

Polymorphisms in human histamine receptor H4 gene are associated with atopic dermatitis

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Summary

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Conflicts of interest

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Background Atopic dermatitis (AD) is a chronic skin disease affecting more than 15% of children and 2% of adults. A strong connection between genetic factors and AD has been described for a long time. Histamine receptor H4 (HRH4) has been shown to be related to different kinds of allergic and autoimmune disorders. However, an association between HRH4 and AD has not yet been reported.

Objectives To examine a possible association between HRH4 and AD.

Methods Genomic DNA from 301 patients with AD and 313 healthy controls was extracted and three exons of HRH4 were sequenced.

Results We found three new single nucleotide polymorphisms (SNPs) in HRH4 which were significantly associated with AD: ss142022671 [odds ratio (OR) 1.87, 95% confidence interval (CI) 1.24–2.81; $P = 0.002$], ss142022677 (OR 4.40, 95% CI 2.42–8.00; $P = 1.5 \times 10^{-7}$) and ss142022679 (OR 4.26, 95% CI 2.38–7.61; $P = 1.3 \times 10^{-7}$). The SNPs ss142022677 and ss142022679 were found to be in strong linkage disequilibrium ($D' = 0.98$; $r^2 = 0.92$). Two-SNP haplotype analysis (ss142022677 and ss142022679) showed that the major AA haplotype was protective against AD (OR 0.22, 95% CI 0.12–0.40; $P = 3.1 \times 10^{-8}$) and the minor TT haplotype was significantly associated with AD (OR 4.13, 95% CI 2.27–7.54; $P = 6.6 \times 10^{-7}$). In addition, in a three-SNP haplotype analysis (ss142022671, ss142022677 and ss142022679), the major TAA haplotype was protective against AD (OR 0.46, 95% CI 0.31–0.69; $P = 0.0001$), while the complementary ATT haplotype was found to be significantly associated with AD (OR 3.81, 95% CI 2.03–7.14; $P = 8.3 \times 10^{-6}$).

Conclusions Polymorphisms of ss142022671, ss142022677 and ss142022679 in HRH4 are associated with AD.

Atopic dermatitis (AD) is a chronic relapsing allergic skin disease. AD affects 15–20% of children and 2–10% of adults.^{1,2} It is characterized by typically distributed eczematous skin lesions and intense pruritus. Many studies that focused on AD pathogenesis have shown that AD is a complex genetic disorder. Family and twin studies supported a significant genetic predisposition to AD.³ Furthermore, genome screens identified a number of chromosomal loci linking to AD including 1q21, 3p24–22, 3q14, 3q21, 13q14, 15q14–15, 17q21, 17q25, 18q21 and 20p.^{4–6} Recently, genome-wide association studies showed that a common variant on chromosome 11q13 is associated with AD.⁷ During the past decade, several genetic studies showed the association of AD with different genes

such as the NLR family genes, C3, CD14 and others.^{8–10} To date, filaggrin gene (FLG) mutations have been identified to be the most widely replicated genetic risks for eczema. More than 30 FLG mutations have been found in different populations.^{11–13}

Histamine plays an important role in allergic and autoimmune diseases. Among the four known histamine receptors (HRH1, HRH2, HRH3 and HRH4), HRH1, HRH2 and HRH4 have been shown to affect inflammation and other immune responses and have been used or proposed to be useful for treating immune and inflammatory diseases.^{14,15} HRH4 is expressed mainly in haematopoietic cells including mast cells, eosinophils, basophils, dendritic cells and T cells. Recently,

Dijkstra *et al.* showed that human inflammatory dendritic epidermal cells expressed a functionally active HRH4, and HRH4 agonists may have beneficial effects in downregulating inflammation which might have implications for the treatment of AD.¹⁶

Many recent studies have shown that HRH4 is related to different allergic disorders and autoimmune diseases, including asthma, colitis and systemic lupus erythematosus.¹⁷ There is accumulating evidence showing that histamine and mast cells which are the main source of histamine are associated with many autoimmune diseases. This effect may be not through HRH1 and HRH2 but through HRH4.¹⁷

The HRH4 gene was mapped to chromosome 18q11.2 by both genomic sequence analysis and radiation hybrid analysis.^{18,19} Haagerup *et al.* showed the linkage of AD to the chromosome locus 18q11–12 via a comprehensive genome scan.^{20,21} So far, no study has shown that HRH4 gene polymorphism was related to AD.

