

Relevance and implication of genetic determinants to asthma pathophysiology

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Purpose of review

The number of single nucleotide polymorphisms (SNPs) found to be associated with asthma and related phenotypes outnumbers those with functional impacts. In this review we briefly described some of the approaches used to investigate functionality of SNPs, and summarized recent findings related to the characterization of functional SNPs in asthma.

Recent findings

For disease-associated SNPs residing in the promoter or 3' untranslated regions, differential protein binding affinity between the major and minor alleles is often the first logical area of investigation. In this review, we described SNPs associated with asthma or related phenotypes in five genes which in the past 12 months have new data implicating potential mechanisms in asthma development.

Summary

Variability in treatment responses poses a great challenge in asthma management. It is established that the genetic makeup of individuals plays a role in asthma development, yet the mechanisms remain unclear. Investigations on the functional impacts of disease-associated SNPs will help us gain insights into potential disease mechanisms, and ultimately lead to effective therapies for those who suffer from asthma.

Keywords

alleles, asthma, polymorphisms, promoter, single nucleotide polymorphisms (SNPs)

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Introduction

Tremendous efforts have been invested in identifying genetic factors that predispose individuals to develop asthma or related traits using linkage analyses and association studies at regional and genome-wide levels. The Human Genetic Epidemiology Navigator database (HuGE Navigator) lists a total of 715 genes, which have been reported to be associated with asthma [1]; however, only a small proportion of these genes have been replicated in at least one other study [2–4]. Inconsistent findings across populations for the vast majority of these reported variants clearly indicate that these variants are likely to be noncausative. This notion is further supported by the recent findings from whole genome-sequencing data that there is a negative relationship between minor allele frequency and the probability that it is functional based on evolutionary conservation and/or residence in functional regions [5]. The rarer the minor allele of a single nucleotide polymorphism (SNP), the more likely it is evolutionarily conserved and/or locates in functional regions (e.g. coding or regions with regulatory potential). Given the minor allele frequencies of the associated SNPs are usually greater than 5%, one might question, 'Do these variants actually increase the risk of

developing asthma?'. Though novel strategies to search for the true genetic risk factors continue to be developed, efforts to evaluate the functional impact of these genetic variants are underway.

Depending on the location of the variation in the gene, the transcribed mRNA and/or coding protein may be altered at the levels of transcription, translation, stability, and structure. An evaluation of published disease or trait associated SNPs identified in genome wide association studies (GWAS) found that these published associated SNPs were found more often in nonsynonymous sites and in 5 kilobase pair (kb)-promoter regions compared with SNPs randomly selected from genotyping arrays implies that the modes of action of the majority of disease associated variants are likely by alterations in structure or mRNA expression [6]. One mechanism is by allelic imbalance, a phenomenon whereby the two alleles of a gene are expressed at different levels. A genome-wide assessment of differential mRNA expression of alleles of a SNP showed that more than 25% of transcripts, coding or noncoding, exhibit allelic imbalance [7]. Furthermore, a genome-wide mRNA expression study [8] using primary peripheral blood CD4+ lymphocytes, showed that approximately 2.6% of the SNPs located within 50 kb

of a transcript with acceptable expression data demonstrated evidence for *cis*-acting regulation. Hence, mRNA expression analyses are essential for evaluating the functions of noncoding SNPs in their role in disease/trait development.

