Microsomal Epoxide Hydrolase, Glutathione S-transferase P1, Traffic and Childhood Asthma

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SUPPLEMENTARY MATERIAL (ONLINE ONLY MATERIAL)

METHODS

Study design and subjects

Children of this study participated in the CHS, which has been described previously.[1, 2] In brief, a total of 6,259 children attending public school in 12 southern California communities were recruited in two cohorts in 1993 and 1996 for the CHS. Mean age at enrollment was 12.1 years (standard deviation (SD) 2.4 years) for cohorts recruited in 1993 and was 9.6 years (SD, 0.4 years) for cohort recruited in 1996. Beginning in 1998, we started collecting buccal samples and by May 2006, we collected and genotyped samples from 3,824 (61.1%) of children. Parents provided informed consent for participants below 18 years of age whereas participants over 18 years of age gave their informed consent. Children who were African-American (n = 162), Asian (n = 166) or belonged mixed race/ethnicity (n = 192) were excluded from the analysis due to insufficient sample sizes for stratified analyses and concern for population stratification. We also excluded 28 children for whom we did not have race/ethnicity information. Sixty-two children with unknown asthma status were also excluded.

Of the 3,214 non-Hispanic and Hispanic white children with available buccal samples, we were unable to obtain genotype results for the two SNPs in the *EPHX1* gene for 90 (2.8%) children. Therefore, our final sample included 3,124 children who were either Hispanic (n = 940) or non-Hispanic (n = 2,124) white with known asthma status. The University of Southern California Institutional Review Board approved the study.

Buccal Cell Collection and Processing

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. Children then swished liquid throughout their mouths and expelled the fluid into a container. The majority of buccal cell specimens were collected at school under the supervision of study staff. The remaining specimens were collected at home and sent to us by mail.

Buccal cell suspensions were centrifuged at 2,000g on the day they were received in the laboratory. The pellets were stored frozen at –20°C until used for DNA extraction, at which time they were resuspended and incubated in 600 µl of lysis solution from a PUREGENE DNA isolation kit (cat #D-5000; GENTRA, Minneapolis, MN) containing 100 µg/ml proteinase K overnight at 55°C. DNA extraction was performed according to manufacturer's recommendations. The DNA samples were resuspended in aqueous solution and stored at –20°C.

Genotyping

EPHX1: Genomic DNA was extracted from buccal mucosal cells using PUREGENE^{TM} DNA purification kit (Gentra Systems, Minneapolis, MN). The genotyping for the exon 3 113Tyr→113His polymorphic site (T→C) of EPHX1 and the exon 4 139His→139Arg (A→G) polymorphic site of EPHX1 was performed using the TaqMan Allelic Discrimination (AD) assay (Applied Biosystems, Foster City, CA). The primers and probes used for these two polymorphisms are presented in TABLE E1. The Taqman genotyping reaction was amplified on a GeneAmp PCR system 9600 (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min), and fluorescence was detected on an ABI PRISM^{TM} 7700 Sequence Detector (Applied Biosystems). In each run 10% of the samples were randomly selected and used for quality control. The results from TaqMan AD assay for exon 3 polymorphic site (T→C)

and the exon 4 (A→G) polymorphic site of EPHX1 were validated using PCR amplification of specific alleles (PASA) method and PCR/RFLP method,[3, 4] respectively.

GSTM1, GSTP1, GSTT1:

GSTM1, GSTT1 and GSTP1 genotypes were determined using real-time PCR using a TaqMan 7700 (Applied Biosystems, Foster City, CA). The forward and reverse primers and probes for GSTM1, GSTT1, and GSTP1 Ile105Val are presented in TABLE E1. The presence or absence of a fluorescent amplification signal was used as an indication of whether the GSTM1 and GSTT1 alleles were present or absent in a particular genomic DNA sample. Samples showing no signal or late cycle number for start of amplification were repeated and further analyzed with primers and probes for the actin gene to verify the presence of amplifiable DNA. The denaturation step during the first cycle was 10 minutes at 95°C. For the remainder of the amplification reaction, we used a two-cycle protocol characterized by 15-second incubation at 95°C followed by 1-minute incubation at 60°C.

