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No Association between Copy Number Variation of the *TCRB* Gene and the Risk of Autism Spectrum Disorder in the Korean Population

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Abstract

Although autism spectrum disorder (ASD) has been thought to have a substantial genetic background, major contributing genes have yet to be identified or successfully replicated. Immunological dysfunction has been suggested to be associated with ASD, and T cell-mediated immunity was considered important for the development of ASD. In this study, we analyzed 163 ASD subjects and 97 normal controls by genomic quantitative PCR to evaluate the association between the copy number variation of the 7q34 locus, harboring the *TCRB* gene, and ASDs. As a result, there was no significant difference of the frequency distribution of *TCRB* copy numbers between ASD cases and normal controls. *TCRB* gene copy numbers ranged from 0 to 5 copies, and the frequency distribution of each copy number was similar between the two groups. The proportion of the individuals with <2 copies of *TCRB* was 52.8% (86/163) in ASD cases and 57.1% (52/91) in the control group (p=0.44). The proportion of individuals with >2 copies of *TCRB* was 11.7% (19/163) in ASD cases and 12.1% (11/91) in the control group (p=0.68). After the effects of sex were adjusted by logistic regression, ORs for individuals with <2 copies or >2 copies showed no significant difference compared with the diploid copy number as reference (n=2). Although we could not see the positive association, our results will be valuable information for mining ASD-associated genes and for exploring the role of T cell immunity further in the pathogenesis of ASD.

Keywords: autism, copy number variation, T cell receptor beta (*TCRB*), 7q34

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Introduction

Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders with the core features of impairment in social interaction and verbal communication and repetitive or stereotyped patterns in behavior. These disorders typically manifest within the first three years of life (Klauck, 2006).

The etiology of ASD is still largely unknown. Growing evidence has suggested a genetic background of ASD development. The concordance rates of ASD in monozygotic twins were reported to be significantly higher than in dizygotic twins, and the recurrence rates in siblings are approximately 10 times higher than in the normal population (Folstein and Rosen-Sheidley, 2001). Cytogenetic alterations were detected in 7.4% of ASDs (Vorstman *et al.*, 2006), and some alterations have been suggested as causative factors of neurodevelopmental disorders (Merikangas *et al.*, 2009). Several studies have revealed that ASDs are associated with genetic factors, such as single gene disorders and genetic polymorphisms, including single nucleotide polymorphisms (SNPs) and copy number variation (CNV), and that multiple genetic factors may work together (McCauley *et al.*, 2005; Santangelo and Tsatsanis, 2005). Significant associations have been reported between ASDs and CNVs of various genes, such as *NLGN3* (Xq13.1), *NLGN4* (Xp22.23), *SHANK3* (22q13.3), and *NRXN1* (2p16.3) (Christian *et al.*, 2008; Cook *et al.*, 2008). However, most of these candidate genes have been neither successfully replicated in independent study populations nor functionally validated as susceptible genes or loci.

Maintaining neuronal cellular connectivity as well as synaptogenesis has been suggested to play a role in the pathologensis of ASD by trafficking of immune cells and mediators through astroglial and microglial cells (Gupta, 2000). T cell receptor beta affects TCR signaling and T cell polarity (McKinney *et al.*, 2010), which consequently affect neuronal activity, development, and plasticity (Syken and Shatz, 2003). Several association

studies reported that an imbalance of the $CD4^{+}$:$CD8^{+}$ T cell ratio was found in children with ASDs (Denny *et al.*, 1996; Sweeten *et al.*, 2003).

Another interesting clue to connect the TCR with ASD is that the 7q34-35 region, where the T cell receptor B variable region (*TCRB*) is located, was found to be rearranged in a patient with mental retardation, anxiety disorder, and autistic features (Dauwerse *et al.*, 2010). In previous reports, the 7q31-34 (Risch *et al.*, 1999) and 7q32.3-36.3 (Schanen, 2006) regions showed association with mental retardation and ASD phenotypic features (Dauwerse *et al.*, 2010; Gu and Lupski, 2008). Based on these reports, it seems that *TCRB* may affect the susceptibility to neurodevelopmental disorders, including ASD.

