Int J Clin Exp Pathol 2016;9(2):2200-2207
www.ijcep.com /ISSN:1936-2625/IJCEP0018613

Original Article

Association of tumor necrosis factor alpha polymorphisms with cervical cancer in a Chinese population

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Received October 27, 2015; Accepted December 24, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: Host genetic factors may confer susceptibility to Cervical Cancer (CC). Tumor necrosis factor alpha (TNF-α) as proinflammatory cytokine participates in the maintenance of immune homeostasis. Allelic variation of immunomodulatory genes is associated with alteration in immune function. This study investigated the relationship between TNF-α-308G>A, -238G>A, and TNFRII-VNTR-322 and cervical cancer in Han China women. Genotypes of those polymorphisms were detected by restriction fragment length polymorphism polymerase Chain Reaction (RFLP-PCR) in 240 cases and 220 controls from the Huaihe Hospital affiliated to Henan University. The variant heterozygote -308 G/A was associated with a 39% decreased risk of cervical cancer (GG vs. A/A; *P* = 0.012; OR = 0.45; 95% CI = 0.21-0.78). Furthermore, compared with dominant variant G/G, the (G/A+A/A) genotypes was significantly associated with a decreased risk of CC (GG vs. G/A+A/A; *P* = 0.025; OR = 0.61; 95% CI = 0.42-0.96). The FIGO stratified analysis showed that the minor variant A/A and combined G/A+A/A of TNF-α-238 G>A and TNF-α-308 G>A increased the risk of the tumor evolution, respectively, (*P* = 0.011; OR = 2.91; 95% CI = 1.11-7.79) (*P* = 0.005; OR = 2.79; 95% CI = 1.28-6.81), (*P* = 0.001; OR = 15.33; 95% CI = (5.18-50.23) (*P* = 0.001; OR = 7.46; 95% CI = 2.61-20.29). There was statistically significant association between the TNF-α polymorphism and the clinical progression of cancer according to the FIGO classification. In this study, the haploview analysis revealed no LD between TNF-α-308G>A, -238G>A. Variations in TNF-α might grant genetic risk factors for cervical cancer in China population. Study on partial genetic factors in CC patients plays crucial role in CC prevention, diagnosis and treatment.

Keywords: Cervical cancer, tumor necrosis factor alpha, polymorphism, risk factor

Introduction

Worldwide, cervical cancer (CC) ranked the second most common cancer among women, accounting for 9% of the total new cancer cases and 8% of the total cancer deaths among women [1]. Infection by an oncogenic human papillomavirus (HPV) is a risk factor for developing cervical cancer [2]. HPV has the ability to target different cellular pathways in order to inhibit host immune responses and to control cell cycle progression, resulting proliferating cells with new phenotypes [3, 4]. Although HPV infection is a necessary factor of CC development, it is not a sufficient cause for the malignancy. In fact, there are a considerable number of females infected with HPV, but never developing CC, indicating that immunological, environmental and genetic factors are also involved in the pathogenesis of cervical precancerous lesions to invasive CC [5-8].

Recently, deficiencies in the immune response to viral infections have been shown to play important role in the individual susceptibility for viral-associated cancer. One of the most important factors in the immune response to viral infections is proinflammatory cytokines which modulate the immunologic response and have been implicated in the development of cancer [9, 10]. Tumor necrosis factor-alpha (TNF-α), secreted mainly by activated macrophages, is an extraordinarily pleiotropic cytokine with a central role in immune homeostasis, inflammation, and host defense [10, 11]. TNF-α is also involved in the defense against HPV infection, modulating viral replication. However, the role of TNF-α in cancer is not well understood. De-

regulated TNF-α expression within the tumor microenvironment appears to be beneficial for malignant cell tissue invasion, migration, and ultimately metastasis formation. There is growing body of evidence that TNF-α has different functions depending on its concentration: lower concentrations seem to protect the cell from viral infections, while higher concentrations seem to deregulate the immune response acting as cancer promoter [12, 13].

