Published in final edited form as:

Blood Cells Mol Dis. 2007 ; 39(3): 353–360. doi:10.1016/j.bcmd.2007.06.013.

Human Chitotriosidase Polymorphisms G354R and A442V Associated with Reduced Enzyme Activity

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Abstract

A common polymorphism in the chitotriosidase gene (*CHIT1*) exists in which a 24 bp duplication in exon 10 results in aberrant splicing and deletion of 87 nucleotides. In this study the gene frequency was found to be 0.56 (n=2054) in subjects of Asian ancestry, 0.17 (n=984) in subjects of European ancestry and 0.07 (n=536) in subjects of African ancestry. Notably, the median enzyme activity in wildtype subjects (TT) was much higher in subjects of European ancestry (2.69 mU/ml, n=202 subjects), than subjects of African (2.57 mU/ml, n=230 subjects) ($P < 0.0001$) and Asian ancestry (0.86 mU/ml, n=114 subjects) ($P < 0.0001$). The question of why chitotriosidase deficiency exists at such a high frequency is a challenging one. We postulated that if there were a selective advantage for chitotriosidase deficiency then there would be polymorphisms that would be associated with reduced enzyme activity independent of the 24 bp duplication. We found that the G102S and the A442G polymorphisms found occurring in subjects of all ancestries were not significantly associated with a reduction of enzyme activity. In contrast, the G354R ($P < 0.0001$) and the A442V ($P = 0.0013$) polymorphisms occurring predominantly in subjects of African ancestry were significantly associated with reduced chitotriosidase activity. We also investigated the possibility that chitotriosidase deficiency was associated with tuberculosis or with atopy, including allergic rhinitis, contact dermatitis, food or drug allergies and asthma.

Keywords

chitotriosidase; polymorphism; tuberculosis; allergy; deficiency

Introduction

Chitin, a polymer of N-acetyl glucosamine and glucosamine, is the second most abundant structural compound found in nature, only cellulose being more widespread. It is present in fungi, nematodes, insects and shellfish. Chitin can be hydrolyzed by a number of chitinases. In man, chitotriosidase (*CHIT1*) is a chitinase primarily expressed in neutrophils and macrophages [1] *CHIT1* is present in normal plasma [2] and it was found that its activity was increased more than 1000-fold in some patients with Gaucher Disease. [3]. Plasma *CHIT1* activity is also elevated, although to a much lesser degree, in other lysosomal storage diseases [4], thalassemias [5], and in the serum of patients with arteriosclerosis [6]. *CHIT1* mRNA

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expression is markedly upregulated following stimulation by prolactin [7], tumor necrosis factor alpha, gamma interferon, and lipopolysaccharide [8].

CHIT1 is thought to play a role in innate immunity. *In vitro* studies with recombinant human chitotriosidase have demonstrated that CHIT1 lyses the hyphae tip of *Mucor rouxii* and inhibits the growth of hyphae of *Candida albicans* [1]. *In vivo*, neutropenic mice challenged with lethal doses of *Candida albicans* or Aspergillosis exhibited increased survival when treated with human recombinant chitotriosidase [1]. Recent studies by Di Luca *et al* demonstrated that human CHIT1 is capable of cooperating with the *Plasmodium falciparum* (malarial) chitinase to break down the chitin containing peritrophic membrane in the *Anopheles* mosquito midgut [9]. These studies all demonstrate that human CHIT1 has chitinase activity against natural substrates. Nevertheless, epidemiologic studies in man have not been as clear in demonstrating a role for CHIT1 in protection against fungal and parasitic infection.

A common polymorphism in the chitotriosidase gene exists in which a 24 bp duplication in exon 10 results in aberrant splicing and deletion of 87 nucleotides [10]. The resulting phenotype is chitotriosidase deficiency. The deficiency is very uncommon in Africa where parasitic disease is endemic. The deficiency is more common in Continental France [11], Sicily [12] and Asia [13]. This has led many to suggest that the difference in gene frequency is due to a selective pressure to maintain a functional chitotriosidase gene. Nevertheless, two studies have come to opposing conclusions in demonstrating an association with chitotriosidase deficiency and susceptibility to filarial infections in Papua New Guinea [14], and India [15] despite the fact that filariasis is endemic in both populations. Studies have also demonstrated a lack of association of chitotriosidase deficiency and incidence of malaria in Taiwan [13] and in areas around the Mediterranean [11]. Furthermore, in a study with immunocompromised leukemia patients, there was a lack of association of chitotriosidase deficiency with candidiasis infections [16–18]. Thus, it is likely that other mammalian chitinases, such as acidic mammalian chitinase (AMCase) expressed in alveolar macrophages and intestinal epithelia [19], are sufficient to protect humans from fungal infections in the absence of chitotriosidase.

