Research article

The (CTG)n polymorphism in the NOTCH4 gene is not associated with schizophrenia in Japanese individuals

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Abstract

Background: The human NOTCH4 gene is a candidate gene for schizophrenia due to its chromosomal location and neurobiological roles. In a British linkage study, NOTCH4 gene polymorphisms were highly associated with schizophrenia. In a Japanese case-control association study, however, these polymorphisms did not show significant associations with schizophrenia. We conducted a case-control study with Japanese subjects to explore an association between the triplet repeat polymorphism in the NOTCH4 gene and schizophrenia, including subtypes of schizophrenia, longitudinal disease course characteristics, and a positive family history for psychoses.

Methods: We examined the (CTG)n repeat polymorphism in the NOTCH4 gene among 100 healthy Japanese individuals and 102 patients with schizophrenia (22 paranoid, 38 disorganized, 29 residual, 64 episodic, 31 continuous, 42 with prominent negative symptoms, and 46 with positive family histories) using a polymerase chain reaction-based, single-strand conformational polymorphism analysis.

Results: Five different alleles consisting of 6, 9, 10, 11, and 13 repeats of CTG (Leu) in patients with schizophrenia, and 4 alleles consisting of 6, 9, 10, and 11 repeats in controls were found. No significant differences in genotype or allele frequencies of repeat numbers were found between controls and patients. In addition, there were no associations between the polymorphism and schizophrenia subtypes, longitudinal disease course characteristics, or positive family history of the patients.

Conclusions: Our data suggest a lack of association between the NOTCH4 gene triplet repeat polymorphism and schizophrenia in Japanese individuals.

Background

NOTCH activity affects the implementation of differentiation, proliferation, and apoptotic programs, influencing organ formation and morphogenesis [1]. The formation of neuronal contacts results in activation of NOTCH receptors, leading to the restriction of neuronal growth and

a subsequent arrest in differentiation [2]. NOTCH functions as a genetic switch between neuronal and glial fates through its negative regulation of the glial cell deficient/glial cells missing (glide/gcm) gene, the gene required to induce gliogenesis in glial precursors [3].

The human NOTCH4 gene is located on chromosome 6p21.3 [4], and several linkage studies have suggested that a susceptibility locus for schizophrenia is present on chromosome 6p [5, 6, 7, 8]. In situ hybridization studies have determined that in embryonic and adult life, NOTCH4 transcripts are primarily restricted to endothelial cells [9]. NOTCH4 is not necessary for embryonic development, as NOTCH4-deficient mice develop normally. However, this gene and the NOTCH1 gene have partially overlapping roles during embryogenesis in mice; both NOTCH1-mutant and NOTCH1/NOTCH4-double mutant embryos have severe defects in angiogenic vascular remodeling [10].

Using linkage disequilibrium mapping of the human major histocompatibility complex (MHC) region in 80 British parent-offspring trios, Wei and Hemmings [11] found that NOTCH4 was highly associated with schizophrenia. The A-to-G substitution in the promoter region (SNP2) and the (CTG)n repeat in exon 1 of NOTCH4 were considered possible candidate sites conferring susceptibility. A Japanese case-control association study [12] reported that these polymorphisms did not show significant associations with schizoaffective disorder or schizophrenia in Japanese individuals; further, no associations were found between the polymorphisms and subcategories of schizophrenia or a positive family histry of psychoses.

We conducted a case-control study using Japanese subjects to explore an association between the (CTG)n repeat polymorphism in the NOTCH4 gene and schizophrenia, and to examine subtypes, longitudinal disease course characteristics, and a positive family history of psychoses. We found no association between the NOTCH4 gene triplet repeat polymorphism and schizophrenia in this patient population.