In this study, we analyzed 163 ASD subjects and 97 normal controls by genomic quantitative PCR (qPCR) to evaluate the association between the 7q34 locus, harboring the *TCRB* region, and ASDs and the potential diagnostic usefulness of *TCRB* in the Korean population.

Methods

Study subjects

We recruited 163 ASD children (137 boys and 26 girls) who were diagnosed with ASD from the Department of Child Psychiatry of Gyeongsang National University Hospital, Ghil Hospital and Seoul National University Hospital. Each proband with ASD was initially screened by two board-certified child psychiatrists according to DSM-IV criteria (American Psychiatric Association, 1994) and assessed using the Autism Diagnostic Interview-Revised (ADI-R) and the Korean version of the Autism Diagnostic Observation Schedule (ADOS). Probands with chromosomal abnormalities, such as fragile X syndrome or other neurological or medical conditions suspected to be associated with ASDs, were not included. All parents or legal guardians of probands provided written informed consent for participation in this study. The normal control samples consisted of 97 Korean adult males who were free of any psychiatric or congenital disorders. This study was approved by the Institutional Review Boards of the participating institutions.

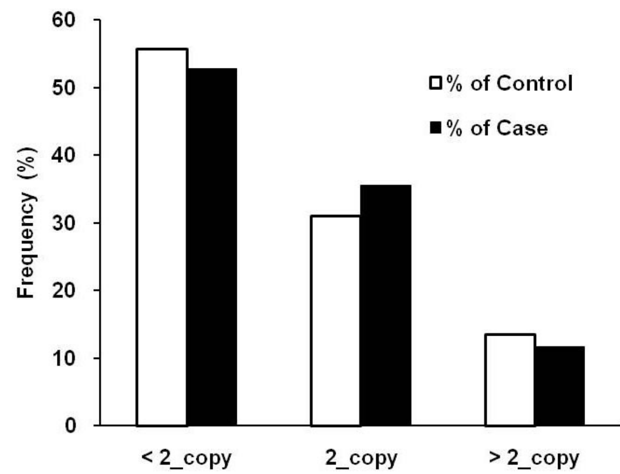

Fig. 2. Frequency distributions of *TCRB* gene copy numbers in ASD cases (solid bar) and normal controls (open bar).