Several common single-nucleotide polymorphisms (SNPs) have been identified in the promoter region of the TNF-α gene which can regulate the transcription and the production of TNF-α such as TNF-α-308 G/A (rs1800629) and -238 G/A (rs361525) polymorphisms seeming to play a role in the balance of TNF-α levels [14]. Up to now, numerous studies of genetic epidemiology have assessed the association of TNF-α gene polymorphisms and risk of CC in different populations, but conflicting results were obtained due to the heterogeneity of the genetic background among populations [15-21]. Furthermore, this support the need for replication studies among all ethnic groups.

Taken together the association of TNF-α gene polymorphisms and CC, the aim of the present study was to investigate the distribution of SNP in TNF-α and its relationship with risk of cervical cancer development in China.

Patients and methods

Subjects

A total of 240 patients with invasive cervical cancer, confirmed by cervical biopsy were enrolled in this study from Department of Gynecology the Huaihe Hospital affiliated to Henan University. The control group comprised 220 healthy blood donor volunteers. All subjects are Han Chinese. Cancer diagnosis was established by clinical examination and biopsy findings, and confirmed by two senior SAI pathologists. Tumors were staged according to International Federation of Gynecology and Obstetrics (FIGO) classification. The inclusion criteria for controls were as follows: no symptoms or history of other pulmonary diseases; no symptoms or history of atopy; negative skin prick test results with a battery of common aeroallergens; and absence of first-degree relatives with a history of asthma or atopy; without autoimmune or inflammatory disease. Demographic and clinical data were collected from cases and controls using a unified questionnaire.

Written informed consents conforming to the tenets of the Declaration of Helsinki were obtained from each participant prior to the study. The Medical Ethics Committee of the Affiliated Hospital of the Huaihe Hospital affiliated to Henan University, approved this study.

Blood collection

Peripheral venous blood was collected from all participants in EDTA-containing bottles. For patients, blood collection was done prior to radiation or chemotherapy. Genome DNA from whole blood cells of each sample was extracted by using Blood Genomic DNA Miniprep Kit (Axygen, USA) according to the manufacturer's instructions.

Genotyping of SNP in TNF-α

Genotyping for the TNF-α polymorphisms in genomic DNA was performed using the PCR and restriction fragment length polymorphism (RFLP). The genomic region encompassing the rs1800629 and rs361525 polymorphism was amplified with appropriate primers as previously described [22-24]. Polymerase chain reaction products were generated in a 10 μL reaction volume containing 50 ng of genomic DNA, 1×PCR buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 1 μmol/L of each primer, and 0.25 U of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA). Cycling conditions consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds and a final elongation step at 72°C for 1 minute. Polymerase chain reaction products were digested with 2 U of *NcoI* restriction enzyme at 37°C, according to the manufacturer's instructions (New England BioLabs, Ipswich, MA).

Statistic analysis

Data were statistically described in terms of mean ± standard deviation (SD), or frequencies (number of cases) and percentages as required depending on their distribution. The Hardy-Weinberg equilibrium (HWE) was assessed for

Table 1. Anthropometric and biochemical data of patients and healthy controls

All women	Patients n (%) 240	Control n (%) 220
Age on diagnostic (year)		
30-40	27 (11.25%)	35 (15.09%)
41-50	63 (26.25%)	53 (22.08%)
51-60	64 (26.67)	93 (38.75%)
61-70	54 (22.5%)	27 (12.27)
≥70	33 (13.75%)	12 (5.45%)
Married status		
+/-[a]	228/12	218/2
Hormonal contraception		
+/-[b]	205/35	215/5
Status of menopause		
Premenopausal	68 (28.33%)	53 (24.09%)
Postmenopausal	172 (71.67%)	167 (75.91%)
FIGO staging		
Stage I	70 (29.00%)	
Stage II	93 (38.70%)	
Stage III	61 (25.60%)	
Stage IV	16 (6.70%)	
Histological type		
Squamous cell carcinoma	195 (81.08%)	
Adenocarcinoma	37 (15.61%)	
Sarcoma	8 (3.31%)	

