell line, containing high levels of *ABCF2*, was more resistant to cisplatin-induced apoptosis compared to its control cell line; whereas the NRF2 knockdown cell line with low levels of *ABCF2*, was more sensitive to cisplatin treatment than its control cell line. Furthermore, transient overexpression of *ABCF2* in the parental cells decreased apoptosis and increased cell viability following cisplatin treatment. Conversely, knockdown of *ABCF2* using specific siRNA notably increased apoptosis and decreased cell viability in cisplatin-resistant cells treated with cisplatin. This data indicates that the novel NRF2 target gene, *ABCF2,* plays a critical role in cisplatin resistance in ovarian cancer, and that targeting *ABCF2* may be a new strategy to improve chemotherapeutic efficiency.

Keywords

ABCF2; *NRF2*; Cisplatin; Chemoresistance; Ovarian cancer

INTRODUCTION

Ovarian cancer is the most lethal type of gynecological cancer with a five-year survival rate less than 25% [1,2]. Cytoreductive surgery plus platinum-based chemotherapy is the first line of treatment, but due to chemoresistance, this therapy has limited efficacy [3]. Nuclear factor erythroid 2 (NF-E2)-related factor 2 (NRF2) overexpression has been reported to correlate with chemoresistance in a variety of cancer types [4–6]. Our previous study has shown that high levels of NRF2 also correlate with cisplatin resistance in ovarian cancer cells [7]. However, the major NRF2 target genes contributing to NRF2-dependent cisplatin resistance in ovarian cancer are still not fully characterized.

The ATP-binding cassette (ABC) transporters are a superfamily of proteins that utilize ATP as an energy source to transport various molecules across membranes [8]. The majority of ABC members, has nucleotide-binding domains (NBD) and transmembrane domains (TMD), and function as energy-dependent transporters that efflux cytotoxic drugs out of the cell. The increased efflux activity mediated by ABC transporters has an important impact on pharmacological treatments including chemotherapy [9]. Several ABC transporters have been reported to induce chemoresistance, including: P-glycoprotein (ABCB1), multidrug resistance protein 1-6 (MRP1-6/ABCC1-6), and breast cancer resistance protein (BCRP/ABCG2) [10]. More importantly, functional antioxidant response elements (AREs) have been identified in the promoter region of a subset of ABC transporters including *MRP2*, *MRP3*, *MRP4* and *ABCG2*, which helps define them as NRF2 target genes [11–14]. It is conceivable that these NRF2-target genes contribute to observed chemoresistance through rapid elimination of chemotherapeutic drugs in the cancer types that have high levels of NRF2 [15–17].

A subgroup of the ABC transporter superfamily is the ABCF transporters, which contains three members, ABCF1, ABCF2, and ABCF3 [18,19]. Unlike other subgroups, ABCF

members have NBDs but not TMDs, and thus do not function as transporters of molecules across the membrane. Instead, they are reported to be involved in protein translation and elongation [18,20]. Of the three ABCF subgroup members, ABCF2 is expressed at significantly higher levels in clear cell ovarian cancer than other types, and therefore it may function as a prognostic indicator for clear cell ovarian cancer [21–23]. In addition, *ABCF2* gene amplification was identified in cisplatin-resistant cancer cell lines, indicating a possible role of ABCF2 in modulating cisplatin resistance [24]. However, current studies are limited to correlating ABCF2 expression with clinicopathological features in different tumor tissues, which does not provide direct evidence that ABCF2 contributes significantly to cisplatin resistance. Furthermore, the molecular mechanism by which ABCF2 is overexpressed in clear cell ovarian cancer cells remains unknown.

In this study, we provide strong evidence that *ABCF2* is an NRF2 target gene that contains a functional ARE sequence in the promoter. We have also demonstrated that modulation of ABCF2 levels can alter the sensitivity of ovarian cancer cells to cisplatin. Therefore, our study suggests that targeting ABCF2 could be a promising therapeutic strategy to re-sensitize ovarian cancer cell lines with high levels of NRF2 to cisplatin treatment.

MATERIALS AND METHODS

Ethics statements

This study was approved by the Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University, China, and is in accordance with the 1964 Helsinki declaration, and its later amendments, as well as other comparable ethical standards.

Cells, plasmids and reagents

Human ovarian cancer lines: the parental A2780 and the corresponding cisplatin resistant A2780cp cells were a kind gift from Dr. Anil K. Sood (MD Anderson Cancer Center). HEK293T cells were purchased from American Type Culture Collection (ATCC). All cells were maintained as previously described [25,26]. The flag tagged-NRF2-pCI vector and Renilla luciferase-pGL4.74[hRluc/TK] vector were used as previously reported [26]. Cisplatin was purchased from Hansoh Pharmaceutical Co., Ltd. (Jiangsu, China).

Lentiviral-mediated establishment of stable cell lines

For stable overexpression of NRF2 in A2780 cell lines (A2780-NRF2 & A2780), the pCDH-CMV-MCS-EF1-copGFP vector alone, or containing human NRF2, were utilized. Lentiviral particles packaged with either vector, or the vector with NRF2 were produced by cotransfecting HEK293T cells with the appropriate plasmid and the Mission Lentiviral Packaging Mix (Sigma-Aldrich, MO, USA). Briefly, 1×10^6 HEK293T cells were seeded in a 60mm dish. The next day, cells were cotransfected using Lipofectamine 3000 (Invitrogen, MA, USA), and the packaging mix containing 1.0 μg of pDCE-NRF2 or vector alone, 0.75 μg packaging vector, and 0.25 μg envelop vector. Lentiviral particles were then harvested from the media 48 h after transfection. Next, 1×10^6 A2780 cells were seeded in a 60 mm dish at 70% confluence. On the next day, cells were transduced with newly harvested lentiviral particles containing either the NRF2 plasmid or the control vector plasmid.

Following 24 h of transduction, GFP positive cells were sorted by flow cytometry to obtain successfully transduced cells. Cells were then maintained until the experiments were performed. To obtain stable knockdown of NRF2 in the cisplatin-resistant A2780 cell lines (A2780cp-NRF2-shRNA & A2780cp), pLenR-GPH vector alone or containing human shNRF2 was generated and transduced using the same method describe above. Successfully transduced cells were selected using 3 μg/ml puromycin.

Cell viability and apoptosis

Cell viability was measured using the Cell Counting Kit-8 (Dojin Laboratory, Kumamoto, Japan). Apoptotic cells were detected using either the Annexin V-FITC staining kit (BD Pharmingen, CA, USA) in combination with flow cytometry, or the TUNEL in situ cell death detection kit (TMR red, Roche, IN, USA) [7]. For the TUNEL assay, cell images were randomly captured in 10 different fields for each group. Then all of the cells were counted. The percentage of cells with red staining was determined and represented as an apoptosis ratio.

