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Roles of functional *NFKB1* and *β-TrCP* insertion/deletion polymorphisms in mRNA expression and epithelial ovarian cancer susceptibility

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ABSTRACT. Epithelial ovarian cancer (EOC) is the leading cause of death among all gynecological cancers. Nuclear factor-kappa B (NF-κB) is involved in carcinogenesis and in the development of EOC. The β-transducin repeat-containing protein (β-TrCP) is a positive regulator of the NF-κB signaling pathway. Recent studies have indicated that the -94 ins/del ATTG polymorphism in the promoter region of the *NFKB1* gene, and the 9N ins/del polymorphism in the 3'-untranslated region of the *β-TrCP* gene are associated with increased susceptibility to a variety of cancers. We examined a potential association between these two polymorphisms and EOC. Genotypes were determined for

187 patients with EOC and 221 healthy control subjects, using the MassARRAY system. We found a significant association between the -94 ins/del ATTG genotype distribution and EOC. The frequency of the -94 del ATTG allele was significantly lower in EOC patients compared to healthy controls. The NF-κB mRNA level in cancer tissue was significantly correlated with -94 ins/del ATTG genotypes. Compared to the $ATTG_1/ATTG_1$ phenotype, the NF-κB mRNA level was 2.089 and 1.257 times higher in the $ATTG_2$ (insertion)/$ATTG_2$ homozygote and the $ATTG_1$ (deletion)/$ATTG_2$ heterozygote, respectively. However, we found no evidence of association between the 9N ins/del polymorphism of the *β-TrCP* gene and EOC in this Chinese population. Based on these results, we suggest that the NF-κB -94 ins/del ATTG polymorphism is a risk factor for EOC susceptibility.

Key words: Nuclear factor-kappa B; Polymorphism; Gene expression; Epithelial ovarian cancer; β-transducin repeat-containing protein

INTRODUCTION

Ovarian cancer is the 5th most frequently occurring cancer among women and the leading cause of gynecologic cancer deaths (Bertone-Johnson, 2005; Gulden and Olopade, 2010). Ovarian cancer has a low 5-year survival rate (~30%), primarily due to the lack of early symptoms and diagnostic tests for early-stage disease (Jemal et al., 2010). The pathogenesis of epithelial ovarian cancer (EOC) is multifactorial and, as a polygenic disease, multiple genetic factors may play an important role in disease development and progression (Gulden and Olopade, 2010). While intense research has focused on the molecular mechanisms of EOC development, leading to a great increase in our understanding of the disease process, the contribution of genetic variation to disease predisposition is still poorly understood. Despite this, several genes have been suggested as potential candidates for EOC progression. The identification of genetic risk markers related to EOC is important, as it may allow for the creation of predictive diagnostics for individual and population risks, and for the clarification of pathophysiological mechanisms relevant to EOC.

Nuclear factor-kappa B (NF-κB) is a critical transcription factor that regulates hundreds of genes involved in various biological activities including immune response, apoptosis, and cell-growth control (Karin, 2006). Mammals carry 5 members of the NF-κB family including p50/p105, p65/RelA, c-Rel, RelB, and p52/p100. Although many dimeric forms of NF-κB have been detected, the major form of NF-κB is a heterodimer of the p50 and p65/RelA subunits, encoded by the *NFKB1* and *RELA* genes, respectively (Lin et al., 2007; Annunziata et al., 2010). Recently, NF-κB signaling activation in ovarian cancer development and progression has been studied intensely (Chen et al., 2001; Karin, 2006; Maeda and Omata, 2008). Lin et al. (2007) suggested an important role for NF-κB in the propagation of ovarian cancer cell lines. Annunziata et al. (2010) found the NF-κB pathway is over-activated in aggressive ovarian cancers. In addition, a recent report by Fan et al. (2011) suggested that a functional promoter polymorphism in *NFKB1* gene increases the risk of advanced ovarian cancer in a population from northeast China. The genetic contribution of NF-κB to ovarian

cancer susceptibility must be evaluated to determine if NF-κB is a key component of oncogenesis in the ovary.

