Identification of PCDH1 as a Novel Susceptibility Gene for Bronchial Hyperresponsiveness


1Pediatric Pulmonology and Pediatric Allergology, Beatrix Children’s Hospital, 2Medical Biology, 3Genetics, 4Epidemiology, 5Pathology, and 6Pathology, University Medical Center Groningen, University of Groningen, The Netherlands; 7Center for Human Genomics, Wake Forest University School of Medicine, Winston-Salem, North Carolina; 8Respiratory Disease Area, Novartis Institutes for Biomedical Research, Horsham, United Kingdom; 9Department of Pulmonary and Preventive Medicine, University of Maryland School of Medicine, Baltimore, Maryland; 10Human Genetics Division, School of Medicine, University of Southampton, Southampton, United Kingdom; 11Arizona Respiratory Center, University of Arizona, Tucson, Arizona

Rationale: Asthma is a chronic inflammatory airway disease that affects more than 300 million individuals worldwide. Asthma is caused by interaction of genetic and environmental factors. Bronchial hyperresponsiveness (BHR) is a hallmark of asthma and results from increased sensitivity of the airways to physical or chemical stimulants. BHR and asthma are linked to chromosome 5q31-q33.

Objectives: To identify a gene for BHR on chromosome 5q31-q33.

Methods: In 200 Dutch families with asthma, linkage analysis and fine mapping were performed, and the Protocadherin 1 gene (PCDH1) was identified. PCDH1 was resequenced in 96 subjects from ethnically diverse populations to identify novel sequence variants. Subsequent replication studies were undertaken in seven populations from The Netherlands, the United Kingdom, and the United States, including two general population samples, two family samples, and three case-control samples. PCDH1 mRNA and protein expression was investigated using polymerase chain reaction, Western blotting, and immunohistochemistry.

Measurements and Main Results: In seven out of eight populations (n = 6,168) from The Netherlands, United Kingdom, and United States, PCHD1 gene variants were significantly associated with BHR (P values, 0.005–0.05). This association was present in both families with asthma and general populations. PCDH1 mRNA and protein were expressed in airway epithelial cells and in macrophages.

Conclusions: PCDH1 is a novel gene for BHR in adults and children. The identification of PCDH1 as a BHR susceptibility gene may suggest that a structural defect in the integrity of the airway epithelium, the first line of defense against inhaled substances, contributes to the development of BHR.

Keywords: bronchial hyperresponsiveness; asthma genetics; protocadherin-1; cell adhesion; airway epithelium

Asthma is a chronic inflammatory airway disease that affects more than 300 million individuals worldwide (1). It is characterized by respiratory symptoms, variable airway obstruction, and bronchial hyperresponsiveness (BHR) and is caused by multiple genetic and environmental factors that may interact (2). BHR is a hallmark of asthma and is due to increased sensitivity of the airways to physical or chemical stimulants (cold air and cigarette smoke) and to pharmacological agents, such as methacholine and histamine. BHR has a considerable genetic component (3) and constitutes a risk factor for asthma development, even in subjects without respiratory symptoms (4). Furthermore, BHR is increased in children who are exposed to environmental tobacco smoke (ETS) in utero and in the first years of life (5).

Our initial report of linkage of BHR to chromosome 5q31-q33 in Dutch families with asthma (6) has been confirmed in six other populations for asthma-associated phenotypes (7–12). This finding has been extended by showing that chromosome 5q31-q33 interacts with ETS exposure in utero and/or early childhood in the development of BHR and asthma (13, 14). This region of chromosome 5q31-q33 contains a large number of candidate genes for allergy and asthma, such as Interleukin-13, Interleukin-9, Interleukin-4, CD14, IRF-1, GM-CSF, TIM-1, and the β2-adrenoceptor (15). These genes have been reported to be associated with asthma-associated phenotypes, which strongly suggests that chromosome 5q31-q33 contains multiple asthma susceptibility genes that each contribute to the observed asthma linkage.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Asthma and bronchial hyperresponsiveness (BHR) are caused by multiple genetic and environmental factors. Linkage studies suggest the presence of one or more genes for asthma and bronchial hyperresponsiveness on chromosome 5q31-q33.

What This Study Adds to the Field

This study reports Protocadherin-1 as a novel gene for bronchial hyperresponsiveness on chromosome 5q31-q33. This gene encodes an adhesion molecule expressed in airway epithelium, which may imply that a structural defect in the integrity of the airway epithelium contributes to the development of BHR.

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Correspondence and requests for reprints should be addressed to Gerard H. Koppelman, M.D., Ph.D., Department of Pediatric Pulmonology and Pediatric Allergology, Beatrix Children’s Hospital, University Medical Center Groningen, University of Groningen, P.O. Box 30001, 9700 RB Groningen, The Netherlands. E-mail: g.h.koppelman@bkk.umcg.nl.