Why chitotriosidase deficiency exists at such a high frequency is intriguing. As is usually the case, the possibilities include random drift, on the one hand, and a selective advantage on the other. If chitotriosidase deficiency provided a selective advantage against infection, it is likely that other mutations that also cause chitotriosidase deficiency also exist, albeit at much lower allele frequencies and possibly only as regional mutations. This is the case in most balanced polymorphisms such as Tay-Sachs disease [20], Gaucher disease [21], cystic fibrosis [22], and glucose 6 phosphate deficiency [23], to mention only a few.

In this study, we examined the allele frequency of subjects of European, Asian and African ancestry compared to enzyme activity in order to determine if there was any evidence for chitotriosidase deficiency that was not associated with the presence of the 24 bp duplication in exon 10. We also investigated the possibilities that chitotriosidase deficiency was associated with tuberculosis or atopies, including allergic rhinitis, contact dermatitis, food or drug allergies and asthma.

Materials and Methods

All samples from human subjects were obtained with consent and with approval from The Institutional Review Boards of The Scripps Research Institute and Kaiser Permanente. The DNA samples were part of a large DNA Bank described previously [24,25]. Self reported ethnicities, history of tuberculosis with or without INH treatment, positive tuberculin skin test results and a history of allergies were used.

Chitotriosidase Genotyping

Genotyping for the 24 bp insertion in exon 10 of the chitotriosidase gene was performed with amplification using primers: 5'-GAAGAGGTAGCCAGGCTCTGG-3' and 5'-CTGCCGTAGCGTCTGGATGAG-3' in a 50 μ l reaction containing 1x PCR Buffer (Qiagen), 0.2 mM dNTP's, 150 ng of each primer, 0.15U Taq polymerase (Qiagen) and 5–50 ng of template DNA. The DNA was denatured at 95°C for 4 min for 1 cycle, and amplified at 95°C for 1 min, 60°C for 30s, and 72°C for 30s for 35 cycles. The amplified DNA was separated on an 8% acrylamide/0.5x TBE gel and visualized by ethidium bromide staining. The genotype was determined by the presence or absence of a 195 bp wildtype band or a 219 bp mutant band.

Genotyping for the c. 304G>A (G102S) in exon 4, c.1072G>A (G354R) in exon 10, and the c. 1325C>G/T (A442G or V) in exon 12 polymorphisms was performed by allele specific oligomer hybridization (ASOH). Amplification was performed using the conditions described above. The primers used to amplify exon 4 were: CHIT Ex4F ACATAGGCACTTTTCACACGTC and CHIT exon4R AACAGCCTGGAGCAAAGCTC, for exon 10 as described above, for exon 12: CHIT Ex12F: GAACCTGAGCATGGCCCCAG and CHIT Ex12R TTCCAGGTGCAGCATTTGCAG. The probes used for ASOH were: 304G>A: TGGAAAT TTC G/A GCACTCAG ; for 1072G>A: ACTGGGC G/A GGGCCATA; for 1325C>G/T: TGTGCAG C/G/T GGGGCGG.

Chitotriosidase Activity

Chitotriosidase activity was determined as described [3] by cleavage of an artificial substrate, 4-methyl umbeliferyl (4-MU) fl-D-NN,N'-Triacetylchitotriose. Essentially, 5 μ l of EDTA plasma was incubated with 100 μ l 22 μ M -methyl umbeliferyl (4-MU) β -D-N,N',N'-(MU)- fl-D-NN,N'-triacetylchitotriose in citrate-phosphate buffer (0.1M/0.2M) pH 5.2 for 15 min at 37°C. The reaction was stopped with the addition of 1.9 ml of 0.3M glycine/NaOH pH 10.6. Release of 4-MU by plasma chitotriosidase was measured using a Turner Model 112 fluorometer (excitation filter: 0-51, 7-37; emission filter: 3-73) at 0 and 50% neutral density, as required. Enzyme activity (mU/mL) = fluorescence $\times$ standard $\times$ 2ml/(15 min $\times$ 0.005 ml).

