Characterization of the methylation patterns of MS4A2 in atopic cases and controls

**Background:** It is largely unknown whether epigenetic modifications of key genes may contribute to the reported maternal effects in atopy. The aim of this study was to characterize the methylation patterns of the membrane-spanning 4-domains, subfamily A, member 2 gene (MS4A2) (β-chain of the IgE high-affinity receptor), a key gene in the allergic cascade.

**Methods:** Mass spectrometry and bisulphite sequencing were used to measure the methylation of two potential substrates for epigenetic regulation of MS4A2, namely a predicted promoter and a CpG-rich AluSp repeat. Methylation was measured in DNA extracted from peripheral blood lymphocytes of 38 atopic cases and 37 controls. Cases were positive for atopy, asthma, bronchial hyper-responsiveness and had high IgE levels. Both parents of eight atopic cases were also tested.

**Results:** The AluSp element was highly methylated across all individuals (mean 0.92, range 0.87–0.94), a pattern inconsistent with classical imprinting. Variation in methylation at this locus was not associated with age, sex, daily steroid use or atopic status, and there were no differences in methylation between mothers and fathers of atopic cases. Bisulphite sequencing analysis of the promoter region showed that it was also not imprinted, and there was no evidence for allele-specific methylation, but we were unable to test for association with atopy status.

**Conclusions:** Methylation levels at the AluSp repeat analysed in MS4A2 were inconsistent with classical imprinting mechanisms and did not associate with atopy status. The promoter region was less methylated but further analysis of this region in larger cohorts is warranted to investigate its role in allergic disease.

Disruption of normal epigenetic patterns of key regulatory genes has been associated with cancer (1). This association has been documented in the absence of sequence variants in those genes and in some instances there is evidence to suggest that it reflects a causal link between epigenetic disregulation and disease status (2). This observation, together with previous evidence from mice (3) and plants (4), has led some to suggest that the classic paradigm that decomposes the aetiology of most human traits into genetic and environmental risk factors must be revised to include the effects of epigenetic modifications (5).

This novel view has stimulated interest in understanding to what extent epigenetic mechanisms may also be involved in the regulation of other human complex traits or diseases. We were interested in investigating whether epigenetic modifications are associated with atopy risk and, specifically, with the maternal effects reported for allergic disease. Since Bray’s observation that twice as many children with asthma had atopic mothers as had atopic fathers (6), a number of epidemiological studies have suggested that maternal-specific factors contribute to the development of atopy in the child (7). Such factors may include maternal immunological influences but also potential epigenetic alterations at key genes involved in allergic responses.

An important gene in the allergic cascade is the membrane-spanning 4-domains, subfamily A, member 2 gene (MS4A2, previously named β-chain of the high-affinity IgE receptor, FCER1B), which is mainly expressed by mature mast cells in the lung and by basophils. The β-chain of the IgE receptor is thought to amplify the signal generated by the cross-linking between allergen and receptor-bound IgE, which enhances mast cell and basophil activation and the consequent release of histamine and other pro-inflammatory mediators (8).

Given its key role in allergic disease, both linkage and association studies have been conducted to search for asthma or atopy risk variants in MS4A2. Early linkage studies reported stronger effects for maternally derived alleles in atopic families (9), and this suggested that epigenetic modifications at this locus, such as imprinting,
could represent a mechanism for maternal effects in asthma. However, other studies (e.g. 10, 11) provided conflicting evidence for significant maternal effects at this locus. Furthermore, a gene expression study of the mouse orthologue also failed to find any evidence for parental imprinting (12). Therefore, the existing data suggest that either there are no true maternal effects at MS4A2, or that mechanisms more complex than classical imprinting are involved.

To provide further insight into the potential mechanisms that could underlie the maternal effects reported for atopy and MS4A2 in particular, we sought to characterize the DNA methylation patterns of MS4A2 in both atopic cases and controls. Specifically, we addressed the following questions: (i) What is the methylation state of MS4A2 in DNA extracted from peripheral blood leukocytes? (ii) Are there differences in methylation between individuals, and can these be explained by differences in age, sex or use of steroid medication? (iii) Are there significant differences in methylation levels between atopic and nonatopic individuals? And finally, (iv) Do mothers of atopic offspring have lower MS4A2 methylation levels than fathers?

