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Five known tagging DLL3 SNPs are not associated with congenital scoliosis

A case–control association study in a Chinese Han population

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Abstract

Genetic etiology hypothesis is widely accepted in the development of congenital scoliosis (CS). The delta-like 3 (DLL3) gene, a member of the Notch signaling pathway, was implicated to contribute to human CS. In this study, a case–control association study was conducted to determine the association of single nucleotide polymorphism (SNP) in the DLL3 gene with CS in a Chinese Han Population. Five known tagging SNPs of the DLL3 gene were genotyped among 270 Chinese Han subjects (128 nonsyndromic CS patients and 142 matched controls). CS patients were divided into 3 types: type I—failure of formation (29 cases), type II—failure of segmentation (50 cases), and type III—mixed defects (49 cases). The 5 SNPs were analyzed by the allelic and genotypic association analysis, genotype–phenotype association analysis, and haplotype analysis. Allele frequencies of 5 tagging SNPs (SNP1: rs1110627, SNP2: rs3212276, SNP3: rs2304223, SNP4: rs2304222, and SNP5: rs2304214) in CS cases and controls were comparable and there were no available inheritance models. The SNPs were not associated with clinical phenotypes. Moreover, the 5 makers in the DLL3 gene were found to be in strong linkage disequilibrium (LD). Both global haplotype and individual haplotype analyses showed that the haplotypes of SNP1/SNP2/SNP3/SNP4/SNP5 did not correlate with the disease ($P > 0.05$). Together, these data suggest that genetic variants of the DLL3 gene are not associated with CS in the Chinese Han population.

Abbreviations: CI = confidence interval, CS = congenital scoliosis, DLL3 = delta-like 3, DSL = Delta/Serrate/lag-2, HWE = Hardy–Weinberg Equilibrium, LD = linkage disequilibrium, MAF = minor allele frequencies, OR = odds ratio, SCD = spondylocostal dysostosis, SNP = single nucleotide polymorphism, STD = spondylothoracic dysostosis, tSNPs = tagging single nucleotide polymorphisms.

Keywords: congenital scoliosis, DLL3, genetic association, notch, single nucleotide polymorphism

1. Introduction

Congenital scoliosis (CS) is a lateral curvature of the spine that is caused by a defect present at birth. An estimated incidence of CS is ~0.5 to 1/1000 births.[1,2] Vertebral defects of CS are commonly classified into type I (failure of formation, such as hemivertebrae, wedged verterbra, and butterfly vertebrae), type II (failure of segmentation, such as vertebral fusions and unsegmented bar), and type III (mixed defects, some combination of both).[3] A better understanding of the disease pathogenesis will be helpful to improve the prediction of the clinical course of CS, particularly in children. However, the exact pathogenesis of CS is still largely unknown.

It is attractive to disclose the etiology of CS and vertebral malformations from genetic aspects.[4,5] Studies reported that 3.4% families had at least 2 members with a congenital spine deformity[6] and 1 positive family history was present in 5 cases.[7] Wynne-Davies[8] identified a sibling recurrence risk of 2% to 3% in multiple vertebral defect patients from a study of 300 patients with CS. Multigenerational CS in 1 family was also reported.[9] In addition, chromosome rearrangements, including trisomy 8 mosaicism,[10] and balanced and unbalanced translocations[11] contribute to vertebral defects as well.

The candidate genes contributing to CS and vertebral malformations were also studied.[12–16] The delta-like 3 (DLL3) gene is a member of the Notch signaling pathway and plays important roles in somitogenesis through binding to Notch (a transmembrane receptor).[17,18] DLL3 is a divergent member of Delta/Serrate/lag-2 (DSL) family of Notch ligands. Targeted deletion of DLL3 causes a developmental defect in somite segmentation, and consequently vertebral malformation, closely resembling human spondylocostal dysostosis (SCD).[19] However, the association of DLL3 single nucleotide polymorphism (SNP) with CS is still not confirmed.

As a high throughput genotyping system, SNP stream technology had been widely used in biochemistry and genetic

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medical research.[20,21] Different populations may have different genetic associations with CS. To elucidate the role of the DLL3 in CS susceptibility, we determined the association of SNP in the DLL3 gene with CS in the Chinese Han Population.

