

## Original Article

# CXCL12 rs266085 and TNF- $\alpha$ rs1799724 polymorphisms and susceptibility to cervical cancer in a Chinese population

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**Abstract:** Further research is required to identify single nucleotide polymorphisms (SNPs) associated with cervical cancer. The aim of this study was to assess the association of TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 polymorphisms with susceptibility to cervical cancer in Han Chinese population in Shandong Province. 348 patients with cervical squamous cell carcinoma, including CIS (121) and invasive carcinoma (227), and 351 healthy controls. Genomic DNA was isolated from peripheral blood and genotyping for TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 was carried out using TaqMan SNP Genotyping Assays. TNF- $\alpha$ /rs1799724 polymorphism showed the C-allele was less prevalent among cases as compared to controls (74.3% vs. 92.0%), while the T-allele was more prevalent among cases ( $P=0.000$ , OR=3.99, 95% C.I.: 2.90-5.51). CXCL12/rs266085 polymorphism showed the C-allele was less prevalent among cases as compared to controls (41.2% vs. 49.7%), while the T-allele was more prevalent among cases ( $P=0.001$ , OR=1.41, 95% C.I.: 1.14-1.74). The genotype and allele frequencies of these two SNPs did not differ between CIS and invasive squamous cell carcinoma ( $P>0.05$ ). Moreover, the allele frequencies of rs1799724 were significantly different between controls without or with HPV infection ( $P<0.05$ ). Neither the genotype nor allele frequencies of rs266085 were statistically different between HPV-negative and positive controls. TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 polymorphisms are associated with cervical cancer. C->T polymorphism of these two SNPs and HPV infection are linked to high risk for cervical cancer.

**Keywords:** Cervical cancer, CXCL12, genetic predisposition, polymorphism, susceptibility, tumor necrosis factor-alpha

## Introduction

Cervical cancer is one of the most common cancers affecting women's health worldwide. Personalized prevention in high-risk population is critical for reducing the incidence of this disease. Accumulating evidence indicates that human papillomavirus (HPV) infection is a necessary factor in the development of cervical cancer. High-risk types of HPV are found in over 95% of cervical cancer patients [1-4]. So far, the etiology of cervical cancer is still not clear. The development of cervical cancer is believed to be a multi-step complicated process associated with HPV infection, genetic and environmental factors, as well as other co-factors [5-7]. Although near 30% of sexually active women became infected with genital HPV soon after sexual debut, most women were able to clear the infections at a young age through

induction of cell-mediated immune response [8]. Only a small proportion of women with HPV develop persistent infections that lead to cervical cancer. Research has shown that genetic variations of genes involved in immune responses are linked with cervical cancer risk [9-11]. Single nucleotide polymorphism (SNP) markers are now under extensive study for genetic variations, in addition to restriction fragment length polymorphisms (RFLP) and microsatellite polymorphisms markers. SNP is a DNA sequence variation occurring when a single nucleotide (A, T, C or G) differs in the genome, which includes two types: transition (C->T, G->A), and transversion (C->A, G->T, C->G, A->T). There are approximately 1.4 million SNPs distributed throughout the human genome, with an average density of one in every 500-1000 bases [12]. During genetic association research, a statistically significant difference in genotype,

haplotype, and allele frequencies between controls and cases suggests a link between the genetic marker and disease susceptibility or traits [13]. rs266085 is a SNP marker located between exons 2 and 3 of CXCL12 on chromosome 10q. CXCL12 is a chemokine that directs leukocyte migration and regulates cancer cell metastatic behavior. An association between genetic variations of CXCL12, including SNP rs266085, and cervical cancer risk in an American population has been reported by Maley and coworkers [14]. rs1799724 is an SNP marker located in the TNF- $\alpha$  promoter region (-857) on chromosome 6. Research has shown that several TNF- $\alpha$  SNPs in the promoter region including rs1799724 are associated with susceptibility to cervical cancer [15]. TNF- $\alpha$  is a major pro-inflammatory cytokine that is implicated in some autoimmune and infectious diseases and cancer [16]. Some of the genetic variations of TNF- $\alpha$  modulate its expression levels by transcriptional regulation.

