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Role of *COL9A1* genetic polymorphisms in development of congenital talipes equinovarus in a Chinese population

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ABSTRACT. Talipes equinovarus is a common congenital deformity. *COL9A1* polymorphisms are associated with the development of articular cartilage-related diseases. In the current study, we evaluated the relationship between *COL9A1* rs1135056, rs35470562, and rs592121 genetic polymorphisms and risk of congenital talipes equinovarus. Between January 2013 and July 2015, 87 children with congenital talipes equinovarus and 174 control subjects were recruited from the Fourth People's Hospital of Shaanxi and the First Hospital of Yulin. Genotyping of *COL9A1* rs1135056, rs35470562, and rs592121 was performed using polymerase chain reaction-restriction fragment length polymorphism. Using conditional regression analysis, the AA genotype of *COL9A1* rs35470562 was found to be significantly associated with increased risk of congenital talipes equinovarus compared to the GG

genotype [odds ratio (OR) = 2.60, 95% confidence interval (CI) = 1.06-6.32]. In addition, under a recessive model, rs35470562 AA carriers were observed to be at higher risk for this condition in comparison to individuals with GG or GA genotypes (OR = 2.23, 95%CI = 1.03-5.04). However, no significant relationship was established between the *COL9A1* rs1135056 and rs592121 polymorphisms and congenital talipes equinovarus in any of the genetic models tested. In conclusion, our results indicate that the *COL9A1* rs35470562 variant may contribute to congenital talipes equinovarus susceptibility in the Chinese population examined.

Key words: *COL9A1*; Polymorphism; Congenital talipes equinovarus

INTRODUCTION

Talipes equinovarus is a common congenital deformity (Cardy et al., 2007; Kancherla et al., 2010). The etiology of this condition is not well understood, and many risk factors have been identified, such as skeletal dysplasia, neuromuscular disease and soft tissue contracture (Kancherla et al., 2010). Recent studies have indicated that consanguineous marriage increases congenital talipes equinovarus risk, and about 10% of sufferers have a first- or second-degree family history of the condition (Cardy et al., 2011; Sahin et al., 2013). Therefore, genetic factors may contribute to its development.

Recent study has shown that mutations in collagen IX genes can cause variation in bone marrow hypoplasia, and might be the molecular biological basis of articular cartilage-related diseases, the risk of which previous studies have associated with *COL9A1* expression (Liu et al., 2011; Brachvogel et al., 2013). Polymorphisms in this gene can alter its expression, and thus influence the function of the encoded protein. *COL9A1* sequence variants have been reported to be associated with the development of osteoarthritis, lumbar disc disease, and multiple epiphyseal dysplasia (Czarny-Ratajczak et al., 2001; Jakkula et al., 2005; Janeczko et al., 2014). Currently, only one study has examined the relationship between the *COL9A1* rs1135056 and rs35470562 polymorphisms and risk of congenital talipes equinovarus (Liu et al., 2011). In the current study, we evaluated the association between the rs1135056, rs35470562, and rs592121 *COL9A1* genetic variants and development of this condition.

MATERIAL AND METHODS

Subjects

Between January 2013 and July 2015, 87 children with congenital talipes equinovarus were recruited from the Fourth People's Hospital of Shaanxi and the First Hospital of Yulin. All patient diagnoses were confirmed by X-ray examination. Patients had congenital talipes equinovarus caused by spina bifida and cerebral palsy.

During the same period, 174 control subjects were selected from the outpatient clinics of the same hospitals. All control participants were matched with patients by gender and age (± 5 years), and were free of congenital talipes equinovarus, articular cartilage disease, and acute or chronic infectious diseases.

The demographic and clinical variables of the study subjects were collected from a questionnaire or medical records. The latter comprised involvement of a single foot or both feet. All subjects signed informed consent forms before participation, and our study was approved by the Ethics Committee of the Fourth People's Hospital of Shaanxi and the First Hospital of Yulin.

DNA extraction and genotyping analysis

A peripheral venous blood sample (5 mL) was obtained from each participant after enrollment. DNA extraction was performed using a TIANGEN blood DNA kit (TIANGEN Biotech Co., Ltd., Beijing, China). Genotyping of *COL9A1* rs1135056, rs35470562, and rs592121 polymorphisms was achieved using polymerase chain reaction (PCR)-restriction fragment length polymorphism. Each of these regions was amplified using primers detailed in previous studies (Liu et al., 2011), as follows: rs1135056 forward, 5'-CTAGCATGGGCTCAAACA-3' and reverse, 5'-CCTGGTCAGATGGGAAAT-3'; rs35470562 forward, 5'-ACTGTGGGCACT TATGAA-3' and reverse, 5'-GCAATCTTGGGAGACTTT-3'; rs592121 forward, 5'-TCTTGCT CTATTAGGGAT-3' and reverse, 5'-TAATGTTAGTTGGCTTGC-3'. The restriction enzymes and PCR cycling conditions used and lengths of the resulting products are shown in Table 1. The digested products were separated by electrophoresis on a 3% agarose gel and visualized using an ultraviolet transilluminator.

