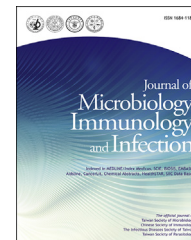


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Original Article

Differential DNA methylation profiles of peripheral blood mononuclear cells in allergic asthmatic children following dust mite immunotherapy

Chuang-Ming Wang ^{a,b,1}, Chia-Bin Chang ^{c,1}, Shiao-Pieng Lee ^d,
Michael W-Y Chan ^c, Shu-Fen Wu ^{e,*}

^a Department of Pediatrics, Ditmanson Medical Foundation Chia-Yi Christian Hospital, Chia-Yi, Taiwan

^b Min-Hwei Junior College of Health Care Management, Tainan, Taiwan

^c Department of Biomedical Sciences and Institute of Molecular Biology, National Chung Cheng University, Chia-Yi, Taiwan

^d Division of Oral and Maxillofacial Surgery, Department of Dentistry, School of Dentistry, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan

^e Center for Innovative Research on Aging Society, National Chung Cheng University, Chia-Yi, Taiwan

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KEYWORDS

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Abstract *Background/Purpose:* Allergen-specific immunotherapy (SIT) is now considered curative to allergic diseases such as asthma. Mechanistically, our previous work showed DNA hypermethylation of cytokine genes, in T-helper cells, in allergic asthmatic children treated with allergen-SIT. In this study, we extended to work to assess possible changes in the DNA methylomes of peripheral blood mononuclear cells (PBMCs), isolated from mite allergen-SIT asthmatic children, to explore further the underlying methylation changes.

Methods: Thirteen allergic asthmatic children who received Der p-SIT, 12 non-SIT allergic asthmatic controls, and 12 healthy controls were enrolled. Bisulfite-converted DNA from Der p-stimulated PBMCs was analyzed using Human Methylation 450 k BeadChip. Pyrosequencing and quantitative real-time PCR were used to validate the DNA methylation levels and the gene expression of individual samples.

Results: We identified 108 significantly differentially methylated regions (DMRs) unique to Der p-treated PBMCs, with 53 probes linked to demethylated DMRs, and 55 probes linked to methylated DMRs. Three associated genes (*BCL6*, *HSPG2*, and *HSP90AA1*), of selected DMRs, were subjected to bisulfite pyrosequencing. Of these, *BCL6* showed significant hypomethylation,

* Corresponding author. Center for Innovative Research on Aging Society, National Chung Cheng University, No. 168, University Road, Min-Hsiung, Chia-Yi, 62102, Taiwan. Fax: +886 5 2722871.

E-mail address: biosfw@ccu.edu.tw (S.-F. Wu).

¹ Chuang-Ming Wang and Chia-Bin Chang contributed equally to this work.

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while *HSPG2* and *HSP90AA1* were hypermethylated in SIT group, compared to the AA group. Furthermore, SIT group had significantly higher gene expression of *BCL6* and lower gene expression of *HSPG2*. KEGG pathway analysis further revealed DMR genes involved in ECM-receptor interactions, asthma, and antigen processing and presentation pathways.

Conclusions: Several DNA regions showed DNA methylation altered by Der p specific immunotherapy, indicating desensitization-associated methylomes. Genes belonging to these SIT-altered pathways may represent therapeutic targets for better clinical management of asthma.

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Introduction

Allergies are on the rise, worldwide, largely due to unknown reasons,¹ and the most common of these is the house dust mite (HDM). As HDMs are invertebrates that lack a digestive system, food is primarily digested by secreted proteases, including the extremely allergenic *Dermatophagoides pteronyssinus* protease (Der p).² Der p, and HDM fecal pellets overall, are easily airborne, and considered to be the most common and sensitizing allergen in the world.^{3,4} Currently, the Der p antigen is generally admixed into immunotherapy cocktails (i.e., “allergy shots”), providing relief to ~ 70% of allergic individuals; however, efficacy often requires continued injections, weekly or biweekly, for several years.⁵

A hallmark of allergy and asthma is the differentiation and activation of type-2 T helper cells (Th2).^{6–8} Moreover, T-cell differentiation, and other immunophenotypes, are largely governed by DNA methylation. Specifically, CpG methylation of the *IL4* and *IFNG* promoters is reported to differ in patients with and without asthma.⁹ Further, DNA methylation changes in Th2 immunity genes have been reported in African American inner-city children.¹⁰ In our previous study, we demonstrated that DNA methylation of cytokine genes increased in T-helper cells in allergic asthmatic children, following treatment with Der p allergen-specific immunotherapy (Der p-SIT).¹¹ Such DNA methylation changes, of cytokine genes in T-helper cells, have been well demonstrated to occur, in allergic asthmatic children, after allergen-SIT.¹¹

In addition to T cells, monocytes are also implicated in the pathogenesis of asthma.¹² For example, studies have found functional differences between peripheral blood mononuclear cells (PBMCs) from patients with asthma, compared to those from healthy subjects.¹³ These anomalous effects of monocyte activation include systemic inflammation, elevated secretion of cytokines, and oxidative stress, with the latter previously associated with asthma.¹⁰ Another recent study reported activation of CD8+, but not CD4+, T cells, in adult patients with severe asthma, whereas patients with non-severe asthma did not differ from controls.¹⁴ In another previous study by our group, we showed that mite allergen immunotherapy altered dendritic cell phenotypes, in allergic asthmatic children.¹⁵

Based on these previous findings, we further hypothesized that methylomic changes in non-T helper cells, in children with allergic asthma, following Der p-SIT. To address that hypothesis, we analyzed genome-wide DNA methylation profiles of PBMCs isolated from allergic asthmatic children, with and without Der p-SIT, and also from healthy children. Further, we searched for differentially methylated regions (DMRs), and then subjected these newfound DMRs to search in the Gene Ontology (GO)¹⁶ and the Kyoto Encyclopedia of Genes and Genomes (KEGG)¹⁷ databases, for genes/pathways likely impacted by SIT-mediated changes in DNA methylation.