Analysis of the single nucleotide polymorphism at codon 105 in the GSTP1 gene was performed using allele-specific probes. The fact that the wavelength of the fluorescent label was different in the two probes allowed distinguishing between amplification products from each allele in a single reaction. The conditions for the PCR were similar to those used for GSTT1 and GSTM1 polymorphisms. Samples showing no signal or late cycle number for start of amplification for either one of these alleles were repeated and further analyzed with primers and probes for the actin gene to verify the presence of amplifiable DNA.

Determining EPHX1 phenotypes

We determined the EPHX1 phenotypes based on methods described by Benhamou et al (TABLE E2).[5]

TABLE E1. Forward and reverse primers and probes for GSTM1, GSTT1, and GSTP1 Ile105Val genotypes

Genes	Primers
EPHX1 Tyr113His (T113C)	Forward: 5'-TGGAAGAAGCAGGTGGAGATTC-3' Reverse: 5'-TGCAAACATACCTTCAATCTTAGTCTTG-3' MGB Probes: T allele: 5'-(6FAM)CAACAGATACCCTCACT-3' C allele: 5'-(VIC)CAACAGACACCCTCA-3'
EPHX1 His139Arg (A139G)	Forward: 5'-ACATCCACTTCATCCACGTGA-3' Reverse: 5'-TAAAACTCGTAGAAAGAGCCGG-3' MGB Probes: A allele: 5'-(6FAM)AGGCCATACCCCGAAG-3' G allele: 5'-(VIC)AGGCCGTACCCCGAA-3'
GSTM1 (null/present)	Forward: 5'-CTTGGAGGAACTCCCTGAAAAG-3' Reverse: 5'-TGGAACCTCCATAACACGTGA-3' Probe: 5'-(6FAM)AAGCGGCCATGGTTTGCAGG-3'
GSTT1 null/present)	Forward: 5'-GTGCAAACACCTCCTGGAGAT-3' Reverse: 5'-AGTCCTTGGCCTTCAGAATGA-3' Probe: 5'-(6FAM)ATGCTGCCCATCCCTGCCC(TAMRA)-3'
GSTP1 (Ile105Val)	Forward: 5'-CCTGGTGGACATGGTGAATG-3' Reverse: 5'-TGCTCACATAGTTGGTGTAGATGA-3' MGB Probes: A allele: 5'-(6FAM)TGCAAATACATCTCCCT-3' G allele: 5'-(VIC)CTGCAAATACGTCTCC-3'

TABLE E2. Determination of EPHX1 phenotypes

EPHX1 His139Arg	*EPHX1 Tyr113His*		
	Tyr/Tyr	*Tyr/His*	*His/His*
Classification based on Benhamou et al (E6)			
His/His	Intermediate	Low	Low
His/Arg	High	Intermediate	Low
Arg/Arg	High	High	Intermediate

TABLE E3. Detectable ORs for different asthma outcomes for the main effects of EPHX1 and GSTP1 functional variant and EPHX1 phenotype

	OR detected with 80% power
EPHX1 Tyr113His	
Ever asthma	0.80
Current asthma	0.77
Early persistent	0.70
Late onset	0.74
EPHX1 His139Arg	
Ever asthma	1.28
Current asthma	1.35
Early persistent	1.47
Late onset	1.40
GSTP1 Ile105Val[†]	
Ever asthma	1.23
Current asthma	1.28
Early persistent	1.37
Late onset	1.32
EPHX1 high phenotype	
Ever asthma	1.40
Current asthma	1.52
Early persistent	1.69
Late onset	1.58

TABLE E4. Detectable ORs for the modifying effects of Ile105Val genotype and residential distance from a major road on EPHX1 high phenotype and lifetime asthma

Interactions	OR detected with 80% power
EPHX1 high phenotype x GSTP1 105 Val/Val genotype	1.62
EPHX1 high phenotype x Residential distance <75m from a major road	2.30
EPHX1 high phenotype x GSTP1 105 Val/Val genotype x Residential distance <75m from a major road	7.20

TABLE E5. Allele and haplotype frequencies in children with no asthma

	N	Minor allele		P_{HWE}*	Haplotype frequencies T113C – A139G				D′†
		Allele	Frequency						
Overall sample					CA	CG	TA	TG	
EPHX1 T113C	2648	C	0.32	0.12	0.24	0.07	0.58	0.10	0.16
EPHX1 A139G	2648	G	0.17	0.18					
GSTP1 A105G	2615	G	0.37	0.52					
Non-Hispanic white									
EPHX1 T113C	1823	C	0.30	0.19	0.22	0.08	0.58	0.12	0.16
EPHX1 A139G	1823	G	0.20	0.24					
GSTP1 A105G	1801	G	0.33	0.99					
Hispanic white									
EPHX1 T113C	825	C	0.35	0.60	0.29	0.06	0.58	0.07	0.19
EPHX1 A139G	825	G	0.12	0.75					
GSTP1 A105G	814	G	0.44	0.05					

* P-values for Hardy-Weinberg equilibrium

† Lewontin's D′, a measure of linkage disequilibrium.

