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Original Article

Genetic association analysis between polymorphisms of *HAIRY-AND-ENHANCER-OF SPLIT-7* and congenital scoliosis

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Abstract: Objective: We explored the association between genetic polymorphisms of HAIRY-AND-ENHANCER-OF-SPLIT-7 (HES7) and congenital scoliosis (CS) in 246 cases of congenital scoliosis and non-congenital controls, in which the age and sex were fully matched. All participants were Chinese Han population. Methods: The genome DNA was extracted from peripheral blood sample. Two SNPs were defined for HES7 using NCBI database. The genotypes of two SNPs were determined by SNP stream UHT Genotyping System. Results: Polymorphisms were found in both SNPs and in accordance with Hardy-Weinberg equilibrium. For SNP rs3027279, the difference of two alleles (C and A) frequencies between CS and control groups Was statistically significant. Analysis also showed the difference of two genotypes (C/C and C/A) frequencies between two groups was significant (c^2=5.857, P<0.05). For SNP rs1442849, both difference of two alleles (A and G) frequencies and difference of three genotypes (G/G, G/A and AA) frequencies between two groups were shown statistically significant. Conclusions: The unconditional Logistic regression analysis showed A/A genotype of SNP rsl442849 may be a protective factor (P=0.018<0.05, OR-0.35, 95% CI=0.17-0.74) for the onset of CS, while C/A genotype of SNP rs3027279 increased the onset risk (P=0.015<0.05, OR=1.93, 95% CI=1.13-3.30) of CS. Linkage disequilibrium analysis demonstrated the existence of linkage disequilibrium between the two SNPs.

Keywords: Congenital scoliosis, single nucleotide polymorphisms, *hairy-and-enhance-of-split-7*, association analysis

Introduction

Scoliosis contains two distinct categories, congenital scoliosis (CS) and idiopathic scoliosis (IS). CS is defined as a lateral curvature of the spine due to a developmental abnormality [1-6]. Even the pathogenesis of CS is not clear yet, but increasingly number of studies support the genetic factor involved in the incidence of CS. Notch signaling pathway is important in regulating the formation and anterior-posterior patterning of the vertebrate somite [5]. *HAIRY-AND-ENHANCER-OF-SPLIT-7* (*HES7*), is a putative Notch effector, encodes a transcriptional repressor. *HES7* deficient mice model showed that the somite derivatives such as vertebrae and ribs are severely disorganized which indicated the Hes7 controls the cyclic expression of lunatic fringe and is essential for coordinated somite segmentation [6]. The mutation in the human *HES7* gene also reported can lead spondylocostal dysostosis (SCD)-by the presence of extensive hemivertebrae, truncal shortening and abnormally aligned ribs [7]. We hypothesized that the mutation of *HES7* may associate with the occurrence of human CS. This study selected *HES7* gene as a candidate, by association analysis to explore the relationship between human *HES7* gene and CS susceptibility.

Materials and methods

Patients

The study population consisted of 123 patients with clinical diagnoses of CS at Peking union hospital from September 2005 to May 2009. Specifically, we included patients aged 2-25 years (average 13.5 years), of both sexes (male 51, female 72), were all excluded from syndromic CS or whole spine deformity. Control group contained 123 cases of non-developmental malformation disease patients.

Table 1. The frequencies of genotype and allele gene of two *HES7* gene SNPs in CS and control group

SNP Site	Genotype/ Allele	Control group		CS group	
		Account	Ratio	Account	Ratio
rs1442849*	G	119	0.484	149	0.611
	A	127	0.516	95	0.389
	G/G	30	0.243	45	0.368
	G/A	59	0.480	59	0.480
	A/A	34	0.276	18	0.148
rs3027279*	C	213	0.866	195	0.793
	A	33	0.134	51	0.207
	C/C	90	0.732	72	0.585
	C/A	33	0.268	51	0.415

*$P<0.05$.

The study was approved by Peking Union Hospital Ethics Committee, and all appropriate informed consent was obtained form from the patient and family for this study.

Genomic DNA extraction

Genomic DNA from patient blood samples was prepared with QIAamp DNA Blood Mini Kit according to the manufacturer's protocol (QIAGEN company, US). Polymerase chain reaction (PCR) was performed with 2 ng of genomic NDA, 0.6 mM primers, 0.5 units AmpliTaq Gold polymerase (Applied Biosystems, US), 2.0 mM $MgCl_2$ and standard buffer.

SNPs selection

According to the US National Biotechnology Information Center (NCBI) database, priority selected *HES7* SNPs which heterozygosity higher than 10% and with missense mutated in exon regions or SNPs located in 3' and 5' control regions. Based on this standard we chosen rs1442849 and rs3027279.