Materials and methods

Study populations

The present study included 301 patients with AD (116 males and 185 females; median age 11 years, range 2–38) and 313 healthy controls (122 males and 191 females; median age 22 years, range 1–45) (Table 1). All patients fulfilled the Hanifin and Rajka diagnostic criteria for AD.²² Healthy controls were individuals without allergic inflammatory disorders. The study was approved by the ethical committee of the Shenzhen Hospital, Peking University. The individuals gave

Table 1 Origin of candidates

	Patients with atopic dermatitis	Control individuals
Guangdong province	83	79
Hunan province	59	57
Guanxi province	38	47
Jiangxi province	31	33
Guizhou province	24	22
Sichuan province	21	19
Hubei province	12	16
Others	33	40
Total	301	313

their written informed consent. The investigations were conducted according to the Declaration of Helsinki principles.

Genotyping methods

Genomic DNA was isolated from the peripheral blood of the candidates using the Genomic DNA Extraction kit (Innogen, Shenzhen, China) according to the manufacturer's instructions. The three exons of HRH4 were amplified by polymerase chain reaction (PCR) using primers as shown in Table 2. The PCR reaction was carried out in a total reaction volume of 20 µL using the following amplification protocol: denaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 25 s and finishing with annealing and extension at 60 °C for 1 min. The PCR products were sequenced using both the forward and reverse primers. The sequencing results were aligned with NCBI sequence NT_010966.13 by NCBI alignment. Duplicate samples were included to check the accuracy of genotyping.

Quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from whole blood by using AxyPrep™ Blood Total RNA MiniPrep kit (Axygen, Union City, CA, U.S.A.) according to the manufacturer's instructions. First strand cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada). Quantitative PCR was performed using the BioRad Chromo4 real-time PCR system (BioRad, Hercules, CA, U.S.A.). The relative HRH4 mRNA level was presented by using the comparative C(T) method^{23,24} with GAPDH as the internal control. Logarithmic transformation [$\log_2 (1 + x)$] was used to transform primary data to normal distribution. Data from three independent experiments were analysed by Student's t-test and $P < 0.05$ was considered statistically significant. The primers for HRH4 are: forward, 5'-GTGGTTAGCATAGGTTATAC-3' and reverse, 5'-ATGCCACTGCACTCCTGC-3'. The primers for GAPDH are: forward, 5'-CAGCCTCAAGATCATCAGCA-3' and reverse, 5'-TGTGGTCATGAGTCCTTCCA-3'.

Protein stability assay

DNA constructs were transfected to 293T cells using Lipofectamine 2000 reagent (Invitrogen, San Diego, CA, U.S.A.)

Table 2 Primers for polymerase chain reaction (PCR) and sequencing of HRH4 (5' to 3')

	Forward	Reverse	PCR product size (bp)
Exon 1	TGGAGATGGATGCATCACAG	TCCCCAGTTGTATACAGTC	566
Exon 2	GCTTGATGAAATACCGTG	ATGGTGCATAGGTGAGTG	513
Exon 3	GTCCACATCGTGATACCCA	GGATACAAAAGAGGATTGAC	853
	GCTGTTTGCTGGGCTCCA	GACAAGCCATACTTTCAAC	911
	AGCGTAGTGTGGGAGAGGA	CTACTGCACTCCAGCCTG	863
	GCCTGGGTGTCAATAAT	CATAGGACCACTCCATTCC	1193

following the manufacturer's protocol. Twenty-four hours later, the cells were continuously treated with cycloheximide (final concentration 10 µg mL⁻¹). The cells were harvested at different time points after cycloheximide treatment. Protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blot.

Statistical analysis

Statistical analysis comparing the allele and genotype distributions was performed using χ^2 test or Fisher's exact test. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated according to Woolf's method, using the SPSS 10.0 software for Windows (SPSS, Chicago, IL, U.S.A.). $P < 0.05$ was considered statistically significant. Linkage disequilibrium pairwise values were obtained by Haploview software (Broad Institute, Cambridge, MA, U.S.A.). Haplotypes were constructed with the expectation-maximization algorithm implemented in the Unphased software package (Frank Dudbridge, Medical Research Council Human Genome Mapping Project Resource Centre, Hinxton, U.K.).

