

Prenatal smoke exposure, DNA methylation, and childhood atopic dermatitis

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Summary

Background The biological mechanisms of how prenatal smoke exposure leading to atopic disorders remain to be addressed. Whether prenatal smoke exposure affects DNA methylation leading to atopic disorders is not clear.

Objective As most children suffering from atopic dermatitis (AD) continue to develop asthma later in life, we explored whether prenatal smoke exposure induces cord blood DNA methylation.

Methods Methylation differences associated with smoke exposure were screened by Illumina Infinium 27K methylation arrays for 14 children from the Taiwan birth panel study cohort initially. Information about development of atopic dermatitis (AD) and risk factors was collected. Cord blood cotinine levels were measured to represent prenatal smoke exposure. CpG loci that demonstrated a statistically significant difference in methylation were validated by methylation-dependent fragment separation (MDFS). Differential methylation in three genes (TSLP, GSTT1, and CYB5R3) was identified through the screen.

Results Among these, only thymic stromal lymphopoietin (TSLP) gene displayed significant difference in promoter methylation percentage after being validated by MDFS ($p = 0.018$). TSLP gene was further investigated in a larger sample of 150 children from the cohort who completed the follow-up study. Methylation status of the TSLP 5'-CpG island (CGI) was found to be significantly associated with prenatal smoke exposure ($OR=3.17$, 95% $CI=1.63-6.19$) and with AD ($OR=2.32$, 95% $CI=1.06-5.11$). The degree of TSLP 5'CGI methylation inversely correlated with TSLP protein expression levels ($r = -0.45$, $P = 0.001$).

Conclusions & Clinical Relevance The effect of prenatal tobacco smoke exposure on the risk for AD may be mediated through DNA methylation.

Keywords atopic dermatitis, cotinine, DNA methylation, prenatal smoke

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Introduction

The prevalence of allergic diseases has been increasing world-wide. The reasons for the increased prevalence remain unclear, but epidemiological studies have demonstrated the importance of environmental exposures in the diseases susceptibility. New studies reinforce the

importance of *in utero* exposures (e.g. allergens, cigarette smoking, pollutants, and dietary nutrients) in fetal immune development and in programming the susceptibility to allergic diseases [1].

In our previous studies, we found prenatal smoke exposure may increase the risk of atopic dermatitis (AD) in the offspring [2]. Multiple large prospective

studies indicate that prenatal exposure to environmental tobacco smoke is associated with impaired respiratory function, transient wheeze and asthma in young children [3–5]. More recent studies demonstrated that maternal smoking during but not following pregnancy contributes to an increased risk of allergic sensitization, recurrent wheezing, and asthma [6–8]. According to the hypothesis of the early origins of chronic disease, exposure to agents that are transmissible from mother to fetus has profound influences on the functional performance of many organs later in life [9]. These works all provide the convincing evidence that prenatal environmental exposures can influence the risk for subsequent atopic disorders.

Prenatal exposures may also have important and permanent effects on DNA methylation. Recent studies suggest that epigenetic regulation, heritable changes in gene expression that occur in the absence of alterations in DNA sequences, may in part mediate the complex gene-by-environment interactions that can lead to allergic diseases [10, 11]. The main epigenetic mediators are DNA methylation, histone modification, and non-coding RNAs [12]. They work on developmental plasticity such that environmental exposures during critical periods might alter the course of cellular differentiation *in utero* to gain adaptive advantages for later-life challenges [13]. Various components of the epigenetic pathway and primarily aberrant DNA methylation patterns are used as potential biomarkers for cancer diagnosis and prognosis [14]. Moreover, a comprehensive assessment of the effect of maternal smoking on cord blood DNA methylation has been performed, although without allergic disease outcomes [15]. Blood DNA methylation has also been reported to alter the risk of AD, although the analysis is restricted to CpG sites in the filaggrin gene and not genomewide [16]. Despite these initial progresses, the mechanisms of how prenatal exposures affect allergic disease development through their actions on DNA methylation and gene expression need to be elucidated. Moreover, there are limited new preventive strategies for allergic disease due to the absence of epigenetic predictive biomarkers. The lifelong effects after preventable exposures indicate an urgent need to understand better the potential mechanisms that mediate these effects.

As AD is a common chronic allergic skin inflammation that has a high prevalence among children and a large proportion of children suffering from AD continue to develop asthma later in life 'atopic march' [17], we explored whether prenatal exposure to tobacco smoke induces aberrant DNA methylation of specific genes that were related to childhood AD. We also searched for epigenetic biomarkers related to both smoking and atopic diseases in children.

Materials and methods

Study population

We recruited 261 mother and child pairs with cord blood in a Taiwan birth panel study (TBPS), as previously described [2]. All of the selected subjects were Han Chinese, with the premise that they were rather homogeneous. Full enrolment into the study required the completion of prenatal smoke exposure monitoring by checking cord blood cotinine levels. The parents were asked by home interview questionnaires at birth for parental age and education levels, family income, parental history of atopic diseases, diets and supplements during pregnancy, history of maternal smoking, and environmental tobacco smoke exposure during pregnancy. From the records of the hospitals, we gathered neonate's health data, such as birth body weight, gestational age, and type of delivery. At 2 years of age, 150 children completed the follow-up study and specimen collection. Information about development of AD was collected. AD cases were defined according to the diagnostic criteria by Hanifin and Rajka [18]. Children not included in these categories were classified as control subjects. Written informed consent was obtained. The study protocol was approved by the institutional review board from National Taiwan University Hospital with the approval number 9561707005 and complied with the principles outlined in the Helsinki Declaration.

Laboratory method

Exposure monitoring. The buffy coat and plasma samples were separated and stored at -80°C . DNA (100–500 ng) was extracted from cord white blood cells. Cord blood cotinine levels were measured by high-performance chromatography–mass spectrometry (HPLC-MS/MS) to stand for prenatal tobacco smoke exposure, as described elsewhere [2]. The cotinine levels below the limit of detection were imputed as 0.04 ng/mL.

