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The role of smoking status and collagen IX polymorphisms in the susceptibility to cervical spondylotic myelopathy

Z.C. Wang[1], J.G. Shi[2], X.S. Chen[2], G.H. Xu[2], L.J. Li[2] and L.S. Jia[2]

[1]Department of Orthopedics, Xinhua Hospital (Chongming), Shanghai Jiaotong University, Shanghai, China
[2]Department of Orthopedics, Changzheng Hospital, Second Military Medical University, Shanghai, China

Corresponding author: L.S. Jia
E-mail: drjialianshun@yahoo.com.cn

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ABSTRACT. We investigated a possible association of collagen IX tryptophan (Trp) alleles (Trp2 and Trp3) and smoking with cervical spondylotic myelopathy (CSM) in 172 Chinese patients and 176 age- and gender-matched controls. The smoking status was evaluated by smoking index (SI). The CSM cases had a significantly higher prevalence of Trp2 alleles (Trp2+) than controls (19.8 *vs* 6.2%, P = 0.002), but the prevalence of Trp3 alleles (Trp3+) was similar between the two groups (23.3 *vs* 21.6%, P = 0.713). Logistic regression analyses showed that the subjects with Trp2+ had a higher risk for CSM. We thus analyzed whether smoking status influenced the association between Trp2 alleles and CSM risk. Among Trp2+ subjects with an SI less than 100, the smoking status did not influence the effect of risk for SCM [odds ratio (OR) = 1.34, 95% confidential interval (95%CI) = 0.85-2.18, P > 0.05]. When SI increased from 101 to 300, the OR for CSM reached 3.34 (95%CI = 2.11-5.67, P = 0.011); when SI was more than 300, the OR for CSM reached 5.56 (95%CI = 3.62-7.36, P < 0.001). Among Trp2-

subjects with SI more than 300, the OR for CSM increased 2.14 (95%CI = 1.15-4.07, P = 0.024). We found a significant association between the Trp2 alleles and CSM risk and smoking amplifies this risk, suggesting that smoking abstinence is important for reducing CSM occurrence in subjects with high genetic risk.

Key words: Smoking; Polymorphisms; Cervical spondylotic myelopathy; Collagen IX

INTRODUCTION

As one type of degenerative intervertebral disc disease, cervical spondylotic myelopathy (CSM) is highly prevalent in clinical practice. The etiology of CSM is complicated, with various environmental risk factors, such as age, gender and occupation (Jumah and Nyame, 1994; Zhang et al., 2008). Previous studies suggested a significant contribution of genetic factors, e.g., genes encoding collagen in the occurrence of spinal disc diseases (Yoo and Origitano, 1998; Garnero et al., 2004; Lucas et al., 2009; Falcon-Ramirez et al., 2011).

As a structural component of nucleus pulposus and annulus fibrosus of intervertebral disc, collagen IX acts as a bridge between collagens and non-collagenous proteins in tissues, which is essential to the proper formation of the collagen type II/IX/XI heteropolymer (Wu and Eyre, 1984; Koelling et al., 2008; Huang et al., 2009; Blumbach et al., 2009; Zhu et al., 2011). Type IX collagen is composed of 3 genetically distinct α polypeptide chains, i.e., α1(IX), α2(IX), and α3(IX), which are encoded by *COL9A1*, *COL9A2*, and *COL9A3*, respectively. An animal study showed that transgenic mice expressing mutant IX (alpha 1) collagen have accelerated intervertebral disc degeneration (Kimura et al., 1996). Clinically, an association between allelic variants in the collagen IX genes and degenerative lumbar disc diseases has also been reported. Collagen IX tryptophan alleles influence the symptomatic degeneration of the lumbar disc in patients with herniated nucleus pulposus, and the Trp2 allele of collagen IX tryptophan alleles has been suggested as a risk factor for the severity of disc degeneration in patients with symptomatic herniated nucleus pulposus of the lumbar spine (Jim et al., 2005; Higashino et al., 2007).

