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BRIEF COMMUNICATION

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# Atopic asthma after rhinovirus-induced wheezing is associated with DNA methylation change in the *SMAD3* gene promoter

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## Abstract

Children with rhinovirus-induced severe early wheezing have an increased risk of developing asthma later in life. The exact molecular mechanisms for this association are still mostly unknown. To identify potential changes in the transcriptional and epigenetic regulation in rhinovirus-associated atopic or nonatopic asthma, we analyzed a cohort of 5-year-old children (n = 45) according to the virus etiology of the first severe wheezing episode at the mean age of 13 months and to 5-year asthma outcome. The development of atopic asthma in children with early rhinovirus-induced wheezing was associated with DNA methylation changes at several genomic sites in chromosomal regions previously linked to asthma. The strongest changes in atopic asthma were detected in the promoter region of *SMAD3* gene at chr 15q22.33 and introns of *DDO/METTL24* genes at 6q21. These changes were validated to be present also at the average age of 8 years.

**KEYWORDS**
asthma, epigenome, rhinovirus, transcriptome, wheezing

## 1 | INTRODUCTION

Rhinovirus has been detected in 20%-40% of children with moderate-to-severe wheezing episodes during the first 2 years of life.[1-3] This etiology is of particular interest due to strong association with recurrent wheezing, prolonged need of asthma controller medication, doctor-diagnosed asthma up to 13 years of age[4-10], and atopic asthma at school age.[11] The suggested explanations for this association are low interferon responses (ie, impaired viral defense), early airway inflammation (ie, broken epithelial barrier; T helper$_2$-polarized

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immune responses) and genetic variations.[12-16] Despite progress in rhinovirus research, the exact molecular mechanisms for this association are still mostly unknown.[17] Furthermore, early predictive biomarkers are needed for the identification of the rhinovirus-infected children with an increased risk for developing asthma and enabling design of effective intervention strategies to prevent asthma.[11,18] For these reasons, we examined epigenomic and transcriptomic changes in 5-year-old children associated with the virus etiology of their first wheezing episode and later asthma status.

## 2 | METHODS

The detailed study protocols are described in the Data S1.

### 2.1 | Clinical study protocol

The study protocol was approved by the Ethics Committee of the Turku University Hospital and for the first 12 months was registered at ClinicalTrials.gov (NCT00731575). At study entry, standard procedures were carried out as previously described.[19] The inclusion criteria were age 3-23 months, delivery at ≥36 gestational weeks, first wheezing episode and a written informed consent from a guardian. Selected children were recruited to regular follow-up visits. Children were diagnosed to have current asthma at the 4-year follow-up visit if they met one or more of the subsequent criteria during the preceding 12 months: doctor-diagnosed asthma, regular use of doctor-prescribed corticosteroid asthma therapy, use of oral corticosteroids for asthma exacerbations, acute asthma attack relieved by repeated use of bronchodilator.[20] Current atopic asthma was defined as asthma with laboratory-verified sensitization (IgE antibodies >0.35 kU/L) at the 4-year follow-up visit. Nonatopic asthma was defined as asthma without these. The statistical analysis is described in Data S1.

### 2.2 | Epigenome and transcriptome analysis

Total RNA (Tempus Spin RNA Isolation kit) and DNA (Qiagen's QIAamp DNA Blood Maxi kit) were isolated from whole blood. The messenger RNA-seq samples were prepared with Illumina TruSeq RNA Sample Preparation kit v2. The reduced representation bisulfite sequencing (RRBS) libraries were prepared with a protocol adapted from Boyle et al.[21,22] For genomic localization of promoters and enhancers H3K4me1, H3K4me3 and H3K27Ac ChIP-seq was carried out from peripheral blood mononuclear cells isolated from 2 reference individuals. Briefly, the chromatin was prepared (truChIP Low Cell Chromatin Kit, Covaris) and ChIP reactions (Auto Histone ChIP-seq kit) were carried out with Diagenode IP-Star SX 8G robot. The libraries were sequenced with Illumina HiSeq2000 platform. The raw data were quality controlled with FastQC[23] and other tools described in the Data S1. The RNA-seq data were analyzed with RNA Express v1.0.0 in Illumina BaseSpace cloud.[24-26] The RRBS data were filtered with Trim Galore! v0.3.3,[27] and reads were aligned to human genome hg19 with bismark v0.12.5[28] and bowtie2 v2.2.3.[29] The methylation calls were extracted with Bismark. Outliers (eg, RnBeads MDS and PCA[30]) were excluded from the analysis. Differentially methylated regions were identified with RADMeth.[31,32] The ChIP-seq data were analyzed in Illumina BaseSpace Cloud with ChIP-Seq BaseSpace Labs tool. In addition, ENCODE ChIP-seq data on H3K4me1, H3K4me3, H3K27me3, and H3K9me3 from PBMCs were utilized in the analysis to examine colocalization of DNA methylation changes with histone marks.[33-35]

### 2.3 | Targeted pyrosequencing

The oligos were designed with Pyromark Software Assay Design 2.0. Samples were prepared, and pyrosequencing was carried out with Qiagen's Pyromark Q24 according to the manufacturer's instructions. The data were analyzed with Pyromark Advanced Software. Unpaired *t* test was used to calculate the statistical differences between study groups. See the Data S1 for more details.

