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Association between reduced copy-number at T-cell receptor gamma (*TCRγ*) and childhood allergic asthma: a possible role for somatic mosaicism

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Abstract

Asthma is a chronic inflammatory disease of the lungs which affects more than 6.5 million American children. A family-based genome-wide association study of copy-number variation identified an association between decreased copy-number at *TCRγ* and childhood allergic asthma. *TCRγ* encodes the T-cell receptor gamma glycoprotein, a cell-surface protein found on T-cells and involved in cell-mediated immunity. Using quantitative real-time PCR, we sought to determine if copy-number variation at *TCRα*, *TCRβ* or *TCRγ* was associated with childhood allergic asthma in an independent cohort of 94 cases and 455 controls using DNA from buccal swabs. Copy-number variation at these loci is well-known, but appears to be dominated by somatic mutations. Genotyping results indicated that copy-number variants at these genes are largely somatic mutations, as inheritance did not show Mendelian consistency. In these mosaic cell populations, copy-number was significantly reduced among asthmatic children at *TCRγ* ($p = 0.0199$), but was not associated at *TCRα* or *TCRβ* ($p = 0.7972$ and 0.8585 , respectively). These findings support the association between reduced copy-number at *TCRγ* and childhood allergic asthma. Further work is needed to resolve whether reduced copy-number at *TCRγ* predisposes individuals to asthma, or whether deletion of this gene is a somatic response to the disease.

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Conflict of Interest Statement

The authors do not have any conflicts of interest, financial or otherwise.

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Keywords

T-cell receptor gamma; Copy Number Variant; allergic asthma; mosaicism

Introduction

Asthma is a chronic inflammatory disease of the lungs, characterized by airway hyper-reactivity, excess mucus secretion, and air flow obstruction and is one of the most common childhood diseases. In the United States in 2005, 6.5 million children under the age of 18 had been diagnosed with asthma.[1] Asthma has long been recognized as having a familial component, as children of allergic and asthmatic mothers are at higher risk of becoming asthmatic than children of unaffected mothers.[2–4] Many candidate genes have been associated with a variety of childhood asthma phenotypes[5–10] and the results of four successful genome wide association studies have recently been reported.[11–14] It appears that many genes are associated with various asthma phenotypes, and that their effects may be modified by environmental exposures.[15]

In studies of disease etiology, recent attention has been given to the role of copy-number variants (CNVs), defined as gains or losses of chromosome regions involving more than 1000 nucleotides (i.e. >1 kb).[16,17] These structural variants can alter gene dosage, and can either be inherited from parents, arise de novo, or be somatically acquired. Somatically acquired CNVs will be harbored by some cells, while other cells remain copy-neutral, resulting in genetic mosaicism.[18]

Genomewide CNV association studies have been performed in the last several years, and significant associations have been found between CNVs and chronic diseases such as lupus, [19] neuroblastoma,[20] tetralogy of fallot,[21] and neuropsychiatric disorders including autism, schizophrenia, and mental retardation.[22]

A recent family-based genomewide association study of CNVs was performed on approximately 400 complete parent-child trios from the CAMP (Childhood Asthma Management Program) Genetics Ancillary Study [CAMP Research Group, 1999].[23] A significant association between copy-number variation at SNP rs2240832 and childhood allergic asthma was identified ($p = 6.05 \times 10^{-7}$). This SNP on chromosome 7 is located in the open reading frame BC072396, which is differentially spliced as either T-cell receptor gamma (*TCR γ*) or T-cell receptor gamma alternative reading frame protein (TARP). Previous studies support a role for T-cells in the pathogenesis of asthma and allergic disease. [24,25]

We sought to replicate the association between copy-number at *TCR γ* and childhood allergic asthma in an independent cohort. T-cells are lymphocytes involved in cell-mediated immunity which have T-cell receptor (TCR) glycoproteins on their surface. Most T-cells have an alpha and a beta TCR chain, however a small proportion instead have a gamma and a delta chain.[26] While CD8+ $\alpha\beta$ T-cells are believed to have pro-inflammatory effects, CD8+ $\gamma\delta$ T-cells can inhibit inflammation.[27,28] Because of these relationships, we also investigated whether copy-number variation at the TCR alpha gene (*TCR α*) and the TCR beta gene (*TCR β*) had any association with the asthma phenotype. We further investigated if copy-number variation at one TCR locus predicted co-occurrence of CNVs at the other TCR loci.