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In this article, we report the identification of protocadherin-1 (PCDH1) as a novel gene for BHR on chromosome 5q31-q33. We used positional cloning to identify this gene in the Dutch population, subsequently identified novel sequence variants, performed genetic replication studies in seven independent populations in The Netherlands, the United States, and the United Kingdom, and showed that PCDH1 mRNA and protein expression is present in airway epithelium, the first line of defense against inhaled allergens and toxic substances known to contribute to asthma development.

METHODS

Study Populations

Recruitment and clinical characterization of all study samples are described in detail in the online data supplement. The primary population consists of 200 families (1,259 individuals) ascertained through probands with asthma who were initially studied between 1962 and 1975 at Beatrixoord Hospital, Haren, The Netherlands (see Table E1 in the online supplement) (16). The Dutch replication samples include an asthma trio population of 407 trios (17), and the longitudinal Vlagtwedde/Vlaardingen cohort study, which includes subjects from the general population who have been tested for BHR at one or more time points during follow-up (n = 418) (Table E2) (18).

Replication samples from the United States include the prospective Children’s Respiratory Study (Tucson, AZ), in which children were tested for BHR to methacholine at ages 11 and 16 (n = 329) (Table E2) (19). Furthermore, three U.S. case-control populations were recruited from three ethnically diverse US populations (African-American [n = 522], Hispanic [n = 246], and white [n = 665]) as part of the Collaborative Study on the Genetics of Asthma (20, 21). BHR measurements to methacholine or reversibility to albuterol were performed in the cases (Table E2) (21).

The replication sample from the United Kingdom is an asthma family study from Southampton, United Kingdom, which includes 341 affected sib pairs with asthma (n = 1508) (22). Bronchial responsiveness was measured in subjects with a baseline FEV1 of greater than 70% predicted using inhaled methacholine (Table E2) (22).

Local medical ethics committees approved these studies, and all participants provided written informed (parental) consent.

Genotyping and Sequence Analysis

Genotyping methods and marker selection are described in the online supplement. Briefly, 22 microsatellite markers were identified on chromosome 5q31–q33 and genotyped in the Dutch family study (14). Subsequently, 103 single nucleotide polymorphisms (SNPs) were selected for fine mapping and genotyped in the Dutch family study (Table E3). The PCDH1 gene (2 kb of the putative promoter region, all exons, and the complete 3’ untranslated region [UTR]) was resequenced in 96 subjects as described previously (23).

Statistical Analysis

Linkage analysis of the microsatellite fine screen markers on chromosome 5q31-33 was performed using GENEHUNTER-PLUS, using the same model as described previously (14).

Association analysis of Dutch families, trios, and UK families was done with the family-based association test (FBAT) using an additive model, using the -e option in case of linkage (24). Case-control analysis was performed using Chi-square tests and analysis of variance, if appropriate. In the Vlagtwedde/Vlaardingen cohort study, the association between PCDH1 SNPs and the occurrence of becoming BHR-positive over time was investigated longitudinally using a Cox regression model (SPSS 14.0), with adjustment for smoking, age at investigation, and FEV1 at baseline. All statistical tests were performed two sided.

PCDH1 expression was investigated using polymerase chain reaction in mRNA obtained from an epithelial cell line 16HBE, and human cells, e.g., blood cells, brain, lung fibroblasts, and cultured airway epithelial cells from patients with asthma. PCDH1 protein expression was investigated using two different antibodies (a polyclonal and a monoclonal antibody) using western blotting and immunohistochemistry as described in the online data supplement.

RESULTS

Linkage analysis using a dense map of 29 microsatellite markers in the linked region on chromosome 5q31-q33 in 200 Dutch families (n = 1,259) revealed significant LOD scores in two regions: HLOD was 3.88 at marker 5q31-33_36 and 3.84 in a region flanked by markers DSS2117 and IL9. In 95 ETS-exposed families, a peak HLOD score of 4.36 was flanked by markers 5q31-33_56 and FFGα (Figure E1). The bivariate co-disequilibrium between markers on 5q in the Dutch population. One hundred three haplotype tagging and functional SNPs were genotyped and FBAT identified significant associations of SNPs in PCDH1 with BHR, and specifically with rs3797054, which encodes a synonymous SNP (Aa750Ala) in PCDH1 (P = 0.005) (Figure 1).

Resequencing of PCDH1 in 96 subjects of ethnically diverse populations revealed 22 SNPs and two insertion deletion polymorphisms (Table E4).

