

ORIGINAL ARTICLE

Genetic variants in *Protocadherin-1*, bronchial hyper-responsiveness, and asthma subphenotypes in German children

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Keywords

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Abstract

Background: Recently, *Protocadherin-1* (*PCDH1*) was reported as a novel susceptibility gene for bronchial hyper-responsiveness (BHR) and asthma. *PCDH1* is located on chromosome 5q31-33, in the vicinity of several known candidate genes for asthma and allergy. To exclude that the associations observed for *PCDH1* originate from the nearby cytokine cluster, an extensive linkage disequilibrium (LD) analysis was performed. Effects of polymorphisms in *PCDH1* on asthma, BHR, and related phenotypes were studied comprehensively.

Methods: Genotype information was acquired from Illumina HumanHap300Chip genotyping, MALDI-TOF MS genotyping, and imputation. LD was assessed by Haploview 4.2 software. Associations were investigated in a population of 1454 individuals (763 asthmatics) from two German study populations [MAGICS and International Study of Asthma and Allergies in Childhood phase II (ISAAC II)] using logistic regression to model additive effects.

Results: No relevant LD between *PCDH1* tagging polymorphisms and 98 single nucleotide polymorphisms within the cytokine cluster was detected. While BHR was not associated with *PCDH1* polymorphisms, significant associations with subphenotypes of asthma were observed.

Conclusion: *Protocadherin-1* polymorphisms may specifically affect the development of non-atopic asthma in children. Functional studies are needed to further investigate the role of *PCDH1* in BHR and asthma development.

Abbreviations:

BHR, Bronchial hyper-responsiveness; CI, Confidence Interval; FEV₁, Forced expiratory volume in 1 second; GWA, Genome-Wide Association Study; IL-13, Interleukin-13; IL-4, Interleukin-4; IL-5, Interleukin-5; ISAAC II, International Study of Asthma and Allergies in Childhood phase II; LD, Linkage Disequilibrium; MAF, Minor Allele Frequency; MAGICS, Multicenter Asthma Genetics in Childhood Study; MALDI-TOF, Matrix Assisted Laser Desorption/Ionization Time of Flight; MMP-9, Matrix metalloproteinase 9; OR, Odds Ratio; *PCDH1*, *Protocadherin-1*; *RAD50*, *RAD50* homolog (*Saccharomyces cerevisiae*); SNP, single nucleotide polymorphism.

Recently, the adhesion molecule *Protocadherin-1* (*PCDH1*) was identified as a novel susceptibility gene for bronchial hyper-responsiveness (BHR) and asthma. The gene is located on chromosome 5q31-33 (1) in close proximity to the cytokine gene cluster which harbors several of the most frequently reported candidate genes for asthma- and allergy-related traits such as *Interleukin-4* (*IL-4*), *Interleukin-5* (*IL-5*), and *Interleukin-13* (*IL-13*) (2). Significant associations between *PCDH1* single nucleotide polymorphisms (SNPs) and BHR have been reported in seven of eight studied populations (1). However, based on the chromosomal position, it could be argued that these associations are attributable to LD between genetic variants in the *PCDH1* gene and the nearby 5q31 cytokine cluster. Thus, the first aim of our study was to define LD in the region comprising *PCDH1* and the cytokine cluster genes *IL-5*, *RAD50*, *IL-13*, and *IL-4*. For these analyses, we used an unbiased, systematic tagging SNP approach including all validated SNPs in the *PCDH1* gene region. For a comprehensive genetic analysis of the *PCDH1* locus, we extended our analyses to more SNPs in the *PCDH1* region than previously reported and investigated not only BHR and asthma but additional phenotypes related to BHR, asthma, and atopy in German children.

Methods

See Data S1.

SNP selection, genotyping, and linkage disequilibrium (LD) analyses

Single nucleotide polymorphisms within the *PCDH1* gene region as well as the cytokine gene cluster on chromosome 5q31-33 were systematically identified using HapMap data (3, http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/), literature search, and sequencing. Haploview 4.2 software (4) was used to select tagging SNPs ($r^2 \geq 0.8$). Genotypes were obtained from Illumina Sentrix HumanHap300 BeadChip genotyping, and imputation was performed as recently described (5). GWAS genotyping had been performed within the GABRIEL consortium (6). Details regarding the genotyping technique and the studied phenotypes are provided in Table S1. Overall, 14 SNPs and one insertion/deletion polymorphism (IVS3_116) in the *PCDH1* gene were genotyped for this study. Genotyping data were retrieved for six SNPs from the BeadChip experiment and for nine polymorphisms from MALDI-TOF MS. Additional four SNPs in the 5q31 cytokine cluster had been re-genotyped as previously reported (5) and were included in the LD analyses. Within the combined dataset, LD between SNPs from the cytokine gene cluster region, containing *IL-5*, *RAD50*, *IL-13*, and *IL-4* (chromosome 5: 131.895.035–132.056.267 according to HapMap), and the *PCDH1* gene (chromosome 5: 141.202.867–141.248.128) was analyzed using the Haploview 4.2 software (4).

