EPIGENETICS
2018, VOL. 13, NOS. 10–11, 1027–1038
https://doi.org/10.1080/15592294.2018.1529517

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RESEARCH PAPER

Association between *AXL* promoter methylation and lung function growth during adolescence

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ABSTRACT

AXL is one of the TAM (*TYRO3, AXL* and *MERTK*) receptor tyrosine kinases and may be involved in airway inflammation. Little is known about how epigenetic changes in *AXL* may affect lung development during adolescence. We investigated the association between *AXL* DNA methylation at birth and lung function growth from 10 to 18 years of age in 923 subjects from the Children's Health Study (CHS). DNA methylation from newborn bloodspots was measured at multiple CpG loci across the regulatory regions of *AXL* using Pyrosequencing. Linear spline mixed-effects models were fitted to assess the association between DNA methylation and 8-year lung function growth. Findings were evaluated for replication in a separate population of 237 CHS subjects using methylation data from the Illumina HumanMethylation450 (HM450) array when possible. A 5% higher average methylation level of the *AXL* promoter region at birth was associated with a 48.4 ml decrease in mean FEV_1 growth from 10 to 18 years of age in the primary study population (95% CI: −100.2, 3.4), and a 53.9 ml decrease in mean FEV_1 growth from 11 to 15 years of age in the replication population (95% CI: −104.3, −3.5). One CpG locus in the promoter region, cg10564498, was significantly associated with decreased growth in FEV_1, FVC and MMEF from 10 to 18 years of age and the negative associations were observed in a similar age range in the replication population. These findings suggest a potential association between *AXL* promoter methylation at birth and lower lung function growth during adolescence.

ARTICLE HISTORY
Received 15 March 2018
Revised 19 August 2018
Accepted 22 September 2018

KEYWORDS
DNA methylation; *AXL*; promoter; lung function; adolescents

Background

Optimal lung function is important for health, as reduced lung function has been associated with increased risks of coronary artery disease and respiratory disease in adults, and with the onset of asthma in adolescents [1,2]. Many factors, environmental, pathological and genetic, may influence lung function [3–6]. These factors may exert their influence through epigenetic mechanisms such as DNA methylation by altering the expression pattern and activity of genes involved in airway development [7,8]. Researchers have identified associations between lung function decline and DNA methylation of the transposable elements LINE-1 [9], and of several candidate genes such as *CRAT, F3, TLR2* and *SERPINA1*, mostly among older subjects [10,11]. DNA methylation at birth may be an early surrogate marker of chronic disease predisposition that is affected by prenatal environmental exposure [12]. However, few studies have investigated the early-life epigenetic marks associated with lung function growth in healthy adolescents.

Methylation of *AXL*, a member of the TAM (*TYRO3, AXL* and *MERTK*) family receptor tyrosine kinases, was identified in our previous work as responsive to prenatal tobacco smoke exposure [13,14]. *AXL* has been implicated in various biological pathways including clearance of apoptotic cells, natural killer cell differentiation, and inhibition of proinflammatory cytokines induced by Toll-like receptors (TLR) [15–17]. Recent evidence suggests that *AXL* is expressed in both human and mouse airway/alveolar macrophages and is critical for effective phagocytosis. Reduced *AXL* expression may contribute to persistent airway inflammation through inefficient clearance of apoptotic cells from inflamed lungs [18,19]. Since defective phagocytosis and subsequent airway inflammation are closely related to accelerated lung function

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Supplementary material data can be accessed here.

decline and chronic lung diseases [3,20], we hypothesized that increased methylation in *AXL* promoter region may be associated with reduced lung function growth even early in life, potentially through repressing its expression and promoting inflammation in the airways.

In this study, we investigated the association between methylation of multiple CpG sites across the regulatory regions of *AXL* at birth and lung function growth from 10 to 18 years of age. Methylation was first assessed using Pyrosequencing in newborn bloodspots from a subset of 923 subjects from the Children's Health Study (CHS) [21–23]. We then sought to replicate the association in a separate population of 237 CHS subjects using methylation levels measured from the Infinium HumanMethylation450 BeadChip (HM450) array, and in whom lung function was assessed from 11 to 15 years of age.

