Tumorigenesis and Neoplastic Progression

Deregulation of IKBKE Is Associated with Tumor Progression, Poor Prognosis, and Cisplatin Resistance in Ovarian Cancer

Jian-Ping Guo,* Shao-Kun Shu,* Lili He,*
Yi-Chun Lee,[†] Patricia A. Kruk,[‡] Seija Grenman,[§]
Santo V. Nicosia,[‡] Gil Mor,[¶] Michael J. Schell,^{||}
Domenico Coppola,** and Jin Q. Cheng*

From the Departments of Molecular Oncology,* Biostatistics,^{||} and Pathology,** H. Lee Moffitt Cancer Center, Tampa, Florida; the State University of New York Downstate Medical Center,[†] New York, New York; the College of Medicine,[‡] University of South Florida, Tampa, Florida; Turku University Hospital,[§] Turku, Finland; and the Yale University School of Medicine,[¶] New Haven, Connecticut

I-kappa-B kinase ϵ (IKBKE; IKK ϵ) has been recently identified as a breast cancer oncogene, and its alteration appears to be an early event in breast cancer development. In this study, we demonstrated that IKK ϵ is frequently overexpressed and activated in human ovarian cancer cell lines and primary tumors. Of 96 ovarian cancer specimens examined, 63 exhibited elevated levels of IKK ϵ . Furthermore, alterations of IKK ϵ were associated with late-stage and high-grade tumors, suggesting a role of IKK ϵ in ovarian tumor progression rather than in tumor initiation. Overall survival in patients with elevated levels of IKK ϵ was significantly lower than patients whose tumors expressed normal levels of IKK ϵ . Moreover, both early and late-stage tumors that overexpressed IKK ϵ conferred a poor prognosis, as compared with those that did not possess elevated IKK ϵ levels. Notably, overexpression of IKK ϵ rendered cells resistant to cisplatin, whereas knockdown of IKK ϵ overcame cisplatin resistance in both A2780CP and C13 cells, which express high levels of endogenous IKK ϵ . Therefore, these data demonstrate for the first time that deregulation of IKK ϵ is a highly recurrent event in human ovarian cancer and could play a pivotal role in tumor progression and cisplatin resistance. IKK ϵ could also serve as a prognostic marker and potential therapeutic target for this malignancy. (*Am J Pathol* 2009, 175:324–333; DOI: 10.2353/ajpath.2009.080767)

The I-kappa-B kinase (IKK) ϵ , also named IKBKE/IKKi, is a serine/threonine protein kinase that belongs to the IKK family. The IKK ϵ protein has 33% and 31% identity at the amino acid level with IKK α and IKK β , respectively, is primarily activated by interferon and mediates interferon signaling.^{1,2} The activated IKK ϵ stimulates the transcription factor interferon regulatory factors 3 and phosphorylates signal transducer and activator of transcription^{1,3,4} IKK ϵ also shares some function with IKK α and IKK β to activate nuclear factor (NF) κ B pathway by phosphorylation and degradation of I κ B α .^{5–7} However, IKK ϵ mainly mediates NF κ B activation induced by interferon, phorbol 12-myristate 13-acetate or the T-cell receptor, but not by tumor necrosis factor and interleukin 1, which activate IKK α and IKK β .

Previous studies have revealed an important role for IKK α and IKK β in tumorigenesis via regulation of various signal transduction pathways.^{8–11} Using complementary genetic approaches, Boehm and colleagues recently identified IKK ϵ as a breast cancer oncogene by dissecting the downstream pathways sufficient for RAS to induce neoplastic transformation. Although RAS can stimulate many pathways, such as the mitogen-activated protein kinase, phosphoinositide 3 kinase, and ral guanine nucleotide dissociation stimulator signaling pathways, a full-blown transformation phenotype of immortalized human cells could be acquired only by the simultaneous expression of constitutively activated mitogen-activated protein kinase kinase together with a myristoylated AKT. Further, by screening a myristoylated kinase expression library for kinases that act in the place of AKT in the RAS-AKT pathway to promote cellular transformation, they identified that the IKK ϵ could substitute constitutively active AKT to induce transformation.¹² Further analysis showed

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Address reprint requests to Jin Q. Cheng at H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, SRB3, Tampa, FL 33612. E-mail: jin.cheng@moffitt.org.