TABLE E6. Associations between *GSTM1* and *GSTT1* polymorphisms and asthma phenotypes

	No asthma	Lifetime asthma		Current asthma		Early persistent asthma (diagnosis by 3 years)		Late onset asthma (diagnosis after 3 years)	
	N	N	OR* (95% CI)	N	OR* (95% CI)	N	OR* (95% CI)	N	OR* (95% CI)
GSTM1									
Present	1300	215	1.0	148		82	1.0	113	1.0
Null	1226	240	1.21 (0.98 to 1.49)	146	1.08 (0.83 to 1.39)	86	1.15 (0.83 to 1.60)	117	1.10 (0.83 to 1.45)
GSTT1									
Present	1992	363	1.0	232		133	1.0	181	1.0
Null	537	91	0.95 (0.74 to 1.24)	60	0.98 (0.72 to 1.34)	31	0.89 (0.58 to 1.34)	50	1.06 (0.76 to 1.49)

*ORs adjusted for age, sex, race/ethnicity, *in utero* exposure to maternal smoking, number of smokers at home, community of residence, parental education, health insurance, and parental history of asthma.

† Data on *GSTM1* and *GSTT1* were unavailable for 140 children.

TABLE E7. Association between EPHX1 phenotypes and asthma among lifelong residents, stratified by *GSTP1 Ile*105*Val* genotype.

EPHX1 phenotypes by asthma status	Residential distance from major road				*P* ‡
	≥75m		<75m		
	N*	OR† (95% CI)	N*	OR† (95% CI)	
No asthma					
Low/intermediate	1511		362		
High	338		65		
Ever asthma					
Low/intermediate	152	1.0	28	1.0	
High	40	1.15 (0.78 to 1.69)	16	**4.16 (1.84 to 9.42)**	**0.009**
Current asthma					
Low/intermediate	105	1.0	20	1.0	
High	27	1.16 (0.73 to 1.85)	11	**3.94 (1.52 to 10.23)**	**0.03**
Early persistent asthma					
Low/intermediate	41	1.0	7	1.0	
High	10	1.05 (0.50 to 2.20)	3	6.80 (0.76 to 60.78)	0.35
Late onset asthma					
Low/intermediate	96	1.0	19	1.0	
High	26	1.23 (0.77 to 1.97)	13	**5.73 (2.25 to 14.57)**	**0.006**

* Children with missing data on residential distance from major road were excluded.

† ORs represent odds ratios for asthma outcomes associated with high EPHX1 phenotype within each stratum of residential distance from major road, and were adjusted for age, sex, race/ethnicity, in utero exposure to maternal smoking, number of smokers at home, community of residence, parental education, health insurance, and parental history of asthma.

‡ The *P* value for the *EPHX1* metabolic phenotypes by residential distance from major road interactions were obtained from likelihood ratio tests from a non-stratified model with appropriate interaction terms and was based on 1 df.

TABLE E8. Association between EPHX1 phenotypes and asthma among lifelong residents, stratified by residential distance from major roads (n = 2483).

Residential distance from major road	*GSTP1 Ile105Val*	*EPHX1* phenotypes	No asthma (N*)	Lifetime asthma (N*)	OR† (95% CI)
≥75m	*Ile/Ile*	Low/Intermediate	582	60	1.0
≥75m	*Ile/Val*	Low/Intermediate	702	73	1.03 (0.71 to 1.50)
≥75m	*Val/Val*	Low/Intermediate	211	18	0.89 (0.50 to 1.58)
≥75m	*Ile/Ile*	High	139	15	1.03 (0.55 to 1.92)
≥75m	*Ile/Val*	High	157	15	0.96 (0.50 to 1.72)
≥75m	*Val/Val*	High	36	8	**2.57 (1.10 to 6.00)**
<75m	*Ile/Ile*	Low/Intermediate	141	11	0.74 (0.37 to 1.47)
<75m	*Ile/Val*	Low/Intermediate	169	14	0.68 (0.36 to 1.30)
<75m	*Val/Val*	Low/Intermediate	48	3	0.63 (0.18 to 2.17)
<75m	*Ile/Ile*	High	31	6	1.77 (0.67 to 4.67)
<75m	*Ile/Val*	High	29	7	**2.67 (1.06 to 6.73)**
<75m	*Val/Val*	High	5	3	**5.50 (1.05 to 28.72)**
					P ‡ = 0.08