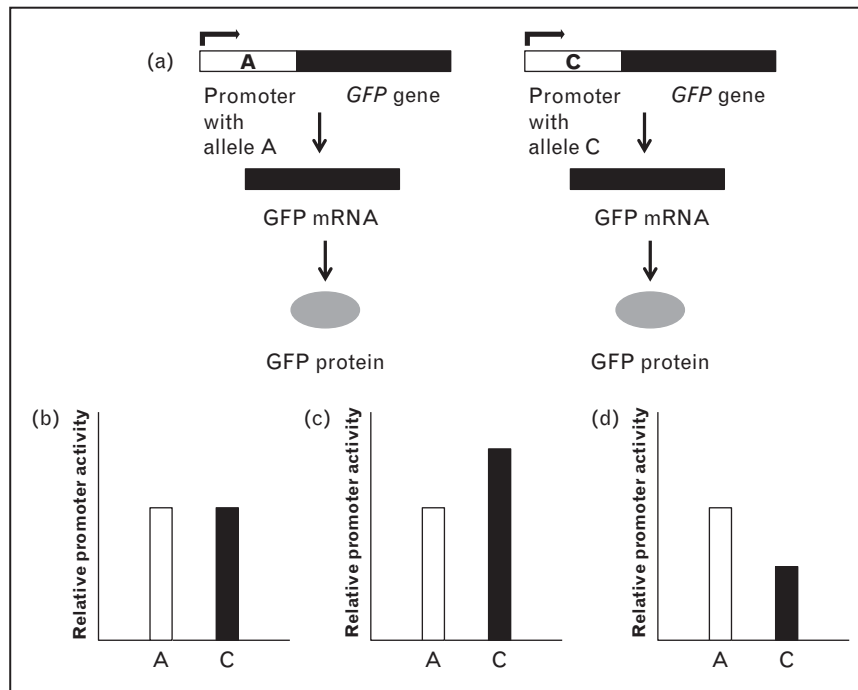
Various approaches have been developed to investigate functional impact of genetic variants. For example, for SNPs residing at promoter regions, promoter assays can be used to evaluate if one allele has a greater ability to transcribe the gene when compared with that of another allele (see Fig. 1). Promoter assays involve introducing cells with short pieces of DNA containing the promoter region of a gene with the major (or minor) allele in question and a reporter gene. This DNA assembly is known as a construct. The reporter gene gives off a visual signal, usually by fluorescence, when translated into a protein. The genes encode the enzyme luciferase and the green fluorescent protein (GFP) are commonly used as reporter genes. By comparing the visual signal given off by the cells with the constructs containing the major allele with those with the minor allele, promoter activity between the two alleles can be evaluated. In addition to promoter assays, potential protein binding sites can be assessed. To investigate the presence of a protein (e.g. transcription factor) binding site at a variant, the upstream and downstream sequence of a SNP can be matched against known protein binding sequences recorded in existing databases (e.g. TFSEARCH [9]). To confirm that the protein binding sites predicted *in silico* are real, *in-vitro* confirmation by electrophoretic mobility shift assay (EMSA) can be used. In EMSA, nucleotide sequence (probe) containing the allele of interest is first mixed with nuclear extracts isolated from cells. Then complexes formed between proteins and probes are detected by gel electrophoresis, which involves separating particles in a solution by using a gel as a sieving medium and electric current as the power. Particles of larger sizes move more slowly down the gel compared with those of smaller sizes. If in the mixture of probes and nuclear extracts there are proteins interacting with the probes, the complexes formed are larger and move relatively slower on a gel than the probes and noninteracting proteins alone. On the gel, a new slower moving band can be seen and this is called a band shift. To identify the binding proteins, antibodies of the suspected proteins can be used to validate the prediction. Proteins bound to antibodies will not bind to the probes, thus no complexes will be formed, and no band shift will be observed on a gel. For example, if protein A is suspected to bind to the probe, then antibodies of protein A can be added to the mixture, if the band shift that was originally observed (in the absence of antibodies) is missing or less intense, then one can conclude that protein A does indeed bind to the probe. Hence, allele specific protein binding affinity can be evaluated by

