Hypermethylation of SHH in the pathogenesis of congenital anorectal malformations

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Abstract

Objective: This study sought to examine promoter methylation and expression of the identified sonic hedgehog (SHH) gene in terminal rectal tissues of children with congenital anorectal malformations (ARMs).

Methods: Tissue samples from the terminal rectum of pediatric patients with ARMs (five cases each of high and intermediate malformation — two cases of rectovesical fistula, two cases of rectourethral prostatic fistula, one case of cloaca with >3 cm common channel, four cases of rectourethral bulbar fistula and one case of imperforate anus without fistula, respectively, and ten cases of low malformation — five cases of perineal fistula and five cases of vestibular fistula, respectively), and patients with non-gastrointestinal tract malformation (six cases, anal stula and one case of imperforate anus without fistula, respectively, and ten cases of low malformation — five cases of perineal fistula and five cases of vestibular fistula, respectively) were collected and divided into three groups: high-intermediate ARM (ARMhi-int), low ARM (ARMlo), and control (Cont.). Real-time RT-PCR was used to detect mRNA expression levels of the verified differentially methylated gene SHH, and bisulfite genomic sequencing was performed to evaluate DNA methylation in the SHH promoter region.

Results: The average methylation levels of the SHH promoter were significantly higher in ARMhi-int (0.850 ± 0.030, P = 0.0036) and ARMlo (0.540 ± 0.053, P = 0.0087) groups than in Cont. group (0.280 ± 0.032). SHH mRNA expression levels were lower in ARMhi-int (0.340 ± 0.015, P = 0.0065) and ARMlo (0.530 ± 0.042, P = 0.0156) groups than in Cont. group (0.870 ± 0.046). The average methylation levels of the SHH promoter were higher in ARMhi-int group than in ARMlo group (0.850 ± 0.030 vs. 0.540 ± 0.053, P = 0.0095), while SHH expression was significantly reduced in ARMhi-int compared to ARMlo group (0.340 ± 0.15 vs. 0.530 ± 0.042, P = 0.0252). The methylation levels of the SHH promoter in ARMhi-int group were negatively correlated with SHH gene expression (r = −0.89, P < 0.01).

Conclusions: The SHH gene, which plays a major role in the development of the anorectum and enteric nervous system, is hypermethylated at its promoter, and this is correlated with low levels of SHH gene expression. This epigenetic modification may therefore be responsible for the observed changes in SHH expression, which could in turn underlie the pathogenesis of congenital ARMs.

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1. Materials and methods

1.1. Subjects

Terminal rectum specimens were obtained from five children with high ARM (one female and 4 males, median age: 114 ± 25 days, two cases of rectovesical fistula, two cases of rectourethral prostatic fistula, and one case of cloaca with >3 cm common channel, respectively), five children with intermediate ARM (one female and 4 males, median age: 108 ± 16 days, four cases of rectourethral bulbar fistula and one case of imperforate anus without fistula, respectively), 10 children with low ARM (5 females and 5 males, median age: 44 ± 60 days, five cases of perineal fistula and five cases of vestibular fistula, respectively), and six children (control group) with non-ARM gastrointestinal malformations (5 males, median age: 520 ± 101 days, anal fistula), who were admitted to the hospital from January to December 2012. The collected specimens were immediately stored at −80 °C to prevent DNA and RNA degradation.

Inclusion criteria for the ARM group were as follows: 1) cases preliminarily diagnosed and treated at the hospital and receiving surgery, from which the terminal rectum specimens were collected; 2) an absence of severe malformations in other organs such as heart, kidneys, and spine. Inclusion criteria for the control group were as follows: 1) infants diagnosed at the hospital without congenital deficiency; 2) cases receiving anorectal surgery for non-ARM disease at the hospital, from which terminal rectum specimens were collected.

Experiments were approved by the hospital ethics committee. Parents of all the subjects were fully informed of the objectives of the study and the procedures involved, and gave their informed, written consent.