* Children with missing data on residential distance from major road and *GSTP1* Ile105Val were excluded.

† ORs adjusted for age, sex, race/ethnicity, *in utero* exposure to maternal smoking, number of smokers at home, community of residence, parental education, health insurance, and parental history of asthma.

‡ The P value for the EPHX1 activity phenotype by residential distance from a major road and by GSTP1 Ile105Val genotype interaction was obtained from likelihood ratio test from a non-stratified model with appropriate interaction terms and was based on 7df.

TABLE E9. Comparison between children who provided buccal samples and those who did not participate in the genetic study.

	Participants (N =3,153)		Non-participants (N = 1,840)		P-value[†]
	N*	(%)	N*	(%)	
Sex					
Girls	1688	(53.5)	886	(48.2)	0.0002
Boys	1465	(46.5)	954	(51.8)	
Age (years)					
≤ 10	1730	(54.9)	718	(39.0)	<0.0001
11-12	593	(18.8)	382	(20.8)	
> 12	830	(26.3)	740	(40.2)	
Ethnicity					
Non-Hispanic white	2172	(68.9)	1137	(61.8)	<0.0001
Hispanic white	981	(31.1)	703	(38.2)	
Annual family income ($)					
< $15,500	372	(13.7)	346	(23.1)	<0.0001
$15,000 - $49,999	1144	(42.0)	658	(44.0)	
≥$50,000	1205	(44.3)	492	(32.9)	
Parent/guardian education					
< 12th grade	362	(11.8)	362	(20.3)	<0.0001
12th grade	595	(19.4)	424	(23.7)	
Some college	1404	(45.7)	732	(41.0)	
College	314	(10.2)	121	(6.8)	
Some graduate	397	(12.9)	146	(8.2)	
Health insurance coverage					
No	444	(14.3)	338	(18.9)[†]	<0.0001
Yes	2660	(85.7)	1453	(81.1)	
Exposure to maternal smoking *in utero*					
No	2553	(82.6)	1366	(76.9)	<0.0001
Yes	537	(17.4)	411	(23.1)	
Number of smokers at home					
None	2153	(70.6)	1035	(58.9)	<0.0001
1	598	(19.6)	458	(26.1)	
2 or more	298	(9.8)	264	(15.0)	
Residential distance from a freeway					
>1500m	1580	(58.6)	806	(52.8)	0.001
1001-1500m	351	(13.0)	236	(15.5)	
500-1000m	437	(16.2)	259	(17.0)	
<500m	327	(12.1)	226	(14.8)	
Asthma					
Never	2726	(86.5)	1632	(88.7)	0.02
Ever	427	(13.5)	208	(11.3)	

*Numbers always do not add up because of missing data

†*P* values are from Pearson chi-square tests). Comparisons were restricted to non-Hispanic and Hispanic white children. Children who had early transient asthma and those with unknown asthma status have been excluded from these comparisons to reflect the population studied.

TABLE E10. Percentage of Hispanic white children in the present study and at study enrollment by communities.

Community	Percentage of Hispanic white within each community	
	Present study	At study enrollment
	(%)	(%)
Alpine	(13.5)	(17.5)
Lake Elsinore	(31.2)	(30.5)
Lake Arrowhead	(17.5)	(18.5)
Lancaster	(33.1)	(34.5)
Lompoc	(27.9)	(30.9)
Long Beach	(32.5)	(39.5)
Miraloma	(42.3)	(43.1)
Riverside	(44.7)	(52.3)
San Dimas	(35.5)	(38.1)
Atascadero	(13.0)	(15.8)
Santa Maria	(68.3)	(70.6)
Upland	(19.0)	(18.8)

* Column percent

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