Immunoblot and immunofluorescence (IF)

Immunoblot and Immunofluorescence analyses were performed as previously described [25]. Antibodies specific for NRF2 (1:1000 for WB, 1:200 for IF, sc-13032), ABCF2 (1:1000 for WB, sc-390496) and β-actin (1:1000, sc-47778) were all obtained from Santa Cruz Biotechnology (TX, USA). The antibody against HA (1:2000) was from Covance (CA, USA). The immunoblot band intensity was analyzed by the Quantity one 4.62 software (Bio-Rad, CA, USA).

Immunohistochemical analysis (IHC)

Paraffin sections of 9 pairs of high-grade serous ovarian cancer (HGSOC) and the contralateral normal ovarian tissues from the same patient were obtained from the tissue bank at the Gynecological and Obstetrics Hospital of Fudan University. Immunohistochemical staining was done according to the manufacturer's instruction (G1210-1, Gugelife, Wuhan, China). The NRF2 and ABCF2 primary antibody was used in a dilution of 1:100; a rabbit immunoglobulin G was used as a negative control. Images were randomly captured taking 10 different fields per group using an Olympus BX53 microscope (Tokyo, Japan). All samples were dual scored by an experienced pathologist based on the sum of the intensity score and the percentage score. Intensity score: 0, negative; 1, weak; 2, moderate; 3, strong; the percentage score is defined as: 1= 0~25%; 2 = 26~50%; 3 = 51~75%; 4 = 76~100%.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time PCR was performed as previously described [7]. The primers used for the measurement of *ABCF2* and β-actin levels were as follows: hABCF2, forward, GGAGCTGGATGCCGACAA, reverse, CTGCATGGCAGGTGTGAAAC; hβ-actin, forward, CCTCGCCTTTGCCGATCC, reverse, CGTGCTCGATGGGGTACTTC. Two independent experiments, each with duplicate samples, were performed.

Microarray analysis

For DNA microarray analyses, 1×10^7 A2780, A2780-NRF2, A2780cp and A2780cp-NRF2-shRNA cells were harvested, and total RNA was extracted and reverse transcribed to cDNA. Then the cDNA was sent out to Kangchen company (Shanghai, China) and analyzed in a whole human genome microarray chip for further analysis (Agilent, CA, US). Each sample was performed in triplicate. The obtained data was analyzed using Gene Ontology for enrichment analysis.

ABCF2 cloning

One putative ARE was identified in the promoter of *ABCF2* using the pDRAW32 1.1.130 software to search for the core ARE sequence (RTGABNNNGCR) [13]. A portion of the human *ABCF2* promoter (−1292 bp to +1069 bp) containing the putative ARE sequence (GTGACTTTGCA) was then amplified by PCR using human genomic DNA as a template and the following primers: forward, GACAGGGTCTAGTTTTGTCAC; reverse, CTGTGGTCTCTCTGCCATTGGC. The amplified fragments were then digested with the BglII and HindIII restriction enzymes, and cloned into the pGL4.22 vector (Promega, WI, USA). Another construct where the region containing the ARE (−893 bp to +1069 bp) was deleted was also amplified from the full-length promoter above using the following primers: forward, GGAGCAATAGCTTCCTTTGCTG; reverse, CTGTGGTCTCTCTGCCATTGGC. Using the wild type promoter of *ABCF2* as the template, the oligo CTCCCTGAGGTCAGGGAGgGcCTTTGCACCATTGCAACTCCAGC was used to perform site-directed mutagenesis of the ARE (GgGcCTTTGCA).

To generate an ABCF2 expression plasmid, the first region of *ABCF2* cDNA was PCR amplified with the following primers: forward, ATGCCCTCCGACCTGGCCAAGAAGA, and reverse, GCTTGGACTTGAGGTGCT. The isolated product was then digested using the restriction enzymes EcoR1 and Not1, and the DNA fragment was cloned into the pCMV-HA vector (Promega, WI, USA). The second region of the ABCF2 cDNA was amplified using the following primers: forward, GGACTTAGATCTCTCACCTTTG; reverse, TCATGGCCTTGGCAGAGATGC. The amplified fragment was again digested using BglII and Not1 and ligated into the plasmid containing the first region of ABCF2 cDNA. Correct cloning of the gene was verified by sequencing.

Dual luciferase assay

The dual luciferase assay was performed according to the manufacturer's instructions (Promega, WI, USA). In brief, 1×10^5 HEK293T cells per well were seeded in a 24-well plate. The next day, 450 ng of NRF2 plasmid DNA and 50 ng of hRluc/TK plasmid were cotransfected using Lipofectamine 3000. Next, 24 h after transfection, media was removed and cells were lysed in passive lysis buffer. Firefly and *Renilla* luciferase values were measured using the dual luciferase assay kit and luminometer (Model TD-20/20, Turner BioSystems, CA, USA). For relative luciferase activity calculation, the Firefly value was normalized to the *Renilla* value.

Small interfering RNA transfection

Human NRF2 siRNA (SI00659737) and nonspecific control siRNA (102728) were purchased from Qiagen (MD, USA). Human ABCF2 specific siRNAs were obtained from RiboBio (Guangzhou, China). The #1, #2 and #3 ABCF2 siRNA sequences were as follows: #1) CCTCTCACTTACCTTTCAT, #2) GGTCGTGAGCGATAAGACA and #3) TGAGCTTCAAGTATACAAA. Human KEAP1 specific siRNA were targeting GGCCUUUGGCAUCAUGAACTT (RiboBio, Guangzhou, China). Hiperfect reagent (Qiagen) was used for siRNA transfections according to the manufacturer's protocol.

Chromatin immunoprecipitation assay (CHIP)

CHIP assay was performed according to the manufacturer's instructions (EZ-CHIP^{TM}, Merck, Germany). In brief, five dishes of nearly 95% confluence A2780cp cells were harvested and cross-linked by 1% formaldehyde in the medium, then incubated at room temperature for 10 min. All cells were washed with cold PBS with protease inhibitor cocktail (Roche) and were suspended in 1 ml SDS lysis buffer containing protease inhibitor cocktail. All samples were sonicated for 27 min and were centrifuged at 15000 *g* for 10 min at 4° C. Solubilized chromatin (100 μL) was diluted with CHIP dilution buffer (900 μL) for CHIP assays. And 100 μL diluted solubilized chromatin was saved for total chromatin input. Chromatin was precleared with protein G-agarose for 60 min at 4° C and then incubated with NRF2 antibody (ab62352, Abcam), or rabbit IgG overnight at 4° C with rotation. Cross-linked immunoprecipitates and total chromatin input were reverse crosslinked and DNA was extracted and purified. Then, 1 μL of DNA was used for PCR with primers specific for the ABCF2 promoter. For PCR amplification, forward primer: 5′-AAATGATCCTCCCACTACGG-3′ and reverse primer: 5′-GAATATCCTCCACCAACGAA-3′ from Gebewiz(Shanghai, China) were used.

Statistical analysis

Data are presented as the mean ± SD. Student's t-test or one way analysis of variance (ANOVA) was used to assess the significance among groups using the SPSS 16.0 software. $P<0.05$ was considered to be a significant difference.

