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Association Analysis of Two Single-Nucleotide Polymorphisms of the *RELN* Gene with Autism in the South African Population

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Background: Autism (MIM209850) is a neurodevelopmental disorder characterized by a triad of impairments, namely impairment in social interaction, impaired communication skills, and restrictive and repetitive behavior. A number of family and twin studies have demonstrated that genetic factors play a pivotal role in the etiology of autistic disorder. Various reports of reduced levels of reelin protein in the brain and plasma in autistic patients highlighted the role of the reelin gene (*RELN*) in autism. There is no such published study on the South African (SA) population. *Aims:* The aim of the present study was to find the genetic association of intronic rs736707 and exonic rs362691 (single-nucleotide polymorphisms [SNPs] of the *RELN* gene) with autism in a SA population. *Methods:* Genomic DNA was isolated from cheek cell swabs from autistic (136) as well as control (208) subjects. The TaqMan® Real-Time polymerase chain reaction and genotyping assay was utilized to determine the genotypes. *Results:* A significant association of SNP rs736707, but not for SNP rs362691, with autism in the SA population is observed. *Conclusion:* There might be a possible role of *RELN* in autism, especially for SA populations. The present study represents the first report on genetic association studies on the *RELN* gene in the SA population.

Introduction

Autism is a severe neurodevelopmental disorder that is characterized by social deficits, impairments in communication, social skills, and repetitive behaviors (Kanner, 1943; Schopler *et al.*, 1986; Scholper and Mesibov, 1988; Bailey *et al.*, 1995). It has onset in early childhood, usually by 3 years of age. Based on studies conducted in different sites in the United States, the current prevalence of autism spectrum disorders (ASDs) is estimated to be one in 150 (Curtin *et al.*, 2010) to one in 91 (Kogan *et al.*, 2009) individuals with a male–female ratio of 4:1. These results are consistent with prevalence rates reported in other countries of Europe and Asia; however, some prevalence estimates are higher (Kogan *et al.*, 2009; Boyle *et al.*, 2011; Kim *et al.*, 2011). Based on the evidence reviewed by Elsabbagh *et al.* (2012), the median of worldwide prevalence estimates of ASDs is 62/10,000. A number of studies have given substantial evidence that points toward the role of genetic (Folstein and Rosen-Sheidley, 2001; Williams and Casanova, 2011; Neale *et al.*, 2012) and environmental factors (Rodier, 2000; Acosta and Pearl, 2003) in the etiology of autism, which further show autism as a multifactorial trait. It has already been clear from various twin studies that the risk of autism is higher among siblings of affected individuals. The heritability values vary from 60% to 90%, emphasizing a higher contribution of genetic factors in autism than other developmental disorders (Rodier, 2000; Folstein and Rosen-Sheidley, 2001; Acosta and Pearl, 2003; Kumar and Christian, 2009). The absence of a clear Mendelian mode of inheritance and low sibling recurrence gives a positive indication that autism is a multilocus disorder with many genes responsible for it (Ritvo *et al.*, 1986; Alcantara *et al.*, 1998; D'Arcangelo and Curran, 1998; Impagnatiello *et al.*, 1998; Kemper and Bauman 1998; Pesold *et al.*, 1998; D'Arcangelo *et al.*, 1999; Risch *et al.*, 1999; Guidotti *et al.* 2000; Hong *et al.*, 2000; Turner, *et al.*, 2000; Costa *et al.*, 2001; Li *et al.*, 2012). Therefore, it is a well-accepted hypothesis (Klauck, 2006) that several susceptibility genes are interacting together with a complex mode of inheritance leading to the typical phenotypes of the ASD. There could be involvement of from three to four genes up to 100 genes (Pickles *et al.*, 1995; Pritchard, 2001; Klauck, 2006). Genome scan data have pointed toward the

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long arm of chromosome 7 as a strong candidate region (Turner *et al.*, 2000). Various association (Alarcon *et al.*, 2008; Arking *et al.*, 2008) and linkage (Alarcon *et al.*, 2002; Laumonnier *et al.*, 2004) studies are being undertaken to screen candidate genes mapping to the long arm of chromosome 7 as susceptibility loci. One such gene is reelin (*RELN*), which maps to 7q22, and has been found to be a positional candidate gene.