Materials and methods
Subjects and clinical characteristics
Methylation levels were measured in 38 unrelated atopic cases and 37 controls (Table S1) ascertained and clinically tested as described elsewhere (13). Atopy was defined by a positive skin prick test to at least one of 11 allergens tested. Cases also had a doctor diagnosis of asthma, positive bronchial hyper-responsiveness (BHR) test and high total serum IgE levels (>400 IU/ml). Controls were unaffected atopy, asthma, positive bronchial hyper-responsiveness (BHR) test and had total IgE levels <100 IU/ml. Both parents of eight atopic probands were also included for analysis. All subjects and the approval of the appropriate ethics committee. This study was performed with the written informed consent from parents of eight atopic probands were also included for analysis. For atopy, asthma, positive bronchial hyper-responsiveness (BHR) test and had total IgE levels <100 IU/ml. Controls were unaffected atopy, asthma, positive bronchial hyper-responsiveness (BHR) test and had total IgE levels <100 IU/ml. Both parents of eight atopic probands were also included for analysis. Methylation analysis

We measured the methylation state of CpG dinucleotides located on the predicted F2 promoter region of MS4A2 (18) and the nearby AluSp repeat (Fig. 1A). The 773 bp target region on chromosome 11 (59 610 274–59 611 046 bp) contains 22 individual CpG dinucleotides, which were grouped for methylation assessment into 17 CpG units as some were in close proximity. CpG methylation was measured in DNA from white blood cells of 93 individuals extracted according to standard procedures. Methylation analysis was performed using the Sequenom Epityper assay as described previously (14). The target region was polymerase chain reaction (PCR) amplified from bisulphite-treated human genomic DNA using three sets of primers (two for the forward strand and one for the reverse strand), resulting in three partially overlapping fragments (Table S2). Standard quality control (QC) filters were applied to the resulting methylation data, both at the CpG and sample level. Specifically, we excluded data for (i) CpG units with unreliable assays (n = 4); (ii) individuals with >10% missing data (n = 2); and (iii) CpG units with inconsistent results between overlapping fragments (n = 1). After QC, quantitative methylation data were available for 91 individuals and 12 CpG units. Based on six CpG units captured by two overlapping fragments, the mean absolute difference between methylation levels obtained by replicate measurements was ~0.1. Given this high reliability, methylation levels were averaged across the replicates for these six CpG sites. Quantitative methylation data were not available for the F2 promoter after the QC filters were applied. Methylation in this region (CpG units 1–4, Fig. 1A) was assessed by bisulphite sequencing in six atopic cases as described previously (15). Primers used for amplification of the analysed region are given in Table S3.

Statistical analysis
Linear regression was used to test the effects of age, sex and steroid daily medication on methylation levels. Nonparametric methods were used to test the association between methylation and atopy (Wilcoxon’s test) or IgE levels (Spearman’s rho) in the sample of unrelated cases and controls. A Wilcoxon’s test was also used to compare the methylation levels between fathers and mothers of the eight trios.

Results
Quantitative methylation data were obtained for 12 CpG units in atopic cases (n = 38) and controls (n = 37), across a CpG rich AluSp repeat located ~1.9 kb upstream the transcription start site of MS4A2 (Fig. 1B). This element was highly methylated in all individuals (mean methylation 0.92, range 0.87–0.94), a pattern that is inconsistent with parental imprinting. There was little variation in methylation between individuals and this was likely due to measurement error ( assay reliability: ± 0.1). Consistent with this observation, age, sex, use of steroid medication, atopy status and total serum IgE levels were not associated with variation in methylation levels (P > 0.05). There were also no significant differences in methylation between fathers and mothers of atopic children (P = 0.5). These results indicate that, at least in peripheral blood lymphocytes, the AluSp repeat is highly methylated, does not show evidence for imprinting and does not associate with atopy status.