Selection of candidate genes by Illumina methylation microarray. Figure 1 shows the study flow chart employed for selecting candidate genes associated with prenatal smoke exposure and AD. The study population was dichotomized into high and low smoke exposures by the median cord blood cotinine levels of the entire cohort (0.12 ng/mL). Initially, we analysed DNA samples of seven gestational age and gender-matched children of high exposure (3 with and 4 without AD) and 7 gestational age and gender-matched children of low exposures (3 with and 4 without AD). The 14 samples were screened with the Illumina Infinium Human Methylation 27 bead chip to comprehensively assess the differences in CpG island methylation between high- and low-exposure

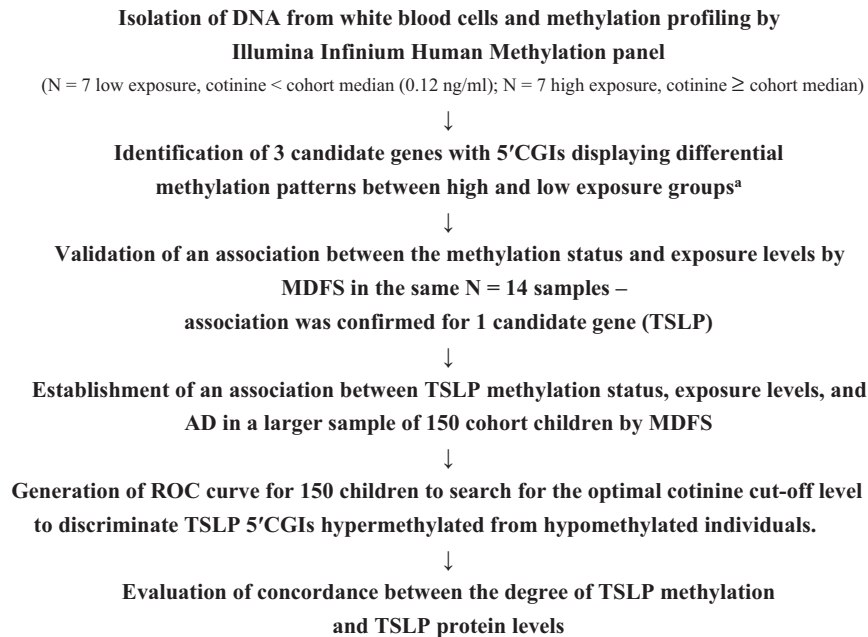


Fig. 1. Study flow chart. ^aSee Figure S2 for details.

groups. This bead chip contained 27578 human CpG methylation markers, and the detailed analysis procedures to identify candidate genes are illustrated in Figure S1. After scanning, quantile normalization was performed to remove potential system biases among different microarray slides. To identify genes showing differential methylation levels between cotinine high and low exposure, Wilcoxon rank sum test was used. Hierarchical clustering was used to illustrate the methylation variations in the samples of cotinine high-exposure group by using the Genesis program (Figure S2) [19]. Moreover, two additional filtering criteria were applied based on Illumina Infinium Assay Methylation Protocol Guide provided by the manufacturer to reduce the false discovery rates caused by the limited sample size. The beta values of candidate target genes after high and low doses cotinine exposure must fall into two groups, showing different methylation levels. The grouping conditions for beta values were as follows [20, 21]. Genes with beta values more than 0.7 were classified into 'high-methylation' group, whereas those with beta values lower than 0.3 were grouped as 'low-methylation'. In addition, genes with beta values more than 0.3 and less than 0.7 were reported as 'middle-methylation'. The second criterion was that the beta value difference must be larger than 0.1 to reflect notable methylation changes. Furthermore, candidate genes should have CpG islands (CGIs) in the promoter region and have reported associations with tobacco smoke exposures or atopic disorders from the literature reviews or NCBI website. These microarray data have been submitted to Gene Expression Omnibus with

the accession number: GSE35222. The DNA isolation was performed by QIAGEN Gentra Puregene Kit (Germantown, MD, USA). The DNA bisulphate conversion and purification were performed by EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA).

Establishment of the methylation status by methylation-dependent fragment separation (MDFS) in a larger sample. After validation of the association between the methylation status and exposure levels by MDFS in initial 14 samples screened by array, we further established an association between methylation status, exposure levels, and AD in a larger sample of 150 cohort children who completed the follow-up study and specimen collection at 2-years-old. The demographic characteristics of the cohort population and study sample were not significantly different from each other (Table S2). The study sample was representative of the cohort population in terms of child's gender, maternal age, median and distribution of cotinine exposure, and percentage of children with AD.

The bisulphate CT conversion rate was > 99%. Meth-Primer software was used to identify CGIs of candidate genes selected from methylation array and to design primers for polymerase chain reaction (PCR) (Table S1). The methylation status was detected with MDFS [22]. Following bisulphite conversion, methylated DNA differed from unmethylated DNA by the presence of multiple 5 mC vs. U bases. A region of interest was PCR amplified using a single set of fluorescent dye (6-FAM)-labelled primers that amplify the DNA regardless of the

methylation status. Finally, the presence of the multiple polymorphisms (C vs. T) leads to differential migration times during fragment analysis by capillary electrophoresis (DNA Analyzer 3730xL, Applied Biosystems., Foster City, CA, USA) so that an amplicon from methylated DNA was resolved from an amplicon from unmethylated DNA. The measurement was performed in duplicate.

Single-nucleotide polymorphism (SNP) genotyping. As some studies reported that SNPs may regulate DNA methylation [23], we searched for SNPs located in the predicted CpG island of TSLP by the database of SNP (dbSNP). The results revealed that only two SNPs, rs11466735 and rs11466736, were located within the target regions. Therefore, we performed the PCR tests to determine their genotypes and assessed their effects on methylation status. The SNPs were identified with CodonCode Aligner Software (CodonCode Corporation, Centerville, MA, USA).