Methods

Study participants

The minimum sample size for various potential analyses were calculated at a predetermined α level of 0.05 and a power of $1-\beta = 0.8$, using the free G*Power 3 software, version 3.1.5 (Heinrich Heine University Duesseldorf, Duesseldorf, Germany), the statistical power analysis was based on the previous studies of the expression of CD203c cell marker.¹¹ According to the fluorescence intensity of CD203c, the sample size was estimated to be approximately 12 cases in each group for 2-way analysis of variance with repeated measurements for 2 groups (intervention and control). A total of 37 children, aged 6–18 years, were enrolled through the Department of Pediatrics at Chia-Yi Christian Hospital, including 13 allergic asthmatic children who received Der p-SIT, 12 non-SIT allergic asthmatic (AA) controls, and 12 healthy controls (HCs).¹¹ All 25 child subjects had a recorded clinical history of asthma and a known Der p allergen-specific IgE (<0.35 kU/ml). Determination of Der p allergen-specific IgE levels was applied by standard ImmunoCAP assay (Phadia, Uppsala, Sweden). Children with total IgE < 100 IU/ml in serum, and no history of allergic symptoms, comprised the HC group. According to specific immunotherapy protocol, SIT group had received SIT in our outpatient clinic for 3–4 years. It was approved by the Ethics Committee of Chia-Yi Christian Hospital in Taiwan (Institutional Review Board No. 103071). The demographic characteristics of the study participants was showed in the [supplementary Table 1](#).

DNA bisulfite conversion and methylation analysis

Genomic DNA was isolated from Der p-stimulated PBMCs, using an AllPrep mini DNA kit (Qiagen, Hilden, Germany). Isolated DNA was then bisulfite-converted using an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. For genome-wide DNA methylation analyses, two 500-ng sample mixes, one from six randomly selected DNA samples, and the other from seven random DNA samples in the SIT group, were subjected to whole-methylome analysis, using Illumina's (San Diego, CA, USA) Infinium Human Methylation 450 k BeadChip, while two mixes of equal DNA sample numbers were also measured from the AA and HC groups.

Global DNA methylation assessment

Approximately 485,000 individual CpG sites, associated with over 24,000 genes, were evaluated for bisulfite-converted DNA binding, to methylated (M) and unmethylated (U) allele intensities, using a BeadArray Reader (Illumina). Beta (β) values were used to represent the proportions of $M/(M + U + 100)$, where 100 in the denominator is a constant offset recommended by Illumina to

regularize β -values, when both methylated and unmethylated intensities are low. The beta value range is then bounded by -1 to 1 , whereby a value of -1 indicates that the CpG is completely unmethylated, and a value of 1 indicates complete methylation.

Differentially methylated regions

Differentially methylated probes were defined by the following criteria: (1) A minimum difference of mean β -values ≥ 0.1 between the SIT, AA, and HC groups; and (2) A p -value < 0.05 between these groups. Genome-wide DNA methylation changes were compared between two sets of allergic asthma children with/without SIT treatment, and healthy controls. From these assays, we identified 108 (AA-SIT) differentially methylated CG sites in this study. The maximum differences of beta value of 20 probes, between the Der p-treated AA and Der p-treated SIT groups, are shown in [Tables 1 and 2](#).

Pathway and network analysis

The selected differentially methylated regions between the SIT and AA groups were assigned Entrez_Gene_IDs using the

Table 1 Top 20 hypermethylated CG sites in SIT, compared to AA PBMCs.

TargetID	gene	position	P value	Difference of β value (SIT-AA)
cg21272897	HSPG2 (heparan sulfate proteoglycan 2)	Gene body	0.038551998	0.36602059
cg24158878	COL11A2 (collagen type XI alpha 2 chain)	Gene body	0.025821467	0.26204435
cg18032705	PREX1 (phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1)	Gene body	0.038645248	0.20683235
cg11973981	ISPD (isoprenoid synthase domain containing)	Gene body	0.001278681	0.20586725
cg24638099	HLA-DRB6 (major histocompatibility complex, class II, DR beta 6)	Gene body	0.049289341	0.1881278
cg23836570	FBRSL1 (fibrosin like 1)	Gene body	0.002799073	0.1805093
cg26882525	SLC36A3 (solute carrier family 36 member 3)	Gene body	0.030255648	0.17902495
cg15897435	MRPL1 (mitochondrial ribosomal protein L1)	Gene body	0.01713076	0.177114
cg01991743	HLA-DPB1 (major histocompatibility complex, class II, DP beta 1)	Gene body	0.032061939	0.1719655
cg16255663	MGMT (O-6-methylguanine-DNA methyltransferase)	Gene body	0.00978087	0.17063035
cg20087192	PXK (PX domain containing serine/threonine kinase like)	promoter	0.001210414	0.1700835
cg24634471	JRK (Jrk helix-turn-helix protein)	promoter	0.033451504	0.16559995
cg23224356	ANKRD36B (ankyrin repeat domain 36 B)	promoter	0.04055948	0.16157865
cg05383619	HLA-DRB1 (major histocompatibility complex, class II, DR beta 1)	Gene body	0.024114689	0.14902235
cg04406784	PKD1 (polycystin 1, transient receptor potential channel interacting)	Gene body	0.014408419	0.14055505
cg19000611	CATSPERB (cation channel sperm associated auxiliary subunit beta)	promoter	0.043921467	0.13565855
cg14711243	ACAD9 (acyl-CoA dehydrogenase family member 9)	promoter	0.01223833	0.135408325
cg20114890	HLA-H (major histocompatibility complex, class I, H)	Gene body	0.027692254	0.1301392
cg09192412	CASP6 (caspase 6)	Gene body	0.027825727	0.12140525
cg02017208	HSP90AA1 (heat shock protein 90 alpha family class A member 1)	3'-UTR	0.005528951	0.1182166

