Whole-genome methylation analysis reveals novel epigenetic perturbations of congenital scoliosis

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INTRODUCTION

Congenital scoliosis (CS) is a congenital disease caused by malformations of vertebrae. Recent studies demonstrated that DNA modification could contribute to the pathogenesis of disease. This study aims to identify epigenetic perturbations that may contribute to the pathogenesis of CS. Four CS patients with hemivertebra were enrolled and underwent spine correction operations. DNA was extracted from the hemivertebra and spinal process collected from the specimen during the hemivertebra resection. Genome-wide DNA methylation profiling was examined at base-pair resolution using whole-genome bisulfite sequencing (WGBS). We identified 343 genes with hyper-differentially methylated regions (DMRs) and 222 genes with hypo-DMRs, respectively. These genes were enriched in the mitogen-activated protein kinase (MAPK) signaling pathway, calcium signaling pathway, and axon guidance in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and were enriched in positive regulation of cell morphogenesis involved in differentiation, regulation of cell morphogenesis involved in differentiation, and regulation of neuron projection development in Biological Process of Gene Ontology (GO-BP) terms. Hyper-DMR-related genes, including IGHG1, IGHM, IGHG3, RNF213, and GSE1, and hypo DMR-related genes, including SORCS2, COL5A1, GRID1, RGS3, and ROBO2, may contribute to the pathogenesis of hemivertebra. The aberrant DNA methylation may be associated with the formation of hemivertebra and congenital scoliosis.

RESULTS

Clinical information

Two of the CS patients were female, and the mean age of patients was 7.75 (from 2 to 14) years old (Table 1). Three of them were classified...
into type 1 CS, and one was classified into type 3 CS. The average angle of main Cobb angle was 66 degrees. All of the patients had hemivertebra (Figure 1).

**DNA methylation differences**

The distribution of global methylation level (Figure 2A) and sequencing depth (Figure 2B) between the hemivertebra and spinal process in the four CS patients were similar. We compared the methylation level distribution of the whole-genome DMRs between the hemivertebra body and the spinal process in each patient (Figure 2C). To characterize the distribution of DMRs, we assigned them to known genomic features, which include promoter, 5′ UTR, 3′ UTR, and intronic and intergenic regions. Among all of the hyper-DMRs, 36% were located in intergenic regions, whereas 14% were located in promoter regions. Similarly, for hypo-DMRs, 40% were located in intergenic regions, and a slightly lower percent (9%) was located in promoter regions (Figure 3). Finally, we identified 343 genes with hyper-differentially methylated regions (DMRs) and 222 genes with hypo-DMRs that are consistent among the four patients (Figure 4). We performed an unsupervised hierarchical clustering analysis using DNA methylation level of 343 hyper-DMR-related genes and 222 hypo-DMR-related genes to characterize the global methylation alterations between the hemivertebra group and spinal process group (Figure 5). We enriched these DMR-related genes and then subjected them to Kyoto Encyclopedia of Genes and Genomes (KEGG) and Biological Process of Gene Ontology (GO-BP) terms for pathway analysis. It revealed that DMR-related genes were enriched in several pathways. Three pathways with the most significant association were mitogen-activated protein kinase (MAPK) signaling pathway, calcium signaling pathway, and axon guidance in KEGG pathways. The top three pathways with the most significant association in GO-BP terms were positive regulation of cell morphogenesis involved in differentiation, regulation of cell morphogenesis involved in differentiation, and regulation of neuron projection development (Figure 6), indicating that these pathways could be involved in the development of hemivertebra in CS patients.

**Validation of candidate DMR-related genes**

To identify the specific pathogenic epigenetic variants, we ranked the recurrent DMR-related genes according to the arithmetic mean value of the methylation difference between hemivertebra body and normal spinal process in each group (Table S1). The top five candidate DMR-related genes with greatest hyper-methylation differences were IGHG1, IGHM, IGHG3, RNF213, and GSE1 (Table 2). The top five candidate DMR-related genes with greatest hypo-methylation differences were SORCS2, COL5A1, GRID1, RGS3, and ROBO2 (Table 2). We hypothesize that the different methylation of DMR-related genes may influence the gene expression by regulating the transcription and be associated with the phenotype of hemivertebra of CS.

