17q21 locus and ORMDL3: an increased risk for childhood asthma

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Genetic variations in the 17q21 locus are strongly associated with childhood nonallergic asthma. Expression of the 17q21 genes, orosomucoid like 3 (ORMDL3) and gasdermin B (GSMDB), is affected by these disease-associated variants. However, until recently, no functional connection of the protein products coded by these genes with asthma was known. Lately, it has been identified that ORMDL3 function has been related to various cellular processes that could be relevant for the pathogenesis of asthma. This includes dysregulation of the unfolded protein response (UPR) associated with airway remodeling and also an effect of ORMDL3-dysregulated sphingolipid synthesis on bronchial hyperreactivity. These findings are crucial for a better understanding of the mechanism of childhood asthma and may lead to asthma therapeutics that target pathways previously not thought to be related to this common pediatric respiratory disease. Furthermore, this may validate the unbiased genome-wide association study (GWAS) approach for complex diseases such as asthma, to better define pathomechanisms and drug targets.

Childhood asthma is a complex genetic disorder (1) that is clinically heterogeneous (2) and poses huge costs to society (3). About half of patients with mild-to-moderate asthma have nonallergic disease (4) and responds poorly to currently available anti-inflammatory therapies (5). Genome-wide association studies (GWAS) have repeatedly and convincingly linked the asthma susceptibility locus 17q21 to nonallergic childhood-onset asthma. The pioneering study by Moffatt *et al.* (6) characterized more than 317,000 single-nucleotide polymorphisms (SNPs) from 994 patients with a history of childhood-onset asthma and 1,243 nonasthmatics using European family and case-referent panels. This study showed multiple markers at the 17q21 locus to have a strong association with childhood-onset asthma (combined P value $< 10^{-12}$), which was then replicated in German and British cohorts. The strongest association was mapped to a 206 kb interval on chromosome 17q21; the SNP with the strongest association within this interval was rs72163891 and has been widely reproducible. The association of SNPs in this region with asthma was stronger for early-onset asthma as shown in all but one study (7).

GENE EXPRESSION

Because polymorphisms in regulating elements could alter gene transcription of factors related to the susceptibility for asthma, the expression of genes coded for in the asthma-associated region on 17q21 was evaluated. Moffatt *et al.* (6) showed that in Epstein-Barr virus–transformed lymphoblastoid cell lines, transcript levels of the orosomucoid like 3 gene (ORMDL3) were strongly and positively associated to rs7216389, the SNP with the strongest association with childhood asthma. This suggested that variants at this asthma susceptibility locus may regulate ORMDL3 expression, which has also been confirmed in rhinovirus-infected blood cells (8). The 17q21 asthma susceptibility locus is located between 35.0 and 35.5 Mb on chromosome 17 and contains at least 15 genes. To date, however, asthma-associated SNPs have been associated with the expression of only four of these genes (9): (i) Ikaros zinc finger protein 3 (IKZF3), involved with the regulation of lymphocyte development; (ii) Gasdermin B (GSDMB), implicated in epithelial cell barrier function; (iii) Mediator of RNA polymerase II transcription subunit 24 (MED24), a component of a transcriptional coactivator complex thought to be required for expression of most genes; and (4) ORMDL3, an endoplasmic reticulum (ER) transmembrane protein involved in regulation of sphingolipid metabolism. GSDMB and ORMDL3 have received the most attention and their genotype-mediated expression is also affected by rhinovirus infection, one of the most common and powerful triggers for asthma exacerbations (8). It has further been suggested that these two genes might be coregulated, as their transcript levels seem connected (10).

ASSOCIATION OF THE 17q21 LOCUS WITH ASTHMA TYPES

The GABRIEL consortium, a multidisciplinary study to identify genetic and environmental causes of asthma in the European Community, followed up the initial identification of the 17q21 locus with a meta-analysis of GWAS from 10,365 subjects with asthma and 16,110 unaffected subjects, all of European descent. SNPs within the ORMDL3/GSDMB asthma-susceptibility locus achieved genome-wide level significance specifically with childhood-onset asthma but not adult-onset asthma (11). Since then, this region has been consistently replicated

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Received 15 April 2013; accepted 9 May 2013; advance online publication 8 January 2014. doi:10.1038/pr.2013.186

as an asthma susceptibility locus in ethnically distinct populations, including Mexicans, Puerto Ricans and African-Americans (12), Japanese (13), Chinese (14,15), Scottish (16), European (16–18), and French Canadians (19). In the United States, the EVE consortium confirmed the 17q21 locus in nine GWAS datasets, representing three major ethnic groups (European American, African American or African Carribean, and Latino ancestry), although no single SNP was associated with asthma in all three ethnic groups (20).