Primer design

For each SNP, primary PCR amplification primers were designed by the web-based software provided at http://www.autoprimer.com/ (Beckman Coulter Inc. Fullerton, CA). PCR primers were designed to amplify a short stretch of DNA (90-150 bp) that included a pair of forward and reverse primer and the single-base extension primer. rs1442849: upstream (5'-TAAGGGACTTGGGCGGGA-3'); downstream (5'-CTGGCTAACATGCTTTGC-3'), exon (5'-AGAGCGAGTGACGCATACTAAGGAGGGCGCGGTTTGGATCTCAGA-3'); rs3027279: upstream (5'-AAAGAGGAGGAGTGAAGGG-3'); downstream (5'-TGTAAACCAGCCAGTGCC-3'), exon (5'-AGCGATCTGCGAGACCGTATGGAGGGGAAGGAGAGAAACTGGCCT-3').

Multiplex PCR

Multiplex PCR and SNP analyses were performed with the GenomeLab SNPstream genotyping platform (Beckman Coulter) and its accompanying SNPstream software suite. The high-throughput SNP genotyping assay was performed within 36 hours in accordance with protocol.

SNPs was achieved with multiplex PCR reactions, each containing 1-2 ng of human genomic DNA, 0.5 μM of each primer, 1 mM deoxynucleotide triphosphates (dNTPs), 10 mM Tris-HCl (pH 8.3), 50 m MKCl, 5 mM $MgCl_2$ and 2 units of AmpliTaq Gold (Perkin Elmer) in a total volume of 25 μl. PCR was performed on a Thermo Cycler (MJ Research) with initial denaturation of the DNA templates and Taq enzyme activation at 96°C for 10 min, followed by 45 cycles of denaturation at 94°C for 60 sec, 55°C for 30 sec, and 72°C for 60 sec. The final extension reaction was at 72°C for 10 min.

Extension and hybridization

Extension reactions and hybridizations to the UHT microarray plates were carried out as described by Bell and colleagues with the SNPware UHT reagent kit and the appropriate extension mix kit containing two dideoxy nuclotides labeled with either BODIPY-fluorescein or TAMRA dye (Beckman Coulter). Finally, the plates were read with the GenomeLab SNPstream UHT Array Imager, and fluorescence intensity was measured with help of the UHTImage software. Intensity was plotted and genotypes were called by the UHT-GetGenos software. After visual inspection of the clusters, manual adjustments were made for some of the assays.

Statistical analysis

All statistical analyses were carried-out using SPSS 13.0 software package (SPSS Inc., Chicago, IL) and SNPstats online software (Beckman Coulter), for Hardy-Weinberg equilibrium of genotype frequencies distribution in CS and control group. R-test for genotype/allele frequencies, and these two loci of linkage dis-

Table 2. Non-conditional Logistic regression analysis of SNPs sites in CS and control group.

Ideal pattern		Genotype	Control group	CS group	OR (95% CI)
rs1442849*	Recessive heredity	G/G-G/A	89 (72.4%)	104 (85.2%)	1.00
		A/A	34 (27.6%)	18 (14.8%)	0.45 (0.24-0.86)
rs3027279*	Dominant heredity	C/C	90 (73.2%)	85 (69.1%)	1.00
		C/A	33 (26.8%)	51 (41.5%)	7.93 (1.13-3.30)

*Non-conditional Logistic regression analysis $P<0.05$.

Table 3. Logistic regression analysis of two SNP sits constructed haplotypes.

Haplotype	SNP		Frequencies		OR (95% CI)
	rs1442849	rs3027279	CS group	Control group	
Hap1	A	C	0.5103	0.3840	1.00
Hap2	G	C	0.3556	0.4087	1.47 (0.96-2.26)
Hap3*	G	A	0.1282	0.2012	2.14 (1.23-3.74)
Hap4	A	A	0.0060	0.0061	1.62 (0.09-27.71)

*$P<0.05$.

equilibrium (LD) analysis. Non-conditional Logistic regression models to assess the single-loci genotype and multi-locus haplotypes associated with the risk of CS occurrence. Calculated the odds ratio (OR), 95% confidence interval (95% CI) and *P* value. Accordance with Akaike Information Criterion (AIC) and Bayes Information Criterion (BIC) to determine the most accurate genetic inheritance pattern from five modes. Inspection standards or =0.05.

Result

Only 1 case failure in SNP rs1442849, the genotyping success rate was 99.6%, SNP rs3027279 genotyping success rate was 100%. These two loci genotype and allele distributions in these two groups are shown in **Table 1**. Detection of genotype in CS group and control group, this distribution is in accordance with the frequencies expected, applying the Hardy-Weinberg principle indicated candidate population is representative.