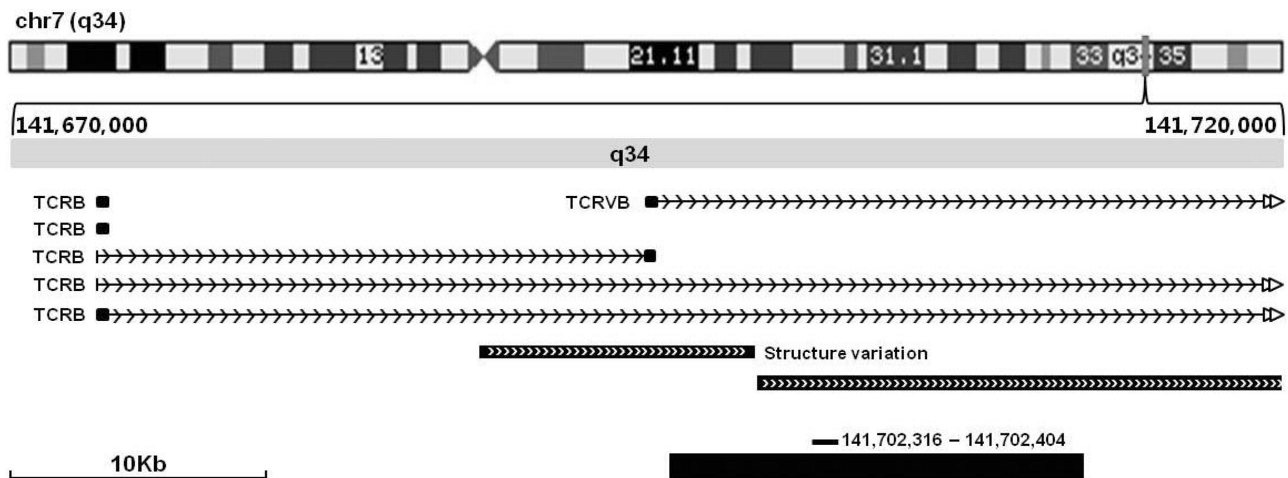

Fig. 1. Scheme of candidate region, referred by the UCSC genome browser. *TCRB* gene clusters stretch throughout the 7q34 region. The thick solid bar (▬) indicates the CNV region, which was reported previously (Redon *et al.*, 2006), and the black arrow bar (>>>) indicates duplication regions as structural variations. The small narrow bar (——) represents the TCBR quantitative PCR amplicon region (141,702,316-141,702,404; NCBI36).

Table 1. Frequencies and odds ratio values of *TCRB* copy numbers

Copy	0_copy	1_copy	2_copies	3_copies	4_copies	5_copies	Total
Control (%)	50 (54.9)	2 (2.2)	28 (30.8)	4 (4.4)	7 (6.6)	1 (1.1)	91 (100)
Case (%)	82 (50.3)	4 (2.5)	59 (35.6)	8 (4.9)	7 (4.3)	4 (2.5)	163 (100)
GM10851=1 copy	<2 copies		2 copies	>2 copies			
Control (%)	52 (57.1)		28 (30.8)	11 (12.1)			91 (100)
Case (%)	86 (52.8)		58 (35.6)	19 (11.7)			163 (100)
χ^2	0.61			0.17			
p-value	0.44			0.68			
Odds ratio (95% CI)	1.25 (0.71～2.21)		1	0.83 (0.35～2.00)			

Selection of the candidate region

To search the CNV region relevant to ASD, the Autism Chromosome Rearrangement Database (ACRD) inn the database of Genome Variants (http://projects.tcag.ca/autism/), the autism gene database AuDB (http://www.mindspec.org/autdb.html), and the UCSC genome browser (http://www. genome.ucsc.edu/) were used. We selected *TCRB* on the 7q34 region as the candidate region, based on an association between ASD and immunity through a literature analysis, compared it with a database of genomic variants, and chose the primer set by in silico PCR in the UCSC browser (Fig. 1). The selected region is located on the *TCRB* gene cluster, and it has been reported that there are many CNVs and duplication sites (Redon *et al.*, 2006).

Quantitative PCR for *TCRB* gene

The primer sequences (141,702,316-141,702,404; NCBI build 36) of the target region are as follows: 5'-TGAGGCAGCCTGT GTGCTTTACTA-3' for forward and 5'-TAGGCTACACATGCCCTGCTACAA-3' for reverse. As a diploid internal reference gene for genomic qPCR, a gene encoding heparan sulfate, 6-O- sulfotransferase 3 (*HS6ST3*), was used, because neither CNV nor segmental duplication in this gene has been observed, according to the Database of Genomic Variants. The primer sequences for the internal control are as follows: 5'-CGCTACCACCACACCAAGCAG-3' for forward and 5'-CCACCTGGCTGTTGTAGTCCTC-3' for reverse. Genomic qPCR was performed using the Mx3000P qPCR system (Stratagene, La Jolla, CA). Twenty microliters of real-time qPCR mixture contained 20 ng of genomic DNA, SYBR Premix (FINNZYMES, Finland), 1× ROX, and 10 pmol primers. Thermal cycling conditions consisted of one hot start cycle of 10 min at 95°C, followed by 40 cycles of 5 sec at 95°C, 10 sec at 55°C, and 20 sec at 72°C. After the PCR reactions, melting curve analysis was performed to confirm specific amplification. NA10851 DNA (Coriell, Camden, NJ, USA) was used as a calibrator. All qPCR experiments were performed in triplicate, and amplification efficiencies for both the target and internal reference genes were evaluated using a standard curve over serial 1:5 dilutions using MxPro Version 3.00 software. To assess efficiency of PCR amplification, the Ct value was determined at the cycle at which the amplicon of *HS6ST3* attained the threshold of the standard curve. Relative copy number was calculated by the $\Delta\Delta$Ct method using the Ct values, as described elsewhere (Yim *et al.*, 2010). The copy number for *TCRB* was defined as $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in threshold cycles for the sample in question, normalized against a reference gene (*HS6ST3*) and expressed relative to the value obtained by NA10851 DNA (individual/NA10851). We assumed the median value of the *TCRB* qPCR ratios in the normal control group to be diploid and adjusted all measured *TCRB* qPCR ratio values accordingly. Then, the adjusted ratio values were rounded off to the nearest integer.