Statistical Analyses

All statistical analyses were performed using Prism GraphPad v. 5.0 (GraphPad Software, Inc., San Diego, CA). All enzyme activity data were analyzed using the Mann Whitney non-parametric test assuming a non-Gaussian distribution. The Fisher's exact test was used for studies examining association of chitotriosidase genotype with disease. All P values are two-tailed.

Results

We documented the 24 bp insertion polymorphism that leads to aberrant mRNA splicing and the deletion of 87 nucleotides of the chitotriosidase (CHIT) gene in subjects of European, Asian and African ancestry and found the highest allele frequency was found among those of Asian (0.56) with lower frequencies among those with European (0.17) and African ancestry (0.07) (Table 1). Across Asia, the highest frequency was in South China followed by Korea, Southeast Asia, Japan, and Taiwan. The subjects with European ancestry was comprised of 43.9% from Western Europe, 3.7% from Northern Europe, 4.1% from Eastern Europe, 4.5% from Southern Europe, 1.0% from Russia and 39.6% of mixed European ancestry.

As shown in Figure 1, the mean CHIT enzyme activity was approximately half normal in heterozygotes (HT) and virtually absent in homozygous mutant (HH) subjects. Notably, the median enzyme activity in wildtype subjects (TT) was significantly higher in subjects of European ancestry (2.69 mU/ml, n=202 subjects), than subjects of African (2.57 mU/ml, n=230

subjects) ($P < 0.0001$) and Asian ancestry (0.86 mU/ml) ($P < 0.0001$). Among homozygous wildtype (TT) or heterozygous (HT) subjects examined, there were clearly some subjects who had little or no enzyme activity, particularly of Asian and African ancestry (Figure 1). Although it is possible that some of these samples lacked enzyme activity due to poor handling at the time of acquisition, it was also possible that other mutations or polymorphisms might exist that would account for the enzyme deficiency.

The DNAs of eleven subjects who were heterozygous or wildtype for the 24 bp insertion polymorphism but exhibited little or no chitotriosidase activity were subjected to direct sequence analyses to identify possible mutations in the coding region or proximal promoter region. We found several polymorphisms that were previously documented in the Genbank SNP Database, A442G or V (exon 12), G354R (exon 10), G102S (exon 4) and a promoter polymorphism -1432A>G. The Q171H polymorphism listed in this database was not observed.

Genotyping analyses were performed in order to examine the allele frequencies of these particular coding region polymorphisms and possible haplotypes. Genotyping analyses revealed that the G102S (c. 304G>A) allele was in marked linkage disequilibrium in repulsion with the H allele (24 bp duplication). The gene frequency of the G102S allele was similar in subjects of different ancestries (Table 2). The G354R (c.1072G>A) allele was never observed with the 24 bp duplication H allele but the low frequency of the G354R allele prevented further linkage analyses. The G354R allele was found predominantly in subjects of African ancestry (Table 2). The A442G (1325C>G) allele was found predominantly but not exclusively with the wildtype T allele in subjects of all ancestries. In contrast the A442V (1325C>T) allele was never observed with the H allele and was found predominantly in subjects of African ancestry (Table 2). In our study, three haplotypes were associated with the H allele out of ten total haplotypes that could be definitively identified due to the presence of homozygosity or heterozygosity in no more than one position (Table 3).

We examined each of these coding region polymorphisms for association with enzyme activity in subjects without enzyme deficiency associated with the 24 bp duplication in exon 10 (i.e. TT wildtype subjects). We found that the G102S (Figure 2) and the A442G polymorphisms were not significantly associated with a reduction of enzyme activity, but the G354R ($P < 0.0001$) (Figure 3) and the A442V ($P = 0.0013$) (Figure 4) polymorphisms were significantly associated with reduced serum chitotriosidase activity.

In an examination of the 492 control subjects and 188 subjects of European ancestry with a positive skin test for tuberculosis, we found a statistically significant excess of subjects who were homozygous for the chitotriosidase deficiency (HH) in the tuberculosis group (gene frequency = 0.07) than the control group (gene frequency = 0.02) (Table 4). Since Asians exhibited a much higher frequency of chitotriosidase deficiency than subjects of European or African ancestry, we decided to determine if the association with chitotriosidase deficiency with tuberculosis susceptibility could be independently verified in this cohort. Of the 931 subjects of Asian ancestry genotyped, 368 had reported a positive TB skin test (Table 4). There was no statistically significant association between chitotriosidase deficiency and susceptibility to tuberculosis as was found in subjects of Asian ancestry.