Results

Characteristics of the study participants

The characteristics of the primary and replication populations are shown in Table 1. There were fewer boys than girls in both the primary study population (46.7% male subjects) and replication population (41.8% male subjects). The primary study population also had more white subjects (46.3% *versus* 32.1%), slightly higher parental education level, more asthmatics (21.5% *versus* 14.8%), more subjects exposed to prenatal tobacco smoke by design (22.9% *versus* 9.0%), and more subjects exposed to environmental tobacco smoke (20.2% *versus* 6.8%). There were very few smokers in both populations. Mean age at first pulmonary function testing was 10.8 and 11.4 years in the primary and replication populations, respectively. There were generally no significant differences for mean height and BMI at age 11, 13 and 15 years between the two populations.

Genomic locations of the *AXL* CpG sites under investigation are shown in Figure 1. Methylation levels at many of the CpG sites were significantly correlated (Table S1 and S2), with CpG sites closer to each other showing stronger correlations. The distribution of methylation at each CpG site in both populations is shown in Table S3. The patterns of lung function growth across the study period are shown in Table S4 and Figure S1.

Table 1. Characteristics of participants with lung function testing.

	Primary study population (N = 923)	Replication population (N = 237)	P-value[a]
Male sex, n (%)	431 (46.7)	99 (41.8)	0.17
Ethnicity, n (%)			0.0002
Hispanic	385 (41.7)	131 (55.3)	
Non-Hispanic white	427 (46.3)	76 (32.1)	
Asian/Black/Other	111 (12.0)	30 (12.7)	
Parental Education, n (%)			0.11
High school or less	272 (30.4)	73 (33.0)	
Some college	395 (44.2)	81 (36.7)	
Finished college/some graduate school	227 (25.4)	67 (30.3)	
Parental history of asthma at study entry, n (%)	229 (25.8)	31 (13.9)	0.0002
Ever MD-diagnosed asthma at first lung function testing, n (%)	198 (21.5)	35 (14.8)	0.02
In-utero tobacco smoke exposure, n (%)	209 (22.9)	21 (9.0)	< 0.0001
Ever smoked cigarettes by age 15, n (%)	82 (8.9)	10 (4.2)	0.02
Passive tobacco smoke exposure by age 15, n (%)	186 (20.2)	16 (6.8)	< 0.0001
Age (year) at first pulmonary function testing, mean (sd)	10.8 (1.1)	11.4 (0.8)	< 0.0001
Height (cm), mean (sd)			
Age 11	148.6 (7.0)	147.8 (7.0)	0.26
Age 13	160.2 (7.8)	159.1 (7.2)	0.12
Age 15	167.3 (8.4)	165.4 (8.2)	0.05
Body mass index (BMI, kg/m^2), mean (sd)			
Age 11	20.4 (4.2)	20.4 (4.1)	0.97
Age 13	21.9 (4.8)	21.7 (4.4)	0.68
Age 15	23.3 (4.6)	23.5 (4.5)	0.78

[a] P-value for testing the difference between the two populations. Derived from a Pearson's Chi-squared test for categorical variables and from an unequal variance 2-sample *t*-test for age, and an equal variance 2-sample *t*-test for height and BMI.

DNA methylation of AXL and lung function growth

We investigated whether *AXL* DNA methylation was associated with lung function growth in both the primary and replication populations. A 5% higher average promoter methylation level was associated with a 48.4 ml lower mean FEV_1 growth from 10 to 18 years of age in the primary study population (95% CI: −100.2, 3.4; P = 0.07) (Figure 2-Panel A and Table 2), and significantly lower mean FEV_1 growth