Study populations

Asthma cases from the MAGIC study (7) were compared to randomly selected children from the cross-sectional

International Study of Asthma and Allergies in Childhood phase II (ISAAC II) study (8). Details of the study population have been given previously (5). Written informed consent was obtained from parents of all children, and study protocols were approved by the ethics committees of participating centers. A total of 1311 (651 asthmatics, 652 controls, eight missing asthma status) subjects had sufficient GWAS data available for analyses, and 1454 samples (763 asthmatics) were successfully re-genotyped for *PCDH1* SNPs.

The ISAAC II study was conducted in Munich and Dresden to assess the prevalence of asthma in school children aged 9–11 years (8). Children were classified as having asthma if a physician's diagnosis of asthma and/or recurrent spastic or asthmatic bronchitis was reported by their parents. Atopy was defined as a positive skin prick test according to ISAAC criteria to one or more of the following allergens: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, mixed grass and mixed tree pollen. A child was considered having allergic rhinitis and/or atopic eczema if parents reported a doctor's diagnosis for these disorders in the parental questionnaire. BHR was assessed in a 50% randomly selected subsample by changes in FEV₁ after inhalation of nebulized, hyperosmolar (4.5%) saline for increasing periods of time using ultrasound nebulizers. Children with a drop in FEV₁ of 15% or more from baseline after hypertonic saline challenge were classified as having BHR.

In the MAGIC study, children were recruited from German and Austrian clinical asthma centers (5). Pediatric pulmonologists diagnosed asthma in 865 children (mean age, 11 years) based on physical examination, clinical history, lung function tests, and allergy testing (skin prick testing and serum IgE measurement). Inhalant and food allergens were assessed by CLA[®] panel (Hitachi Chemical Diagnostics, Inc. Mountain View, California, USA) or AllergyScreen[®] (Medi-wiss Analytic GmbH, Moers, Germany). Atopy was defined as at least one positive result (RAST class equivalent > 2) to any of the following common aeroallergens: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat epithelium, grass pollen, birch pollen. Atopic asthma was defined as asthma and concomitant atopy present according to the criteria above. Non-atopic asthma was stringently specified as asthma and no sensitization (RAST class equivalent of 0) and more permissively as asthma and no RAST class equivalent > 2. The more loose definition of non-atopic asthma was used in an additional analysis presented in the online supplement (Table S2). Both atopic asthma and non-atopic asthma were compared to controls without any asthma or sensitization.

Statistical analysis

Statistical analyses and quality control were performed with PLINK software package (9, <http://pngu.mgh.harvard.edu/~purcell/plink/>) version 1.07 and confirmed using SNP and Variation Suite v7.4 (Golden Helix, Bozeman, MT, USA) (<http://www.goldenhelix.com>). Additive genetic effects were modeled using logistic regression. Per-allele effects are displayed as odds ratios (OR) with 95% confidence intervals (95% CI).

Results

Fine mapping of *PCDH1* and LD in the locus

In the *PCDH1* gene, a total of 18 SNPs with a minor allele frequency ≥ 0.05 were identified by HapMap. These SNPs were captured by 14 tagging SNPs, which cover the genetic information of the gene locus ± 10 kb adjacent of the gene (see Fig. 1b). LD between *PCDH1* SNPs and 98 SNPs in the region of *IL-5*, *RAD50*, *IL-13* and *IL-4*, was evaluated. As indicated in Fig. 1a, polymorphisms within *PCDH1* were not in relevant LD with those in the cytokine cluster.

We investigated associations between tagging SNPs in the *PCDH1* gene and BHR, assessed by hyperosmolar saline inhalation in our cross-sectional study population. We studied BHR using the subset ($n = 344$) of the ISAAC II reference population in which both lung function and BHR were assessed. In contrast to the previous report, none of the genetic variants in *PCDH1* showed any significant association with BHR in our cross-sectional study population (Table 1, left panel). Specifically, SNPs rs3797054, rs3822357, and IVS3_116 were not associated with BHR, as previously reported by Koppelman et al. (1).