RESULTS

NRF2 levels are highly expressed in high-grade serous ovarian cancer tissue and cisplatin resistant A2780 ovarian cancer cell lines

First, immunohistochemical analysis of human tissues from 9 individual patients revealed that the high-grade serous ovarian cancer (HGSOC) tissues had significantly higher levels of NRF2 compared to their normal ovarian tissue (NOT) controls (Figure 1A). The NRF2 IHC score of the cancer tissues is significantly higher than the normal tissues from the same patient ($P<0.01$, Figure 1B). The specificity of the IHC analysis with the NRF2 antibody was confirmed by the absence of staining in the negative control slides (NC). Then the level of NRF2 in the parental and cisplatin-resistant A2780 cells was determined by immunoblot and immunofluorescent analyses. As shown in Figure 1C, the level of NRF2 in cisplatin-resistant A2780 cells (A2780cp) was higher than that in the parental A2780 cells (right

panel). Immunofluorescent analysis confirmed activation of the NRF2 pathway, as NRF2 is predominately localized in the nucleus in A2780cp cells, whereas it is in the cytosol in A2780 cells (left panel). These results indicate that high expression of NRF2 correlates with chemoresistance in ovarian cancer.

NRF2 expression regulates cisplatin sensitivity in ovarian cancer cells

To test the role of NRF2 expression in regulating cisplatin resistance, we stably overexpressed or knocked down NRF2 in the parental (A2780) and cisplatin-resistant (A2780cp) human ovarian cancer cells, respectively. The two pairs of cell lines generated were A2780 (vector control) and A2780-NRF2 (NRF2 overexpression), as well as A2780cp (vector control) and A2780cp-NRF2-shRNA (NRF2 knockdown). The protein levels of NRF2 and its downstream gene NQO1 were measured using immunoblot analysis to confirm NRF2 overexpression or knockdown (Figure 2A and 2B). Sensitivity of these cell lines to cisplatin treatment was compared using a cell viability assay. Cisplatin treatment decreased cell viability in a dose dependent manner in all four lines, with A2780-NRF2 cells being more resistant to cisplatin than the A2780 vector control cells, and A2780cp-NRF2-shRNA cells being more susceptible to cisplatin toxicity than A2780cp vector control cells (Figure 2C and 2D). The cell viability data revealed that the half maximal effective concentration (EC50) of cisplatin at 48 h in A2780-NRF2 and A2780 was 1.97 ± 0.11 μg/ml and 0.64 ± 0.16 μg/ml, respectively ($P<0.05$, Figure 2C); whereas the EC50 of cisplatin in A2780cp-NRF2-shRNA and A2780cp cells was 9.34 ± 0.53 μg/ml and 13.64 ± 0.23 μg/ml, respectively ($P<0.05$, Figure 2D). TUNEL staining of cells treated with cisplatin for 24 h revealed that the number of apoptotic cells in the A2780-NRF2 cell line was 2.19 ± 0.64% compared to 7.07 ± 2.75% in the A2780 vector control cell line ($P<0.01$, Figure 2E), and the number of apoptotic cells in the A2780cp-NRF2-shRNA cell line was 38.81 ± 33.84% compared to 3.4 ± 0.38% in the A2780cp vector control cell line ($P<0.01$, Figure 2F). These data suggest that NRF2 effectively regulates the sensitivity of ovarian cancer cells to cisplatin.

Identification of putative NRF2 target genes by DNA microarray analysis

For microarray analysis, only genes with a ~1.5 fold change in each pair of cell lines were included in the enrichment analysis. Results showed that 859 genes were upregulated in A2780-NRF2 compared to A2780 vector control cells, whereas 396 genes were downregulated in A2780cp-NRF2-shRNA compared to A2780cp vector control cells (Figure 3A). Among the genes that changed, 18 genes were identified to be potentially regulated by NRF2, including *IL18*, *TRMT61A*, *ABCF2*, *HGD*, *NEFH*, *LEO1*, *PRR22*, *TIMP1*, *N4BP2L2*, *TAGLN*, *ZBTB1*, *NFYA*, *FLG*, *SGPP2*, *CCDC39*, and *GTF2IRD2B* (Figure 3A and 3B). It is worth mentioning that within the 396 NRF2 down-regulated genes, there was an enrichment of genes that function in transporter activities, as well as in regulation of cell death (Figure 3C). Additionally, other well-known NRF2 downstream genes were listed in the heatmap from the single pair of stable cell lines (Figure S1).

ABCF2 has an antioxidant response element (ARE) in its promoter region

One of the putative NRF2 target genes identified in the microarray analysis was *ABCF2*, a member of the ABC transporter family, whose expression is elevated in clear cell ovarian

cancer tissues [21–23]. A putative ARE sequence (GTGACTTTGCA) in the *ABCF2* promoter was identified in the −1095 bp to −1085 bp region upstream of the transcription start site (TSS). To determine if *ABCF2* is a direct target gene of NRF2, a firefly luciferase reporter construct containing the promoter region, exon 1, intron 1, and part of exon 2 of the *ABCF2* gene (−1292 bp to +1069 bp) was cloned (Figure 4A). The *ABCF2*-luciferase and TK-*Renilla*-luciferase plasmids were cotranfected along with either an NRF2 plasmid or an empty control vector into HEK293T cells for 24 h; dual luciferase activities were then measured and relative luciferase activity was calculated. The reporter activity was 2.11 ± 0.07 fold higher in cells cotransfected with NRF2 plasmid than the control group ($P<0.01$, Figure 4A). To verify that this putative ARE in the −1095 bp to −1085 bp is a functional ARE, we generated a truncated reporter construct without the putative ARE (−893 bp to +1069), and a mutated ARE-luciferase construct (GTGACTTTGCA to GgGcCTTTGCA) (Figure 4A). The luciferase activity of either the truncated reporter or the mutated ARE reporter construct was no longer enhanced by cotransfection of NRF2 ($P>0.05$, Figure 4B).

To further confirm that NRF2 binds to the putative ARE of the ABCF2 promoter, a CHIP assay was performed in A2780cp cells. As shown in Figure 4B, compared to the positive control RNA polymerase II, the promoter region of ABCF2 also recruited NRF2. Analysis of the NRF2 binding site reveals that the binding region starts from −1213 bp to −1017 bp, which contains the putative ARE, indicating that the ARE sequence (GTGACTTTGCA) located at −1095 bp to −1085 bp is a functional ARE controlled by NRF2.

ABCF2 is directly regulated by NRF2

To further verify that NRF2 controls endogenous *ABCF2* expression, the effect of both stable and transient NRF2 modulation on *ABCF2* mRNA and protein levels was measured. As shown in Figure 5A, the mRNA level of *ABCF2* was 1.2 fold higher in A2780-NRF2 compared to A2780 vector control cell line, and 0.6 fold lower in A2780cp-NRF2-shRNA compared to A2780cp vector control cell line. The protein level of ABCF2 was also higher in A2780-NRF2 and lower in A2780cp-NRF2-shRNA compared to their vector control cell line.

