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Original Article

Transcription factor FOXP3 gene variants affect epithelial ovarian carcinoma in the Han Chinese population

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Abstract: Background: Epithelial ovarian cancer (EOC) is the most common cause of death among gynecological cancers. *FOXP3* gene is the most dependable marker for regulatory T cells (Treg) which play a major role in immune tolerance. The aim of this study was to explore whether the *FOXP3* gene polymorphisms (rs3761548 A/C and rs5902434del/ATT) were associated susceptibility and prognosis for EOC. Methods: A total of 455 ovarian cancer patients and 337 healthy female controls were enrolled. Genotyping of *FOXP3* polymorphisms rs3761548 A/C was determined by polymerase chain reaction-restrictive fragment length polymorphism (PCR-RFLP), while rs5902434 del/ATT was directly visualized in a 6% polyacrylamide gel electrophoresis stained after PCR. Kaplan-Meier method and Cox regression analysis were used to find an association between the *FOXP3* gene and survival of EOC patients. Results: Data showed that AC genotype of *FOXP3* rs3761548 was associated with the high susceptibility of EOC (overdominant model: OR=1.42, 95% CI=1.07-1.89, *P*=0.015), while AA genotype showed lower risk for ovarian cancer compared with CC/AC genotypes (OR=0.45, 95% CI=0.23-0.90, *P*=0.022). In contrast, there were no significant differences for rs5902434 polymorphism of *FOXP3* in ovarian cancer patients and controls. However, del/ATT genotype might be an independent risk factor for EOC prognosis in the dominant (HR=2.60, 95% CI=1.26-5.38, *P*=0.010) and overdominant (HR=2.46, 95% CI=1.31-4.61, *P*=0.005) models. Conclusions: Our findings suggest that rs3761548 could contribute to EOC risk in a Chinese Han population. Rs5902434 polymorphisms might be a marker to identify high risk patients.

Keywords: Epithelial ovarian cancer, FOXP3, gene polymorphisms, regulatory T cell

Introduction

Epithelial ovarian cancer (EOC) comprises approximately 90% of all ovarian neoplasms, which is the most lethal gynecologic malignancy. Studies have shown that EOC is composed of a diverse group of tumors that can be classified based on distinctive morphologic and molecular genetic features [1]. The histological subtypes of EOC include serous, mucinous, endometrioid, clear cell, undifferentiated carcinoma, and malignant mixed mesodermal tumors. Since the majority of ovarian cancer patients are not diagnosed until advanced stages, the five-year survival rate is low. Otherwise, early stage EOC is favorable with a survival rate of approximate 90% [2]. Furthermore, theoretical concerns are that EOC may develop and spread exceptionally rapidly, leaving only a small window of opportunity for early detection. For these reasons, early detection, diagnosis, and clinical cure could theoretically improve outcomes, but highly sensitive and specific biomarkers for diagnostic and prognostic remain a

challenging problem. Thus, improved diagnosis and treatment remain a pressing need for management of EOC [3].

Increasing evidence indicates that EOC is an immunogenic disease which is kept at a delicate balance between pro-inflammatory and suppressive networks [4]. In EOC, the balance between effector and suppressor cells might determine the fate of tumor as well as the clinical outcome of patients. The tumor microenvironment plays a key role in EOC genesis and progression [5], among which Tregs are considered pivotal regulators of immune suppression and peripheral tolerance. Tregs develop in the thymus (natural) or periphery (acquired) and typically express CD4, CD25, and human Forkhead Box P3 (FOXP3) [6]. Their presence influences the occurrence, development, and prognosis of tumors [7].

FOXP3 gene (Gene ID: 50943, MIM number: 300292) is an important member of the family of the forked head transcription factor, located on chromosome Xp11.23 and consisting of 12 exons about 14.39 kb, FOXP3 protein consists of 431 amino acids. Studies have shown that $CD4^+CD25^+$Treg cells are a specific subpopulation of T cells, which might play a major role in the prevention of transplantation tolerance and autoimmunity. *FOXP3* is a characteristic marker of Treg cells except CD4 and CD25 molecules. It is also a key molecule in cell development and function of Treg cells, and contributes to converting naive T cells to Treg-like cells with suppressive activity [8]. Up to now, there have been described 13 single nucleotide polymorphisms (SNPs) in this gene. Among them: rs3761547, rs2232365, rs2294021, rs3761548 and rs5902434 are more common among Asian populations, and locate in the promoter region of FOXP3 gene. Although these loci mutation do not directly change the amino acid sequence of the protein, they can reduce binding to the *FOXP3* gene and the relative transcription factors, consequently affecting the expression level of *FOXP3* in Treg cells [9].