1.2. Grouping criteria

Because methylation status differs for various types of ARM, cases with similar extent of lesions were grouped together and analyzed with one chip, so as to maximize inter-group differences and minimize intra-group variability. Therefore, cases were divided into high-intermediate ARM (ARMhi-int) group, low ARM (ARMlo) group, and the control (Cont.) group, assigned according to a clinical evaluation. Homogenate of terminal rectum samples of patients in each group were prepared in replicate for DNA extraction to reduce intra-sample variability. One chip per group was used. Pair-wise comparisons were made between chip results for ARMhi-int and ARMlo groups, and between the two ARM groups and Cont. group.

1.3. Observed indicators

1.3.1. Whole genome methylation microarray-based scanning and data analysis

Whole genomic DNA extracted from terminal rectum tissue samples was detected using a UV spectrophotometer and by agarose gel electrophoresis. The purity of the DNA sample was assessed from the spectrophotometric reading: an optical density, i.e., absorbance ratio at 260 and 280 nm (OD 260/280) > 1.8 indicated the presence of RNA, which was removed by RNase treatment; OD 260/280 < 1.6 indicated the presence of protein or phenol, which was removed by phenol/chloroform extraction followed by ethanol precipitation.

Genomic DNA was pretreated using the EZ DNA Methylation Kit (Zymo Research Corporation, Irvine, CA, USA), according to manufacturer’s instructions. A whole human genome DNA methylation microarray (Infinium HumanMethylation450 BeadChip Kit; Illumina Inc., San Diego, CA, USA) was used to analyze differential DNA methylation of over 450,000 CpG islands or CpG sites. Methylated and unmethylated probes were designed for each CpG site. Calibration and differential analysis were performed for the methylation sites of differentially expressed genes obtained from the chip.

1.3.2. Real-time reverse transcriptase PCR (RT-PCR) and bisulfite sequencing

Real-time RT-PCR was used to quantify mRNA expression levels, using the PrimerScript RT reagent Kit (Takara Inc., Otsu, Japan), SuperReal PreMix (SYBR Green) was used for the PCR reaction (TIANGEN Biotech (Beijing) Co., Ltd., Beijing, China). DNA was purified using the QIAamp DNA Mini Kit (Qiangen China (Shanghai) Co., Ltd., Shanghai, China), and bisulfite sequencing was performed using the EpiTect Bisulfite Kit (Qiagen) to determine DNA methylation levels in the promoter region of the differentially expressed *SHH* gene.

1.4. Statistical analysis

Statistical analysis was performed using SPSS 14.0 software (SPSS Inc., Chicago, USA). All data are expressed as mean ± standard deviation. Inter-group differences were analyzed using the chi-square test or one-way analysis of variance. P values < 0.05 were considered statistically significant.

2. Results

2.1. Quality assessment of genomic DNA from terminal rectum tissue samples

The assessment of DNA quality by UV spectrophotometry (Table 1) and agarose gel electrophoresis was used to select samples for the microarray analysis.

2.2. Identification of differentially methylated sites in the human genome by microarray analysis

After applying correction methods and performing differential analysis, 32,731 sites differentially methylated in congenital ARM samples were selected. Of these, 15,141 sites representing 5419 genes were hypomethylated, and 17,590 sites representing 5524 genes were hypermethylated.

After applying restrictive conditions (i.e., screening of genes associated with the development and differentiation) according to gene function, the hypermethylation of many genes associated with development and differentiation, such as *SHH, Wnt5a, Fgf10*, and *Cdx1*, was observed (Fig. 1, Table 2).