Next, we analyzed the effect of *PCDH1* tagging SNPs on asthma in a case-control setting using the MAGICs and ISAAC II data. *PCDH1* polymorphism rs7719391 was significantly associated with asthma diagnosis (Table 2). As childhood asthma may be a heterogeneous syndrome rather than a single disease entity, we further investigated if *PCDH1* polymorphisms may be associated with specific subphenotypes of asthma. Thus, we analyzed *PCDH1* SNP effects on atopic

and non-atopic asthma separately. None of the SNPs was associated with atopic asthma. In contrast, two SNPs (rs2974704 and rs11167761) were significantly associated with non-atopic asthma. Polymorphism rs7719391 showed a trend to an association with non-atopic asthma ($p = 0.0509$).

As SNP rs3822357 and IVS3_116 were recently reported to be associated with eczema (10), we also analyzed associations between *PCDH1* polymorphisms and atopy, atopic eczema, and allergic rhinitis in the cross-sectional ISAAC population (Table 1). However, we could not detect any significant associations with atopy, and only a weak trend was observed for rs7719391 and rs3935792. No association between *PCDH1* polymorphisms and other atopic diseases such as atopic eczema and allergic rhinitis was found.

Because this study was primarily designed as a replication study, correction for multiple testing was not applied for those phenotypes previously investigated. Testing for the subphenotypes of atopic and non-atopic asthma, one would expect 1.5 positive associations at a significance level of 0.05 in 30 tests. We observed two significant and one borderline associations all clustering in the analysis for non-atopic asthma, suggesting the presence of non-random effects with this subphenotype of asthma.

Discussion

Recently, *PCDH1* was identified as a novel susceptibility gene for BHR (1). *PCDH1* is located on chromosome 5q31-33, which also harbors the cytokine gene cluster containing several known candidate genes for asthma and allergy such as

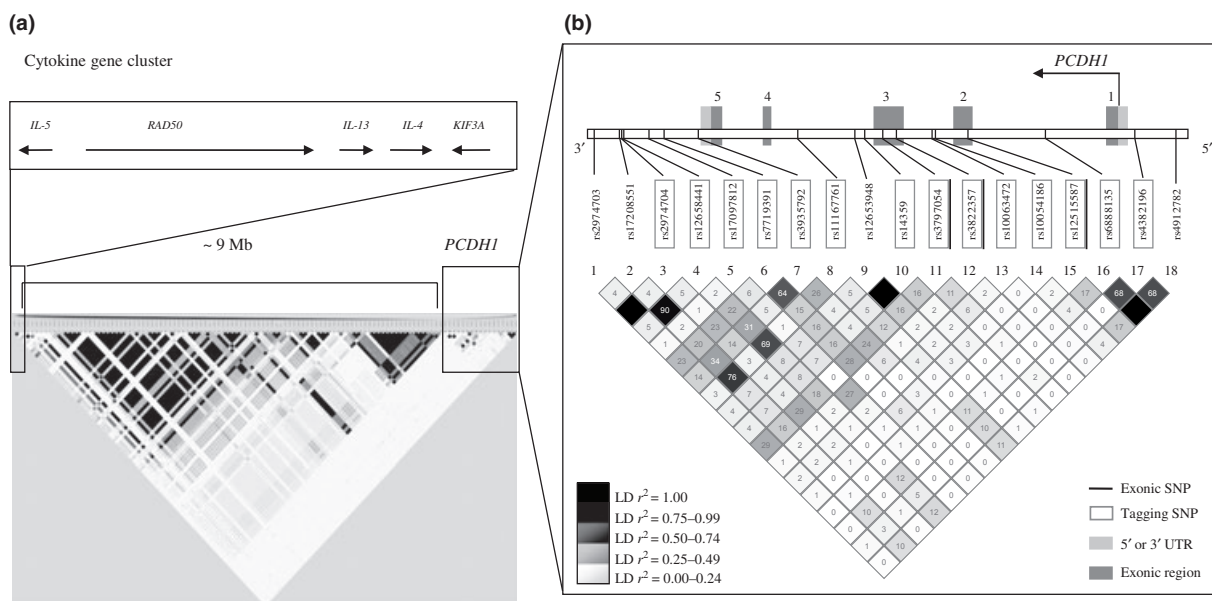


Fig 1 Linkage disequilibrium (LD) between SNPs in the cytokine gene cluster and *Protocadherin-1* (*PCDH1*). (a) LD between the cytokine cluster (Chr 5: 131.895.035–132.056.267) containing *IL-5*, *RAD50*, *IL-13*, and *IL-4* and the *PCDH1* (Chr 5: 141.248.128–141.202.867), including ± 10 kb borders. A schematic representation of the genes within the cytokine cluster is shown above the LD plot. (b) LD of *PCDH1* isoform 2 precursor ± 10 Kb border. Eighteen SNPs captured by 14 tagging SNPs (framed) were selected from HapMap (MAF ≥ 0.05 ; $r^2 \geq 0.8$) in CEU population. Exonic SNPs are underlined.

