

Sex- and age-dependent DNA methylation at the 17q12-q21 locus associated with childhood asthma

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Received: 23 January 2013 / Accepted: 18 March 2013 / Published online: 2 April 2013
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Abstract Chromosomal region 17q12-q21 is one of the best-replicated genome-wide association study (GWAS) hits and associated with childhood-onset asthma. However, the mechanism by which the genetic association is restricted to childhood-onset disease is unclear. During childhood, more boys than girls develop asthma. Therefore, we tested the hypothesis that the 17q12-q21 genetic association was sex-specific. Indeed, a TDT test showed that in the Saguenay-Lac-Saint-Jean familial collection, the 17q12-q21 association was significant among male, but not among female asthmatic subjects. We next hypothesized that the bias in the genetic association resulted from sex-specific and/or age-dependent DNA methylation at regulatory regions and determined the methylation profiles of five 17q12-q21 gene promoters using the bisulfite sequencing methylation assay. We identified a single regulatory region within the *zona pellucida* binding protein 2 (*ZPBP2*) gene, which showed statistically significant differences between

males and females with respect to DNA methylation. DNA methylation also varied with age and was higher in adult males compared to boys. We have recently identified two functionally important polymorphisms, both within the *ZPBP2* gene that influence expression levels of neighboring genes. Combined with the results of the present work, these data converge pointing to the same 5 kb region within the *ZPBP2* gene as a critical region for both gene expression regulation and predisposition to asthma. Our data show that sex- and age-dependent DNA methylation may act as a modifier of genetic effects and influence the results of genetic association studies.

Introduction

One of the best-replicated GWAS hits for predisposition to asthma maps to the 17q12-q21 chromosomal region (Bouzigon et al. 2010; Madore et al. 2008; Moffatt et al. 2007, 2010; Torgerson et al. 2011) (Fig. 1a). A distinct feature of this region is its association with childhood asthma (Halapi et al. 2010; Moffatt et al. 2007), with higher significance of association among children that were exposed to cigarette smoke (Brauner et al. 2011; Flory et al. 2009; Smit et al. 2009; van der Valk et al. 2012). Interestingly, the sex ratio in asthmatic children is significantly biased in favor of the male sex, whereas it is reversed among adults (reviewed in Kynk et al. 2010; McCallister and Mastronarde 2008). Sex differences in genetic association have been documented for several asthma-associated genomic regions (Aschard et al. 2009; Hunninghake et al. 2010; Loisel et al. 2011), but were not detected for the 17q12-q21 alleles in study populations from Europe, Australia and Korea (Halapi et al. 2010). However, in a smaller study of adult asthma, a 4 single nucleotide polymorphism (SNP) haplotype in the

Electronic supplementary material The online version of this article (doi:10.1007/s00439-013-1298-z) contains supplementary material, which is available to authorized users.

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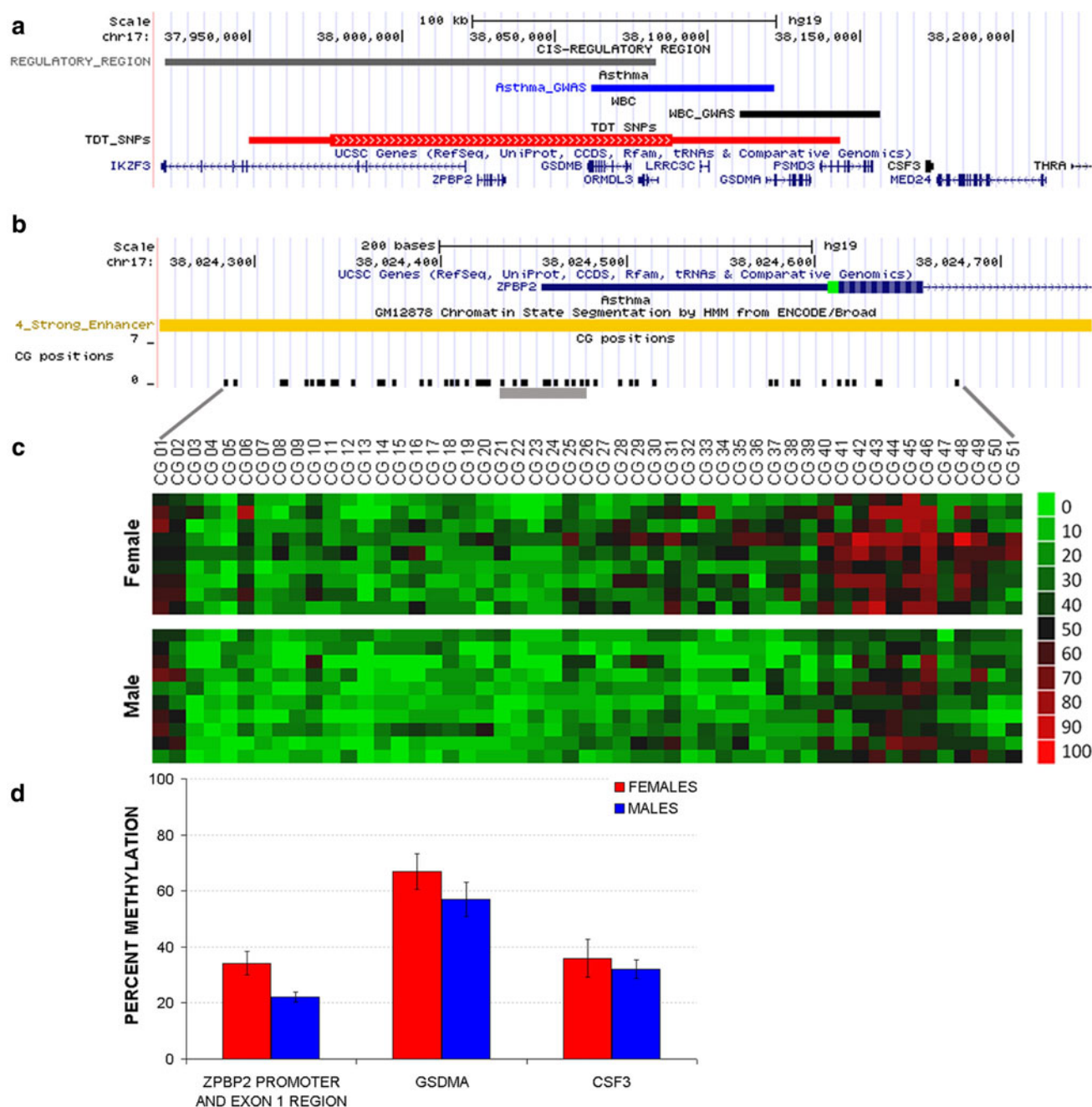