Statistical analysis

After validation of the selected genes by MDFS, independent t tests were performed to assess differences in methylation percentage (Met%) between high- and low-exposure groups with different disease status in a larger sample of 150 cohort children who completed the follow-up study and specimen collection at 2-years-old. ROC curve was generated to search for the optimal cotinine level to discriminate hypermethylated from hypomethylated individuals. TSLP methylation status was dichotomized into hypermethylated and hypomethylated with cut-off value of median of promoter methylation percentage (29.6%) while performing the ROC analysis. Logistic regression was performed to evaluate the association between methylation status and AD. Spearman correlation was performed to estimate the relationship between cotinine levels and methylation

percentage and between methylation percentage and TSLP protein expression levels in 150 cord blood plasma samples. Selection of the confounders that were left in the model was based on the literature and the standard statistical procedures showed a 10% change in point estimate. All tests assumed a two-sided alternative hypothesis and a 0.05 significance level. All analyses were conducted using SAS software version 9.1 (SAS Institute, Cary, NC, USA).

Results

Selection of the most relevant candidate genes

Finally, only TSLP and CYB5R3 met the filtering criteria and were further selected for validation (Table 1). In addition to statistical approaches, GSTT1, a gene related with *in utero* smoke exposure and cotinine metabolism [24], was also selected for investigations due to its largest beta value changes in the microarray (Ratios: 2.67 in the high- vs. the low-exposure group).

The methylation array indicated that there were differential methylation statuses in the three genes (TSLP, GSTT1, and CYB5R3) between the high- and low-exposure groups. Therefore, these three genes were selected as our candidates. Literature reviews showed that they were related to immune responses (thymic stromal lymphopoietin; TSLP, Gene ID: 85480), cotinine metabolism (Glutathione S-transferase theta-1; GSTT1, Gene ID: 2952), and oxidative-reductive reaction (cytochrome b5 reductase 3; CYB5R3, Gene ID: 1727) (Table 1). Of these candidates selected by the methylation array, only TSLP gene exhibited significant difference in methylation status between high- and low-exposure groups after being validated by MDFS ($P = 0.018$). The percentage of promoter methylation (Met%) as measured by MDFS of the candidate genes tested on 14 samples is presented in Table 2.

Table 1. Candidate genes identified by using microarray

Gene	Cytoband	Gene ID	CpG Loci	Gene function	Delta beta*	Avg beta in high group [†]	Avg beta in low group [‡]
<i>TSLP</i>	5q22.1	85480	cg15739437	Expressed in the skin keratinocytes and related to immune responses; control the survival, growth, differentiation, and effector function of tissues and cells	0.100	0.283	0.383
<i>GSTT1</i>	22q11.2	2952	cg24330042	Expressed in the respiratory tract and involved in asthma pathogenesis, including oxidant defences, xenobiotic metabolism, cotinine metabolism, and detoxification of hydroperoxides.	0.204	0.123	0.327
<i>CYB5R3</i>	22q13.31-qter	1727	cg20727362	Catalysis of an oxidation-reduction reaction	0.154	0.581	0.735

*High-dose cotinine exposure minus low-dose cotinine exposure.

[†]Average beta values of samples in high cotinine exposure group.

[‡]Average beta values of samples in low cotinine exposure group.

Table 2. Percentage of promoter methylation (Met%) of the candidate genes by cotinine exposure ($n = 14$)

Sample		TSLP		GSIT1		CYB5R3	
Low exposure	Cotinine level (ng/mL)	Met% [†]	<i>P</i> value	Met%	<i>P</i> value	Met%	<i>P</i> value
Y008	<0.04	44.2	0.018*	14.6	0.096	32.9	0.324
NT-0283	<0.04	30.5		13.9		29.6	
NT-0268	<0.04	44.4		14.1		40.3	
NT-0290	<0.04	30.3		42.1		32.5	
NT-0285	<0.04	40.4		13.5		35.6	
NT-0297	<0.04	30.9		1.3		38.7	
NT-0281	<0.04	54.4		16.7		35.8	
High exposure	Cotinine level (ng/mL)	Met%		Met%		Met%	
NT-0197	0.21	29.6		93.9		34.0	
Y039	0.32	30.4		37.0		33.8	
NT-0201	0.35	29.3		12.3		29.9	
NT-0309	1.73	20.1		31.4		42.0	
NT-0199	3.91	28.7		42.9		51.4	
B041	8.53	29.3		23.0		42.7	
N029	38.90	29.2		18.7		34.2	

* $P < 0.05$ [†]Met% is the percentage of methylated cytosine in the CGIs determined by MDFS.

Association of methylation status with exposure in the larger sample & Receiver Operating characteristics (ROC) curve analysis

TSLP gene was further investigated for the relationships between prenatal smoke exposure and 5'CpG island methylation status and for analyses of methylation status and AD in a larger sample of 150 children from the cohort. ROC curve was generated to search for the optimal cotinine level to discriminate TSLP methylation status. TSLP methylation status was dichotomized into hypermethylated and hypomethylated with cut-off value of median of promoter methylation percentage (29.6%) while performing the ROC analysis. The cotinine cut-off level that best distinguished TSLP 5'CGI hypermethylation vs. hypomethylation status was determined to be 0.10 ng/mL (Fig. 2). This cut-off point had a sensitivity of 67% and specificity of 74%. The Area Under ROC curve (AUC) value was 0.72 suggesting fair separability of the subjects with hypermethylated vs. hypomethylated TSLP 5'CGI.