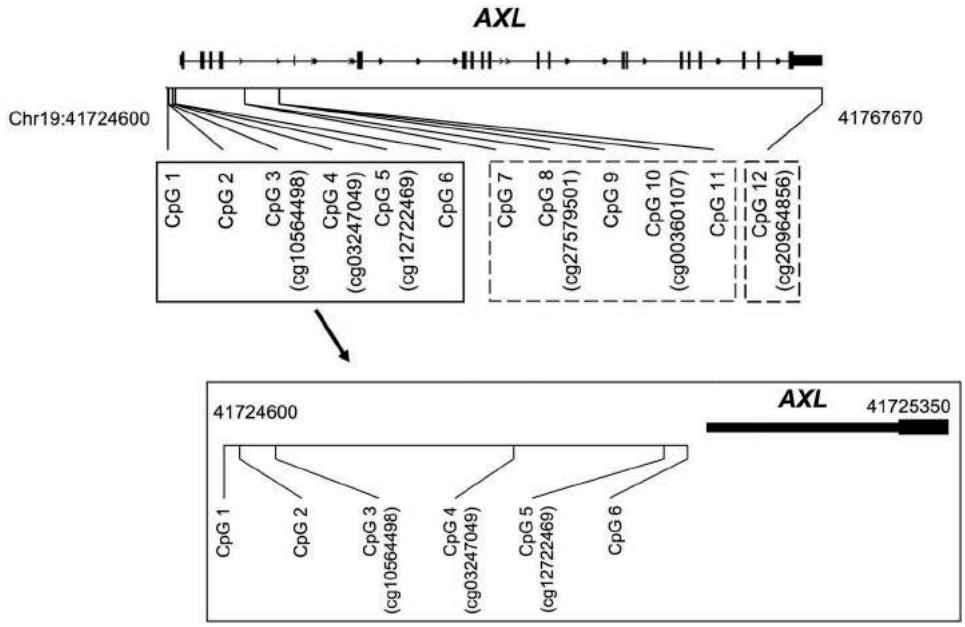

Figure 1. Genomic locations of *AXL* CpG sites under investigation.
Solid black box: CpG sites in the promoter region (CpG1-CpG 6); dashed gray box: CpG sites in the gene-body region (CpG 7-CpG 11); dashed black box: CpG site in the 3'untranslated region (CpG 12).Cg number in parenthesis: corresponding CpG locus in HM450 array.

from 11 to 15 years of age in the replication population (β= -53.9; 95% CI: −104.3, −3.5; P = 0.04) (Figure 2-Panel D and Table 2). Similar negative associations with MMEF growth were observed in both populations. A 5% higher average promoter methylation level was associated with a 89.6 ml/sec lower mean 8-year MMEF growth (95% CI: −192.8, 13.7; P = 0.09) and the association with growth from 11 to 15 years was even stronger (β= -166.2; 95% CI: −278.4, −54.1; P = 0.004) (Table 2). We also investigated whether the relationship between *AXL* promoter methylation and lung function growth differed by sex (Table 2). Although there was no significant interaction, a stronger association was observed for boys than girls in both populations. For instance, in the primary study population, a 5% higher average promoter methylation was associated with a 69.4 ml decrease in mean 8-year FEV_1 growth in boys (95% CI: −139.9, 1.1; P = 0.05) but only a 33.0 ml decrease in girls (95% CI: −94.1, 28.0; P = 0.29). In the replication population, a significant association between *AXL* promoter methylation and FEV_1 growth from 11 to 15 years of age was also observed only in boys (β= -93.6; 95% CI: −166.1, −21.1; P = 0.01) but not in girls (β= -17.5; 95% CI: −87.8, 52.8; P = 0.62). There was no significant interaction between methylation and child's ethnicity in both populations (Table S5).

In the primary study population, a 5% higher average promoter methylation level was significantly associated with a 61.6 ml lower mean FVC growth from 10 to 18 years of age (95% CI: −117.5, −5.7; P = 0.03) (Table 2). The association between *AXL* promoter methylation and lower mean FVC growth was marginally significant in the replication population, yet still suggesting the same trend (β= -44.6; 95% CI: −97.2, 8.1; P = 0.10).

Methylation at several individual CpG sites in the promoter region was also associated with lower lung function growth (Figure S2-Panel A). For example, methylation at CpG 3 was significantly associated with lower growth in FEV_1, FVC and MMEF from 10 to 18 years of age in the primary study population (Table S6). Methylation at CpG 2, which is located near CpG 3, was also significantly associated with lower mean 8-year FVC growth, and lower attained FEV_1 and FVC levels at 18 years of age. In the replication sample, we observed associations between CpG 3 methylation with lower growth in FEV_1 and MMEF from 11 to 15 years