Fig. 1 Analysis of sex-specific effects in the asthma-associated region 17q12-q21. **a** Relative positions of the asthma-associated and neutrophil count-associated GWAS regions, the *cis*-regulatory haplotype and the region tested using TDT in the SLSJ asthmatic familial collection. The thick line indicates the interval between the two SNPs that reached the significance level of 0.005. All features are shown in the context of the UCSC Genome Browser (<http://genome.ucsc.edu>). **b** Positions of CGs analyzed for methylation in the promoter region of the *ZPBP2* gene shown as *tics*. The gray bar shows the 11 CG-region analyzed using a pyrosequencing assay. The yellow bar indicates the location of a strong enhancer based on the data from Ernst et al. (2010), Ernst and Kellis (2010). **c** DNA methylation of the *ZPBP2*

promoter region in the peripheral blood cells of nine females and ten males. Each row corresponds to results from a single individual. Each square represents a single CG. Color represents level of methylation at each CG position with red corresponding to high methylation and green to low methylation level, as indicated on the right. **d** Effect of sex on average DNA methylation levels in three gene promoters with high variance in methylation. Red bars show average methylation levels in females, blue bars show average methylation levels in males. Error bars show 95 % confidence intervals. For *ZPBP2*, methylation patterns were determined in ten males and nine females; for *GSDMA*—in three males and six females; for *CSF3*—in seven males and three females

region showed association with allergic asthma in adult males, but not females (Hrdlickova and Holla 2011). Moreover, several studies found association between asthma and 17q12-q21 alleles in adults (Hrdlickova and Holla 2011; Madore et al. 2008). These facts suggest the possibility that the 17q12-q21 locus harbors genetic elements that act in a sex-specific and/or age-dependent fashion and contribute to susceptibility to asthma.

Expression of three genes located within the 17q12-q21 region: gasdermin B (*GSDMB*), ORM1-like 3 (*Saccharomyces cerevisiae*) (*ORMDL3*) and zona pellucida binding protein 2 (*ZPBP2*), is allele-specific with 2- to 4-fold allelic differences in expression levels (Moffatt et al. 2007; Verlaan et al. 2009a, b). It was therefore hypothesized that predisposition to asthma resulted from increased/decreased expression of one or all of these genes, which in turn was due to *cis*-acting regulatory polymorphisms in the region (Moffatt et al. 2007; Verlaan et al. 2009a). The *cis*-regulatory haplotype that controls *ORMDL3* and *GSDMB* expression in lymphoblastoid cell lines (LCLs) was mapped to a 160-kb region that spans *IKZF3*, *ZPBP2*, *GSDMB* and *ORMDL3* genes (Verlaan et al. 2009a) (Fig. 1a). The two most common *cis*-regulatory haplotype variants are termed for convenience HapA and HapB. The HapA haplotype variant carries alleles that are associated with asthma (Moffatt et al. 2007) and is present in almost two-thirds of the population, however, not every single HapA haplotype carrier develops asthma. This may be explained by differences in environmental exposures and genetic background at other unlinked susceptibility loci. It is also possible that inter-individual variation in DNA methylation is responsible for at least some of the phenotypic variance found in individuals that carry the same asthma-predisposing HapA haplotype.

Here, we asked two questions: (a) whether the genetic association between asthma and the 17q12-q21 alleles was stronger among males; and (b) if inter-individual, age-dependent or sex-specific variation in DNA methylation of regulatory elements could be responsible for phenotypic differences between affected and unaffected individuals that carry the asthma-predisposing alleles.

To answer these questions, we analyzed the genetic association between asthma and 17q12-q21 alleles considering sex stratification and DNA methylation profiles at gene promoters in the Saguenay-Lac-Saint-Jean (SLSJ) asthma familial collection.

Materials and methods

Subjects

The SLSJ asthma familial collection comprises 1,214 samples distributed in 240 extended families (see Table 1).

The familial collection consists of firstly recruited probands ($n = 240$) and their relatives who joined the study ($n = 974$). These extended families were subdivided into 484 nuclear families for statistical analyses. Clinical evaluation and completion of a standard respiratory questionnaire was done for all subjects according to the American Thoracic Society's recommendations (American Thoracic Society 1987) and are described in greater detail in Begin et al. (2007). Participants were defined as having asthma if (1) they had a reported history of asthma (validated by a physician), or (2) they showed asthma-related symptoms and a positive PC₂₀ [provocative challenge that induces a 20 % fall in the forced expiratory volume in 1 s (FEV₁)] at the time of recruitment. Subjects were deemed atopic if they had at least one positive response on skin prick test (wheal diameter ≥ 3 mm at 10 min) to one of the 24 common airborne allergens: (1) cat, dog, horse, cow and feathers; (2) dust; (3) *D. farinae* and *D. pterionisus*; (4) grass, weed, ambrosia, timothy, rye grass; (5) tree mix, birch, maple, oak, elm; (6) *Cladosporium*, *Hormodendrum*, *Alternaria alternata*, *Alternaria tenuis*, *Aspergillus* and *Penicillium*. The work was done in accordance with the Canadian Tri-Council Policy Statement on Ethical Conduct for Research Involving Humans. The Centre de Santé et de Services Sociaux de Chicoutimi and University Hospital local ethics committee and the MUHC Research Ethics Board approved the study and all subjects gave their informed consent.