Materials and Methods

Ethics Statement

The study was approved by the Yale Human Investigation Committee and appropriate informed consent was obtained from human subjects.

Study Population

Women who participated in an earlier study of asthma in pregnancy (1997–2000)[29] were re-contacted to invite participation in a follow-up study (Perinatal Risk of Asthma in Infants of Asthmatic Mothers [PRAM]) of asthma development in the children on their six birthday. All women who reported a physician diagnosis of asthma at the time of enrollment in the pregnancy study and who delivered a singleton infant were selected for PRAM (N = 873) of whom 832 were eligible (English-speaking, living in CT, MA or NY at last pregnancy interview, and child alive at age 6). In addition, all women who reported asthma symptoms or medication use but had no physician diagnosis of asthma were also selected for PRAM (n=449 of whom 426 met eligibility criteria). A random sample of 550 women who did not have asthma, asthma symptoms, or asthma medication use during pregnancy and who delivered singleton infants were also selected for PRAM (n = 549 eligible). Of 1807 women who were eligible, a total of 1505 (83.3%) were enrolled when their child was six years of age (± 3 months).

From the PRAM study group, 607 children met strict phenotype criteria and were selected for genotyping. After removing samples with insufficient DNA or DNA of poor quality, 94 children with allergic asthma and 455 controls were successfully genotyped at one or more of the three T-cell loci (Figure 1). Patient characteristics are shown in table 1.

Definition of cases and controls

To examine the genetic influences on asthma development in these children, we conducted a nested case-control study selected from the entire PRAM population. Children were defined as cases and controls according to strict criteria. Cases were allergic asthmatics with early-onset persistent asthma defined as having physician diagnosed asthma *and* wheeze at ages one and six years *and* asthma medication use *and* allergies as reported by the mother. Controls had no diagnosis of asthma or reactive airway disease *and* no symptoms at ages one or six years *and* no asthma medication use *and* no allergies.

DNA Collection

All DNA samples were obtained through buccal swab collection using the Whatman OmniSwab. At the end of the home interview, the research assistant instructed the mother to collect the sample from the child using two swabs, one for each side of the child's mouth, by rubbing the pad at the head of the swab over the sides of the cheeks and in the gutter area of the mouth. Maternal and paternal DNA samples were also collected, when possible. Each swab head was then ejected from the stem into a 2 mL microcentrifuge tube labeled with the study number, date of collection, and side of mouth (left or right). The samples were stored at -20°C until the DNA was extracted.

DNA quantitation

Using real-time quantitative PCR, 607 samples were assayed for DNA concentration using the ABI 7900HT cyclor and RNaseP detection reagents (Applied Biosystems). Reactions were run according to ABI recommendations. In brief, 1 μL of genomic DNA was aliquoted onto a 384-well plate in 3 replicate wells for each sample and dried overnight. A VIC-labeled RNaseP detector was used to determine absolute DNA quantity per μL of sample by

comparing it to serial dilutions of a known concentration run on the same plate. The RNaseP gene is known to exist as a single copy per haploid genome. Average cycle thresholds were calculated for each sample and converted into absolute DNA concentrations using the SDS v2.2.2 software (Applied Biosystems; Foster City, CA). A total of 574 samples (94.6%) were determined to have sufficient DNA of adequate quality to undergo copy-number analysis.