Smoking is another common risk factor for spinal diseases. Nicotine has an overall detrimental effect on cultured nucleus pulposus disc cells *in vitro*. Researchers observed a significant inhibition of nucleus pulposus cell proliferation and extracellular matrix synthesis, suggesting that nicotine in tobacco smoke may have a role in the pathogenesis of disc degeneration (Akmal et al., 2004). In an animal model treated with passive cigarette smoking, intervertebral discs exhibited cracks, tears, and misalignment of the annulus fibrosus and increased interleukin-1beta expression. These changes were partially irreversible after cessation of smoking, the amount of mucin (proteoglycan) in the nucleus pulposus and annulus fibrosus tended to increase, suggesting that smoking-induced intervertebral disc degeneration can be repaired by cessation of smoking (Nemoto et al., 2006).

The gene-environment interaction is more important in determining disease predisposition than each alone. To date, the effect of smoking, genetic variant of collagen IX and their interaction on the susceptibility of CSM has not been studied in a clinical setting. We planed to analyze the role of smoking and collagen IX SNPs as well as their interaction, in the occurrence of CSM in a Chinese cohort.

SUBJECTS AND METHODS

Subject enrollment

A total of 172 consecutive patients diagnosed with CSM were enrolled in the study. The diagnoses were established on the basis of findings from the history and physical examination, which were confirmed by magnetic resonance imaging (MRI). Exclusion criteria included congenital cervical anomalies, trauma, prior cervical surgery, rheumatoid arthritis, infections, tumors, ankylosing spondylitis, ossification of the posterior longitudinal ligament, diffuse idiopathic skeletal hyperostosis, and any other inflammatory disease involving the cervical spine. The control sample consisted of 176 gender- and age-matched Chinese individuals with negative MRI findings. The clinical characteristics including gender, age, weight, height, body mass index (BMI), profession desk work time per day, and family history of intervertebral degenerative disc disease were collected. The smoking index (SI) was calculated according to the following: SI = average number of cigarette consumed per day * smoking years. According to SI, all the subjects were divided into 4 groups, namely group I (non-smokers), group II (including smokers with SI less than 100), group III (including smokers with SI between 101 and 300), and group IV (including smokers with SI more than 300). The study protocol was approved by the Ethics Committee of the hospital. Written informed consent was obtained from all patients before participation in the study.

Polymorphism genotyping

Fasting blood samples were collected from the patients. Genomic DNA was extracted from 2 mL peripheral blood using commercially available extraction kit (TaKaRa, Japan). Primers for DNA amplification and sequencing were designed manually, according to the genomic sequence flanking exon 19 of the COL9A2 gene or exon 3 of the COL9A3 gene (Matsui et al., 2003). PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems, USA) with an initial denaturation at 95°C for 8 min, followed by 35 cycles at 95°C for 30 s, 58°C for 10 s and 72°C for 30 s, and a final step at 72°C for 5 min. Five microliters of PCR products was treated with 2 U shrimp alkaline phosphatase and 10 U exonuclease I (Amersham, Buckinghamshire, UK) at 37°C for 15 min, followed by incubation at 80°C for 15 min for enzyme inactivation. Sequencing reactions were performed using the ABI Prism BigDye Terminator kit (Applied Biosystems) in a GeneAmp PCR system 9700 with an initial denaturation at 96°C for 5 min, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Purified products were analyzed on an ABI Prism 3100 multicapillary sequencer (Applied Biosystems).

Statistical analysis

The enrolled subjects were divided into two groups according to the presence or absence of the Trp allele: Trp2 or 3 (+) and Trp2 or 3 (-). The genotype data of all tested SNPs were used to estimate Hardy-Weinberg equilibrium by comparison of genotype frequencies within the case and control groups by χ^2 tests. The interaction effect between smoking and SNPs was analyzed by the multinomial regression model. P values were calculated with the SPSS statistical software (SPSS Statistics 17.0; 2008 SPSS Inc., Chicago, IL, USA).

RESULTS

Table 1 shows the clinical characteristics of CSM subjects and controls. There was no significant difference in age, gender, and BMI. CSM subjects had a higher percentage of family history (32.0 *vs* 23.3%, P = 0.034). The mean desk work time in CSM subjects was significantly higher than that in controls (2.8 ± 0.7 *vs* 1.1 ± 0.6 h/day, P < 0.001). By chi-square analyses, we observed a significant difference between the smoking distributions in case and control groups. Cases had a markedly higher prevalence of moderate (individuals with SI between 101 and 300) and heavy smokers (individuals with SI more than 300) compared to controls (P = 0.015).