To determine whether patients with atopy have an increased prevalence of chitotriosidase deficiency we studied patients who reported having one or more of the following medical indications: allergic rhinitis, contact dermatitis, drug or food allergies, or asthma. We found that there were fewer Asian subjects homozygous for chitotriosidase deficiency than wildtype controls (10.1% vs 6.6%) that suffered from 3 or more atopic conditions, suggesting that chitotriosidase deficient subjects were less likely to suffer from atopy (Table 5A). A similar

result was seen for subjects of European ancestry although the data were not statistically significant (Table 5B).

Discussion

In the current study, we genotyped 1027 subjects of Asian ancestry, 305 subjects of European ancestry and 268 subjects of African ancestry. We found that the frequencies of the 24 bp duplication leading to an in frame deletion of 87 nucleotides and enzyme deficiency were significantly different between the groups, being highest among Asians and lowest among African subjects. The observed allele frequency of subjects of European ancestry (0.17) was comparable to previously published allele frequencies in European subjects that ranged from 0–27%. The mutant HH genotype frequency in our study was 3.0% in comparison to the reported range from 0–9% in other studies with subjects of European ancestry [11]. The observed distribution of genotypes fell within Hardy Weinberg predictions. We also found that the mean enzyme activity for wildtype (no 24 bp duplication) subjects of European descent to be higher than subjects of African descent and lowest in subjects of Asian descent. This is in contrast to what has been reported by Barone *et al* [26] who observed that African Blacks exhibited higher CHIT enzyme activity than Caucasians (geographic origin not stated).

We identified two polymorphisms in chitotriosidase, G354R and A442V found predominantly in subjects of African ancestry to be associated with reduced chitotriosidase activity. The glycine at position 354 is conserved between human, mice, and frog suggesting a selective pressure to maintain a glycine in this position. The alanine at position 442 is a glycine in mouse and frog. Our data demonstrate that an A442G polymorphism in humans was not associated with reduced chitotriosidase activity but that the A442V polymorphism was associated with reduced enzyme activity. The presence of the G354R and A442V polymorphisms in man at measurable frequencies demonstrate that there are polymorphisms in chitotriosidase other than the 24 bp duplication that are associated with reduced enzyme activity. Our data also indicate that there may be additional polymorphisms, possibly in the promoter region, or cryptic splice sites that might be associated with reduced enzyme activity, particularly in subjects of Asian ancestry and wildtype for the 24 bp duplication.

Previous attempts to associate a clinical phenotype with partial or complete chitotriosidase deficiency have been based upon the hypothesis that deficient individuals are at some type of disadvantage, such as increased susceptibility to infection with microorganisms that contained chitin, for example, candida [17,18], filarial [14,15] “parasitic diseases” [12], and gram negative infections in children with leukemia [16]. While most of the previous studies designed to show that chitotriosidase deficiency causes increased susceptibility to infection have been negative, even in the studies in which statistically significant results are reported, the differences observed are small and unconvincing. Perhaps having chitotriosidase deficiency is not a disadvantage but rather a selective advantage. Given the very high gene frequency for the main mutant allele, and, as shown in the present study other polymorphic alleles that decrease chitotriosidase activity, it seems more likely that in humans selection has occurred in the direction of decreasing or eliminating the activity of this enzyme, and thus it seems reasonable to seek an advantage not of sufficient enzyme activity, but rather of deficient enzyme activity. Indeed, among some Asian populations the allele frequency of the insertion mutation is higher than that of the allele without the insertion, and thus the so-called mutant H allele might properly be considered to be the wildtype.