Results

We found three new single nucleotide polymorphisms (SNPs) in HRH4, and their positions relative to NCBI genomic contig NT_010966.13 are: 3529644T/A, 3546581A/T and 3546582A/T. These three SNPs were submitted to NCBI, and their accession numbers are ss142022671, ss142022677 and ss142022679, respectively.

Table 3 shows HRH4 ss142022671, ss142022677 and ss142022679 genotype and allele frequencies in patients with AD and healthy controls. Genotype frequencies were in Hardy–Weinberg equilibrium in the patients and controls. Statistically significant differences were observed in both

genotype and allele frequencies for all three SNPs in the patients with AD as compared with the controls.

SNPs ss142022677 and ss142022679 were found to be in strong linkage disequilibrium ($D' = 0.98$; $r^2 = 0.92$), as they are in consecutive positions (Fig. 1). Linkage disequilibrium between rs142022671, which is 16 937 bp away, and rs142022677 ($D' = 0.80$; $r^2 = 0.51$) and rs142022679 ($D' = 0.81$; $r^2 = 0.54$), respectively, was less strong.

So, we performed a haplotype analysis using only two SNPs, ss142022677 and ss142022679 (Table 4). The major AA haplotype was found at a higher frequency among the control subjects (OR 0.22, 95% CI 0.12–0.40; $P = 3.1 \times 10^{-8}$), suggesting this haplotype to be protective against AD. In contrast, the minor TT haplotype was more frequent among patients with AD (OR 4.13, 95% CI 2.27–7.54; $P = 6.6 \times 10^{-7}$), suggesting this haplotype to be significantly associated with AD.

Next, a three-SNP haplotype analysis was performed (Table 4). We found that the major TAA haplotype was protective against AD (OR 0.46, 95% CI 0.31–0.69; $P = 0.0001$). In addition, the complementary ATT haplotype was found to be significantly associated with AD (OR 3.81, 95% CI 2.03–7.14; $P = 8.3 \times 10^{-6}$), while the minor AAA haplotype was irrelevant to AD (OR 0.75, 95% CI 0.41–1.37; $P = 0.35$).

SNP ss142022671 is in intron 1 of HRH4, 35 bp upstream of the transcription start site of HRH4. This site is located inside a consensus transcription regulatory factor binding motif for Ets-1, a γ -interferon responsive element.¹⁸ We examined whether the SNP ss142022671 affects the transcription of HRH4. By quantitative PCR analysis, we found that HRH4 mRNA levels were significantly higher in the candidates with the SNP ss142022671 than in those without (Fig. 2).

SNPs ss142022677 and ss142022679 are consecutively located in exon 3. The TT haplotype results in the change of Lys to Leu at aa376, while the single SNP at either site results

Table 3 Genotype and allele frequencies of HRH4 single nucleotide polymorphisms (SNPs) ss142022671, ss142022677 and ss142022679 in patients with atopic dermatitis and healthy controls

SNP, population	No. of subjects	Genotype frequency, n (%)			P-value	Allele frequency, n (%)			OR (95% CI)
		Major homozygote	Heterozygote	Minor homozygote		Major allele	Minor allele	P-value	
ss142022671									
Genotype or allele		TT	TA	AA		T	A		
Cases	301	237 (78.8)	60 (19.9)	4 (1.3)	0.01	534 (88.7)	68 (11.3)	0.002	1.87 (1.24–2.81)
Controls	313	274 (87.6)	38 (12.1)	1 (0.3)		586 (93.6)	40 (6.4)		
ss142022677									
Genotype or allele		AA	TA	TT		A	T		
Cases	301	248 (82.4)	51 (16.9)	2 (0.7)	1.0×10^{-6}	547 (90.9)	55 (9.1)	1.5×10^{-7}	4.40 (2.42–8.00)
Controls	313	299 (95.5)	14 (4.5)	0 (0.0)		612 (97.8)	14 (2.2)		
ss142022679									
Genotype or allele		AA	TA	TT		A	T		
Cases	301	246 (81.7)	53 (17.6)	2 (0.7)	8.4×10^{-7}	545 (90.5)	57 (9.5)	1.3×10^{-7}	4.26 (2.38–7.61)
Controls	313	298 (95.2)	15 (4.8)	0 (0.0)		611 (97.6)	15 (2.4)		

OR, odds ratio; CI, confidence interval.