The β-transducin repeat-containing protein (β-TrCP), encoded by the *β-TrCP* gene, is a positive regulator of NF-κB signaling (Ougolkov et al., 2004). It functions in phosphorylation-dependent ubiquitination as one of 4 subunits in the SCF ubiquitin ligase complex, which targets β-catenin and IκBα for proteasomal degradation (Frescas and Pagano, 2008). It is well established that the ubiquitin-proteasome pathway is important in regulating protein signaling pathways that are involved in tumorigenesis. Previous studies have shown that β-TrCP possesses mainly oncogenic characteristics, and overexpression of β-TrCP is associated with several cancers (Kudo et al., 2004; Ougolkov et al., 2004). However, whether the overexpression of β-TrCP is associated with advanced ovarian cancer is still unclear. Further, the genetic contribution of *β-TrCP* to ovarian cancer susceptibility has not been investigated.

These studies suggest that NF-κB and β-TrCP are involved in the molecular pathology of tumorigenesis, potentially including those of ovarian origin. We report the evaluation of 2 functional polymorphisms in the *NF-κB* and *β-TrCP* genes in advanced ovarian cancer. We selected a -94 insertion/deletion ATTG (rs28362491) located between 2 key promoter regulatory elements in *NFKB1*, and a 9-bp (AACAGTGGA) insertion/deletion (rs16405) in the 3'-untranslated region (UTR) of *β-TrCP* for this case-control study.

MATERIAL AND METHODS

Subjects

Patients with pathologically confirmed EOC (N = 187; mean age of 51.2 ± 17.4) were recruited from among the inpatient population of the Department of Obstetrics and Gynecology at Xijing Hospital. Enrolled patients had no other malignancies. Clinical characteristics including age at diagnosis, FIGO stage, histological type, and tumor grade were obtained from medical records. Concurrently, 221 age-matched healthy controls (mean age of 49.8 ± 6.6) were recruited from the population of healthy women undergoing regular gynecological examination at Xijing Hospital. All participants are northern Han Chinese and not genetically related. Written informed consent was obtained from all participants. The study was approved by the Ethical Committee of Fourth Military Medical University, Xi'an, China.

Genotyping

Peripheral blood sample (3-5 mL) was collected from each subject in EDTA tubes. Genomic DNA was extracted with the TIANamp Blood DNA Kit (TIANGEN, Beijing, China) and stored at -20°C. Genotyping of the *NFKB1* and *β-TrCP* polymorphisms were performed by MassARRAY (Sequenom Inc., San Diego, CA, USA), which employs matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). Primers were designed using the Sequenom software (as shown in Table 1) and the extension reaction produced allele-specific products with masses differing by 30 Da, or approximately 1 nucleotide. Primer extension and PCR were performed according to manufacturer protocols, using iPLEX enzyme (Sequenom) and HotStarTaq DNA polymerase (Qiagen). Genotype identification was

performed in real time with the MassARRAY RT version 3.0.0.4 software and analyzed using the MassARRAY Typer version 3.4 software (Sequenom).

Table 1. Primer sequences used for genotyping the *NFKB1* gene SNPs with the MALDI-TOF Sequenom platform.

Polymorphism sites	Forward primers (5'→3')	Reverse primers (5'→3')	Extension primers (5'→3')
rs28362491	ACGTTGGATGCTCCGTGCTG CCTGCGTTC	ACGTTGGATGTAGGGAAG CCCCCAGGAAG	CGCCTGCCGGGCCCAAT
rs16405	ACGTTGGATGATGATGAGAG TATCATGTC	ACGTTGGATGAGTGAAGA TTTCAGCCCCAG	GCAAGTGACCCTGTGGC

Quantitative RT-PCR

Real-time RT-PCR was performed to determine NF-κB (p50) mRNA levels in EOC tissues. Total RNA was extracted from tumor tissue specimens with different genotypes using the RNAiso Plus kit (Takara, Shiga, Japan). cDNA was synthesized with oligo(dT)$_{15}$ primer and Superscript II (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcription-PCRs (qRT-PCRs) (25 µL) contained 2 µL cDNA, 12.5 µL SYBR Green (Applied Biosystems, Foster City, CA, USA), and the appropriate primers. Product accumulation was monitored by SYBR Green fluorescence on an ABI Prism 7000 Sequence Detection System. Relative expression was determined from a standard curve of serial dilutions of cDNA samples. Forward and reverse primers for NF-κB (p50) and β-actin were described previously (Wang et al., 2010). Data were normalized to β-actin as an internal control.