This research was designed according to the International HapMap Project (<http://www.hapmap.org>). In this study, we investigated four SNPs including rs266085, rs1800630, rs2430561, and rs179972 located on two candidate genes CXCL12 and TNF- $\alpha$  in Chinese Han woman, and found that rs1799724 and rs266085 are likely associated with cervical cancer. We aimed to assess the association of TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 polymorphisms with cervical cancer susceptibility and clinical characteristics in Han Chinese population by investigating the genotype and allele frequencies. To our knowledge, this is the first case-control study in Chinese population on the association between these two SNPs and cervical cancer.

### Materials and methods

A total of 699 peripheral blood samples were collected and analyzed, including 348 incident cervical cancer cases and 351 healthy women who were TCT (cervical thinprep cytologic test)-negative during health screening as controls. All subjects underwent TCT and HPV subtype tests. Diagnostic Pathology was used as the criteria for final diagnosis.

#### *Study population*

The cervical cancer group was comprised of 348 patients with cervical squamous cell carcinoma

including squamous cell carcinoma in situ (121) and invasive squamous cell carcinoma (227). Patients were randomly recruited in five hospitals in Shandong, China, between January 2011 to October 2012. All patients were newly diagnosed incident cervical squamous cell carcinoma cases and histopathologically confirmed. The exclusion criteria included previous history of cancer as well as chemo- or radiotherapy. All patients were female Han Chinese in Shandong Province, 27.7 to 67.0 years of age. Data regarding demographic characteristics, occupational exposure, family history of cancer, and smoking, sexual, and reproductive histories were obtained from all participants during research interviews using a structured questionnaire similar to the one used in a cervical cancer case-control study in Seattle, USA [17]. Written consent was obtained from all participants.

The control group was comprised of 351 age- and region-matched married females, 26.0 to 69.0 years of age, randomly selected during the same time period as the case study from healthy individuals with no history of cancer. Frequency matching of controls to cases was used in the design of this study.

This study was approved by the Ethical Committees of the hospitals the patients were attending. Data obtained using structured questionnaires were saved into databases for cases and controls.

#### *HPV test*

HPV test was conducted using HPV GenoArray test kit (Kaipu biochemistry Co. Ltd. China; SFDA registration certificate number S200-60011) and flow-through hybridization gene chip technology. After sample DNA extraction, PCR amplification, flow through DNA hybridization, chromogenic reaction, and reading of results, we genotyped 15 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68) and 6 low-risk HPV types (6, 11, 42, 43, 44, and CP8304).

#### *SNP genotyping*

Genotyping of TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 SNPs was carried out using TaqMan SNP Genotyping Assays.

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**Table 1.** Demographic characteristics and HPV infection rates in cases and controls

Groups	N	Age	Age at first sex	Number of pregnancies	Number of births	Professionals	Smoking rate	HPV infection rate
Cases	348	47.5 $\pm$ 17.5	24.5 $\pm$ 2.5	4.7 $\pm$ 2.3	2.4 $\pm$ 1.3	43.10%	20.40%	91.1% (317/348)
Controls	351	48.0 $\pm$ 18.5	24.6 $\pm$ 3.7	5.1 $\pm$ 2.8	2.3 $\pm$ 1.7	46.20%	17.40%	18.8% (66/351)

HPV: human papillomavirus.

**Table 2.** Comparison of rs1799724 and rs266085 genotype and allele frequencies in cervical cancer cases and controls

SNP	Case (%)		Control (%)		OR (95 CI)	P-value	Model		
	N=348	N=351					Model	OR (95% CI)	P-value
rs1799724									
Genotype									
CC/CC	215 (61.8)	301 (85.8)	1.00 (reference)				Dom	3.72 (2.57-5.38)	0.000
TC/TC	87 (25.0)	44 (12.5)	2.77 (1.85-4.14)		0.000		Rec	8.76 (3.69-20.79)	0.000
TT/TT	46 (13.2)	6 (1.7)	10.73 (4.50-25.58)		0.000		Add	3.00 (2.22-4.05)	<.0001
Alleles									
CC Alleles	74.3	92.0	1.00 (reference)		0.000				
TT Alleles	25.7	8.0	3.99 (2.90-5.51)						
rs266085									
Genotype									
CC/CC	73 (21.0)	66 (18.8)	1.00 (reference)				Dom	0.87 (0.60-1.27)	0.472
TC/TC	141 (40.5)	217 (61.8)	1.70 (1.15-2.53)		0.008		Rec	2.61 (1.85-3.67)	0.000
TT/TT	134 (38.5)	68 (19.4)	1.78 (1.14-2.77)		0.010		Add	1.43 (1.15-1.77)	0.001
Alleles									
CC Alleles	41.2	49.7	1.00 (reference)		0.000				
TT Alleles	58.8	50.3	1.41 (1.14-1.74)						