How could a deficiency of chitotriosidase confer a selective advantage? Chitins have been shown to stimulate macrophages to produce IL-12, IL-18, and TNF α [27] and has been shown to act as an immunological adjuvant [28]. Shibata *et al* demonstrated that chitins had TH-1 adjuvant activity in developing immunity against a mycobacterial antigen [29]. This suggests

that chitotriosidase might modulate the circulating concentration of chitins and regulate immune responses. Thus, chitotriosidase deficiency might provide individuals with an advantage in mounting an immune response against mycobacteria including tuberculosis. Studies in mice by Shibata et al [30] demonstrated that intratracheal administration of chitin reduced lung epithelial response to ragweed. Chitin was recently shown by Reese *et al* [31] to induce the tissue accumulation of innate immune cells such as eosinophils and basophils which express IL-4. These data suggest that chitinase might have an adverse effect on allergic responses. This may indeed be the case since polymorphisms in the chitinase AMCase have been associated with asthma [32]. Nevertheless, polymorphisms in chitotriosidase have been shown to not be associated with asthma [33].

Exhibiting a positive TB skin test is a surrogate indicator for identifying subjects who have been infected with the tuberculosis mycobacterium and were able to mount an immune response. Hypothetically, if chitotriosidase deficiency, would in effect, increase plasma chitin levels, then we might observe an increase in immune reactivity toward tuberculosis in subjects. Although among subjects of European ancestry we observed a higher percentage of homozygotes for the chitotriosidase deficient genotype in the tuberculosis group as compared to the control group, this was not observed in subjects of Asian ancestry. Accordingly, it seems unlikely to us that resistance to infection with tuberculosis has been a selective factor in causing a high gene frequency for the chitotriosidase deletion.

We addressed the question as to whether chitotriosidase deficiency had any association with atopic allergies, including allergic rhinitis, contact dermatitis, drug or food allergies, and asthma. We found that subjects homozygous for chitotriosidase deficiency were less likely to suffer from atopic allergies.

In conclusion, our data reveal that there are polymorphisms other than the 24 bp duplication occurring at low allele frequencies in subjects of African ancestry that are associated with reduced chitotriosidase activity. Our hypothesis, suggesting that there might have been a selective advantage for the presence of chitotriosidase deficiency resulting in the high frequency of the 24 bp duplication in all subjects, is supported by the presence of additional polymorphisms that result in reduced chitotriosidase activity. Suggestive data have been obtained that chitotriosidase deficiency may protect against atopy by modulating the immune response, but more studies in an independent group of patients are required.

Acknowledgments

This is manuscript 18949-MEM from The Scripps Research Institute. Supported by the National Institutes of Health grants DK53505-09, PO1 CI000095-04 and the Stein Endowment Fund.

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Figure 1. CHIT1 Enzyme activity and genotype of the 24 bp duplication in exon 10

The genotype of the 24 bp duplication in exon 10, homozygous wildtype (TT), heterozygous mutant (HT) and homozygous mutant (HH) were analyzed for enzyme activity as described in *Methods*. All enzyme activity levels of zero (0) were assigned a value of 0.01 in order to include them for the log₁₀ scale. Non-parametric analyses were performed using the Mann-Whitney test. All P values are two-tailed. The line indicates the median enzyme activity.

Figure 2. CHIT1 enzyme activity and G102S genotype. All subjects are wildtype for the 24 bp duplication

The G102S (nt 304 G>A) genotypes were determined by allele specific oligomer hybridization as described in *Methods*. The genotypes are: homozygous G102 (nt 304 GG), homozygous S102 (nt 304 AA) and heterozygous G102S (nt 304 GA). All enzyme activity levels of zero (0) were assigned a value of 0.01 in order to include them for the $\log_{10}$ scale. Non-parametric analyses were performed using the Mann-Whitney test. All P values are two-tailed. The line indicates the median enzyme activity.

African Ancestry CHIT nt 1072 G>A (G354R)

	GG	GA	AA
Median	2.27	0.90	0.08
n	143	33	2

P Value GG vs GA <0.0001

Figure 3. CHIT1 enzyme activity and G354R genotype. All subjects are wildtype for the 24 bp duplication

The G354R (nt 1072 G>A) genotypes were determined by allele specific oligomer hybridization as described in *Methods*. The genotypes are: homozygous G354 (nt 1072 GG), homozygous R354 (nt 1072 AA) and heterozygous G354R (nt 1072 GA). All enzyme activity levels of zero (0) were assigned a value of 0.01 in order to include them for the log₁₀ scale. Non-parametric analyses were performed using the Mann-Whitney test. All P values are two-tailed. The line indicates the median enzyme activity.