We then investigated whether more subtle epigenetic mechanisms could be present at this locus, such as allele-specific methylation. We focused on the nearby predicted F2 promoter that contains three CpG units and two known sequence variants (Fig. 1A), and used bisulphite sequencing to carefully characterize the methylation patterns in six atopic individuals (Fig. S1). Overall, this region showed lower methylation (mean 0.64, range 0.55–0.83) than the AluSp repeat (Fig. 1B). Of the six individuals analysed, one was heterozygote for rs2583477 and so we were able to compare methylation levels between the two inherited alleles (T and C). The T allele was less methylated than the C allele (0.48 vs 0.73), but these differences were not significant (P = 0.09). Thus, there was no evidence for either parental imprinting or allele-specific methylation at the predicted F2 promoter.
Methylation of MS4A2 in atopic cases and controls

![DIAGRAM]

**Figure 1.** (A) Location and DNA sequence of the 773 bp target region in the 5’ end of MS4A2 selected for quantitative methylation analysis. The target region included a predicted F2 promoter (green) and an AluSp repeat (blue), with a total of 22 individual CpG dinucleotides, which were grouped for methylation assessment into 17 CpG units (boxes) as some were in close proximity. Quantitative methylation data were not available after quality check (QC) for five of these CpG units (unshaded boxes). Two single nucleotide polymorphisms in the predicted F2 promoter are highlighted in blue (rs2847659 and rs2583477). (B) Quantitative methylation results for the 12 CpG units that passed QC. For each CpG unit, a data point corresponds to the methylation level for a given individual, coded in red for atopic cases, green for controls and white for parents of atopic offspring. Horizontal bars for each CpG unit represent the mean methylation for cases (green) or controls (red). For CpG units 1–4, quantitative methylation data were not available after QC. Instead, methylation was measured through bisulphite sequencing in six atopic cases (cf. Fig. S1). The mean methylation for each CpG based on this analysis (i.e. mean proportion of methylated CpGs across six individuals) is shown by a horizontal black line, with the 95% confidence interval around the mean represented by the grey box.

**Discussion**

It has been suggested that epigenetic modifications at key loci such as MS4A2 may provide a molecular mechanism for the reported maternal effects in atopy. Although appealing, this hypothesis has not been directly tested in humans. Yotsumoto et al. (12) measured allele-specific expression of MS4A2 in the mouse and found that both...
maternal and paternal alleles were expressed, indicating that parental imprinting was not present in the mouse. However, as shown for other genes, the possibility remained that imprinting may be present in humans (16) or that more subtle epigenetic regulation mechanisms may exist (17).

Given this general lack of knowledge regarding the epigenetic regulation of MS4A2 expression and the potential maternal effects on atopy, we sought to characterize the CpG methylation pattern of a 5’ region that included two potential substrates for epigenetic effects, a predicted promoter and a CpG rich AluSp repeat. The latter was highlighted by Traherne et al. (18) as a potential locus for epigenetic regulation of MS4A2. We hypothesized that if imprinting was indeed present at this locus, we would observe methylation levels around 50% across the region, reflecting the presence of both 100% methylated and 100% unmethylated alleles for each individual.

Methylation patterns were inconsistent with parental imprinting mechanisms. The AluSp repeat was highly methylated, with little variation between individuals. This pattern of methylation is typically associated with gene suppression and so was unexpected given the documented role of this gene in allergy and the proportion of atopic cases in our sample. One possible explanation for this may be that MS4A2 is likely to be expressed at high levels only in <1% of peripheral blood leukocytes (in basophils). Another possibility, however, is that only key CpG sites will undergo demethylation prior to gene transcription as documented for IL4 (19), another key regulatory gene in allergic disease.

Candidate CpG sites in MS4A2 for more restricted but potentially functional relevant methylation changes are located in the predicted F2 promoter, which in the bisulphite sequencing analysis showed lower levels of methylation when compared to the AluSp repeat. As we were unable to obtain quantitative methylation data for this region in the larger group of cases and controls, we could not test whether variation in methylation correlated with atopy status and total IgE levels. Further analysis of this region in larger cohorts is warranted.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1. Results for the bisulphite sequencing analysis of the first four CpG units of the target region, including the three CpG sites of the predicted F2 promoter (CpG units 1–3).

Table S1. Characteristics of the study participants.

Table S2. Location, primers and DNA sequence of the three fragments used for the Sequenom Mass-Spectrometry methylation analysis.

Table S3. Primer sequences used for bisulphite sequencing analysis of the predicted F2 promoter.

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