Methods

DNA Samples

Informed written consent was obtained from subjects prior to the study according to research protocols approved by the Ethics Committee of Tsukuba University. Schizophrenic patients (N = 102) were examined; this group consisted of 61 men (mean age, 46.2 ± 12.0 years; mean age at onset of schizophrenia, 25.2 ± 7.4 years) and 41 women (mean age, 47.5 ± 15.5 years; mean age at onset of schizophrenia, 27.0 ± 10.6 years) who matched the DSM-IV criteria for schizophrenia [13]. Patients were further divided into subtypes (22 paranoid, 38 disorganized, 4 catatonic, 29 residual, and 9 undifferentiated), and classified as to longitudinal course specifiers (64 episodic, 31 continuous, 5 single episode, and 2 other or unspecified). Furthermore, 42 patients were categorized with prominent negative symptoms, and 46 patients had a family history of psychoses in first- or second-degree relatives.

The control group consisted of 100 unrelated healthy volunteers (30 men, mean age 32.1 ± 11.4 years and 70 women, mean age 43.1 ± 12.0 years) who were hospital employees living in the same city as the patients. Each volunteer was interviewed by 2 psychiatrists in order to rule out subjects with a family history of mental illness. All patients and controls were of Japanese origin.

Genomic DNA samples were prepared from whole blood collected in disodium ethylenediamine tetra-acetic acid (EDTA; 3 mg/L) according to the sodium iodide method (DNA Extractor WB Kit, Wako Pure Chemical Industries, Osaka, Japan).

Polymerase Chain Reaction Conditions

A set of polymerase chain reaction (PCR) primers that spanned the (CTG)n repeat region in exon 1 was used to produce DNA fragments. The primers used were NOT-LR-F (forward): 5'-CCCTGCCTGAAGAGGGACAG-3' and NOT-LR-R (reverse): 5'-TCTGGGTCTGACCACT-GAGAC-3'. These primers were designed using information from a previous report [4] and the GenBank sequence (accession number: U89335). The 5'-terminus of NOT-LR-F was labeled with indodicarbocyanine fluorescent dye (Amersham Pharmacia Biotech, Uppsala, Sweden) for fluorescence-based, single-strand conformational polymorphism (SSCP) analysis.

The PCR mixture contained the following: 0.5 ng genomic DNA, 0.25 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 U of Taq DNA polymerase (HotStarTaq, QIAGEN, Hilden, Germany) in a final volume of 25 μl.

The amplification reaction was performed as follows: an initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step of 72°C for 10 minutes (GeneAmp 9600, PE Applied Biosystems, Foster City, CA).

PCR products were visualized by ethidium bromide staining under UV light after electrophoresis on 2% agarose gels.

Table 1: Allele frequencies of the CTG repeat in controls and schizophrenic patients, including subtypes, course specifiers and positive family history, and results of Fisher's exact test and Monte Carlo method

Subjects (n)	Alleles (%)					Fisher's test 6R vs 9-13R	Monte Carlo method
	6R	9R	10R	11R	13R	P value	P value
Controls (100)	38 (19.0)	72 (36.0)	78 (39.0)	12 (6.0)	0 (0.0)	0.7095	0.9547
Schizophrenic patients (102)	42 (20.6)	67 (32.8)	80 (39.2)	14 (6.9)	1 (0.5)	0.7095	0.9547
Subtypes							
Paranoid (22)	10 (22.7)	13 (29.5)	17 (38.6)	4 (9.1)	0 (0.0)	0.5378	0.7538
Disorganized (38)	21 (27.6)	27 (35.5)	23 (30.3)	4 (5.3)	1 (1.3)	0.1392	0.3808
Residual (29)	8 (13.8)	18 (31.0)	28 (48.3)	4 (6.9)	0 (0.0)	0.4386	0.6666
Others (13)	3 (11.5)	9 (34.6)	12 (46.1)	2 (7.7)	0 (0.0)	0.4304	0.7869
Course							
Episodic (64)	24 (1 8.8)	48 (37.5)	45 (35.2)	10 (7.8)	1 (0.8)	1.0000	0.9207
Continuous (31)	15 (24.2)	15 (24.2)	28 (45.2)	4 (6.5)	0 (0.0)	0.3709	0.4709
Others (7)	3 (21.4)	4 (28.6)	7 (50.0)	0 (0.0)	0 (0.0)	0.7346	0.8137
Prominent negative symptoms (42)	17 (20.2)	26 (31.0)	34 (40.5)	6 (7.1)	1 (1.2)	0.8696	0.8388
Positive family history (46)	19 (20.7)	31 (33.7)	34 (37.0)	7 (7.6)	1 (1.1)	0.7522	0.9002

6R, 9R, 10R, 11R and 13R indicate 6, 9, 10, 11 and 13 repeats of CTG, respectively.