The *RELN* gene consists of 65 exons spanning ~450 kb and is mapped at chromosome 7q22. Reelin protein is found in the spinal cord, brain, blood, and other body organs and tissues. It plays a pivotal role in the development of the cerebral cortex, cerebellum, hippocampus, and several brainstem nuclei (Persico *et al.*, 2001). This gene functions as a signaling protein that regulates brain development during neuronal migration, formation of cortical layers, and synaptic plasticity (Rice and Curran, 2001). One of the most distinct effects of the autosomal recessive mutation of *RELN* gene is being observed in reeler, a natural mutant mouse: severe neuroanatomical abnormalities such as inverted cortical lamination, abnormal positioning of neurons, cerebellar hypoplasia, and aberrant orientation of cell bodies and nerve fibers and reduced Purkinje cell number (Falconer, 1951; Caviness and Sidman, 1973; Goffinet, 1979, 1984, 1992) have been observed. These abnormalities also overlap with the cytoarchitectural defects seen in the autopsied brains of autistic subjects (Bauman, 1991; Courchesne, 1997; Bailey *et al.* 1998; D'Arcangelo and Curran, 1998; Kemper and Bauman, 1998; Gillberg, 1999). Additionally, studies (Goffinet, 1992; Fatemi, 2004) have also reported association between mutations in the *RELN* gene and significant learning disability, hypoplastic cerebellum, ataxia, and cognitive decline in both man and mouse. Several other studies have also confirmed the absolute requirement of the *RELN* gene in correct cell positioning and proper formation of brain (Weeber *et al.*, 2002; Tissir and Goffinet, 2003). This indicates that *RELN* abnormalities could contribute to the etiology of several neurogenetic diseases. Reelin protein levels are significantly reduced in multiple brain areas of patients with schizophrenia and bipolar disorder. This suggests that persisting low Reelin levels in the developed brain increase vulnerability to schizophrenia, bipolar disorder, lissencephaly syndrome, and others, thus inducing damage (Impagnatiello *et al.*, 1998; Fatemi *et al.*, 2000, 2001; Hong *et al.*, 2000; Kim and Webster, 2009). Similar abnormalities have also been seen for Reelin levels in autistic individuals. Brain levels of Reelin protein and mRNA among postmortem superior frontal, parietal, and cerebellar cortices of age-, gender-, and postmortem interval-matched autistic and control subjects were investigated (Fatemi, 2004). These individuals were not suffering from any other disorders. Reductions in Reelin protein and mRNA, Dab-1 mRNA, and elevations in *RELN* receptor and very-low-density lipoprotein receptor mRNA were observed, which demonstrate impairments in the *RELN* signaling system in autism, which further accounts for some of the brain structural and cognitive deficits observed in the disorder. In addition, the *RELN* gene is within the autism susceptibility locus 1 (AUTS1), and blood levels of unprocessed Reelin are significantly reduced in autistic twins, their fathers, their mothers, and their phenotypically normal siblings versus controls (Fatemi *et al.*, 2001, 2002). Similar observations have been reported by Lugli *et al.* (2003), which strongly suggest a genetic predisposition for inheriting Reelin deficiency in progeny of individuals who might be carrying *RELN* mutations. Low or undetectable levels of blood Reelin protein among affected children also show delays in neurologic and cognitive development such as little or no language and inability to sit or stand unsupported, hypotonia, myopia, nystagmus, and generalized seizures (Hong *et al.*, 2000). All these features make *RELN* an attractive candidate gene for autism. Based on this evidence, a number of genetic association studies have been carried out, which have given both positive and negative evidence for having association with the disorder (Persico *et al.*, 2001; Krebs *et al.*, 2002; Zhang *et al.*, 2002; Bonora *et al.*, 2003; Li *et al.*, 2004; Skaar *et al.*, 2004; Serajee *et al.*, 2006). However, linkage and the association between single-nucleotide polymorphisms (SNPs) of *RELN* and autism in the South African (SA) population have not been investigated. In view of this lacuna, the present study was undertaken to investigate the possible effect of *RELN* SNPs (rs362691and rs736707) in the SA autistic and control populations.

Materials and Methods

Ethics clearance

The study protocol was approved by Western Cape Ethics Committee (SR: 5/09/32) of University of the Western Cape, and Western Cape Education Department. Consent was also obtained from principals of schools and parents to allow the collection and genetic analysis of DNA samples, in accordance with the Declaration of Helsinki.