The protein level of ABCF2 in A2780cp was 12.1 fold higher than in A2780 cells (Figure 5B). Silencing KEAP1 with siRNA in A2780 increased the protein levels of NRF2, as expected (Figure 5C). Moreover, this also caused an increase in ABCF2 protein levels. Consistently, silencing NRF2 with siRNA in A2780cp decreased ABCF2 protein levels. Taken together, these results indicate that ABCF2 expression is controlled by NRF2 (Figure 5D).

ABCF2 plays a key role in cisplatin resistance in ovarian cancer cells

To determine if ABCF2 is a critical downstream mediator of NRF2-dependent cisplatin resistance in ovarian cancer cells, cell viability assays were performed. In A2780 cells, stable overexpression NRF2 decreases sensitivity to cisplatin (Figure 5E, left). However, ABCF2 knockdown with siRNA slightly increased cisplatin sensitivity of A2780-NRF2 cells (Figure 5E, left). Conversely, knockdown of NRF2 with siRNA in A2780cp cells increases cisplatin sensitivity, whereas ABCF2 overexpression in A2780cp-NRF2-shRNA cells

restores cisplatin resistance (Figure 5E, right). Further, the parental A2780 cells were transiently transfected with HA-tagged ABCF2. Overexpression of ABCF2 in A2780, as verified by immunoblot analysis, decreased sensitivity to cisplatin compared to cells transfected with an empty control vector (Figure 6A, 48 h EC_{50}: 6.50 ± 0.09 μg/ml and 6.23 ± 0.14 μg/ml, respectively). Conversely, siRNA knockdown of ABCF2 in the cisplatin-resistant A2780cp cells reduced ABCF2 protein levels and increased cisplatin toxicity (Figure 6B, 48 h EC_{50}: 23.63 ± 2.98 μg/ml and 30.75 ± 2.30 μg/ml, respectively). Next, we determined the percentage of apoptotic cells in response to cisplatin treatment using Annexin V/PI staining and flow cytometry. ABCF2 overexpression in itself has no effect on apoptosis, but reduced apoptosis in A2780 cells treated with cisplatin (6.15 ± 0.21% vs 8.15 ± 0.07%, $P<0.01$, Figure 6C). ABCF2 knockdown using siRNA in A2780cp cells had no effect on apoptosis, but resulted in a higher percentage of apoptotic cells in A2780cp cells treated with cisplatin (44.15 ± 5.53 % vs 23.26 ± 3.78%, $P<0.01$, Figure 6D).

Finally, we analyzed the expression of ABCF2 in patient samples. Immunohistochemical staining showed that the high-grade serous ovarian cancer tissues had significantly higher levels of ABCF2 compared to their normal ovarian tissue controls (Figure 6E). The IHC score of the cancer tissues is also higher than that of the normal tissues ($P<0.01$, Figure 6F).

Taken together, these results suggest that the expression of ABCF2 correlates with cisplatin resistance in ovarian cancer.

DISCUSSION

Cisplatin resistance is a major obstacle for successful treatment of ovarian cancer [1,3]. NRF2 has been reported to contribute to chemoresistance in a variety of different cancer cell types [4–6]. While our previous studies have indicated the involvement of NRF2 in cisplatin resistance in ovarian cancer cells, the major NRF2 target genes contributing to the NRF2-dependent cisplatin resistance in ovarian cancer are still not fully characterized [7].

High-grade serous ovarian cancer (HGSOC) is one of the most malignant types of ovarian cancer, and has been associated with a high potential to develop chemoresistance [27,28]. Immunohistochemical staining of patient-derived samples revealed much higher levels of NRF2 in HGSOC tissues compared to normal tissue controls, indicating a potential correlation of NRF2 levels with chemoresistance in ovarian cancer tissue (Figure 1A and 1B). Next, we analyzed NRF2 expression levels in ovarian cancer A2780 cells and confirmed that cisplatin-resistant A2780cp have higher NRF2 than parental A2780 (Figure 1C). To further understand how NRF2 regulates cisplatin chemoresistance in ovarian cancer cells, we stably overexpressed or knocked down NRF2 in the parental and cisplatin-resistant human ovarian cancer cells, respectively, and compared their response to cisplatin. NRF2 overexpression in A2780 cells significantly decreased cisplatin toxicity, while knockdown of NRF2 in A2780cp cells resulted in an increase in cisplatin-induced cell death. TUNEL analysis also revealed that the percentage of apoptotic cells decreased with NRF2 overexpression and increased with NRF2 knockdown (Figure 2C and 2D). These data confirm that NRF2 contributes to cisplatin resistance in ovarian cancer.

As a transcription factor, NRF2 exerts its biological effects, including chemoresistance, through direct activation of its target genes that contain an antioxidant response element (ARE), or indirect activation through upregulation of its downstream genes [29,30]. For example, NRF2-dependent activation of heme oxygenase 1 (HO-1) is associated with cisplatin resistance in A549 lung cancer cells [31,32]. Furthermore, the NRF2-dependent activation of a number of ATP binding cassette transporters, such as ABCB6, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, and ABCG2, have also been linked to chemoresistance [10]. To determine other NRF2 target genes that enhance chemoresistance, microarray analysis of two pairs of cell lines with different amounts of NRF2 expression was carried out, and 18 putative NRF2-regulated genes were identified (Figure 3). Among these genes, we chose *ABCF2* for further characterization since it has been reported to correlate with chemoresistance in clear cell ovarian cancer [21–23], and identified a functional ARE in the promoter region of *ABCF2*, which defines it as a novel NRF2 target gene (Figures 4 and 5).

ABCF2 possesses nucleotide-binding domains, but has no transmembrane domains, which makes it different from other members of the ATP binding cassette family since it cannot function as a membrane transporter [18,19,24]. Previous studies have shown that ABCF2 expression correlates with cisplatin resistance in human bladder cancer T24 cell lines [24] and clear cell ovarian cancer [22]. ABCF2 was also indicated to be a potential marker for clear cell adenocarcinomas of the ovary and the uterine corpus [23,33]. However, the precise molecular mechanism by which ABCF2 mediates cisplatin resistance is not clear. Our study provides evidence for a direct role of ABCF2 in mediating cisplatin resistance, since *ABCF2* overexpression rendered A2780 cells more resistant to cisplatin and *ABCF2* knockdown rendered resistant A2780 cells more sensitive to cisplatin (Figure 6).