SSCP Analysis

A DNA sequencer (ALF express, Amersham Pharmacia Biotech) was used to perform fluorescence-based SSCP analysis. PCR products were mixed with loading buffer containing 99.5% deionized formamide and 0.5% blue dextran. The solution was denatured at 97°C for 5 minutes, then immediately cooled on ice. Single-stranded PCR products were analyzed by electrophoresis on 7% polyacrylamide gels (49:1, acrylamide:bisacrylamide ratio) containing 7 M urea in 0.5X Tris-borate-EDTA buffer at 50°C. The data were analyzed using the software package Fragment Manager (Amersham Pharmacia Biotech).

PCR Product Sequencing

PCR products showing altered band patterns by SSCP analysis were purified by centrifugation to recover the DNA (Microcon tube, Millipore, Bedford, MA). DNA sequences of the PCR products were directly determined using a Genetic Analyzer (ABI PRISM TM 310, PE Applied Biosystems) after termination-dideoxy-cycle sequencing (Sequencing Reaction Kit-FS, PE Applied Biosystems) with the forward primer (NOT-LR-F).

Statistical Analysis

Deviation of genotype distribution as derived from the Hardy-Weinberg equilibrium equation was calculated using the chi-square test for goodness of fit. Association analyses were performed using Fisher's exact probability test (2-sided) using InStat-2.01 (GraphPad Software, San Diego, CA). Simulations using the Monte Carlo method [14] were performed using ARLEQUIN (version 2.00, University of Geneva) software.

Results and Discussion

A triplet repeat (CTG)n polymorphism in the NOTCH4 gene was identified by SSCP analysis and confirmed by direct sequencing of PCR products. As shown in Table 1,

5 different alleles consisting of 6, 9, 10, 11, and 13 repeats (6R, 9R, 10R, 11R, and 13R) of CTG (Leu) in patients with schizophrenia, and 4 alleles consisting of 6R, 9R, 10R, and 11R in controls were found. No significant differences in genotype or allele frequencies of repeat numbers were found between controls and patients. In addition, there were no associations between the polymorphism and subtypes, longitudinal disease course characteristics, or family history of psychoses.

The British linkage study [11] revealed that an excess of the $(CTG)_{10}$ allele was transmitted to affected offspring by their parents. In our comparison of the frequency of the 10R allele with the other alleles (6R+9R+11R+13R), no significant differences were found between controls and patients with schizophrenia.

Previous study [12] and our study using random samples found no association between the polymorphism and schizophrenia. Possible reasons for disagreement with the British study [11] depend on the differences of ethnicity, population admixture, sample size and methodology. Wei and Hemmings [11] used the Transmission Disequilibrium Test, while our study employed a case-control design, which might produce false positive or negative findings due to stratification problems [15]. Further study using a larger sample size and the investigation of more SNPs in coding regions will be necessary to confirm the relationship between the NOTCH4 gene and schizophrenia.

Conclusions

Our study suggests a lack of association between the triplet repeat (CTG)n polymorphism in the NOTCH4 gene and schizophrenia in Japanese patients.

List of abbreviations used

NOTCH1,4; Notch (*Drosophila*) homolog of 1, 4

SNP; single nucleotide polymorphism

DSM-IV; diagnostic and statistical manual of mental disorders, 4th edn

PCR; polymerase chain reaction

SSCP; single-strand conformational polymorphism

Competing interests

None declared

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