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Heritability and role for the environment in DNA methylation in *AXL* receptor tyrosine kinase

Carrie V. Breton,* Muhammad T. Salam and Frank D. Gilliland

Departments of Preventive Medicine; Keck School of Medicine; University of Southern California; Los Angeles, CA USA

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Abbreviations: CHS, children's health study; IQR, interquartile range; NK cells, natural killer cells

DNA methylation in *AXL*, a receptor tyrosine kinase relevant in cancer and immune function, is reportedly highly heritable. We present evidence to suggest that heritability of DNA methylation in *AXL* is variable, dependent on population characteristics and cell type studied. Moreover, environmental exposures in utero, particularly exposure to maternal smoking, contributes to variation in DNA methylation of select CpG loci that can affect calculations of heritability. Children exposed to maternal smoking in utero had a 2.3% increase (95% CI 0.3, 4.2) in DNA methylation in *AXL*, which was magnified in girls as compared to boys. These results present compelling evidence that environmental exposure to tobacco smoke during pregnancy may alter DNA methylation levels in subtle but potentially important ways and that these changes are persistent years after birth.

AXL, a receptor tyrosine kinase, was originally identified as a transforming gene in human leukemias[1] and has since been shown to be functionally relevant in many other cancers.[2] More recently, studies have shown that *AXL* also plays a role in immune response, with activation of *AXL* receptors having immunosuppressive activity. Another study demonstrated that stimulation of Gas6, the ligand that binds to *AXL*, reduces inducible nitric oxide synthase and interleukin-1β expression in microglia cells.[3] Interference with the binding of Gas6 to *AXL* was also shown to markedly reduce natural killer (NK) cell differentiation from $CD34^+$ hematopoietic progenitor cells and impair IFNgamma production by NK cells.[4]

Gene expression of *AXL* has been correlated with DNA methylation levels in an Sp1/Sp3 transcription factor binding site in the promoter, suggesting DNA methylation plays a key role in determining gene function.[5,6] CpG methylation at this site has been reported to have high heritability.[7] Recent evidence also suggests *AXL* is an imprinted gene whose expression is maternally transmitted in some but not all individuals and dependent on DNA methylation in the locus.[8] Despite its reported high heritability, methylation levels in *AXL* may be susceptible to environmental insults and the degree to which DNA methylation in *AXL* is susceptible to the external environment and epigenetic drift[9,10] remains an interesting and largely unanswered question.

Recently, we reported that DNA methylation in the *AXL* promoter is susceptible to environmental stimuli. In a study of 272 children who participated in the Children's Health Study (CHS),[11,12] children who were exposed to maternal smoking in utero had increased DNA methylation at a CpG locus located in the same Sp1/Sp3 binding site compared to children who were not exposed.[13] In this report, we present additional data replicating these results in an independent population of 173 children who participated in a case-control study of asthma nested within the CHS and we demonstrate that the effect is largely restricted to girls. We also provide estimates of heritability of DNA methylation at this locus. Characteristics of the study population are described in **Table 1**.

DNA methylation was measured in buccal cell DNA. Children were provided with two toothbrushes and instructed to brush their teeth with the first one. They were instructed to gently brush the buccal mucosa with the second toothbrush. The brush was then placed in a leak proof container that was filled with an alcohol-based fixative. Buccal cell suspensions were centrifuged at 2,000 g on the day they were received in the laboratory. DNA was then extracted using a PUREGENE DNA isolation kit (cat #D-5000; GENTRA, Minneapolis, MN) following the manufacturer's recommendations.

DNA methylation was measured using bisulfite-polymerase chain reaction (PCR) Pyrosequencing assay using the HotMaster Mix (Eppendorf, Hamburg, Germany) and the PSQ HS 96 Pyrosequencing System (Biotage AB, Uppsala, Sweden),[14] as described in previous work.[15] The output from Pyrosequencing is reported as a percent of DNA methylation at the CpG locus. Mean and SD from four technical replicates was 31.7% (2.9%) yielding a coefficient of variation of 9%. The CpG site in *AXL* was located in the promoter at -223 base pairs upstream of the transcription start site in part of the core promoter region located

*Correspondence to: Carrie V. Breton; Email: breton@usc.edu
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Table 1. Population characteristics for the 173 CHS children, by in utero smoking exposure status

			Unexposed		Exposed	
	N (percent)		N (percent)		N (percent)	
Female	73	42.2	40	40.8	33	44.0
Male	100	57.8	58	59.2	42	56.0
Ethnicity						
Non-Hispanic White	113	65.3	69	70.4	44	58.7
Hispanic White	45	26.0	23	23.5	22	29.3
Other	15	8.7	6	6.1	9	12.0
Exposed to maternal smoking in utero	75	43.4	---	---	75	43.4
Timing of exposure to maternal smoking						
1 month prior to pregnancy	69	39.9	---	---	69	39.9
1st trimester	54	31.2	---	---	54	31.2
2nd trimester	43	24.9	---	---	43	24.9
3rd trimester	41	23.8	---	---	41	23.8
Exposed to paternal smoking in the home during pregnancy*	65	37.6	16	16.3	49	65.3
Dr. diagnosed asthma by age 5*	82	47.4	59	60.2	23	30.7
			Mean (SD)			
Age at study entry	11.4	2.4	11.5	2.5	11.2	2.4

*Chi-square test of difference across groups was <0.0001.