Table 1 Associations of 14 tagging *Protocadherin-1* (*PCDH1*) SNPs with bronchial hyper-responsiveness (BHR), atopy, allergic rhinitis, and atopic eczema in the cross-sectional ISAAC II study (n = 763)

No	SNP	Reference allele	Allele frequency	BHR (n = 71/273)		Atopy (n = 192/536)		Allergic rhinitis (n = 80/665)		Atopic eczema (n = 131/603)	
				OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
3	rs2974704*	A	0.10	1.23 (0.67–2.23)	0.509	1.21 (0.80–1.82)	0.374	1.19 (0.67–2.09)	0.556	1.06 (0.65–1.72)	0.828
4	rs12658441†	C	0.24	0.95 (0.59–1.54)	0.850	1.06 (0.79–1.41)	0.717	1.02 (0.67–1.55)	0.938	1.14 (0.82–1.58)	0.445
5	rs17097812	T	0.07	1.25 (0.60–2.59)	0.545	1.30 (0.81–2.08)	0.275	1.57 (0.87–2.85)	0.138	0.86 (0.49–1.50)	0.588
6	rs7719391	C	0.37	1.03 (0.71–1.50)	0.883	0.79 (0.62–1.01)	0.057	0.76 (0.54–1.08)	0.131	0.96 (0.72–1.27)	0.769
7	rs3935792	C	0.49	0.86 (0.59–1.27)	0.458	1.26 (0.98–1.60)	0.067	1.02 (0.72–1.44)	0.921	0.84 (0.64–1.11)	0.214
8	rs11167761	A	0.16	0.97 (0.58–1.61)	0.904	1.08 (0.78–1.49)	0.657	0.70 (0.42–1.18)	0.185	0.88 (0.60–1.30)	0.516
10	rs14359‡	C	0.20	0.82 (0.48–1.38)	0.447	1.18 (0.87–1.60)	0.278	1.33 (0.88–2.02)	0.176	0.85 (0.59–1.22)	0.386
11	rs3797054	G	0.37	1.24 (0.84–1.84)	0.273	0.97 (0.76–1.24)	0.822	0.85 (0.60–1.20)	0.344	0.95 (0.71–1.25)	0.697
12	rs3822357	T	0.06	1.29 (0.61–2.75)	0.504	1.26 (0.79–2.02)	0.332	1.27 (0.68–2.39)	0.453	0.58 (0.29–1.13)	0.108
13	rs10063472	T	0.20	1.29 (0.82–2.02)	0.274	0.97 (0.73–1.31)	0.864	0.67 (0.42–1.07)	0.096	0.90 (0.64–1.28)	0.573
14	rs10054186	C	0.21	0.97 (0.62–1.54)	0.912	0.95 (0.72–1.27)	0.741	0.99 (0.67–1.48)	0.980	1.11 (0.80–1.53)	0.533
15	rs12515587	G	0.14	0.67 (0.38–1.18)	0.164	0.89 (0.62–1.26)	0.510	1.00 (0.62–1.62)	0.994	0.92 (0.62–1.38)	0.703
16	rs6888135§	A	0.48	1.25 (0.85–1.85)	0.258	0.96 (0.76–1.23)	0.763	1.28 (0.91–1.80)	0.159	1.05 (0.79–1.38)	0.756
17	rs4382196	C	0.44	0.86 (0.57–1.29)	0.460	1.06 (0.82–1.37)	0.657	0.80 (0.56–1.16)	0.239	0.95 (0.71–1.27)	0.725
**	IVS3_116	del	0.07	0.66 (0.28–1.54)	0.333	0.69 (0.41–1.15)	0.152	0.78 (0.36–1.60)	0.507	1.22 (0.66–1.90)	0.669

CI, Confidence interval; OR, Odds ratio; No, corresponds to SNP location in Fig. 1b; Numbers of affected and unaffected individuals per phenotype are given in brackets; SNPs No 3, 8, 11, 12, 13, and 16 were genotyped with Illumina Sentrix HumanHap300 BeadChip, all others with MALDI-TOF MS.

*Tagging SNP for rs2974703 ($r^2 = 1.00$).

†Tagging SNP for rs17208551 ($r^2 = 0.90$).

‡Tagging SNP for rs12653948 ($r^2 = 1.00$).

§Tagging SNP for rs4912782 ($r^2 = 1.00$).