Association analysis

A transmission disequilibrium test (TDT) was performed to identify association between SNPs and asthma using pLINK software. To be included in the analyses, a SNP had to fulfill these criteria: follow the Hardy–Weinberg equilibrium ($p > 0.05$), have a minor allele frequency higher than 0.05, do not show Mendelian errors and present a call rate higher than 95 %. According to the number of independent SNPs, the corrected threshold for significance was fixed at 0.005 (Nyholt 2004). Haplotypes were defined using linkage disequilibrium (LD) measures between SNPs (D') (Barrett et al. 2005) with Haploview software (version 4.2, <http://www.broadinstitute.org-haploview/haploview>). Haplotype-specific associations were also investigated using pLINK software (version 1.07, <http://pngu.mgh.harvard.edu/~purcell/plink/gplink.shtml>). SNP- and haplotype-specific association analyses were also performed after filtering out male or female offspring of the nuclear families in order to identify SNPs that showed sex-specific association with asthma. According to the quality criteria, 24 SNPs were included in the analyses. According to the number of independent TagSNPs, which is 11, the corrected threshold for significance was fixed to 0.005 after a

Table 1 Phenotypic description of subjects recruited in the Saguenay-Lac-Saint-Jean asthma familial collection

	All samples (<i>n</i> = 1,214)	Probands (<i>n</i> = 240) ^a	Family members (<i>n</i> = 974)		
			All (<i>n</i> = 974)	Without asthma (<i>n</i> = 615) ^b	With asthma (<i>n</i> = 353) ^b
M:F ratio	1:1.17	1:1.12	1:1.18	1:1.12	1:1.34
Age [mean (range)] ^c	38 (0–93)	17 (3–62)	43 (0–93)	46 (3–93)	39 (2–83)
Age median	39	15	43	46	40
Smoking status [<i>n</i> (%)] ^d					
Non-smoker	646 (54)	202 (86)	444 (46)	259 (43)	182 (52)
Ex-smoker ^d	314 (26)	9 (4)	305 (32)	204 (34)	100 (29)
Smoker	235 (20)	25 (11)	210 (22)	142 (23)	68 (19)
Passive smoking [<i>n</i> (%)] ^e					
Childhood	280 (61)	121 (60)	159 (63)	76 (56)	81 (70)
In utero	113 (22)	57 (28)	56 (18)	28 (17)	27 (20)
FEV ₁ [% pred. (SD)] ^f	94 (43)	93 (35)	95 (45)	99 (48)	88 (38)
PC ₂₀ [(SD)] ^g	6.85 (5.2)	2.36 (3.45)	9.28 (5.07)	19.2 (3.61)	3.14 (4.35)
IgE [(SD)] ^h	125 (5)	238 (5)	104 (5)	79 (4)	165 (5)
Asthma [<i>n</i> (%)] ^b	592 (49)	239 (100)	353 (36)	0 (0)	353 (100)
Allergy [<i>n</i> (%)] ⁱ	670 (57)	189 (80)	481 (51)	241 (40)	239 (70)
Rhinitis [<i>n</i> (%)] ^j	372 (31)	102 (43)	270 (28)	131 (21)	138 (39)
Atopic dermatitis [<i>n</i> (%)] ^k	478 (40)	120 (50)	358 (37)	188 (31)	169 (48)

^a Probands are first affected family member recruited in the familial collection and family members referred to other family members who joined the study

^b Present asthma or past documented clinical history of asthma. The reported mean age of onset is 7 years among the probands and 22 years among the asthmatic family members. Asthma phenotype is available for 968 of the 974 family members

^c Mean and median age calculated for 1,212 subjects [240 probands and 972 family members (614 without asthma and 353 with asthma)]

^d Smoking status available for 1,195 subjects [236 probands and 959 family members (605 without asthma and 350 with asthma)]. Ex-smokers are defined as subject who stopped smoking since 1 year or more

^e Childhood passive smoking available for 456 subjects [202 probands and 254 family members (136 without asthma and 116 with asthma)] and passive smoking in utero available for 508 subjects [204 probands and 304 family members (165 without asthma and 138 with asthma)]

^f FEV₁ = mean calculated for the forced expiratory volume in 1 s in % of predicted value and SD calculated for 931 subjects [207 probands and 724 family members (422 without asthma and 299 with asthma)]

^g PC₂₀ = provocative concentration of methacholine that induces a 20 % fall in FEV₁. Geometric mean and SD calculated for 903 subjects [201 probands and 702 family members (421 without asthma and 277 with asthma)]

^h IgE = immunoglobulin E serum concentration. Geometric mean and SD calculated for 1,012 subjects [226 probands and 786 family members (468 without asthma and 314 with asthma)]

ⁱ Defined as at least one positive response on skin prick testing (wheal diameter > 3 mm at 10 min). Allergy phenotype is available for 1181 subjects [236 probands and 945 family members (598 without asthma and 342 with asthma)]

^j Rhinitis phenotype is available for 1,208 subjects [239 probands and 969 family members (613 without asthma and 352 with asthma)]

^k Defined as a personal history of urticaria, eczema or both. This phenotype is available for 1,209 subjects [240 probands and 969 family members (613 without asthma and 352 with asthma)]

correction for multiple testing based on the spectral decomposition (SpD) of matrices of pairwise LD between SNPs (Nyholt 2004).