2. Materials and methods

2.1. Subjects

A total of 128 unrelated CS patients (55 boys and 73 girls, mean age: 12.90 years) without known syndromes and 142 nonscoliosis subjects (61 boys and 81 girls, mean age: 13.39 y) were recruited from Peking Union Medical College Hospital (PUMCH) and Beijing Friendship Hospital (PFH) between October 2005 and September 2010. The subjects from the nonsyndromic CS group and the control group were from Han Chinese population. The nonscoliosis subjects including trauma (11%), infectious disease (82%), and healthy patients (7%) were applied as the controls. All control subjects were frequency matched to the cases on age (±3 years) and sex. The diagnosis of CS was through clinical and radiological (x-ray, CT and MRI) examinations. The 128 CS patients were classified into 3 types: type I (failure of formation including hemivertebrae and wedged verterbra; 29 cases), type II (failure of segmentation mainly including unsegmented bar; 50 cases), and type III (mixed defects of the 2 symptoms; 49 cases).[1] The following subjects were excluded from this study: idiopathic scoliosis patients, neuromuscular scoliosis patients, and scoliosis patients with known syndrome. The known syndrome included Klippel–Feil syndrome (short neck, low posterior hairline, and fusion of cervical vertebrae), Goldenhar's syndrome (associated with craniofacial anomalies, including microtia and epibulbar dermoids because of abnormal branchial arch development), incontinentia pigmenti (hyperpigmented whorls and streaks associated with eye, skin, hair, nail, teeth, and central nervous system abnormalities), and the VACTERL association (Vertebral malformations, Anal atresia, Cardiac malformations, TracheoEsophageal fistula, Renal and Radial anomalies, and Limb defects). All control subjects underwent clinical and radiological examinations. Those with a family history of scoliosis, congenital deformities, neuromuscular diseases, skeletal dysplasia, connective tissue abnormalities, or mentally retardation were excluded from the control group. Informed consent was obtained from each subject and the protocols were approved by the Ethics Committee of PFH and PUMCH. Four-milliliter vein blood from each subject was collected and genomic DNA was extracted using QIAamp DNA Blood Mini Kit.

2.2. SNP identification and selection

Based on genotype data from the International HapMap project (http://www.Hapmap.org), the tagging SNPs (tSNPs) in the "CHB+JPT" ethnic group analysis panel were selected using Haploview 4.1 software (Availability: http://www.broad.mit.edu/mpg/haploview/). The minor allele frequency (MAF) of all selected SNPs was >5%. We eventually selected 5 tagging SNPs including SNP1 (rs1110627), SNP2 (rs3212276), SNP3 (rs2304223), SNP4 (rs2304222), and SNP5 (rs2304214). The gene locations of the 5 tagging SNPs are shown in Table 1.

2.3. Genotyping

Genotyping was carried out by SNP stream technology (Beckman Coulter SNP Stream, Germany). Briefly, SNP stream technology consisted of fidelity polymerase-mediate reaction, single base primer extension and microarray methods.[22] Details of the characteristics of patients, 5 studied SNPs, and the primer sequences are listed in Tables 1 and 2.

2.4. Statistical analysis

In each SNP analysis, Hardy–Weinberg equilibrium (HWE) using the goodness-of-fit χ^2 test was applied. The genotype–phenotype association analysis and allelic and genotypic association analysis

Table 1

Information of the 5 tagging SNPs of DLL3 and the primers used in this study.