Association of methylation status with exposure and AD

The degree of TSLP 5'CGI methylation (Met%) for AD and non-AD is mean $\pm$ SD 25.10 $\pm$ 6.53% vs. 30.41 $\pm$ 10.65% ($P = 0.006$). The degree of methylation was inversely associated with cotinine levels ($r = -0.50$, $P < 0.001$). Moreover, Table 3 shows the association of TSLP 5'CGI methylation status with exposure categories and AD. TSLP 5'CGI methylation status was significantly associated with prenatal smoke

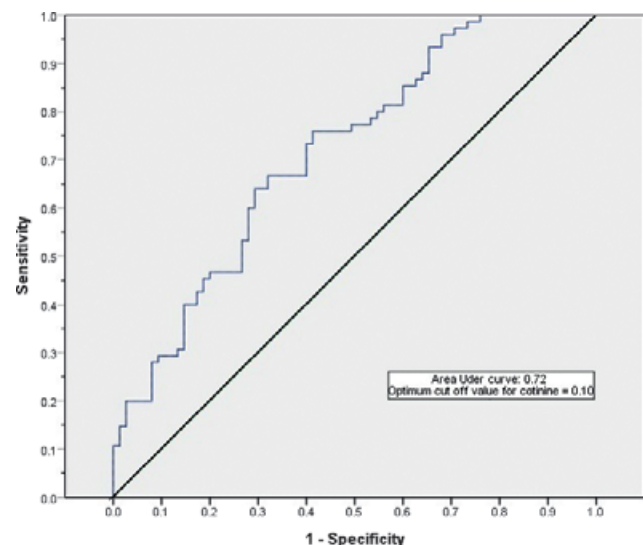


Fig. 2. Receiver operating characteristics (ROC) curves for 150 children on cotinine levels and methylation status of TSLP gene. The cotinine exposure cut-off value that best distinguished TSLP 5'CGIs hypermethylation vs. hypomethylation status was 0.10 ng/mL, with sensitivity of 67%, and specificity of 74%. The area under ROC curve was 0.72.

exposure (OR = 3.17, 95% CI = 1.63–6.19), given the cotinine level $\geq$ 0.10 ng/mL compared to the cotinine level $<$ 0.10 ng/mL. TSLP 5'CGI hypomethylated group was found to be positively associated with AD in children (OR = 2.32, 95% CI = 1.06–5.11), compared with hypermethylated group.

To further demonstrate whether the TSLP 5'CGI methylation status affects its protein expression, we checked

Table 3. The association of TSLP 5'CGI methylation status with characteristics, exposure categories, and AD ($n = 150$)

TSLP 5'CGI Methylation Status	Hypomethylated [†] ($n = 75$)	Hypermethylated ($n = 75$)	Subjects ($n = 150$)	Odds Ratios (95% CIs)
Gender (% Males)	29 (53.7%)	35 (56.5%)	64 (55.2%)	0.90 (0.43–1.86)
Maternal age ≥ 34	7 (25.9%)	20 (31.3%)	27 (29.7%)	0.77 (0.28–2.11)
Caesarean Delivery	21 (38.9%)	21 (33.9%)	42 (36.2%)	1.24(0.58–2.65)
Birth weight < 2500 g	3 (4.7%)	4 (6.3%)	7 (5.5%)	0.73 (0.16–3.38)
Parental atopy (%Yes) [‡]	23 (32.9%)	15 (22.4%)	38 (27.7%)	1.49 (0.54–4.14)
Cotinine 0.10 (%) [*]	50 (66.8%)	29 (38.7%)	79 (52.7%)	3.17 (1.63–6.19)
AD (% Yes) [*]	23 (30.7%)	12 (16.0%)	35 (23.3%)	2.32 (1.06–5.11)

^{*} $P < 0.05$

[†]TSLP methylation status was dichotomized into hypermethylated and hypomethylated with cut-off value of median of promoter methylation percentage (29.6%) while performing the ROC analysis.

[‡]Either father or mother reported that he/she ever had physician-diagnosed atopic dermatitis, allergic rhinitis, or asthma.

cord blood plasma TSLP protein. The median (range) was 9.41 (2.49) ng/mL. We found that TSLP protein level and the degree of TSLP 5'CGI methylation (Met%) exhibited the significantly inverse relationship ($r = -0.45$, $P = 0.001$).

Association of TSLP SNPs around the CpG island and methylation status

We genotyped two SNPs, rs11466735 and rs11466736, around the predicted TSLP CpG island (Table S1) and assessed their effects on methylation status. We found that all samples had 'GG' genotype in rs11466735. Only one sample had the heterozygous genotype 'AG' in rs11466736. Therefore, the genotyping data suggest that these SNPs may not play an important role in regulating the methylation status of TSLP.

Discussion

This is the interesting study linking tobacco smoke exposure with atopic disorders via epigenetic mechanisms. It is reported that prenatal smoke exposure may increase the risk of AD in children, and tobacco smoke can induce epigenetic changes [2, 11]. In this study, we went further to investigate the possible linking mechanism. We found TSLP a candidate gene whose methylation status was related to prenatal tobacco smoke exposure and was further associated with childhood atopic disorders. Moreover, TSLP 5'CGI methylation was significantly associated with its downstream protein expression.

Multiple epidemiological studies have suggested that allergic disease risk is determined by prenatal exposure to pollutants and allergens that modify later-life T helper cell allergic phenotype [11]. Epigenetic modifications of regulatory genes crucial to the development of allergic diseases have been put forth as a possible mechanism [11]. In our study, we found that prenatal tobacco

smoke exposure was associated with changes in DNA methylation. In a recent study, Perera *et al.* correlated maternal exposure to traffic-related polycyclic aromatic hydrocarbons with methylation of the CpG island of acyl-CoA synthetase long-chain family member 3 (ACSL3) and with the development of asthma symptoms in children [25]. Breton *et al.* reported that prenatal tobacco smoke exposure may increase gene-specific methylation and consequently influence gene expression and disease phenotypes across the life course [26]. Joubert *et al.* also identified a set of genes with methylation changes present at birth in children whose mothers smoked during pregnancy [15]. Combined, these findings implicate that prenatal environmental exposures do have the potential to alter DNA methylation status [27].