Sodium bisulfite sequencing methylation assay

DNA methylation assays for the rs4795397 region, *ZPBP2*, *ORMDL3* and *GSDMA* proximal promoters were

performed as described earlier (Berlivet et al. 2012). Conditions for the *GSDMB* and *CSF3* promoter methylation assays are provided in Supplementary Table 1S. As a rule the assays were designed to encompass at least one SNP; however, the *ORMDL3* promoter region did not contain a polymorphism. For most samples, at least two independent PCR reactions were used for cloning. Seven to 24 (average 18) sequenced clones per sample were used to

determine the individual's methylation profile. Clone sequences were analyzed using BiQ Analyzer software (Max-Planck-Institute for Informatics, Saarbrücken, Germany) and bead charts were generated by QUMA (QUantification tool for Methylation Analysis) web-based application (RIKEN Center for Developmental Biology, Kobe, Japan).

Pyrosequencing methylation assay

Thirty-seven subjects (Table 2) were selected for analysis based on two characteristics: (1) being homozygous for the HapA haplotype at the 17q12-q21 asthma-associated region; and (2) being under the age of 16 years. Forty-three males older than 16 (henceforth referred to as “adult” males) were also selected for analysis of the effect of age and affected status on DNA methylation. Subject characteristics are presented in Table 2.

Subjects' DNA was extracted from PBMC cells (Blood and cell culture Midi kit, Qiagen, Canada). Sodium bisulfite DNA conversion was performed using Epitect Bisulfite Kit from QIAGEN (#59104, Canada). 600 ng of DNA was used for each sample. Bisulfite-treated DNA was then amplified by PCR using the Pyromark PCR kit from QIAGEN (#978703, Canada). Methylation of 11 CGs in the promoter region of the *ZPBP2* gene (Fig. 1b) was analyzed. This region was selected based on results from the sodium bisulfite sequencing methylation assay and its overlap with the putative enhancer region (Berlivet et al. 2012; Ernst and Kellis 2010; Ernst et al. 2010) and transcriptional start site of the gene (Fig. 1b). PCR and pyrosequencing primers were designed using the PyroMark Assay Design 2.0 (QIAGEN, Canada) (primers: F 5'-GTG ACGTACTAGCAACGAGAGAGTAAGGGGTGA GGAG, R: 5'-TAGCAGGATACGACTATCTCCCCACCTCCTCC ACAATA, seq: 5'-ATCCCCTCCCCTACCC). Methylation profiles were assessed by pyrosequencing using the Pyromark Gold Q24 Reagents by QIAGEN (#978802, Canada). Each sample was analyzed in duplicate. To assess if methylation levels of CGs were correlated and could be analyzed as one signal, Spearman correlation were performed between CGs giving $\rho > 0.65$. Principal component analysis was performed and the correlation between the 11 CGs (variance explained by the first component = 80.54 %) confirmed. A two-tailed Student *t* test was used to assess the difference of methylation levels for the different phenotypes (age, sex, asthma, smoking in utero). *P* values were adjusted using a Bonferroni correction to compensate for the number of tests performed. The new *P* value significance was set at $0.05/3 = 0.0167$ for comparison of methylation levels for the number of independent phenotypes (three) using a matrix spectral decomposition (matSpD) approach (Nyholt 2004).

Results

Sex-specific association between 17q12-q21 alleles and asthma

SNPs of the 17q12-q21 region, specifically between 37.9 and 38.1 Mb, were extracted from the GWAS that was earlier performed on the SLSJ asthma collection using the pLINK software (Moffatt et al. 2010). Briefly, the previous GWAS study included 1,214 subjects that were genotyped using Illumina 610KQuad array (Illumina, San Diego, USA) at the Centre National de Génotypage (Evry, France). The 0.2 Mb region was selected for analysis based on the previous GWAS published by Moffatt et al. (2007). It spans part of the regulatory haplotype region and several asthma-associated GWAS SNPs reported in different studies (Ferreira et al. 2010; Moffatt et al. 2007, 2010) (Table 3). Neutrophils play an important role in the pathogenesis of asthma [reviewed in (Fox et al. 2010; Haslett et al. 1989)] and the GWAS region associated with white blood cell/neutrophil counts (Crosslin et al. 2011, Nalls et al. 2011, Okada et al. 2010, Reiner et al. 2011, Soranzo et al. 2009) overlaps with the asthma-associated 17q12-q21 region (Fig. 1a). Therefore, we also included the region of high association with neutrophil count (Crosslin et al. 2011) in our analyses (Fig. 1a; Table 3).

Transmission analysis of 26 SNPs of the 17q12-q21 locus in members of asthmatic families from the SLSJ region showed association among males but not females (Table 3). After filtering for quality control, 24 of the 26 SNPs of the 0.2 Mb locus on the 17q12-q21 chromosomal region were included in the analyses (Table 3). TDTs show significant associations before correction between nine SNPs and asthma for the analysis that considers all offspring. These same nine SNPs are also associated before correction with asthma when only male children are considered and two of these SNPs (rs9303277, rs4795405) are significant after statistical correction. Rs9303277 is located in the third intron of the *IKZF3* gene, whereas rs4795405 is 4.5 kb upstream of the *ORMDL3* gene promoter. No association was found between the 17q12-q21 locus and asthma in the analysis that considers only female offspring. Haplotype analyses did not show significant association after correction. The size of the sample was not big enough to determine if the genetic association was present in girls younger than 16 compared to adult females.