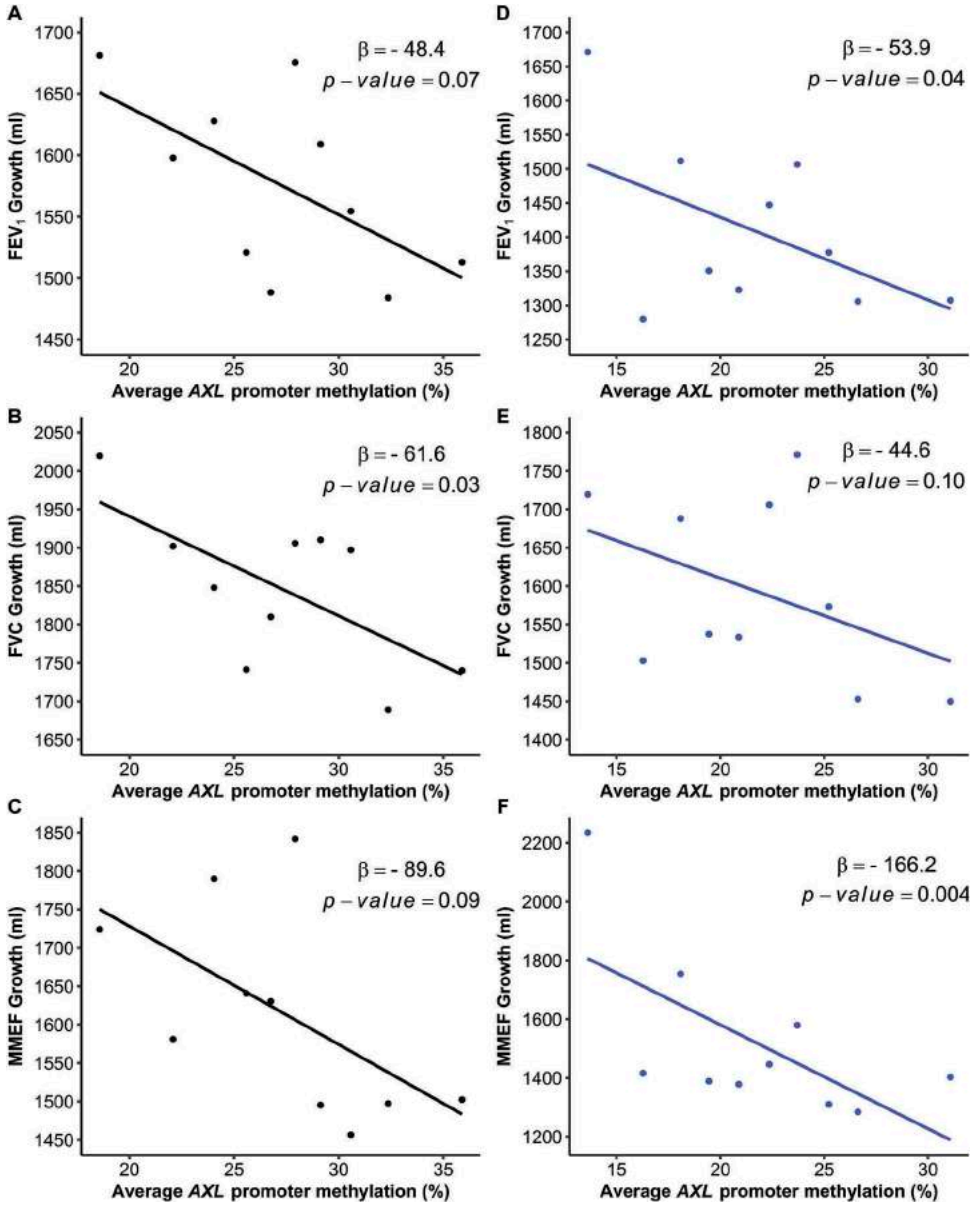

Figure 2. Mean lung function growth versus the average methylation level of *AXL* promoter region.

AXL promoter methylation levels were first categorized into equal-sized groups based on deciles. The mean growth in FEV_1, FVC and MMEF from 10 to 18 years of age in the primary study population (Panel A-C) and 11 to 15 years of age in the replication population (Panel D-F) are plotted against the corresponding levels of methylation in each decile. The corresponding coefficients and P-values are shown.FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; MMEF, maximal midexpiratory flow rate.

of age (Table S7). No associations between lung function and methylation of specific CpG sites in the gene-body region or 3′ untranslated region were observed (Table S6).

Since asthma itself is associated with lower lung function growth in children [24] and the prevalence of asthma in the primary study population was higher, we also conducted sensitivity analyzes in non-asthmatic subjects (Table S8). The observed associations were generally consistent with using all subjects.

AXL promoter methylation and expression in lung

AXL was previously reported to be expressed at very low levels in blood [25]. Thus, we evaluated

Table 2. Association between lung function growth and *AXL* promoter methylation stratified by sex in the primary study population (N = 923) and replication population (N = 237)[a].