Table 1. Restriction enzymes, polymerase chain reaction (PCR) cycling conditions, and lengths of products used to genotype *COL9A1* rs1135056, rs35470562, and rs592121 single nucleotide polymorphisms (SNPs).

SNP	Restriction enzyme	PCR cycling conditions
rs1135056	*Sma*I	One cycle at 94°C for 3 min; 35 cycles of denaturation at 94°C for 50 s, annealing at 54°C for 50 s, and extension at 72°C for 50 s; and one final cycle at 72°C for 5 min
rs35470562	*Mbo*II	One cycle at94°C for 3 min; 35 cycles of denaturation at 94°C for 50 s, annealing at 51°C for 40 s, and extension at 72°C for 50 s; and one final cycle at 72°C for 10 min
rs592121	*Hae*III	One cycle at 94°C for 3 min; 35 cycles of denaturation at 94°C for 50 s, annealing at 49°C for 40 s, and extension at 72°C for 50 s; and onefinal cycle at 72°C for 10 min

Statistical analysis

Demographic variables in the patient and control groups were compared by the chi-square test, which was also used to estimate conformity of *COL9A1* rs1135056, rs35470562, and rs592121 genotype distributions to Hardy-Weinberg equilibrium (HWE). The relationship between these polymorphisms and congenital talipes equinovarus was analyzed using conditional logistic regression analysis. Odds ratios (ORs) and corresponding 95% confidence intervals (95%CIs) were used as a measure of risk. All statistical analyses were conducted using SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, NY, USA).

RESULTS

The mean ages in the congenital talipes equinovarus patient and control groups were 4.25 ± 5.74 and 4.56 ± 5.15 years, respectively (Table 2). In both groups, 62.07% of participants were boys. Of the patients, 11.49% had a family history of the condition. Using the chi-square test, this variable was found to significantly differ between the two study groups (chi-square = 20.80, $P < 0.001$). However, no significant differences were established in terms

of age (chi-square = 0.44, P = 0.33), gender (chi-square = 0.00, P = 1.00), maternal smoking (chi-square = 3.19, P = 0.07), or maternal drinking (chi-square = 3.54, P = 0.06).

Table 2. Demographic characteristics of congenital talipes equinovarus patients and controls.

Variable	Patients (N = 87)	%	Controls (N = 174)	%	Chi-square test	P value
Mean age, years	4.25 ± 5.74		4.56 ± 5.15		0.44	0.33
Gender						
Female	33	37.93	66	37.93		
Male	54	62.07	108	62.07	0.00	1.00
Family history of congenital talipes equinovarus						
No	77	88.51	174	100.00		
Yes	10	11.49	0	0.00	20.80	<0.001
Maternal smoking						
Never	68	78.16	151	86.78		
Yes	19	21.84	23	13.22	3.19	0.07
Maternal drinking						
Never	80	91.95	169	97.13		
Yes	7	8.05	5	2.87	3.54	0.06
Talipes equinovarus						
Single foot	51	58.62				
Both feet	36	41.38				

The distributions of *COL9A1* polymorphism genotypes in each group are shown in Table 3. rs1135056 (chi-square = 1.38, P = 0.50), rs35470562 (chi-square = 5.52, P = 0.06), and rs592121 (chi-square = 0.24, P = 0.89) genotype frequencies did not significantly vary between congenital talipes equinovarus patients and controls. These variants conformed to HWE in both groups (rs1135056: P = 0.21 in controls; rs35470562: P = 0.48 in controls; and rs592121: P = 0.43 in controls).

Table 3. Distribution of *COL9A1*rs1135056, rs35470562, and rs592121genotypes in each study group.

SNP	Patients	%	Controls	%	Chi-square	P	Controls	
							Chi-square (HWE)	P (HWE)
rs1135056								
AA	32	36.78	74	42.53				
AG	37	42.53	73	41.95				
GG	18	20.69	27	15.52	1.38	0.50	1.57	0.21
rs35470562								
GG	30	34.48	78	44.82				
GA	41	47.13	80	45.98				
AA	16	18.39	16	9.20	5.52	0.06	0.49	0.48
rs592121								
AA	28	32.18	60	34.48				
AG	40	45.98	80	45.98				
GG	19	21.84	34	19.54	0.24	0.89	0.62	0.43

SNP = single nucleotide polymorphism; HWE = Hardy-Weinberg equilibrium.

Using conditional regression analysis, the AA genotype of *COL9A1* rs35470562 was observed to be significantly associated with increased risk of congenital talipes equinovarus compared to the GG genotype (OR = 2.60, 95%CI = 1.06-6.32; Table 4). In a recessive model, carriers of the AA genotype of this polymorphism were revealed to be at higher risk of congenital talipes equinovarus in comparison to individuals with the GG and GA genotypes (OR = 2.23, 95%CI = 1.03-5.04). However, no significant relationship was apparent between the *COL9A1* rs1135056 and rs592121 variants and risk of this condition under any genetic model.