**IVS3_116 is not published in HapMap and it is not displayed in Fig. 1b.

IL-4, *IL-5*, and *IL-13* in close vicinity. To investigate the possibility that the original signal observed for *PCDH1* may be a result of extensive long-range LD with the cytokine gene cluster, we dissected the relationship between 18 polymorphisms found in the 45 Kb spanning region of *PCDH1* and 161 Kb of cytokine cluster containing 98 SNPs. Using information obtained from our large dataset, we could not detect any LD between the two regions, indicating independent patterns of inheritance between the *PCDH1* gene locus and the cytokine gene cluster. This information is important to interpret previous and current results obtained from the *PCDH1* gene locus.

In addition, we aimed to replicate the previously reported association between *PCDH1* variants and BHR as well as asthma in this study. To cover all validated *PCDH1* SNPs, we performed a systematic tagging SNP approach using HapMap data, leading to the identification of 14 *PCDH1* tagging SNPs (MAF ≥ 0.05). Out of these polymorphisms, three had not been investigated previously (rs2974704, rs12658441, rs4382196) in asthma and other atopic disorders. Thus, this study extends the SNP coverage of the gene considerably and allows for more comprehensive analyses of the locus.

We were not able to replicate the association between *PCDH1* SNPs and BHR as previously reported (1). This may be due to the fact that our assessment of BHR by hypertonic saline inhalation targets a type of BHR in which *PCDH1* is

not involved. In addition, the population for which we had BHR data available was rather small, and thus, power was limited. Also, in contrast to previous reports, we assessed BHR in a cross-sectional population, and thus, BHR in the general population may be less connected to asthma than when assessed in an asthma case-control population (1).

To address the question if *PCDH1* is associated with asthma *per se*, we investigated tagging SNPs within the MAGICs and ISAAC II case-reference study population using an additive genetic model as described in the initial report (1). SNP rs7719391 showed a protective effect against asthma development. When subphenotype analyses were conducted, two SNPs (rs2974704 and rs11167761) were significantly associated with non-atopic asthma, and rs7719391 showed a borderline effect. We used a stringent definition of non-atopic asthma, not allowing for any sensitization in those subjects. However, most asthmatics with pulmonary symptoms triggered by allergy display a higher degree of sensitization, and when we performed an analysis where weakly sensitized children with RAST class equivalents of ≤ 2 were considered as non-atopic asthmatics, five SNPs showed significant associations in that analysis (see Table S2).

Protocadherin-1 codes for Protocadherin-1, a membrane protein, localized at cell-cell boundaries. Recently, it has been shown very elegantly that it is expressed in airway epithelial cells and that its mRNA expression is higher in

Table 2 Association of 14 *Protocadherin-1* (*PCDH1*) tagging SNPs with asthma, atopic, and non-atopic asthma in the combined MAGICs and ISAAC II case–reference study population (n = 1454*)

No	SNP	Reference allele	Allele frequency	Asthma (n = 763/683)		Atopic asthma (n = 548/426)		Non-atopic asthma (n = 113/426)†	
				OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
3	rs2974704‡	A	0.10	1.08 (0.83–1.40)	0.583	1.33 (0.98–1.82)	0.071	0.46 (0.22–0.94)	0.032
4	rs12658441§	C	0.24	1.12 (0.94–1.35)	0.215	1.15 (0.92–1.44)	0.221	1.24 (0.88–1.73)	0.220
5	rs17097812	T	0.07	0.91 (0.68–1.24)	0.560	0.99 (0.68–1.44)	0.959	1.08 (0.60–1.97)	0.792
6	rs7719391	C	0.37	0.85 (0.73–0.99)	0.039	0.84 (0.69–1.01)	0.066	0.73 (0.53–1.00)	0.051
7	rs3935792	C	0.49	1.08 (0.93–1.26)	0.313	1.09 (0.91–1.31)	0.361	1.18 (0.88–1.59)	0.271
8	rs11167761	A	0.16	1.10 (0.88–1.36)	0.403	1.08 (0.82–1.41)	0.586	1.55 (1.03–2.34)	0.037
10	rs14359¶	C	0.20	1.03 (0.85–1.25)	0.750	0.92 (0.73–1.16)	0.478	0.93 (0.64–1.35)	0.692
11	rs3797054	G	0.37	0.89 (0.76–1.04)	0.143	0.96 (0.79–1.17)	0.698	0.79 (0.56–1.09)	0.151
12	rs3822357	T	0.06	0.80 (0.58–1.12)	0.191	0.89 (0.60–1.32)	0.556	0.74 (0.36–1.53)	0.415
13	rs10063472	T	0.20	1.08 (0.89–1.31)	0.416	1.23 (0.97–1.55)	0.088	0.89 (0.59–1.34)	0.566
14	rs10054186	C	0.21	0.86 (0.72–1.03)	0.096	0.83 (0.67–1.04)	0.109	1.09 (0.77–1.54)	0.633
15	rs12515587	G	0.14	0.95 (0.76–1.18)	0.638	0.92 (0.71–1.19)	0.529	0.79 (0.50–1.24)	0.305
16	rs6888135**	A	0.48	0.99 (0.84–1.16)	0.871	0.98 (0.81–1.18)	0.812	1.07 (0.77–1.47)	0.702
17	rs4382196	C	0.44	1.12 (0.95–1.31)	0.170	1.18 (0.98–1.43)	0.087	1.07 (0.78–1.46)	0.673
††	IVS3_116	del	0.07	0.92 (0.68–1.25)	0.591	0.82 (0.56–1.18)	0.277	1.25 (0.72–2.15)	0.427