OR, odds ratios under a recessive model (OR=1 indicates no association between the gene and the disease; OR>1 indicates risk factor for the disease; OR<1 indicates protective factor against the disease); CI, confidence interval; Dom, dominant model; Rec, recessive model; Add, additive model.

### DNA extraction from peripheral blood lymphocytes

EDTA-anti-coagulated peripheral venous blood samples (4-5 ml) were preserved at -20°C. Genomic DNA from blood lymphocytes was isolated using a DNA extraction kit (Fujifilm, Japan) according to the manufacturer's protocol.

### Materials and equipment

TaqMan SNP Genotyping Assays, TaqMan Genotyping Master Mix, and ABI 7900HT Fast Real-Time PCR System were purchased from Applied Biosystems, USA. 386-well PCR plates were purchased from Roche Diagnostics, Germany.

### Preparation of real-time PCR primers, probes and reaction

100  $\mu$ M stock solutions were prepared for TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 for

ward and reverse primers and P1, P2 probes by adding appropriate amount of nuclease-free water to the powder. Stock solutions were then diluted 1:10 to make 10  $\mu$ M working solutions. PCR master mix without DNA was made by mixing 5  $\mu$ l of 2X TaqMan Universal Master Mix, 0.2  $\mu$ l of forward primer (10  $\mu$ M), 0.2  $\mu$ l of reverse primer (10  $\mu$ M), 0.25  $\mu$ l of Probe1 (10  $\mu$ M), 0.25  $\mu$ l of Probe 2 (10  $\mu$ M), 0.2  $\mu$ l of ROX dye, and 2.9  $\mu$ l of nuclease-free water for each reaction. 9  $\mu$ l of PCR master mix was loaded into each well of a 384-well plate. Then 1  $\mu$ l (10 ng) of DNA template was added for each reaction. 3 min initial denaturation at 95°C followed by 40 cycles of 95°C denaturation for 15 sec and 60°C annealing/extension for 1 min.

### Statistical analysis

The fitness to the Hardy-Weinberg equilibrium was tested by chi-square test using Haploview 4.2 software. The association between geno-

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**Table 3.** Comparison of rs1799724 and rs266085 genotype and allele frequencies in cervical cancer in situ and invasive cervical cancer

SNP	In situ (%)		Invasive (%)		Model		
	N=121	N=227	OR (95 CI)	P-value	Model	OR (95% CI)	P-value
<b>rs1799724</b>							
Genotype							
CC/CC	75 (62.0)	140 (61.7)	1.00 (reference)		Dom	0.99 (0.63-1.55)	0.96
TC/TC	32 (26.4)	55 (24.2)	1.09 (10.65-1.82)	0.76	Rec	0.80 (0.41-1.56)	0.51
TT/TT	14 (11.6)	32 (14.1)	0.82 (0.41-1.63)	0.56	Add	0.95 (0.69-1.29)	0.73
Alleles							
CC Alleles	75.2	73.8	1.00 (reference)	0.68			
TT Alleles	24.8	26.2	0.93 (0.65-1.33)				
<b>Rs266085</b>							
Genotype							
CC/CC	25 (20.6)	48 (21.1)	1.00 (reference)		Dom	1.03 (0.60-1.77)	0.92
TC/TC	55 (45.5)	86 (37.9)	1.23 (0.68-2.22)	0.50	Rec	0.74 (0.47-1.17)	0.20
TT/TT	41 (33.9)	93 (41.0)	0.85 (0.46-1.55)	0.59	Add	0.89 (0.66-1.19)	0.43
Alleles							
CC Alleles	43.4	40.8	1.00 (reference)	0.40			
TT Alleles	56.6	59.2	0.87 (0.64-1.20)				

types and disease risks was evaluated by multiple logistic regression analysis using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA), and expressed as odds ratio (OR) and their 95% confidence intervals (CI). Genotype and allele frequencies were compared between controls and cervical squamous cell carcinoma cases as well as between squamous cell carcinoma in situ and invasive squamous cell carcinoma.  $P < 0.05$  was considered statistically significant.