Sex-specific DNA methylation patterns in the promoter region of the *ZPBP2* gene

In principle, a sex bias in the genetic association with asthma could result from the action of hormones, sex-chromosome linked genetic modifiers or from sex

Table 2 Phenotypic description of subjects whose DNA methylation was assessed using pyrosequencing assays

Characteristics	Subjects under 16 (<i>n</i> = 37)	Adult subjects older than 16 (<i>n</i> = 43)
Mean age [years (range)]	10 (5–16)	50 (19–80)
Sex (male:female)	1:1.06	1:0
FEV ₁ [% predicted (SD)] ^a	99.5 (26.1)	92.0 (20.1)
PC ₂₀ [mg/ml (SD)] ^b	5.79 (3.65)	1.11 (0.67)
Serum IgE [μg/l (SD)] ^c	188.49 (3.71)	119.75 (5.87)
Exposure to tobacco in utero, <i>n</i> (%)	13 (36.1)	N/A
Asthma, <i>n</i> (%) ^d	23 (62.2)	21 (48.8)

^a FEV₁ = mean calculated for the forced expiratory volume in one second [data not available for 8 (under 16) and 8 adult subjects]

^b PC₂₀ = geometric mean of provocative concentration of methacholine that induces a 20 % fall in FEV₁ [data not available for 15 (under 16) and 11 (adult) subjects]

^c IgE = geometric mean of immunoglobulin E serum concentration [data not available for 9 (under 16) and 8 (adult) subjects]

^d Present asthma or past documented clinical history of asthma

Table 3 Genetic associations between SNPs of the 0.2 Mb interval on the 17q12-q21 region and asthma considering all offspring or considering male or female offspring only in the Saguenay-Lac-Saint-Jean asthma familial collection

SNP	Position (hg19) ^a	Gene ^a	A1:A2	All				Males				Females			
				OR	T:U	χ ²	<i>p</i> ^b	OR	T:U	χ ²	<i>p</i> ^b	OR	T:U	χ ²	<i>p</i> ^b
rs17676191	37949924	<i>IKZF3</i>	G:A	1.34	79:59	2.90	0.089	1.39	39:28	1.81	0.179	1.29	40:31	1.14	0.286
rs9303277	37976469	<i>IKZF3</i>	T:C	0.74	141:191	7.53	0.006	0.62	57:92	8.22	0.004	0.85	84:99	1.23	0.268
rs2060941	37982883	<i>IKZF3</i>	T:G	1.05	65:62	0.07	0.790	0.96	27:28	0.02	0.893	1.12	38:34	0.22	0.637
rs9635726	38020141	<i>IKZF3</i>	T:C	1.20	113:94	1.74	0.187	1.35	58:43	2.23	0.136	1.08	55:51	0.15	0.698
rs12150079	38025417	<i>ZBP2</i>	A:G	0.74	113:152	5.74	0.017	0.64	46:72	5.73	0.017	0.84	67:80	1.15	0.284
rs11557467	38028634	<i>ZBP2</i>	T:G	0.77	144:187	5.59	0.018	0.64	58:91	7.31	0.007	0.90	86:96	0.55	0.459
rs2872507	38040763		A:G	0.75	131:174	6.06	0.014	0.63	53:84	7.02	0.008	0.87	78:90	0.86	0.355
rs8067378	38051348		G:A	0.78	144:184	4.88	0.027	0.64	58:90	6.92	0.009	0.91	86:94	0.36	0.551
rs2305480	38062196	<i>GSDMB</i>	A:G	0.74	128:172	6.45	0.011	0.63	52:83	7.12	0.008	0.85	76:89	1.02	0.312
rs2290400	38066240	<i>GSDMB</i>	C:T	0.76	141:186	6.19	0.013	0.63	57:90	7.41	0.006	0.88	84:96	0.80	0.371
rs7216389	38069949	<i>GSDMB</i>	C:T	0.76	145:191	6.30	0.012	0.65	59:91	6.83	0.009	0.86	86:100	1.05	0.305
rs3744246	38084350	<i>3' of ORMDL3</i>	T:C	0.83	70:84	1.27	0.259	0.67	24:36	2.40	0.121	0.96	46:48	0.04	0.837
rs4795402	38085385		A:C	0.88	95:108	0.83	0.362	0.72	34:47	2.09	0.149	1.00	61:61	0.00	1.000
rs4795405	38088417		T:C	0.75	125:166	5.78	0.016	0.60	46:77	7.81	0.005	0.89	79:89	0.60	0.440
rs8079416	38092713		C:T	1.16	164:141	1.73	0.188	1.33	80:60	2.86	0.091	1.04	84:81	0.05	0.815
rs1007654	38111354		A:G	0.85	139:164	2.06	0.151	0.78	58:74	1.94	0.164	0.90	81:90	0.47	0.491
rs7219080	38114516		A:C	0.83	137:166	2.78	0.096	0.76	58:76	2.42	0.120	0.88	79:90	0.72	0.398
rs3902025	38119254		G:T	0.83	145:174	2.64	0.104	0.76	62:82	2.78	0.096	0.90	83:92	0.46	0.496
rs3894194	38121993	<i>GSDMA</i>	A:G	1.16	163:140	1.75	0.186	1.32	82:62	2.78	0.096	1.04	81:78	0.06	0.812
rs3859192	38128648	<i>GSDMA</i>	T:C	1.03	167:163	0.05	0.826	1.23	86:70	1.64	0.200	0.87	81:93	0.83	0.363
rs921651	38133922	<i>GSDMA</i>	G:A	0.93	76:82	0.23	0.633	0.73	27:37	1.56	0.211	1.09	49:45	0.17	0.680
rs8075668	38137623	<i>PSMD3</i>	T:C	0.99	160:161	0.00	0.956	0.83	69:83	1.29	0.256	1.17	91:78	1.00	0.317
rs11655584	38139024	<i>PSMD3</i>	T:C	1.25	40:32	0.89	0.346	0.73	11:15	0.62	0.433	1.71	29:17	3.13	0.077
rs4065321	38143548	<i>PSMD3</i>	C:T	0.92	164:179	0.66	0.418	1.07	81:76	0.16	0.690	0.81	83:103	2.15	0.143

A1:A2 minor allele (A1) and major allele (A2) frequencies, OR odds ratio, SNP polymorphisms, T:U transmitted minor allele count and untransmitted allele count, χ² Chi-square value