Note: [a](+) Married, (-) Single; [b](+) Yes, (-) No; FIGO = International Federation of Gynecology and Obstetrics.

each variation to identify the deviation. The differences of the genotypes and alleles of TNF-α between patients and normal controls were evaluated by using Pearson Chi-square test. Exact test was used instead when the expected frequency is less than 5. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. Unpaired Student's t test or Mann-Whitney tests were used for two-group comparisons. Linkage disequilibrium (LD) analysis and haplotype reconstruction was performed using Haploview 4.2 (http://www.broad.mit.edu/mpg/haploview). Logistic regression analysis was performed in order to determine the OR and 95% CI. Statistical analysis of data was performed using the SPSS software package 16.0 (SPSS Inc. USA). *P*-value less than 0.05 was considered statistically significant.

Results

In this study, 240 patients with CC (100 males and 140 females) and 220 controls (106 males and 114 females) were screened for rs18006-29 and rs361525 polymorphisms using PCR-RFLP methods. Demographic and tumor characteristics of the study population are listed in **Table 1**. The median age of patients with CC was 53.28 years, and mean age of matched controls was 55.58 years. There were no significant differences between two groups with regarded to gender and age distribution. Among the 240 patients, 228 (95%) are married, 205 (85.42%) have used hormonal contraceptives and 41 (17.08%) have a cancer history in their family. According to the menopausal status of CC patients, our samples were divided into two subgroups; 68 (28.33%) were pre-menopausal and 172 (71.67%) patients were post-menopausal. Diagnoses of squamous cell carcinoma were detected by histopathological examination as International Federation of Gynecology and Obstetrics (IFGO), the distribution of the sample according to the FIGO statue is as follow; stage I (29.00%), (38.70%), III (25.60%) and IV (6.70%). Three histological types were identified: squamous cell carcinoma (81.08%), adenocarcinoma (15.61%) and sarcoma (3.31%).

Firstly, the frequency of genotypes and alleles of rs1800629 and rs361525 were detected in CC patients and controls. HWE of rs1800629 and rs361525 in patients and controls were listed in **Table 2**, and the results showed allelic distribution of rs18006-29 and rs361525 were not deviated from HWE in both case and control populations. The genotypic and allelic frequency of rs361525 did not show significant difference between CC patients and normal controls. In contrast, a significant difference of genotype distribution at the rs1800629 was revealed. These were summarized as follows. The variant heterozygote rs-1800629 was associated with a 39% decreased risk of cervical cancer (GG vs. AA; P = 0.012; OR = 0.45; 95% CI = 0.21-0.78). Furthermore, compared with dominant variant GG, the (GA+AA) genotypes was significantly associated with a decreased risk of CC (GG vs. GA+AA; P = 0.025; OR = 0.61; 95% CI = 0.42-0.96). Among the three polymorphisms, no significant correlation was observed upon carrier analysis using the dominant model.

Then, a case-only, analysis was carried out to investigate whether any possible relationship

Table 2. Genotype and allele frequency of rs361525 and rs1800629 and Pearson's chi-square test in patients and normal control

Genotype/Allele	Patients (n = 240)	Controls (n = 220)	*P*-value	OR (95% CI)
	HWE* *P* = 0.25	HWE *P* = 0.21		
rs361525				
GG	160	181	0.630	0.915 (0.656-1.330)
GA	73	37	0.074	0.832 (0.681-1.687)
AA	7	2	0.142	4.562 (0.536-37.422)
G	393	369	0.358	0.941 (0.578-1.192)
A	87	41		
rs1800629				
GG	110	121	0.025	0.612 (0.421-0.963)
GA	66	25	0.061	0.932 (0.501-1.712)
AA	64	74	0.314	0.929 (0.610-1.634)
G	286	267	0.110	0.782 (0.571-1.107)
A	194	173		

*Chi-square test for deviation from the Hardy-Weinberg equilibrium (a value of *P*<0.001 was regarded as a deviation from the HWE).