Key points

- The genetic association observed between variants of ORM1-like 3 (ORMDL3) and asthma may likely be the result of differential interaction between nucleosomes and active chromatin, mediated by allelic specific binding affinity of variant rs12936231 to CCCTC-binding factor (CTCF).
- Two variants (rs2038366 and rs3753194) at the promoter region of Sphingosine-1-phosphate receptor 1 (S1PR1) exhibited differential promoter activities between the common and minor alleles; hence, S1PR1 may contribute to asthma phenotype via transcriptional regulation of the gene.
- A variant (rs1046295) at the 3' end of PHD finger protein 11 (PHF11) may influence asthma outcome via transcriptional regulation and alternative splicing of the gene.
- Allelic imbalance in transcription dictated by the promoter variant rs1361600 of factor III (F3) may explain the elevated F3 protein level observed in the sputum of asthma subjects compared with healthy control subjects.
- Allelic and ethnic specific methylation at variant rs731236 of vitamin D receptor (VDR) have been suggested to explain the inconsistent genetic association observed between VDR and tuberculosis susceptibility in different populations; a similar mode of action may underlie the contribution of VDR to asthma risk.

comparing band shifts and intensities between the probes containing the major and minor alleles.

In addition to evaluation of allele specific promoter activity and protein binding site, DNA methylation variability between the major and minor alleles can also be evaluated. DNA methylation refers to the addition of a methyl group ($-CH_3$) to the 5-carbon of a cytosine, thus converting the cytosine to 5-methylcytosine. DNA methylation alters transcription factor binding to the DNA sequence by physically blocking the DNA from binding to transcription factor, and subsequently affecting expression of the gene under regulation. Regions of the gene rich in cytosine-phosphate-guanine dinucleotides (CpGs) called CpG islands are hotspot for DNA methylation. CpG islands of the genomic region can be mapped *in silico*, and variants which reside on a CpG island can be tested for allelic specific methylation activity using polymerase chain reaction (PCR) based methods such as bisulfite sequencing [8*,9,10]. Bisulfite sequencing involves treating DNA with bisulfite. Bisulfite converts cytosine to uracil and leaves 5-methylcytosine unconverted, and subsequent sequencing of the target regions can distinguish cytosine from methylcytosine via uracil conversion [10] (see Fig. 2).

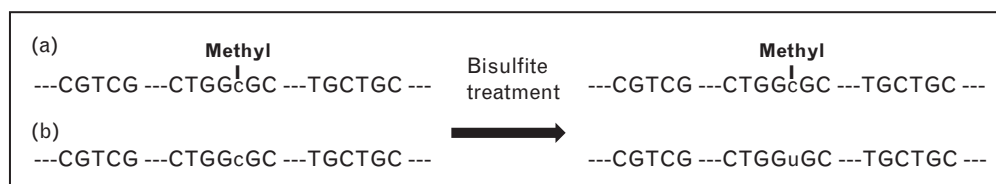
Figure 1 Promoter assay using green fluorescent protein

Part (a) illustrates the constructs used to evaluate allele specific promoter activity. The left construct consists of a promoter region with the sequence surrounding the SNP of interest, and allele A of an A>C SNP is engineered into this construct. The reporter gene assembled into this construct is the gene encodes GFP. During transcription, the transcription machinery uses the promoter of the construct to transcribe GFP, translation of GFP follows and green fluorescent protein is synthesized. The right construct is identical to that of the left, except at the promoter region the allele C is engineered. Green fluorescent protein is also synthesized by the right construct. The fluorescence signal given by GFP synthesized using the two constructs can be compared as a measure of promoter activity. Relative promoter activity can be plotted for the two constructs and differences in promoter activity can be evaluated. If allele A has the same promoter activity as allele C, the relative promoter activity will be the same as represented by Part (b). If allele C has a greater promoter activity than allele A, the graph will be similar to the one shown in Part (c). If allele C has a lesser promoter activity than allele A, the graph will be similar to Part (d).

In addition to transcriptional regulation, posttranscriptional regulation is also likely to contribute to asthma risk and will require different approaches (e.g. proteomic studies) for evaluation, which will not be discussed here. In this review, we will summarize the recent discovery of the asthma associated SNPs whose functional impact has been characterized.