^a SNP position in base pairs and gene name according to the National Center for Biotechnology Information Build GRCh37 (hg19). NHGRI GWAS catalog SNPs associated with asthma are shown in bold and underlined

^b Significant *p* values before correction are shown in bold and significant *p* values after correction (significant threshold of 0.005) are shown in bold and underlined

differences in DNA methylation at specific regulatory regions (El-Maarri et al. 2011; Sawalha et al. 2012). Here, we tested the possibility that the sex-specific differences in the genetic association of asthma with 17q12-q21 alleles resulted from sex-specific differences in DNA methylation. We established the methylation profiles of five gene promoters: *ZBP2* [two regions were tested: (a) proximal promoter and exon 1 (Fig. 1b) and (b) the *rs4795397* region], *GSDMB*, *ORMDL3*, *GSDMA* and colony stimulating factor 3 (granulocyte) (*CSF3*), in peripheral blood DNA samples from members of the SLSJ asthmatic familial collection using the bisulfite sequencing methylation assay. These DNA samples were part of the collection that was used in genetic association studies and therefore the 17q12-q21 genotypes of these individuals were established earlier (Moffatt et al. 2010). *ZBP2*, *GSDMB*, and *ORMDL3* show allelic differences in expression in LCLs and therefore may not be excluded as candidate causal genes in predisposition to asthma (Verlaan et al. 2009a, b). *GSDMA* and *CSF3* are not expressed in LCLs and hence allelic effects on their expression could not be assessed nor ruled out. As a first step, promoter methylation patterns were established in DNA samples from three males and three females of the SLSJ asthmatic familial collection. The results of these methylation assays are shown in Table 4 and Supplementary Figure 1S. The *ORMDL3* promoter was unmethylated in all tested samples. The *GSDMB* promoter and the *rs4795397* region were highly methylated with little variation between sexes, whereas the *GSDMA*, *CSF3* and *ZBP2* (Supplementary Figure 1S) promoters showed inter-individual variation and were tested in additional individuals. The *ZBP2* promoter was analyzed in a total of nine females and ten males that were not selected for age, 17q12-q21 genotype or asthmatic phenotype (Fig. 1b, c). Males had significantly lower average methylation levels of the *ZBP2* promoter region compared to females ($p < 0.05$) (Fig. 1c, d). DNA methylation of *GSDMA* and *CSF3* promoters varied among individuals, but did not show statistically significant sex-specific differences when the number of samples was increased (t test statistics, $p = 0.0987$ for *GSDMA* and $p = 0.2821$ for *CSF3*) (Fig. 1d).

To validate the sex-specificity of the *ZBP2* promoter DNA methylation results, we compared average DNA methylation levels throughout the 11 CG sites flanking the transcriptional start site of the *ZBP2* gene using a pyrosequencing DNA methylation assay (Fig. 1b, indicated by a gray bar). *ZBP2* DNA methylation was evaluated in 18 males and 19 females that were younger than 16 years of age (age threshold selected based on Moffatt et al. 2010) and homozygous for the HapA haplotype. Females had higher DNA methylation levels at all 11 CG sites and these differences were statistically significant (Table 5). The average methylation levels across the 11 CGs were 35 % in

girls and 26 % in boys, a difference of 9 %. In utero exposure to tobacco alone or in combination with sex had no effect on methylation in this region (Table 5). Sex-specific differences in methylation levels in the *ZBP2* promoter region may be responsible for the sex-specific genetic association in the SLSJ asthma familial collection.

DNA methylation levels increase with age in males

The 17q12-q21 region was associated with childhood asthma in several independent studies (Halapi et al. 2010; Moffatt et al. 2007, 2010). We tested the possibility that DNA methylation levels of the *ZBP2* promoter may also increase with age and thereby reduce the role of this regulatory DNA element in the development of asthma and hence reduce the significance of the genetic association. Average DNA methylation levels across 11 CG sites in 18 boys younger than 16 (age range 5–16) and 43 adult men (age range 19–80) were established. The average *ZBP2* methylation level in adult men showed slight but statistically significant increase compared to the methylation level in young boys (30 vs 26 %) (t test statistics, $p = 0.0012$).

DNA methylation levels of the *ZBP2* promoter are similar in affected and unaffected males

Since lower methylation in the *ZBP2* promoter region is found in boys compared to girls or adult men and women, one could expect that, in general, affected individuals would have lower methylation in this region compared to unaffected individuals. To test the hypothesis, we compared the average methylation levels of the *ZBP2* promoter region in two age groups of asthmatic and non-asthmatic males that were homozygous for the asthma-associated haplotype HapA. No statistically significant differences in the mean DNA methylation levels between affected and unaffected individuals were found (t test statistics, $p = 0.10$ in the boys group and $p = 0.36$ in the adult men group) (Table 5).

Thus, methylation levels of the *ZBP2* proximal promoter are significantly higher among females of any age and adult males. We therefore propose that DNA methylation attenuates the genetic effect of the 17q12-q21 alleles on asthma development and explains why significant genetic association is found among those groups that have reduced methylation of the *ZBP2* promoter, i.e., males and children.