Table 3. Correlations between rs361525 and rs1800629 polymorphisms with early (I+II) vs. advanced (III+IV) stages of cervical cancer

		FIGO stages		*P*-value	OR (95% CI)
		Early stages I+II	Advanced stage III+IV		
rs361525	GG	130	30	-	Reference
	GA	30	43	0.075	2.43 (0.80-7.37)
	AA	2	5	0.011	2.91 (1.11-7.79)
	GA+AA	34	46	0.005	2.79 (1.28-6.81)
rs1800629	GG	89	21	-	Reference
	GA	53	13	0.097	2.61 (0.72-9.73)
	AA	20	44	0.001	15.33 (5.18-50.23)
	GA+AA	73	57	0.001	7.46 (2.61-20.29)

Note: Total number of cervical cancer cases of early stages (I+II) = 162 and of advanced stages (III+IV) = 78; OR: age-adjusted odds ratio; CI: confidence interval.

between rs1800629 and rs361525 gene polymorphisms and the stages of CC; early stage (stages I+II; n = 162 cases) and advanced stage (stages III+IV; n = 78 cases). Our data showed that the minor variant AA and combined GA+AA; increased the risk of the tumor evolution for rs361525 and rs1800629, respectively, (*P* = 0.011; OR = 2.91; 95% CI = 1.11-7.79) (*P* = 0.005; OR = 2.79; 95% CI = 1.28-6.81), (*P* = 0.001; OR = 15.33; 95% CI = 5.18-50.23) (*P* = 0.001; OR = 7.46; 95% CI = 2.61-20.29). There was statistically significant association of the incidence TNF-α mutations and the clinical progression of cancer according to the FIGO classification (**Table 3**). Furthermore, haplotype analysis including the two TNF-α SNPs studied revealed that the prevalence of all the haplotypes constructed was comparable between cases-controls and no LD between rs1800629 and rs361525 (**Table 4**).

Discussion

Cervical cancer is a cancer arising from the cervix. It is due to the abnormal growth of cells that have the ability to invade or spread to other parts of the body. Early on, typically no symptoms are seen. Later symptoms may include abnormal vaginal bleeding, pelvic pain, or pain during sexual intercourse [25]. While bleeding after sex may not be serious, it may also indicate the presence of cervical cancer. Worldwide, cervical cancer is both the fourth-most common cause of cancer and the fourth-most common cause of death from cancer in women. In 2012, an estimated 528,000 cases of cervical cancer occurred, with 266,000 deaths. This is about 8% of the total cases and total deaths from cancer. About 70% of cervical cancers occur in developing countries. In low-income countries, it is the most common cause of cancer death. In developed countries, the widespread use of cervical screening programs has dramatically reduced rates of cervical cancer. In medical research, the most famous cell line known as HeLa was developed from cervical cancer cells of a woman named Henrietta Lacks [26].

It has been established that cytokines have a crucial role in tumors progression and are considered as the important candidates for genetic host markers in the susceptibility to develop-

Table 4. Haplotype analysis of SNPs rs1800629 and rs361525 for association with cervical cancer cases in China

Haplotype	Freq (cases)	Freq (controls)	χ^2	*P* Fisher's	*P* Pearson's	*P* (Fisher) Monte Carlo	*P* (Pearson) Monte Carlo	OR (95% CI)
AA	0.145	0.125	0.342	0.413	0.622	0.951	0.95	1.171 (0.789-1.755)
AG	0.218	0.258	1.541	0.346	0.246	0.82	0.84	0.811 (0.568-1.156)
GA	0.373	0.283	1.988	0.534	0.254	0.821	0.89	1.218 (0.867-1.713)
GG	0.436	0.458	0.315	0.515	0.545	0.968	0.96	0.909 (0.663-1.242)