## 3 | RESULTS

### 3.1 | Patient characteristics

Originally, 124 first-time wheezing children were enrolled of whom 77 (62%) children participated in the 4-year follow-up visit (Table S1). Of these, 48 most representative children were selected for the transcriptome and epigenome studies according to the virus etiology of their first severe wheezing episode, and their 5-year asthma outcome as follows: rhinovirus + asthma + (n = 16), rhinovirus + asthma − (n = 16), and rhinovirus − asthma − (n = 16). Three study subjects from the latter group were excluded after quality analysis. Further details of patient characteristics are shown in Tables S2 and S3.

### 3.2 | DNA methylome and transcriptome changes in rhinovirus-associated wheezing and asthma

Comparison of the children with rhinovirus + atopic asthma + (n = 11) vs rhinovirus + asthma − (n = 16) revealed methylation changes (median change ±20% and FDR ≤0.05) in 17 genomic regions associated with atopic asthma (Table 1). The strongest changes were observed in the region chr6:110720838-110720871 located in the introns of genes D-aspartate oxidase (*DDO*) and methyltransferase like 24 (*METTL24*), and region chr15:67356671-67356696 located 1511 bp upstream of the transcription start site of SMAD family member 3 (*SMAD3*) gene, close to active promoter mark (H3K4me3). Similarly, comparison of the children with rhinovirus + nonatopic asthma + (n = 5) vs rhinovirus + asthma − (n = 10) revealed differences in 13 genomic regions (Table 1). The strongest change associated with nonatopic asthma was observed in the site chr9:139860386-139860387 overlapping enhancer mark H3K4me1, 9.12 kb upstream of the gene prostaglandin D2 synthase (*PTGDS*).

Interestingly, no differences in gene expression (fold change cut off ±1.4, FDR ≤0.05) were observed to be associated with either atopic or nonatopic asthma at the time of sampling, although for example *SMAD3* gene was detected to be expressed in the blood of the study subjects.

### 3.3 | Targeted validation and stability of the DNA methylation changes

The CpG methylation changes associated with atopic asthma at the *DDO/METTL24* intron (chr6:110720839-110720905) and *SMAD3* promoter (chr15:67356631-67356721) with RRBS (Table 1) were confirmed with targeted pyrosequencing of the samples collected at the average age of 5 years (Figure 1A, Figure S1). Importantly, analysis of the samples collected from the same children at the average age of 8 years revealed that the methylation changes associated with atopic asthma in these 2 regions were still present 3 years later ($P \leq 5.08E-04$; Figure 1B).

## 4 | DISCUSSION

We detected epigenetic changes in several genomic regions in children who had suffered early rhinovirus-induced wheezing, and which associated with later onset of asthma. Interestingly, among the strongest changes associated with atopic asthma was DNA

**TABLE 1** The differentially methylated genomic regions associated with rhinovirus-induced wheezing and asthma (median methylation difference ≥20, FDR ≤0.05)