Potential functional SNPs significantly associated with BHR were genotyped in seven replication populations from the Netherlands (adults), the United States (adults and children), and the United Kingdom (children), displaying significant associations in two Dutch, one UK, and three US populations (Table 1). First, the association found between rs3797054 (T allele) and BHR was consistently observed in the parents of the Dutch families (using the probands with asthma as cases and unaffected spouses in a case-control design) and in the children of the Dutch families (using FBAT). The association of rs3797054 with BHR was replicated in participants of the population-based Children’s Respiratory Study in Tucson, Arizona, who were investigated for BHR at ages 11 and 16 years (19). Moreover, rs3797054 was associated with the development of BHR in adults from a longitudinal adult population-based study in the Netherlands (Figure 2) (18). Second, a 3-bp insertion/deletion polymorphism (IVS3-116) in the 3’ UTR region of exon 3 was associated with BHR in two Dutch populations and with asthma and BHR in two US case-control populations. Third, the major allele of rs3822357, which encodes Ala514Thr localized in the fifth cadherin repeat, was associated with BHR in the US Children Respiratory Study (19), and with BHR in a UK population of 341 families ascertained through affected siblings with asthma (22). Interestingly, PCDH1 Ala514Thr was associated with BHR when ETS exposure in utero and the first years of life was taken into account in the latter population (Table 1). However, there was no strong evidence for gene by smoking interaction in the Dutch or the United States Tucson populations.

In addition to BHR, we investigated PCDH1 SNPs in asthma defined by an algorithm (14) and observed significant association of rs3797054 and asthma (P = 0.003) in the 200 Dutch families. Haplotype analysis did not further improve these results.
By polymerase chain reaction, we identified high PCDH1 mRNA expression in human brain, airway epithelial cell lines (16HBE), and in primary epithelial cell cultures of patients with asthma. Weak mRNA expression was observed in airway fibroblasts, peripheral blood mononuclear cells, and granulocytes (Figure 3). Western blot analysis showed consistent and specific PCDH1 protein expression in bronchial epithelial cell line 16HBE and primary epithelial cell cultures of patients with asthma using different polyclonal and monoclonal antibodies. Proteins of 150–160 kD were identified with a protein weight consistent with previous observations (Figure 4) (27). Immunohistochemistry using the PCDH1 polyclonal antibody showed a specific expression pattern of PCDH1 protein in the apical part of differentiated airway epithelial cells lining the airway lumen and in the membrane of macrophages in lung resection specimens of a patient with bronchitis as well as airway wall

**Figure 1.** Linkage disequilibrium ($D'$) and association of single nucleotide polymorphisms at chromosome 5q31-q33 and bronchial hyperresponsiveness in the Dutch family study.
biopsies in patients with asthma (Figure 5). PCDH1 expression was also observed between epithelial cells at the apical side of the epithelium.

**DISCUSSION**

This study identified *PCDH1* as a novel gene for BHR in children and adults. Interestingly, we have provided evidence that *PCDH1*, particularly rs3797054, is important in the development of BHR in a sample of families with asthma. In addition, *PCDH1* is associated with BHR in two population-based samples from the Netherlands and the United States, irrespective of the presence of asthma. Moreover, we present strict replication of a second gene variant in *PCDH1* in subjects without respiratory symptoms (4). Based on these data, we hypothesize that mechanisms related to the function of *PCDH1* play an early role in asthma pathogenesis.

To interpret these findings, several strengths and limitations need to be considered. First, to our knowledge this is the first gene that is identified by positional cloning for an intermediate phenotype of asthma, BHR. *PCDH1* gene variants are associated with BHR in families with asthma as well as two general populations not ascertained for asthma. Previous epidemiological data have shown that BHR is a risk factor for asthma, even in subjects without respiratory symptoms (4). Based on these data, we hypothesize that mechanisms related to the function of *PCDH1* contribute to susceptibility to BHR and subsequent asthma development. This may implicate that *PCDH1* dysfunction plays an early role in asthma pathogenesis.

Second, in all study populations, a direct agonist of BHR was used (either histamine or methacholine). Our findings suggest that PCDH1 function is not specific for BHR induced by either methacholine or histamine. Previous work has indicated that BHR to histamine and methacholine is highly correlated (29).

We suggest that further work should be performed on the role of *PCDH1* in indirect measures of BHR, such as exercise or adenosine-monophosphate.