that IKK ϵ is frequently altered in breast cancer cell lines and patient-derived tumors.¹²

In the present report, we demonstrate frequent over-expression and activation of IKK ϵ in human ovarian cancer cell lines and primary tumors. The alterations of IKK ϵ are more frequently detected in high grade and late stage ovarian tumors. Further, patients with ovarian cancer with low levels of IKK ϵ have a better outcome than the patients whose tumors express elevated levels of IKK ϵ regardless of tumor stages. Ectopic expression of IKK ϵ renders cells resistant to cisplatin (CDDP) whereas knockdown of IKK ϵ sensitizes cells to CDDP-induced cell death. These findings suggest that IKK ϵ is an ovarian cancer oncogene that not only plays an important role in CDDP resistance but also serves as a prognostic marker and therapeutic target for this malignancy.

Materials and Methods

Cell Lines, Tumor Specimens, and Ovarian Tissue Microarray

Human immortalized ovarian surface epithelial cell lines HIOSE-MCC3 and HIOSE118 were cultured in medium 199/MCDB 105 (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT) and 10 μ g/ml Gentamicin (Gibco, NY). Ovarian cancer cell lines (OVCAR3, OVCAR4, OVCAR5, OVCAR8, C13, OV2008, A2780S, A2780CP, OVCAR420, OVCAR433, R567 and P70) were maintained RPMI-1640 medium with 10% fetal bovine serum. OV2008 and C13 were derived from the same cell line whereas A2780S and A2780CP were from another line following CDDP selection. OV2008 and A2780S are sensitive to CDDP, and C13 and A2780CP are resistant to CDDP.^{13,14} All of the primary human ovarian cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center. Each sample contained at least 80% tumor cells, confirmed by microscopic examination. The tissues were snap-frozen and stored at -70°C . The ovarian specimens included 45 serous, 8 mucinous, and 3 endometrioid epithelial ovarian carcinomas, 11 clear cell carcinomas, 2 transitional cell carcinomas, 11 low malignant potential (eg, borderline) carcinomas, and 18 cystadenomas, as well as 20 normal ovaries. Clinical stage, histological grade¹⁵ and follow-up data are available for the majority of these patients. The ovarian tissue microarray, which includes 5 normal ovarian tissues and 22 ovarian cancers with stage, grade, and histological diagnosis, was purchased from US Biomax.

Plasmid, Virus, and Antibodies

The pCMV-Myc-IKK ϵ was created by PCR amplification of IKK ϵ using pRK5-IKK ϵ , kindly provided by Dr. Ivan Dikic (Goethe University Medical School, Frankfurt, Germany) as a template. The PCR products were digested and subcloned to BamH1-EcoRI sites of pCMV3b vector (Stratagene). Resulting pCMV-Myc-IKK ϵ plasmid was confirmed by DNA sequencing. IKK ϵ lentiviral short hair-

pin (sh)RNAs (pLK0.1) were purchased from Open Biosystems. Lentiviruses were packaged as previously described.¹⁶ Briefly, the lentiviral shRNA and the package plasmids were co-transfected into 80% confluence HEK293FT cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 4 hours of transfection, culture medium was replaced and cells were incubated for another 48 hours. The lentiviruses (conditional medium) were obtained by filtering medium through a 0.45 μ m filter and stored at -80°C . The ovarian cancer cells were infected with the lentiviruses (1:1 conditional medium and fresh medium) and treated with puromycin for stable knock-down cell lines. Anti-IKK ϵ antibody was from Sigma and antibodies against poly(ADP-ribose) polymerase, caspase 3, and actin were purchased from Cell Signaling.

Immunoblotting, Southern Blot Analysis, Immunohistochemistry, and Enzyme-Linked Immunosorbent Assay

Immunoblotting and Southern blot analyses were performed as previously described.¹⁷ Briefly, cells and the frozen tissues were lysed by a lysis buffer. Equal amounts of protein were separated on SDS-polyacrylamide electrophoresis gel and probed with appropriate antibody indicated in figure legend. Detection was performed with the ECL Western Blotting Analysis System (Amersham, Piscataway, NJ) and the quantification was performed with ImageJ program from the National Institutes of Health. Southern blots were prepared by digestion of 10 μ g of DNA with EcoRI and detected with a random primer [³²P]dCTP-labeled IKK ϵ cDNA probe. Autoradiographs were quantified relative to β -actin using Image-Quant software.