Between high and low smoke exposure groups, we found that TSLP gene exhibited significant differential methylation. Moreover, the cotinine cut-off level that best distinguished TSLP 5'CGI methylation status was 0.10 ng/mL by ROC curve. Consistent with previous studies, cotinine concentrations were reported to be < 0.1 ng/mL for low tobacco smoke exposure and ≥ 0.1 ng/mL for high exposure [28, 29]. This biological rational further supports our hypothesis that if exposure is higher than the cut-off value, methylation process may trigger gene expression regulation in allergic disease pathogenesis. Nafee *et al.* reported that smoking was associated with reduced folate levels, and folate-deficient diets were associated with DNA methylation in mice [30]. Smoke exposure might cause epithelial injury and facilitate allergens entry into the submucosa. Injury to the epidermis may also induce expression of TSLP [31]. As a result, this might increase the contact of allergens with dendritic cells and provoke the Th2-type immune responses through TSLP expression [32]. Therefore, smoke exposures may trigger keratinocytes to produce TSLP. It is interesting that intradermal injection of mice with TSLP plus antigen results in asthma after subsequent lung challenge with that same antigen alone

[33]. Fehervari et al. also indicated that skin-derived TSLP may represent an important factor that triggers progression from AD to asthma, driving the so-called 'atopic march' from skin to lung tissue [34]. Smelter et al. suggested that tobacco smoke exposure might induce TSLP expression in the mouse lung in an oxidative stress-dependent and TNF- α receptor I-dependent manner, thereby promoting the development of asthma [35]. Moreover, some previous studies regarding methylation and smoke exposure found that the methylation of AHRR, CYP1A1, GFI1, F2RL3, GPR15, ORAI2, FNDC8, and NCAPD3 was significantly related to smoke exposure [15, 36–38]. However, we failed to find these associations in our study. These discrepancies may be as a result of different age and ethnicity, different filtering criteria, statistical method, study design or different mechanism, and clinical presentation of diseases.

TSLP was a candidate gene whose methylation status was related to smoke-associated atopic disorder. TSLP gene is located in 5q22.1 which has been shown to be associated with regions of the AD susceptibility loci. It works to skew the immune system towards Th2 dominance by activating DCs that promote differentiation of naïve T cells to Th2 cells by an OX40 ligand–OX40 interaction [39]. Li et al. demonstrated that TSLP produced by keratinocytes is absolutely required in pathogenesis of AD triggered by topical vitamin D3 analogue [40]. TSLP may also activate Langerhan cells of the epidermis and induce the production of pro-inflammatory cytokines, potentially causing AD. These further support our finding that TSLP 5'CGI methylation status was associated with AD in children. Thus, TSLP 5'CGI methylation changes are expected to diminish the regulation function of immune system and trigger the development of Th2-type allergic inflammation in AD.

We found that TSLP 5'CGI methylation status was different depending on the cotinine levels. The hypomethylated TSLP 5'CGI was positively associated with AD. Moreover, the degree of TSLP 5'CGI methylation and the level of TSLP protein exhibited inverse relationship. This implicated that high smoke exposure might lead to TSLP 5'CGI hypomethylation. Consequently, hypomethylated TSLP 5'CGI is associated with increased gene expression and elevated TSLP protein (Figure S3). Elevated TSLP protein might activate epidermal Langerhan cells, promoting the development of AD. Consistent with our study, Lee et al. reported increased serum TSLP in children with AD [41]. TSLP was also highly expressed in the lesional skin of AD patients [42]. It was induced by extracellular double-stranded RNA via a NF- κ B-dependent pathway, leading to AD [43]. In addition to triggering AD, increase expression of TSLP also aggravates experimental allergic asthma induced by ovalbumin challenge. As TSLP may trigger progression from epidermal-barrier defects to asthma, under-

standing the mechanism of TSLP in atopic march may help us prevent or treat AD and asthma.

This study has some potential limitations. First, cord blood samples were used for candidate identification. Whether cord blood methylation reflects the methylation status in other organs is not clear. However, it is challenging to obtain immune cells or keratinocytes in fetus. Moreover, the methylation alterations may occur not only in immune system but also in fetal circulating white blood cells [11]. As the cord blood contains stem cells that provide abundant T cells which are important producers for allergic disease mediators, it may serve as a reasonable surrogate in our study. Second, as individual RNA samples were not available and high-quality RNA isolation in cord blood was difficult because of its limited cellular components, we assessed the concordance between the extent of gene methylation and its translation levels in protein isolated from cord blood plasma instead. Our results demonstrated good concordance for TSLP methylation and its protein expression. This further confirmed the role of this newly discovered epigenetic marker.

One of the strengths of this study is its cohort study design with specimens linking to well-collected clinical and environmental exposure data. Such cohort-driven epigenetic research has the potential to address key questions concerning the influence of early life disease risk factors and environmental exposures on susceptibility to atopic disorders. Another strength is that we applied a two-step approach – an Illumina methylation array to identify candidate gene methylation followed by MDFS to confirm the candidate gene methylation status in a larger sample. This stepwise approach allowed us to detect the most promising candidates cost effectively. Moreover, MDFS is a high-throughput method and is more sensitive and rapid than pyrosequencing to quantitatively detect DNA methylation [22].

In conclusion, we afford the evidence that prenatal exposure to tobacco smoke increases the risk for childhood atopic disorders and that this may be mediated through alterations in DNA methylation. This helps to understand the aetiology and new approaches for early prevention or therapeutic intervention in childhood allergic diseases. Further large follow-up and genome-wide approach studies are necessary to discover the mechanisms for lifelong effects of the atopic march after environmental exposures.

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Conflict of interests

The author declares no conflict of interests.

