Identification of Overexpression and Amplification of *ABCF2* in Clear Cell Ovarian Adenocarcinomas by cDNA Microarray Analyses

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Abstract

Purpose: Patients with ovarian clear cell adenocarcinoma generally have a poor response to combination chemotherapy and have overall poorer prognosis than patients with other histologic types of ovarian cancer. Genetic changes in this group of cancer have not been thoroughly explored. Identification of these changes may provide us new therapeutic targets to treat this disease.

Experimental Design: Genomic and expression array analyses were applied on 30 clear cell ovarian cancer cases and 19 serous cases using a 10,816-element cDNA microarray platform. Further validation and clinical correlation studies were done on differentially expressed genes that are related to chemoresistance.

Results: Based on array analyses, 12 genes showed a significant increase in DNA and mRNA copy number and 5 genes showed a significant decrease in DNA and RNA copy number in clear cell tumors compared with those in the serous type. One of the genes was *ABCF2*, which belongs to the ATP-binding cassette gene superfamily and has been shown to amplify in other tumor types. Validation studies were done using real-time quantitative PCR and immunohistochemistry. The results showed significantly higher *ABCF2* DNA and mRNA copy number and protein levels in clear cell cases compared with those in serous cases. Furthermore, in 20 clear cell cases with chemoresponse data available, ABCF2 cytoplasmic staining was significantly higher in nonresponders than that in the responders (60.0% versus 28.5%; $P = 0.0002$).

Conclusions: These data suggest that ABCF2 protein may be a prognostic marker for ovarian clear cell ovarian adenocarcinoma.

Ovarian cancer is the fifth most common form of cancer in women in the United States, accounting for 4% of the total number of cancer cases and 25% of those cases occur in the female genital tract. It was estimated that 14,300 deaths would be caused by ovarian cancer in the year 2005 (1). Ovarian cancer can be subdivided into four major histologic types. Among them, clear cell ovarian cancer, which constitutes 5% to 10% of ovarian cancer cases, differs from the other histologic types with respect to its clinical characteristics (2, 3). This type of tumor is thought to sometimes arise from endometriosis and many of the patients present the disease at early stages (2, 3). Clear cell type is usually more resistant to systemic chemotherapy than other types and has a worse prognosis (4, 5). In fact, in current clinical practice, patients with clear cell type ovarian cancer are treated as those with high-grade neoplasms (6). The molecular pathobiology of clear cell type ovarian cancer remains largely unknown.

Recent studies showed that 25% to 75% of clear cell type ovarian cancer showed increased DNA copy numbers on 8q11-q13, 8q21-q22, 8q23, 8q24-qter, 17q25-qter, and 20q13-qter and decreased copy number on 19p by chromosome comparative genomic hybridization (CGH; ref. 7). However, changes in the DNA copy number on the gene level have not been identified. Using oligonucleotide array expression profiling, Schwartz et al. reported that clear cell type ovarian cancer has a molecular signature that distinguishes it from other histologic types. A total of 73 genes, with expression levels 2- to 29-fold higher in clear cell type than in other histologic types, were identified (8). However, in this report, only eight clear cell specimens were included and DNA copy number changes were not analyzed. The cDNA array platform for CGH analysis is a powerful tool to identify DNA copy number changes on the gene-by-gene basis (9, 10). In addition, we can use the same

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platform to analyze RNA levels simultaneously. Previously, we have successfully adopted this method to identify changes in both DNA and RNA levels from microdissected tumor specimens (11). In this study, we reported the use of the same approach to identify differential DNA copy number abnormalities (CNA) and expression patterns in clear cell ovarian cancer compared with those identified in the serous type. We further validated the expression of one of the genes called *ABCF2* and correlated its expression with clinical outcomes in patients with clear cell ovarian cancers.

Materials and Methods

Clinical samples. A total of 27 clear cell ovarian adenocarcinomas and 19 serous ovarian adenocarcinomas were included in this study. All patient-derived specimens were collected and archived under protocols approved by the institutional review boards of the parent institutions. Clinical data of these samples are shown in Table 1. Normal female DNA (Promega, Madison, WI) was used as the reference for CGH analysis, and RNAs isolated from a pool of 10 normal ovarian epithelial cell (human ovarian surface epithelium) cultures were used as the reference for expression profiling.