Study group

The present study group comprised of 57 autistic Black, 42 autistic mixed ancestry, and 43 autistic White unrelated SA children, while 107, 66, and 48 were control children, respectively. With respect to gender classification, 129 males and 13 females were autistic, while among controls, the number was 89 and 132, respectively. Samples were collected from only autistic individuals studying in schools for children with special needs located in Cape Town and Gauteng Province (South Africa), and control samples were collected from healthy school children from primary and high schools in Cape Town. The diagnosis was carried out strictly on the basis of the criteria outlined by Diagnostic and Statistical Manual of Mental Disorders, 4th edition (American Psychiatric Association, 1994), and the Childhood Autism Rating Scale was administered for the assessment of the cases (Schopler *et al.*, 1986). Selection of all subjects in the study was based on clinical and psychological evaluation by an experienced psychologist and psychiatrist. Detailed history and health information of the subjects were collected by a team comprising psychiatrist, psychologist, speech therapist, neurologist, pediatrician, and researchers. In the present study, individuals suffering from Asperger syndrome, schizophrenia, bipolar syndrome, and other developmental as well as neurological disorders were excluded. None of the subjects in the studied group were taking specific drugs.

Sample collection

Children participating in the study were swabbed by taking a sterile swab (Puritan Sterile Polyester Tip Applicators; Manta Forensics) and rubbing it against the inside of their cheek for 1 min and placing it into a labeled sterile 15-mL falcon tube (Boeco). This was done for both cheeks.

DNA extraction

DNA was extracted from the swabs using the BuccalAmpTM DNA Extraction Kit (Epicentre) according to the manufacturer's suggestion. This solution was then stored at 4°C, and the stock was stored at −20°C. The concentration of each DNA sample was determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies).

Genotyping

Genotypes were determined using the TaqMan® SNP-Validated Genotyping Assay (Applied Biosystems). This is a real-time polymerase chain reaction (PCR) method that accumulates amplified product during the exponential phase of the PCR cycle. Combining thermal cycling, fluorescence detection, and application-specific software, it enables the cycle-by-cycle detection of the increase in the amount of nucleic acid sequences.

SNP selection and primer design

The exonic SNP (rs3622691) and intronic SNP (rs736707) on the *RELN* gene were identified from a previous experiment carried out by Serajee *et al.* (2006). The SNP sequence was blasted against the *Homo sapiens* genome using the BLAST tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were then synthesized from Applied Biosystems according to the specifications as indicated by the File Builder application. The TaqMan Custom Genotyping Assay probes (40×) were labeled VIC and FAM to the following sequences respectively:

Exon 22: 5′-TTCTTTGGGTGATTTCATCCTG-3′ and 5′-CCGTCTCTGT TTGTATGTGCG-3′

Intron 59: 5′-GCAGGGCTGACAGGTTACAC-3′ and 5′-TGGTCTCCTC TATCAAAGTT GGC-3′

PCR preparation

All samples were diluted with double-distilled water (ddH_2O) to a final concentration of 20 ng/μL and then aliquoted individually into 96-deep-well plates (Applied Biosystems). The first row of each 96-deep-well plate contained the negative control, which consisted of 50 μL ddH_2O. A PCR mastermix was prepared by adding 1650 μL of TaqMan Universal Genotyping Mastermix (Applied Biosystems) to a microfuge tube. To the tube, 165 μL of 20× TaqMan SNP Custom Genotyping Assay (Applied Biosystems) was added, along with 825 μL of SABAX water. The solution was then vortexed for 10 s. The Microfuge epMotion 5070 automated pipetting system (Applied Biosystems) was then used to transfer the samples and mastermix into the 384-well plate (Applied Biosystems) yielding a final volume of 5 μL. The Perkin Elmer 7900 PCR System (Applied Biosystems) was used for PCR amplification. PCR parameters were as follows: initial denaturation for 10 min at 95°C; denaturation for 15 s at 92°C; annealing for 1 min at 60°C 40 cycles; and extension for 1 min at 60°C. Results from the amplified PCR products were viewed using the Applied Biosystems 7900HT Real-Time PCR System.

Statistical analysis

Fisher's exact test was used to compare the gender distributions as well as the distribution of ethnic groups, between the autistic cases and controls. The genetic association with autism was tested with logistic regression models, enabling us to adjust for gender and ethnicity. Haplotypes of the two SNPs were inferred and analyzed using the methods described by Schaid *et al.* (2002). All statistics were done using R (freely available from http://r-project.org) and the R package genetics and haplo.stats. p-Values < 0.05 are described as significant, except for the Hardy–Weinberg tests, where we used a critical value of p-value < 0.01.