Table 2 Top 20 hypomethylated CG sites in SIT, compared to AA PBMCs.

TargetID	gene	position	P value	difference of β value (SIT-AA)
cg03446092	MTG1 (mitochondrial ribosome associated GTPase 1)	Gene body	0.005966237	-0.1063403
cg10549986	RSAD2 (radical S-adenosyl methionine domain containing 2)	Gene body	0.038134647	-0.10696595
cg21210642	TRIM14 (tripartite motif containing 14)	promoter	0.048097862	-0.1084464
cg18146226	BCL6 (B-cell CLL/lymphoma 6)	Gene body	0.036491495	-0.10904885
cg05533223	XIST (X inactive specific transcript)	Gene body	0.045224802	-0.1110039
cg02923224	FHIT (fragile histidine triad)	Gene body	0.019517942	-0.11892905
cg23291158	KCNMA1 (potassium calcium-activated channel subfamily M alpha 1)	Gene body	0.035127298	-0.12046705
cg18001722	GRK5 (G protein-coupled receptor kinase 5)	Gene body	0.035566443	-0.1303345
cg02512559	CFDP1 (craniofacial development protein 1)	Gene body	0.025680526	-0.13326975
cg20141578	BCL2L14 (BCL2 like 14)	promoter	0.021783985	-0.13398205
cg11601967	MAD1L1 (MAD1 mitotic arrest deficient like 1)	Gene body	0.01516843	-0.13974855
cg12161182	NINJ2 (ninjurin 2)	Gene body	0.047797825	-0.1443866
cg27286337	INPP5A (inositol polyphosphate-5-phosphatase A)	Gene body	0.047598859	-0.15372995
cg02715072	TNXB (tenascin XB)	Gene body	0.042975953	-0.1540811
cg18763100	HLA-DMA (major histocompatibility complex, class II, DM alpha)	Gene body	0.0405042	-0.17527595
cg15550113	OGFR (opioid growth factor receptor)	Gene body	0.03283779	-0.1932183
cg13935315	ZNF443 (zinc finger protein 443)	Gene body	0.015947004	-0.20269845
cg24506221	GSTM1 (glutathione S-transferase mu 1)	promoter	0.041885048	-0.2111475
cg21130926	SULF2 (sulfatase 2)	promoter	0.038844132	-0.2239418
cg19405842	PRKCZ (protein kinase C zeta)	promoter	0.034833617	-0.3025489

freely accessible web tool, Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8, for gene annotation.¹⁸ Consequently, we entered lists of 108 gene IDs, associated with DMRs, to analyze their KEGG¹⁹ pathway annotations. From the significant pathway lists ($p < 0.05$), we further selected those consisting of at least two genes (Table 3). The functional relevance of our selected probes was also investigated by analysis of their Gene Ontology (GO) terms²⁰ (<http://app.aporc.org/NOA/>). The resulting GO gene lists, including numerous biological processes, cellular components, and molecular functions, are shown in Table 4 ($p < 0.05$).

Locus-specific bisulfite sequencing validation of microarray findings

For pyrosequencing, bisulfite-converted DNA was used to amplify the differentially methylated sites, using PCR and sequencing with specific primers. The position of PCR

primers located at the exon 8 of BCL6 gene, or the exon 36 of HSPG2 gene, or the 3'-UTR of HSP90AA gene. These designed primers were listed in [supplementary Table 2](#). Pyrosequencing was performed on a PyroMark Q24 sequencer, using the reagents and protocols supplied by the manufacturer (Qiagen).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from Der p-stimulated PBMCs using GENEzol™ TriRNA Pure Kit (Geneaid, New Taipei city, Taiwan) according to the manufacturer's protocol. cDNA was synthesized using MMLV Reverse Transcription Kit (Protech Technology Enterprise, Taipei, Taiwan). qRT-PCR with a SYBR Green on StepOne cycler (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) was used for amplification of selected genes. Expression of each gene was determined relative to that of GAPDH, and the relative

Table 3 KEGG pathway analysis of different methylation region.