Table 1. Clinical characteristics of cervical spondylotic myelopathy subjects and controls.

	Case (N = 172)	Control (N = 176)	P
Age at diagnosis (years)	47.7 ± 7.5	47.1 ± 6.9	ns
BMI (kg/m^2)	24.5 ± 3.6	23.9 ± 2.7	ns
Gender (male, %)	96 (55.9%)	88 (51.2%)	ns
Family history (N, %)	55 (32.0%)	40 (23.3%)	0.034
Desk work time (h/day)	4.3 ± 0.7	3.1 ± 0.6	<0.001
Smoke index			
I	23 (13.4%)	47 (26.7%)	0.015
II	45 (26.1%)	58 (33.0%)	
III	56 (32.6%)	44 (25.0%)	
IV	48 (27.9%)	27 (15.3%)	

BMI = body mass index; ns = nonsignificant.

Table 2 shows the presence or absence of the Trp alleles in cases and controls. We observed that CSM cases had a markedly higher prevalence of Trp2 alleles than did controls (19.8 *vs* 6.2%, P = 0.002). However, for the prevalence of Trp3 alleles, there was no significant difference between cases and controls (23.3 *vs* 21.6%, P = 0.713).

Table 2. Presence or absence of the Trp alleles in cases and controls.

	Case	Control	Chi-square	P
Trp2(+)	34 (19.8%)	11 (6.2%)	14.6	0.002
Trp2(-)	138 (80.2%)	165 (93.8%)		
Trp3(+)	40 (23.3%)	38 (21.6%)	0.139	0.713
Trp3(-)	132 (76.7%)	138 (78.4%)		

Logistic regression analyses showed that smoking was an independent risk factor for CSM. Taking non-smokers as a reference, the odds ratio (OR) for CSM in moderate smokers (individuals with SI between 101 and 300) was 2.22 [95%CI (confidence interval) = 1.78-3.24, P = 0.013] while the OR in heavy smokers (individuals with SI more than 300) reached 3.35 (95%CI = 2.67-5.45, P = 0.05), respectively. Logistic regression analyses also showed that the subjects with Trp2+ had a higher risk for CSM than those without. The OR for CSM in Trp2+ carriers was 1.78, 95%CI = 1.13-3.35, P = 0.048. However, the presence of the Trp3 alleles did not affect CSM risk (OR = 1.02, 95%CI = 0.79-1.84, P = 0.087).

Given the influence of Trp2+ on CSM risk, we further analyzed the interaction be-

tween the Trp2 alleles and smoking status in determining CSM risk. Our data showed that the effect of Trp2 alleles was significantly affected by smoking status. The risk factor for CSM in TRP2+ subjects markedly increased when combined with smoking status. For these subjects with SI less than 100, the risk for CSM was 1.34 (95%CI = 0.85-2.18, P > 0.05). When SI increased from 101 to 300, the risk for CSM reached 3.34 (95%CI = 2.11-5.67, P = 0.011); when SI was more than 300, the risk for CSM reached 5.56 (95%CI = 3.62-7.36, P < 0.001). For Trp2- subjects with SI more than 300, the risk for CSM increased to 2.14 (95%CI = 1.15-4.07, P = 0.024) (Table 3).

Table 3. Interaction between smoking and Trp2 alleles on CSM risk.

	OR	95%CI	P
Trp2+*SI			
<100	1.34	0.85-2.18	0.067
101-300	3.34	2.11-5.67	0.011
>300	5.56	3.62-7.36	<0.001
Trp2-*SI			
<100	1.21	0.78-1.54	0.167
101-300	1.15	0.98-1.48	0.057
>300	2.14	1.15-4.07	0.024

SI = smoking index; OR = odds ratio; 95%CI = confidence interval at 95%.