For immunohistochemistry, IKK ϵ -transfected OV2008 and IKK ϵ -knockdown C13 cells were cytospun, fixed, and immunostained with anti-IKK ϵ C-terminal antibody (Sigma), which has been previously used for immunohistochemistry.¹² Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M/L sodium citrate buffer (pH 6.0) in a microwave oven after dewaxing and rehydration. The Vectastain ABC Kit for rabbit IgG (Vector Laboratories) was used to immunostain the tissue sections with anti-IKK ϵ C-terminal antibody.¹² Endogenous peroxidase and biotin were blocked, and sections were incubated overnight at 4°C with 1:250 dilution of IKK ϵ antibody. The remainder of the staining procedure was performed according to the manufacturer's instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of non-immune rabbit IgG on control sections. The percentage of cells showing hybridization was scored by two independent senior pathologists as follows: 0 or negative = 0% to 5% positive cells; 1 or weak = $\leq$ 25% positive cells; 2 or moderate = 26% to 75% positive cells; 3 or strong = $>$ 75% positive cells. Positive rates relative to the total number of cells were calculated for each case. The scoring result was transformed into a two-tiered scale: low (negative and weak)

and high (moderate and strong). Enzyme-linked immunosorbent assay (ELISA) was performed with Protein Detection ELISA anti-human ABTS system (KPL) following manufacture's instruction. The data were analyzed with *t*-tests.

In Vitro Protein Kinase Assay

The immunoprecipitation was performed by incubation of 200 μ g protein lysate with anti-IKK ϵ antibody. The immunoprecipitates were subjected to *in vitro* kinase assay. The reaction was performed with kinase buffer (20 mmol/L Hepes, pH 7.6, 10 mmol/L MgCl₂, 50 mmol/L NaCl, 0.1 mmol/L sodium vanadate, 20 mmol/L β -glycerolphosphate, and 1 mmol/L dithiothreitol), in the presence of 10 μ Ci [³²P]ATP, 50 μ mol/L unlabeled ATP, and myelin basic protein as exogenous substrate. After incubation at 30°C for 30 minutes, the reaction was stopped by adding protein-loading buffer and separated by 12.5% SDS-polyacrylamide gel electrophoresis. The relative amounts of incorporated radioactivity were determined by autoradiography.

Cell Viability and Annexin V-Fluorescein Isothiocyanate Apoptosis Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and Annexin V labeling were performed as previously described.¹⁷ Briefly, cells were diverted into 96-well plate with 1×10^5 cells/well. Following 24 hours incubation, cell were treated with different amounts of CDDP for 18 hours and then assayed for total cell viability (MTT assay). Apoptosis was analyzed with Annexin V-fluorescein isothiocyanate apoptosis detection kit following manufacture's instruction (BD Pharmingen).

Statistic Analysis

Correlations of the IKK ϵ overexpression with the tumor grade, stage, and histology were analyzed by χ^2 testing. For survival analysis, optimal cut-point was selected using X-tile program as described previously.¹⁸ Monte Carlo simulations were used to adjust for multiple looks in optimal cut-point selection.¹⁹ Hazard ratios were assessed using the univariate and multivariate Cox proportional hazards model (Log-rank test at $\alpha = 0.05$) based on the cut-point value determined by the X-tile. The regression analyses were adjusted for International Federation of Gynecology and Obstetrics (FIGO) stage (I, II, III, and IV), histological type of tumor (serous and other histological types), and histological grade (high and low). Confidence intervals (95% CI) for the corresponding parameters in the multivariate Cox regression model are presented. Patients' deaths of non-related ovarian cancer diseases were censored in the survival analyses at the date of death. These analyses were performed using the SPSS 11.5 Statistical Software and $P \leq 0.05$ was considered statistically significant.