Primer selection

For the *TCR γ* gene, primers were chosen to target the center of the significant copy-number variable region reported from the genome-wide scan (ABI primer ID Hs04326912_cn).[23] An additional qPCR probe (Hs03646230_cn), located 1730 bp downstream of the first *TCR γ* probe, was also genotyped to support copy-number findings in this gene. Primers for the *TCR α* and *TCR β* genes (ABI primer IDs Hs03307199_cn and Hs04339988_cn, respectively) were chosen to target the centers of copy-number deletions which were reported to be significantly increased in frequency among neuroblastoma cases in a recent genome-wide study.[20] The primers targeting the *TCR α* gene were positioned upstream of the TCR delta gene (*TCR δ*), which is contained within the *TCR α* locus, to avoid attributing copy-number differences to the effects of the wrong gene.

TaqMan® Copy-number Genotyping

Copy-number genotyping was performed using real-time quantitative PCR and commercially available reagents (Applied Biosystems) following the manufacturer's recommendations. Cases and controls were assigned to one of five plates for genotyping using 1:5 blocked randomization. Plates 1, 2, and 3 contained 19 cases and 95 controls, while plates 4 and 5 contained 20 cases and 96 controls. Five nanograms of sample DNA was added to each of three replicate wells and dried overnight. Two calibrator samples available from Coriell Cell Repositories (NA15510 and NA10851) have been characterized extensively with respect to CNV content and were included in all plates analyzed to ensure genotyping consistency. A minimum of eight wells were left without DNA as "no template controls" to set maximum limits for cycle threshold values. A copy-number reference assay containing two primers and a VIC and TAMRA dye-labeled probe assayed copy-number at the RNaseP locus. This was amplified in a multiplex reaction with a copy-number target assay containing two primers and a FAM dye-labeled MGB probe which targeted a region in one of the TCR genes. Plates were run on the ABI 7900HT machine using the manufacturer's recommended PCR cycling conditions.

Cycle thresholds were calculated using the SDS v2.2.2 software with the autobaseline on and a manual C_T threshold of 0.20. Wells with a cycle threshold exceeding 32.5 for either the target or the reference probe were excluded from analysis. 549 of 574 samples had 2 or more replicates successfully amplify in at least one TCR gene assay and were included in the final analysis. This included 94 cases and 455 controls.

C_T values for the target and reference assay were imported into CopyCaller™ Software version 1.0 (Applied Biosystems; Foster City, CA). A comparative C_T ($\Delta\Delta C_T$) relative quantification analysis of the real-time data was performed. First, the difference between the target and reference C_T value is determined for each well and then averaged across sample replicates. The ΔC_T values of the samples are used to calculate copy-number at the target locus relative to that at the RNaseP control locus, which is known to exist at one copy per haploid genome. In the primary analysis, these continuous copy-number values among the cases were compared to those among the controls. These values can also be categorized into discrete copy-number classes using a maximum-likelihood algorithm in the CopyCaller™

Software. Because discrete CNV classes are inappropriate when somatic mosaicism exists, categorized copy-number values were used only in a secondary analysis.

Statistical analyses

The primary statistical test utilized to compare copy-number values was the Mann-Whitney U test, also known as the Wilcoxon rank-sum test. All p-values reported in this study are two-sided.

Using a maximum-likelihood algorithm available in Applied Biosystems CopyCaller™ Software, copy-number values were also grouped into one of three categories: deletion (0 or 1 copy), copy-neutral (2 copies), or amplification (≥ 3 copies). The algorithm was conditioned to treat two copies as the most commonly occurring value. Odds ratios were calculated and tested for a linear trend using the one-degree-of-freedom Cochran-Armitage unconditional test statistic.

In order to determine if copy-number variation at one T-cell receptor locus was predictive of that at another T-cell receptor locus, correlation coefficients were calculated for the association between copy-number values and compared against the null ($r = 0$). Statistical analyses were performed using SAS version 9.1 (SAS Institute; Cary, NC).

Tests of Mendelian inheritance

To determine if the CNVs identified in this study are inherited, or if they are somatic mutations, a subset of individuals determined to have CNVs at TCR loci and the parents of these individuals were genotyped on the same real-time qPCR plate ($n = 27$ trios). Child genotypes were compared to parental genotypes to check for Mendelian consistency. Lack of Mendelian consistency in CNV inheritance was considered indicative of somatic mutations, as germline CNVs are known to be stably inherited [17,30] and de novo germline events are quite rare [31].