References

- Prescott SL, Clifton V. Asthma and pregnancy: emerging evidence of epigenetic interactions in utero. *Curr Opin Allergy Clin Immunol* 2009; 9:417–26.
- Wang IJ, Hsieh WS, Wu KY *et al*. Effect of gestational smoke exposure on atopic dermatitis in the offspring. *Pediatr Allergy Immunol* 2008; 19:580–6.
- Alati R, Al Mamun A, O'Callaghan M, Najman JM, Williams GM. In utero and postnatal maternal smoking and asthma in adolescence. *Epidemiology* 2006; 17: 138–44.
- Magnusson LL, Olesen AB, Wennborg H, Olsen J. Wheezing, asthma, hayfever, and atopic eczema in childhood following exposure to tobacco smoke in fetal life. *Clin Exp Allergy* 2005; 35:1550–6.
- Burke H, Leonardi-Bee J, Hashim A *et al*. Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. *Pediatrics* 2012; 129:735–44.
- Keil T, Lau S, Roll S *et al*. Maternal smoking increases risk of allergic sensitization and wheezing only in children with allergic predisposition: longitudinal analysis from birth to 10 years. *Allergy* 2009; 64:445–51.
- Lannero E, Wickman M, Pershagen G, Nordvall L. Maternal smoking during pregnancy increases the risk of recurrent wheezing during the first years of life (BAMSE). *Respir Res* 2006; 7:3.
- Tanaka K, Miyake Y, Sasaki S, Ohya Y, Hirota Y. Maternal smoking and environmental tobacco smoke exposure and the risk of allergic diseases in Japanese infants: the Osaka Maternal and Child Health Study. *J Asthma* 2008; 45:833–8.
- Padmanabhan V. Environment and origin of disease. *Rev Endocr Metab Disord* 2007; 8:67–9.
- Slaats GG, Reinius LE, Alm J, Kere J, Scheynius A, Joerink M. DNA methylation levels within the CD14 promoter region are lower in placentas of mothers living on a farm. *Allergy* 2012; 67:895–903.
- Martino D, Prescott S. Epigenetics and prenatal influences on asthma and allergic airways disease. *Chest* 2011; 139:640–7.
- Tang WY, Ho SM. Epigenetic reprogramming and imprinting in origins of disease. *Rev Endocr Metab Disord* 2007; 8:173–82.
- Swanson JM, Entringer S, Buss C, Wadhwa PD. Developmental origins of health and disease: environmental exposures. *Semin Reprod Med* 2009; 27:391–402.
- Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI. Oxidative stress, DNA methylation and carcinogenesis. *Cancer Lett* 2008; 266:6–11.
- Joubert BR, Håberg SE, Nilsen RM *et al*. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environ Health Perspect* 2012; 120:1425–31.
- Ziyab AH, Karmaus W, Holloway JW, Zhang H, Ewart S, Arshad SH. DNA methylation of the filaggrin gene adds to the risk of eczema associated with loss-of-function variants. *J Eur Acad Dermatol Venereol* 2012. doi: 10.1111/jdv.12000. [Epub ahead of print].
- Spergel JM. Epidemiology of atopic dermatitis and atopic march in children. *Immunol Allergy Clin North Am* 2010; 30:269–80.
- Hanifin J, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol* 1980; 92:4.
- Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. *Bioinformatics* 2002; 18:207–8.
- Perez C, Martinez-Calle N, Martin-Subero JI *et al*. TET2 mutations are associated with specific 5-methylcytosine and 5-hydroxymethylcytosine profiles in patients with chronic myelomonocytic leukemia. *PLoS ONE* 2012; 7: e31605.
- Nishino K, Toyoda M, Yamazaki-Inoue M *et al*. Defining hypo-methylated regions of stem cell-specific promoters in human iPS cells derived from extra-embryonic amnions and lung fibroblasts. *PLoS ONE* 2010; 5:e13017.
- Boyd VL, Moody KI, Karger AE, Livak KJ, Zon G, Burns JW. Methylation-dependent fragment separation: direct detection of DNA methylation by capillary electrophoresis of PCR products from bisulfite-converted genomic DNA. *Anal Biochem* 2006; 354:266–73.
- Rønneberg JA, Tost J, Solvang HK *et al*. GSTP1 promoter haplotypes affect DNA methylation levels and promoter activity in breast carcinomas. *Cancer Res* 2008; 68:5562–71.
- Hong YC, Lee KH, Son BK, Ha EH, Moon HS, Ha M. Effects of the GSTM1 and GSTT1 polymorphisms on the relationship between maternal exposure to environmental tobacco smoke and neonatal birth weight. *J Occup Environ Med* 2003; 45:492–8.
- Perera F, Tang WY, Herbstman J *et al*. Relation of DNA methylation of 5'-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma. *PLoS ONE* 2009; 4:e4488.
- Breton CV, Byun HM, Wenten M, Pan F, Yang A, Gilliland FD. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med* 2009; 180:462–7.
- Patel MM, Miller RL. Rapid DNA methylation changes after exposure to traffic particles: the issue of spatio-temporal factors. *Am J Respir Crit Care Med* 2009; 180:1030.
- Kharrazi M, DeLorenze GN, Kaufman FL *et al*. Environmental tobacco smoke and pregnancy outcome. *Epidemiology* 2004; 15:660–70.
- Donnenfeld AE, Pulkkinen A, Palomaki GE, Knight GJ, Haddow JE. Simultaneous fetal and maternal cotinine levels in pregnant women smokers. *Am J Obstet Gynecol* 1993; 168:781–2.
- Nafee TM, Farrell WE, Carroll WD, Fryer AA, Ismail KM. Epigenetic control of fetal gene expression. *BJOG* 2008; 115:158–68.
- Angelova-Fischer I, Fernandez IM, Donnadieu MH *et al*. Injury to the stratum corneum induces in vivo expression of human thymic stromal lymphopoietin in the epidermis. *J Invest Dermatol* 2010; 130:2505–7.
- Nakamura Y, Miyata M, Ohba T *et al*. Cigarette smoke extract induces thymic