	Primary study population		Replication population	
	Growth from 10 to 18 years of age		Growth from 11 to 15 years of age	
Lung function measurement	Coefficient (95% CI)	P value	Coefficient (95% CI)	P value
FEV_1 (ml)				
Overall	−48.4 (−100.2 to 3.4)	0.07	−53.9 (−104.3 to −3.5)	**0.04**
By sex				
Girls	−33.0 (−94.1 to 28.0)	0.29	−17.5 (−87.8 to 52.8)	0.62
Boys	−69.4 (−139.9 to 1.1)	**0.05**	−93.6 (−166.1 to −21.1)	**0.01**
Interaction p-value	0.37		0.14	
FVC (ml)				
Overall	−61.6 (−117.5 to −5.7)	**0.03**	−44.6 (−97.2 to 8.1)	0.10
By sex				
Girls	−45.0 (−112.3 to 22.3)	0.19	−45.5 (−119.2 to 28.3)	0.22
Boys	−84.0 (−161.9 to −6.0)	**0.03**	−45.3 (−122.4 to 31.7)	0.25
Interaction p-value	0.40		0.99	
MMEF (ml/sec)				
Overall	−89.6 (−192.8 to 13.7)	0.09	−166.2 (−278.4 to −54.1)	**0.004**
By sex				
Girls	−75.3 (−194.4 to 43.8)	0.22	−87.0 (−244.6 to 70.5)	0.28
Boys	−110.7 (−249.3 to 28.0)	0.12	−249.7 (−410.5 to −88.9)	**0.003**
Interaction p-value	0.65		0.16	

the correlation between *AXL* promoter methylation and mRNA expression levels using histologically normal lung tissue samples based on HM450 array and RNA sequencing data from TCGA (Figure S3). Average promoter methylation showed negative correlation with expression (r = -0.40, P = 0.03). Although these data need to be interpreted with caution because they were generated in an older population enriched with smokers, they showed preliminary evidence that higher methylation might be associated with lower *AXL* expression level in the adult lung.

Lastly, we integrated chromHMM and RNA-seq data from the Roadmap Epigenomics Project to display a functional annotation of CpG sites in the promoter region in relevant tissues and cell types (Figure S2-Panel B). The CpG loci evaluated in the *AXL* promoter region are located adjacent to enhancers in both fetal and adult lung fibroblast cells (yellow in chromHMM tracks), but are located within transcriptionally-repressed region in cord blood primary T cells. The observed chromatin state patterns are similar in fetal and adult lung cells. As indicated from RNA-seq data, the CpG sites under investigation are located within or near the transcriptionally active region in both fetal and adult lung, and a consistent pattern is observed in adult blood progenitor cells, though at much lower levels. These data suggest that epigenetic alterations in this region of *AXL* may be associated with active patterns for transcription and chromatin state of this gene throughout life, supporting the use of blood as surrogate marker of *AXL*'s activity in fetal and adult lung.

Discussion

We showed that increased average methylation of the *AXL* promoter region at birth was associated with lower lung function growth during adolescence, as measured by the growth in FEV_1, FVC and MMEF from the ages of 10 to 18 years. Similar associations were observed for the average promoter methylation and specifically, CpG 3 in this region in a separate population of 237 CHS subjects, despite differences in the age at which lung function was measured as well as differences in some baseline characteristics of the two populations.

AXL and other TAM receptors are broadly expressed by cells of the vascular, nervous, immune and reproductive systems [26]. Although the role of *AXL* in the pathogenesis of cancers and cardiovascular events is well-characterized [27,28], little is known about its role in pulmonary homeostasis, which requires a balance between adequate responses to pathogens and control of inflammatory processes arising from accumulation of

apoptotic cells and cellular products [29]. Inefficient clearance of apoptotic cells in the airways may lead to accumulation of necrotic cell debris, subsequent uncontrolled inflammatory responses, and ineffective resolution of lung inflammation upon microbial and viral infection [30]. Recent evidence from mouse models suggests that *Axl* is expressed in airway macrophages [18] – the main cell populations responsible for phagocytosis in the respiratory tract [30] – and is essential for macrophage function through clearance of apoptotic cells and cell debris and inhibiting influenza-induced inflammation [18]. Similar findings were reported in human patients with moderate-to-severe asthma, suggesting a critical role of *AXL* in clearing apoptotic cells from the inflamed lung [19].