| Genomic location (hg19) | No. of sites | Median meDiff (%) | Genomic element | Chromatin marks (±1 kb) | Closest gene (Symbol) |
|---|---|---|---|---|---|
| Differentially methylated genomic regions associated with atopic asthma | | | | | |
| chr15:67356671-67356696 | 2 | 41.68 | Intergenic | H3K4me1, -me3 | *SMAD3* |
| chr10:116273998-116274013 | 2 | 33.70 | Intronic | H3K4me1 | *ABLIM1* |
| chr2:158287048-158287070 | 2 | 29.13 | Intronic | H3K4me1[a] | *CYTIP* |
| chr7:73456938-73456960 | 4 | 28.34 | Exon-intron | – | *ELN* |
| chr13:110384309-110384326 | 4 | 28.07 | Intergenic | H3K4me3[a] | *LINC00676, IRS2* |
| chr7:35302562-35302563 | 1 | 27.40 | Intergenic | H3K27me3[a] | *TBX20* |
| chr2:31806387-31806402 | 2 | 25.17 | Intergenic | H3K27me3 | *SRD5A2* |
| chr16:89674059-89674095 | 3 | 23.19 | Intergenic | – | *DPEP1* |
| chr17:80542056-80542086 | 3 | 21.92 | Exon-intron | – | *FOXK2* |
| chr19:9711129-9711155 | 2 | 21.65 | Intergenic | – | *ZNF561* |
| chr7:66211672-66211673 | 1 | 21.28 | Intronic | H3K4me1[a] | *RABGEF1* |
| chr7:1005102-1005133 | 5 | 21.24 | Exon, intron | H3K4me1[a] | *COX19, ADAP1* |
| chr14:106437030-106437045 | 2 | −20.36 | Exon-intron | – | *abParts, ADAM6* |
| chr11:123388388-123388389 | 1 | −20.64 | Intergenic | – | *GRAMD1B* |
| chr1:143279717-143279732 | 2 | −20.98 | Intronic | – | *CR936796* |
| chr6:29831764-29831935 | 4 | −24.13 | Intronic | – | *HLA-G, HLA-H* |
| chr6:110720838-110720871 | 3 | −48.19 | Intronic | H3K4me3 | *DDO, METTL24* |
| Differentially methylated genomic regions associated with nonatopic asthma | | | | | |
| chr9:139860386-139860387 | 1 | 45.95 | Intergenic | H3K4me1[a] | *PTGDS* |
| chr6:144329349-144329433 | 4 | 35.90 | Intron, exon | H3K4me1, -me3[a] | *PLAGL1* |
| chr1:3292550-3292562 | 2 | 34.38 | Intron | – | *PRDM16* |
| chr19:2546695-2546719 | 6 | 32.88 | Intron | – | *GNG7* |
| chr7:138726331-138726332 | 1 | 26.95 | Intergenic | – | *ZC3HAV1* |
| chr7:48241582-48241586 | 2 | 24.72 | Intron | – | *ABCA13* |
| chr6:169977857-169977884 | 3 | 24.09 | Intron | – | *WDR27* |
| chr11:67051767-67051780 | 2 | 23.21 | Exon | H3K4me1[a], -me3[a] | *ADRBK1* |
| chr18:77906226-77906417 | 4 | 22.51 | Intron | H3K4me1[a], -me3[a] | *PARD6G-AS1* |
| chr6:20320057-20320136 | 4 | 20.95 | Intergenic | H3K4me1, -me3[a] | *E2F3* |
| chr9:45439874-45439891 | 3 | −23.22 | Intergenic | H3K4me1, -me3 | *LOC102723709* |
| chr4:3572886-3572887 | 1 | −33.30 | Intergenic | – | *LINC00955* |
| chr4:49150172-49150193 | 4 | −34.46 | Intergenic | H3K4me1, -me3, H3K27me3 | *CWH43* |

[a]Differentially methylated region overlaps with the histone mark.

methylation alteration in the promoter of *SMAD3* gene in a well-known asthma locus at chr 15q22.33.[36] SMAD3 protein has an important function in the regulation of immune responses and together with the other components of the transforming growth factor beta (TGFβ) signaling regulate fibrosis in airways.[37] While our study was in progress, DeVries et al[38] reported that hypermethylation of *SMAD3* promoter is associated with asthma of children of asthmatic mothers further confirming importance of this epigenetic change in the molecular pathology of asthma. In addition, we detected changes in *DDO/METTL24* genes at 6q21 and in *HLA* locus at chr 6p21.1-22.3 previously associated with asthma.[39] *HLA-G* gene, 33 kb upstream from the differentially methylated region, is an asthma susceptibility gene,[40] and its expression is induced by allergens.[41] Several other differentially methylated sites were also in the proximity of genes, such as *ELN*[42] or *CYTIP*,[43,44] previously linked to lung or immune cell functions, or viral infections, therefore having potential functional significance in the atopic asthma.

Among the strongest changes associated with nonatopic asthma was DNA methylation change linked to *PTGDS* gene. PTGDS