KEGG name	KEGG ID	p-value	gene No	Gene list
ECM-receptor interaction	KEGG-04512	3.10E-02	3	COL11A2, HSPG2, TNXB
Asthma	KEGG-05310	4.00E-03	3	HLA-DMA, HLA-DPB1, HLA-DRB1
Antigen processing and presentation	KEGG-04612	1.70E-03	5	HSP90AA1, HLA-DMA, HLA-DPB1, HLA-DRB1, PTPRN2

Table 4 Gene ontology analysis of different methylation regions.

category	GO category	GO ID	p-value	gene No	Gene list	
Biological Processes	antigen processing and presentation	GO:0019882	9.70E-04	4	HLA-DRB1; HLA-DPB1; HLA-H; HLA-DMA	
		GO:0042981	9.80E-04	9	PREX1; MGMT; CASP6; PRKCZ; ZNF443; BCL6; KCNMA1; BCL2L14; CFDP1	
	mitochondrial transport	GO:0006839	0.001	3	GRPEL1; HSP90AA1; SLC25A37	
		GO:0006461	0.0043	6	TSPAN4; PREX1; HSP90AA1; PRKCZ; GTF2A1; KCNMA1	
	protein complex assembly	GO:0001952	0.0046	2	PRKCZ; BCL6	
		GO:0007155	0.0214	6	PKD1; COL11A2; HSPG2; NINJ2; TNXB; CFDP1	
	Cellular Components	MHC protein complex	GO:0042611	4.50E-04	4	HLA-DRB1; HLA-DPB1; HLA-H; HLA-DMA
		fibrillar collagen extracellular matrix part	GO:0005583	4.90E-04	2	COL11A2; TNXB
GO:0044420			0.0047	3	COL11A2; HSPG2; TNXB	
integral to plasma membrane		GO:0005887	0.0329	7	HLA-DRB1; TSPAN4; IL17RA; PKD1; HLA-H; NINJ2; PTPRN2	
Molecular Functions	interleukin-17 binding	GO:0019975	0.0029	1	IL17RA	
		GO:0003908	0.0029	1	MGMT	
	methylated-DNA-[protein]-cysteine S-methyltransferase activity	GO:0005543	0.0192	3	PREX1; PXK; GRK5	
		GO:0030911	0.0089	1	HSP90AA1	
	TPR domain binding					

fold change was calculated by the $\Delta\Delta C_t$ method. Specific primers for mRNA expression level were listed in [supplementary Table 3](#). The position of these primers included the exon 7–8 of BCL6 gene, the exon 34–36 of HSPG2 gene, and the exon 1–2 of HSP90AA1 gene.

Statistical analysis

Statistical comparisons were performed using one-way ANOVA followed by Kruskal–Wallis test between the different subject groups. GraphPad Prism Software version 5 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analysis. A p-value <0.05 was considered statistically significant.

Results

Genome-wide DNA methylation profile changes between SIT and AA groups

The distribution of DNA methylation data of Der p-treated PBMCs from allergen-specific immunotherapy (SIT),

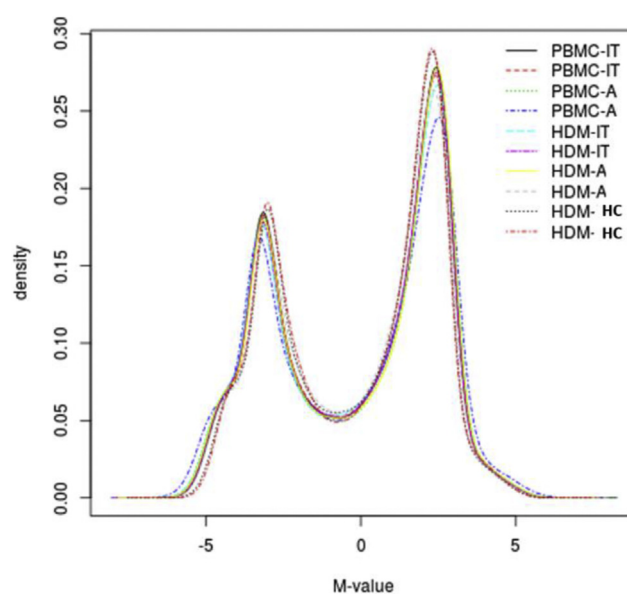


Fig. 1. The distribution of DNA methylation data. The M value of normalized data among the three groups (SIT, AA and HC) stimulated with Der p (HDM) or without Der p (PBMC).