Results

Frequent Overexpression and Activation of IKK ϵ in Ovarian Cancers

A recent study has demonstrated IKK ϵ as a breast cancer oncogene. Amplification of IKK ϵ was detected in ~16% breast cancers and overexpression of IKK ϵ protein was observed in nearly half of primary breast tumors.¹² Since IKK ϵ resides in chromosome 1q32 that is amplified in different tumors including ovarian cancer,^{20,21} we examined if IKK ϵ is altered in ovarian carcinoma. Immunoblotting analysis revealed elevated levels of IKK ϵ protein in 11 of 14 ovarian cell lines, including 12 ovarian cancer and two immortalized human ovarian surface epithelial cell lines (Figure 1A). Further, overexpression of IKK ϵ protein and mRNA was detected in 45 of 74 frozen ovarian cancer specimens (60.8%) whereas only 1 of 20 normal ovarian tissues exhibited detectable IKK ϵ protein (Figure 1, B and C and Table 1). ELISA analysis also revealed significant elevated levels of IKK ϵ in ovarian carcinomas. The relative expression of IKK ϵ in ovarian tumor specimens ranged from 0.110 to 0.412 and the median value was 0.236 (Figure 1D). Further, Southern blot analysis showed moderate increase in DNA levels in 5 of 52 (9.6%) primary ovarian tumors examined (Figure 1E and data not shown), which is lower than that in breast cancer.¹²

Since IKK ϵ is a serine/threonine kinase and its function depends on its kinase activity,¹² we next investigated whether the protein changes correspond to increases in IKK ϵ kinase levels. Indeed, *in vitro* kinase assay revealed that IKK ϵ kinase activity was elevated in cell lines and ovarian tumors that exhibited overexpression of IKK ϵ protein (Figure 1F). However, three tumors with increased protein levels of IKK ϵ did not show elevated kinase activity, suggesting that these specimens might not have been promptly frozen after surgical removal or deregulation of its upstream activator(s).

To further demonstrate overexpression of IKK ϵ protein in ovarian cancer cells, we performed immunohistochemical staining with anti-IKK ϵ antibody in the same series of ovarian tumors used for immunoblotting analysis, as well as in 22 additional cases in the tissue microarray. We first evaluated the specificity of anti-IKK ϵ antibody¹² by immunostaining myc-IKK ϵ -transfected OV2008 and IKK ϵ -knockdown C13 cells (Figure 2A). Figure 2B shows high intensity signals in IKK ϵ -expressing OV2008 and parental C13, but not pcDNA-transfected OV2008 and IKK ϵ -knockdown C13 cells, suggesting that the anti-IKK ϵ antibody specifically recognizes IKK ϵ . Immunostaining of primary ovarian tumors revealed elevated levels of IKK ϵ in 63 of 96 tumors examined. Because ovarian cancer has been shown to be derived from ovarian surface epithelial cells, fallopian tube epithelium, and/or endometrium, we next evaluated expression levels of IKK ϵ in these tissues. A total of 20 normal ovaries, 10 fallopian tubes, and 10 normal endometrial tissues were examined and showed low or undetectable levels of IKK ϵ (Figure 2C and Table 1). In addition, elevated levels of IKK ϵ were observed in only 1 of 11 low malignant potential carci-

noma, and 2 and 18 cystadenomas (Table 1). These findings further suggest that IKK ϵ is frequently altered in ovarian carcinoma.

IKK ϵ Overexpression Is Associated with Clinical Features

We next examined the relationships between overexpression of IKK ϵ and different clinicopathological features of ovarian cancer. We noted that IKK ϵ is more frequently overexpressed in serous (39/55, 70.9%) and clear cell (11/13; 84.6%) adenocarcinomas than in other histological types of ovarian cancer (Table 1). However, statistical analysis shows no significant difference of IKK ϵ alteration

between serous and mucinous carcinoma ($P = 0.064$; Tables 1 and 2), whereas the frequency of aberrant IKK ϵ is significantly higher in ovarian cancer than cystadenoma, low malignant potential, and normal ovary ($P < 0.001$; Table 1). Further, the frequency of IKK ϵ overexpression was relatively low in low grade ovarian tumors and increased in late stage and high grade tumors ($P = 0.021$ and $P = 0.037$; Table 2), suggesting that alterations of IKK ϵ could be involved in ovarian cancer progression rather than initiation.