Results

In the subset of genotyped parent-child trios, predicted discrete copy-number values did not consistently demonstrate Mendelian inheritance at any of the three TCR genes (table 2), consistent with previous reports of CNVs at these loci.[20] This indicates that these CNVs are not inherited, and are likely not germline events. Instead these appear to be somatic mutations which exist in a mosaic state in some human cells.

In all, 94 children with allergic asthma and 455 controls were successfully genotyped for copy-number variation at one or more of the three TCR genes. Copy-number values were not normally distributed, as determined using the Shapiro-Wilk test statistic (p-values all < 0.001). Distributions of copy-number values at each locus can be seen in figure 2. Cases had significantly lower copy-number values than controls at *TCR γ* ($p = 0.0199$), but copy-number values at *TCR α* and *TCR β* were not significantly different between cases and controls ($p = 0.7972$ and 0.8585 , respectively). Genotyping an additional probe in the *TCR γ* gene supported this association, as copy-number was again significantly lower in cases than controls ($p = 0.0488$). Results of this analysis can be seen in table 3.

When the copy-number values were categorized into discrete copy-number classes (deletion, copy-neutral, duplication) using the maximum-likelihood algorithm available in the CopyCaller™ Software, no significant differences could be detected between the case and control groups.

It has been previously reported that deletions at T-cell receptor loci tend to co-occur in individuals.[20] To determine if copy-number at one TCR locus was correlated with the copy-number at another TCR locus, correlation statistics were calculated between copy-number values at each locus. Copy-number at *TCRβ* was significantly positively correlated with copy-number at *TCRα* and both *TCRγ* probes ($r = 0.781, 0.216$ and 0.418 , respectively; $p < 0.0001$ for each). Copy-number at *TCRα* was not significantly correlated with the first *TCRγ* probe ($r = -0.063$; $p = 0.147$), but was weakly correlated with the second *TCRγ* probe ($r = 0.194$; $p < 0.0001$).

Discussion

A genomewide study of copy-number variation previously reported that copy-number at *TCRγ* is associated with childhood allergic asthma.[23] This study genotyped samples on the Illumina HumanHap550 BeadChip and intensity data from the 550,000 genotyped SNPs were used to infer copy-number at each position. Their data indicated that asthmatics had reduced copy-number at *TCRγ* compared to healthy parents, and that detected CNVs appeared to be somatic events.[32,33] In our study, individuals with childhood allergic asthma had significantly lower copy-number values at *TCRγ* than controls, while values did not significantly differ between cases and controls at *TCRα* and *TCRβ*. Our results support the association between childhood allergic asthma and reduced copy-number at the T-cell receptor gamma gene.

Two defining features of chronic asthma are airway hyperresponsiveness and airway inflammation.[34] In humans, CD8+ T cells that bear the $\alpha\beta$ T-cell receptor typically have pro-inflammatory effects, whereas CD8+ T cells bearing the $\gamma\delta$ T-cell receptor can inhibit inflammation.[27,28] This finding has led to the hypothesis that defective or altered $\gamma\delta$ T-cell function may contribute to development of an allergic asthma phenotype.[25] We did not seek to genotype the TCR delta gene (*TCRδ*) because it is located within the telomeric end of the *TCRα* locus and variation at this position could influence expression of either gene, making results difficult to interpret.

Our results indicate that children with allergic asthma do appear to have reduced copy-number at *TCRγ* compared to non-asthmatic controls, reflecting somatic copy-number differences in a mosaic cell population. Interestingly, $\gamma\delta$ T-cells are unique among T-cells in that they have anti-inflammatory effects.[35] Because these are somatic mutations, we can not determine if these CNVs are a cause of allergic asthma, or if instead they represent an immunologic response to the disease. Our study of CNVs at TCR genes utilized DNA derived from buccal cells and demonstrates that these somatic events are not limited to blood-derived DNA. As the buccal mucosa contains leukocytes, including $\alpha\beta$ and $\gamma\delta$ T-cells [36], it is not surprising that the somatic events observed in blood-derived DNA are also observed in DNA from buccal swabs.