Based on this background, we hypothesized that variants in genes activated in suppressive immune cells might associate with occurrence, development, or prognosis of EOC patients. The objective of this study was to explore the influence of *FOXP3* (rs3761548 and rs5902434) on the prevalence of EOC for patients attending a hospital-based clinic in southwest district of China and its correlation with established clinical information and clinical prognostic factors.

Materials and methods

Study population and inclusion/exclusion criteria

This study was approved by the West China Second University Hospital of Sichuan University Ethics Committee and all subjects had written informed consent to participate. A hospital based case-control study included 455 unrelated patients with EOC and 337 healthy women as controls. Patients from the West China Second University Hospital of Sichuan University were enrolled in the current study after an informed consent during 2007 to 2016. Clinical and follow-up data of patients were abstracted from telephone calls every six months for five years. All the diagnosis of patients was confirmed by histological examination of tissue from resected specimens. Patients with borderline ovarian tumors, two or more different malignancies, autoimmune and infectious diseases, or metastasized cancer from other origins which might affect the final results were excluded. The control subjects had no genetic relationship with each other from a routine health survey in the same hospital, excluding the one with personal or family history of EOC or other severe diseases. All subjects were Han population living in southwest of China.

Tissue collection, DNA extraction

Genomic DNA of each patient was isolated from a paraffin section of the tumor tissue sample by genomic DNA isolation kit (Bioteke, Beijing, China). DNAs of healthy individuals was extracted from 200 μl EDTA-anticoagulated peripheral blood sample by a whole-blood DNA isolation kit (Peking, China). The procedure was performed according to the instructions of the manufacturer and the products were stored at -20°C until needed.

Genotyping of FOXP3 polymorphisms

Genotyping was performed using the PCR-RFLP method. In brief, the primer sequences were: F: 5'-GAAGGGCAAATTGAAGACCA-3', R: 5'-GGTGCTGAGGGGTAAACTGA-3' for rs3761548; F:

Table 1. Characteristics of the studied population

	Patients	Controls
Sample size, N	455	337
Age ± SD, years	51.1±9.7	54.2±13.1
Clinical histology, N		
Serous ovarian cancer	377	
Other types	78	
Tumor stage, N		
I	51 (11.2%)	-
II	49 (10.8%)	-
III	331 (72.7%)	-
IV	24 (5.3%)	-
Tumor grade, N		
G1-G2	57 (12.5%)	-
G3	376 (82.6%)	-
NA	22 (4.9%)	-

5'-CC-CTGCCCATGCATTAAGTA-3', R: 5'-TACCCA-GCTACCGTGATTCC-3' for rs5902434. DNA fragments containing the polymorphisms were amplified in a total volume of 10 μl, including 5 μl Mix, 0.10 μl each primer, 100 ng of genomic DNA and 3.8 μl distilled water. The PCR conditions were 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, with a final elongation at 72°C for 10 min for both SNPs. PCR products were digested overnight for rs3761548 with specific restriction enzyme; whereas rs5902434 didn't require digestion.

The above products were visualized in a 6% polyacrylamide gel electrophoresis stained by the 1.5 g/L argent nitrate to detect the quality and the quantity of the products: Pst I for rs3761548 allele C was digestable, appearing two fragments of 123 bp and 24 bp, allele A is digestable and the fragment is still 147 bp. Insert fragment length of rs5902434 was 102 bp. About 10% of the samples were randomly selected to carry out the repeated assays with the results being 100% concordant.

Survival analysis

The patients' clinical characteristics (**Table 1**) were evaluated for clinical prognostic factors, including age, tumor stage (I, II, III, IV), tumor grade (G1, G2, G3) and histological type (serous ovarian cancer, no-serous ovarian cancer). We obtained follow-up data by reviewing the inpatient charts, contacting patients or correspondence. As a result, 246 patients were available for survival analysis with a range of 2-204 months.

Statistical analysis

Differences of genotype and allele frequency between the cases and controls were evaluated using chi-square test, with odds ratios (ORs) and 95% confidence intervals (95% CI). Pearson's chi-square test was used to test Hardy-Weinberg equilibrium among the cases and controls. Genotypic association tests were done in a case-control pattern assuming codominant, dominant, recessive or overdominant genetic models and was performed using SNP States.

