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Association of the genetic polymorphisms of *NFKB1* with susceptibility to ovarian cancer

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ABSTRACT. Nuclear factor-κB (NF-κB), a transcription factor that is activated by various stimuli, is associated with the pathogenesis of several cancers. One functional polymorphism, -94 insertion/deletion ATTG (rs28362491), in the human *NFKB1* gene (one member of the NF-κB gene family) is associated with increased risk of various cancers. However, only one study has reported that rs28362491 is significantly associated with ovarian cancer. The aim of this study was to analyze the association between single nucleotide polymorphisms (SNPs) and haplotypes in the *NFKB1* gene and the risk of ovarian cancer in a Chinese population. We examined the potential association between ovarian cancer and 15 SNPs (rs28362491, rs3774932, rs1598856, rs230531, rs230530, rs230528, rs230521, rs230498, rs230539, rs1005819, rs3774956, rs4648055, rs4648068, rs3774964, rs3774968) of the *NFKB1* gene using the MassARRAY system. Participants included 411 patients with ovarian cancer and 438 healthy controls. The results showed that the allelic or genotypic frequencies of three polymorphisms, including rs28362491 (promoter region), rs230521 (intron 4), and rs4648068 (intron 12), in the patients with ovarian cancer, were significantly different from those in the healthy controls. Strong linkage disequilibrium was observed in four blocks (D' > 0.9).

Significantly more A-C (block 2: rs230528-rs230521) haplotypes (P = 0.0003 after Bonferroni's corrections) and G-A-A (block 4: rs4648068-rs3774964-rs3774968) haplotypes (P = 0.021) were found in the patients with ovarian cancer. These findings point to a role of the *NFKB1* polymorphism in patients with ovarian cancer among a Chinese Han population, and may be informative for future genetic or biological studies on ovarian cancer.

Key words: Nuclear factor-κB; Single nucleotide polymorphisms; Ovarian cancer

INTRODUCTION

Ovarian cancer is the leading cause of death among all gynecological cancers. Its early symptoms are often not apparent, and its early diagnosis is therefore difficult, resulting in a low 5-year survival rate of approximately 30% (Jemal et al., 2010). As a polygenic disease, the pathogenesis of ovarian cancer is multifactorial, which means that multiple genetic factors may be associated with its development and progression (Gulden and Olopade, 2010). Previous studies have suggested that polymorphisms in nuclear factor-κB (NF-κB), a transcription factor that is activated by various stimuli, may be associated with ovarian cancer (Yang et al., 2014).

NF-κB is a major transcription regulator of immune response, apoptosis, and cell-growth control genes, and is involved in the pathogenesis of several cancers (Naugler and Karin, 2008; Karin, 2009), including epithelial ovarian cancer (EOC) (Hernandez et al., 2010). There are five members of the NF-κB family in mammals: p50/p105, p65/RelA, c-Rel, RelB, and p52/p100. The major form of NF-κB is a heterodimer of the p50 subunit, which is encoded by the *NFKB1* gene (Chen et al., 1999). The p50 subunit inhibits cell apoptosis by regulating several survival genes, such as *bcl-2* homologue *A1* (Karsan et al., 1996), *PAI-2* (Kumar and Baglioni, 1991), and the *IAP* gene family (LaCasse et al., 1998). Certain anti-apoptosis proteins, such as Bcl-xL, are upregulated through the NF-κB signaling pathway (Bernal-Mizrachi et al., 2006; Glauert et al., 2008). Furthermore, the p50 signaling pathway participates in cellular proliferation by regulating interleukin-5 (Yang et al., 1998), mitogen-associated protein kinase (Yu et al., 2009), and cyclin D1 (Shukla et al., 2005).

The human *NFKB1* gene encoding p50 is located on chromosome 4q24 and encodes a 50-kDa DNA-binding protein (Sun and Zhang, 2007; Yu et al., 2009). The first potential functional *NFKB1* polymorphism is rs28362491 (-94 insertion/deletion ATTG), which is located between two putative key promoter regulatory elements. An increasing number of studies have reported an association between rs28362491 and cancer risk, although conflicting results have been obtained (Lewander et al., 2007; Cai et al., 2013; Li et al., 2013). Moreover, only one study reported that rs28362491 in *NFKB1* was significantly associated with ovarian cancer (Huo et al., 2013). In fact, a limited number of single nucleotide polymorphisms (SNPs) could not effectively capture the true causative SNPs in the *NFKB1* gene owing to the weak linkage disequilibrium (LD) between them. Thus, to exactly identify the association of *NFKB1* SNPs with ovarian cancer, it is necessary to use a more powerful technique that can identify the SNPs as precisely as possible.