Table 1. Clinical features of ovarian cancer cases

Case no.	Histology	Age (y)	Clinical stage	Histologic grade
BWH-451	Clear cell adenocarcinoma	54	IV	1
BWH-539	Clear cell adenocarcinoma	57	Ic	3
BWH-671	Clear cell adenocarcinoma	37	IIc	2
BWH-725	Clear cell adenocarcinoma	62	Ia	1
BWH-805	Clear cell adenocarcinoma	67	III	3
BWH-810	Clear cell adenocarcinoma	53	IIIc	1
BWH-855	Clear cell adenocarcinoma	51	IIIc	3
BWH-857	Clear cell adenocarcinoma	39	IIIc	2
BWH-1048	Clear cell adenocarcinoma	40	IIIc	2
BWH-1288	Clear cell adenocarcinoma	67	Ic	3
BWH-1411	Clear cell adenocarcinoma	78	IIIc	3
BWH-1484	Clear cell adenocarcinoma	51	Ib	3
BWH-1640	Clear cell adenocarcinoma	49	IIIc	3
BWH-1638	Clear cell adenocarcinoma	50	III	1
OCGH-63	Clear cell adenocarcinoma	55	Ic	3
OCGH-65	Clear cell adenocarcinoma	57	Ic	2
OCGH-74	Clear cell adenocarcinoma	55	Ia	2
OCGH-92	Clear cell adenocarcinoma	39	IIb	1
OCGH-98	Clear cell adenocarcinoma	55	IIIc	1
OCGH-108	Clear cell adenocarcinoma	60	Ic	3
OCGH-110	Clear cell adenocarcinoma	52	Ic	1
OCGH-113	Clear cell adenocarcinoma	62	IIc	2
OCGH-120	Clear cell adenocarcinoma	54	IIIc	2
OCGH-122	Clear cell adenocarcinoma	56	Ic	1
OCGH-123	Clear cell adenocarcinoma	59	Ia	3
OCGH-126	Clear cell adenocarcinoma	54	Ic	3
BWH403	Serous cystadenocarcinoma	67	Ia	1
BWH437	Serous cystadenocarcinoma	42	Ia	3
BWH662	Serous cystadenocarcinoma	59	III	3
BWH800	Serous cystadenocarcinoma	59	IIIc	3
BWH 1231	Serous cystadenocarcinoma	56	III	3
BWH715	Serous cystadenocarcinoma	61	IIIc	3
BWH879	Serous cystadenocarcinoma	46	III	3
BWH489	Serous cystadenocarcinoma	73	IIIb	1
BWH765	Serous cystadenocarcinoma	73	III	3
BWH333	Serous cystadenocarcinoma	59	Ic	1
BWH398	Serous cystadenocarcinoma	52	Ic	3
BWH416	Serous cystadenocarcinoma	42	Ia	1
BWH484	Serous cystadenocarcinoma	54	Ib	3
BWH565	Serous cystadenocarcinoma	68	Ic	1
BWH627	Serous cystadenocarcinoma	58	Ia	3
BWH689	Serous cystadenocarcinoma	43	Ic	3
BWH808	Serous cystadenocarcinoma	57	Ia	3
OCGH-130	Serous cystadenocarcinoma	55	II	3

Microdissection and RNA and DNA extraction. Microdissection was done as described previously (11). Approximately 15,000 tumor cells were used in each case. For DNA extraction, dissected cells were collected into 50 μL cell lysis buffer [1× expand high-fidelity buffer (Boehringer Mannheim, Mannheim, Germany) containing 4 mg/mL proteinase K] and incubated for 72 hours at 55°C. The proteinase K was inactivated by heating at 95°C for 10 minutes before PCR. Total RNA extraction was done using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA).

DNA and RNA amplification. DNA amplification was done using the DOP-PCR master kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. PCR was done in a ABI 9600 thermocycler (Applied Biosystems, Foster City, CA). RNA was amplified using a modified single-round T7 RNA amplification protocol. In brief, total RNA (600 ng) was first incubated with 1 μL T7 primer (5′-GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGA-GATTTTTTTTTTTTTTTTTTVN-3′, 200 ng/μL) in a total volume of 50 μL for 3 minutes at 70°C. First-strand cDNA synthesis was then done by incubating 5 μL primer annealed sample and 5 μL first-strand master mix containing 2 μL of 5× first-strand buffer, 1 μL of 0.1 mol/L DTT, 0.5 μL DEPC water, 0.5 μL of 10 mmol/L deoxynucleotide triphosphate mix, 0.5 μL RNase inhibitor, and 0.5 μL Moloney murine leukemia virus (200 units/μL) for 1 hour and 15 minutes at 37°C. Subsequently, second-strand cDNA synthesis was done by incubating the10 μL first-strand reaction with 65 μL second master mix, which contained 46 μL DEPC water, 15 μL of 5× second-strand buffer, 1.5 μL of 10 mmol/L deoxynucleotide triphosphate mix, 0.5 μL *Escherichia coli* DNA ligase (10 units/μL), 1.5 μL *E. coli* DNA polymerase I (10 units/μL), and 0.5 μL *E. coli* RNase H (2 units/μL) for 2 hours at 16°C and then for 15 minutes at 70°C. The entire 75 μL cDNA sample was loaded onto a ChromaSpin TE-200 spin column (BD Biosciences, San Diego, CA), which was centrifuged for 5 minutes at 2,900 rpm (700 × *g*) in an Eppendorf centrifuge. Purified cDNA collected was lyophilized, dissolved in 8 μL RNase-free water, and incubated at 70°C for 10 minutes. *In vitro* transcription was subsequently done by incubating the 8 μL postlyophilization cDNA product with 12.2 μL master mix containing 2 μL of 10× T7 reaction buffer, 6 μL of 25 mmol/L rNTP mix, 2 mL of 100 mmol/L DTT, 0.2 μL RNase inhibitor (40 units/mL), and 2 μL T7 RNA polymerase for 3 hours at 37°C. The amplified RNA was purified on a RNeasy Mini column (Qiagen) according to the manufacturer's protocol. The purified amplified RNA was quantified by the RiboGreen RNA Quantitation Reagent (Molecular Probes, Eugene, OR).

Microarray analyses. Tumor DNAs (1.5 μg) and normal female control DNAs were labeled with fluorescein-12-dCTP and biotin-11-dCTP, respectively, using the Random Primers DNA Labeling System (Invitrogen, Carlsbad, CA) as described (11). Amplified RNA (1 μg) prepared from normal human ovarian surface epithelium cells and microdissected tumor tissue specimens were labeled with the MicroMax TSA labeling and detection system (Perkin-Elmer, Boston, MA) as described (12). The relative fluorescent level or fluorescent ratio, which represents the relative amount of target sequences in the probe mix, is analyzed by comparing the fluorescent intensity of corresponding individual spots after local background subtraction and normalization. The average local background and SD over all the array spots were also calculated. A 1.4-fold cutoff for amplification and a 0.7-fold cutoff for deletion, which could achieve a 95% specificity in identifying differential DNA CNAs, were used as described previously (11).