- stromal lymphopoietin expression, leading to T(H)2-type immune responses and airway inflammation. *J Allergy Clin Immunol* 2008; 122:1208–14.
- 33 Han H, Xu W, Headley MB *et al.* Thymic stromal lymphopoietin (TSLP)-mediated dermal inflammation aggravates experimental asthma. *Mucosal Immunol* 2012; 5:342–51.
- 34 Fehervari Z. TSLP underlies the atopic march. *Nat Immunol* 2012; 13:533.
- 35 Smelter DF, Sathish V, Thompson MA, Pabelick CM, Vassallo R, Prakash YS. Thymic stromal lymphopoietin in cigarette smoke-exposed human airway smooth muscle. *J Immunol* 2010; 185:3035–40.
- 36 Suter M, Ma J, Harris AS *et al.* Maternal tobacco use modestly alters correlated epigenome-wide placental DNA methylation and gene expression. *Epigenetics* 2011; 6:1284–94.
- 37 Wan ES, Qiu W, Baccarelli A *et al.* Cigarette smoking behaviors and time since quitting are associated with differential DNA methylation across the human genome. *Hum Mol Genet* 2012; 21:3073–82.
- 38 Breitling LP, Yang R, Korn B, Burwinkel B, Brenner H. Tobacco-smoking-related differential DNA methylation: 27K discovery and replication. *Am J Hum Genet* 2011; 88:450–7.
- 39 Wang YH, Liu YJ. Thymic stromal lymphopoietin, OX40-ligand, and interleukin-25 in allergic responses. *Clin Exp Allergy* 2009; 39:798–806.
- 40 Li M, Hener P, Zhang Z, Ganti KP, Metzger D, Chambon P. Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. *J Invest Dermatol* 2009; 129:498–502.
- 41 Lee EB, Kim KW, Hong JY, Jee HM, Sohn MH, Kim KE. Increased serum thymic stromal lymphopoietin in children with atopic dermatitis. *Pediatr Allergy Immunol* 2010; 21:e457–60.
- 42 Soumelis V, Reche PA, Kanzler H *et al.* Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002; 3:673–80.
- 43 Vu AT, Chen X, Xie Y *et al.* Extracellular double-stranded RNA induces TSLP via an endosomal acidification- and NF-kappaB-dependent pathway in human keratinocytes. *J Invest Dermatol* 2011; 131:2205–12.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The workflow chart outlining the identification of the differentially methylated genes in a step-by-step manner.

Figure S2. The heatmap of 84 genes showing significantly different methylation profiles in the group after

cotinine high exposure.

Figure S3. Possible smoke exposure effects and hypothesis in our study.

Table S1. Candidate gene methylation information, the CpG island prediction and primers design.

Table S2. Population characteristics

Supplement

Table S1 Candidate gene methylation information, the CpG island prediction and primers design.

The CGIs (shaded in blue in the DNA sequence) were identified in silico based on a CG content $\geq$ 60%, with an observed/ expected ratio of 0.6 according to MethPrimer definitions. The PCR-amplified region was indicated by a line with double heads and methylation status of this region was detected by methylation-dependent fragment separation (MDFS) via DNA Analyzer 3730xL and GeneMapper v4.0 software. For every PCR product, one CpG island was analyzed.

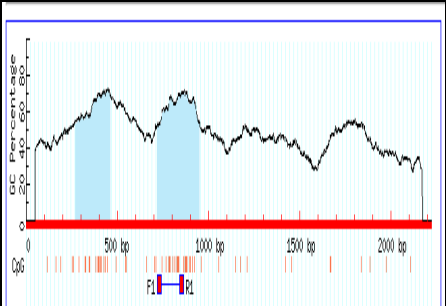
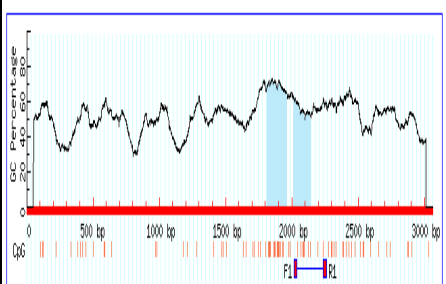
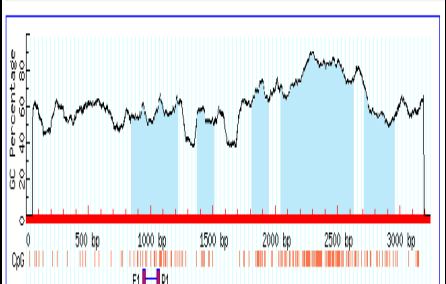
Gene ID Chr Band	CpG island Prediction	Gene Ontology
		Primers
TSLP 85480 5q22.1	 chr5: 110,405,999-110,406,220	Expressed in the skin keratinocytes and related to immune responses; control the survival, growth, differentiation and effector function of tissues and cells F1:FAM-TGTGATATTTGTTGGGATTTATATA R1:TACTCCTCCCTAACTTAACTCATAC
GSTT1 2952 22q11.2	 Chr22: 24,384,060-24,384,281	Expressed in the respiratory tract and involved in asthma pathogenesis, including oxidant defenses, xenobiotic metabolism, and detoxification of hydroperoxides. F1:FAM-GAGTTTGTTGATTTTTTTTGGTTTT R1:ATATACCTACCTATCCCCTTCCTAACTT
CYB5R3 1727 22q13.31-qter	 Ch22:22,437,080-22,437,226	Catalysis of an oxidation-reduction reaction F1:FAM-TGTAATTTTAGTATTTTGGGAGGT R1:ATAACTAACAACACAAACACCCAC

Table S2 Population characteristics

Category	Full cohort (N=261)	Study sample (N=150)	Array sample (N=14)	<i>p</i> -value
Gender (% Males)	54.7	55.2	71.4	0.443
Maternal age (mean $\pm$ SD)	29.65 $\pm$ 5.16	31.49 $\pm$ 4.15	30.57 $\pm$ 4.31	0.710
Cesarean Delivery %	45.8	36.2	42.9	0.224
Birth weight<2500gm %	6.9	5.5	7.1	0.865
Parental atopy(% Yes)	21.6	27.7	14.3	0.278
Cotinine ng/ml (mean $\pm$ SD)	2.78 $\pm$ 18.16	2.74 $\pm$ 18.48	3.86 $\pm$ 10.36	0.976
AD (% Yes)*	19.2	23.3	35.7	0.243

Figure S1 The workflow chart outlining the identification of the differentially methylated genes in a step-by-step manner.