To verify the putative association between the *NFKB1* SNPs and ovarian cancer, in the present study we investigated the association between 15 SNPs (rs28362491, rs3774932,

rs1598856, rs230531, rs230530, rs230528, rs230521, rs230498, rs230539, rs1005819, rs3774956, rs4648055, rs4648068, rs3774964, rs3774968) of the *NFKB1* gene and the risk of ovarian cancer in a Chinese Han population.

MATERIAL AND METHODS

Subjects

All the patients with ovarian cancer underwent careful clinical examinations, including hysteroscopy or hysterosalpingography, transvaginal ultrasound, serial endometrial biopsies, and analyses of tissue antibodies and autoantibodies. Four hundred and ten patients with pathologically confirmed ovarian cancer (mean age of 49.3 ± 8.2 years) were recruited from the in-patient department of gynecology in our hospital from January 2008 to April 2013. Patients with other malignancies were excluded. Four hundred and forty-two women (mean age of 50.6 ± 6.5 years) were recruited as healthy controls in the medical examination center of our hospital. Exclusion criteria were: taking other prescribed medications that could affect the central nervous system; history of seizures, hematological diseases, or severe liver or kidney damage; smoking; hypertension; and previous use of oral contraceptives. All participants were from a non-genetically related Chinese Han population in Zhejiang Province (China). The study was performed according to the guidelines of the Medical Ethics Committee of our hospital (Wenzhou, China). Written informed consent was obtained from all the participants.

SNP selection

A total of 15 SNPs located on the *NFKB1* gene with a genomic size of 116 kb were selected for genotyping. Marker selection was done based on previous studies (Cai et al., 2013; Li et al., 2013), and preliminary analysis was performed using HapMap data (HapMap data release 27). We examined tagSNPs in the Chinese Han population of Beijing using the Haploview software v4.2, with a minor allele frequency cut-off of ≥5%. The LD pattern of the *NFKB1* gene was determined in the Chinese population using the preliminary data from HapMap. The SNPs were further analyzed in an association study.

Genotyping

Peripheral blood (3-5 mL) was collected from the subjects and preserved in tubes coated with ethylenediaminetetraacetic acid. Genomic DNA was extracted using a TIANamp Blood DNA Kit (TIANGEN Biotech, Beijing, China) and stored at -4°C until use. Genotyping was carried out for all SNPs using the MassARRAY platform (Sequenom Inc., San Diego, CA, USA). Primer extension and polymerase chain reaction were performed according to the manufacturer instructions, using iPLEX enzyme (Sequenom Inc.) and HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). The completed genotyping reactions were spotted onto a 384-well SpectroCHIP (Sequenom Inc.) using a MassARRAY Nanodispenser (Sequenom Inc.), and determined by a matrix-assisted laser desorption ionization time-of-flight mass spectrometer. Genotype calling was performed in real-time with the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom Inc.).

Statistical analysis

All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Each SNP was tested for deviation from Hardy-Weinberg equilibrium using the Pearson chi-square test or the Fisher exact test. Differences between the cases and controls in the frequency of the alleles and genotypes were evaluated by the Fisher exact test or the Pearson chi-square test. Subject age was treated as a covariate in binary logistic regression. P values were calculated based on codominant, dominant for the rare allele, heterosis, and recessive for the rare allele models of inheritance. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI) in independent association between each locus and the presence of ovarian cancer. Haplotype blocks were defined according to the criteria developed by Gabriel et al. (2002). Pairwise LD statistics (D' and r^2) and haplotype frequency were calculated, and haplotype blocks were constructed using Haploview 4.0 (Barrett et al., 2005). The significance of any haplotypic association was evaluated using a likelihood ratio test, followed by a permutation testing that compared estimated haplotype frequencies in cases and controls (Zhao et al., 2000, 2002; Curtis et al., 2006). Bonferroni's correction was used to adjust the test level when multiple comparisons were conducted, and the P value was divided by the total number of loci.