The phenotypic relationship of asthma with atopy and allergic sensitization is well established, but the exact nature of this relationship still remains unclear. The overlap between allergy, atopy, and asthma is highest in childhood asthma, with estimates for up to 80% of asthmatic children being allergic, but seems lower in adults (60%) (21). It remains unknown, however, if asthma and atopy are a result of the same disease process, or if they represent distinct disease entities occurring concomitantly with different causes (1). Analyses by the GABRIEL consortium revealed no association between the ORMDL3/GSDMB locus and IgE levels, a commonly used indicator for atopy (11). This has been replicated in numerous studies (8,9,13,18,22,23). The absence of any overlap between the most highly associated asthma-susceptible SNPs and serum IgE at any locus tested suggests separate, genetically distinct pathways. Instead, 17q21 SNPs have been correlated with bronchial hyperreactivity (9), a cardinal feature of asthma. This suggests that the disease susceptibility originating from the 17q21 locus affects airway reactivity independent of allergic sensitization.

LINKING GENE(S) TO FUNCTION(S)

None of the genes in the asthma-associated 17q21 region would have been associated to asthma without the unbiased GWAS approach. This approach is an opportunity to discover novel targets and pathomechanisms but has often been both a challenge and a curse for complex diseases. This is particularly true for ORMDL3. Until recently, little was known about its overall cellular function in mammalian cells, as most of its function had been studied in yeast. There, ORMDL functions as regulator of sphingolipid synthesis (24). Since the asthma-associated SNPs lead to increased cellular ORMDL3 protein expression, it would suggest that an asthma phenotype related to ORMDL3 should be associated with a gain-of-protein function. To date, the following mechanisms of how increased ORMDL3 could be related to asthma have been proposed (**Figure 1**): (i) ORMDL3 is involved in ER-mediated Ca^{2+} signaling and activation of the unfolded protein response (UPR), leading to epithelial cell remodeling through its effect on the sarco/endoplasmic reticulum CaATPase (SERCA) (25,26) and (ii) ORDML3 influences sphingolipid metabolism to directly affect bronchial reactivity (27).

ORMDL3 AND INTRACELLULAR CALCIUM HOMEOSTASIS

ORMDL3 is widely expressed in both fetal and adult mammalian tissues including lung epithelial cells (26,28). In mouse lungs, expression can be increased by a variety of stimuli, such as allergens, tobacco smoke, and lipopolysaccharides (26). In lung

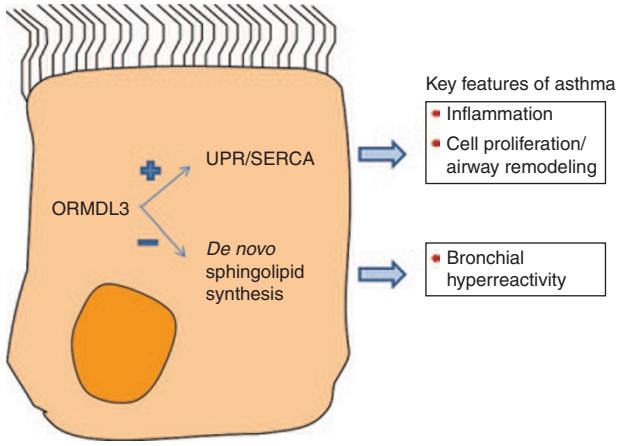

Figure 1. Proposed mechanisms for the role of ORMDL3 in asthma pathogenesis in an airway epithelial cell. ORMDL3, orosomucoid like 3; SERCA, sarco/endoplasmic reticulum Ca ATPase; UPR, unfolded protein response.