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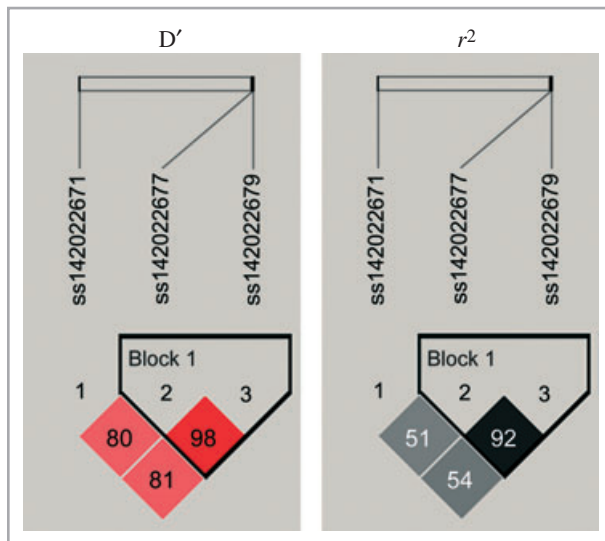


Fig 1. Linkage disequilibrium between the three HRH4 single nucleotide polymorphisms (SNPs). SNP 1 is ss142022671, SNP 2 is ss142022677, and SNP 3 is ss142022679.

in a stop codon (ss142022677) or Ile (ss142022679), respectively. To examine the biological effect of these variants, we made several constructs with mutations at the corresponding sites. We tested the protein stability of these mutations and, as shown in Figure 3, the truncated protein (ss142022677 with stop codon at aa367) was much more stable than the others. The stability of the other mutations (ss142022679 and double mutation both resulted in a single amino acid change) was similar to that of wild-type.

Discussion

The expression pattern of HRH4 implies that it has a role in immune and inflammatory responses. The clearest expression has been in eosinophils, mast cells and dendritic cells, which

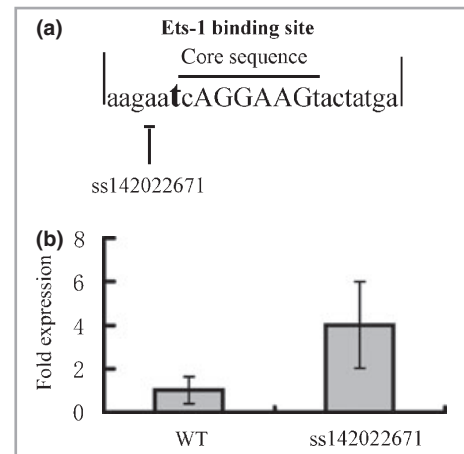


Fig 2. HRH4 mRNA levels are higher in the population with the single nucleotide polymorphism ss142022671. (a) The sequence of the Ets-1 binding site in the HRH4 promoter region. The sequence analysis was performed on the Genomatix website. (b) Total RNA was isolated from blood samples, and then reverse transcribed. SYBR green-based quantitative polymerase chain reaction was used to measure the relative mRNA levels of HRH4. Logarithmic transformation [$\log_2(1 + x)$] was used to transform primary data to normal distribution. The mean mRNA level in the wild-type (WT) population was set at 1. Data from three independent experiments were analysed by Student's *t*-test. Results are shown as mean $\pm$ SD, and the difference between WT and ss142022671 is significant at $P < 0.05$.

are the most important cell types involved in immune disorders and inflammation.^{19,25–27} HRH4 activation promotes the accumulation of inflammatory cells (particularly eosinophils and mast cells) to sites of allergic inflammation.²⁵ Experiments in mice showed that mast cells from wild-type and HRH3-deleted mice migrated in response to histamine, while mast cells from HRH4-deleted mice did not, indicating that HRH4-mediated chronic inflammatory effects of histamine may be essential in inflammatory disorders. Histamine as well as the HRH4 agonist clobenpropit induced an enhanced chemotaxis