Discussion

Our genetic analysis detected genetic association between asthma and 17q12-q21 alleles among males independent of

Table 4 Summary of DNA methylation analysis of six regulatory regions in the peripheral blood DNA from members of the Saguenay-Lac-Saint-Jean asthma familial collection

Genomic region	Genome coordinates hg19	Common SNP	Number of individuals tested (females/males)	Average methylation in percents (range)	DNA methylation varies with sex of individuals
ZBPB2 promoter, rs4795397 region	Chr17:38023682-38023857	rs4795397	6 (3/3)	86 (72–97)	No
ZBPB2 promoter	Chr17:38024254-38024701	rs11557466	6 (3/3)	27 (19–40)	Yes
GSDMB promoter, transcript variant 1	Chr17:38073347-38073654	rs111767450	6 (3/3)	96 (91–98)	No
ORMDL3 promoter	Chr17:38083808-38084106	none	6 (3/3)	0 (0–1)	No
GSDMA promoter	Chr17:38119132-38119397	rs3902025	6 (3/3)	63 (50–78)	No
CSF3 exon and intron 1	chr17:38171579-38171964	rs2227322	6 (3/3)	35 (27–43)	No

Table 5 Comparison of average methylation levels of the *ZBPB2* promoter region by sex, asthmatic phenotype and exposure to tobacco in utero

Group	Asthma (both sexes), <i>n</i>	Sex, <i>n</i>	Male/asthma <i>n</i>	Female/asthma <i>n</i>	Smok. IU, <i>n</i>	Male/Smok. IU, <i>n</i>	Female/Smok. IU, <i>n</i>
Subjects <16 years old (<i>n</i> = 37)	23 asthmatics/14 controls	18 males/19 females	10 asthma/8 controls	13 asthma/6 controls	13 exposed/23 non exposed	5 exposed/12 non exposed	6 exposed/11 non exposed
Means (%)	31.1/29.0	25.9/34.5	26.9/24.6	34.4/34.9	30.3/30.1	25.3/26.1	34.5/34.5
SD	5.0/5.8	2.9/3.3	2.8/2.7	3.8/2.2	6.1/5.2	1.9/3.4	4.9/2.7
<i>P</i> value	0.2555	6.6540E–10	0.1004	0.7585	0.9121	0.6495	0.9988
Adult male subjects > 16 years old (<i>n</i> = 43)	N/A	N/A	21 asthma/22 controls	N/A	N/A	N/A	N/A
Means			29.2/30.8				
SD			5.25/3.7				
<i>P</i> value			0.3573				

Smok. IU exposed to tobacco smoke in utero, N/A not applicable, SD standard deviation

The numbers in the cells represent the *p* values from two-tailed *t* test statistics. Due to the number of tests, correction for multiple testing set the significance level at $p = 0.05/3 = 0.0167$ (3 independent phenotypes). Significant *P* values are shown in bold

their age, but not females. Our results contradict the conclusions of Halapi et al. (2010) who did not find an effect of sex on genetic association, but are consistent with the results of Hrdlickova and colleagues (Hrdlickova and Holla 2011) that show statistically significant association in male subjects only.

Variation in DNA methylation profiles has been suspected to contribute to predisposition to asthma (Kabesch et al. 2010) and reported for a number of genes implicated in predisposition to asthma (Breton et al. 2011; Ferreira et al. 2009; Perera et al. 2009; Torrone et al. 2012); associated with specific phenotypes such as wheezing (Baccarelli et al. 2012; Morales et al. 2012) or certain triggers of asthma, including allergy to dust mites (Pascual et al. 2011), smoking (Sood et al. 2012) or exposure to

traffic-related polycyclic aromatic hydrocarbons (Nadeau et al. 2010; Perera et al. 2009). However, sex-specific or age-dependent DNA methylation has not been addressed in these studies. Here, we demonstrate that the DNA methylation profiles of the *ZBPB2* proximal promoter region and exon 1 are both sex-specific and age-dependent. The differences in methylation levels are very modest: 9 % between sexes and 4 % between boys and adult men. The sex bias in DNA methylation that we find is consistent with the results from a recent study where analysis of sex-specific differences in eQTLs in lymphoblastoid cell lines showed different regulation of the *ZBPB2* gene in males and females (Dimas et al. 2012). Small differences in methylation levels in the range of less than 5 % between groups have been reported in other studies (Karmaus et al.

2013; Munthe-Kaas et al. 2012). They may reflect a difference of a larger magnitude in a sub-population of the tested cell sample (peripheral blood cells in this work) or different proportions of functionally important cell populations that are relevant for asthma development (e.g., T-regulatory cells, or Th2 lymphocytes) in the sample that was analyzed.

Earlier we have demonstrated that the *ZPBP2* genic region contains functional regulatory polymorphisms that influence gene expression levels in the 17q12-q21 region (Berlivet et al. 2012; Verlaan et al. 2009a). However, none of the previously reported SNPs with best genome-wide associations ((Ferreira et al. 2010; Moffatt et al. 2010) overlap with the *ZPBP2* gene (Fig. 1). Hence, it was possible that distinct genetic mechanisms were responsible for predisposition to asthma and regulation of gene expression in LCLs. Our methylation results demonstrate the importance of the *ZPBP2* promoter region for susceptibility to asthma and point to DNA methylation as the molecular mechanism that may be responsible for the sex and age effects detected in genetic association studies. Our data

also suggest that the effect of the 17q12-q21 alleles on asthma susceptibility may be attenuated by DNA methylation. This may reduce the significance of the genetic association results as females or adult male carriers of the asthma-predisposing alleles may not be affected due to higher methylation levels of the *ZPBP2* gene. Our previous work suggests that the *ZPBP2* promoter and exon 1 harbor an enhancer element (Berlivet et al. 2012). It is plausible that sex- and age-dependent methylation of an enhancer may affect gene expression in the 17q12-q21 region and modify susceptibility to developing asthma. We therefore speculate that DNA methylation may act as a protective mechanism against development of asthma in a locus-specific fashion. Similar effects of DNA methylation on the expressivity of phenotypes have been documented in mouse models and in human disease (Fu et al. 2012; Morgan et al. 1999; Oates et al. 2006);

To the best of our knowledge, this is the first instance when sex-specific and age-dependent DNA methylation in a GWAS locus is implicated in modulating the disease status for a common genetic disorder. The fact that our