epithelial cells, *in vitro* expression can be increased by interleukin-4 (IL-4) and interleukin-13 (IL-13) but not tumor necrosis factor-α (26). Interestingly, overexpression of ORMDL3 in airway epithelial cells activates activating transcription factor 6 (ATF6), one of the three signaling branches of the UPR in response to ER stress. This appears to be accompanied by increases in metalloproteases (MMP-9, ADAM-8), CC chemokines (CCL-20), CXC chemokines (IL-8, CXCL-10, CXCL-11), and oligoadenylate synthetase (OAS). These findings suggest that ORMDL3 is an allergen- and cytokine-inducible gene that may regulate the expression of chemokines, metalloproteinases, and OAS through activation of the UPR and may thus be linked to inflammatory and remodeling responses in asthma (26). Activation of the UPR has been implicated in other inflammatory and immune-related diseases other than asthma, such as inflammatory bowel disease, chronic obstructive pulmonary disease, and diabetes, as well as environmental stressors such as tobacco smoke, an important trigger for asthma exacerbations. It is unclear, if activation of the UPR is a primary underlying mechanism of asthma or a reaction to chronic inflammation or environmental insults (29).

Proper assembly and folding of ER proteins is dependent on appropriate ER Ca^{2+} levels, with alterations of these resulting in activation of the UPR. The UPR is a system of highly conserved signaling pathways, which sense the needs and capacity of the ER to maintain protein quality control and homeostasis. The ER responds to unfolded proteins in its lumen by activating intracellular signal transduction pathways which allow for expansion of the ER protein folding machinery. If these needs are not met, apoptosis is induced (29). The ER stress response is also involved in inflammation through the activation of transcriptional regulators of genes involved in the inflammatory response (30). Of the three primary UPR transcription pathways such as, ATF6, double-stranded RNA-activated protein kinase (PKR)–like endoplasmic reticulum kinase (PERK), and inositol requiring kinase 1 (IRE1), ATF6 has been shown to regulate SERCA (26) and interleukin-6 (IL-6) (31), both of which have been implicated in the pathogenesis of asthma.

SERCA belong to the P-type ATPase family to actively transport cations across membranes and play a key role in Ca^{2+} signaling by restoring free Ca^{2+} to baseline levels after cell activation and by replenishing sarcoplasmic reticulum stores (32). The SERCA2b isoform is mainly expressed in smooth muscle cells and has been shown to be the predominate isoform in airway smooth muscle cells (25). Smooth muscle responses to a variety of stimuli are controlled by changes in the concentration of free cytosolic Ca^{2+}. Alterations in the control of smooth muscle cell Ca^{2+} concentration might be an important contributor to airway hyperresponsiveness in asthma (33). Decreased SERCA2 expression as well as a diminished ability of SERCA2 to replete sarcoplasmic reticulum (SR) Ca^{2+} stores has been seen in asthmatic airway smooth muscle, suggesting that Ca^{2+} handling in airway smooth muscle may be abnormal in asthma and that disruption of SERCA2 contributes to the increased proliferation and enhanced cytokine expression in airway smooth muscle (25). However, some of the data relating ORMDL3 to this has been conflicting: Miller *et al.* (26) showed that overexpression of ORMDL3 selectively activates the ATF6 and that knockdown of ATF6 decreases expression of SERCA. By contrast, Cantero-Recasens *et al.* (34) showed that overexpression of ORMDL3 impaired cytosolic Ca^{2+} clearance, resulting in an inhibitory effect on SERCA activity. The group also showed coimmunoprecipitation of ORMDL3 and SERCA and that ORMDL3 activates the UPR through the PERK pathway with no effect on the IRE1 pathway (34). In contrast, Hsu *et al.* (35) found no association of ORMDL3 expression with UPR activation or with changes in IL-6 and IL-8. In addition, a study in a T-cell line demonstrated that ORMDL3 decreased cell activation by decreasing store-operated Ca^{2+} entry, an important mechanism of Ca^{2+} homeostasis and activation in immune cells (36). This would suggest that higher ORMLD3 expression is associated with decreased T-cell activation. Taken together, these studies strongly suggest a relationship between ORMDL3, SERCA, regulation of intracellular Ca^{2+}, and the UPR, which is still unfolding. Of note is that none of these studies address why ORMDL3 polymorphisms would be linked more to nonallergic asthma, nor do they study a potential effect on sphingolipids, the class of cellular lipids that is regulated by ORMDL3.