Survival rates were calculated with the Kaplan-Meier method, with the association between *FOXP3* SNPs genotypes and patients, outcome from the date of primary diagnosis until recurrence or death evaluated by the log rank test. We also used multivariate survival analysis carried out by Cox regression analysis to determine the independent effects on recurrence and survival. A level of $P<0.05$ was considered to be statistically significant. SPSS for Windows™ 22.0 (SPSS, Inc., Chicago, IL, USA) was used for the statistical analyses.

Results

Association between FOXP3 gene polymorphisms and EOC

Genotype frequencies of controls were in agreement with that expected under the Hardy-Weinberg equilibrium. Genotype distributions and allele frequencies of *FOXP3* in patients and controls were shown in **Table 2**. A significant difference in rs3761548 polymorphism among patients and healthy people was presented (P=0.010, OR=1.35, 95% CI=1.01-1.80 in codominant genetic model; *P*=0.022, OR=0.45, 95% CI=0.23-0.90 in recessive model; P=0.015, OR=1.42, 95% CI=1.07-1.89 in overdominant model). While for rs5902434 gene polymorphism, there was no significant difference observed between patients and controls.

Results were stratified by tumor stage, tumor grade, histology type, and age with these two SNPs are presented in **Tables 3**, **4**. In statistical analyses stratified by histology type, the

Table 2. Genotypic frequencies of the *FOXP3* polymorphisms between patients and controls and their associations with the risk of EOC

Model	rs3761548					rs5902434				
	Genotype	Patients N (%)	Controls N (%)	OR (95% CI)	*P*-value	Genotype	Patients N (%)	Controls N (%)	OR (95% CI)	*P*-value
Codominant	CC	218 (47.9%)	179 (53.1%)	1.00		del/del	170 (37.6%)	140 (72.4%)	1.00	
	AC	223 (49%)	136 (40.4%)	**1.35 (1.01-1.80)**	**0.010**	del/ATT	218 (47.9%)	144 (42.7%)	1.24 (0.91-1.68)	0.35
	AA	14 (3.1%)	22 (6.5%)	0.52 (0.26-1.05)		ATT/ATT	66 (14.5%)	53 (15.7%)	1.02 (0.67-1.56)	
Dominant	CC	218 (47.9%)	179 (53.1%)	1.00		del/del	171 (37.6%)	140 (41.5%)	1.00	
	AC/AA	237 (52.1%)	158 (46.9%)	1.23 (0.93-1.63)	0.15	del/ATT + del/del	284 (62.4%)	197 (58.5%)	1.18 (0.88-1.57)	0.26
Recessive	CC/AC	441 (96.9%)	315 (93.5%)	1.00		ATT/ATT + del/ATT	389 (85.5%)	284 (84.3%)	1.00	
	AA	14 (3.1%)	22 (6.5%)	**0.45 (0.23-0.90)**	**0.022**	del/del	66 (14.5%)	53 (15.7%)	0.91 (0.61-1.35)	0.63
Overdominant	CC/AA	232 (51%)	201 (59.6%)	1.00		ATT/ATT + del/del	237 (52.1%)	193 (57.3%)	1.00	
	AC	223 (49%)	136 (40.4%)	**1.42 (1.07-1.89)**	**0.015**	del/ATT	218 (47.9%)	144 (42.7%)	1.23 (0.93-1.64)	0.15
Allele	C	659 (72%)	494 (73%)	1.00		del	560 (62%)	424 (63%)	1.00	
	A	251 (28%)	180 (27%)	0.96 (0.77-1.20)	0.69	ATT	350 (38%)	250 (37%)	0.94 (0.77-1.16)	0.58

N corresponds to the number of individuals.