Based on the results from the microarray, the *SHH* gene was selected for further analysis of promoter methylation status and gene expression.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample Name (group)</th>
<th>Concentration (ng/μl)</th>
<th>A260/A280</th>
<th>A260/A230</th>
<th>Volume (μl)</th>
<th>Total (μg)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ARMlo</td>
<td>227.90</td>
<td>1.88</td>
<td>2.47</td>
<td>90</td>
<td>27.51</td>
<td>Partially degraded</td>
</tr>
<tr>
<td>2</td>
<td>ARMlo</td>
<td>446.00</td>
<td>1.88</td>
<td>2.33</td>
<td>90</td>
<td>40.14</td>
<td>Partially degraded</td>
</tr>
<tr>
<td>3</td>
<td>ARMhi-int</td>
<td>382.40</td>
<td>1.87</td>
<td>2.28</td>
<td>90</td>
<td>34.42</td>
<td>Partially degraded</td>
</tr>
<tr>
<td>4</td>
<td>ARMhi-int</td>
<td>506.00</td>
<td>1.87</td>
<td>1.97</td>
<td>90</td>
<td>45.54</td>
<td>Partially degraded</td>
</tr>
<tr>
<td>5</td>
<td>Cont.</td>
<td>304.70</td>
<td>1.85</td>
<td>1.86</td>
<td>90</td>
<td>27.42</td>
<td>Partially degraded</td>
</tr>
<tr>
<td>6</td>
<td>Cont.</td>
<td>254.60</td>
<td>1.85</td>
<td>1.86</td>
<td>90</td>
<td>22.91</td>
<td>Partially degraded</td>
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</table>
2.3. Determination of transcript expression level and promoter methylation of SHH gene in terminal rectum tissue samples

2.3.1. Relative expression levels of SHH mRNA by real-time RT-PCR (Fig. 2)

The expression levels of SHH mRNA were significantly reduced in ARMhi-int (0.340 ± 0.015, P = 0.0065) and ARMlo (0.530 ± 0.042, P = 0.0156) groups relative to Cont. group (0.870 ± 0.046). The levels of SHH mRNA in ARMhi-int group were significantly lower than in ARMlo group (P = 0.0252).

2.3.2. Average methylation levels in the promoter region of the SHH gene by bisulfite genomic sequencing

The bisulfite sequencing PCR (BSP)-amplified region (~80 to −118 bp) of the SHH gene containing the TATA box is shown in Fig. 3. The average methylation levels of the amplified region in ARMhi-int, ARMlo, and Cont. groups were 0.850 ± 0.030, 0.540 ± 0.053, and 0.280 ± 0.032, respectively (Fig. 4). The average levels of SHH promoter methylation were significantly higher in ARMhi-int (P = 0.0036) and ARMlo (P = 0.0087) groups than in Cont. group. The average methylation levels were also higher in ARMhi-int group than in ARMlo group (P = 0.0095).

3. Discussion

Due to the diverse etiological factors and complex pathological changes of congenital ARM, the specific mechanisms that lead to the development of this condition during embryogenesis are poorly understood. The general view is that ARM has a multifactorial etiology [7,8], with genetic predisposition being a possible contributing factor. Some findings [7,9-13] suggest that epigenetic mechanisms may underlie the development of ARM. The objective of the present study was to establish an epigenetic profile of ARM, specifically by examining DNA methylation status and differential gene expression in ARM.