### Results

**DNA extraction from peripheral lymphocytes:** Genomic DNA was isolated from peripheral blood lymphocytes. DNA samples (699 total;  $\geq 20 \mu\text{g}/\mu\text{l}$ ).

#### *Characteristics of cervical cancer cases and controls*

The observed genotype distributions for the TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 polymorphisms in both controls and cervical cancer patients were both in agreement with the Hardy-Weinberg equilibrium ( $P > 0.05$ ), indicating that samples were representative of the real populations. These two SNPs were independent, with no linkage disequilibrium (LD:  $r^2 < 0.8$ ). No statistically significant difference was observed between patients and controls

regarding demographic characteristics, occupational exposure, and sexual and reproductive histories ( $P > 0.05$ ). The rate of HPV infection in cervical epithelial cells was significantly higher in cervical cancer group as compared to controls ( $P < 0.01$ ) (Table 1).

#### *Comparison of rs1799724 and rs266085 allele frequencies in cervical cancer cases and controls*

The genotyping results were shown in Table 2. TNF- $\alpha$ /rs1799724 showed significant association with cervical cancer. C-allele frequencies of rs1799724 in cervical cancer patients and controls were 74.3% and 92.0%, respectively. The T-allele was more prevalent among cervical cancer patients as compared to controls ( $P = 0.000$ , OR=3.99, 95% C.I.: 2.90-5.51). CXCL12/rs266085 showed significant association with cervical cancer as well. The C-allele frequencies of rs266085 in cervical cancer patients and controls were 41.2% and 49.7%, respectively. Similarly, the T allele was more prevalent among cervical cancer patients as compared to controls ( $P = 0.001$ , OR=1.41, 95% C.I.: 1.14-1.74). For both TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 polymorphisms, TT + AT were risk genotypes while CC was a non-risk genotype.

*Comparison of rs1799724 and rs266085 genotype and allele frequencies in cervical cancer in situ and invasive cervical cancer*

The associations between allele frequencies and invasive cervical cancer risks were assessed using multiple logistic regression analysis (**Table 3**). Neither TNF- $\alpha$ /rs1799724 nor CXCL12/rs266085 showed significant differences in genotype and allele frequencies between in situ and invasive cervical cancer ( $P>0.05$ ). The T-allele of rs1799724 was not prevalent among cervical cancer patients as compared to carcinoma in situ (OR=0.93, 95% C.I.: 0.65-1.33). The T-allele of rs266085 was also not prevalent among cervical cancer patients as compared to carcinoma in situ (OR=0.87, 95% C.I.: 0.64-1.20).

*Comparison of rs1799724 and rs266085 genotype and allele frequencies in HPV-negative and HPV-positive subgroups within controls or cervical cancer cases*

Within the control group, we found significant difference in TNF- $\alpha$ /rs1799724 allele frequencies, but not genotype frequencies, between HPV-negative and HPV-positive subgroups ( $P<0.05$ ). No significant differences were observed in rs1799724 genotype or allele frequencies between HPV-negative and HPV-positive subgroups ( $P>0.05$ ) in cervical cancer cases. Genotype or allele frequencies of CXCL12/rs266085 did not significantly differ between HPV-negative and HPV-positive subgroups in either controls or cases.

**Discussion**

In the present study, we explored whether the TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 are associated with cervical cancer susceptibility in a Chinese population in Shandong Province, according to the information on HapMap database. After genotyping of DNAs from 351 controls and 348 patients, we found that there is an association between TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 polymorphism and cervical cancer risk in Chinese Han women population. Our work provides further evidence supporting a role of TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 in cervical carcinogenesis.