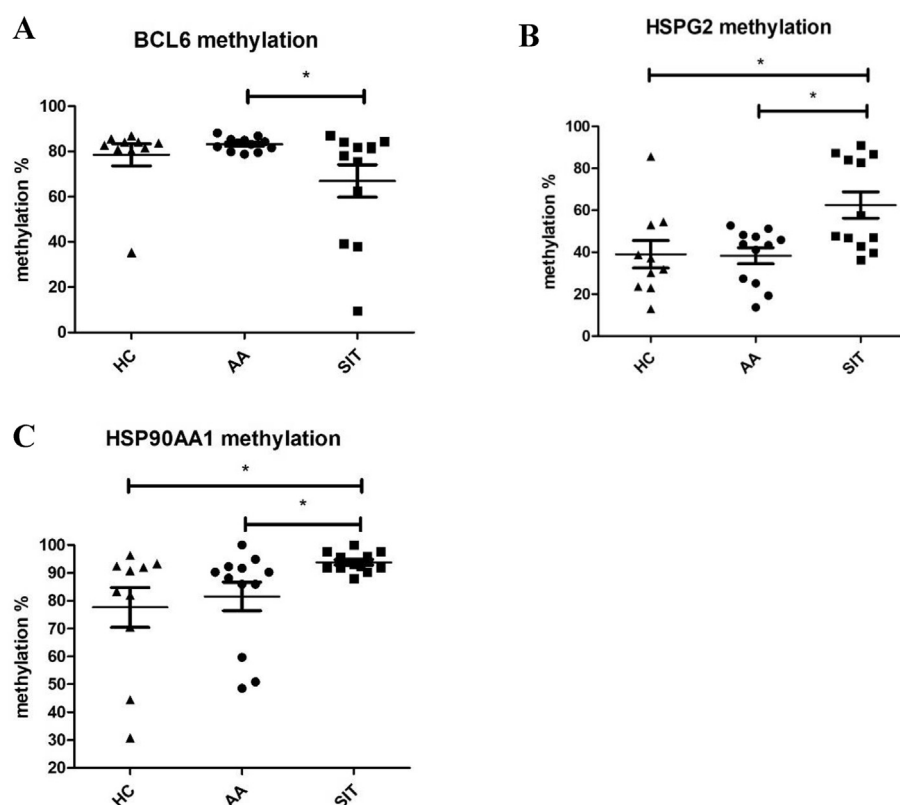


Fig. 2. DNA Methylation levels of selected differentially methylated sites in the SIT, AA and HC groups. The methylation statuses of selected probe regions, including (A) *BCL6*, (B) *HSPG2*, and (C) *HSP90AA1* were investigated, using bisulfite-DNA pyrosequencing, of PBMC DNA obtained from SIT ($n = 12$), AA ($n = 12$), and HC ($n = 10$) children. Horizontal bars represent the means, and vertical bars indicate the standard errors of the means. SIT: ■, AA: ●, HC: ▲, * $p < 0.05$. Statistical comparisons of (A) to (C) were analyzed using a one-way ANOVA followed by Kruskal–Wallis test.

untreated allergic asthma (AA), and healthy control (HC) subjects was shown in Fig. 1. Genome-wide DNA methylation changes were compared between two sets of Der p-treated PBMCs from SIT and AA groups, respectively, using a high-density microarray. Using a cutoff p -value of ≤ 0.05 , and an absolute difference in beta value (SIT-AA) ≥ 0.1 , we identified 108 gene-associated DMRs from Der p-treated PBMCs from the SIT vs. AA groups, respectively. Among associated genes, 53 were exclusively linked to hypomethylated DMRs, and 55 were linked to hypermethylated DMRs. The maximum difference of beta value and p -value ≤ 0.05 of 20 probes between AA and SIT are shown in Tables 1 and 2. Several genes involved in the extracellular matrix (ECM) were hypermethylated in SIT patients, including *HSPG2* (heparin sulfate proteoglycan 2) and *COL11A2* (collagen type XI alpha 2 chain). Other categorized genes were found to comprise the MHC class II protein complex, including *HLA-DRB6* (major histocompatibility complex, class II, DR beta 6), *HLA-DPB1* (major histocompatibility complex, class II, DP beta 1), and *HLA-DRB1* (major histocompatibility complex, class II, DR beta 1). Other significantly hypomethylated genes, from SIT PBMCs, encoded antioxidant enzymes, including *GSTM1* (glutathione S-transferase mu 1), and regulators of apoptosis, including *BCL2L14* (BCL2 like-14) and *BCL6* (B-cell CLL/lymphoma-6).

Pathway and network analysis of differentially methylated genes between SIT and AA PBMCs

To investigate the pathway and network interactions underlying differential DNA methylation between the SIT and AA groups, gene ontology (GO) terms, and KEGG pathway analysis, were used. From the input list of 108 DMRs from SIT vs. AA PBMCs, Tables 3 and 4 list significant terms (adjusted $p < 0.05$), and their associated genes that reside within their DMRs, including the distinct GO categories of antigen processing and presentation, regulation of apoptosis, and extracellular matrix. Likewise, KEGG pathway analysis revealed genes involved in ECM-receptor interactions, asthma, and antigen processing and presentation pathways (Table 3).

Validation of selected probes by pyrosequencing and qRT-PCR

To validate the DNA methylation levels of individual samples, we selected three probes (cg21272897, cg02017208, cg18146226), from Tables 1 and 2, to perform DNA pyrosequencing. Some genes associated with the three probes were previously correlated to allergic asthma and Th2