This is the first paper investigating the role of epigenetic regulation of *AXL* in lung function growth during adolescence. Understanding predictors of lung function growth is important considering the maximal attained lung function predicts risk of subsequent cardiovascular and respiratory conditions during adult life [31,32]. Because we previously found that *AXL* methylation was associated with risk of asthma-related symptoms in childhood [25], and asthma may affect the rate of lung function growth [24], we performed sensitivity analyzes in subjects who were never diagnosed with asthma. Similar associations in non-asthmatics indicate that the epigenetic regulation of *AXL* on lung function growth may act independently of asthma status and probably through different biological pathways. The question of whether *AXL* regulates lung homeostasis mainly through the apoptosis pathways as opposed to also exerting negative control over pro-inflammatory cytokines and TLR signaling in the airway [16,26] remains unclear. Our results also support a stronger association between *AXL* promoter methylation and lower lung function growth among boys than girls. Despite the non-significant differences, the findings highlight the need for investigating whether there are sex-specific effects.

The *AXL* promoter region under investigation in this paper overlapped with the core promoter region (−556 to −182 upstream to translational start codon), which is a known Sp1/Sp3 transcription factor binding site. Methylation within or in close proximity to this core region is negatively associated with *AXL* gene expression in cultured colon cancer cells [33]. Due to the low expression of *AXL* in blood [34], we were not able to test whether *AXL* methylation correlated with mRNA expression in our population. Nonetheless, average promoter methylation of *AXL* was shown to correlate with lower mRNA level in normal lung tissue samples from the TCGA database, suggesting a potential link between higher promoter methylation and lower expression level of this gene. Moreover, *in silico* analyzes using data from the Roadmap Epigenomics Project suggest that this region may be associated with active transcription in the lung since it is located adjacent to enhancers in both fetal and adult lung fibroblast cells.

The strengths of this study include the analysis of DNA methylation using Pyrosequencing, a highly reproducible method that accurately quantifies DNA methylation [35], and the replication of results in a separate population using a different assay. The measurement of DNA methylation utilized archived newborn bloodspots, which constitute a unique and underutilized resource of biospecimens that represent essentially every child born in the countries with newborn screening programs [36]. The cost, ease of transportation and storage of bloodspot cards point to new opportunities for using the neonatal biobanks available worldwide to facilitate population-based studies on epigenetic modifications at birth [37–39]. We also utilized the longitudinal measurements of pulmonary function and covariates data, collected in a consistent manner throughout the study period, to study the lung function growth prospectively. The temporal separation of DNA methylation assessment (at birth) and lung function growth measurement (during adolescence) removes the possibility of reverse causation.

Some limitations are noteworthy. First, the replication population was a different wave of CHS recruited in a different decade but not an external population. Since our analyzes were based mainly on Hispanic and non-Hispanic whites as well as non-smokers, it remains to be investigated whether our findings can be extrapolated to other ethnicities or to active smokers. The methylation sites measured in the replication

population did not capture the exact range of the promoter region and this may also limit the generalizability of our results. Secondly, as in any epidemiologic study, the observed effects could be biased by some unknown confounders associated with both DNA methylation levels and lung development. Since DNA methylation was only measured from newborn bloodspots in our study, we were not able to evaluate whether the methylation pattern in *AXL* still persists during adolescence, or whether there is any correlation between *AXL* methylation in blood and other more pathologically-relevant tissues such as lung or airway epithelial cells. Nonetheless, the epigenetic changes at birth may reflect a summary of *in-utero* environmental exposures that affect different tissues systematically. The subtle shifts in methylation occurring during fetal development reflected in newborn blood may serve as early biomarkers for the disruption of pathways involved in lung function growth later in life.

In conclusion, our results suggest a potential association between *AXL* promoter DNA methylation at birth and lower lung function growth during adolescence. Future studies on gene-specific methylation will improve our understanding of the relationship between epigenetic changes and lung development.

Methods

Study population

This study was conducted in participants from the Children's Health Study, a longitudinal study of respiratory health of children in southern California [21–23]. Based on our ability to link CHS subjects with California birth records and to obtain a newborn bloodspot, a subset of 923 children was selected for an epigenetic study in which DNA methylation at multiple CpG loci on *AXL* was assessed using Pyrosequencing. Subjects were recruited either as fourth-grade students in 1996 (cohort D) or as kindergarten and first-grade students in 2003 (cohort E). The sample selected was enriched with subjects exposed to prenatal tobacco smoke. The replication population included 237 CHS subjects from cohort E who had previously participated in a substudy of childhood cardiovascular health [40], for whom California birth records could be linked and at least 700ng of DNA was available from a dried newborn bloodspot. In these subjects bloodspot DNA methylation was measured with the HM450 array.