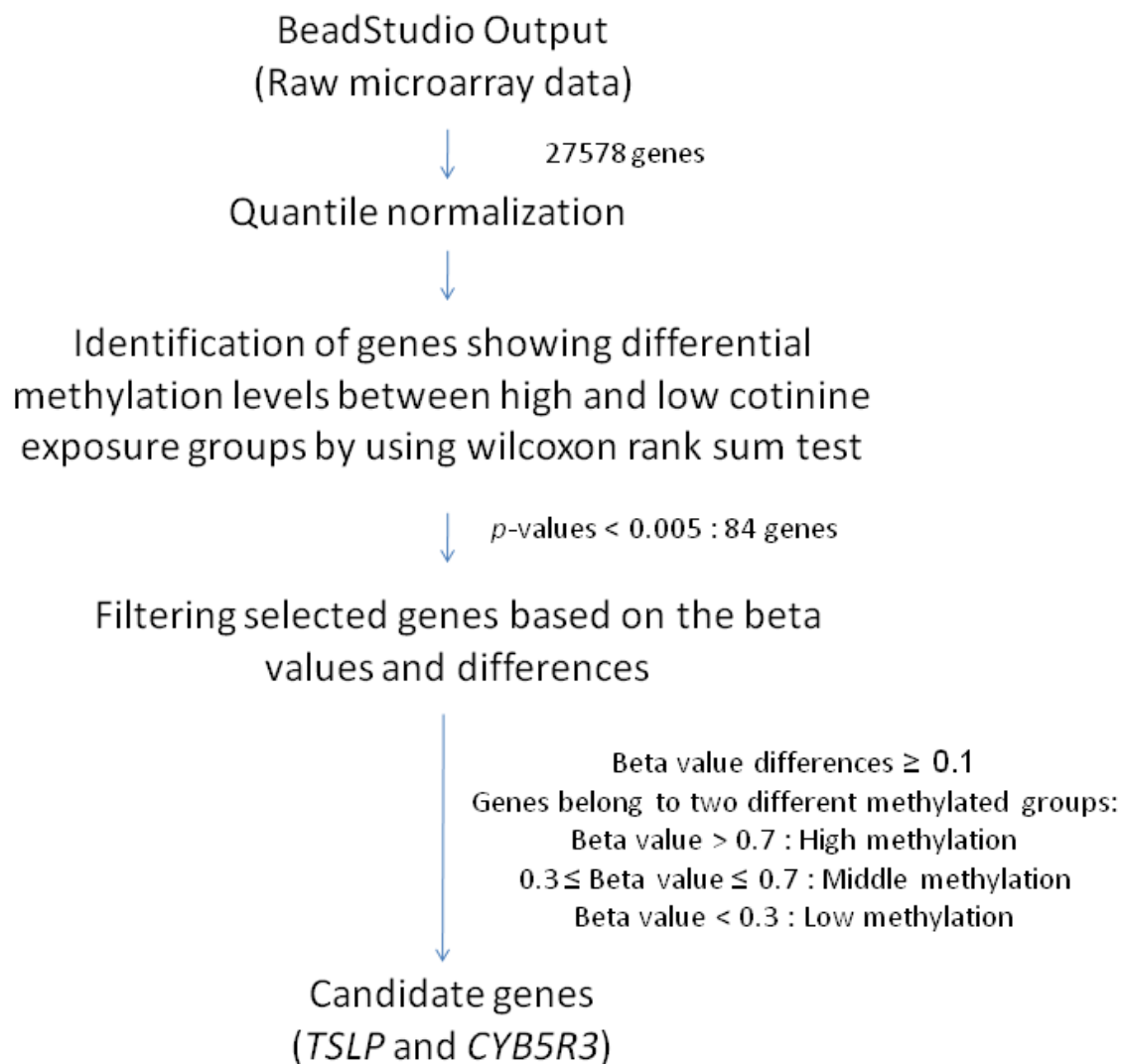


Figure S2 The heatmap of 84 genes showing significantly different methylation profiles in the group after cotinine high exposure. The input data were the beta values in the samples exposing to high dose cotinine after Z-transformation. Two calculation steps were applied here. For each gene, the raw beta values in the high dose cotinine exposure group minus the median in the low dose cotinine exposure group first and further divide the resulting values by the standard deviation of the same gene in the low dose cotinine exposure group. Hierarchical clustering with average linkage method was utilized to illustrate the methylation changes.

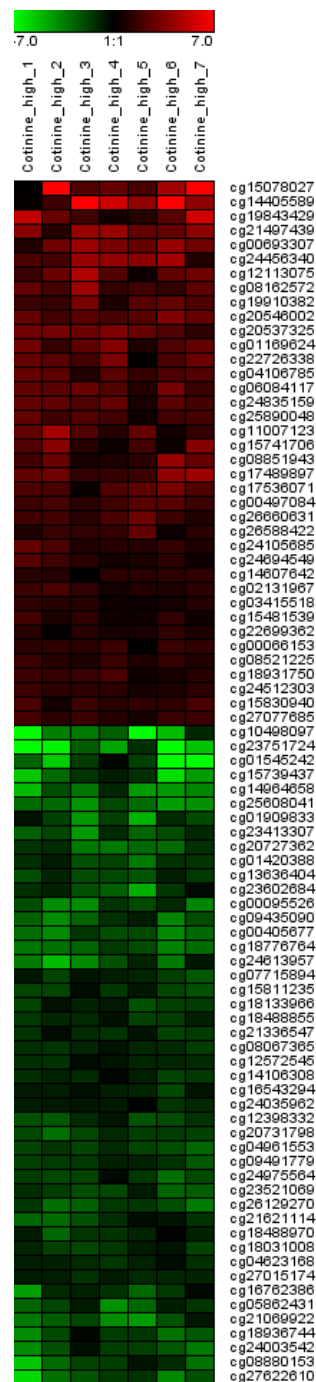


Figure S3 Possible smoke exposure effects and hypothesis in our study. Determining the underlying mechanism may have consequences of diagnosis and treatment of atopic disorders.