SNPs location	SNP ID	Nucleotide substitution, M/m	Primer sequence
Exon5	1 (rs1110627)	C/T	Forward 5′-TGTTCACAGAAGCCATGCT-3′ Reverse 5′-GACGTTGGTGTTCCCTTTC-3′ SNP primers 5′-AGATAGAGTCGATGCCAGCTAGGGYTGCAGCCTGCTCGGCACACC-3′
Intron4	2 (rs3212276)	G/A	Forward 5′-AGAGGGTTCAAACACGTAGTTCT-3′ Reverse 5′-TCGCTTTTCTTCCTAATGAAGTT-3′ SNP primers 5′-CGACTGTAGGTGCGTAACTCAAGGAAAAAGAGACAAGGGCACCGA-3′
Intron2	3 rs2304223	C/G	Forward 5′-AAGACTGAAGACACTCACCTCC-3′ Reverse 5′-AACTCTGGCCTTCATTGAGTACT-3′ SNP primers 5′-AGGGTCTCTACGCTGACGATAAGGTGCCCTGGTTGGGTGAAGGAA-3′
Intron2	4 (rs2304222)	A/G	Forward 5′-AGCAATGGMCATCACCCT-3′ Reverse 5′-AGGTTTCGATGATGAAAGAGAA-3′ SNP primers 5′-GGCTATGATTCGCAATGCTTGAACTCTGGCCTTCATTGAGTACTT-3′
Exon6	5 (rs2304214)	C/T	Forward 5′-ATTGCGGCATGGCTGCAG-3′ Reverse 5′-TTGTGTGTCGGGGGTGCA-3′ SNP primers 5′-GCGGTAGGTTCCCGACATATTCTCACAGTTGGAGCCTTGGAAACC-3′

M/m = major allele and minor allele, SNP = single nucleotide polymorphism.

Table 2

Characteristics of the subjects in CS and control groups.

Characteristics	CS, n = 128	Control, n = 142	*P*
Age, y (mean± SD),	12.91 ± 4.36	13.39 ± 4.28	0.117
Male, n (%)	55 (42.97)	61 (42.96)	0.999
Rib deformity, n (%)	73 (57.48)	—	—
Spinal canal dysplasia, n (%)	43 (33.59)	—	—
Kyphosis, n (%)	63 (49.61)	—	—
Vertebral deformation, n (%)		—	—
Single	35 (27.34)		
Multiple	93 (72.66)		
Type, n (%)		—	—
Type I	29 (22.66)		
Type II	50 (39.06)		
Type III	49 (38.28)		

CS = congenital scoliosis, SD = standard deviation, Type I = failure of formation including hemivertebrae and wedged verterbra, Type II = failure of segmentation mainly including unsegmented bar, Type III = mixed defects.

were performed by χ^2 test (SPSS v17.0). The following statistical analyses were performed by the SNP stats software (Availability: http://bioinfo.iconcologia.net/SNPstats). Pairwise linkage disequilibrium (LD) among SNPs was analyzed using Lewontin's $D\prime$ statistic and the squared correlation statistic r2. SNPstats software was used to calculate the LD coefficient and to define haplotype blocks. In this study, the SNP pairs were considered to be in LD when $D\prime \geq 0.7$ and $r2 \geq 0.7$. The association analysis of haplotypes was similar to that of genotypes with logistic regression and results were shown as odds ratio (OR) and 95% confidence interval (CI). The most frequent haplotype was automatically selected as the reference category and haplotypes were pooled together in a group. $P < 0.05$ was considered significant.

3. Results

3.1. Hardy–Weinberg equilibrium test

Initially, we found that all 5 SNPs had MAF >5%. The distributions of the alleles of all the 5 SNPs met HWE in controls and SC cases (goodness-of-fit χ^2 test, $P > 0.05$, Table 3).

3.2. Single nucleotide polymorphism analysis

As shown in Table 3, the allele frequencies in CS cases and controls (CS/Control) were as follows: SNP1C: 145/165, SNP1T: 109/119, SNP2G: 238/255, SNP2A: 18/29, SNP3C: 229/255, SNP3G: 19/29, SNP4A: 146/266, SNP4G:10/18, SNP5C: 238/255, and SNP5T: 18/29. There were no significant differences between CS case group and control group. Therefore, the polymorphism of all 5 SNPs may not be correlated with the occurrence of CS.

Table 3

Genotype frequencies of subjects in congenital scoliosis (CS) and control groups.

Genotypes	CS, n = 128 (%)	Control, n = 142 (%)	*P*
rs1110627*			0.274
C/C	39 (30.71)	52 (36.62)	
C/T	67 (52.76)	61 (42.96)	
T/T	21 (16.54)	29 (20.42)	
rs3212276			0.292
A/A	1 (0.78)	1 (0.70)	
G/A	16 (12.50)	27 (19.01)	
G/G	111 (86.72)	114 (80.28)	
rs2304223†			0.587
C/C	106 (85.48)	114 (80.28)	
G/C	17 (13.71)	27 (19.01)	
G/G	1 (0.81)	1 (0.70)	
rs2304222			0.410
A/A	118 (92.19)	125 (88.03)	
G/A	10 (7.81)	16 (11.27)	
G/G	0 (0.00)	1 (0.70)	
rs1110627			0.292
C/C	111 (86.72)	114 (80.28)	
C/T	16 (12.50)	27 (19.01)	
T/T	1 (0.78)	1 (0.70)	

CS = congenital scoliosis.
* 1 patient's data missing.
† 4 patients' data missing.