Real-time quantitative PCR. Both DNA and mRNA copy numbers were validated using TaqMan real-time PCR amplification with TaqMan Universal PCR Master Mix or TaqMan One-Step RT-PCR Master Mix and an ABI Prism 7000 Sequence Detection System (Applied Biosystems). All results were normalized to the amount of β-actin DNA or rRNA (Applied Biosystems). Primers and probes used were as follows: β-actin, probe: 6FAM-CTACGAGCTGCCTGACGGCCAGG-TAMRA, primers: forward 5′-GATGGCCACGGCTGCTT and reverse 5′-ACCGCTCATTGC-CAATGG. TaqMan rRNA control reagents (Applied Biosystems) were used as an internal control for DNA validation. Primers and probes used for each analysis were as follows: ABCF2, probe: 6FAM-CCTCG-CGGATCTTGCATGGACTG-TAMRA, primers: forward 5′-GGAGCTG-GATGCCGACAA-3′ and reverse 5′-CTGCATGGCAGGTGTGAAAC-3′. For DNA, amplification was done using 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. For RNA, amplification was done using 48°C for 30 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. DNA and mRNA quantification was assessed by the fluorescence intensity emitted after PCR reaction. The difference in the fluorescence between tubes with both the internal control amplification and the test amplification was compared for tumor and normal control samples.

Establishment of a polyclonal anti-ABCF2 antibody. The polyclonal anti-ABCF2 antibody was generated by injecting the purified full-length ABCF2 fusion protein into two rabbits. The specificity of the antibody was determined by Western blot and immunohistochemical analyses on human embryonic kidney cells (293T) transfected with an expression vector pcDNA3.1 or with the vector containing either a full-length *ABCF2* coding sequence or a full-length *ABCF2* coding sequence with a Myc and a His tag. In brief, the open reading frame encoding the human *ABCF2* gene was amplified from the pET28a(+) containing *ABCF2* using the forward primer 5′-AATAGGATCCAC-CATGCCCTCCGACCTGGC-3′ and the reverse primer 5′-AATAACTAGT-CACGTTGTGGGTCCTCTTGG-3′. The PCR product was ligated in frame into the *Bam*HI and *Spe*I sites of the mammalian expression vector pcDNA3.1/Myc-His A (Invitrogen), which encodes a COOH-terminal Myc epitope and six His polypeptides. To exclude the His tag, the reverse primer 5′-AATAACTAGTTCACACGTTGTGGGTCCTCTTG-3′, including the stop codon in front of *Spe*I sites, was designed. The pcDNA3.1 vector alone or vectors containing the *ABCF2* sequence and the Myc-His-tagged *ABCF2* sequence, pcDNA3.1/ABCF2 and pcDNA3.1/ABCF2mH, respectively, were then transiently transfected in human embryonic kidney cells (293T) grown in DMEM supplemented with 10% FCS, 1 unit/mL penicillin, and 1 μg/mL streptomycin using LipofectAMINE (Invitrogen). After 3 days, cells were lysed according to the method of Laemmli (13). SDS-PAGE was done with a 12.5% polyacrylamide gel. Electrophoresed proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with PBS containing 5% bovine serum albumin and 0.1% NaN_3, the membrane was reacted with an anti-human ABCF2 polyclonal antibody or a mouse anti-Myc antibody (MBL, Nagoya, Japan) for 1 hour at room temperature. Peroxidase-conjugated anti-rabbit IgG or peroxidase-conjugated anti-mouse IgG (MBL) was then added and incubated for 1 hour at room temperature. Finally, the enzyme activity was detected using the enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

For immunolocalization of ABCF2 protein *in vitro*, cells transfected with different constructs as described above in air-dried cytocentrifuge preparations were fixed in 4% paraformaldehyde at room temperature for 15 minutes. After pretreatment with 0.2% Triton X-100, cells were incubated with a rabbit anti-ABCF2 polyclonal antibody or normal rabbit serum at room temperature for 30 minutes. Cells were washed with PBS and incubated with biotin-labeled anti-rabbit IgG (American Gualex, San Clemente, CA) at room temperature for 30 minutes. After washing with PBS, cells were incubated with phycoerythrin-conjugated streptavidin (Beckman Coulter, Fullerton, CA) at room temperature for 30 minutes. Cells were then washed with PBS and water and were mounted in ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen). In addition, cells were also incubated with a mouse anti-Myc tag monoclonal antibody (MBL) or normal mouse IgG1 at room temperature for 30 minutes. Cells were washed with PBS and incubated with phycoerythrin-conjugated anti-mouse IgG (Beckman Coulter) at room temperature for 30 minutes. Cells were then washed with PBS and water and were mounted in

ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole. Cells were examined under a Leica DMIRE2 fluorescent microscope.

Immunohistochemistry. Immunolocalization of the ABCF2 protein was done using a polyclonal anti-ABCF2 antibody generated by injecting the purified full-length ABCF2 fusion protein into the rabbits as described above. In brief, histologic sections (4 μm) were affixed to glass slides, dewaxed, and rehydrated. The sections were then incubated in 3% hydrogen peroxide for 10 minutes at room temperature to quench endogenous peroxidase activity. The sections were reacted with the ABCF2 antibody at 4°C overnight. The peroxidase activity for all

Table 2.