Table 4 Frequencies of the most common ($> 2\%$) haplotypes formed by the HRH4 single nucleotide polymorphisms (SNPs) in patients with atopic dermatitis (AD) and healthy controls

Haplotype	Patients with AD, n (%)	Healthy controls, n (%)	P-value	OR (95% CI)
Two-SNP haplotype				
AA	542 (90.0)	611 (97.6)	3.1×10^{-8}	0.22 (0.12–0.40)
TT	52 (8.6)	14 (2.2)	6.6×10^{-7}	4.13 (2.27–7.54)
Others ($< 2\%$)	8 (1.4)	1 (0.2)	–	–
Three-SNP haplotype				
TAA	523 (86.9)	585 (93.4)	0.0001	0.46 (0.31–0.69)
ATT	45 (7.5)	13 (2.1)	8.3×10^{-6}	3.81 (2.03–7.14)
AAA	19 (3.2)	26 (4.2)	0.35	0.75 (0.41–1.37)
Others ($< 2\%$)	15 (2.4)	2 (0.3)	–	–

SNPs of the two-SNP haplotype are ss142022677 and ss142022679, and those of the three-SNP haplotype are (in sequence) ss142022671, ss142022677 and ss142022679. OR, odds ratio; CI, confidence interval.

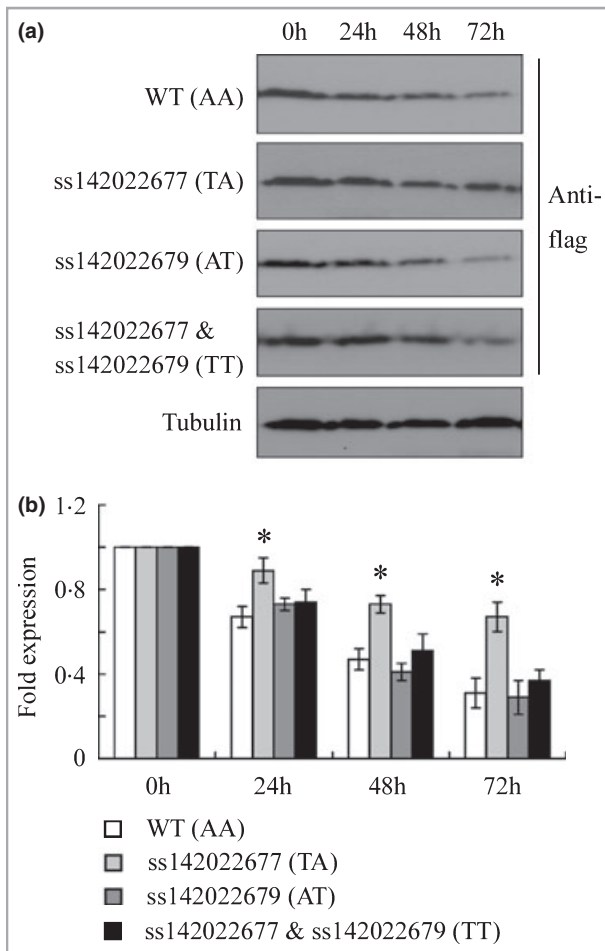


Fig 3. Protein stability assay for the single nucleotide polymorphisms ss142022677 and ss142022679. (a) Wild-type (WT) and mutated DNA constructs were transfected to 293T cells. The cells were treated with cycloheximide and harvested 24, 48 and 72 h later. Proteins were subjected to Western blot using anti-flag (for HRH4 constructs) or antitubulin antibodies. (b) Results from three independent experiments were quantified, and are shown as mean $\pm$ SD. Left to right for each time point: WT, ss142022677, ss142022679 and double mutation. The protein level at 0 h was set at 1. * $P < 0.05$ vs. the others.

in the skin dendritic cell migration assay. The enhanced chemotaxis was blocked by the HRH4 antagonist JNJ777120.²⁸ Thus, HRH4 represents an interesting candidate gene for chronic inflammatory skin diseases such as AD due to its important role for inflammatory and immunological processes. Our research is the first study examining the association of HRH4 SNPs with AD, although histamine has been identified as a key molecule for allergic diseases for a long time. We found three new SNPs in HRH4, and all these SNPs were significantly associated with AD.