ORM1-like 3

The chromosomal region 17q21.1, where the genes ORM1-like 3 (ORMDL3) and gasdermin B (GSDMB) are located, has initially been shown to be associated with childhood asthma in a GWAS of European children from the United Kingdom and Germany [11]. The gene ORMDL3 was identified in 2002 and it encodes the

Figure 2 Bisulfite conversion of cytosine to uracil

In the presence of a C>A SNP, methylation may occur at allele C or may not occur. To assess if methylation is present at allele C, bisulfite can be used to convert nonmethylated allele C, as in sequence (b), to uracil. Bisulfite cannot convert methylated allele C, as in sequence (a), to uracil. Methylated allele C remains as methylated allele C. Amplification of the target sequence and subsequent sequencing can determine if methylation occurs at allele C.

ORM1-like protein 3 [12]. ORMDL3 is a transmembrane protein localized to the endoplasmic reticulum, and recently it has been shown that overexpression of ORMDL3 in HEK293 and Jurkat T-cell lines reduced Ca^{2+} level in the endoplasmic reticulum, enhanced phosphorylation of eukaryotic initiation factor 2 α (eIF2 α), and elevated gene expressions of immunoglobulin heavy chain-binding protein (BIP) and early growth response factor 1 (EGR1); all are key steps of the unfolded-protein response (UPR) pathway [13,14,15]. Using B-cell-derived Epstein-Bar virus (EBV)-transformed lymphoblastoid cell lines (LCLs) obtained from the probands and siblings of the initial GWAS families, SNP rs7216389 was found to be associated with childhood asthma and transcript abundance of ORMDL3 [11]. Recently, other SNPs in the area of SNP rs7216389 have been shown to be associated with mRNA expression of ORMDL3 and its neighboring GSDMB genes in white blood cell [16]. In the past 3 years, association between SNPs within the ORMDL3 gene and its neighboring genes of the same linkage disequilibrium block and asthma have been reported across different racial and ethnic groups [16–27]. These associations were primary with childhood onset asthma [28]. Intensive investigation of the functional impacts of variants in the region demonstrated that alleles of SNP rs12936231 (C>G) have different association with nucleosomes and its DNA region is associated with active chromatin [29]. The G allele binds significantly to more CCCTC-binding factor (CTCF) proteins than the C allele; given that CTCF is involved in chromatin looping and nucleosome repositioning [30,31], the associated gene expression of ORMDL3 and its neighboring genes could likely be the result of interactions initiated by the binding of CTCF to the G allele. At present, it remains unknown how ORMDL3 plays a role in asthma pathogenesis. It is widely speculated that ORMDL3 may play a role in asthma pathogenesis via its participation in UPR, a process that can trigger Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and c-Jun N-terminal kinase and initiate inflammation [32–34].

Sphingosine-1-phosphate receptor 1

The sphingosine-1-phosphate receptor 1 (S1PR1) gene was cloned and sequenced in 1990 from human umbilical endothelial cells [35]. It is located on chromosome 1p21 and encodes the protein sphingosine-1-phosphate receptor 1 [35]. The S1PR1 protein was recently discovered, by immunocytochemistry, to be expressed differently in the lungs of asthmatic and nonasthmatic subjects [36[•]]. The protein was expressed roughly six-fold less in the pulmonary vessels of asthmatic than nonasthmatic subjects. In the same study, the S1PR1 gene was resequenced and SNPs were genotyped in one European American and two African American populations. Gene level replication was observed in all three populations but the associated

SNPs were different; two SNPs reside in the promoter region and two in introns. Follow-up transcription factor motif analysis suggested putative transcription factor binding sites for SNPs rs2038366 (G>T) and rs3753194 (T>C) in the promoter region and functional assays confirmed that nucleotide changes at these two sites resulted in biological changes. SNP rs3038366 resides at a putative epidermal growth factor receptor (EGFR) binding site and SNP rs59317557 (C>G) resides at that of early growth response 2 (Egr2) site. Under basal condition, the minor allele of SNP rs2038366 (allele T) confers a weaker promoter activity compared with the common allele (allele G). When activated with epidermal growth factor (EGF), the promoter activity of allele T was weaker than that of allele G. On the contrary, the rare G allele of SNP rs59317557 confers a stronger activity with or without stimulation of Egr2 binding growth factor when compared with the common allele C.