Table 2. The effect of maternal smoking in utero on % DNA methylation of *AXL* in 173 CHS children, by sex

	Difference in % methylation*	95%CI	
Overall	2.3	0.3	4.2
By sex**			
Boys	1.3	-1.5	4.0
Girls	3.4	0.6	6.2

*Adjusted for plate, ethnicity, sex, age, paternal smoking in utero and asthma status. **Interaction p value = 0.02.

-556 to -182, which is a known Sp1/Sp3 transcription factor binding site.[6]

In this study, we found that children exposed to maternal smoking in utero had a 2.3% increase in DNA methylation in the *AXL* promoter (95% CI 0.3, 4.2) after adjustment for ethnicity, sex, age, paternal smoking in the house during pregnancy, asthma status and experimental plate. The association was magnified in girls, where exposure was associated with a 3.4% increase in DNA methylation (95% CI 0.6, 6.2) and no effect was seen in boys (**Table 2**). When we evaluated timing of maternal smoking exposure, similar results were seen across all trimesters. However, we cannot rule out the possibility that exposure during a specific trimester is most biologically relevant. Rather, the consistency of effect estimates across trimesters reflects the fact that if a mother smokes, she tends to smoke throughout the entire pregnancy.

We also assessed heritability of the *AXL* locus in 16 monozygotic and 20 dizygotic twins in the CHS. These twins were not part of the 173 CHS participants in the study reported above. Heritability was calculated using Falconer's formula and using variance components analysis.[16,17] Mean age of CHS twins at time of buccal cell collection was 10.5 years old. DNA methylation of *AXL* was right-skewed (**Fig. 1**) with a median (IQR) of 7.7% (5.0%). DNA methylation levels did not differ significantly by sex. The Intra class Correlation Coefficient in monozygotic twins (n = 8 pair) was 0.44 and in dizygotic twins (n = 10 pair) was 0.48, yielding a heritability estimate of -0.08. Heritability estimated using analysis of variance was -0.12 with a p-value of 0.68. The heritability we observed was not due to an artifact in DNA methylation measurement from a single nucleotide polymorphism located at the CpG site evaluated.

Our estimates of heritability of *AXL* DNA methylation suggest that parental transmission of epigenetic marks in our population studied does not play a large role, relative to the environment, in DNA methylation level at this locus. In addition, our maternal smoking results present compelling evidence that environmental exposure to tobacco smoke during pregnancy may alter DNA methylation levels in subtle but potentially important ways, and that these changes are persistent years after birth. If *AXL* is an imprinted gene, then we might hypothesize that exposure to tobacco smoke could alter DNA methylation during gamete development or post-fertilization of the embryo, or cause loss of imprinting at times in which the epigenotype is undergoing developmental change in a manner similar to the observed effects of diet on the imprinted gene *IGF2* in a mouse model.[18] Environmental exposures may also affect DNA methylation in a sex-specific manner, as in the Agouti A^{vy} mice model in which DNA methylation associated with fur color is maternally transmitted.[19]

The results presented in this report appear contradictory to those published in a 2009 report by Boks et al. suggesting that DNA methylation of the same locus in *AXL* is highly heritable.[7] The authors investigated the heritability of DNA methylation in