CI, Confidence interval; OR, Odds ratio; No, corresponds to SNP location in Fig. 1b; Numbers of affected and unaffected individuals per phenotype are given in brackets; SNPs No 3, 8, 11, 12, 13, and 16 were genotyped with Illumina Sentrix HumanHap300 BeadChip, all others with MALDI-TOF MS. Significant p-values are indicated in bold.

*Eight of 1454 subjects were excluded from the analyses because of missing information for their asthma status.

†The stringent definition of non-atopic asthma using RAST class equivalent of 0 was applied.

‡Tagging SNP for rs2974703 ($r^2 = 1.00$).

§Tagging SNP for rs17208551 ($r^2 = 0.90$).

¶Tagging SNP for rs12653948 ($r^2 = 1.00$).

**Tagging SNP for rs4912782 ($r^2 = 1.00$).

††IVS3_116 is not published in HapMap and it is not displayed in Fig. 1b.

primary bronchial epithelial cells cultured under air–liquid interface conditions in comparison with submerged cultures (11). Asthma is a chronic inflammatory airway disease, and local inflammation is present even when the affected individual is not atopic. Our data show association of the *PCDH1* gene variants specifically with non-atopic asthma and they may suggest that this gene plays a role in the epithelial inflammation independent of atopy and may lead to changes in the differentiation and structure of the bronchial epithelial layer. Further functional studies are needed.

It is not surprising that SNP effects may be related to specific forms of asthma. The major asthma susceptibility signal so far identified in chromosome 17q21 correlates with childhood onset asthma but not adult asthma (6, 7). Furthermore, we could recently show that a SNP allele in *MMP-9* was associated with non-atopic asthma (12). Thus, exploring the influence of *PCDH1* polymorphisms on different subphenotypes of asthma and BHR may help to shed light on the complex mechanisms of asthma development and its relationship with BHR. As the associations with subphenotypes are novel, they have to be taken with caution. Replication of these observations is needed before conclusions can be drawn.

Taken together, our study indicated very specific effects of *PCDH1* SNPs on non-atopic asthma, while an effect of genetic variants in *PCDH1* on BHR was not replicated in our small underpowered subpopulation with BHR data available. Based on the association results, its expression in the airway epithelium and its function as an adhesion molecule, *PCDH1*, remain an interesting candidate gene for asthma and the intermediate phenotype of BHR. In light of our novel findings and previous results, one may hypothesize that *Protocadherin-1* plays a role in non-atopic asthma and possibly in specific forms of BHR. Thus, new knowledge on *PCDH1* function could also elucidate the mechanisms how BHR is related to asthma and how distinct forms of asthma develop.

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Authors contributions

Antoaneta A. Toncheva contributed to study design development and execution, data interpretation, and manuscript preparation; Kathrin Suttner drafted the first version of the manuscript and contributed to data interpretation; Sven Michel performed statistical analyses and contributed to data interpretation; Norman Klopp, Thomas Illig, and Tobias Balschun performed the MALDI-TOF MS genotyping; Christian Vogelberg, Andrea von Berg, Albrecht Bufe, Andrea Heinzmann, Otto Laub, Ernst Rietschel, Burkhard Simma, Thomas Frischer, and Jon Genuneit contributed to data collection; Erika von Mutius contributed to the study design of ISAAC and MAGICs and to data collection. Michael Kabesch developed the study design, participated in the collection of data, data analysis, and wrote the final version of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Table S1. Description of the technique used for genotyping for each of the studied phenotypes with the respective numbers of cases and controls in the analysis.

Table S2. Comparison of stringent and loose definitions of non-atopic asthma in the combined MAGICs and ISAAC II case-reference study population (n = 1454^{II}).