Table 4. Association between *COL9A1* rs1135056, rs35470562, and rs592121 polymorphisms and risk of congenital talipes equinovarus.

SNP	Patients	%	Controls	%	OR (95%CI)	P value
rs1135056						
Co-dominant						
AA	32	36.78	74	42.53	Reference	
AG	37	42.53	73	41.95	1.17 (0.64-2.17)	0.59
GG	18	20.69	27	15.52	1.54 (0.69-3.38)	0.24
Dominant						
AA	32	36.78	74	42.53	Reference	
AG+GG	55	63.22	100	57.47	1.27 (0.73-2.24)	0.37
Recessive						
AA+AG	69	79.31	147	84.48	Reference	
GG	18	20.69	27	15.52	1.42 (0.69-2.88)	0.30
rs35470562						
Co-dominant						
GG	30	34.48	78	44.82	Reference	
GA	41	47.13	80	45.98	1.33 (0.73-2.44)	0.32
AA	16	18.39	16	9.20	2.60 (1.06-6.32)	0.04
Dominant						
GG	30	34.48	78	44.82	Reference	
GA+AA	57	65.52	96	55.18	1.54 (0.88-2.74)	0.60
Recessive						
GG+GA	71	81.61	158	90.8	Reference	
AA	16	18.39	16	9.2	2.23 (1.03-5.04)	0.03
rs592121						
Co-dominant						
AA	28	32.18	60	34.48	Reference	
AG	40	45.98	80	45.98	1.07 (0.57-2.02)	0.82
GG	19	21.84	34	19.54	1.20 (0.54-2.60)	0.62
Dominant						
AA	28	32.18	60	34.48	Reference	
AG+GG	59	67.82	114	65.52	1.11 (0.62-2.00)	0.71
Recessive						
AA+AG	68	78.16	140	80.46	Reference	
GG	19	21.84	34	19.54	1.15 (0.57-2.25)	0.66

SNP = single nucleotide polymorphism; OR = odds ratio; CI = confidence interval.

DISCUSSION

In our study, we investigated the role of the *COL9A1* rs1135056, rs592121, and rs35470562 polymorphisms in the development of congenital talipes equinovarus, finding the AA genotype of the latter to be associated with increased risk of this condition.

Collagen IX can combine with tissue inhibitors of matrix metalloproteinases, growth factors, and cartilage cell-surface receptors, and plays an important role in protecting articular cartilage and maintaining its internal stability (Parsons et al., 2011; Brachvogel et al., 2013). It has been reported that mutations in collagen IX genes are associated with the development of skeletal abnormalities (Posey et al., 2008). Therefore, *COL9A1* polymorphisms may be implicated in the pathogenesis of articular cartilage-related diseases.

Previous studies have investigated the relationship between *COL9A1* sequence variations and risk of such conditions, such as Kashin-Beck disease, multiple epiphyseal dysplasia, and osteoarthritis (Snelgrove et al., 2005; Itoh et al., 2006; Shi et al., 2015). Shi et al. (2015) performed a study involving 274 Kashin-Beck disease patients and 248 healthy controls, reporting a significant relationship between *COL9A1* rs6910140 and risk of this

pathology. However, other investigations have generated contrasting results. For instance, Czarny-Ratajczak et al. (2001) failed to establish a significant association between mutations in *COL9A1* and multiple epiphyseal dysplasia risk in the Finnish population, while Jakkula et al. (2005) identified no significant link between cartilage collagen genes (*COL2A1*, *COL9A1*, *COL9A2*, and *COL9A3*) and early-onset osteoarthritis.

Only one previous publication (Liu et al., 2011) has tested the connection between *COL9A1* polymorphism and congenital talipes equinovarus risk. In an analysis incorporating 118 children with this condition and 100 normal controls, the authors reported that COL9A1 protein is highly expressed in individuals with congenital talipes equinovarus, and the rs1135056 genetic variant is associated with its pathogenesis. In another study conducted by Liu et al. (2007), the expression of *COL9A1* mRNA was found to be significantly higher in congenital talipes equinovarus patients than in healthy controls. In the present study, no association between *COL9A1* rs1135056 and risk of congenital talipes equinovarus was evident, but a significant correlation was established between rs35470562 and this disease.

Certain limitations to our study should also be considered. First, the relatively small sample size involved reduces the ability of statistical tests to detect differences between groups. Second, the participants were recruited from only one hospital, which may be a source of selection bias in our analysis.

In conclusion, our results indicate that the *COL9A1* rs35470562 polymorphism may contribute to congenital talipes equinovarus risk in the Chinese population examined. Further research including a greater number of subjects is needed to confirm our findings.

Conflicts of interest

The authors declare no conflict of interest.

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