Results

For SNP rs362691, the present study group was comprised of typed cases (136, unrelated autistic children) and typed controls (193, unrelated healthy children), while the respective figures for SNP rs736707 were 129 and 208, respectively. For statistical correction, some of the cases and controls were excluded from the actual number of collected subjects. Both groups (cases as well as controls) were summarized according to the gender and ethnicity distribution. The gender distribution differed highly significantly between cases and controls ($p < 0.0001$); thus, all further analyses were adjusted for gender. There was no significant difference between cases and control groups ($p = 0.7557$) with respect to ethnicity distribution. The genotype distributions differed significantly between the ethnic groups (results not shown); therefore, all further analyses were adjusted for ethnicity too. The genotypic distributions of both SNPs, in both autistic and controls, in all three ethnic groups (Table 1), conformed to the Hardy–Weinberg equilibrium. The observed allelic, genotype, and inferred haplotype counts and frequencies as well as the p-values for association tests, after adjusting for gender and ethnicity, are given in Table 2. For SNP rs736707, there is a significant difference ($p = 0.0413$) in the allelic distribution between autistic and control subjects. The estimated risk of autism with any AA genotype is 1.18-fold of that with GG (95% confidence interval: 1.01–1.39, $p = 0.0347$).

For SNP rs362691, no significant difference has been detected for allelic or genotypic frequencies between autistic and controls. The rs362691–rs736707 G-A haplotype was significantly ($p = 0.038$) over-represented in cases (freq = 0.64) compared to controls (freq = 0.54) (Fig. 1).

Table 1. Genotypic Frequencies in the Three Ethnic Groups, Stratified by Autism Status in South African Population in Comparison to Normal Individuals

	Ethnicity					
	Black		*Mixed*		*White*	
Genotype	*Autistic*	*Control*	*Autistic*	*Control*	*Autistic*	*Control*
rs362691						
Typed	51	90	42	57	43	46
C/C	0.02	0	0.02	0.02	0.02	0.02
G/C	0.08	0.11	0.17	0.24	0.12	0.22
G/G	0.90	0.89	0.81	0.74	0.86	0.76
rs736707						
Typed	51	107	37	53	41	48
A/A	0.39	0.37	0.65	0.32	0.51	0.46
A/G	0.39	0.42	0.27	0.49	0.49	0.48
G/G	0.22	0.21	0.08	0.19	0	0.06

Table 2. The Observed Allele, Genotype, and Inferred Haplotype Counts and Frequencies as Well as the *p*–Values for Association Tests, After Adjusting for Gender and Ethnicity for South African Autistic and Normal Populations, Are Presented

Genotype	*Autistic subjects*	*Control subjects*	p-*Value*
rs362691			
Typed	136 (0.97)	193 (0.89)	
Allelic frequency			
G	250 (0.92)	348 (0.90)	0.3352
C	22 (0.08)	38 (0.10)	
Genotype frequency			
G/G	117 (0.86)	157 (0.81)	0.3588
G/C	16 (0.12)	34 (0.18)	
C/C	3 (0.02)	2 (0.01)	
rs736707			
Typed	129 (0.92)	208 (0.96)	
Allelic frequency			
A	180 (0.70)	252 (0.61)	0.0413[a]
G	78 (0.30)	164 (0.39)	
Genotype frequency			
A/A	65 (0.50)	79 (0.38)	0.1064
A/G	50 (0.39)	94 (0.45)	
G/G	14 (0.11)	35 (0.17)	
rs362691-rs736707 haplotype			
C-A	(0.06)	(0.06)	0.7655
G-A	(0.64)	(0.54)	0.0388[a]
C-G	(0.02)	(0.04)	0.0916
G-G	(0.28)	(0.36)	0.1270

[a]Represents statistical significance $p < 0.05$.

Discussion

In light of the paucity of data, the present study attempts to decipher the role of the *RELN* gene polymorphisms in autism and is the first report on such association in the SA population. We have carried out a systematic analysis of allelic and genotypic distribution of two SNPs (rs362691 and rs736707) of the *RELN* gene among autistic and control groups.