We found that both TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 were significantly associat-

ed with cervical cancer. The C-allele of rs1799724 was less prevalent among cases as compared to controls (74.3% vs. 92.0%), while the T-allele of rs1799724 was more prevalent among cases ( $P=0.000$ , OR=3.99, 95% C.I.: 2.90-5.51). Similarly, the C-allele of rs266085 was less prevalent among cases as compared to controls (41.2% vs. 49.7%), while the T allele of rs266085 was more prevalent among cases ( $P=0.001$ , OR=1.41, 95% C.I.: 1.14-1.74). No significant difference was observed between patients and controls regarding demographic characteristics, occupational exposure, and sexual and reproductive histories ( $P>0.05$ ). The rate of high-risk HPV infection in cervical epithelial cells was significantly higher in cervical cancer group (91.1%) as compared to controls (18.8%) ( $P<0.01$ ). TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 were independent SNPs, with no linkage disequilibrium (LD:  $r^2<0.8$ ). For both TNF- $\alpha$ /rs1799724 and CXCL12/rs266085 polymorphisms, TT + AT were risk genotypes and CC was a non-risk genotype.

We postulate that the C->T polymorphism at rs1799724 of the TNF- $\alpha$  gene may lead to increased susceptibility for cervical cancer by altering the expression levels of TNF- $\alpha$ , resulting in impaired immune responses and thus persistent HPV infection and subsequent cervical carcinogenesis. This hypothesis is supported by research in other cancers. Johnson LG et al. reported that polymorphisms in genes in the chromosome 5 cytokine cluster are associated with cervical cancer risk [18]. De Oliveira JG et al. evaluated the association between gene polymorphisms in cytokines, including TNF- $\alpha$ /rs1799724 (-857), and risk for gastric cancer [16], and found that several polymorphisms including rs1799724 C->T polymorphism are associated with the development of gastric cancer, indicating that the combined effect of polymorphisms in inflammatory genes may potentiate gastric cancer risk. However, in another case-control study, Danforth KN et al. did not observe any association of TNF- $\alpha$ /rs1799724 with prostate cancer risk [19]. In the present study, we found that high-risk HPV infection in cervical epithelial cells occurs in the majority of cervical cancer patients (91.1%). The rs1799724 C->T polymorphism along with cervical inflammatory lesions and progressive cervical intraepithelial neoplasia caused by persistent high-risk HPV infection [2, 4] may

contribute to the development of cervical cancer.

CXCL12 (also known as SDF-1) is a chemokine that mediates immune and inflammatory responses. Recent research has shown that CXCL12 is expressed in tumor stromal cells including endothelial cells and infiltrated macrophages, dendritic cells, lymphocytes, and NK cells [20]. CXCL12 has been implicated in HIV infection, tumor invasion and metastasis [21]. Therefore CXCL12 and its receptor CXCR4 have received a lot of attention in research on cancer risks and metastasis. Kryczek et al. reported that, of the 14 chemokines studied, only CXCL12 receptor CXCR4 is expressed in ovarian carcinoma cells, and that CXCL12 is involved in tumor angiogenesis and malignant ascites [22]. Maley SN et al. have reported an association between cervical cancer risk and SNP or haplotype variations of CXCL12 including SNP rs266085 in an American population in western Washington state [14]. Interestingly, they found that rs266085 minor allele A (T on the other DNA stand) is inversely associated with cervical cancer under a recessive model. In contrast, we report here that C->T polymorphism of CXCL12/rs266085 is associated with increased risk for cervical cancer. The reason for the disparity between our study and the report by Maley SN is not clear. One possible explanation is different ethnic backgrounds of subjects: We investigated the association between rs266085 and cervical cancer in a Chinese population while their study was carried out in an American population.

In summary, we observed that TNF- $\alpha$ /rs-1799724 and CXCL12/rs266085 polymorphisms are strongly associated with cervical cancer development in China Han women Chinese. This further implicates TNF- $\alpha$ /rs-1799724 and CXCL12/rs266085 as a cervical cancer-related gene. However, the functional consequence of this genetic alteration in cervical carcinogenesis remains to be determined.

### Disclosure of conflict of interest

None.

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