Statistical analyses

Allele and genotype frequencies of each polymorphism and Hardy-Weinberg equilibrium were evaluated by the Pearson chi-square test or the Fisher exact test. The normalized expression values of NF-κB (p50) were analyzed by one-way ANOVA followed by the Tukey test. Unconditional logistic regression was used to calculate the odds ratio and 95% confidence interval in independent association between each locus and the presence of epithelial ovarian cancer. All statistical analysis was carried out in SPSS 11.5 (IBM, Armonk, NY, USA). Bonferroni's correction was used in multiple testing, and the P value was divided by the total number of loci. The P-value threshold was set at 0.025.

RESULTS

Of the 187 EOC participants in this study, 106 (56.68%) were FIGO stage III, 99 (52.94%) were serous-papillary, and 132 (70.59%) were G3 tumor grade (Table 2). The allele and genotype frequencies and statistical analysis of the 2 polymorphisms in case and control participants are listed in Table 3. The genotype and allele distribution of both polymorphisms were in agreement with Hardy-Weinberg equilibrium. We found a strong linkage between the -94 ins/del ATTG genotype distribution and EOC (P = 0.008 after Bonferroni's correction). The frequency of the -94 del ATTG allele was significantly lower in EOC patients compared to healthy controls (χ^2 = 10.028, P = 0.002, OR = 0.632, 95%CI = 0.475-0.840). No signifi-

cant differences were found in the distribution of genotype and allele frequencies of the 9N ins/del polymorphism between EOC and healthy subjects ($P > 0.05$).

Table 2. Descriptive characteristics of the cases (N = 187).

Variable	Cases [N (%)]
FIGO stage	
I	36 (19.25)
II	16 (8.56)
III	106 (56.68)
IV	29 (15.51)
Histological type	
Serous-papillary	99 (52.94)
Mucinous	20 (10.70)
Endometrioid	42 (22.46)
Clear cell	8 (4.28)
Adenocarcinoma	7 (3.74)
Mix and other	11 (5.88)
Tumor grade	
G1	17 (9.09)
G2	30 (16.04)
G3	132 (70.59)
Unknown	8 (4.28)

Table 3. Distribution of genotype and allele frequencies of the *NFKB1* and *β-TrCP* gene insertion/deletion (ins/del) polymorphisms in epithelial ovarian cancer patients (N = 187) and controls (N = 221).

Position	Genotype/allele	Controls [N (%)]	Case [N (%)]	OR (95%CI)
rs28362491	-94ins ATTG/-94insATTG	71 (32.13)	83 (44.39)	1.00 (Reference)
	-94ins ATTG/-94delATTG	103 (46.61)	82 (43.85)	0.681 (0.443-1.046)
	-94del ATTG/-94delATTG	47 (21.26)	22 (11.76)	0.400 (0.220-0.728)
	$\chi^2 = 9.610$, P = 0.008*			
	-94 ins ATTG allele	245 (55.43)	248 (66.31)	0.632 (0.475-0.840)
	-94 del ATTG allele	197 (44.57)	126 (33.69)	
	$\chi^2 = 10.028$, P = 0.002*			
rs16405	9N ins/ins	20 (9.05)	22 (11.76)	1.00 (Reference)
	9N ins/del	97 (43.89)	88 (47.06)	0.825 (0.442-1.613)
	9N del/del	104 (47.06)	77 (41.18)	0.673 (0.343-1.320)
	$\chi^2 = 1.739$, P = 0.419			
	9N ins	137 (31.00)	132 (35.29)	1.214 (0.906-1.627)
	9N del	305 (69.00	242 (64.71)	
	$\chi^2 = 1.694$, P = 0.193			

*P values retained statistical significance after Bonferroni's correction ($P < 0.05$). OR = odd ratio; 95%CI = 95% confidence interval.