Table 2

<table>
<thead>
<tr>
<th>Target ID.</th>
<th>ARMhi-int group</th>
<th>ARMlo group</th>
<th>Cont. group</th>
<th>DiffScore</th>
<th>Delta.Beta</th>
<th>UCSC Gene.Name</th>
</tr>
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<tbody>
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<td>cg11824921</td>
<td>0.3424138</td>
<td>0.2397785</td>
<td>0.1374957</td>
<td>32.56393</td>
<td>0.2022828</td>
<td>SHH</td>
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<tr>
<td>cg26003934</td>
<td>0.3270936</td>
<td>0.228761</td>
<td>0.122168</td>
<td>33.61734</td>
<td>0.2007082</td>
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<tr>
<td>cg01777121</td>
<td>0.7249916</td>
<td>0.2211806</td>
<td>0.1579615</td>
<td>47.38047</td>
<td>0.1530301</td>
<td>WNT5A</td>
</tr>
<tr>
<td>cg0589796</td>
<td>0.6583272</td>
<td>0.4843845</td>
<td>0.3413216</td>
<td>28.51171</td>
<td>0.1700629</td>
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<td>cg08822220</td>
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<td>0.5934273</td>
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<td>20.08161</td>
<td>0.2106838</td>
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<tr>
<td>cg045719184</td>
<td>0.5274238</td>
<td>0.1953744</td>
<td>0.0877542</td>
<td>39.43468</td>
<td>0.107589</td>
<td>WNT5A</td>
</tr>
<tr>
<td>cg05921397</td>
<td>0.6838637</td>
<td>0.623097</td>
<td>0.5183616</td>
<td>18.47041</td>
<td>0.1655021</td>
<td>CDX1</td>
</tr>
<tr>
<td>cg08046044</td>
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<td>0.1577074</td>
<td>0.0547227</td>
<td>27.93838</td>
<td>0.1029816</td>
<td>5UT</td>
</tr>
<tr>
<td>cg09229893</td>
<td>0.6249503</td>
<td>0.387467</td>
<td>0.2447952</td>
<td>35.31581</td>
<td>0.1428485</td>
<td>CDX1</td>
</tr>
<tr>
<td>cg03967901</td>
<td>0.854644</td>
<td>0.6746433</td>
<td>0.4635338</td>
<td>31.37993</td>
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<td>CDX1</td>
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<tr>
<td>cg16204420</td>
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<tr>
<td>cg16536714</td>
<td>0.5585532</td>
<td>0.5170862</td>
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<td>CDX1</td>
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<td>cg20344911</td>
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<td>HOXC8</td>
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<td>cg05922306</td>
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<td>0.06461178</td>
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<td>0.2437666</td>
<td>HOXC8</td>
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<td>cg07781427</td>
<td>0.1038339</td>
<td>0.043222</td>
<td>0.001165898</td>
<td>25.78116</td>
<td>0.1026673</td>
<td>HOXC8</td>
</tr>
</tbody>
</table>

Note: 1st Exon = CpG within the first exon of the gene; 3'UTR = In the 3' UTR of the gene; 5'UTR = In the 5' untranslated region of the gene; AVG_Beta = Methylation level (beta) of the CpG locus in the group of samples; Body = CpG within the body of the gene beyond the first exon; Delta Beta = Control AVG_Beta - Case AVG_Beta; Gene. Name = CpG locus coordinates gene name from NCBI database; Signal A = Signal intensity of the unmethylated (A) probe; Signal B = Signal intensity of the methylated (B) probe; TargetID = Identifies the probe name; TSS1500 = Between 200 and 1500 bp upstream of the TSS; TSS200 = Within 200 bp upstream of the transcriptional start site; UCSC REFGENE GROUP; CpG location coordinates from UCSC database; SHH = sonic hedgehog.
patients using microarray technology. The results presented here lay the groundwork for future studies of how DNA methylation may alter transcriptional events during embryogenesis, which can lead to the development of a debilitating condition such as ARM.

The bisulfite-modified 450 K microarray used in this study has high reproducibility, with a repeatability coefficient greater than 95%[14,15]. The chip contains more than 450,000 CpG islands or CpG island shores. The selection of CpG sites and the design of probes for Illumina methylation microarray are based on the results of a human epigenome project pilot study. All selected CpG sites are representative of the methylation status of the CpG islands. Furthermore, these sites have been verified by methylation-specific PCR and BSP, and the results obtained using this microarray are consistent with those of other low-throughput methylation detection methods, thus providing validity to these results.

The Illumina methylation microarray has been widely used to investigate tumor-related diseases, birth defects, and diabetes [16–19]. The present study is the first to use a DNA methylation microarray in the study of ARM. Although portion of the samples’ DNA in this study was degraded, the presence of the integrity of DNA was determined by agarose gel electrophoresis and the methylation analysis was not affected. The screen identified 32,731 differentially methylated sites and of these, 15,141 representing 5419 genes were hypomethylated, while 17,590 sites representing 5524 genes were hypermethylated. It is known that during embryonic development, the rectum migrates from its original position to a final position at the anus; blocking this migration can result in ARM [20]. Therefore, the screening conditions were refined to identify genes associated with development and differentiation, and revealed a host of such genes including SHH, Wnt5a, Fgf10, and Cdx1, all of which were hypermethylated (Fig. 1).