We also investigated the association of the expression of IKK ϵ with patient outcome using a recently developed statistical method called X-tile.¹⁸ All 74 patients with available survival data were randomly divided into

Table 1. Expression of IKK ϵ in Ovarian Specimens

Histology	<i>n</i>	Low/no	High/moderate	<i>P</i> value
Serous carcinoma	55	17	39	
Mucinous carcinoma	24	12	12	
Endometrioid carcinoma	4	3	1	
Clear cell and transitional carcinoma	13	2	11	
Borderline carcinoma (low malignant potential)	11	10	1	0.001*
Cystadenoma	18	16	2	0.001†
Normal	20	19	1	0.001‡

Expression of IKK ϵ protein in total 145 clinical ovarian specimens. *P* value was calculated by χ^2 test.

*†‡Aberrant expression of IKK ϵ in ovarian cancer is significantly higher than LMP, cystadenoma, and normal ovary.

Figure 2. Immunohistochemical staining reveals elevated IKKε in ovarian cancer cells. **A:** Western blot. Following transfection of IKKε in OV2008 cells and knockdown of IKKε in C13 cells, cells were lysed and immunoblotted with indicated antibodies. **B:** Specificity of anti-IKKε antibody. The IKKε-manipulated OV2008 and C13 cells were fixed, cytopun, and immunostained with anti-IKKε antibody (1:250, Sigma), which has previously been used for immunohistochemistry.¹² **C:** Immunohistochemical staining of the paraffin sections prepared from a normal ovary (**a**), fallopian tube (**b**), normal endometrial tissue (**c**), serous and mucinous papillary tumors (**d** and **e**), an invasive serous adenocarcinoma (**f**), and an invasive transitional adenocarcinoma (**g**) with anti-IKKε antibody.

“training” and “validation” sets for cross-validation analysis. Based on ELISA IKKε protein levels (range from 0.110 to 0.412), 0.221 was determined by X-tile as the optimal cut-point in the training set (Figure 3A–C).

Table 2. IKKε Expression and Clinic Pathological Characteristics

Variable	IKKε		<i>P</i> value
	Low/no	High/moderate	
Age			
≤58	13	10	0.202
>58	31	40	
FIGO stage			
Early (I/II)	21	20	0.021
Late (III/IV)	16	36	
Histology			
Serous	16	39	0.064
Mucinous	12	12	
Grade			
I/II	20	29	0.037
III/IV	9	37	

Analysis with χ^2 test.

Cross-validation analysis revealed that overexpression of IKKε (≥ 0.221) in validation set had poor overall survival (OS; $P = 0.0313$; Figure 3, A and D). Further, Kaplan-Meier analysis of all 74 patients demonstrated a statistically significant negative correlation between OS and IKKε expression level ($P = 0.021$; Figure 3E). To extend this observation, we separated the patients into two groups by stage [eg, late stages (III/IV) and early stages (I/II)] and asked whether within each subgroup IKKε expression predicts patient survival. In both groups, high IKKε expression was still a prognostic factor (Figure 3, F and G), although with the small sample size, the result was not statistically significant in early stage tumors ($P = 0.067$; Figure 3G). In addition, we also evaluated the IKKε expression impact on OS in different grade and histological tumors; we found that both groups with elevated IKKε appear to have poor OS while P values did not reach significance (data not shown).

Further univariate analysis revealed that several factors, including FIGO stage, grade, and IKKε expres-

Figure 3. Poor overall survival in patients with elevated levels of IKBKE expression. **A–D:** X-tile analysis. Seventy-four patients with available survival data were randomly separated to two groups with the X-tile program, ie, a training set ($n = 37$; **left of A**) and a matched validation set ($n = 37$; **right of A**). The optimal cut-point highlighted by the **arrow** in (**A**) is shown on a histogram of the training set (**B**), and a Kaplan-Meier plot (**C**). P values were determined with the cut-point defined in the training set, and then applied to the validation set (**D**). **E:** Overall survival (OS) in patients with high levels of IKBKE ($n = 44$) versus the remaining patients ($n = 30$) was plotted by the Kaplan-Meier method. Statistical comparison of survival between groups with the log-rank statistic analysis suggests that patients whose tumors express elevated levels of IKBKE had poor survival compared with those with low levels of IKBKE ($P = 0.021$). **F** and **G:** OS was also analyzed in patients with late stage (**F**) and early stage (**G**) ovarian tumors expressing high versus low levels of IKBKE.