Recently, some studies elucidated the function of HRH4 in different cell types. Horr *et al.* showed a constitutive down-regulation of STAT1 in peripheral blood mononuclear cells from atopic individuals. This may be due to histamine acting on HRH4 to inhibit STAT1 activation and drive the Th2 polar-

ization.²⁹ A decrease of Th2-type immune responses-associated interleukin 12p70 production was also mediated via HRH4.²⁶ These previous results indicated that signalling transduction of HRH4 is essential in inflammatory response. In our study, the two SNPs (ss142022677 and ss142022679) are consecutively located in exon 3 with strong linkage disequilibrium. The TT haplotype results in the change of Lys to Leu at aa376, while the single SNP at either site results in a stop codon (ss142022677) or Ile (ss142022679), respectively. The truncated protein (ss142022677 with stop codon at aa367) was much more stable than the others, suggesting that polymorphism ss142022677 possesses biological effects. On the other hand, the change of amino acid residue (ss142022679 and double mutation both resulted in single amino acid change) may possibly affect the intracellular signal transduction of HRH4 and also change the chemotaxis of inflammatory cells. But, so far in our study, the subsequent cellular events induced by the mutated HRH4 protein need to be elucidated further. This provides us a future direction of study on HRH4.

In the two-SNP (ss142022677 and ss142022679) and three-SNP (ss142022671, ss142022677 and ss142022679) haplotype analysis, the major TAA haplotype (OR 0.46, 95% CI 0.31–0.69; $P = 0.0001$) and AA haplotype (OR 0.22, 95% CI 0.12–0.40; $P = 3.1 \times 10^{-8}$) had similar protective effects against AD, while the complementary ATT haplotype (OR 3.81, 95% CI 2.03–7.14; $P = 8.3 \times 10^{-6}$) and TT haplotype (OR 4.13, 95% CI 2.27–7.54; $P = 6.6 \times 10^{-7}$) were both associated with AD. The statistical results did not change with consideration of the SNP ss142022671. This is not unexpected as the other two SNPs (ss142022677 and ss142022679) were located in consecutive positions, and SNP ss142022671 was 16 937 bp away.

However, it is possible that these SNPs are in linkage disequilibrium with other disease-causing variants in a nearby gene. According to information from Ensembl genome browser, one gene named IMPACT (Chr 18: 22 006 609–22 033 492) is located in the vicinity of HRH4 (Chr 18: 22 040 593–22 059 920). HapMap data showed 22 SNPs in IMPACT. We examined IMPACT, but no SNP in IMPACT was found to associate with AD (data not shown). Thus, the association signal should be centred on HRH4.

In this study, we found that SNP ss142022671 affects the transcription of HRH4, implying a possible association of the SNP ss142022671 with higher allergic susceptibility. In general, HRH4 expression level is rather low and may be regulated by inflammatory stimuli.¹⁷ Upregulation of HRH4 may result in a stronger response to histamine, and subsequently induce accumulation of inflammatory cells.

In conclusion, the results of this study suggest that genetic variation in HRH4 contributes to the pathogenesis of AD. Therapeutic targeting of HRH4 has already been suggested as a promising new opportunity to modulate allergic responses. Future research on the function of this receptor might help to identify new candidates for therapeutic targeting. Also, the information on SNPs of HRH4 may provide a potential criterion for diagnosis of AD, or help define the population with high

susceptibility to AD. On the other hand, our study only represents the Chinese population. Although China possesses one-fifth of the world's total population, in the future it would be extremely important to perform replication studies in other countries. Also, more functional studies are needed to illustrate the consequences of these SNPs.

What's already known about this topic?

- Atopic dermatitis is a chronic skin disease with strong association with genetic factors.

What does this study add?

- Three new single nucleotide polymorphisms in the histamine receptor H4 gene (HRH4) were found to be significantly associated with atopic dermatitis, indicating a role for HRH4 in development of this disease.

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