Third, we used the positional cloning approach that has been previously used to identify *DPP10* and *PHD11* (25, 26). This approach is based on identification of a genetic association with a microsatellite marker used for linkage analyses, to subsequently fine map the region that is in possible linkage disequilibrium (LD) with this marker and the gene of interest. Our fine mapping results indicate that we have adequately screened the region for SNPs in other genes that may be in LD with *PCDH1*, given the extent of LD of about 100 kb in this region in the Dutch population (Figure 1).
Fourth, we identified PCDH1 in a primary family population, in which linkage to BHR was reported (6, 14). We did not correct for multiple testing, but rather performed extensive replication studies of significant and/or functional SNPs in seven independent populations. We interpret the consistent signal in parents and offspring in the Dutch families as internal validation of this genetic association in the Dutch families. Moreover, the strict replication of two PCDH1 gene variants with the same risk allele being associated with BHR in the same direction in four independent populations provides strong support for a role of PCDH1 in BHR (28). Specifically, we found strict replication with regard to phenotype and genotype for Ala750Ala and IVS3_116. Loose replication with regard to genotype was observed for Ala514Thr. The reasons for this allelic heterogeneity are yet to be determined, but include the presence of multiple functional SNPs, other SNPs that are in linkage disequilibrium with the associated SNPs, or gene by environment interaction. Because gene variants in 5q31-q33 have been shown to interact with ETS exposure in utero/early life, we investigated the association of PCDH1 with BHR in ETS exposed and nonexposed children in four populations. These gene–environment interaction analyses (Table E3) in the study cohorts revealed evidence of gene–environment interaction for PCHD1 in the UK family cohort only, but not in the other populations. However, the power to detect such an interaction was low.

We suggest characterizing the functional role of these gene variants in PCDH1. Ala750Ala and IVS3_116 are localized in the 3’UTR of exon 3 and may affect mRNA stability or splicing, whereas Ala514Thr is localized in the fifth cadherin repeat of the extracellular domain and may affect cell–cell adhesion.

The Protocadherin 1 gene (PCDH1, PC42) has five exons and encodes multiple mRNA isoforms through alternative splicing (Table E5). There are two annotated isoforms: a three-exon isoform and a five-exon isoform. The three-exon isoform lacks the major part of the cytoplasmic domain, which encodes conserved signaling sequences CM1, CM2, and CM3 (22). Protocadherins are believed to play an important role in homologous cell adhesion and organ development, in particular within the neural system (30). Overexpression of PCDH1 induces calcium-dependent cell–cell adhesion and membrane expression of PCDH1 in a mouse fibroblast L cell assay (27). Protocadherin 1, like PCDH, belongs to the β1-protocadherin family of transmembrane proteins. β1-Protocadherins are characterized by six or seven cadherin repeats in the extracellular region, and three conserved regions designated CM1, CM2, and CM3 in the intracellular domain (31). The conserved region CM2 interacts with protein phosphatase 1 (PP1α) in PCDH7 (31). Interestingly, PP1α plays an important role in lung development, as inhibition of PP1α in a mouse model led to impaired lung development and branching morphogenesis (32). Finally, in a skin keratinocyte wounding model, PCDH1 mRNA was significantly up-regulated 24 hours after wounding, compatible with a role of PCDH1 in epithelial repair (33).

The expression pattern of PCDH1 is consistent with expression in the apical adhesion complex of airway epithelial cells. We therefore hypothesize that PCDH1 plays a role in epithelial integrity of the airways and that loss of function of PCDH1 is associated with increased BHR, which may lead to symptomatic asthma (34). It is tempting to speculate that PCDH1 dysfunction may provide a functional explanation for the observed epithelial vulnerability and increased epithelial shedding in asthma (35). Further investigations will address the functional relevance of genetic variations in PCDH1 in epithelial cell adhesion and its interaction with environmental tobacco smoke exposure. Moreover, these findings may be relevant for other diseases in which the integrity of the epithelium is a potential pathogenetic mechanism, such as atopic dermatitis (36) and celiac disease (37). We therefore suggest performing genetic studies of PCDH1 in these diseases.

In conclusion, this is the first report of a gene specifically identified for HR, an important hallmark of asthma. Further investigations in PCDH1 function may provide novel insight

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![Figure 3](image3.png)

**Figure 3.** mRNA expression of PCDH1 in various tissues and cells. GAPDH = glyceraldehyde 3 phosphate dehydrogenase; PBMC = peripheral blood mononuclear cell.

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![Figure 4](image4.png)

**Figure 4.** Western blot of epithelial cell line 16 HBE, and primary epithelial cell culture of a patient with asthma using a monoclonal and a different polyclonal antibody. Upper lane, PCDH1, lower lane, β-actin. One experiment of n = 4 is shown. Protein expression was identified using two different antibodies, a monoclonal (Abnova, Taiwan) (left panel) and a polyclonal antibody (Eurogentec, Liege, Belgium) (middle panel). The signal of the polyclonal antibody was blocked by preincubation with peptide (right panel).
declares that a patent on PCDH1 has been filed as US patent 7122311 and PCT patent application WO 03/008640 A2 and assigned to Novartis Pharmaceuticals.

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References


Figure 5. Protein expression of PCDH1 in airway epithelial cells and macrophages on (A, B) lung, and (C) airway wall biopsy of patient with asthma. Expression is observed (A, C) at the apical part of airway epithelial cells, and (B) in macrophages.

Conflict of Interest Statement: G.H.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.A.M. into its role in the integrity of the airway epithelium in BHR and asthma development.