DNA methylation is a common form of epigenetic modification and plays an important role in maintaining the normal physiological state of cells. Changes in DNA methylation pattern can cause chromosomal instability and abnormal gene expression, thereby leading to the development and progression of disease. In general, DNA methylation is negatively correlated with, and thus suppresses, gene expression. Abnormal methylation status could entail hypermethylation, which refers to the presence of a methyl mark at a site that is not normally methylated, and hypomethylation, which is the demethylation of a site that is methylated in normal tissue [21,22]. This study found that a considerable number of genes and loci associated with development and differentiation had altered methylation patterns in ARM tissue samples; in particular, the SHH gene, which is widely acknowledged as having a critical role in ARM pathogenesis [5,6], was differentially methylated and expressed in the ARM samples examined (Fig. 2).

Table 3
<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Relative expression levels of SHH mRNA</th>
<th>Average methylation levels of SHH promoter</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARMhi-int</td>
<td>10</td>
<td>0.340 ± 0.015*</td>
<td>0.850 ± 0.030</td>
<td>−0.89</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>ARMLo</td>
<td>10</td>
<td>0.530 ± 0.042**</td>
<td>0.540 ± 0.053</td>
<td>−0.56</td>
<td>&lt; 0.08</td>
</tr>
<tr>
<td>Cont.</td>
<td>6</td>
<td>0.870 ± 0.046</td>
<td>0.280 ± 0.032</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Compared with Cont. group, * p < 0.01, ** p > 0.05.

Fig. 3. Methylation levels of the promoter region of the SHH gene in terminal rectum of ARM and controls. (A) Schematic diagram showing the BSP-amplified region (−80 to −118 bp) of the SHH promoter for bisulfite genomic sequencing. (B) Average methylation levels of the amplified region in ARMLo-int (0.540 ± 0.053); (C) Average methylation levels of the amplified region in ARMLo (0.540 ± 0.053); (D) Average methylation levels of the amplified region in Cont. (0.280 ± 0.032).

Fig. 4. Average methylation levels in the promoter region of the SHH gene in tissue samples from the terminal rectum of ARM and control groups as determined by bisulfite genomic sequencing.
Based on the evidence linking changes in hedgehog signaling to the abnormal development of the gastrointestinal tract, a possible molecular basis for these changes was examined in ARM patients using RT-PCR to evaluate changes in SHH expression and microarray analysis to examine differential methylation of the SHH gene. The results indicated that SHH gene expression was reduced in congenital ARM patients (Fig. 2), consistent with previous reports [6,23]. In addition, this study also found that the average methylation levels of the SHH promoter were significantly increased in ARM patients (Fig. 4) and negatively correlated with the transcript levels (Table 3). Interestingly, both SHH expression and methylation levels were correlated with the severity of ARM; that is, the high-intermediate ARM group showed greater differences in these parameters with respect to control subjects than did patients in the low ARM group. This may imply that the hypermethylation of the SHH promoter in the terminal rectum of ARM patients was responsible for the down-regulation of SHH transcript expression, which could underlie the development of this condition. However, the specific mechanism responsible for SHH promoter hypermethylation is an aspect that requires further investigation. Some common causes of DNA hypermethylation include the loss of factors protecting CpG islands from methylation; the overexpression of DNA methyltransferase; the disruption of DNA replication timing; and external factors such as diet, environment, infection, among others [24].

In this study, since the results of the human genome methylation microarray analysis provided huge amount of information, it was difficult to accurately describe the overall methylation and expression status of a certain gene. So only the results of preliminary screening were obtained. However, the results of this study provide novel insight into the possible etiology of ARM, and new directions for future investigations. In addition to the observed changes in the SHH gene, changes in expression and methylation status were observed for many genes – specifically those involved in development and differentiation – in terminal rectum tissue samples from patients with congenital ARM. Understanding the role of these genes and their interaction with SHH can provide a more complete picture of ARM pathogenesis, and offer a number of factors that can potentially be targeted for regulation in order to prevent the development of this debilitating condition. However, the specific mechanism of how SHH gene hypermethylation is involved in the pathogenesis of ARM remains to be investigated.

Statement

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Shan Zheng
January 20, 2014

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