Because this genomic region undergoes somatic rearrangement in T-cells,[37] the association observed by Ionita-Laza, et al. may reflect inter-individual variation in the proportion of somatically rearranged cells among all white blood cells, rather than germline copy number variation. Although the DNA samples used in our study were obtained from buccal swabs and not from blood-derived DNA, genetic mosaicism can still exist in these cell populations. Therefore, calculated copy-number values were not classified into discrete copy-number classes in the primary analysis. Continuous copy-number values represent the average copy-number within a mosaic cell population.

The primary statistical test utilized in this study to compare copy-number values between cases and controls was the Mann-Whitney U test. This test statistic is most appropriate

because it can identify significant differences in copy-number between two groups without imposing discrete copy-number values on the samples (e.g. 1 copy, 2 copies, 3 copies). In the presence of genetic mosaicism, discrete copy-number classes would not reflect the underlying biological phenomenon in which some cells are copy-number variable and others copy-neutral, resulting in an intermediate copy-number value. Because the copy-number values should vary around whole numbers, with increased variability reflecting greater levels of mosaicism, an assumption of normality cannot be made and a non-parametric test is appropriate.

The significant correlations observed between copy-number at various T-cell receptor genes may be the result of similar recombinogenic events occurring at these loci in a given individual. Another possibility is that this correlation reflects the level of genetic mosaicism in the cell population, regardless of the underlying copy-number. For instance, an individual with a mosaic deletion of one TCR gene and a mosaic duplication of another TCR gene may have correlated copy-number values at these two positions simply because their buccal cell population has remained largely wild-type, thus pushing the average copy-number of each gene closer to two. Thus, correlation could exist due to an individual's underlying level of mosaicism, without two genes having undergone similar recombination events.

A recent study of somatic CNVs in peripheral-blood-derived DNA identified genomic regions which had discordant CNV profiles within monozygotic twin-pairs.[38] Somatic mosaicism for pathogenic mutations may be more common than previously thought, and is currently not well-studied outside of cancer epidemiology. Our study joins studies of cancer, and a study phobic disorders,[39] in implicating a role for somatically mosaic copy-number variants in disease etiology.

Further work is needed in order to resolve whether reduced copy-number at *TCR γ* predisposes individuals to asthma, or whether CNVs at this position arise as a result of the disease. If these somatic mutations do arise as a result of the disease, it will be valuable to determine if this is a coincidental marker of the asthma phenotype, or if deletion of this gene is a somatic response intended to alter disease progression or severity through establishment of a cell population which is mosaic for *TCR γ* copy-number.

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Abbreviations

<i>TCRγ</i>	T-cell receptor gamma
<i>TCRα</i>	T-cell receptor alpha
<i>TCRβ</i>	T-cell receptor beta
CNVs	Copy Number Variants
SNP	Single nucleotide polymorphism
TARP	T-cell receptor gamma alternative reading frame protein
TCR	T-cell receptor
PCR	Polymerase chain reaction

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Figure 1.
Flow of individuals from recruitment through genotyping

Figure 2. Distribution of individuals' average somatic copy-number in mosaic buccal cell populations at each of three TCR genes

Somatic copy-number was determined using real-time quantitative PCR. C_T values for the target and reference assay were imported into CopyCaller™ Software version 1.0 (Applied Biosystems; Foster City, CA) and analyzed using comparative C_T ($\Delta\Delta C_T$) relative quantification. Continuous copy-number values can be interpreted as the average copy-number within a mosaic cell population. Control values appear as gray circles, while case values appear as black triangles ($N = 94$ cases, 454 controls). Copy-number values did not differ significantly by case/control status at *TCRα* or *TCRβ* ($p = 0.7972$ and 0.8585 , respectively). Cases had significantly lower copy number values at *TCRγ* ($p = 0.0199$), as demonstrated by the larger proportion of case values (versus control values) falling below the copy-neutral value of 2.0 in the figure. The *TCRγ* copy-number values plotted here are those determined using the first *TCRγ* probe (ABI primer ID Hs04326912_cn).