At present it remains unclear how these two SNPs influence asthma pathogenesis *in vivo*. However, proteins which interact with S1PR1 have all been shown to be important factors in asthma. Its ligand, sphingosine-1-phosphate (S1P), has been found to regulate airway hyperresponsiveness, airway remodelling, and vascular permeability [37–40]. EGFR and its ligands have been shown to be involved in airway hyperresponsiveness, neutrophilic inflammation and airway remodelling [41–44]. Vascular endothelial growth factor has been shown to be elevated in sputum of asthmatic subjects and can increase expression of S1PR1 in endothelial cells [45,46]. Subsequently S1PR1 can activate nitric oxide synthase 3 (NOS3) and lead to vasodilation and angiogenesis [47]. Therefore, one can speculate that genetic variants of S1PR1 mediate various asthma related outcomes via alterations in angiogenesis and vascular permeability in the airways.

PHD finger protein 11

The PHD finger protein 11 (PHF11) gene was cloned in 1999 [48] and it encodes the protein plant homeo domain (PHD) finger protein 11 [48]. The gene was mapped to chromosome 13q14.1 and it has been identified as an asthma gene by positional cloning in 2003 [49]. Gene level associations between SNPs toward the 3' end of PHF11 and asthma and related traits have been observed in several adult and children populations [49–52]. Recently, SNP rs1046295 (G>A) has been shown to have a functional effect [53^{••}]. In-vitro assays in human B-cell line detected a strong binding affinity for octamer-binding transcription factor 1 (Oct-1) with the minor allele A than allele G. Subtle yet statistically significant over-expression of allele A was also observed in EBV immortalized lymphoblastoid cell isolated from blood. In an earlier study [54] similar over-expression of allele A was also observed in T-Helper type 1 (T_H1) but not T_H2 cells

isolated and activated from healthy adult subjects. Furthermore, SNP rs1046295 is correlated with the PHF11 isoform that lacks exon II, resulting in a non-functional transcript in T_H1 cells. However, given its location at the 3' UTR, it is unlikely to be affecting the splicing event directly. The genetic and functional data suggest that SNP rs1046295 mediates asthma and related traits by influencing transcription and, via linkage disequilibrium, splicing of PHF11. PHF11 is a transcription factor that has been shown recently that its over-expression can increase the binding of NF- κ B to the interferon γ (IFNG) promoter and increased the subsequent transcription of IFNG [54]. In the same study the knock-down of PHF11 mRNA brought out three effects: decreased transcription of T_H1 cytokines interferon- γ , IL-2 and CD28 co-receptor in T cells, reduced NF- κ B import to the nucleus in activated T cells, and reduced T-cell viability [54,55^{*}]. The low serum IgE, asthma, and atopy associated G allele may increase the risk through PHF11 expression reduction, subsequently suppressing NF- κ B mediated T_H1 response and reducing T-cell viability [54,55^{*}].