In the risk estimation analysis (Table 4), no SNPs showed significant difference between different genotypes suffering from CS and specific deformed site.

3.3. Genotype–Phenotype analysis

One hundred twenty-eight CS cases had 3 phenotypes: type I (failure of formation, 29 cases), type II (failure of segmentation, 50 cases), and type III (mixed defects, 49 cases). Genotype or allele frequency of the 5 SNPs did not show association with clinical phenotypes in the case group (χ^2 test; $P > 0.05$).

Table 4

Risk estimation of patients with different genotypes suffering from congenital scoliosis and specific deformed site.

			Specific deformed sites					
	Congenital scoliosis		Spinal canal dysplasia		Rib deformity		Kyphosis	
Genotypes	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
rs1110627								
T/T+C/C vs C/T	0.674 (0.418–1.091)	0.108	0.654 (0.330–1.299)	0.224	0.621 (0.352–1.097)	0.100	0.685 (0.377–1.242)	0.212
rs3212276								
A/A+G/A vs G/G	0.624 (0.323–1.203)	0.156	0.931 (0.389–2.226)	0.872	0.494 (0.213–1.146)	0.096	0.500 (0.206–1.214)	0.120
rs2304223								
G/G+G/C vs C/C	0.691 (0.362–1.322)	0.263	0.994 (0.413–2.388)	0.988	0.582 (0.258–1.310)	0.187	0.582 (0.249–1.359)	0.207
rs2304222								
G/G+G/A vs A/A	0.623 (0.274–1.416)	0.255	0.552 (0.154–1.979)	0.355	0.420 (0.215–1.307)	0.122	0.490 (0.158–1.520)	0.209
rs1110627								
T/T+C/T vs C/C	0.624 (0.323–1.203)	0.156	0.931 (0.389–2.226	0.872	0.494 (0.213–1.146)	0.096	0.500 (0.206–1.214)	0.120

CI = confidence interval, OR = odd ratio.

Figure 1. Linkage disequilibrium analysis in the DLL3 gene. *D* is the deviation between the expected haplotype frequency and the observed frequency; *D* is a proportion of the maximum value of *D*, which scaled in [−1, 1] range; R is the correlation coefficient between alleles. DLL3 = delta-like 3.

3.4. Estimation of LD

SNPstats calculation of pairwise measurements of LD among the 5 SNPs revealed strong LD ($D > 0.75$) in the selected SNP1/SNP2/SNP3/SNP4/SNP5 of the DLL3 gene in Chinese Han population. D and r2 for 5 tagging SNPs in DLL3 are shown in Fig. 1. Therefore, we constructed SNP1/SNP2/SNP3/SNP4/SNP5 as a haplotype block located in DLL3. The frequencies of the estimated haplotypes are presented in Table 5 and Fig. 1.

3.5. Haplotype association analysis

The association analysis of the haplotypes with CS is shown in Table 6. We found that the haplotype of SNP1/SNP2/SNP3/SNP4/SNP5 showed no significant association with CS ($P = 0.67$).

4. Discussion

In previous studies, the functional roles of DLL3 in CS were discussed. Mutations in DLL3 cause SCD type I associated with severe axial skeletal malformations, including malformed vertebrae and ribs.[23] Maisenbacher et al[24] found 1 missense variant (S225N) of DLL3 in 46 patients, suggesting a limited contribution of DLL3 to CS. Giampietro et al[25] sequenced the DLL3 gene in 50 patients with congenital vertebral malformations and found a Caucasian male patient with VACTERL (vertebral, cardiac, renal, limb anomalies, anal atresia, and tracheo-esophageal fistula) manifestations was heterozygous for a "G" to "A" missense mutation, resulting in change from glycine to arginine at codon 269. Together, these findings raise the possibility that the DLL3 gene may be a potential susceptibility gene for congenital vertebrae defects. In this present study, we tested polymorphism of the DLL3 gene in the pathogenesis of CS in Chinese Han population. All 5 tagging SNPs in the DLL3 gene were selected as the gene markers for association analysis. However, we found negative results regarding the roles of DLL3 polymorphism in CS.