To test whether the transcription of NF-κB (p50) is regulated by the -94 ins/del polymorphism, we measured the expression of NF-κB (p50) mRNA in EOC tissues with different genotypes using real-time RT-PCR. Statistical analysis revealed significant effects of genotype on levels of p50 mRNA [$F(2, 44) = 7.963$, $P < 0.01$]. *Post-hoc* analysis revealed p50 mRNA levels in EOC tissue from patients homozygous for $ATTG_2/ATTG_2$ were significantly higher than in patients homozygous for $ATTG_1/ATTG_1$ ($P < 0.01$). A heterozygous effect on mRNA levels was also observed, $ATTG_1/ATTG_2$ ($P < 0.05$); however, there was no significant difference in p50 mRNA levels between heterozygous $ATTG_1/ATTG_2$ and homozygous $ATTG_1$ (Figure 1).

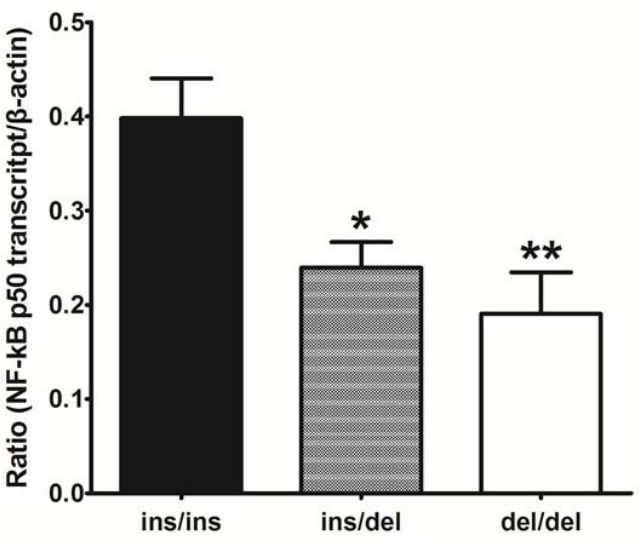

Figure 1. Transcript levels of NF-κB (p50) in epithelial ovarian cancer tissues of differing genotypes. Results are reported as a ratio of p50 transcript to β-actin. The mean ± standard error (N = 8) is shown. *P < 0.05 (one-way ANOVA followed by the Tukey test).

DISCUSSION

We examined the genetic influence of insertion/deletion variants in the *NF-κB* and *β-TrCP* genes in this case-control study of EOC. We identified a significant association between a functional polymorphism (-94 ins/del ATTG) in the promoter region of *NFKB1* and an increased risk of EOC. Carriers of the $ATTG_2$ allele (insertion) were 1.583-fold more likely to have EOC in comparison to non-carriers. Moreover, our study is the first to demonstrate that mRNA levels of NF-κB from EOC tissues significantly correlated with the -94 ins/del ATTG genotype. The highest levels of NF-κB were observed in EOC $ATTG_2$ homozygous tissues. In addition, 9N ins/del, a functional insertion/deletion polymorphism in the 3'-UTR of *β-TrCP*, was not associated with susceptibility to EOC.

The ubiquitin-proteasome pathway is thought to play an important role in tumorigenesis (Mani and Gelmann, 2005). β-TrCP family members are components of the ubiquitin ligase complex targeting IκBα for proteasomal degradation, and are thus positive regulators of NF-κB (Basak and Hoffmann, 2008). Several lines of evidence indicate β-TrCP possesses mainly oncogenic characteristics (Fuchs et al., 2004; Westbrook et al., 2008). Indeed, overexpression of *β-TrCP* has been reported in multiple tumors (Kudo et al., 2004). As one of the major transcriptional regulators of cell-growth control, apoptosis, and immune response genes, all positively regulated by β-TrCP, NF-κB is critically involved in carcinogenesis and the development of several cancers (Naugler and Karin, 2008; Karin, 2009), including EOC (Hernandez et al., 2010). Association of rs16405 in *β-TrCP* with *β-TrCP* mRNA expression has been reported (Chen et al., 2010). The presence of the 9-bp insertion allele in the 3'-UTR of *β-TrCP* may disrupt miR-920 binding, leading to relatively higher *β-TrCP* levels, which would result in increased NF-κB activity. However, we were unable to find an association be-

tween this functional insertion/deletion polymorphism of *β-TrCP* and advanced ovarian cancer. This result may support data suggesting the activation of NF-κB signaling by increased *β-TrCP* expression inhibits tumor metastasis (Ougolkov et al., 2004).