Gene name	Gene symbol	Map	Accession no.	CGH analysis ratio	Expression analysis *t* (*P*)
A. Amplified and overexpressed genes					
Clear cell adenocarcinoma					
Neurogranin (protein kinase C substrate, RC3)	*NRGN*	11q24	NM_006176	3.03	2.78 (0.004)
ADP-ribosyltransferase 3	*ART3*	4p15.1-p14	NM_001179	2.68	3.37 (0.01)
ATP-binding cassette, subfamily F (GCN20)	*ABCF2*	7q35-q36	NM_007189	2.58	2.21 (0.02)
Zinc finger protein 207	*ZNF207*	17q11.2	NM_003457	2.27	3.55 (<0.001)
Cytochrome P450, subfamily VII, subfamily	*CYP7B1*	8q21.3	NM_004820	1.65	2.46 (0.001)
KIAA1140 protein	*KIAA1140*	2p21	AK_046740	1.59	2.15 (0.002)
Leucine zipper – like transcriptional regulator 1	*LZTR1*	22q11.21	NM_006767	1.48	4.94 (<0.001)
Hypoxia-inducible protein 2	*HIG2*	7q32.2	NM_013332	1.48	2.11 (0.021)
Kinesin family member 5A	*KIF5A*	12q13	NM_004984	1.47	2.88 (0.004)
ATPase, Na^+/K^+ transporting, β3 polypeptide	*ATP1B3*	3q22-q23	NM_001679	1.43	2.18 (0.018)
Transcription factor 21	*TCF21*	6q23	NM_198392	1.42	2.62 (0.007)
KIAA0955 protein	*KIAA0955*	19q13.33	AB_023172	1.41	2.12 (0.002)
Serous cystadenocarcinomas					
Interleukin-15 receptor, α	*IL15RA*	10p15-p14	NM_002189	2.20	−2.23 (0.017)
Hepatocyte nuclear factor 3, α	*HNF3A*	14q12-q13	NM_004496	1.93	−3.06 (0.002)
Small proline-rich protein SPRK	*SPRK*	1q21	AW_135226	1.49	−2.50 (0.009)
Cysteine-rich protein 1 (intestinal)	*CRIP1*	7q11.23	NM_001311	1.44	−2.93 (0.003)
Podocalyxin-like	*PODXL*	7q32-q33	NM_005397	1.42	−2.95 (0.003)
GCIP-interacting protein p29	*p29*	1p36.13-p35.1	NM_015484	1.42	−2.61 (0.007)
Neuron-specific protein	*D4S234E*	4p16.3	NM_014392	1.41	−2.21 (0.017)
DKFZP564D177 protein	*NIPSNAP3A*	9q31	NM_015469	1.41	−2.71 (0.005)
B. Deleted and underexpressed genes					
Clear cell adenocarcinoma					
Kallikrein 6 (neurosin, zyme)	*KLK6*	19q13.3	NM_0002774	0.63	−3.22 (0.002)
KIAA0575 gene product	*GREB1*	2p25.1	NM_148903	0.69	−2.90 (0.003)
ESTs	—	12q24.1	—	0.69	−3.72 (<0.001)
Prepronociceptin	*PNOC*	8q21	NM_006228	0.71	−3.87 (<0.001)
ESTs	—	19q13.4	—	0.71	−3.30 (0.001)
Serous cystadenocarcinomas					
Fibrinogen, A α polypeptide	*FGA*	4q28	NM_000508	0.09	2.28 (0.015)
Meningioma expressed antigen 6	*MGEA6*	14q13.3	NM_203356	0.12	4.07 (<0.001)
Aldo-keto reductase family 1, member C4	*AKR1C4*	10p15-p14	NM_001818	0.12	3.97 (<0.001)
Ligase III, DNA, ATP dependent	*LIG3*	17q11.2-q12	NM_013975	0.13	3.66 (0.001)
Methyl-CpG-binding domain protein 1	*MBD1*	18q21	NM_015846	0.18	2.71 (0.005)
Retinol-binding protein 4, plasma	*RBP4*	10q23-q24	NM_006744	0.20	3.28 (0.001)
Insulin-like growth factor 1 receptor	*IGF1R*	15q26.3	NM_000875	0.22	2.62 (0.007)
Insulin-degrading enzyme	*IDE*	10q23-q25	NM_004969	0.24	2.21 (0.017)
Glypican 3	*GPC3*	Xq26.1	NM_004484	0.26	3.01 (0.003)
Spectrin, β, nonerythrocytic 1	*SPTBN1*	2p21	NM_003128	0.28	322 (0.001)
LIM and senescent cell antigen-like domains 1	*LIMS1*	2q12.3	NM_004987	0.29	2.22 (0.017)
Mutated in colorectal cancers	*MCC*	5q21-q22	NM_002387	0.29	2.11 (0.022)
Carboxypeptidase M	*CPM*	12q15	NM_001874	0.29	3.16 (0.002)
Hexosaminidase A (α polypeptide)	*HEXA*	15q23-q24	NM_000520	0.29	2.40 (0.01)
Homo sapiens mRNA; cDNA DKFZp434K152	*DKFZp434K152*	1p34.2	AL_157461	0.30	2.42 (0.01)

NOTE: $t = [\log_2(\text{clear cell}) - \log_2(\text{serous})] / \text{SE}[\log_2(\text{clear cell}) - \log_2(\text{serous})]$.