African Ancestry CHIT nt 1325 C>G/T (A442G or V)

Figure 4. CHIT1 enzyme activity and A442G and A442V genotypes. All subjects are wildtype for the 24 bp duplication

The A442G (nt 1325 C>G) and A442V (nt 1325 C>T) genotypes were determined by specific oligomer hybridization as described in *Methods*. The genotypes are: homozygous A442 (nt 1325 CC), homozygous G442 (nt 1325 GG), heterozygous A442G (nt 1325 CG), heterozygous A442V (nt 1325 CT), homozygous V442 (nt 1325 TT). All enzyme activity levels of zero (0) were assigned a value of 0.01 in order to include them for the log₁₀ scale. Non-parametric analyses were performed using the Mann-Whitney test. All P values are two-tailed. The line indicates the median enzyme activity.

Table 1

Allele Frequency of CHIT1 24 bp insertion in exon 10 associated with chitotriosidase deficiency in different ethnic groups.

CHIT1	24 bp insertion
	Allele Frequency
European	164/984 (0.17)
African	36/536 (0.07)
Asian	1150/2054 (0.56)
Korea	47/80 (0.58)
Japan	176/326(0.54)
North China	18/36 (0.50)
South China	173/272 (0.64)
Southeast Asia	581/1020 (0.57)
Taiwan	18/40 (0.45)
India	27/74 (0.37)
Middle East	9/26 (0.35)

Table 2

Allele frequencies of CHIT G102S, G354R, A442G and A442V polymorphisms in different ethnic groups.

CHIT1	nt 304 G>A (G102S)	nt 1072 G>A (G354R)	nt 1325 C>G (A442G)	nt 1325 C>T (A442V)
	Allele Frequency	Allele Frequency	Allele Frequency	Allele Frequency
European	49/180 (0.27)	1/426 (0.002)	65/598 (0.11)	2/598 (0.003)
African	39/150 (0.26)	39/432 (0.09)	58/522 (0.11)	44/522 (0.08)
Asian	213/904 (0.24)	0/362 (0.0)	16/382 (0.04)	0/382 (0.0)

Table 3

List of CHIT1 Haplotypes. Haplotypes were identified from four polymorphic positions in exon 4, 10 and 12.

Haplotype	Exon 4 304 G/A	Exon 10 24 bp H/T	Exon 10 1072 G/A	Exon 12 1325 C/G/T
1	G	H	G	C
2	G	H	G	G
3	A	H	G	C
4	G	T	G	C
5	A	T	G	C
6	A	T	G	G
7	G	T	A	G
8	G	T	A	T
9	G	T	G	G
10	G	T	G	T

Table 4

Association of the CHIT1 24 bp insertion and Lung Disease in subjects of European Ancestry and Asian ancestry. Two tailed P values were determined using Fisher's Exact Test.

CHIT1	24 bp insertion	No TB	+ TB skin test	P value
European Ancestry	allele frequency	164/984 (0.17)	87/376 (0.2314)	0.0078
	homozygous mutant frequency	12/492 (0.02)	13/188 (0.07)	0.0103
Asian Ancestry	allele frequency	629/1126 (0.5586)	403/736 (0.5476)	ns
	homozygous mutant frequency	176/563 (0.3126)	81/368 (0.2201)	ns

Table 5

Table 5A. All Asians – Atopic * Conditions				
N=1021				
	Chitotriosidase Genotype			p **
	HH (n=327)	HT (n=489)	TT (n=205)	
Has at least 1 atopic condition	176 (53.8%)	275 (56.2%)	127 (62.0%)	0.065
Has 3 or more atopic conditions	33 (10.1%)	53 (10.8%)	34 (16.6%)	0.028

Table 5B. All Whites – Atopic * Conditions				
N= 492				
	Chitotriosidase Genotype			p **
	HH (n=12)	HT (n=140)	TT (n=340)	
Has at least 1 atopic condition	8 (66.7%)	83 (59.3%)	211 (62.1%)	0.99
Has 3 or more atopic conditions	0 (0%)	25 (17.9%)	58 (17.1%)	0.36

* Includes asthma, eczema, allergy to medications, allergy to cosmetics, allergic rhinitis/hay fever, and frequent sneezing, watering, or nasal congestion

** Chi square for TT vs. HH

* Includes asthma, eczema, allergy to medications, allergy to cosmetics, allergic rhinitis/hay fever, and frequent sneezing, watering, or nasal congestion

** Fisher's exact test TT vs. HH