Concurrent with the 9-bp ins/del polymorphism in *β-TrCP*, we also evaluated the -94 ins/del ATTG polymorphism in the promoter region of *NFKB1*, which was initially observed in patients with ulcerative colitis (Karban et al., 2004). The presence of this 4-bp deletion results in loss of binding to nuclear proteins, leading to inhibition of NF-κB promoter activity (Karban et al., 2004). The -94 ins/del ATTG promoter polymorphism of *NF-κB* increases susceptibility to multiple cancers (Sun and Zhang, 2007; Lo et al., 2009; Zhou et al., 2009, 2010; Tang et al., 2010). Our results confirm the previously reported positive association of the 4-bp insertion allele with EOC in a Chinese population (Fan et al., 2011). The molecular mechanisms underlying our observations remain unclear; however, one possible explanation is that variants in *NF-κB* may lead to altered gene expression in the ovarian tumor.

Variations in DNA sequences contribute to individual differences in disease susceptibility. Previously published findings on the role of the -94 ins/del ATTG polymorphism on *NF-κB* promoter activity strengthen the hypothesis that the risk allele of *NF-κB* may cause aberrantly increased levels of p50/p105 NF-κB, which in turn results in transactivation of anti-apoptosis genes important for cell survival (Karban et al., 2004). Indeed, at least 5 studies have reported that presence of the insertion allele is associated with increased cancer risk and aggressive tumor behavior. Lin et al. (2007) have suggested a role for NF-κB in the propagation of ovarian cancer cell lines. Moreover, a recent report revealed that over-activated NF-κB may contribute to the development of EOC, and that the p50 subunit of NF-κB is significantly associated with poor overall survival in women with EOC (Annunziata et al., 2010). In this study, transcript levels of NF-κB in EOC tissues were correlated with different -94 ins/del ATTG genotypes. Our results showed that EOC tissues homozygous for $ATTG_2$ had the highest level of NF-κB (1.661- and 2.089-fold higher than heterozygous $ATTG_1/ATTG_2$ and homozygous $ATTG_1$, respectively). Based on our data and the NF-κB pathway's critical role in EOC development and aggressiveness, it is logical to assume that the risk allele of *NF-κB* may cause over-activation of NF-κB, increasing susceptibility to EOC by enhancing tumorigenesis, inhibiting apoptosis, and repressing the immune response. We examined the association between this polymorphism and NF-κB activation; however, the *in vivo* situation is likely to be more complex, as NF-κB could be regulated by different and multiple mechanisms (Chen et al., 2008; Dai et al., 2009). The precise mechanism by which the insertion/deletion alters promoter activity and NF-κB levels *in vivo* requires further investigation.

It should be noted that reports concerning the association of -94 ins/del polymorphism in *NF-κB* with disease have been conflicting. For instance, previous studies demonstrated an association of the deletion allele with ulcerative colitis (Borm et al., 2005), but others have not identified this polymorphism as a predisposing factor to this inflammatory intestinal disorder (Fyhn, 1988; Oliver et al., 2005). The discrepancy between studies could be the result of regional and ethnic differences in genotypes, and the sample size included in these studies. Since our study and Fan et al. (2011) suggest the polymorphism is associated with predisposition to EOC among Chinese, and the insertion allele increases EOC susceptibility, further research is needed to confirm and extend these findings in a different and larger ethnic population. These studies should help reveal the mechanism by which the *NF-κB* gene promoter polymorphism influences the EOC phenotype.

In summary, our study demonstrates that the -94 ins/del polymorphism in the *NF-κB* promoter, but not the 9 bp ins/del polymorphism in the 3'-UTR of *β-TrCP*, may influence EOC susceptibility, possibly through altered *NF-κB* gene expression, which is likely involved in the pathogenesis of EOC.

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