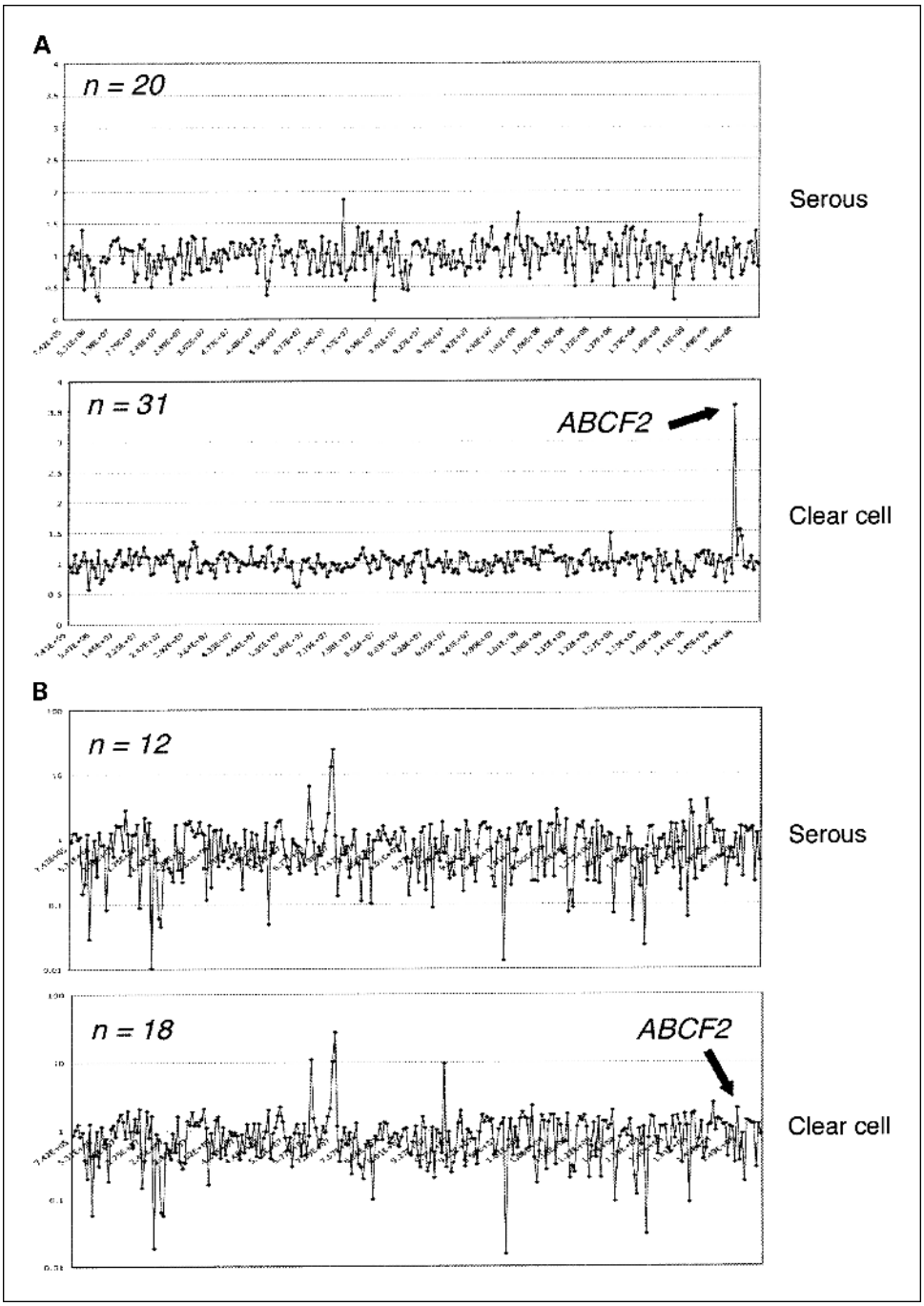


Fig. 1. *A,* array CGH profile of chromosome 7 in serous cystadenocarcinoma and in clear cell adenocarcinoma. *B,* expression profile of genes located chromosome 7 in serous cystadenocarcinoma and in clear cell adenocarcinoma.

proteins was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxide for 2 to 10 minutes at room temperature. The sections were then counterstained with hematoxylin. The slides were read by two independent pathologists, who were blinded to the clinical background of the patients. Positive cells were counted for ABCF2 protein in nuclear or cytoplasm by examining at least 1,000 tumor cells. Levels of ABCF2 were scored based on the percentage of cells with positive nuclear or cytoplasmic staining.

Evaluation of ABCF2 expression and chemotherapy response in patients with clear cell adenocarcinoma. Chemorespond data from a total of 20 patients with clear cell ovarian cancer were available. Among them, 17 cases had at least one measurable tumor lesion documented radiographically after primary surgery. The remaining three cases were recurrent cases who had at least one measurable tumor lesion documented radiographically. Tumor response was evaluated according to WHO criteria. The response was assessed from the product of the two largest perpendicular diameters using the following criteria: complete response was defined as the disappearance of all detectable lesions with no new lesions for at least 4 weeks; partial response was defined as ≧50% reduction of the sum of the products of measurable lesions for at least 4 weeks. Progressive disease was defined as a ≧25% increase in the sum of the products of all measurable lesions, reappearance of any lesion that had disappeared, or appearance of a new lesion. No change was defined as any outcome that did not qualify as response or progression. Both complete response and partial response patients were defined as responder.

Statistical analysis. Comparison of the mean of log expression between clear cell type and serous type was done by unpaired t test at

the 3% level of significance. When the test statistic of a gene was positive and significant, that gene was defined as the overexpressed gene. When the test statistic of a gene was negative and significant, that gene was defined as the underexpressed gene. Quantitative PCR data and immunochemistry scores were compared using nonparametric Mann-Whitney *U* test. The level of critical significance was considered to be $P < 0.05$. Significances in the correlation between array and quantitative PCR data and between DNA and mRNA copy numbers were determined by Spearman's correlation analysis.

Results

Identification of amplification and overexpression of ABCF2 in clear cell tumors by cDNA microarray. Using the same cDNA array platform, we first identified genes with CNA, which significantly overrepresented in clear cell tumors compared with those in the serous tumors. Further selection of genes that were simultaneously overexpressed relative to that in human ovarian surface epithelium was done. A total of 12 genes were identified (Table 2A). Subsequently, we identified genes with CNA, which significantly underrepresented in clear cell tumors compared with those in the serous tumors. Further selection of genes that were simultaneously underexpressed relative to that in human ovarian surface epithelium was done. A total of five genes were identified (Table 2B). Using the same approach, we next identified genes with CNA, which significantly overrepresented in serous tumors compared with those in clear cell tumors. Further selection of genes that were simultaneously overexpressed relative to that in human ovarian surface epithelium was done. A total of eight genes were identified (Table 2A). Finally, we identified genes with CAN, which significantly underrepresented in serous tumors compared with those in clear cell tumors and a total of 15 genes were identified (Table 2B).