Written questionnaires were completed by children's parents at study entry and were updated annually throughout the study thereafter. The questionnaires were used to obtain personal, parental and socio-demographic characteristics with respect to the child, which included the child's age, sex, race, ethnic origin, level of parental education, and *in-utero* as well as post-natal tobacco smoke exposure.

The study was approved by the Institutional Review Board of the University of Southern California.

Pulmonary-function testing

Trained technicians measured subjects' weight and height, and supervised performance of pulmonary-function maneuvers. Details of the testing protocol have been published previously [22]. Three measures of pulmonary-function were analyzed for each child: forced vital capacity (FVC), forced expiratory volume in the first second (FEV_1), and maximal midexpiratory flow rate (MMEF). Pulmonary-function testing was performed annually from approximately 10 to 18 years of age with the use of rolling-seal spirometers in cohort D. Cohort E subjects were tested for pulmonary-function every other year when they were approximately 11, 13 and 15 years of age with the use of pressure transducer-based spirometers. Information on asthma status and personal smoking status was collected at the time of each pulmonary-function testing.

DNA methylation

DNA methylation was measured in newborn bloodspots (NBS) obtained as part of the routine California Newborn Screening Program from the California Department of Public Health Genetic Disease Screening Program. The NBS were stored by the state of California at −20 degrees Celsius. A single complete newborn bloodspot for each requested participant was mailed to us and stored in our lab at −80 degrees Celsius upon receipt. Laboratory personnel

performing DNA methylation analysis were blinded to study subject information. Using a disposable scalpel, 1/2 of the dried bloodspot in the Guthrie card was cut out and further divided into 4 pieces each equivalent to 1/8 spot. The pieces were transferred to two microcentrifuge tubes each containing two of the 1/8 spots. DNA was extracted using the QiaAmp DNA Micro kit (Cat # 56,304; Qiagen Inc, Valencia, CA). The procedure consisted of lysis of the sample followed by binding of the DNA in the lysate to the Minielute column. The column was then washed and the purified DNA was eluted with 60 μl of buffer AE and stored at −80 degrees Celsius.

Eleven CpG loci (CpG 1–3, and 5–12) spanning the regulatory regions of *AXL* were selected for Pyrosequencing assays (Figure 1); five (CpG 3, 5, 8, 10 and 12) were located at positions corresponding to positions in the HM450 array. Methylation of CpG 1 was previously reported to be associated with prenatal tobacco smoke exposure [13,14]. Pyrosequencing assays were conducted as previously described [25]. PCR primers were designed by EpigenDx Inc. (http://www.epigendx.com) to cover the loci of interest. Methylation assays (assays ADS6525-FS, ADS8094-FS2, ADS6528-FS, ADS8097-FS, and ADS6570-FS) were performed by EpigenDx Inc. using the PSQ96HS system (Pyrosequencing, Qiagen) according to standard procedures as described in previous work [41,42]. 500 ng of genomic DNA extracted from each sample was bisulfite treated using the EZ DNA Methylation Kit™ (Zymo Research, Irvine, CA, USA) and was purified according to the manufacturer's protocol. The methylation level was determined using QCpG software (Pyrosequencing, Qiagen) and was reported as percent of DNA methylation for each CpG locus.

For replication analyzes, two CpG loci (CpG 3 (cg10564498) and CpG 5 (cg12722469)) were selected for replication based on results with the primary study population. We additionally selected another HM450 array targeting locus in the promoter region (CpG 4 (cg03247049)) to further investigate this region (Figure 1). For HM450 assays, 700 to 1000 ng of genomic DNA from each sample was treated with bisulfite using the EZ-96 DNA Methylation Kit™ (Zymo Research, Irvine, CA, USA), according to the manufacturer's recommended protocol and eluted in 18 µl. The Infinium HM450 data was processed as previously reported [25], compiled for each locus and was expressed as beta (β) values. Methylation data was extracted for CpG 3, 4 and 5.

Genotyping

Details of genotyping assays, data processing and assessment of admixture were previously described [43]. Genotypes of SNPs in *AXL* and its surrounding region (1 kb upstream and downstream) were extracted from the CHS genome-wide genotypic data. 28 tagged SNPs were identified with a pair tag $r^2 > 0.8$ in Haploview using all available CHS samples (N = 3845) and were included in the analyzes [44]. Admixture was assessed using the program STRUCTURE from a set of ancestral informative markers that were scaled to represent the proportion of African American, Asian, Native American and White [45].