sion, were associated with poor clinical outcome ($P < 0.05$; Table 3). Of them, IKBKE expression was a relative strong predictor of poor clinical outcome with a hazard ratio of 1.813 (95% CI, 1.043 to 3.151, $P = 0.029$). A multivariate Cox proportional hazards model with following variables: age, FIGO stage, grade, histology, and IKBKE expression, was used to analyze their simultaneous association with OS. As expected, the best multivariable model predictive of OS showed that FIGO stage was the strongest independent predictor of outcome with a hazard ratio of 2.196 (95% CI, 1.127 to 4.197, $P = 0.027$, Table 3). Moreover, IKBKE expression also appeared to be an independent predictor with a

hazard ratio of 1.664 (95% CI, 0.882 to 3.085). The P value that did not reach statistic significance in multivariate Cox analysis ($P = 0.056$; Table 3) could be due to the limited patient number examined. Taken collectively, these results suggest that elevated levels of IKBKE are associated with poor prognosis in ovarian cancer.

IKBKE Plays a Significant Role in CDDP Resistance

Platinum-based chemotherapy is the cornerstone of the treatment of epithelial ovarian cancer. Despite good initial

Table 3. Five-Year Survival Analysis of Patients with Ovarian Tumor Examined

Variable	Univariate analysis		Multivariate analysis	
	HR* (95% CI) [†]	P value	HR (95% CI)	P value
Age				
>58 years vs. ≤58	0.978 (0.591–1.582)	0.820	1.017 (0.621–1.533)	0.780
FIGO stage				
Late (III/IV) vs. Early (I/II)	2.558 (1.478–4.530)	0.015	2.196 (1.127–4.197)	0.027
Histology				
Serous tumor versus other type	1.532 (0.674–3.121)	0.077	1.586 (0.866–2.907)	0.135
Differentiation grade				
III/IV versus I/II	1.948 (1.102–3.443)	0.022	1.417 (0.708–2.838)	0.325
IKBKE				
≥0.221 vs. <0.221	1.804 (1.121–3.541)	0.029	1.664 (0.882–3.085)	0.056

*HR: Hazard ratio.

[†]CI: confidence interval.

Figure 4. Ectopic expression of IKKε renders cells resistance to CDDP. **A:** CDDP-sensitive OV2008 cells, which express low level of IKKε, were transfected with pCMV-Myc-IKKε and pCMV vector. After G418 selection, stably transfected cells were immunoblotted with anti-Myc (**top**) and actin (**bottom**) antibodies. **B:** IKKε- and vector-transfected OV2008 cells were cultured in a 96-well plate. Cell growth was evaluated by cell counting at 24, 48, 72, and 96 hours. **Asterisks** indicate that IKKε-OV2008 cell proliferation is significantly higher than control cells ($P < 0.05$). **C–E:** Myc-IKKε-stably transfected cells and control cells were treated with indicated amounts of CDDP for 18 hours and assayed by MTT (**C**), immunoblotting with indicated antibodies (**D**), and Annexin V-labeling/FACS (**E**). Each experiment was repeated three times. * $P < 0.05$, ** $P < 0.02$.

response rates, the majority of patients ultimately relapse with resistant disease. Increased anti-apoptotic activity in resistant cells is one of the major mechanisms of CDDP-resistance. We and others have previously shown that elevated AKT kinase pathway plays a significant role in CDDP resistance.²² Since IKKε replaces AKT to induce human immortalized cell transformation,¹² we next evaluated if deregulation of IKKε in ovarian cancer contributes to CDDP resistance. As shown in Figures 1A and 4A, CDDP sensitive OV2008 (IC_{50} 10 μmol/L) exhibits undetectable IKKε, whereas its derivative C13, which is resistant to CDDP (IC_{50} 40 μmol/L),²² expresses relative high levels of IKKε. Thus, we stably transfected OV2008 cells with pCMV-Myc-IKKε and vector alone as control (Figure 4A) and observed that cell proliferation rate was increased in IKKε-transfected OV2008 cells (Figure 4B). Further, IKKε-expressing OV2008 and control cells were treated with CDDP for 18 hours. The cell survival was assessed with MTT assay and apoptosis was detected by Western blot analysis of poly(ADP-ribose) polymerase cleavage and Annexin V-labeling followed flow cytometry. As shown in Figure 4, C–E, ectopic expression of IKKε significantly reduced the sensitivity of OV2008 cells to CDDP-induced cell death and apoptosis (eg, from IC_{50} 10 μmol/L in OV2008 to 20 μmol/L in IKKε-OV2008 cells). In addition, all cell lines, except A2780S (IC_{50} = 10 μmol/L), that express high levels of IKKε have $IC_{50} \geq 15$ μmol/L of CDDP (Table 4).^{22–24}