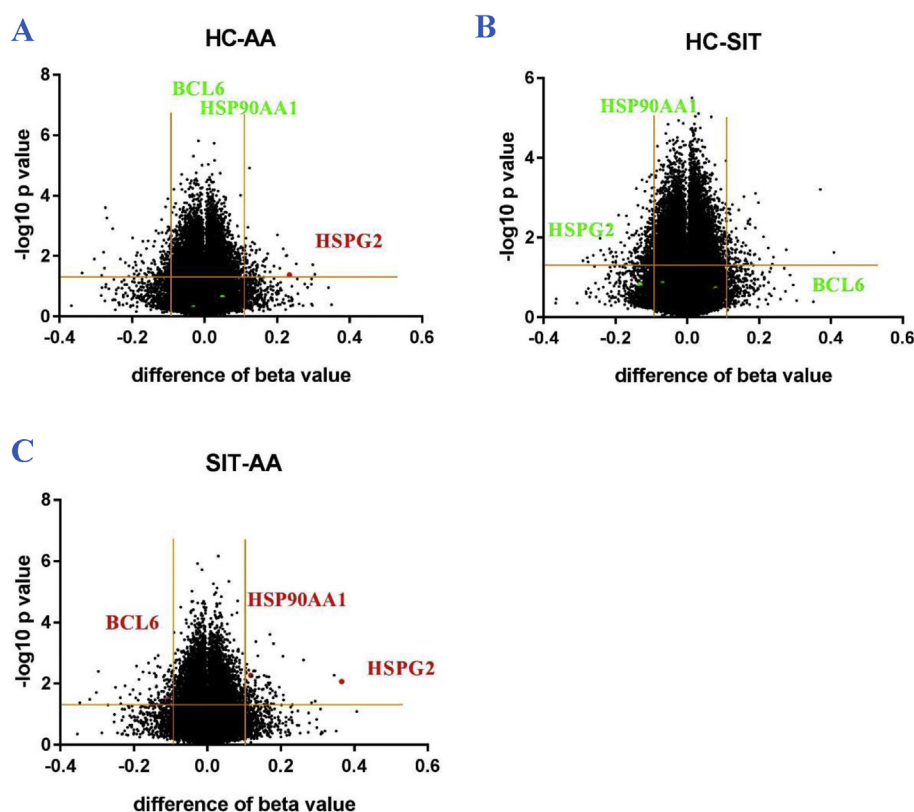


Fig. 3. The volcano plot of the genome-wide DNA methylation profile between SIT, AA, and HC. A comparison of the SIT, AA, and HC groups in the DNA methylation level was represented by the difference of β -value. The orange vertical lines were represented by the range of difference of mean β -values ≤ 0.1 . The orange horizontal line represented the statistical significance in the DNA methylation level (upper, this line means $p < 0.05$). (A) HC vs. AA: the red plot indicated the HSPG2 cg21272897 probe; whereas, the green plots represented the BCL6 cg18146226 and HSP90AA1 cg02017208 probes. (B) HC vs. SIT: the green plots represented the HSPG2 cg21272897, BCL6 cg18146226, and HSP90AA1 cg02017208 probes. (C) SIT vs. AA: the red plot represented the HSPG2 cg21272897, BCL6 cg18146226, and HSP90AA1 cg02017208 probes.

inflammation.^{21–24} Significant hypomethylation of the anti-apoptosis gene, *BCL6*, in the SIT group (Fig. 2A), was consistent with our identified DMRs (from Table 2), while hypermethylation of *HSPG2* (Heparan Sulfate Proteoglycan 2) and *HSP90AA1* (Heat Shock Protein 90 Alpha family class A member 1), in SIT PBMCs (Fig. 2B and C), was similar to Table 1. The volcano plot of the genome-wide DNA methylation profile between SIT, AA, and HC subjects were represented in Fig. 3 (HC vs. AA, Fig. 3A; HC vs. SIT, Fig. 3B; SIT vs. AA, Fig. 3C). We found that the HC and SIT groups had similar methylation levels at *BCL6* cg18146226, *HSP90AA1* cg02017208, and *HSPG2* cg21272897 probes (Fig. 3B). Of note, the methylation level of *BCL6* was downregulation after immunotherapy (Fig. 3C). Moreover, both the HC and SIT groups showed significantly higher methylation levels than the AA group at *HSPG2* cg21272897 probe (Fig. 3A and C). However, the DNA methylation levels of the promoter region were similar between the HC, AA, and SIT groups in *BCL6*, *HSPG2*, and *HSP90AA1*, as shown in supplementary Figure 1.

To confirm these findings, RNA was extracted from Der p stimulated PBMCs of AA, SIT or control groups and subjected to qRT-PCR. *BCL6* was significantly increased in SIT group (Fig. 4A). *HSPG2* and *HSP90AA1* was decreased in SIT

group (Fig. 4B and C), although *HSP90AA1* did not show significant difference (Fig. 4C). These results indicate that Der p-SIT alters DNA methylation levels, of genes, in distinct pathways, and thus, our identified differentially methylated probes merit further examination.

Discussion

In this study, we compared genome-wide DNA methylation profiles of PBMCs from Der p-SIT and AA control groups, with the former demonstrating 108 differentially methylated regions (DMRs), linked to genes such as *HSPG2*, *HLA-DRB6*, *HLA-DPB1*, *HLA-DRB1*, *GSTM1*, *BCL2L14*, and *BCL6*. These 108 DMRs mapped to several networks and pathways involving antigen processing and presentation, regulation of apoptosis, and extracellular matrix remodeling. Selected probes were then validated by bisulfite DNA pyrosequencing and qRT-PCR.