Table 3. Association between the genotype distribution of rs3761548 polymorphism of *FOXP3* gene and clinical features of EOC

Clinical features	rs3761548											
	Genotype			Genetic model								
	C/C	A/C	A/A	Codominant (C/C vs. A/C vs. A/A)		Dominant (C/C vs. A/C-A/A)		Recessive (C/C-A/C vs. A/A)		Overdominant (C/C-A/A vs. A/C)	
				OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Tumor stage											
I-II	36	43	3	0.92 (0.55-1.53)	0.93	0.91 (0.55-1.51)	0.72	0.88 (0.23-3.32)	0.85	0.93 (0.56-1.43)	0.77
III-IV	164	175	12	0.84 (0.22-3.26)							
Tumor grade											
G1- G2	23	32	2	1.49 (0.80-2.77)	0.43	1.49 (0.81-2.75)	0.19	1.24 (0.26-5.93)	0.79	1.44 (0.79-2.64)	0.23
G3	177	186	13	1.55 (0.31-7.70)							
Histology											
Serous	168	195	14	**1.92 (1.04-3.54)**	0.072	**1.98 (1.08-3.61)**	**0.025**	2.35 (0.28-19.81)	0.38	**1.80 (0.99-3.30)**	**0.050**
Others	32	23	1	3.27 (0.38-3.61)							
Age											
<50	86	105	6	0.81 (0.55-1.17)	0.51	0.82 (0.56-1.21)	0.32	1.25 (0.43-3.58)	0.68	0.80 (0.55-1.17)	0.25
≥50	114	113	9	1.11 (0.83-3.26)							

Boldfaced values indicate a significant difference at the 5% level.

Table 4. Association between the genotype distribution of rs5902434 polymorphism of *FOXP3* gene and clinical features of EOC

Clinical features	rs5902434										
	Genotype			Genetic model							
				Codominant (del/del vs. del/ATT vs. ATT/ATT)		Dominant (del/del vs. del/ATT+ATT/ATT)		Recessive (del/del+ del/ATT vs. ATT/ATT)		Overdominant (del/del+ ATT/ATT vs. del/ATT)	
	del/del	del/ATT	ATT/ATT	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Tumor stage											
I-II	39	29	14	**2.23 (1.28-3.89)**	**0.014**	**1.90 (1.15-3.15)**	**0.013**	0.82 (0.42-1.59)	0.55	**2.10 (1.25-3.53)**	**0.004**
III-IV	121	179	51	1.23 (0.60-2.53)							
Tumor grade											
G1-G2	19	29	9	1.34 (0.69-2.63)	0.68	1.32 (0.70-2.50)	0.38	1.07 (0.47-2.44)	0.87	1.25 (0.68-2.30)	0.46
G3	141	179	56	1.26 (0.51-3.15)							
Histology											
Serous	18	31	7	0.70 (0.37-1.37)	0.44	0.79 (0.42-1.48)	0.45	1.39 (0.57-3.37)	0.45	0.68 (0.38-1.24)	0.21
Others	142	177	58	1.14 (0.43-3.01)							
Age											
<50	68	100	29	0.77 (0.51-1.18)	0.48	0.81 (054-1.20)	0.29	1.05 (0.61-1.79)	0.86	0.80 (0.54-1.17)	0.25
≥50	92	108	36	0.91 (0.51-1.63)							

Boldfaced values indicate a significant difference at the 5% level.

Table 5. Association between SNPs in *FOXP3* and survival for EOC patients

SNP/Genotype		Alive/Dead	HR (95% CI)[a]	*P*-value[a]	Recurrence/Non-recurrence	HR (95% CI)[a]	*P*-value[a]
rs3761548							
	CC	87/21			41/67		
	CA	107/23			43/87		
	AA	7/1			3/5		
	Codominant		1.38 (0.78-2.46)	0.26		1.20 (0.80-1.80)	0.37
	Dominant		1.31 (0.70-2.42)	0.39		1.24 (0.81-1.91)	0.31
	Recessive		0.87 (0.20-3.63)	0.95		0.87 (0.21-3.63)	0.85
	Overdominant		1.16 (0.62-2.15)	0.63		1.25 (0.82-1.92)	0.29
rs5902434							
	del/del	80/12			33/59		
	del/ATT	86/27			41/72		
	ATT/ATT	35/6			13/28		
	Codominant		0.75 (0.50-1.12)	0.17		1.41 (0.89-2.24)	0.14
	Dominant		**2.60 (1.26-5.38)**	**0.010**		0.95 (0.52-1.74)	0.87
	Recessive		1.16 (0.49-2.79)	0.73		0.95 (0.52-1.74)	0.87
	Overdominant		**2.46 (1.31-4.61)**	**0.005**		1.42 (0.90-2.25)	0.13

[a]Adjusted by age, clinical histology, clinical stage and tumor grade. Boldfaced values indicate a significant difference at the 5% level.

increased risk associated with allele A carriers of rs3761548 tended to be more evident in serous ovarian cancer patients (P=0.025, OR=1.98, 95% CI=1.08-3.61). No significant difference in the genotype distribution of rs5902434 between different tumor stage, grade and age, was observed. Stratified analyses by tumor stage showed that advanced tumors with del/ATT heterozygous of rs5902434 had the higher EOC risk (P=0.014, OR=2.23, 95% CI=1.28-3.89). Del carriers (del/del+ del/ATT genotypes) were associated with III-IV in EOC, while no significant association between rs5902434 and other stratifications was observed.