In conclusion, we have identified *ABCF2* as a novel NRF2 target gene. We have also provided proof-of-concept *in vitro* evidence that targeting ABCF2 may be a promising approach to improve the efficacy of cisplatin in treating ovarian cancer. As such, future studies should focus on identifying the molecular mechanisms by which ABCF2 affects the cisplatin response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABCF2	ATP-binding cassette subfamily F member 2
NRF2	Nuclear factor erythroid 2 (NF-E2)-related factor 2

NQO1	NAD(P)H dehydrogenase (quinone) 1
KEAP1	Kelch-like ECH-associated protein 1
ARE	Antioxidant response element
ABC	ATP binding cassette
NBD	Nucleotide binding domain
TMD	Transmembrane domain
TSS	Transcription start site
IF	Immunofluorescence
IHC	Immunohistochemical
qRT-PCR	Quantitative real time polymerase chain reaction
CHIP	Chromatin immunoprecipitation
siRNA	Small interfering RNA
PI	Propidium iodide
FITC	Fluorescein isothiocyanate
HGSOC	High-grade serous ovarian cancer
NOT	Normal ovarian tissue
NC	Negative control
SD	Standard deviation

References

1. Jayson GC, Kohn EC, Kitchener HC, Ledermann JA. Ovarian cancer. Lancet. 2014; 384(9951): 1376–1388. [PubMed: 24767708]
2. Kehoe S, Hook J, Nankivell M, et al. Primary chemotherapy versus primary surgery for newly diagnosed advanced ovarian cancer (CHORUS): an open-label, randomised, controlled, non-inferiority trial. Lancet. 2015; 386(9990):249–257. [PubMed: 26002111]
3. Chan JK, Brady MF, Penson RT, et al. Weekly vs. Every-3-Week Paclitaxel and Carboplatin for Ovarian Cancer. N Engl J Med. 2016; 374(8):738–748. [PubMed: 26933849]
4. Wang XJ, Sun Z, Villeneuve NF, et al. NRF2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of NRF2. Carcinogenesis. 2008; 29(6):1235–1243. [PubMed: 18413364]
5. Jiang T, Chen N, Zhao F, et al. High levels of NRF2 determine chemoresistance in type II endometrial cancer. Cancer Res. 2010; 70(13):5486–5496. [PubMed: 20530669]
6. Hayden A, Douglas J, Sommerlad M, et al. The NRF2 transcription factor contributes to resistance to cisplatin in bladder cancer. Urol Oncol. 2014; 32(6):806–814. [PubMed: 24837013]
7. Bao LJ, Jaramillo MC, Zhang ZB, et al. NRF2 induces cisplatin resistance through activation of autophagy in ovarian carcinoma. Int J Clin Exp Pathol. 2014; 7(4):1502–1513. [PubMed: 24817946]

8. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem. 2002; 71:537–592. [PubMed: 12045106]
9. Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res. 2001; 42(7):1007–1017. [PubMed: 11441126]
10. Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev. 2003; 55(1):3–29. [PubMed: 12535572]
11. Vollrath V, Wielandt AM, Iruretagoyena M, Chianale J. Role of NRF2 in the regulation of the Mrp2 (ABCC2) gene. Biochem J. 2006; 395(3):599–609. [PubMed: 16426233]
12. Xu S, Weerachayaphorn J, Cai SY, Soroka CJ, Boyer JL. Aryl hydrocarbon receptor and NF-E2-related factor 2 are key regulators of human MRP4 expression. Am J Physiol Gastrointest Liver Physiol. 2010; 299(1):G126–G135. [PubMed: 20395535]
13. Singh A, Wu H, Zhang P, Happel C, Ma J, Biswal S. Expression of ABCG2 (BCRP) is regulated by NRF2 in cancer cells that confers side population and chemoresistance phenotype. Mol Cancer Ther. 2010; 9(8):2365–2376. [PubMed: 20682644]
14. Canet MJ, Merrell MD, Harder BG, et al. Identification of a functional antioxidant response element within the eighth intron of the human ABCC3 gene. Drug Metab Dispos. 2015; 43(1):93–99. [PubMed: 25349122]
15. Ji L, Li H, Gao P, et al. NRF2 pathway regulates multidrug-resistance-associated protein 1 in small cell lung cancer. PLoS One. 2013; 8(5):e63404. [PubMed: 23667609]
16. Chen P, Zeng H, Wang Y, et al. Low dose of oleanolic acid protects against lithocholic acid-induced cholestasis in mice: potential involvement of nuclear factor-E2-related factor 2-mediated upregulation of multidrug resistance-associated proteins. Drug Metab Dispos. 2014; 42(5):844–852. [PubMed: 24510383]
17. Wang X, Campos CR, Peart JC, et al. NRF2 upregulates ATP binding cassette transporter expression and activity at the blood-brain and blood-spinal cord barriers. J Neurosci. 2014; 34(25): 8585–8593. [PubMed: 24948812]
18. Kerr ID. Sequence analysis of twin ATP binding cassette proteins involved in translational control, antibiotic resistance, and ribonuclease L inhibition. Biochem Biophys Res Commun. 2004; 315(1): 166–173. [PubMed: 15013441]
19. Liu X, Li S, Peng W, et al. Genome-Wide Identification, Characterization and Phylogenetic Analysis of ATP-Binding Cassette (ABC) Transporter Genes in Common Carp (Cyprinus carpio). PLoS One. 2016; 11(4):e153246.
20. Allikmets R, Gerrard B, Hutchinson A, Dean M. Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the expressed sequence tags database. Hum Mol Genet. 1996; 5(10):1649–1655. [PubMed: 8894702]
21. Tsuda H, Ito YM, Ohashi Y, et al. Identification of overexpression and amplification of ABCF2 in clear cell ovarian adenocarcinomas by cDNA microarray analyses. Clin Cancer Res. 2005; 11(19 Pt 1):6880–6888. [PubMed: 16203778]
22. L'Esperance S, Popa I, Bachvarova M, et al. Gene expression profiling of paired ovarian tumors obtained prior to and following adjuvant chemotherapy: molecular signatures of chemoresistant tumors. Int J Oncol. 2006; 29(1):5–24. [PubMed: 16773180]
23. Nishimura S, Tsuda H, Ito K, et al. Differential expression of ABCF2 protein among different histologic types of epithelial ovarian cancer and in clear cell adenocarcinomas of different organs. Hum Pathol. 2007; 38(1):134–139. [PubMed: 16996567]
24. Yasui K, Mihara S, Zhao C, et al. Alteration in copy numbers of genes as a mechanism for acquired drug resistance. Cancer Res. 2004; 64(4):1403–1410. [PubMed: 14973057]
25. Bao L, Jaramillo MC, Zhang Z, et al. Induction of autophagy contributes to cisplatin resistance in human ovarian cancer cells. Mol Med Rep. 2015; 11(1):91–98. [PubMed: 25322694]
26. Sun Z, Huang Z, Zhang DD. Phosphorylation of NRF2 at multiple sites by MAP kinases has a limited contribution in modulating the NRF2-dependent antioxidant response. PLoS One. 2009; 4(8):e6588. [PubMed: 19668370]
27. Bonome T, Lee JY, Park DC, et al. Expression profiling of serous low malignant potential, low-grade, and high-grade tumors of the ovary. Cancer Res. 2005; 65(22):10602–10612. [PubMed: 16288054]

28. Narod SA, Boyd J. Current understanding of the epidemiology and clinical implications of BRCA1 and BRCA2 mutations for ovarian cancer. Curr Opin Obstet Gynecol. 2002; 14(1):19–26. [PubMed: 11801872]