Because clear cell tumors in general have a more chemoresistant phenotype compared with the serous type, we therefore selected genes that are have been shown to be related to chemoresistance for further validation. *ABCF2*, a member of the ATP-binding cassette family, was selected because overexpression and amplification of several members of this family have been shown to correlate with chemoresistance in different cancer types (14). Array CGH analysis showed a significantly higher fold increase in CNA in clear cell tumors than in serous tumors ($P = 0.016$; Table 2; Fig. 1A). Expression profiling also showed a significantly higher level of *ABCF2* expression in clear cell tumors than in the serous type ($P = 0.02$; Fig. 1B).

Quantitative PCR and quantitative reverse transcription-PCR were used to validate the microarray data. Significantly higher fold increase in CNA was identified in clear cell tumors than in serous tumors ($P < 0.001$; Fig. 2A). When 1.5 for amplification and 0.5 for deletion were used as cutoffs, *ABCF2* gene was amplified in 57.1% (16 of 28) of clear cell types and 11.1% (2 of 18) of serous type ($P = 0.002$). Relative gene expression of *ABCF2* was significantly higher in clear cell type than it in serous type ($P < 0.001$; Fig. 2B).

The relationship between DNA and RNA levels of *ABCF2* identified by the array and the quantitative PCR platform was evaluated. There was a significant correlation between CNA and expression levels of ABCF2 in the microarray analysis ($r = 0.418$; $P = 0.027$). Quantitative PCR also showed a significant correlation between DNA and RNA copy number ($r = 0.426$; $P = 0.006$).

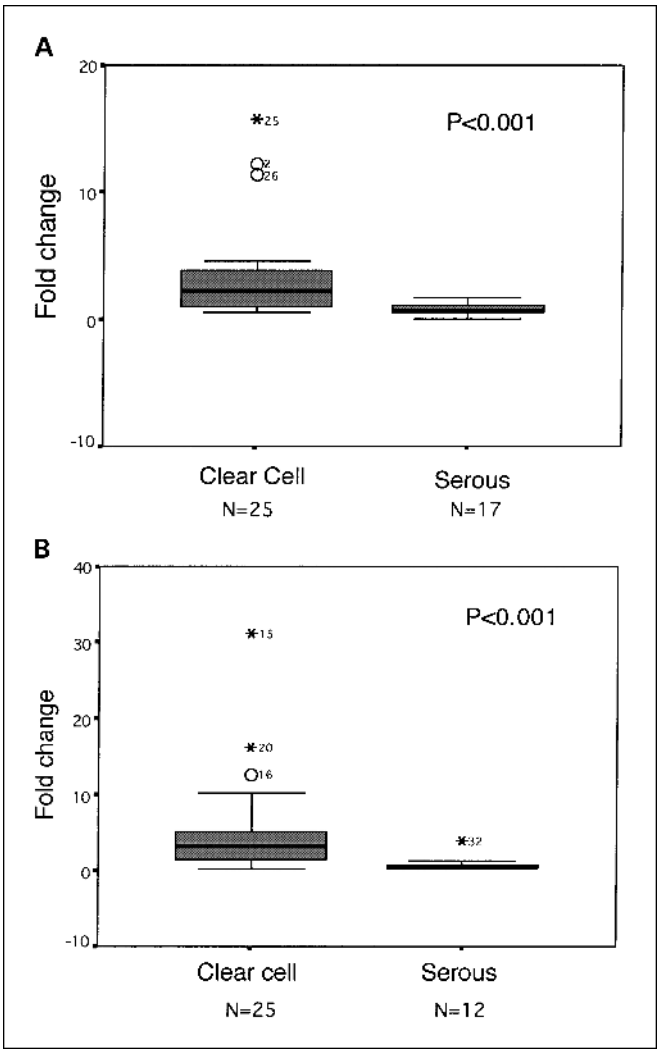
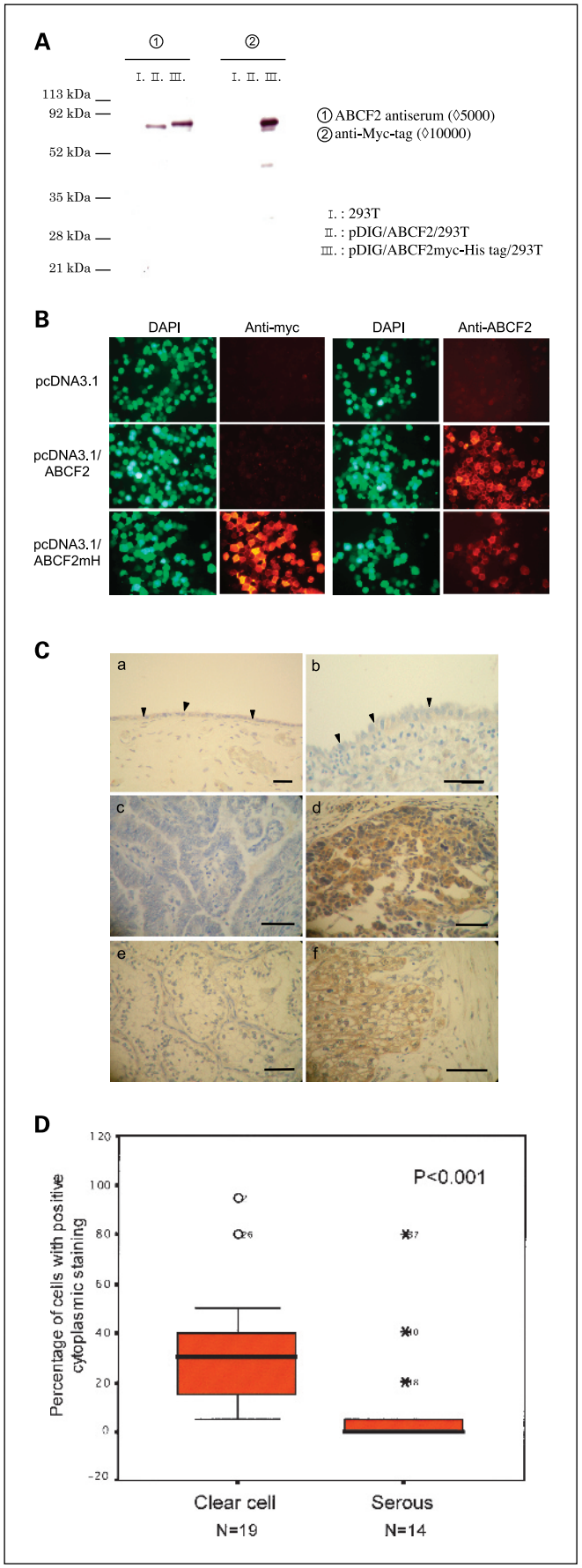