**SORCS2** encodes Sortilin Related VPS10 Domain Containing Receptor 2 (SORCS2), which is one family member of the vacuolar protein sorting 10 (VPS10) domain-containing receptor proteins. It is indicated that Sorcs2 was prominently expressed in the vertebrae of mice embryo. In a genome-wide association study (GWAS), a locus

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**Table 1. Characteristics of the enrolled CS patients**

| ID     | Gender | Age (y) | CS classification | Main Cobb angle | Hemivertebra location |
|--------|--------|---------|-------------------|-----------------|-----------------------|
| DISCO552 | F      | 12      | 1                 | 73              | T4                    |
| DISCO644 | M      | 14      | 3                 | 75              | T7                    |
| DISCO799 | F      | 3       | 1                 | 56              | L3                    |
| DISCO831 | M      | 2       | 1                 | 80              | L1                    |

CS, congenital scoliosis; F, female; M, male.
of SORCS2 is significantly associated with insulin-like growth factor-binding protein-3 (IGFBP-3), which is involved in bone metabolism.\textsuperscript{16} We hypothesized that the abnormal methylation could influence the expression of SORCS2, which could induce the malformation of vertebrae.

**COL5A1** encodes an alpha chain for one of the low-abundance fibrillar collagens. Mutations of this gene could lead to Ehlers-Danlos syndrome (EDS).\textsuperscript{17} EDS is a kind of connective tissue disorder, characterized by severe muscle hypotonia at birth, joint hypermobility, hyperelastic skin, osteopenia, increased bone fragility, and progressive scoliosis.\textsuperscript{18,19} Although the patients in our study manifested with only hemivertebra, the abnormal methylation of **COL5A1** could lead to abnormal expression of collagen, which may increase the risk of scoliosis development.

**RGS3** encodes Regulator of G-protein signaling 3, which is an antagonism of G protein and interacts with ephrin.\textsuperscript{20} It is postulated that the dislocation of RGS3 could impair the function of Ephrin-B-RGS cell fate signaling complex.\textsuperscript{21} The ephrin signaling within the osteoclast and osteoblast lineages could promote or inhibit cell differentiation in bone remodeling.\textsuperscript{22} Different methylation level of RGS3 could influence bone remodeling and have an effect on the formation of hemivertebra.

**DISCUSSION**

In general, genetic variants and epigenetic modifications are pivotal causes to embryonic somatogenesis failure.\textsuperscript{2,26} Environmental factors, including hypoxia, are validated principal regulators of fibroblast growth factor (FGF) signaling, which is an essential pathway in the pathogenesis of CS.\textsuperscript{2} Up until now, the epigenetic modification of CS is still unclear. In this study, we present the epigenetic landscape of CS, or more specifically hemivertebra. According to previous studies, DNA methylation can predispose to a wide range of congenital developmental diseases.\textsuperscript{10,11} Some studies also indicated that DNA methylation is related to scoliosis.\textsuperscript{12–14} However, there is no study investigating the relation between the whole-genome DNA methylation pattern and CS.

Therefore, we performed whole-genome bisulfite sequencing (WGBS) to detect the whole-genome DNA methylation between the hemivertebra body and normal spinal process of CS patients. Potential pathological signal pathways and DMR-related genes were identified. DMR-related genes with differently methylated level were enriched in several signaling pathways, including the MAPK signaling pathway, calcium signaling pathway, and axon guidance in KEGG pathways, and positive regulation of cell morphogenesis involved in differentiation, regulation of cell morphogenesis involved in differentiation, and regulation of neuron projection development in GO-BP terms.

The MAPK signaling pathway is a critical pathway regulating the proliferation and differentiation of multiple cell types, including osteoprogenitor cells.\textsuperscript{27} Mutations of the genes involved in the MAPK signaling pathway are associated with multiple diseases manifesting scoliosis, such as Noonan syndrome,\textsuperscript{28} Costello syndrome,\textsuperscript{29} and
cardiofaciocutaneous (CFC) syndrome. Transcriptional regulation, including miRNA and DNA methylation in genes involved in the MAPK signaling pathway, was also associated with scoliosis. Thus, we hypothesized that the abnormal DNA methylation of the MAPK signaling pathway-related genes could lead to the hemivertebra development.

Calcium metabolism is very important in bone formation. The abnormal calcium signaling pathway could lead to osteoblast dysfunction and scoliosis. Previous studies also proved that the abnormal transport of calcium ion is involved in scoliosis. Previous studies indicated that the proteins of axon guidance could guide growing axons during development. The defective axon guidance could lead to several diseases manifesting scoliosis. Thus, the abnormalities of these pathways could have potential to increase the risk for hemivertebra.