Airway oxidative stress is also well-implicated in the pathogenesis of asthma.²⁵ For example, the antioxidant superfamily of glutathione S-transferases (GSTs), including the subclasses GSTP1 and GSTM1, is normally expressed in the lungs, to help reduce lung epithelial cell inflammation

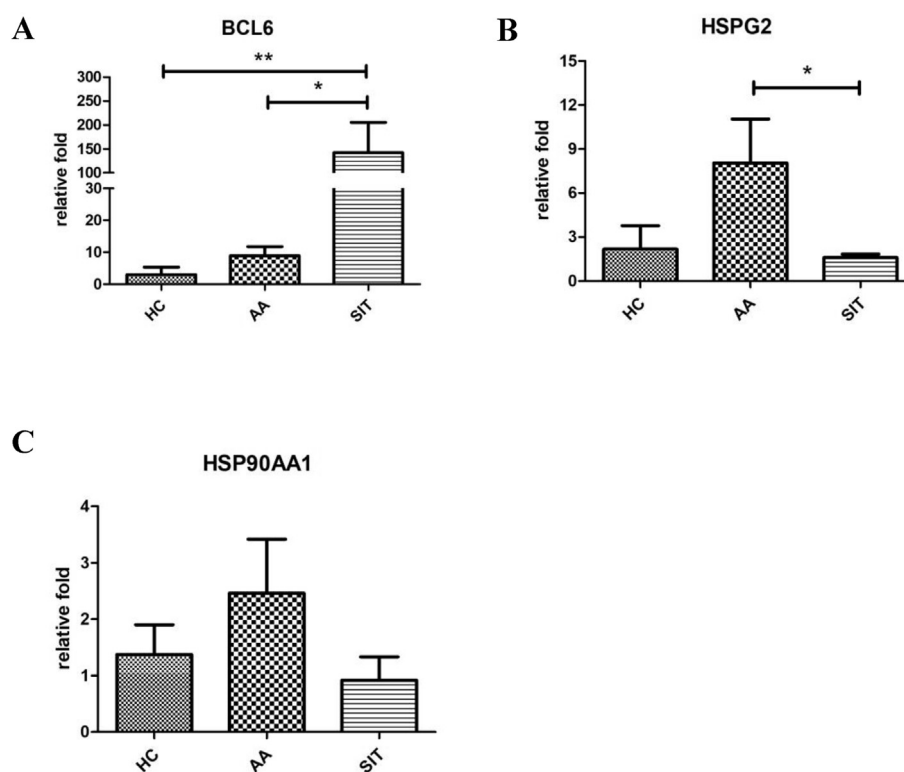


Fig. 4. mRNA level of *BCL6*, *HSPG2* and *HSP90AA1* in the SIT, AA and HC groups. Der p stimulated PBMCs were obtained from SIT ($n = 10$, right panel), AA ($n = 10$, middle panel), and HC ($n = 5$, left panel) children. The RNA expression level including (A) *BCL6*, (B) *HSPG2*, and (C) *HSP90AA1* were assessed using qRT-PCR as Materials and Methods described. Horizontal bars represent the means, and vertical bars indicate the standard errors of the means. * $p < 0.05$ and ** $p < 0.01$. Statistical comparisons of (A) to (C) were analyzed using a one-way ANOVA followed by Kruskal–Wallis test.

and tissue injury generated by reactive oxygen species (ROS).^{26,27} ROS also induces the synthesis of HSP90 heat shock family proteins, molecular chaperones involved in oxidative repair that are upregulated in moderate asthmatics, as compared to mild asthmatics.^{28,29} HSP90 α also was shown to mediate HDM-induced human bronchial epithelial barrier dysfunction.²⁴ Our data revealed that the HSP90 Alpha family class A member-1 (HSP90AA1) gene was significantly hypermethylated in SIT-treated children, combining our data with above researches we speculate that SIT-influenced DNA methylation may relate with bronchial epithelial barrier functions.

Fibrosis and airway obstruction, including airway extracellular matrix (ECM) remodeling and hyperresponsiveness are hallmarks of chronic asthma.³⁰ Previous studies have also characterized a population of circulating, inflammation-inducing mesenchymal cells, fibrocytes.³¹ Often in response to IL-4 or IL-13, fibrocytes produce numerous ECM proteins/enzymes, including, collagens 3/5/6, versican, heparan sulfate proteoglycan 2 (HSPG2), and hyaluronan (HA), involved in fibrocyte matrix deposition and its effects on airway smooth muscle contractility/relaxation,³² and many of these we found hypermethylated following Der p-SIT. The most hypermethylated gene we observed, in Der p-SIT PBMCs, was the proteoglycan-encoding gene *HSPG2*. SIT-influenced ECM protein HSPG2 may associate with airway remodeling. Moreover, our previous publication showed that after Der p challenge,

peripheral blood mononuclear cells from the SIT group, compared with the non-SIT AA group, produced lower levels of IL-4 and IL-5.¹¹ It indicated that after Der p immunotherapy, it can reduce Th2 cytokines to influence the proteoglycan encoding gene *HSPG2*.

Bcl6 prevents mouse development of asthma by attenuating Th2 cytokine production,³³ via binding to an *IL4* intron,³⁴ and its genetic variants significantly associate with marked atopy, characterized by high levels of HDM-specific IgE.³⁵ In our study, *BCL6* was significantly hypomethylated in SIT-treated children, likely attenuating Th2 cytokine production.

Genetic variants in the *HLA-DP* locus are associated with the risk of pediatric asthma in Asian populations, especially the *HLA-DPB1* alleles.³⁶ Analogously, we showed that *HLA-DPB1*, *HLA-DRB1* and, *HLA-DRB6* showed significant increased methylation in SIT patients compared to AA patients. Because the *HLA-DPB1*, *HLA-DRB1*, and *HLA-DRB6* polymorphisms all associate with asthma,^{36–38} their methylation changes, in SIT patients may represent a mechanistic therapeutic target against the pathogenesis of asthma.