DISCUSSION

A previous study showed that CSM is a genetically predisposed disease. Several genes encoding collagen were reported to be implicated in the development and progression of CSM. In this study, we investigated the role of Trp alleles in collagen IX and smoking status, as well as their interaction, in determining the risk of CSM in a Chinese cohort. We found that both smoking and the presence of Trp allele predisposed the occurrence of CSM. Meanwhile, a strong positive interaction between smoking status and the presence of Trp alleles on CSM risk was noted. The risk of developing CSM in subjects with Trp alleles increased with the amount of smoking. Our results suggest the importance of smoking abstinence in reducing CSM occurrence in subjects who are genetically at high risk.

As a relatively minor component of the nucleus pulposus, collagen IX is essential to the proper formation of the collagen type II/IX/XI heteropolymer (Eyre et al., 2002). The Trp2 is located in the middle of α2(IX) COL2, within a few residues of the site of cross-linking between the α3(IX) chain and type II collagen, interfering with this molecular interaction. Individuals with Trp2 allele may have a less cross-linked collagen network and may allow more enzymatic cleavage of the disc matrix. A recent study showed that having the Trp2 allele resulted in a 6-fold increase in the risk of severe disc degeneration in the Japanese population. The alpha2 type IX collagen tryptophan polymorphism was associated with the severity of disc degeneration in younger patients with herniated nucleus pulposus of the lumbar spine. These results suggest that the Trp2 allele is a risk factor for intervertebral disc diseases (Higashino et al., 2007). The patients with the Trp2 allele were more flexible, and more often tended to have a radial tear in a nonherniated disc than their control counterparts (Karppinen et al., 2002).

It should be noted that the frequency of Trp2 alleles in patients with intervertebral disc diseases was different among the Finnish, Greeks, Chinese, and Japanese. Trp2 was found

only in affected individuals (4%) in a Finnish population and was absent in a Greek population (Paassilta et al., 2001; Kales et al., 2004), while in a south Chinese population, the Trp2 allele was present in 20% of the population and was associated with an increased risk of developing degenerative lumbar disease, which was consistent with the Trp 2 allele findings in our study (Jim et al., 2005). The contrasting Trp2 allele frequencies between the Finns and Chinese are the first indication that the genetic risk factors for degenerative disc disease vary between ethnic groups.

Several studies have demonstrated the important role of smoking as a cause in the occurrence and development of degenerative intervertebral disc disease. Cigarette smoking significantly affects the circulatory system outside the intervertebral disc and significantly deteriorates the cellular uptake rate and metabolite production within the disc. Tobacco smoke inhalation increases local production and release of inflammatory cytokines and resultant decrease in chondrocyte activity. Smoking causes adverse morphologic changes at the histological level, including reduced cell proliferation, disrupted cell architecture, disintegration of cells and extracellular matrix; intervertebral discs exhibited cracks, tears, and misalignment of the annulus fibrosus, and increased fibrous tissue was seen in the nucleus pulposus.

We postulate that smoke increased the risk of developing CSM by the following mechanisms: 1) Smoke interrupts the collagen metabolism of intervertebral disc tissues. In animals receiving passive smoke, immunohistochemistry revealed the presence of type I collagen in the extracellular matrix, rather than the normal type II collagen. Collagen genes were down-regulated remarkably after 7 weeks of smoking, which suggests an interaction effect between smoking and collagen synthesis in pathogenesis of disc degeneration. 2) Smoke amplifies the local inflammation in intervertebral disc tissues. Researchers have reported that tobacco smoke inhalation increases local production and release of inflammatory cytokines such as increased interleukin-1beta expression, which leads to a decrease in chondrocyte activity in the lumbar discs (Oda et al., 2004).

Some limitations of the current study should be addressed. First, this is a small scale case-control study, which involved only 172 cases and 176 controls. Further study with larger samples is warranted. Second, we did not consider secondary smoking exposure for cases and their matched controls. Subjects with low smoking index may have extensive secondary smoking exposure. In addition, previous studies showed that smokers or secondary smokers have more transversion mutations versus transition mutations. Thus, smoking abstinence may not reduce CSM occurrence. Third, due to financial limitations, we were unable to perform gene sequencing to verify the accuracy of genotyping of Trp2 and 3 in this study.

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