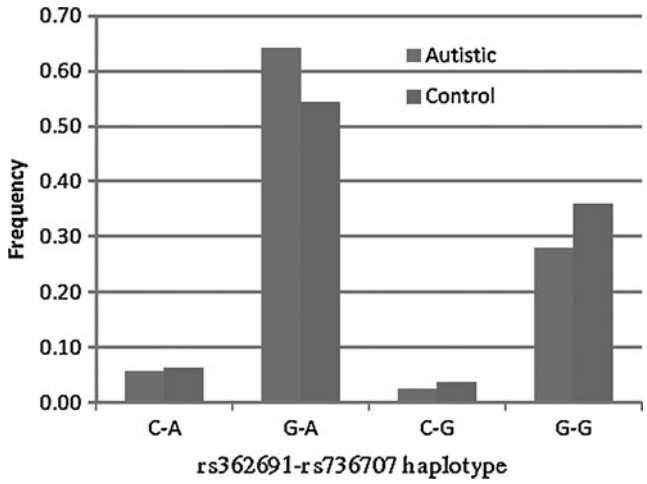

FIG. 1. Observed frequencies of all rs362691-rs736707 haplotypes in autistic and control groups.

To date, a number of studies have investigated association of the SNPs of the *RELN* gene with autism in various populations (Persico *et al.*, 2001; Krebs *et al.*, 2002; Zhang *et al.*, 2002; Bonora *et al.*, 2003; Devlin *et al.*, 2004; Skaar *et al.*, 2004; Serajee *et al.*, 2006). The conflicting results obtained through these studies demand replication of *RELN* association studies in larger groups from different populations worldwide. In the present study, for rs736707, we estimated 1.18 times more risk of autism with AA genotype as compared to GG. Similar findings for SNP rs736707 have also been reported by Serajee *et al.* (2006). SNP rs362691 located on exon 22 is a C/G transversion and is responsible for nonsynonymous amino acid change of leucine 997 valine in reelin protein. We detected a significant rs362691–rs736707 haplotype, although we did not observe a significant association of SNP rs362691 with autism. A significant difference in transmission of the C/G alleles of SNP in exon22 (rs362691) was reported by Serajee *et al.* (2006). For this SNP, the G and C allele frequencies were quite different from frequencies reported by Dutta *et al.* (2008) in an Indian population and by Serajee *et al.* (2006) among Caucasian families of Autism Genetics Resource Exchange. In an earlier report by Bonora *et al.* (2003), among autistic patients selected from International Molecular Genetic Study of Autism Consortium (IMGSAC) multiplex families of the European autistic population, the G allele was identified as the minor allele, while in the SA population (present study), the G allele was observed to be the major allele. Similar G allele frequencies have been observed in various Asian, European, and African populations, which assumes homogenous allelic distribution in these populations (http://genecards.qfab.org/cgi-bin/snps/snp_link.pl?rs_number=362691&file=/data/GeneCards_2.41.1/cards_usr/entries/RE/card_RELN.txt;&kind=AlleleFreqData;&chrom=7). To some extent, disparity in allelic frequencies reflects racial differences, which might be responsible for the inconsistent results.

Dutta *et al.* (2008) studied six SNPs, including rs362691 and rs736707, in an Indian population and concluded that there is no association of the *RELN* polymorphism with ASDs. Bonora *et al.* (2003) also found no evidence of association of rs362691 with autism among IMGSAC samples. On the other hand, Serajee *et al.* (2006) found that rs736707 was in significant transmission disequilibrium among Caucasian autistic subjects. We cannot comment about transmission of alleles, as we took samples from individuals only. Further, in a case–control study, Li *et al.* (2008) studied eight SNPs of the *RELN* gene and detected a positive association of intron 59 (rs736707) with autism in the Chinese Han Population. Heterogeneity between study groups in clinical features and gene–environment interactions may also be responsible for the inconsistency of results. Dutta *et al.* (2008) reported a significant association of SNP rs362691 with epilepsy in an Indian population, while they did not find any evidence of association with SNP rs736707. These apparent controversies w.r.t association studies of *RELN* gene markers with autism might be explained on the basis of genetic complexity of the disorder. It has also been hypothesized that autism is a disorder of polygenic inheritance so the effect of a single SNP could be subtle. Therefore, more studies, including more SNPs of *RELN*, should be investigated (He *et al.*, 2011). The present study has certain limitations. Only two SNPs of the *RELN* gene have been investigated; thus, the results of the present study should be

interpreted with caution. The replication of these results using a larger study group and more markers in ethnically distinct populations is important to have additional evidence of a possible role of *RELN* in autism, especially for SA populations.

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Author Disclosure Statement

No competing financial interests exist.

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