29. O'Connell MA, Hayes JD. The Keap1/NRF2 pathway in health and disease: from the bench to the clinic. Biochem Soc Trans. 2015; 43(4):687–689. [PubMed: 26551713]

30. Menegon S, Columbano A, Giordano S. The Dual Roles of NRF2 in Cancer. Trends Mol Med. 2016; 22(7):578–593. [PubMed: 27263465]

31. Furfaro AL, Traverso N, Domenicotti C, et al. The NRF2/HO-1 Axis in Cancer Cell Growth and Chemoresistance. Oxid Med Cell Longev. 2016; 2016:1958174. [PubMed: 26697129]

32. Kweon MH, Adhami VM, Lee JS, Mukhtar H. Constitutive overexpression of NRF2-dependent heme oxygenase-1 in A549 cells contributes to resistance to apoptosis induced by epigallocatechin 3-gallate. J Biol Chem. 2006; 281(44):33761–33772. [PubMed: 16950787]

33. Nishimura S, Tsuda H, Miyagi Y, et al. Can ABCF2 protein expression predict the prognosis of uterine cancer? Br J Cancer. 2008; 99(10):1651–1655. [PubMed: 19002184]

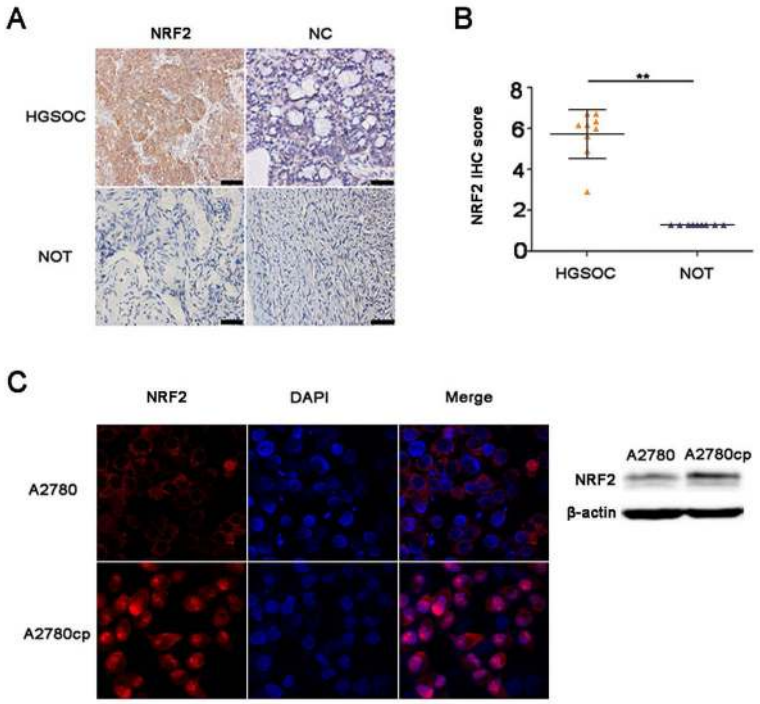

Figure 1.

NRF2 expression in high-grade serous ovarian cancer tissue and A2780 ovarian cancer cells. (A) Representative IHC images of NRF2 levels in 9 pairs of HGSOC and corresponding normal ovarian tissue samples (magnification, 400x). The scale bars represent 50 μm. Negative control (NC): rabbit immunoglobulin G. NOT: normal ovarian tissues. (B) NRF2 IHC score in HGSOC and corresponding NOT samples. ***P*<0.01. (C) IF staining of NRF2 was performed in A2780 and A2780cp cell lines. The red signal represents NRF2 (magnification, 400x) (left panel). Total cell lysates were collected for immunoblot analysis of NRF2 (right panel).

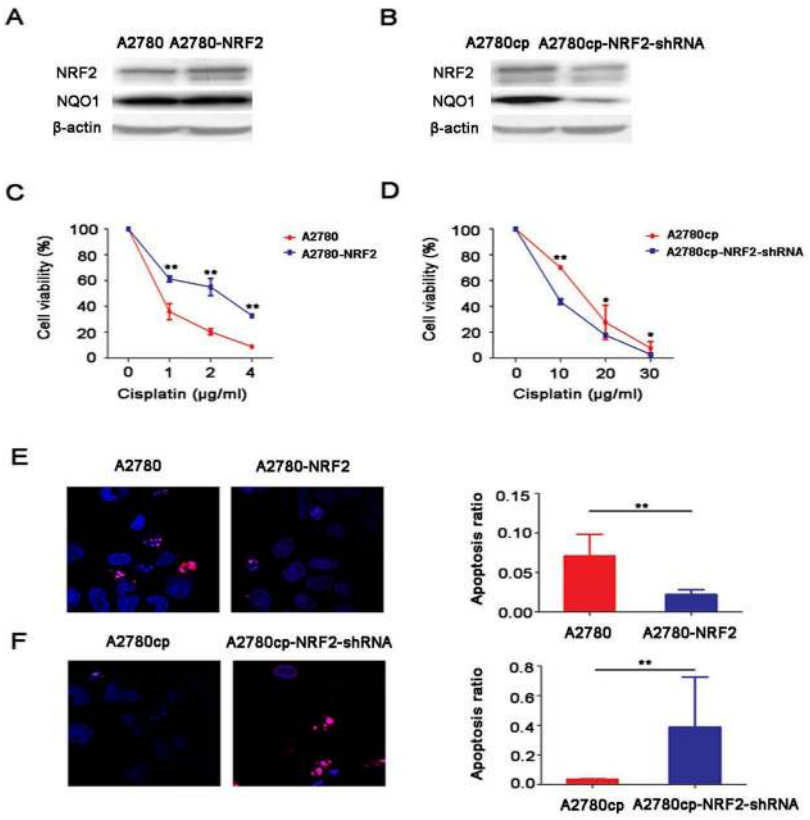

Figure 2.

Establishment of NRF2 overexpression and knockdown stable cell lines. (A and B) A2780 cells were transfected with NRF2 plasmid or NRF2 shRNA by lentiviral packaging system to generate the stable cell lines. NRF2 and NQO1 protein levels were detected in A2780, A2780-NRF2, A2780cp, and A2780cp-NRF2-shRNA by immunoblot analysis. (C and D) Cell viability assay. A2780-NRF2 and A2780 cells (C) and A2780cp-NRF2-shRNA and A2780cp cells (D) were treated with the indicated doses of cisplatin for 48 h to determine cell viability. (E and F) Analysis of apoptosis. A2780-NRF2 and A2780 cells were treated with 1 μg/ml cisplatin (E) and A2780cp-NRF2-shRNA and A2780cp cells were treated with 10 μg/ml cisplatin (F) for 24 h to determine apoptosis by TUNEL assay. All data are presented as mean ± SD from three independent experiments, n=3. **P*<0.05. ***P*<0.01.