Since CDDP-resistant C13 (IC_{50} 40 μmol/L) and A2780CP (IC_{50} 45 μmol/L)^{22–24} cells express relative

high levels of IKKε (Figure 1A), we further examined the role of IKKε in CDDP resistance by stable knockdown of IKKε in these two cell lines. The cells were infected with lentiviruses expressing five shRNAs of IKKε, four of which correspond to coding region, and the other targets the 3'UTR region of IKKε. The cells infected with lentiviral vector alone were used as controls. After selection with puromycin, stably IKKε-knocked down cells were established (Figures 5A and 6A). Cell growth rate was decreased in IKKε-knocked down cells (Figures 5B and 6B). Following treatment with CDDP for 18 hours, MTT, caspase-3 cleavage, and annexin V/FACS analyses revealed that knockdown of IKKε sensitizes both C13 (IC_{50}

Table 4. Compared IKKε Protein Levels with Cisplatin (CDDP) IC_{50}

Cell lines	IKKε (OD490)	CDDP IC_{50} (μM)
HOSE118	0.2	10
OVCA3	0.68	45
OVCA4	0.7	38
OVCA8	0.6	33
A2780S	0.4	10
A2780CP	0.7	45
A2780CP-sh-IKKε	0.3	30
C13	0.58	40
C13-sh-IKKε	0.32	20
OV2008	0.08	10
OV2008-IKKε	0.34	20
OVCA420	0.9	55
OVCA433	0.5	45

Figure 5. Knockdown of IKK ϵ in C13 cells reduces CDDP resistance. **A:** CDDP-resistant C13 cells were infected with lentiviruses expressing 5 shRNAs of IKK ϵ or lentiviral vector alone as a control. After selection with puromycin, stably IKK ϵ -knocked down cells and control cells were immunoblotted with the indicated antibodies. **B:** IKK ϵ -knockdown (sh-IKK ϵ) and control cells were seeded in a 96-well plate and cell growth was examined at the indicated time points. **C:** IKK ϵ -knocked down cells and control cells were treated with the indicated amounts of CDDP for 18 hours and then subjected to an MTT assay. **D and E:** Following CDDP treatment, cells were subjected to Western blot analysis with the indicated antibodies (**D**) and an Annexin V/FACS assay (**E**). The experiments were repeated thrice in triplicates samples. * $P < 0.05$.

20 μ mol/L) and A2780CP (IC_{50} 30 μ mol/L) to CDDP-induced total cell death and apoptosis (Figures 5, C–E and 6, C–E). These results indicate that overexpression of IKK ϵ in ovarian cancer could play an important role in CDDP resistance.

Discussion

Ovarian cancer is the leading cause of gynecological cancer mortality. The standard treatment for ovarian cancer consists of primary surgery aiming at maximal cytoreduction, and chemotherapy with a platinum-taxane combination. However, the 5-year survival rate for stage III and IV disease remains 20% to 30%.^{25,26} Accumulated studies have demonstrated that deregulation of oncogenes (such as RAS, HER2, AKT, Aurora-A etc) and tumor suppressors (such as TP53, BRCA1/2, PTEN etc) as well as DNA repair genes are implicated in ovarian carcinogenesis and chemoresistance.^{26–32} In the present report, we showed frequent overexpression of IKK ϵ in ovarian cancer. Deregulation of IKK ϵ is associated with high grade and advanced stage tumors, as well as poor clinical outcomes. Overexpression of IKK ϵ renders cells resistant to CDDP, whereas knockdown of IKK ϵ considerably overcomes CDDP resistance.

Our data show that overexpression of IKK ϵ is much more common than its gene amplification. This might be due to the limited sensitivity of Southern blot for detection of DNA amplification. In breast cancer, how-

ever, Boehm et al, using fluorescence *in situ* hybridization for IKK ϵ amplification, found that 13 of 17 cell lines and 10 of 21 breast cancers showed increased expression of IKK ϵ protein in the absence of 1q32 copy-number changes.¹² These data suggest that gene amplification is not the primary mechanism for overexpression of IKK ϵ in a significant fraction of breast and ovarian tumors.