ORMDL3 AND SPHINGOLIPIDS

A role of orm proteins in the regulation of sphingolipid synthesis has been well established in yeast for many years (24,28,37–39) and has recently been demonstrated in human cells (40,41). ORMDL proteins act as negative regulators of sphingolipid synthesis by interacting with serine palmitoyl-coenzyme A transferase (SPT) (38). SPT catalyzes the condensation of serine and palmitoyl-coenzyme A, the rate-limiting step of the *de novo* sphingolipid synthesis (**Figure 2**). The reaction product, 3-ketosphinganine is instable. It is converted to sphinganine that is metabolized by distinct ceramide synthases to dihydroceramides. A dihydroceramide desaturase generates ceramides that also originate from catabolism of complex sphingolipids (i.e. sphingomyelin, gangliosides) (42,43). Sphingolipids are a diverse and complex category of lipids due to their numerous

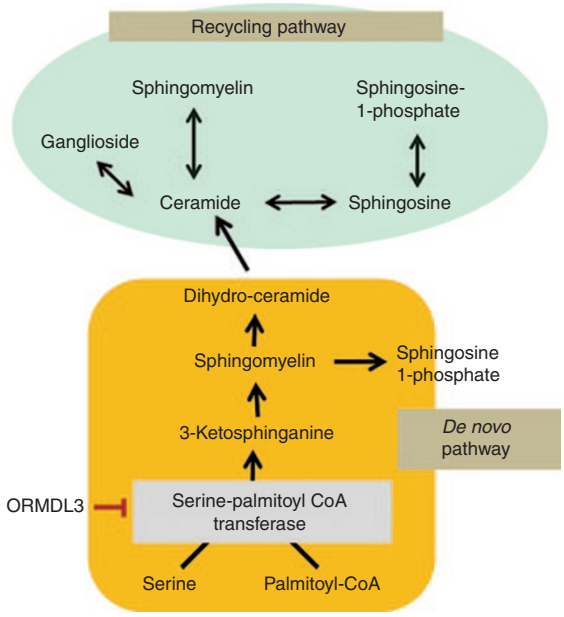

Figure 2. Sphingolipid synthesis. Highlighted are *de novo* and recycling pathways of sphingolipid metabolism.

variations in the sphingoid bases, fatty acids, and head groups (37,44,45). Several sphingolipids, in particular, ceramides, sphingosine, and sphingosine 1-phosphate (S1P), function as bioactive signaling molecules (37,44). The regulation of sphingolipid metabolism is incompletely understood and likely complex through multiple interconnected mechanisms (37). Although *de novo* sphingolipid synthesis may only play a minor contribution to the total cellular sphingolipid pool (46), homeostatic regulators of *de novo* synthesis such as ORMDL stabilize cellular sphingolipid levels in the face of external perturbations (37,38,47). Previous studies linking asthma to sphingolipids have been centered on inflammatory and allergic mechanisms related to the sphingolipid mediator S1P (48–53). S1P is involved in mast cell degranulation and airway hyperresponsiveness in allergic asthma models (53–56) and has been a focus on the development of sphingolipid-based anti-inflammatory agents (51,57–59).

The asthma-associated ORMDL3 SNPs are associated with higher expression of ORMDL. Knock-down of ORMDL1, 2, and 3 in mammalian cells increases ceramides, products of sphingolipid synthesis (38). Therefore, asthma-associated SNPs are expected to negatively regulate SPT resulting in inhibited *de novo* sphingolipid synthesis (46). A recent study from our laboratories suggest that impaired *de novo* sphingolipid synthesis leads to airway hyperreactivity in mouse lungs and both human and murine bronchial rings. In addition, we observed altered magnesium homeostasis and contractile response to magnesium (27). SPT activity in the lung was decreased using myriocin, a specific inhibitor of SPT, or using a genetic model of SPT-haploinsufficient mice. The associated increased airway hyperreactivity was not associated with inflammation or mucus hyperplasia. These findings are also supported by the recently reported association of SNPs in the 17q21 locus with

bronchial hyperreactivity without atopy (9). Airway remodeling in asthma is commonly considered to be a consequence of sustained airway inflammation (60). This assumption has recently been challenged in such that a genetically predisposed asthmatic respiratory tract reacts in a certain way to environmental (allergic or inflammatory) stimuli (61–63). Our study suggests that genetically altered sphingolipid homeostasis could be one of those predispositions. Genetic deficiency of SPT has not been observed in humans. Of note is a recent description of two nonrelated children with a currently undefined metabolic defect and encephalopathy characterized by low serine concentrations (the substrate for SPT) in serum and CSF, both of whom were also suffered from severe asthma (64).