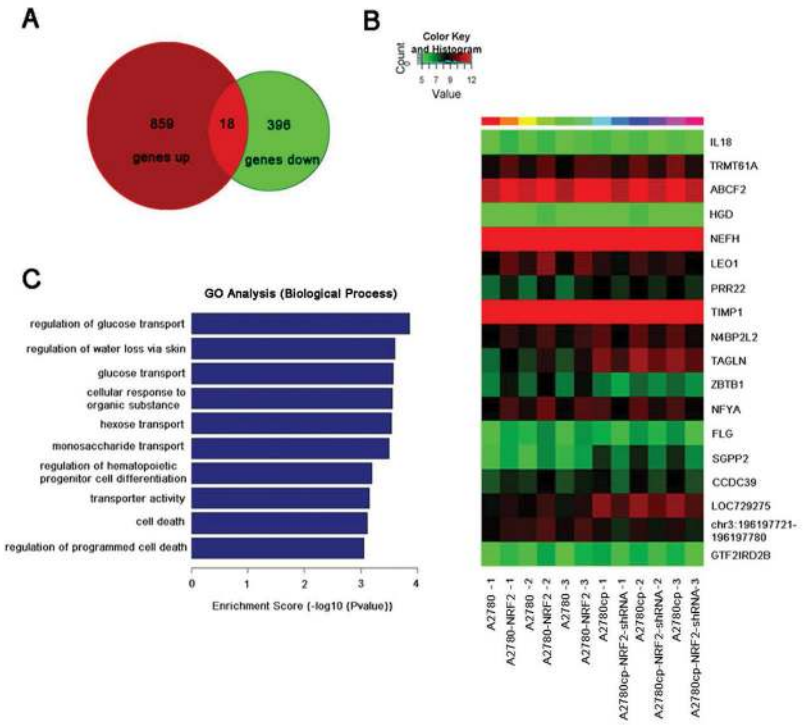

Figure 3.

Microarray analysis of NRF2 stable cell lines. (A) Venn diagrams of upregulated and downregulated genes in A2780-NRF2 vs. A2780 and A2780cp-NRF2-shRNA vs. A2780cp cells. (B) Heat map of the 18 genes that significantly changed in both sets of stable cell lines. (C) Gene Ontology enrichment analysis of the biological process of the downregulated genes from the knockdown stable cell lines. Each cell sample was done in triplicate.

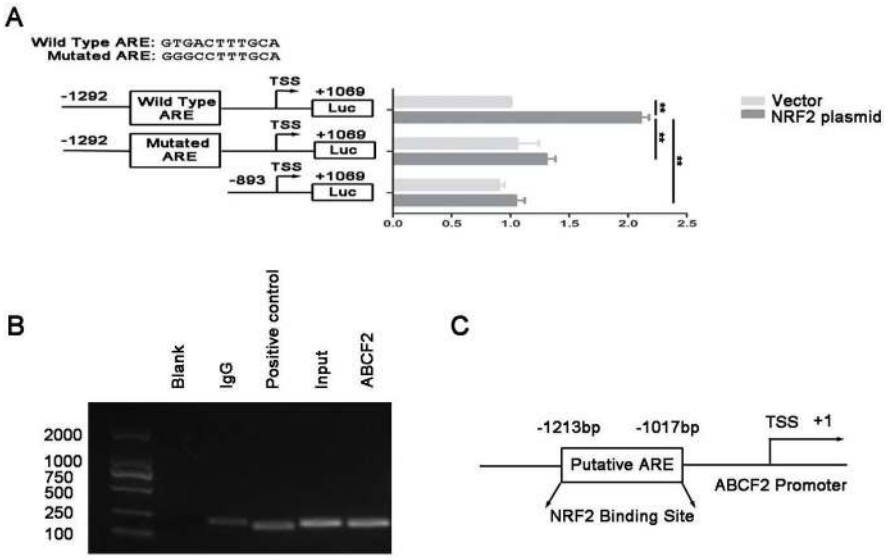

Figure 4.

ARE identification in the promoter of ABCF2. (A) Schematic representation of the luciferase constructs generated and their relative luciferase activities. (1) Wild-type ARE-luciferase plasmid: DNA fragment containing a putative ARE (−1292 bp to +1069 bp). (2) Mutated ARE-luciferase plasmid: two nucleotides in the wild-type ARE-luciferase plasmid were mutated (GTGACTTTGCA to GgGcCTTTGCA). (3) No ARE-luciferase plasmid: DNA fragment without the putative ARE (−893 bp to +1069 bp). These constructs were cotransfected with either a NRF2 plasmid or a control vector into HEK293T cells for 24 h, and dual luciferase assay was performed. Samples were prepared in triplicate. Data are represented as mean ± SD. $^{**}P<0.01$. (B) CHIP assay was performed in A2780cp cells. Rabbit immunoglobulin G as negative control. RNA polymerase II as positive control. (C) Schematic representation of the NRF2 binding site which starts from −1213 bp to −1017 bp in ABCF2 promoter region.

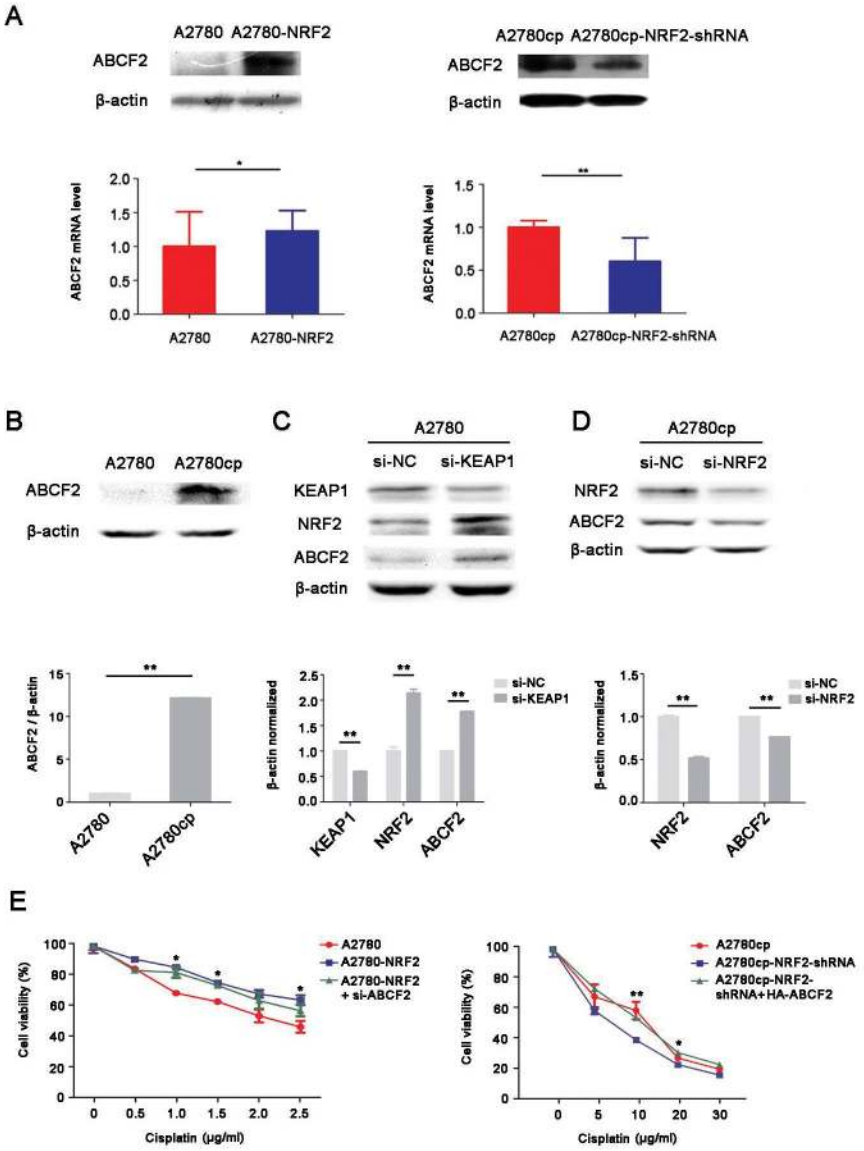

Figure 5.