Among members of IKK kinase family, IKK α and IKK β are more close to each other with 52% identity and 63% similarity at amino acid level. IKK ϵ is a more distinct member from IKK α and IKK β .^{33,34} It has been well documented that IKK α and IKK β form a complex with scaffold protein IKK γ (also called NEMO) and activate NF κ B pathway through phosphorylation and degradation of I κ B proteins, which leads to cell survival, growth, differentiation, angiogenesis, inflammation and immune response.^{33,34} However, recent studies showed that nuclear IKK α has pro-apoptotic and tumor suppressor function that is independent of NF κ B signaling. On CDDP treatment, ataxia-telangiectasia mutated kinase phosphorylates IKK α at Ser473, which leads to IKK α accumulation in the nucleus where it binds to and stabilizes p73. This process is critical for CDDP-induced apoptosis.^{35–38} Moreover, IKK α inhibits squamous cell carcinogenesis by acting as a major cofactor in a transforming growth factor β -Smad2/3 signaling pathway that is Smad4 independent.^{37,38} Unlike IKK α , IKK ϵ is amplified/overexpressed in a number of breast cancers, and functions as an oncogene.¹² Ec-

Figure 6. Decrease in CDDP resistance in A2780CP cell by knockdown of IKKε. A2780CP cells were treated and assayed following the procedures described in Figure 5. The cells were subjected to immunoblotting (A), cell growth (B), cell viability (C), and apoptosis (D and E) analyses. **P* < 0.05.

topic expression of IKKε transforms human immortalized mammary epithelia¹² as well as murine NIH3T3 fibroblasts (Guo and Cheng, unpublished data). While IKKε phosphorylates IκB and mediates NFκB activation induced by PMA,^{5–7} it has been shown to activate interferon regulatory factors 3 and 7, as well as signal transducer and activator of transcription 1 and plays a critical role in the interferon-inducible antiviral transcriptional response.^{1,3,4} Our data show that IKKε has anti-apoptotic function and its overexpression attributes to CDDP resistance. While the mechanism by which IKKε promotes cell growth and survival remains elusive, the activation of the NFκB pathway has been shown to play a pivotal role in IKKε-induced transformation.¹²

Accumulated studies demonstrated that aberrant changes of a number of genes are associated with CDDP resistance in ovarian cancer.^{22–24} While our data show that IKKε protein levels generally correlated with CDDP IC₅₀ in ovarian cancer cell lines examined, the imperfect match in some cell lines could be due to other genetic alterations in these cells. For instance, A2780S is sensitive to CDDP (IC₅₀ 10 μmol/L) but expresses comparable IKKε, as compared with CDDP resistant cell A2780CP. Whereas knockdown of IKKε in A2780CP cells decreases IKKε protein expression even lower than A2780S (Figure 6), the CDDP IC₅₀ (30 μmol/L) of A2780CP/shRNA-IKKε is still higher than that of A2780S (eg, 10 μmol/L; Table 4). This could be due to the fact that A2780S expresses wild-type TP53 whereas A2780CP has mutant TP53. In contrast,

OV2008 and C13 cells have wild-type TP53, and partial knocked-down IKKε sensitizes C13 cells to CDDP-induced apoptosis. These findings suggest that TP53 and/or other genetic changes cooperate with IKKε to regulate CDDP resistance in ovarian cancer.

The data obtained from breast cancer have implicated that alterations of IKKε might be an early event in the development of some breast cancers due to the fact that amplification of this locus was observed in 4 of 10 ductal carcinoma *in situ*.¹² However, our study shows that elevated level of IKKε is more frequently observed in late stage and high grade tumors. Deregulation of IKKε is associated with poor prognosis. This discrepancy could be due to either different role of IKKε in ovarian and breast cancers or the limited number of breast cancer (eg, 30 cases) examined.¹²

In summary, our findings indicate that overexpression of IKKε is a highly recurrent event in ovarian cancer and could serve as not only a prognostic marker and pathogenetic factor but also as a critical therapeutic target. Future investigations are required for determining the mechanism by which IKKε is up-regulated in human ovarian cancer as well as its role in ovarian carcinogenesis *in vitro* and *in vivo*.

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