In SNP rs1442849, there were significant differences in the genotype frequencies and allele frequencies in CS group compared with the control group ($P<0.05$; **Table 1**). The single-loci unconditional Logistic regression analysis showed that the ideal genetic model is recessive inheritance mode. Compared with the G/G-G/A genotype, the occurrence of A/A genotype CS significantly decreased ($P=0.013<0.05$, OR=0.45, CI=0.24-0.86; **Table 2**).

In SNP rs3027279, there also were significant differences in the genotype frequencies and allele frequencies in CS group compared with the control group ($P<0.05$; **Table 1**). The single-loci unconditional Logistic regression analysis showed that the ideal genetic model is recessive inheritance mode. Compared with the C/C genotype, the occurrence of C/A genotype CS significantly decreased ($P=0.015<0.05$, OR=1.93, CI=1.13-3.30; **Table 2**).

There is an intense linkage disequilibrium between these two loci ($D'=0.923$, $r^2=0.3812$), these loci constructed four haplotypes are Hap1-AC, Hap2-GC, Hap3-GA and Hap4-AA. The multiple-loci unconditional Logistic regression analysis showed haplotype Hap1-AC as the reference baseline, the occurrence rate of Hap3-GA increased CS is 2.14 (95% CI=1.23-3.74, $P=0.008<0.05$; **Table 3**).

Discussion

CS is classified by orthopedists as a failure of segmentation, failure of formation and mixed defects. The occurrence mechanism of congenital scoliosis (CS) is not clear yet, genetic and environmental factors influencing spinal development. Developmental related gene mutation and pregnancy environmental factors may play a role in the abnormalities associated with human CS. Multiple genetic loci and/or modifying genes is widely considered that contribute toward the development of CS in recently years [8].

Occasional familial clusters often associated with segmentation defects. CS has underlying genetic mechanism, and that a single genetic defect can result in a predisposition to different types of spinal deformities. Since CS is usually a sporadic condition that generally not possible

to use conventional genetic linkage studies to identify chromosome regions that contribute to the development of this disorder. Therefore, disease candidate gene association analysis was utilized in this study.

Knocking-out or mutation mouse model experiments indicated that multiple genes may lead the development of vertebral deformation. Most of these genes are associated with the Notch pathway, their inactivating mutations can result in semi-vertebrae, fused ribs, segmentation defects and thoracic vertebrae fusion. Notch pathway is a crucial signaling pathway in cell-cell boundaries formation. Presently study of human genes indicated that *MESP2*, *HES7*, *LFNG*, *DLL3* and other genes related with human spine and rib deformity [6, 9-11]. But all cases are reported in spondylocostal dysostosis (SCD), mainly for serious segmentation failure of whole spine and ribs which means.

However, CS, generally and studied in this study, means non-syndromic and mild spine/rib deformity result in scoliosis. There is still no report that Notch pathway genes mutation whether relevant with this non-syndromic CS occurrence.

HES7, a *bHLH* gene which downstream Notch effectors, essential for somitogenesis, displays cyclic expression of mRNA in the presomitic mesoderm (PSM), whereas Notch signaling is essential for somitogenesis. Spatial comparison revealed that Hes7 and Lunatic fringe (Lfng) transcription occurs in the Hes7 protein-negative domains [12, 13]. Hes7 expression in the PSM is dynamic and variable, even in embryos at the same stage [14]. Thus, it is likely that Hes7 expression is cyclic in the PSM and observed in two bilateral domains: rostral and caudal stripes which result in severe deformity of spine and ribs [13].

Human *HES7* located on the 17th chromosome p13.1 [15]. In this study, the selected sites rs302729 SNP loci is located in the 5' UTR and the rs1442849 located in 3'UTR, which may be involved in the role of mRNA transcription and splicing. Our research analyzed the impact of *HES7* gene to Chinese Han population patients suffer from CS from single SNP loci and multi-SNP haplotype level. The results reflect that there is a correlation between the *HES7* gene rs1442849 and rs302729 polymorphisms in Chinese Han population and the susceptibility of CS. Haplotype analysis showed that in these two SNP loci haplotypes constructed, the frequency of common haplotype Hap3-GA in CS group is higher than the control group, further analysis embodied that the haplotype Ha3-GA is significantly related to the risk of CS occurrence.

To sum up prevent states, the SNP locus rs3027279 and re1442849 polymorphism of HES7 gene in Chinese Han Population may be associated with susceptibility to non-syndromic CS.

Disclosure of conflict of interest

None.

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