RESULTS

The genotype frequency and distribution of the 15 SNPs were in agreement with Hardy-Weinberg equilibrium. Pairwise LD analyses of the patients with ovarian cancer and healthy controls revealed that SNPs rs3774932 and rs1598856, SNPs rs230528 and rs230521, SNPs rs3774956 and rs4648055, and SNPs rs4648068, rs3774964, and rs3774968 were located in haplotype blocks 1, 2, 3, and 4, respectively (D' > 0.9; Figure 1).

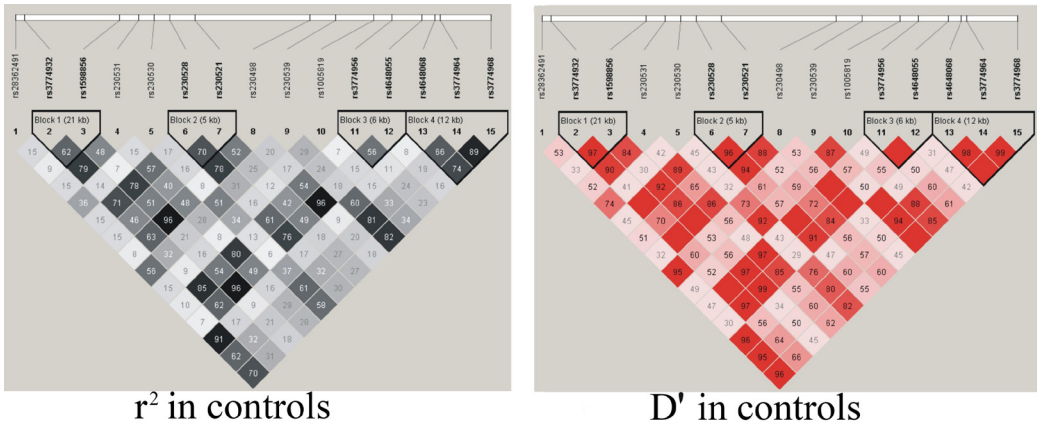

Figure 1. Linkage disequilibrium (LD) plot of the 15 single nucleotide polymorphisms (SNPs) in the *NFKB1* gene. Values in squares are the pairwise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e., perfect LD between a pair of SNPs). Empty squares indicate D' = 1 (i.e., complete LD between a pair of SNPs).

The distributions of genotype and the allele frequencies of the 15 SNPs are listed in Table 1.

Table 1. Genotypic and allelic frequencies of NFKB1 polymorphisms in the controls and patients with ovarian cancer.