We also found several common DMR-related genes manifested with the same methylation tendency. Hyper-DMR-related genes, including *IGHG1, IGHM, IGHG3, RNF213*, and *GSE1*, and hypo-DMR-related genes, including *SORCS2, COL5A1, GRID1, RGS3*, and *ROBO2*, were the common DMR-related genes with greatest mean methylation differences.

There are several limitations in this study. First, the abnormal methylation can cause transcriptome disorder in target tissues. In our study, the transcriptome data were not detected; further studies are needed to explore the relationship between abnormal methylation modifications and unfaithful activation of key genes. Second, although this is the pilot, and to our knowledge the first study elucidating the relation between epigenetic modification and congenital scoliosis, the sample size of the study was relatively small. Further studies with larger sample size are necessary to validate our findings.

MATERIALS AND METHODS

Patients and materials

Four patients with CS were recruited from Peking Union Medical College Hospital (PUMCH) from July 2015 to January 2016, as a part of the Deciphering Disorders Involving Scoliosis and Comorbidities (DISCO) study (http://www.discostudy.org/). All the patients had no relationship to each other. All the CS patients manifested with hemivertebra and underwent hemivertebra resection. Parts of the hemivertebra body and normal spinal process were collected from the specimen during the surgery. Genomic DNA was extracted from those specimens using DNeasy Blood & Tissue Kits (QIAGEN, Eastwin Scientific, Beijing, China) according to the manufacturer’s instructions. Written informed consent was obtained from all the participants or their parents. The Ethical...
Review Board of Peking Union Medical College Hospital approved this study.

**WGBS and data analysis**

After the extraction of genomic DNA, WGBS was performed and data analysis was conducted as described in our previous study. DMRs between the hemivertebra body and the spinal process were identified using MethPipe (v.3.4.3) with significantly differentially CpGs ≥ 5 and a minimal number of 10 CpGs that the DMR spans.

Table 2. Top 5 common DMR-related genes with greatest mean hyper- and hypo-methylation differences in the CS patients

| DMR-related genes | Gene ID | DNA methylation direction in HV | Mean methylation difference |
|-------------------|---------|---------------------------------|-----------------------------|
| IGHG1             | ENSG00000211896 | hyper                          | 0.39                        |
| IGHM              | ENSG00000211899 | hyper                          | 0.33                        |
| IGHG3             | ENSG00000211897 | hyper                          | 0.33                        |
| RNF213            | ENSG00000173821 | hyper                          | 0.28                        |
| GSEI              | ENSG00000131149 | hyper                          | 0.24                        |
| SORCS2            | ENSG00000184985 | hypo                           | −0.25                       |
| COL5AJ            | ENSG00000130635 | hypo                           | −0.23                       |
| GRID1             | ENSG00000182771 | hypo                           | −0.21                       |
| RGS3              | ENSG00000138835 | hypo                           | −0.21                       |
| ROBO2             | ENSG00000185008 | hypo                           | −0.20                       |

According to the methylation level difference, all the DMRs were classified into two groups, the hypermethylation group and hypomethylation group. ChIPseeker (v.1.14.0) Bioconductor package was used to evaluate the genomic position distribution of DMRs. Every DMR was assigned to a related RefSeq gene from 3 kb downstream to 3 kb upstream of the TSS. The DMR-related genes were defined as genes with at least one DMR mapped to it. In the case of multiple DMRs being mapped to the same gene, we retained the DMR with the largest absolute methylation difference to represent the gene. Hypo/hyper-DMR-related genes observed among four patients were identified as recurrent DMR-related genes. Hierarchical clustering analysis was performed to characterize the global methylation alterations between the hemivertebra group and spinal process group using the R package. Then, the recurrent DMR-related genes were ordered by the arithmetic mean value of the methylation difference of corresponding DMRs in four groups. All of the common DMR-related genes were enriched at Gene Ontology (GO) and KEGG using clusterProfiler (v.3.6.0) Bioconductor package.
SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
N.W. conceived the project. G.L., H.Z., and N.W. performed the research and analyzed and interpreted the data. G.L. drafted the manuscript. Z.Y., S.Z., S.I., Y.N., X.L., S.W., and Y.Y. helped sample collection. S.L., T.J.Z., and Z.W. performed phenotyping of patients. H.Z., Z.Y., and S.Z. helped with analysis and interpretation of the data. Y.N. provided technique support. Z.W. and T.J.Z. offered professional discussions and instructions. T.J.Z., Z.W., and N.W. conceived and designed the study, revised the manuscript, and provided the final approval of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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