Fig. 2. *A,* comparison of DNA copy number of *ABCF2* gene between clear cell adenocarcinoma and serous cystadenocarcinoma. *B,* comparison of mRNA expression of ABCF2 between clear cell adenocarcinoma and serous cystadenocarcinoma. The box is bounded above and below by the 75th and 25th percentiles, and the median is the line in the box. Whiskers are drawn to the nearest value not beyond a standard span from the quartiles; points beyond (outliers) are drawn individually, where the standard span is 1.5 × (interquartile range).

Immunolocalization of ABCF2. To confirm the specificity of the anti-ABCF2 antibody, Western blot analysis was done on cell lysates prepared from 293T cells transfected with pcDNA3.1 vector alone or with vector containing the *ABCF2* sequence and the Myc-His-tagged *ABCF2* sequence, pcDNA3.1/ABCF2 and pcDNA3.1/ABCF2mH, respectively. The results showed that the anti-ABCF2 antibody recognized a single 70- to 75-kDa protein band in 293 cells transfected with pcDNA3.1/ABCF2 and a band with slightly higher molecular weight in cells transfected with pcDNA3.1/ABCF2mH. Furthermore, when the anti-Myc antibody was used, a protein band was only observed in cells transfected with pcDNA3.1/ABCF2mH (Fig. 3A). Immunohistochemical analysis showed strong cytoplasm ABCF2 staining as well as weak nuclear staining in cells transfected with the pcDNA3.1/ABCF2 and the pcDNA3.1/ABCF2mH constructs compared with the mock transfectants using the anti-ABCF2 antibody. Furthermore, strong cytoplasm Myc staining and weak nuclear staining were observed only in cells transfected



with pcDNA3.1/ABCF2mH using the anti-Myc tag antibody (Fig. 3B). These data strongly suggest that the anti-ABCF2 antibody is specific to the ABCF2 protein, which is predominantly located in the cytoplasm of the cells.

Using the anti-ABCF2 antibody, immunolocalization of ABCF2 protein was then done on 5 normal ovaries, 5 endometriotic cysts, 22 clear cell cases, and 15 serous cases. Epithelial cells lying the surface of all the normal ovaries and endometriotic cysts had undetectable ABCF2 staining in both the nuclei and the cytoplasm. In 86.4% (19 of 22) of clear cell cases, ABCF2 immunoreactivity was detected in both nuclei and cytoplasm of cancer cells (Fig. 3C). In 13.6% (3 of 22) of clear cell cases, ABCF2 protein was negative in both nuclei and cytoplasm of cancer cells. In 20% (3 of 15) of serous cell cases, ABCF2 immunoreactivity was detected in both nuclei and cytoplasm of cancer cells. In 46.7% (7 of 15) of serous cell cases, ABCF2 protein staining was negative. In 26.7% (4 of 15) of serous cell cases, ABCF2 protein was detected only in nuclei of cancer cells, and in 6.7% (1 of 15) of serous cell cases, ABCF2 protein was detected only in the cytoplasm of cancer cells.

At least 1,000 tumor cells were examined for positive nuclear or cytoplasm ABCF2 protein staining. There was no significant difference in the number cells with positive nuclear ABCF2 staining between clear cell and serous cases ($P = 0.181$). However, levels of cytoplasmic ABCF2 staining were significantly higher in the clear cell cases compared with that in serous cases ($P < 0.001$; Fig. 3D).

In addition, the relationship between ABCF2 protein expression and both DNA and RNA copy number was determined. Both DNA copy number and relative mRNA expression significantly correlated with levels of ABCF2 protein cytoplasmic expression ($r = 0.483$; $P = 0.005$ and $r = 504$; $P = 0.006$, respectively).

Relationship between ABCF2 expression and chemotherapy response. ABCF2 protein levels in 20 patients were used to correlate with chemotherapy response. Median age was 55 years (range, 37-82 years). Sixteen cases were stage III, 1 case was stage IV, and 3 cases were recurrent cases. Seventeen primary cases had at least one measurable tumor lesion documented radiographically after primary surgery. Three recurrent cases had at least one measurable tumor lesion documented radiographically after second cytoreductive surgery. All patients received platinum-based chemotherapy. The relationship between ABCF2 expression and chemotherapy response was shown in Fig. 4. ABCF2 cytoplasmic staining was significantly higher in nonresponder than that in responder [60.0% (95%

Fig. 3. *A,* Western blot analyses on cell lysates prepared from 293T wild-type (*lane I*) or 293T transfected by pcDNA3.1 carrying ABCF2 (*lane II*) or Myc-His-tagged ABCF2 (*lane III*) using an anti-ABCF2 polyclonal antibody or an anti-Myc tag monoclonal antibody. *B,* immunolocalization of ABCF2 and Myc tag proteins in 293T cells transfected with pcDNA3.1 vector alone or with vectors containing ABCF2 or Myc-His-tagged ABCF2. *C,* immunolocalization of ABCF2 protein in normal ovaries, endometriotic cysts, and ovarian cancer tissue. *a,* negative immunostaining of ABCF2 in the surface epithelium of a normal ovary (*arrowhead*). *b,* negative immunostaining of ABCF2 in the epithelial lying of an endometriotic cyst (*arrowhead*). *c,* positive immunostaining of ABCF2 in serous cystadenocarcinoma. *d,* negative immunostaining of ABCF2 in serous cystadenocarcinoma. *e,* positive immunostaining of ABCF2 in clear cell adenocarcinoma. *f,* negative immunostaining of ABCF2 in clear cell adenocarcinoma. *Bar*, 50 μm. *D,* Comparison of protein expression of ABCF2 between clear cell adenocarcinoma and serous cystadenocarcinoma. The box is bounded above and below by the 75th and 25th percentiles, and the median is the line in the box. Whiskers are drawn to the nearest value not beyond a standard span from the quartiles; points beyond outliers are drawn individually, where the standard span is 1.5 × (interquartile range).