Variable	ID	Position	MAF	Controls (N = 442)		Ovarian cancer (N = 410)		P value[a]	OR (95%CI)
				No.	%	No.	%		
rs28362491	102500998:102501001	Promoter	0.458					0.003	
Del/Del				122	27.6	95	23.2	0.138	1.264 (0.927-1.724)
Ins/Del				235	53.2	195	45.1	0.104	1.251 (0.955-1.639)
Ins/Ins				85	19.2	120	31.7	0.001	0.575 (0.418-0.791)
Per Ins allele				405	45.8	445	54.3	0.001	1.367 (1.130-1.653)
rs3774932	102503036	Promoter	0.391					0.848	
AA				162	36.7	149	36.3	0.543	0.917 (0.693-1.213)
AG				214	48.4	194	47.3	0.857	1.025 (0.783-1.341)
GG				66	14.9	67	16.3	0.572	1.113 (0.768-1.611)
Per G allele				346	39.1	328	40.0	0.717	1.037 (0.854-1.259)
rs1598856	102524958	Intron 1	0.492					0.199	
CC				123	28.0	102	25.1	0.265	0.839 (0.620-1.136)
TC				201	45.7	211	51.8	0.083	1.269 (0.969-1.662)
TT				116	26.4	94	23.1	0.303	1.177 (0.863-1.607)
Per T allele				433	49.2	399	49.0	0.939	0.993 (0.820-1.201)
rs230531	102529220	Intron 1	0.396					0.095	
TT				156	35.3	174	42.4	0.265	1.171 (0.887-1.545)
TC				222	50.2	180	43.9	0.364	0.883 (0.674-1.156)
CC				64	14.5	56	13.7	0.728	1.071 (0.727-1.578)
Per C allele				350	39.6	292	35.6	0.090	0.844 (0.693-1.027)
rs230530	102532823	Intron 2	0.437					0.198	
TT				136	30.8	150	39.0	0.073	0.771 (0.579-1.025)
CT				226	51.1	193	44.6	0.238	1.176 (0.898-1.539)
CC				80	18.1	67	16.3	0.495	1.132 (0.792-1.618)
Per C allele				386	43.7	317	38.7	0.495	1.132 (0.792-1.618)
rs230528	102536428	Intron 3	0.474					0.563	
CC				125	28.3	104	25.4	0.231	0.830 (0.613-1.125)
AC				215	48.6	213	52.0	0.277	1.161 (0.887-1.520)
AA				102	23.1	93	22.7	0.893	1.022 (0.742-1.408)
Per A allele				419	47.4	399	48.7	0.603	1.052 (0.870-1.272)
rs230521	102542171	Intron 4	0.404					0.013	
CC				163	36.9	191	51.5	0.007	1.465 (1.112-1.930)
GC				201	45.5	164	40.0	0.148	0.818 (0.622-1.074)
GG				78	17.6	55	8.5	0.090	1.383 (0.951-2.013)
Per G allele				357	40.4	274	33.4	0.003	0.741 (0.608-0.903)
rs230498	102568446	Intron 5	0.497					0.362	
GG				122	27.6	109	26.6	0.895	0.980 (0.724-1.326)
GA				201	45.5	205	50.0	0.200	1.193 (0.911-1.562)
AA				119	26.9	96	23.4	0.239	1.205 (0.884-1.645)
Per A allele				439	49.7	397	48.4	0.607	0.951 (0.787-1.151)
rs230539	102574375	Intron 5	0.423					0.100	
AA				139	31.6	128	31.4	0.887	1.021 (0.765-1.363)
GA				230	52.3	191	46.9	0.169	0.828 (0.633-1.084)
GG				71	16.1	88	21.6	0.036	0.694 (0.493-0.917)
Per G allele				372	42.3	367	45.1	0.243	1.121 (0.925-1.359)
rs1005819	102583148	Intron 5	0.346					0.998	
CC				186	42.1	172	42.0	0.701	0.948 (0.772-1.245)
TC				206	46.6	192	46.8	0.836	0.972 (0.742-1.273)
TT				50	11.3	46	11.2	0.959	1.011 (0.661-1.548)
Per T allele				306	34.6	284	34.6	0.994	1.001 (0.820-1.222)
rs3774956	102587369	Intron 9	0.355					0.868	
TT				184	41.6	178	43.4	0.812	0.967 (0.737-1.271)
CT				202	45.7	181	44.1	0.863	1.024 (0.781-1.342)
CC				56	12.7	51	12.4	0.915	1.022 (0.681-1.535)
Per C allele				314	35.5	283	34.5	0.663	0.957 (0.184-1.168)

Continued on next page

Table 1. Continued.

Variable	ID	Position	MAF	Controls (N = 442)		Ovarian cancer (N = 410)		P value[a]	OR (95%CI)
				No.	%	No.	%		
rs4648055	102594156	Intron 10	0.494					0.924	
AA				123	27.8	111	27.1	0.608	0.924 (0.684-1.249)
AG				201	45.5	192	46.8	0.672	1.060 (0.809-1.388)
GG				118	26.7	107	26.1	0.843	1.031 (0.760-1.399)
Per G allele				437	49.4	406	49.5	0.974	1.003 (0.829-1.213)
rs4648068	102597148	Intron 12	0.454					0.0005	
AA				123	27.8	95	23.2	0.120	1.278 (0.938-1.743)
AG				237	53.6	192	45.6	0.048	1.313 (1.002-1.719)
GG				82	18.6	123	31.2	0.0001	0.531 (0.386-0.731)
Per G allele				401	45.4	438	53.2	0.001	1.381 (1.141-1.671)
rs3774964	102598330	Intron 13	0.449					0.126	
AA				127	28.7	125	31.7	0.578	0.920 (0.685-1.235)
GA				233	52.7	190	45.1	0.064	1.290 (0.986-1.690)
GG				82	18.6	95	23.2	0.097	0.755 (0.542-1.053)
Per G allele				397	44.9	375	45.7	0.733	1.034 (0.854-1.251)
rs3774968	102609955	Intron 17	0.472						
AA				115	26.0	122	31.2	0.224	0.830 (0.615-1.121)
GA				237	53.6	193	45.6	0.056	1.300 (0.993-1.702)
GG				90	20.4	95	23.2	0.321	0.848 (0.621-1.174)
Per G allele				417	47.2	383	46.7	0.848	0.982 (0.811-1.187)