There were limitations to this study. First, this is a retrospective study but not a prospective study. The DNA methylomes of PBMCs before and after SIT couldn't be showed in this study. Second, the DNA methylomes of respiratory epithelium should be performed to represent therapeutic targets for better clinical management of

asthma. However, children's respiratory epithelium is hard to obtain, especially our patients during SIT is quite healthy except for asthma. Another potential limitation of the study is some of the samples obtained from children were not enough. The DNA methylomes of T cells and B cells and the protein level of BCL6 and HSPG2 genes couldn't be showed in this study since insufficient blood was obtained from children.

In conclusion, we show that allergen-specific immunotherapy (SIT), with the house dust mite cysteine protease Der p affects PBMCs DNA methylomes. Such methylome remodeling likely affects genes involved in extracellular matrix remodeling, and HLA-class II and oxidative stress pathways, possible decreasing inflammation. Thus, decreasing airway inflammation, by down-regulation of IL-4 and IL-5, likely reduces airway remodeling and oxidative stress, causing hypermethylation of *HSPG2*, and hypomethylation of *GSTM1* and *HSP90AA1*. These findings demonstrate that significant changes in the DNA methylomes of monocytes, after Der p-SIT, may provide new insight into mechanistic development of allergies, and facilitate personalized therapy of allergic asthma.

Declaration of Competing Interest

The authors declare that they have no conflicts of interests.

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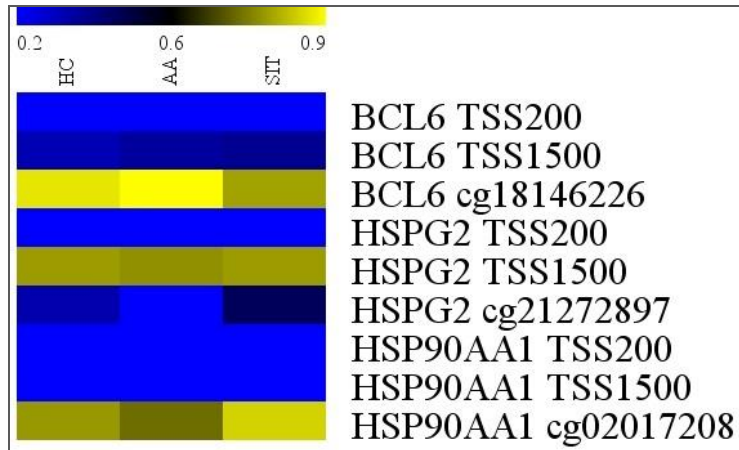
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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmii.2020.06.004>.

Supplementary Material



Supplementary Figure 1. The heat map of methylation levels of the selected probes. The upper bar indicates that the β -values ranged from 0.2 to 0.9. The lower panel indicates the healthy control (HC, left), allergic asthma (AA, middle), and subcutaneous immunotherapy (SIT, right) groups. These selected probes, including BCL6, HSPG2, and HSP90AA1 genes and the regions upstream to TSS (TSS200 or TSS1500), were analyzed.

Supplementary Table 1. Study participant characteristics.

Results are expressed as mean $\pm$ SD. *p value <0.05 between SIT and AA groups.

Characteristic	Allergic asthma group (n=12)	SIT group (n=13)
Age, year	10.49 $\pm$ 1.67	12.37 $\pm$ 2.72
Sex, M/F	8/4	7/6
Asthma severity level (mild/moderate persistent asthma) before SIT	5/7	6/7
Asthma control test (ACT) score before SIT	22.58 $\pm$ 2.39	23.61 $\pm$ 1.66
Serum total IgE (kUa/l)	978.75 $\pm$ 691.43	1543.38 $\pm$ 3341.3
Serum specific IgE (kUa/l)	79.11 $\pm$ 24.41	55.58 $\pm$ 33.83
Serum IgG4 (mga/l)	0.923 $\pm$ 0.71*	11.57 $\pm$ 8.45*
sIgE/IgG4 ratio	106.4 $\pm$ 43.22*	6 $\pm$ 3.69*

Supplementary Table 2. Pyrosequencing primer list.

*: biotinylated primer. Y denotes the detection site of CpG.

Detected gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Sequencing Primer (5'→3')	Sequence to Analyze
BCL6	GTATAAGGGG ATTGAGTGGGA TTT*	CCAACCTAAAA ACCCACACT	ATTTATACAAAT AAACAAAAT	AAACAAACYAACYYA ACYAA CAAAC
HSPG2	AGGTGGTGGA GTTGGTTATA	ACCCCAACCC AAAAATTC*	GGGAGATTTTTA GGTTGATTG	YGTGATGTTGGTYGT TAGYGGTAYGGAGG AGAA
HSP90AA1	AGGGAATGAA AAGTTTAAAAA GTAGA	ATTCAATACTC TACAATTCCTC TAATA*	ATAATAAGATTT GGTTTATTTAGG	TATTYGGTTTGATAGT TAAATATTTTAGATTA TAAAGTTAATATTATG TTATATAYGTTTTATA GTGTA

Supplementary Table 3. Quantitative real-time PCR primer list.

gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
BCL6	CTCAGATTCTAGCTGTGAGAAC	CTTGTAGCGGAAGGAGGCCT
HSPG2	CGATGTGCAGATCACGGGCA	GTGGCCCGGATCAGGAGCTCAT
HSP90AA1	TATAAGGCAGGCGCGGGGGT	GGTTTCCTCAGGCATCTTGGCTA