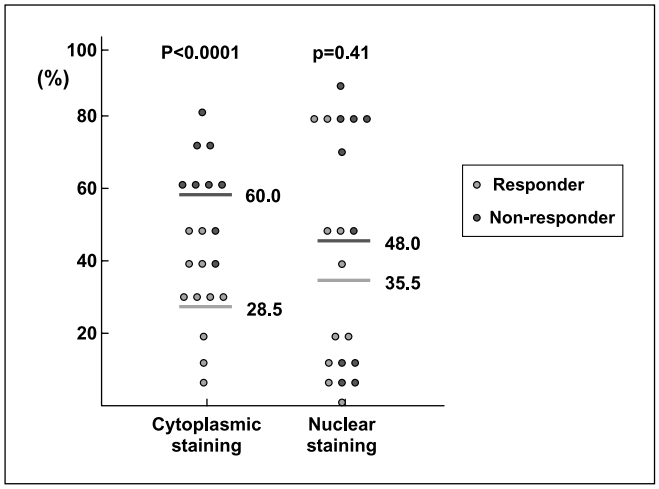


Fig. 4. Comparison of ABCF2 protein expression between responders and nonresponders.

CI, 51.7-68.3) versus 28.5% (95% CI, 18.7-38.3); $P < 0.0001$]. However, there was no significant relationship between ABCF2 nuclear staining and chemotherapy response [48.0% (95% CI, 22.0-74.0) versus 35.5% (95% CI, 14.5-56.5)].

Discussion

Clear cell adenocarcinoma is usually more resistant to systemic chemotherapy than other histologic types of ovarian cancer and patients with clear cell type ovarian cancer have worst prognosis (4, 5). In fact, in current clinical practice, clear cell ovarian cancers are treated as high-grade neoplasms (6). The overall clinical response rate to platinum-based chemotherapy was reported to be 72.5% in patients with serous type; however, only 11.1% of the patients with clear cell type responded to platinum-based chemotherapy (15). Schwartz et al. reported that *GPX3*, *GLRX*, and *SOD2* were highly expressed in clear cell type ovarian cancer. They suggested that high levels of these proteins and perhaps other antioxidant proteins in clear cell tumors may render these tumors to be more resistant to chemotherapy (8). One of the important mechanisms of drug resistance is a decrease in the accumulation of the drug caused by enhanced drug efflux mediated by ABC transporter. The correlation between expression of these ABC transporter genes or proteins and drug response has been examined in epithelial ovarian cancer. However, the conclusions are conflicting (16–20). In addition, to our knowledge, there are few reports that examine the mechanism of chemoresistance in clear cell type of ovarian cancer. In this type of ovarian cancer, low proliferation rates of tumor cells were reported to contribute to its survival. In addition, both *MDR1* and *MRP3* expression have not been shown to be related to the prognosis of patients with clear cell ovarian cancer (21, 22). Ohishi et al. showed that the mRNA levels of *MRP1* and *MRP3* were related to the prognosis of serous type ovarian cancer and mRNA levels of *MRP3* were significantly higher in clear cell type than that in serous type (23). In spite of all these studies, the mechanism of chemoresistance in clear cell type ovarian cancer still remains unclear.

In this study, we showed that ABCF2 protein is predominantly located in the cytoplasm of cells. We showed that both *ABCF2* gene and protein expression were significantly correlated with gene amplification especially in clear cell ovarian cancer. Furthermore, we also showed that cytoplasmic ABCF2 expression was significantly correlated with chemotherapy response despite the small number of cases. These data suggest that ABCF2 expression may contribute to the chemoresistant phenotype of clear cell ovarian cancer. However, the role of ABCF2 in conferring chemoresistance in cancer cells is unclear. Yasui et al. reported that *ABCF2* gene is amplified in a chemoresistant ovarian cell line (t24/cDDp10), which had chromosome gain at 7q34-36 (14). Besides, there are no other reports to our knowledge showing the mechanism of ABCF2 in chemoresistance. ABCF2 protein is a member of the ABCF transporter superfamily and the GCN20 subfamily (24). Like other members of the ABCF family, ABCF2 contains a pair of nucleotide-binding domain but without any transmembrane domains (25, 26), suggesting that it unlikely functions as a transporter located on the cell membrane as other members of the ABC family. This is further confirmed by our immunohistochemistry data showing predominantly cytoplasmic localization of the protein. The functions of many of these twin nucleotide-binding domain proteins remain unknown. Kerr suggested that a mechanistic similarity exists between eukaryotic members of the ABCF family, which are involved in the control of translation initiation and elongation. These proteins may also have functional similarities to prokaryotic ABCF proteins, which have been shown to be involved in translational control, antibiotic resistance, and RNase L inhibition (26). ABCF may induce factors related with chemoresistance. Further study will be required to delineate the role of ABCF2 in chemoresistance.

In conclusion, ABCF2 protein is a potential prognostic marker for clear cell ovarian cancer and its expression correlates with chemoresponse in patients with clear cell ovarian cancer. Further functional studies of ABCF2 in clear cell ovarian cancer pathogenesis are under way.

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