A tag SNP is characterized by high-linkage disequilibrium (the nonrandom association of alleles at ≥2 loci).[26] It is not necessary to genotype every SNP in a chromosomal region to identify genetic variation. Especially, tag SNPs have advantages in whole-genome SNP association studies in which hundreds of thousands of SNPs across the entire genome are genotyped. For this reason, the International HapMap Project uses tag SNPs to discover genes responsible for certain diseases.[27] In this study, we selected all 5 tagging SNPs in the DLL3 gene with the (MAFs >0.1. All 5 tagging SNPs were genotyped and they were in HWE in control and CS groups. However, we did not find any significant difference in the alleles or genotypes of all 5 SNPs between the cases and controls. In genotype–phenotype analysis, we did not get any positive SNP either. In the haplotype association analysis, the haplotype of SNP1/SNP2/SNP3/SNP4/SNP5 showed no association with CS either. These data suggested that the DLL3 gene might not be a susceptible gene for CS in the Chinese Han population.

The development process of somites, namely somitogenesis, depends on the molecular oscillations of the products of the so-called cyclic genes. The underlying network involves the Wnt, Fgf/Mapk, Notch signaling pathways, and the T-box genes.[28] Mutations that disrupt the patterning of individual somites have dramatic effects on malformations of the ribs and vertebrae. On the basis of mouse–human synteny analysis, Giampietro et al[29] identified 27 loci for CS, 21 of which cause vertebral malformations in the mouse. Mutations in Notch signaling pathway genes, DLL3, MESP2, LFNG, and HES7,[30] can result in monogenic autosomal recessive forms of spondylocostal dysostoses (SCDs), which are a heterogeneous group of axial skeletal disorders characterized by multiple segmentation defects of the vertebrae. In addition, a recessive null mutation in the MESP2 gene[31] was also identified in patients with spondylothoracic dysostosis (STD). Although DLL3 is critical for mammalian somitogenesis,[32] the polymorphisms of DLL3 were not associated with the individual susceptibility to CS.

Table 5

Linkage disequilibrium tests (*D'*) among rs1110627, rs3212276, rs2304223, rs2304222, and rs2304214.

Site	rs3212276	rs2304223	rs2304222	rs2304214
rs1110627	0.999	0.999	0.999	0.999
rs3212276	–	1.000	1.000	1.000
rs2304223	–	–	1.000	1.000
rs2304222	–	–	–	1.000

Table 6

Haplotype analysis among SNPs and congenital scoliosis.

Haplotype	CS (freq)	Control (freq)	OR (95% CI)	*P*
C A G A T	8.00 (0.033)	10.98 (0.039)	0.836 (0.331–2.113)	0.704
C A G G T	10.00 (0.041)	18.00 (0.063)	0.626 (0.283–1.383)	0.243
C G C A C	123.01 (0.500)	136.02 (0.479)	1.088 (0.773–1.531)	0.628
T G C A C	104.99 (0.427)	118.98 (0.419)	1.033 (0.731–1.459)	0.855

Loci chosen for haplotype analysis: rs1110627, rs3212276, rs2304223, rs2304222, and rs2304214.
Global haplotype association *P*-value = 0.666; frequency <0.03 was ignored in analysis.
CI = confidence interval, CS = congenital scoliosis, OR = odd ratio.

There were some limitations in our present study. One limitation was the relatively small sample size. Increasing the sample size will increase power to detect underlying susceptibility genes or loci to some extent. Another limitation could be because of correction of multiple genetic models and the method of multiple testing. It is noteworthy that this is the first association analysis showing that CS is not associated with the DLL3 gene SNP in Chinese Han population. Therefore, it is necessary to replicate the findings in large sample size study or in other population.

In this study, all 5 tagging SNPs polymorphisms of DLL3 were not associated with the individual susceptibility to CS and no model was accepted as the best inheritance model. Our study suggested that genetic variants of the DLL3 gene were not associated with CS and the development of CS in the Chinese Han population.

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