In silico analyses in publicly available data

To evaluate the association between *AXL* promoter methylation and mRNA expression, we downloaded *AXL* methylation profiling data in 29 histologically normal tissue samples from cases with lung adenocarcinoma (LUAD) or lung squamous cell carcinoma (LUSC) from the TCGA dataset [46]. All samples had both methylation profiling (HM450 array) and RNA-seq (Illumina HiSeq) data. The mean age was 65.9 years (SD: 12.39) and 75.9% of the subjects were males. 51.7% of the subjects were moderate to heavy smokers. Promoter methylation average was calculated from average methylation of CpG 3 (cg10564498), CpG 4 (cg03247049) and CpG 5 (cg12722469). Spearman correlation coefficient was calculated to evaluate the correlation between promoter methylation and mRNA level.

R packages coMet and snp.plotter were used to graphically display additional information about CpGs, including genomic location and functional annotation from the Roadmap Epigenomics Project [47–49].

Statistical analyses

The methylation status of *AXL* promoter was defined as the average of CpG 1, 2, 3, 5 and 6 in

the primary study population and the average of CpG 3, 4 and 5 in the replication population. Although defined differently for the two populations, these CpG sites are located in the same genomic region (Figure 1) and the correlations between them in the same population were relatively high (Tables S1 and S2).

To evaluate the association between *AXL* methylation and lung function growth from 10 to 18 years of age in the primary study population, all available pulmonary-function measurements for each subject were used to estimate lung function growth curves. We used a previously-reported linear spline model to account for the nonlinear pattern of growth during adolescence, with knots placed at ages 12, 14 and 16 years [50,51]. The model was fitted for each outcome and CpG individually and adjusted for child's age, sex, sex*age interaction, ethnicity, height, height squared, body-mass index (BMI), BMI squared, city of residence at study entry, history of asthma, parental history of asthma, and maternal smoking during pregnancy. Random effects were included to account for multiple measurements contributed by each subject. Additional adjustment for *AXL* genetic polymorphisms, methylation plate, admixture, cohort, parental education level, second-hand smoke exposure, wheezing symptoms, personal smoking status, field technician, and preterm birth did not change the effect estimates by more than 10% and were removed from final models. Visual inspection of residual values did not identify any departures from model assumptions. Estimates of association between methylation with 8-year lung function development (from 10 to 18 years of age) and with mean attained lung function at both 10 and 18 were obtained. Sensitivity analyzes were conducted to evaluate the association in non-asthmatic subjects, defined as subjects who were never diagnosed with asthma before the last pulmonary function testing visit. To assess whether associations between lung function growth and methylation are modified by sex, an interaction term between sex and methylation was included in the regression models, and Wald tests were used to compute interaction p-values. Interaction between methylation and child's ethnicity was also evaluated.

A similar mixed-effect linear spline model was used in the replication population, with knots placed at ages 12 and 14 years and the same adjustment variables. Further adjustment for estimated cord blood cell type proportions showed minimal effects and were not included in the model. Due to the limited availability of lung function measurements, the model was constructed to yield estimates of the association between methylation and 4-year lung function development (from 11 to 15 years of age). Effect modification by sex was also tested.

All tests assumed a two-sided alternative hypothesis and were conducted using SAS statistical analysis software (version 9.4). An alpha level of 0.05 was used to determine statistical significance.

Acknowledgments

We would like to express our sincere gratitude to Steve Graham and Robin Cooley at the California Biobank Program and Genetic Disease Screening Program within the California Department of Public Health for their assistance and advice regarding newborn bloodspots. The biospecimens and/or data used in this study were obtained from the California Biobank Program, (SIS request number(s) 479)" Section 6555(b), 17 CCR. The California Department of Public Health is not responsible for the results or conclusions drawn by the authors of this publication.

This research was supported by NIEHS grants 4R01ES022216, K01ES017801 and P30ES007048.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Institute of Environmental Health Sciences [4R01ES022216];National Institute of Environmental Health Sciences [P30ES007048]; National Institute of Environmental Health Sciences [K01ES017801].

Competing financial interests

The authors declare that they have no competing interests.

Author contributions

CB conceived and designed the study. CB, JM, KS, LD, and RU supervised the project. LG analyzed the data and wrote the manuscript. All authors edited and approved the manuscript.

Data availability

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