[a]P value was calculated by 2 x 3 and 2 x 2 chi-squared tests based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance. Alpha value is adjusted by Bonferroni's correction and statistically significant results (P < 0.003). MAF = minor allele frequency in controls.

The distributions of haplotype frequencies are listed in Tables 2-5.

Table 2. NFKB1 haplotype in block 1 frequencies and the results of their associations with risk of ovarian cancer.

	Haplotype[a]		Frequency (%)		P value[a]
ID	rs3774932	rs1598856	Cases	Controls	
HAP1	A	C	0.487	0.502	0.673
HAP2	G	T	0.400	0.386	0.695
HAP3	A	T	0.113	0.106	0.784

[a]Based on comparison of frequency distribution of all haplotypes for the combination of SNPs. Alpha value is adjusted by Bonferroni's correction and statistically significant results (P < 0.025).

Table 3. NFKB1 haplotype in block 2 frequencies and the results of their associations with risk of ovarian cancer.

	Haplotype		Frequency (%)		P value[a]
ID	rs230528	rs230521	Cases	Controls	
HAP1	C	C	0.509	0.519	0.808
HAP2	A	G	0.330	0.376	0.158
HAP3	A	C	0.157	0.078	0.0003

[a]Based on comparison of frequency distribution of all haplotypes for the combination of SNPs. Alpha value is adjusted by Bonferroni's correction and statistically significant results (P < 0.025).

Table 4. NFKB1 haplotype in block 3 frequencies and the results of their associations with risk of ovarian cancer.

Haplotype			Frequency (%)		P value[a]
ID	rs3774956	rs4648055	Cases	Controls	
HAP1	T	A	0.502	0.506	0.899
HAP2	C	G	0.342	0.355	0.674
HAP2	T	G	0.153	0.139	0.517

[a]Based on comparison of frequency distribution of all haplotypes for the combination of SNPs. Alpha value is adjusted by Bonferroni's correction and statistically significant results (P < 0.025).

Table 5. NFKB1 haplotype in block 4 frequencies and the results of their associations with risk of ovarian cancer.

Haplotype[#]				Frequency (%)		P value[a]
ID	rs4648068	rs3774964	rs3774968	Cases	Controls	
HAP1	G	A	A	0.529	0.451	0.021
HAP2	A	G	G	0.459	0.447	0.757

[a]Based on comparison of frequency distribution of all haplotypes for the combination of SNPs. [#]Haplotypes with frequency <0.05 were excluded. Alpha value is adjusted by Bonferroni's correction and statistically significant results (P < 0.0167).

The difference in the distribution of genotype frequencies of rs28362491 between the patients with ovarian cancer and healthy controls was significant (P = 0.003). The patients with ovarian cancer had a significantly higher frequency of the $ATTG_2$ allele (insertion, P = 0.001, OR = 1.367, 95%CI = 1.130-1.653). The analysis revealed an association between the rs230521 genotype distribution and ovarian cancer (P = 0.013). The patients with ovarian cancer had a significantly lower frequency of the G allele (P = 0.003, OR = 0.741, 95%CI = 0.608-0.903). There was a significant between-group difference in the genotype distribution of rs4648068 (P = 0.0005). The patients with ovarian cancer had a significantly lower frequency of the T allele of rs4648068 (P = 0.001, OR = 1.381, 95%CI = 1.141-1.671).

Significantly more A-C (block 2: rs230528-rs230521) haplotypes (P = 0.0003 after Bonferroni's corrections) were found in ovarian cancer subjects. Compared with the healthy controls, significantly more G-A-A (block 4: rs4648068-rs3774964-rs3774968) haplotypes (P = 0.021) were found in the patients with ovarian cancer. However, this association was no longer significantly different after Bonferroni's corrections (P > 0.0167).