ment of different cancers including CC [27]. Among them, TNF-α system is potent cell mitogens potent cell mitogens [28-33]. TNF-α gene is located in the class III region of the human major histocompatibility complex (MHC) on chromosome 6p21 [34, 35]. Among the several single nucleotide polymorphisms (SNPs) identified in TNF-α, rs1800629 and rs361525 are the most extensively studied. The A allele of this polymorphism can lead to high binding affinity of nuclear factors to the TNF promoter, resulting in a high level of transcription activity and secretion levels of TNF-α. So, it was suggested to have a significant functional effect. A number of studies have tried to determine whether the polymorphism of TNF-α rs1800629 and rs361525 influences TNF-α expression, susceptibility to CC, but no accordant result was obtained due to the heterogeneity of the genetic background among populations [36-39]. Whether genetic variations of the TNF-α and conferred susceptibility to CC patients in Chinese was puzzled.

In this context, we analyzed TNF-α gene SNP rs1800629 and rs361525 in 240 CC patients and 220 matched controls from Henan, China. In order to exclude the gender bias, the percentage of males was extremely similar in patients and controls. No difference was found between genotypic and allelic frequency of rs361525 in CC patients and normal controls. This result was similar with other previous reported in five CC case-control studies [14, 40-43]. However, the overall results of a recent meta-analysis in which data from those studies were pooled, the rs361525 A allele was associated with a decreased risk of CC. This discrepancy is might ascribed to the simple size and ethnic background analyzed in each study. In addition, Barbasian et al. have also reported a luck of association between TNF-α rs361525 polymorphism and CC in Argentina [20]. Larger prospective studies assessing the association of TNF-α rs361525 with CC are needed to get better picture of the role of its polymorphism with CC.

TNF-α rs1800629 polymorphism was suggested to have a significant functional effect, with the A allele being associated with higher constitutive and inducible levels of transcription for TNF-α than the G allele [44]. The A allele of this polymorphism has been reported to be correlated with an increase in transcription activity and secretion levels of TNF-α [45, 46]. The TNF-α rs1800629 A allele leads to high binding affinity of nuclear factors to the TNF promoter and gives a high level of gene transcription. However, TNF-α rs1800629 is a widely investigated variant with risk of CC with conflicting results. Here, our result suggested that carriers of GA and GA+AA genotypes have a decreased risk of CC. Moreover, recently meta-analysis reported that TNF-α rs1800629 polymorphism is associated with an increased risk of CC. Subgroup analysis by ethnicity further showed that there was a significant association of this polymorphism and increased risk of CC in Asians and in Caucasian and African populations [15, 47]. However, a large number of studies have been performed to evaluate TNF-α rs1800629 polymorphism as risk factor for CC and the findings were inconclusive. These controversial results could be explained by differences in ethnic compositions; genetic background and sizes of samples between analyzed populations. It is evident that more extensive studies with larger samples and using patients with different genetic makeup should provide additional insights and improve our understanding of rs1800629 variant in etiology of cervical cancer.

Furthermore, our results demonstrated the AA and combined GA+AA genotypes increased the risk of the tumor evolution. In this context, Ahmed et al. showed that TNF-α levels were

correlated to advanced tumor stage. Correlation with tumor stage indicates that TNF-α might have a role in tumor development and progression [48].

In summary, our results suggested an increased risk for CC with rs1800629 AA and AG+AA genotypes. Future studies using patients with different ethnic backgrounds should provide additional insights and improve our understanding of the TNF-α gene variants in cervical carcinogenesis, which may in future lead to better prediction of individuals who are at risk of CC.

Acknowledgements

We sincerely thank Ma Ke Ph.D. student at Medical College of Qingdao University and state key laboratory of brain cognitive science in institute of biophysics, CAS for his assistance with data analysis and manuscript revision. We thank all the participants in this study. This study was supported by Training Program Foundation for Talent of the Huaihe Hospital affiliated to Henan University (HNU201426148).

Disclosure of conflict of interest

None.

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