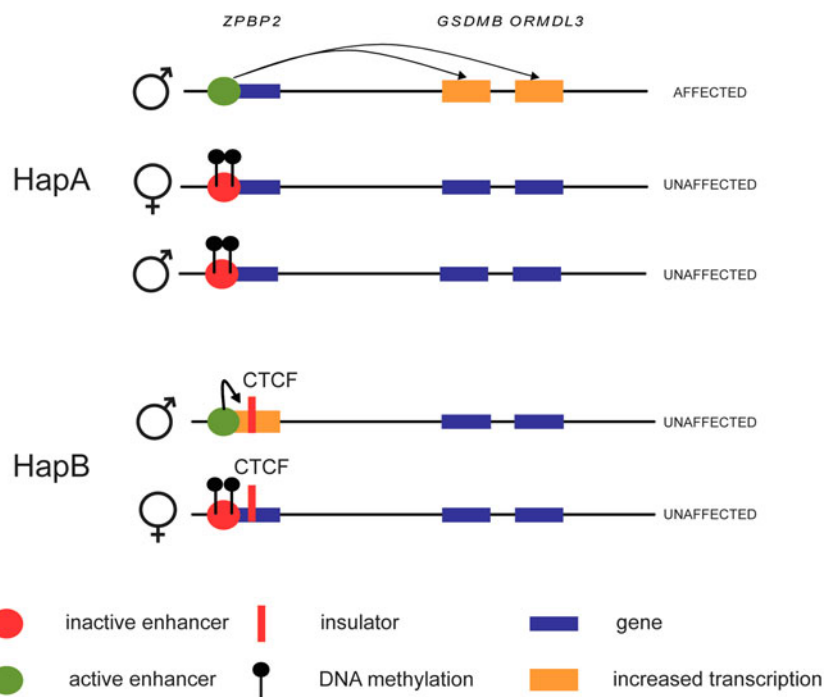


Fig. 2 Model of interaction between genetic polymorphisms and variation in DNA methylation in the 17q12-q21 region. Two common haplotypes termed HapA (harboring alleles associated with childhood asthma) and HapB (harboring alleles that are not associated with asthma) show association with expression levels of *ORMDL3*, *GSDMB* and *ZPBP2*. HapA is associated with increased levels of *ORMDL3* and *GSDMB*, but reduced expression of *ZPBP2*, while HapB is associated with lower expression of *ORMDL3* and *GSDMB* and higher expression of *ZPBP2*. In the HapA haplotype, the SNP rs12936231 removes a CTCF-binding site, which is present in the HapB haplotype. We hypothesized that this polymorphic CTCF-

binding region acts as an insulator preventing interaction between the enhancer located within the 5' region of *ZPBP2* and the promoters of *ORMDL3* and *GSDMB*. Its loss in the HapA allele allows the *ZPBP2* enhancer to stimulate transcription of *ORMDL3* and *GSDMB*. However, methylation of the *ZPBP2* enhancer region in females and adult males attenuates its effect on *ORMDL3* and *GSDMB* expression even in the absence of the CTCF insulator. As increased expression of *ORMDL3* and/or *GSDMB* is suspected to increase the risk of childhood asthma, *ZPBP2* methylation may be reducing the risk

results are consistent with the natural history of asthma with respect to sex and age strengthens the value of the present study.

In our previous work, we hypothesized that the *ZBP2* promoter region harbored one or several enhancers that acted at distance to increase expression of several distantly located genes and that the asthma-associated haplotype HapA carried the more powerful variants of these enhancers (Berlivet et al. 2012). Intron 5 of the *ZBP2* gene contains a polymorphic CTCF-binding site that may potentially act as an insulator preventing the interaction between the *ZBP2* enhancer and the promoters of two distal genes, *GSDMB* and *ORMDL3* (Verlaan et al. 2009a). This polymorphic CTCF site is present in the non-asthma-associated haplotype HapB, but is lost in the asthma-associated haplotype HapA. We hypothesized that the loss of the CTCF site permitted distant enhancer–promoter interactions on the HapA allele. To accommodate the role of DNA methylation of the *ZBP2* promoter/enhancer region in predisposition to asthma, here we further refine our model and propose that the effect of the asthma-associated genetic variant is most pronounced when the enhancer element is hypomethylated, i.e., in boys that carry the HapA haplotype (Fig. 2). Conversely, increased methylation of the enhancer element attenuates its effect on gene expression and thereby reduces the risk of asthma, e.g., in females and adult males including those that carry the asthma-predisposing HapA haplotype. Therefore, due to methylation of the *ZBP2* promoter/enhancer region the genetic association with asthma is significant in children and in males. Our model highlights the modifying potential of inter-individual variation in DNA methylation and may be useful for the understanding of the role of DNA methylation in other loci and in relation to other diseases.

Acknowledgments The authors want to thank the families for their valuable participation. The work was supported by funds from the Canadian Institutes for Health Research (A.N. and C.L.). C.L. holds the Canada Research Chair on Genetic Determinants in Asthma; is the director of the Asthma Inflammation and Remodeling Strategic Group of the Respiratory Health Network of the FRQS and is a member of AllerGen NCE. A.A. is a recipient of the National Guard Health Affairs Saudi Arabia scholarship. The authors are grateful to Jacky Leung for technical assistance and Dr. Luigi Bouchard for the equipment sharing and technical support.

Web Resources

UCSC Genome Bioinformatics/UCSC Genome Browser <http://genome.ucsc.edu/>

National Human Genome Research Institute <http://www.genome.gov>

BiQ Analyzer software (Max-Planck-Institute for Informatics, Saarbrücken, Germany) <http://biq-analyzer.bioinf.mpi-inf.mpg.de>

Quantification tool for methylation analysis (RIKEN Center for Developmental Biology, Kobe, Japan) <http://quma.cdb.riken.jp>

Simple Interactive Statistical Analysis <http://www.quantitativeskills.com/sisa/about/instr.htm>

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