Gene polymorphisms of FOXP3 with patients' outcome

Many factors that affected the prognosis of EOC, including age, tumor stage, tumor grade, and histological type, were involved in the multivariate survival analysis to adjust the difference between the two loci SNPs of *FOXP3* gene and the outcome of EOC. 246 patients were included in the study based on the follow-up results. Of those, 45 patients died and 87 had a recurrence, and the results in rs5902434 of *FOXP3* revealed the risk of death increased significantly (HR=2.60, 95% CI=1.26-5.38, P=0.010 in dominant model; HR=2.46, 95% CI=1.31-4.61, *P*=0.005 in overdominant model), while the distinction for rs3761548 of FOXP3 gene in recurrence-free survival or overall survival was not statistically significant (shown in **Table 5**).

Kaplan-Meier survival curves were used to evaluate different patients' recurrence-free survival and overall survival on FOXP3 in the univariate analysis. Patients with rs5902434 del/ATT genotype had a higher risk for death than patients with del/del and ATT/ATT genotype (P=0.036) (**Figure 1**). However, rs3761548 of FOXP3 was not an indication of prognosis in EOC.

Discussion

Gene polymorphism-disease association studies are of increasing importance from the aspect of understanding multifactorial diseases such as EOC [10]. The topic was closely proven in an article recently published by Bridget Charbonneau in the USA [7], finding associations between overall survival in EOC and SNPs in genes related to Treg activation, migration, and function. Independent studies by several laboratories have established that *FOXP3* plays a critical role for the maintenance and function of regulatory Treg, which functions through immunosuppression and immune tolerance for preventing autoimmune disease and guards against uncontrolled reaction to external fac-

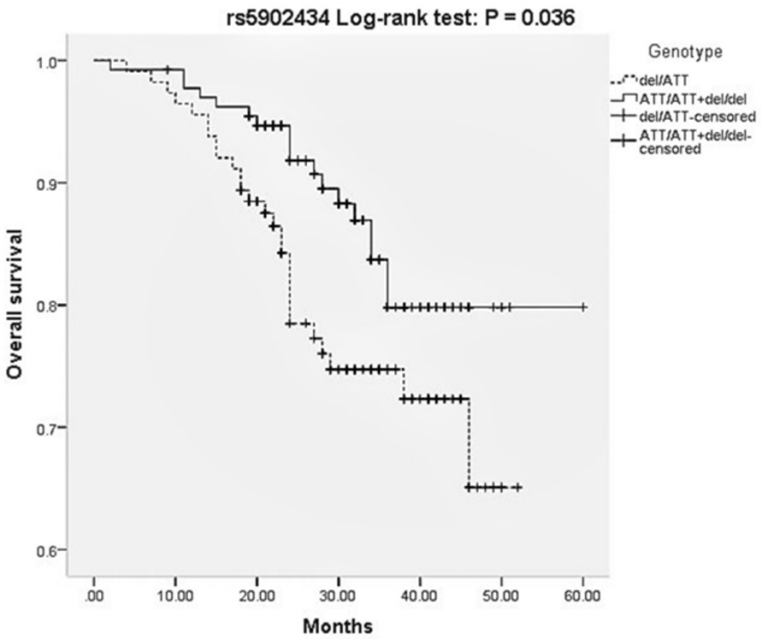

Figure 1. Kaplan-Meier survival curves showed that patients with rs5902434 del/ATT genotype had a higher risk for death than patients with del/del and ATT/ATT genotype (P=0.036).

tors. *FOXP3*-expressing Treg attenuate autoimmunity as well as immunity against autoimmune and infectious diseases [11]. Initially, *FOXP3* gene polymorphisms had been shown to be associated with these diseases, such as Graves' disease [12, 13], rheumatoid arthritis [14], systemic sclerosis [15], allergic rhinitis [16], SLE [17], and type 1 diabetes [18].