NRF2 regulates ABCF2 expression. (A) ABCF2 mRNA and protein levels in A2780-NRF2 and A2780 cells, A2780cp-NRF2-shRNA and A2780cp cells. (B) The protein level of ABCF2 in A2780 and A2780cp cells. (C) A2780 cells were transfected with 5 nM KEAP1 siRNA or nonspecific control siRNA for 48 h. (D) A2780cp cells were transfected with 5 nM NRF2 siRNA, or nonspecific control siRNA for 48 h. Cell lysates were subjected to immunoblot analysis. Band intensity was analyzed by densitometry and values were normalized to β-actin. (E) Cell viability assay. A2780, A2780-NRF2 and A2780-NRF2 cells transfected with ABCF2 siRNA for 48 h, A2780cp, A2780cp-NRF2-shRNA and A2780cp-NRF2-shRNA transfected with HA-ABCF2 for 24 h were treated with doses of cisplatin for 48 h to determine cell viability. Data are represented as mean ± SD, from two independent experiments, n = 3. **P*<0.05. ***P*<0.01.

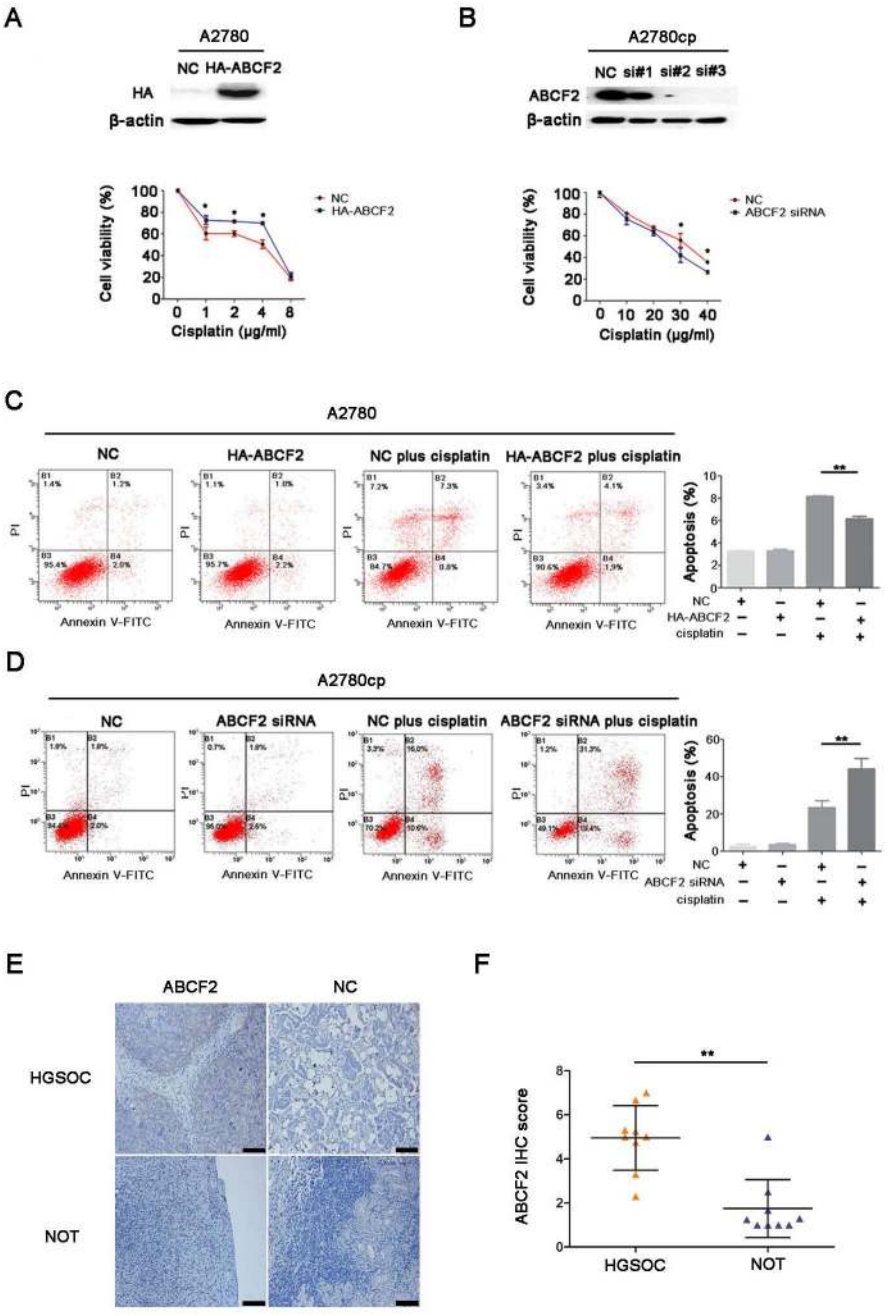

Figure 6.

Role of ABCF2 in cisplatin resistance of ovarian cancer cells. (A and B) Cell viability assay. A2780 cells were transfected with a HA-ABCF2 plasmid or an empty vector for 24 h (A), and A2780cp cells were transfected with ABCF2 siRNA or nonspecific siRNA for 48 h (B), then treated with the indicated doses of cisplatin for 48 h to determine cell viability. Data are represented as mean ± SD from three independent experiments, n = 3. *P<0.05. (C and D) Analysis of apoptosis. A2780 cells were transfected with HA-ABCF2 or control vector, and either left untreated or treated with cisplatin (1 μg/ml) for 24 h (C) and A2780cp cells were transfected with #3 ABCF2 siRNA or nonspecific control siRNA, and either left untreated or

treated with (30 μg/ml) for 24 h (D). Apoptosis was determined with Annexin V-PI and flow cytometry. $^{**}P<0.01$. (E) Representative IHC images of ABCF2 levels in HGSOC and corresponding normal ovarian tissue samples (magnification, 400x). The scale bars represent 50 μm. (F) ABCF2 IHC score in HGSOC and corresponding normal ovarian tissue samples. $^{**}P<0.01$.