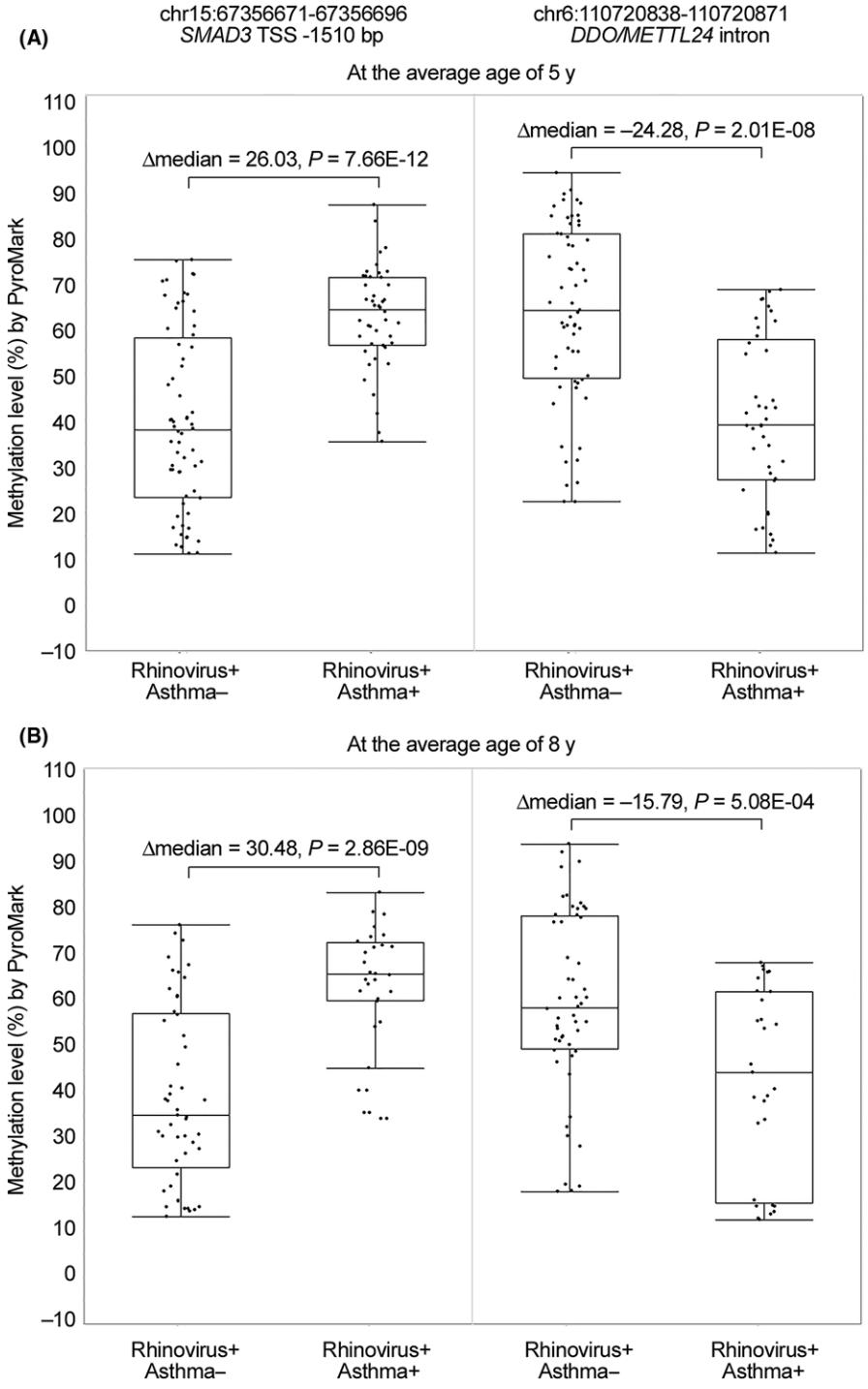


**FIGURE 1** Validation and stability of the DNA methylation changes associated with atopic asthma. The strongest DNA methylation changes associated with atopic asthma (rhinovirus + atopic asthma + vs rhinovirus + atopic asthma −) were validated with targeted pyrosequencing (A) at the average age of 5 y and (B) at the average age of 8 y in samples collected from the same children. In addition, to outlier box plots, the distribution of DNA methylation levels of individual CpG sites within the indicated region for each individual is shown in the figure. In addition, median methylation differences and *t* test *P*-values are shown in the figure

catalyzes synthesis of prostaglandin D2, which mediates development and symptoms of asthma by recruiting Th2 cells and inducing contraction of airways smooth muscle cells.[45,46] Prostaglandin D2 enhances proinflammatory actions of macrophages and subsequent neutrophil activation.[47] Therefore, altered regulation of *PTGDS* may be important in the development of nonatopic asthma.

In conclusion, our results demonstrate that epigenetic changes associated with rhinovirus-induced early wheezing and asthma can be detected in peripheral blood. The strongest changes associated with atopic asthma were localized in the genomic regions previously associated with asthma[15,16,36] and importantly for *SMAD3* promoter and in *DDO/METTL24* gene were detected at the age of both 5 and 8 years. Although we did not detect significant changes in the transcriptomes at the time of measurement, it is possible that the regulation of the affected genes is altered, for example in response to antigen challenge. Alternatively, the DNA methylation changes detected in blood reflect functional changes present in other tissues, such as airways.

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## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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