DISCUSSION

The activation of NF-κB signaling is frequently found in the pathogenesis of ovarian cancer (Chen et al., 2001; Karin, 2006; Lin et al., 2007; Maeda and Omata, 2008; Annunziata et al., 2010). Our results provide direct evidence that a genetic change in *NFKB1* is linked to ovarian cancer in humans, and extend the list of variants that may affect the development of ovarian cancer (Huo et al., 2013).

The study of *in vitro* promoter expression indicated that the *ATTG2* allele may increase the mRNA expression of the *NFKB1* gene, resulting in the production of p50/p105 NF-κB protein (Karban et al., 2004). The study indicated that individuals homozygous for *ATTG2* had a 2.560-fold risk of developing cervical squamous cell carcinoma compared with

those homozygous for *ATTG1*. Individuals with the *ATTG2* allelotype had a 1.493-fold risk of cervical squamous cell carcinoma compared with those carrying the *ATTG1* allelotype (Zhou et al., 2010). A recent study by Fan et al. (2011) suggested that a functional promoter polymorphism in the *NFKB1* gene increases the risk of advanced ovarian cancer in a population from Northeast China. In addition, a meta-analysis of all eligible studies in 2010 suggested that the deletion allele serves as a protective or risk allele for cancer susceptibility among Asians or Caucasians, respectively (Zou et al., 2011). We identified a significant association between a functional polymorphism (rs28362491) in the promoter region of *NFKB1* and an increased risk of ovarian cancer. Individuals with the *ATTG2* allele (insertion) had a 1.367-fold risk of ovarian cancer compared with non-carriers. Interestingly, the study by Huo et al. (2013) demonstrated that the mRNA level of NFKB1 in EOC tissues significantly correlated with the -94 insertion/deletion ATTG genotype; the highest level of NFKB1 was observed in EOC *ATTG2* homozygous tissues. This correlation may be associated with the enhanced expression and activity of p50. It has been reported that the insertion allele is associated with an increased activity of the *NFKB1* promoter and enhanced *NFKB1* mRNA expression (Karban et al., 2004; Riemann et al., 2007). Indeed, at least five studies have reported that the presence of the insertion allele is associated with increased cancer risk and aggressive cancer behavior. Lin et al. (2007) have suggested a role for NF-κB in the propagation of ovarian cancer cell lines. Moreover, a recent report has revealed that the overactivation of NF-κB may contribute to the development of EOC, and that p50 is significantly associated with the overall poor survival rate of women with EOC (Annunziata et al., 2010).

In this case-control association study, the G alleles of *NFKB1* rs230521 were strongly associated with the decreased risk of ovarian cancer, and the T allele of *NFKB1* rs4648068 was associated with decreased risk of ovarian cancer. To the best of our knowledge, this is the first study that reports a significant association of two SNPs (rs230521 and rs4648068) in the *NFKB1* gene with ovarian cancer. To some extent, this finding further supports a role of *NFKB1* promoter polymorphism in ovarian cancer. Our studies could help reveal the mechanism by which the *NFKB1* gene polymorphisms influence the ovarian cancer phenotype. We further investigated the interaction among polymorphisms and observed strong LD. Haplotype analysis revealed that more A-C (rs230528-rs230521) and G-A-A (rs4648068-rs3774964-rs3774968) haplotypes were found in the patients with ovarian cancer, which indicated that these two haplotypes of the *NFKB1* gene displayed a risk effect. These results suggest that people with these two haplotypes of the *NFKB1* gene are more prone to develop ovarian cancer. This finding further supports a role of *NFKB1* polymorphisms in ovarian cancer, with differences in the populations of the association between ethnic groups.

The main strengths of this study include: a systematical screening of the functional SNPs in the promoter region, 5'- and 3'-untranslated region, exons of the *NFKB1* gene, and the homogeneity of the study subjects representing the Chinese Han population. The potential limitation of this study is the lack of data proving the positive association observed for rs230521 and rs4648068. Furthermore, the association of the serum level of NFKB1 with ovarian cancer still needs to be investigated.

In conclusion, these findings encourage future efforts aimed at identifying functional polymorphisms within, and close to, the *NFKB1* gene using a systemic approach in a larger sample set. Our study could improve the understanding of the mechanism of ovarian cancer, and help in the development of more efficient therapy.

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