Statistical analysis

Statistical procedures were performed using Stata software (version 10.0; Stata Corporation, College station, TX, USA). The χ^2-test was used to test allelic differences between the case and control groups. $p<0.05$ was considered to be statistically significant.

Results

All measured copy number ratio values in *TCRB* and the controls were converted to integers, as described above. There was no significant difference in the frequency distribution of *TCRB* copy number between ASD cases and normal controls. As shown in Table 1, *TCRB* copy numbers ranged from 0 to 5 copies, and the frequency distribution of each copy number was similar between the two groups. The proportion of individuals with <2 copies of *TCRB* was 52.8% (86/163) in ASD cases and 57.1% (52/97) in the control group (p-value=

0.44) (Fig. 2). The proportion of individuals with >2 copies of *TCRB* was 11.7% (19/163) in ASD cases and 12.1% (11/91) in the control group (p-value=0.68). To estimate the odds ratio (OR) of ASD, we chose the diploid copy number group as a reference point. After the effects of sex were adjusted by logistic regression, ORs for individuals with <2 copies or >2 copies showed no significant difference compared with the diploid copy number as reference (n=2). Since the age variable showed a strong colinearity with the case-control status, only the effect of sex was adjusted through logistic regression. The adjusted OR was also insignificant (1.05, 95% CI 0.80-1.38).

Discussion

Immunological dysfunction has been suggested to be associated with ASD (Folstein and Rosen-Sheidley, 2001; Rutter, 2005). Especially, T cell-mediated immunity has been considered important for the development of ASD (Cohly and Panja, 2005). To maintain immune balance against numerous MHC molecules in the central nervous system, the rejoining of the TCR plays a role in T cell responses. In autistic children and their families, autoimmune disorders, such as asthma, have been reported to be more common than in the normal population (van Gent *et al.*, 1997). Also, elevated levels of IFN-γ in postmortem autistic patients have suggested a possible association between the pathogenesis of ASDs and impairment of T cell-mediated immunity (Vargas *et al.*, 2005).

Although CNV has been suggested as a new type of genomic diversity among normal individuals, including Koreans (Freeman *et al.*, 2006; Yim *et al.*, 2010), it also seems to play a certain role in disease susceptibility. A number of significant CNVs on chromosome 7 and 15 have been suggested to be associated with ASD (Marshall *et al.*, 2008). In addition, among copy number alterations, changes on chromosome 7 have been reported to be associated with various psychiatric diseases, such as ASD and mental retardation. For example, complex rearrangements on chromosome 7q were identified in patients with mental retardation, anxiety disorder, and autistic features (Dauewerse *et al.*, 2010). One of the key ASD-associated genes, named *CNTNAP2*, is located on 7q35 (Arking *et al.*, 2008).

Our target locus is a variable region in *TCRB*. Abnormalities in *TCRB* have been reported to be associated with immunological defects (Currier *et al.*, 1998; Zhao *et al.*, 1994). Based on the previous findings described above, we hypothesized that the CNV of the *TCRB* gene, located on 7q34, may be associated with ASDs. We explored this possibility by genomic qPCR, targeting the *TCRB* gene, but we did not find any evidence of association.

There are several limitations of this study. First, we examined one locus to quantify the DNA copy number dosage of *TCRB* (7q34). To represent the copy number status of this gene more reliably, several more qPCR probes must be examined in the same study subjects. Second, the study size was not large enough to make a definite conclusion.

In conclusion, there was no significant difference in *TCRB* gene copy number between ASDs and normal individuals. An additional SNP analysis might be useful and add some more information. Although we could not see a positive association, our results will be valuable information for mining ASD-associated genes and for exploring the role of T cell immunity further in the pathogenesis of ASD.

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