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SUPPORTING INFORMATION

GENETIC VARIANTS IN *PROTODHERIN-1*, BRONCHIAL HYPERRESPONSIVENESS AND ASTHMA SUBPHENOTYPES IN GERMAN CHILDREN

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Study populations

This study was performed using asthma cases derived from the Multicenter Asthma Genetics in Childhood (MAGIC) study (E1) and reference children randomly selected from the cross-sectional International Study of Asthma and Allergies in Childhood Phase II (ISAAC II) in Munich and Dresden (E2). Written informed consent was obtained from the parents of all children from both populations and the study protocols were approved by the ethics committees of each participating center.

ISAAC II

The ISAAC II study (E2) was conducted in Germany (Munich and Dresden) to assess the prevalence of asthma in school children at 9–11 years of age. A random subset of 800 German children was selected for a GWA study as previously described (E3). This random subset was investigated in this study for BHR, atopy, allergic rhinitis and atopic eczema and served as a reference to asthma cases derived from the MAGIC study in the GABRIEL consortium GWA study.

The sensitivity to common aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat epithelium, mixed grass and mixed tree pollen) in ISAAC II was assessed by skin prick test (SPT) and by *Sx1* test panel for specific IgE antibodies (ImmunoCAP, Phadia) against inhalatory allergens (local grass pollen, rye pollen, birch pollen, mugwort pollen, *Dermatophagoides pteronyssinus*, cat dander, dog dander, *Cladosporium herbarum*). The measurements from *Sx1* testing were in the range between 0.35-100 IU/ml.

To be consistent with the previous calculations on atopy in the ISAAC II study, the assessment of the atopy phenotype (Table1 from main body manuscript) was based on the

SPT results. A child was considered atopic if a wheal reaction ≥ 3 mm occurred to one or more allergens after subtraction of the negative control.

Next, to match the atopy definition of the MAGICS cohort which is based on specific IgE measurements (see section MAGICS below) more closely, the *Sx1* measurements additionally were used when ISAAC and MAGIC were analyzed together. In this case measurement of at least 3.5 IU/ml in the *Sx1* test was the criterion to consider a child atopic. To be assigned as non-atopic, a negative SPT was mandatory. Along with SPT a *Sx1* measurement of not more than 0.35 IU/ml was applied as a stringent criterion. For more loose cut-off a *Sx1* measurement of less than 3.5 IU/ml was applied (see the calculations for both criteria in table E2).

A child was considered as having allergic rhinitis and/or atopic eczema if a doctor's diagnose for these disorders was reported in the parental questionnaire.

Lung function was measured by MasterScope version 4.1 (Jäger, Würzburg, Germany) according to ISAAC II protocols. A minimum of two baseline spiromograms were recorded and the higher of two reproducible (within 5%) measurements of FEV₁ was recorded as baseline FEV₁. Bronchial reactivity was assessed in a 50% randomly selected subsample by changes in FEV₁ after inhalation of nebulized, hyperosmolar (4.5%) saline for increasing periods of time using ultrasound nebulizers (DeVilbiss Sunrise Medical, Langen, Germany). All children with a drop in FEV₁ of 15% or more from baseline after hypertonic saline challenge were classified as having bronchial hyperresponsiveness (BHR).

MAGICS

In the MAGIC study, 1255 children were recruited from seven German and Austrian clinical asthma centers as previously described (E1). Pediatric pulmonologists diagnosed asthma in

865 children (mean age 11 years) based on the physical examination, clinical history, lung function tests and allergy testing (skin prick testing and serum IgE measurement).

Atopy was assessed using the CLA[®] Allergen-Specific IgE Atopy 20 panel (Hitachi Chemical Diagnostics, Inc.) or AllergyScreen[®] (Mediwiss Analytic GmbH) for semiquantitative determination of circulating allergen-specific IgE concentrations in human serum. Since both techniques were based on a non-isotopic modification of the original RAST method, it allows classification of the semiquantitative results according to a system similar to the one used in RAST testing. Subjects in the MAGIC study were assigned as atopic if at least one positive result (RAST class equivalent >2) to any of the following common allergenes: *Dermatophagoides pteronyssinus*, *Dermatophagoide farinae*, cat epithelium, grass pollen, birch pollen, was obtained. Two different criteria were applied to define non-atopic asthma for the asthmatic individuals. First, a stringent criterion of complete lack of sensitization (RAST class equivalent of 0) was used. Additionally, a more permissive definition was used assigning all subjects with measurements resulting in RAST class equivalents of ≤ 2 to the group of non-atopic asthmatics. Calculations for both criteria are shown in table E2.