Table 1

PRAM cohort (CT, MA and NY) 1997–2000: demographic and phenotype data for genotyped individuals

	Asthmatics ^a (n=94)		Controls ^b (n=455)	
	N	%	N	%
Mother asthma ^c				
No	22	23.91	283	62.75
Yes	70	76.09	168	37.25
Child's race				
White	43	45.74	358	78.68
African-American	13	13.83	30	6.59
Hispanic	20	21.28	38	8.35
Bi-racial	18	19.15	29	6.37
Child's eczema ^c				
No	42	45.16	371	82.44
Yes	51	54.84	79	17.56
Sibling asthma ^c				
None	40	45.45	349	77.38
1 or more	48	54.55	102	22.62
Gender ^c				
Male	50	64.10	189	45.65
Female	28	35.90	225	54.35

^a Asthmatic defined as allergic asthmatics with early-onset persistent asthma including physician diagnosed asthma *and* wheeze at ages one and six years *and* asthma medication use *and* allergies as reported by the mother

^b Controls defined as having no diagnosis of asthma or reactive airway disease *and* no symptoms at ages one or six years *and* no asthma medication use *and* no allergies

^c Demographic and phenotype data not available for all patients

Table 2Parent-child trio genotypes^a

Child's genotype	Number of trios genotyped	Trios showing Mendelian inheritance
TCR α deletion	5	1
TCR α duplication	6	3
TCR β deletion	4	2
TCR β duplication	5	1
TCR γ deletion ^b	0	NA
TCR γ duplication	7	1

^a For a copy-neutral child, the only parental genotype which is inconsistent with Mendelian inheritance is a homozygous deletion. Therefore, parents of children determined to be copy-neutral were not genotyped because such trios are overwhelmingly uninformative for testing Mendelian inheritance of CNVs.

^b No children with TCR γ deletions had paternal DNA available. TCR γ copy-number genotypes presented here are those determined using the first TCR γ probe (ABI primer ID Hs04326912_cn).

Table 3Statistical analyses of copy-number genotyping^a results at each of three TCR genes

	N	Median	10 th %ile	90 th %ile	Points above pooled median	Expected under H ₀	Mann-Whitney p-value ^b	Median Test p-value ^c
TCRα	Case	91	1.98	1.59	2.36	45.90		
	Control	441	1.98	1.52	2.35	220.10	p = 0.7972	p = 0.9343
	Pooled	532	1.98	1.53	2.35	NA		
TCRβ	Case	90	2.01	1.49	2.49	47.00		
	Control	437	1.98	1.43	2.45	218.10	p = 0.8585	p = 0.6284
	Pooled	527	2.00	1.44	2.47	NA		
TCRγ_1	Case	94	1.95	1.81	2.19	34.57		
	Control	454	1.99	1.80	2.26	227.00	p = 0.0199	p = 0.0044
	Pooled	548	1.98	1.80	2.26	NA		
TCRγ_2	Case	91	1.93	1.63	2.16	39.75		
	Control	435	1.99	1.66	2.25	217.50	p = 0.0488	p = 0.1805
	Pooled	526	1.98	1.65	2.25	NA		

^a Somatic copy-number was determined using real-time quantitative PCR. CT values for the target and reference assay were imported into CopyCallerTM Software version 1.0 (Applied Biosystems; Foster City, CA) and analyzed using comparative CT ($\Delta\Delta CT$) relative quantification. These continuous copy-number values can be interpreted as the average copy-number within a mosaic cell population. Two different primer sets were used to genotype TCR γ , targeting positions separated by 1730 bp.

^b The Mann-Whitney test (a.k.a. Wilcoxon rank-sum test) ranks copy-number values and tests whether those among cases are equally distributed compared to those among controls.

^c The Median test assesses whether the median copy-number value among cases is equal to the median copy-number value among controls.