Sphingolipids affect the force of skeletal muscle contraction (65,66) and smooth muscle proliferation (67). The effect of altered cellular sphingolipids on airway smooth muscle cells is not known. The association of decreased *de novo* sphingolipid synthesis with alterations in cellular magnesium homeostasis and the altered contractile sensitivity to magnesium could provide a mechanistic link of decreased *de novo* sphingolipid synthesis to smooth muscle function. Asthma has been associated with lower intracellular magnesium concentrations (68,69), and intravenous or nebulized magnesium sulfate ($MgSO_4$) is used to treat asthma exacerbations (68,70). This therapeutic approach has been very controversial with variable efficacy. In light of these new findings, it could be speculated that response to $MgSO_4$ depends on the 17q21 genotype.

ENVIRONMENTAL INFLUENCE AND GENETIC SUSCEPTIBILITY

The development and clinical course of childhood asthma is impacted by environmental exposures. Environmental exposures linked to asthma risk include tobacco smoke, day care attendance in early childhood, exposure to farms and farm animals, respiratory viral infections, allergens, and others (1). Although the exact mechanisms by which environmental exposures affect asthma remain unclear, epigenetic mechanisms likely play an important role, affecting the regulation of genes accessible for transcription through the modification of DNA and DNA-associated proteins. Early viral infections are well-known, but poorly understood, asthma risk factors, and it appears that variants at the 17q21 locus may enhance the association between early respiratory infections and childhood asthma. In a study by Smit *et al.* (71), the association between early viral infection and asthma showed a greater than twofold difference in odds ratio in individuals who were homozygous for minor alleles at the ORMDL3-associated SNPs. Respiratory infections with rhinovirus, one of the most potent triggers of asthma exacerbations, but not with respiratory syncytial virus were associated with a >10-fold increase in odds ratio for childhood asthma in individuals with the at-risk genotype rs7216389-TT (8). This highlights the interaction between genetic susceptibility and a common environmental risk factor. The increased risk for early-onset asthma conferred by the 17q21 locus was further increased by early exposure to tobacco smoke (18) and also confirmed in a study analyzing the relationship between early-onset asthma, viral infection, and tobacco smoke exposure (71).

Exposure to farm environments seems to have a protective effect on asthma and the development of allergies (72). DNA methylation patterns observed in cord blood, which were not found at 4 y of age in a large rural birth cohort, showed hypermethylation in loci related to ORMDL3, and also signal transducer and activator of transcription 6 (STAT6), in asthmatics from nonfarm environments as compared with nonasthmatic farm children (74). This suggests that epigenetic influences present at birth, but not after 4 y of age, may influence disease susceptibility. Interestingly, the asthmatic farmers' children had a significant decrease in methylation of the ORMDL3 loci over time as compared with increasing methylation in asthmatic children of nonfarmers, suggesting an environmental effect on epigenetic changes in the 17q21 locus (74).

CONCLUSIONS

None of the current medications for asthma are disease modifying or curative (73). Despite tremendous efforts to find an underlying cause for asthma that can be targeted therapeutically, none of these have yet been successful. GWAS studies using the most unbiased approach to find the genetic origin of asthma have also not yet been successful. Remarkably though is the consistency with which the 17q21 locus and within it ORMDL3 has been associated with asthma. Without this approach, it is unlikely that the genes in this region would have been primarily identified to be associated with asthma. It is becoming increasingly clear that this locus is associated with early-onset asthma and bronchial hyperreactivity, independent of allergic sensitization and wheezing phenotypes. Although the functional connection of altered ORMDL3 expression has not been made in humans, recent experimental data linking this protein's effect on asthma to altered calcium homeostasis, UPR, airway remodeling, and sphingolipid synthesis open new therapeutic targets for asthma at its basic mechanisms. Current asthma therapies rely mainly on bronchodilators for symptom relief and on anti-inflammatory actions of steroids and other anti-inflammatory medications. Identifying novel pathways that could be therapeutically manipulated based on a patient's genotype is an important step toward a more personalized medicine approach to asthma therapies.

STATEMENT OF FINANCIAL SUPPORT

J.G.O. is supported by a grant UL1 TR000457 from the National Center for Advancing Translational Sciences, National Institutes of Health. No other financial assistance was received to support this study.

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