Children diagnosed as asthmatics with genotyping data from the GABRIEL consortium GWA study are analyzed here and compared to randomly selected children from the ISAAC study as previously described (E4). For the genotyping with Illumina Sentrix HumanHap300 BeadChip 1 311 (651 asthmatics) passed all subsequent quality controls. Due to continuous quality checks, drop outs and update of consent in a follow up study, the number of children from MAGICS contributing to this analysis is slightly smaller than that in previous studies (E1, E4)

Regenotyping by MALDI-TOF MS was successfully conducted for 1 454 individuals (763 asthmatics). Please, note that there is a difference between the total number of subjects genotyped by the above mentioned techniques. This is due to the still ongoing recruitment in the MAGIC study at the time of chip genotyping set up as well as due to the presence of drop outs which failed the quality controls in the chip genotyping even though successfully processed in MALDI-TOF MS genotyping. Therefore, the number of individuals analyzed for each of the investigated phenotypes varies due to the method of genotyping as described in table E1.

Table E1 Description of the technique used for genotyping for each of the studied phenotypes with the respective numbers of cases and controls in the analysis

Phenotype	Population where phenotype was assessed	BeadChip		Maldi-Tof MS	
		Cases	Controls	Cases	Controls
BHR	50% random sample of ISAAC II	68	265	71	273
Atopy	ISAAC II	183	515	192	536
Allergic rhinitis	ISAAC II	77	636	80	665
Atopic Eczema	ISAAC II	123	578	131	603
Asthma	MAGICS and ISAAC II	651	652	763	683
Atopic asthma	MAGICS and ISAAC II	468	408	548	426
Non-atopic asthma stringent definition	MAGICS and ISAAC II	98	408	113	426
Non-atopic asthma loose definition	MAGICS and ISAAC II	143	408	167	426

Table E2 Comparison of stringent and loose definitions of non-atopic asthma in the combined MAGICS and ISAAC II case-reference study population (n = 1,454^{II})

№	SNP	Reference allele	Allele frequency	<i>Non-atopic asthma stringent definition^a</i> (n = 113/426)		<i>Non-atopic asthma loose definition^b</i> (n = 167/426)	
				OR (95% CI)	P-value	OR (95% CI)	P-value
3	rs2974704*	A	0.10	0.46 (0.22-0.94)	0.032	0.41 (0.22-0.78)	0.007
4	rs12658441†	C	0.24	1.24 (0.88-1.73)	0.220	1.24 (0.92-1.67)	0.167
5	rs17097812	T	0.07	1.08 (0.60-1.97)	0.792	0.96 (0.56-1.63)	0.867
6	rs7719391	C	0.37	0.73 (0.53-1.00)	0.051	0.68 (0.51-0.89)	0.006
7	rs3935792	C	0.49	1.18 (0.88-1.59)	0.271	1.30 (1.00-1.68)	0.049
8	rs11167761	A	0.16	1.55 (1.03-2.34)	0.037	1.54 (1.07-2.22)	0.019
10	rs14359‡	C	0.20	0.93 (0.64-1.35)	0.692	1.07 (0.78-1.46)	0.693
11	rs3797054	G	0.37	0.79 (0.56-1.09)	0.151	0.69 (0.52-0.93)	0.013
12	rs3822357	T	0.06	0.74 (0.36-1.53)	0.415	0.79 (0.43-1.45)	0.446
13	rs10063472	T	0.20	0.89 (0.59-1.34)	0.566	0.86 (0.60-1.23)	0.413
14	rs10054186	C	0.21	1.09 (0.77-1.54)	0.633	0.97 (0.71-1.31)	0.825
15	rs12515587	G	0.14	0.79 (0.50-1.24)	0.305	0.88 (0.60-1.28)	0.495
16	rs6888135§	A	0.48	1.07 (0.77-1.47)	0.702	0.99 (0.75-1.31)	0.933
17	rs4382196	C	0.44	1.07 (0.78-1.46)	0.673	1.11 (0.85-1.46)	0.448
**	IVS3_116	del	0.07	1.25 (0.72-2.15)	0.427	0.83 (0.49-1.41)	0.497

CI - Confidence interval; OR – Odds ratio; № - corresponds to SNP location in figure 1B; Numbers of affected and unaffected individuals per phenotype are given in brackets; SNPs № 3, 8, 11, 12, 13 and 16 were genotyped with Illumina Sentrix HumanHap300 BeadChip, all others with MALDI-TOF MS; * tagging SNP for rs2974703 ($r^2=1.00$); † tagging SNP for rs17208551 ($r^2=0.90$); ‡ tagging SNP for rs12653948 ($r^2=1.00$); § tagging SNP for rs4912782 ($r^2=1.00$); ^{II} Eight out of 1,454 subjects were excluded from the analyses due to missing information for their asthma status; ** IVS3_116 is not published in HapMap and it is not displayed in Figure 1B; ^a For the stringent definition of non-atopic asthma RAST-class equivalent of 0 was used; ^b For the loose definition of non-atopic asthma RAST-class equivalents of ≤ 2 were used.

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