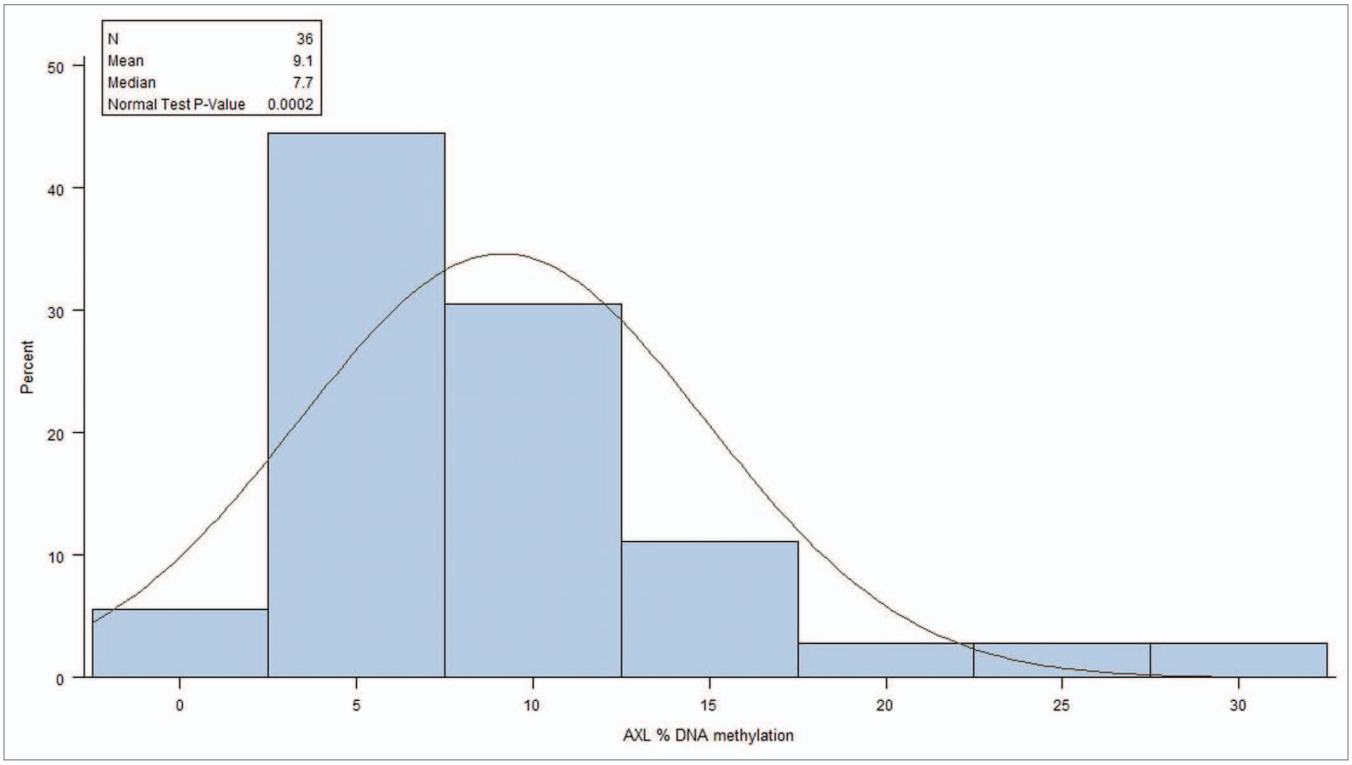

Figure 1. Histogram of *AXL* methylation in 36 twins.

92 twins using the Illumina GoldenGate methylation assay containing 1,505 CpG loci. They found that 96 of 431 CpG probes investigated yielded significant heritability, of which *AXL* was the highest, with a reported heritability of 0.94. However, estimates of broad-sense heritability of *AXL* methylation should be interpreted with caution. Use of such heritability estimates may be misleading, given the recent evidence that *AXL* may be imprinted in some tissues, because such heritability estimates are based on the assumption of classical Mendelian inheritance. Choufani et al. found some evidence of polymorphic imprinting, which complicates this evaluation even further, since in some individuals *AXL* may be monoallelically expressed and in others biallelically expressed.[8] Differences in underlying genetic distributions between populations could yield vastly different estimates of heritability under this scenario.

Differences in the tissue types used for DNA methylation analysis may also explain differences in heritability. DNA methylation is known to vary by tissue type and we used buccal cell DNA, whereas Boks et al. measured DNA methylation in peripheral blood samples.[15]

The populations studied differed and the contrasting findings could represent differences in genetic and/or environmental variation underlying the estimates. The CHS twins were children whose ages ranged from 7–17 years at the time of buccal DNA collection, whereas the twins studied in Boks et al. ranged in age from 18–57 years old. Boks et al. studied healthy twin volunteers of primarily Dutch origin, whereas our population of children was comprised mostly of non-Hispanic and Hispanic white children. While we did not observe age or ethnicity to be strongly related to methylation, whether differences in ethnicity or related exposures affect heritability of DNA methylation in general is unknown.

In summary, DNA methylation in *AXL* depends on environmental exposure and may also reflect imprinting in some individuals making the usefulness of calculating broad-sense heritability problematic. Nevertheless, the external environment has great potential to influence CpG methylation levels in small but potentially meaningful ways. In particular, environmental exposures that occur in utero have the potential to affect DNA methylation patterns before birth, and imprinted genes may be particularly susceptible to such exposures because only a single allele is active. Thus, any environmentally-induced epigenetic changes will have greater impact on gene expression. Investigation into the effects of environmental exposures on epigenetic changes is a largely unexplored area of research, and one that holds great promise for helping to elucidate biological mechanisms underlying exposure-disease associations observed in epidemiologic studies.

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