Coagulation factor III

The gene factor III (F3) was cloned in 1987 and it encodes the coagulation factor III, also known as tissue factor [56]. F3 is mapped on chromosome 1p22-p21 [57,58]. F3 is involved in the initiation of the blood coagulation pathway by forming a complex with coagulation factor VII (F7), and subsequently leading to the generation and activation of coagulation Xa (FXa) and thrombin (FIIa), as reviewed by [59]. Although the promoter polymorphism rs1361600 (A>G) of the F3 gene has been investigated and found to be associated with adult-onset asthma in Japanese population only, its known biological effect in F3 transcription and the role of tissue factor in the coagulation system deserves a discussion. Recently, the homozygous rare (GG) genotype of SNP rs1361600 has been found to increase the risk of developing adult-onset asthma by three-fold in a primary population and two-fold in a replication population, with the replication population at borderline significance [60^{**}]. Follow-up *in vitro* functional assay detected 30% stronger promoter activity in the presence of the allele G compared with allele A in human lung epithelial and umbilical vein endothelial cells. In lung epithelial cells, allele G binds to a nuclear protein with greater affinity, although the identity of the nuclear protein is unknown. These *in-vitro* data suggest that the risk allele is associated with higher expression of F3, which is consistent with earlier findings that tissue factor protein level is roughly four times higher in the sputum of stable asthma subjects compared with healthy subjects [61].

Tissue factor, a membrane-bound glycoprotein, when activated, upregulates thrombin production, which is found to be elevated in sputum of asthmatic subjects

[61]. Thrombin acts as a serine protease and has been previously shown to promote proliferation of cultured airway smooth muscle cells via matrix metalloproteinase (MMP)14 and MMP2 dependent pathway, and to promote procollagen production and tissue fibrosis, suggesting that tissue factor, via thrombin, plays a role in airway remodeling, a clinical feature of asthma [61–64].

Vitamin D receptor

The vitamin D receptor (VDR) gene was cloned in 1988 and it encodes the vitamin D receptor protein [65]. It is located on chromosome 12q12-q14 [66,67]. VDR exerts genomic and nongenomic effects by binding to 1,25(OH)₂D₃ (vitamin D₃). The interaction of vitamin D₃ with VDR modulates many biological activities of the neural, immune, and endocrine systems, including calcium and phosphorous homeostasis, apoptosis and cell differentiation (reviewed in [68–70]). Polymorphisms of the VDR gene have been found to be associated with asthma and related traits in many genetic association studies [71–75], and lack of associations has also been reported [76,77]. Until recently, the only SNP in VDR with a well-characterized functional effect is the translation start polymorphism (rs10735810 A>G, also known as *FokI*). The minor C allele encodes a protein 3 amino acid longer compared with the common G allele, and the alleles bring out a different pattern of response to vitamin D [78,79]. However, the SNPs which have been implicated in asthma and related traits tend to reside at the 3' end of VDR. Of these SNPs, rs731236 (T>C) (also known as *TaqI*) has been found to be associated with asthma and related traits in several populations [71–73,80]. Recently, Andraos *et al.* [81^{**}] discovered that, in respect to tuberculosis susceptibility, SNP rs731236 resides on a CG rich island. In lymphoblastoid cell lines, the C allele is always methylated. In addition to allele-specific methylation level, ethnicity-specific methylation level was also observed. Although the functional characterization of SNP rs731236 was performed in the context of tuberculosis susceptibility, epigenetic effect and methylation variability may exist in asthma susceptibility.

Conclusion

Since the publication of the first draft of the human genome in 2000, the number of SNPs implicated in asthma and related traits have soared tremendously. However, discoveries of the functional impact of these SNPs lag behind in speed and number. Hurdles in characterizing the function of disease-associated SNPs include validity of causality, tissue/organ specific expression of proteins, epistasis, and epigenetic. In this review, we chose to discuss SNPs which have been shown to behave differently *in vitro* in an allelic specific manner and whose encoding proteins have functional data to support their role in asthma pathogenesis. Although we

discussed these SNPs and their in-vitro behaviors in isolation, we must keep in mind that these SNPs do not exert their effects alone, but in frameworks with other variants, genes, and environment. At present, characterization of functional variants are done for SNPs, other types of variations such as copy number variants, inversion, and methylation variable positions are gaining attentions as probable variants contributing to disease pathogenesis [82–84].

Acknowledgements

Conflicts of interest

None declared.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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