On the other hand, accumulating data have demonstrated that *FOXP3* was disrupted in a broad-spectrum of cancer cells, including breast [19-21], pancreatic [22], lung [23], liver [24], prostate [25] and colorectal cancer [26]. However, research of correlations between *FOXP3* gene polymorphisms and susceptibility or prognosis of EOC have been under reported.

In our current study, we investigated for the first time the association between two loci SNPs of *FOXP3* gene (rs3761548 and rs5902434) with the susceptibility and overall survival in EOC patients. Results demonstrate that the frequency of AA genotype in rs3761548 of patients was significantly lower than controls, indicating a decreased risk of EOC development, compared with patients with CC/AC genotype. AC heterozygous genotype showed a statistically significant hazardous effect on EOC, however, indicating a greater risk in dominant model compared with CC genotype and overdominant model compared with CC/AA genotype. Nevertheless, in one study of triple negative breast cancer in Brazil, AC genotype was a protective factor while AA was associated with risk [20]. Meanwhile, studies showed that the AC/AA genotype of rs3761548 was significantly increased in female patients with severe UC [9] and thyroid cancer [27]. Saxena D et al. [8] demonstrated that the mutant haplotype carriers of rs3761548 showed an increased risk for IRM cases. Hiroto Katoh summarized *FOXP3* as an important immunological regulator as well as an important tumor suppressor, its disruption conferred growth benefit to cancer cells [11]. These findings were different from our results. It is noteworthy that Jahan P showed AC genotype at higher risk to develop vitiligo in female participants [28]. This important discovery was consistent with our findings. Meanwhile, one study demonstrated that females homozygous for the rare *FOXP3* rs3761548 allele AA were protected against allergic rhinitis in Chinese [10], further validating the results of our study. Furthermore, stratified analysis revealed that the SNP rs3761548 of FOXP3 was associated with serous ovarian cancer, while the SNP rs5902434 of *FOXP3* was associated with tumor stage of EOC. Both of these two SNPs were not correlated with tumor grade and age. There has been no unified opinion on the relevance of the *FOXP3* gene and the clinical parameters. Considering the sample size and racial difference, further studies were needed.

In Kaplan-Meier survival and multivariate analysis, no statistically significant differences were shown between rs3761548 SNPs and the recurrence-free survival or overall survival of EOC patients. Similarly, rs5902434 del/ATT, located in the *FOXP3* gene promoter, may also

affect the *FOXP3* T-cell-specific function by negatively affecting *FOXP3* transcriptional activity and function. In most studies rs5902434 was focused on examining autoimmune disease susceptibility but no unified theory has emerged since rs5902434 has scarcely been associated with cancer. The rs5902434 ATT genotype was considered as a risk factor for patients with renal disorders and systemic lupus erythematosus in Taiwan [17]. Furthermore, the rs5902434 del/ATT allelic distribution in Crohn's disease groups was found to be slightly different from that in control groups in a non-Hispanic white population. However, no association was found between rs5902434 del/ATT and psoriasis in Han Chinese populations [29, 30]. This contradiction might be explained by the sample size and the ethnic differences in genotype frequencies. However, our study showed that rs5902434 of *FOXP3* gene was not correlated with EOC susceptibility, suggesting it did not play an essential role in the pathogenesis of EOC. It was surprising that Kaplan-Meier survival curves of patients with del/ATT genotype had shorter overall survival, which we speculated that rs5902434 might be used to monitor the prognosis of EOC. In multivariate analysis we found that the del/ATT +ATT/ATT genotype showed significantly shorter overall survival of EOC compared with the del/del genotype and similar to del/ATT genotype compared to del/del+ ATT/ATT genotype. Above all, del/ATT genotype might be an independent risk factor for the prognosis of EOC. Hermans C in a study showed that $FOXP3^+$ cells located within lymphoid aggregates surrounding the EOC were strongly associated with reduced survival time [31], which was consistent with our results.

In summary, we provide evidence that FOXP3 polymorphisms have a significant effect on the risk for developing and prognosis of EOC in the Han Chinese women. However, it is still very important to examine a larger number of samples from different populations and to investigate the proportions of Treg cells and expression among different genotypes in EOC patients to obtain more reliable conclusions.

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Disclosure of conflict of interest

None.

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