Keywords; Tumor necrosis factor-α; Schizophrenia; Case-control study; Transmission disequilibrium test; Cytokine

1. Introduction

Schizophrenia is a complex genetic disorder and affects approximately 1% of the population worldwide. The pathogenesis of schizophrenia is still unclear; however, cytokines might be implicated in the etiology or pathology of schizophrenia (for review; Nawa et al., 2000). Tumor necrosis factor-α (TNF-α), a pleiotrophic cytokine, exerts neuroprotective and neurodegenerative effects in brain (for review; Venters et al., 2001). Several studies have shown that blood concentrations and *in vitro* production of TNF-α were significantly higher in patients with schizophrenia than in healthy controls (Monteleone et al., 1997; Kowalski et al., 2001; Theodoropoulou et al., 2001), whereas some studies failed to find this increase (Haack et al., 1999; Erbağci et al., 2001). Buka et al. (2001) have reported that the elevated TNF-α levels of maternal serum at the time of birth were associated with schizophrenia and related psychotic disorders in offspring, although Brown et al. (2004) have revealed a significant association between maternal interleukin-8 but not TNF-α levels during the second trimester and the risk of schizophrenia spectrum disorders in offspring. Interestingly, Skurkovich et al. (2003) reported a case of schizophrenia whose negative symptoms improved with antibodies to

TNF-α and to interferon-γ. Thus, these findings suggest that cytokines including TNF-α are likely related to the pathogenesis of schizophrenia.

Wilson et al. (1997) demonstrated that the minor A-allele of a polymorphism at the position -308 in promoter region of the *TNF-α* gene, located on chromosome 6p21.1-21.3, is much more powerful transcriptional activator than the major G-allele. Recently, several studies have shown that the -G308A polymorphism of the *TNF-α* gene is associated with schizophrenia (Boin et al., 2001; Meira-Lima et al., 2003; Schwab et al., 2003; Tan et al., 2003). However, other studies have failed to find this association (Riedel et al., 2002; Handoko et al., 2003; Pae et al., 2003; Tsai et al., 2003; Duan et al., 2004; Hashimoto et al., 2004; Hänninen et al., 2005; Kampman et al., 2005; Pae et al., 2006). This inconsistency requires further investigations. Therefore, we performed a case-control association study and the transmission disequilibrium test (TDT) analysis in Japanese subjects to assess whether the *TNF-α* gene promoter polymorphisms could be implicated in vulnerability to schizophrenia.

2. Methods

2.1 Subjects

All participants were Japanese living in Niigata Prefecture or Fukushima Prefecture. Patients meeting the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV) criteria for schizophrenia were recruited from 14 hospitals as follows: Iizuka Hospital, Kohdo Hospital, Matsuhama Hospital, Minamihama Hospital, Niigata Prefectural Psychiatric Center, Niigata University Hospital, Niitsu-Shin-ai Hospital, Ohjima Hospital, Sado General Hospital, Sagata Hospital, Seki Hospital, Shirone-Kensei Hospital, Shirone-Midorigaoka Hospital, and Suehirobashi Hospital. The diagnosis of schizophrenia had been assigned based on all available sources of information including unstructured interviews, clinical observation and medical records, and subsequently reassessed by an experienced psychiatrist (T.M. or N.K.). Controls were mainly recruited from the staffs of participating hospitals and not assessed with any structured psychiatric interview; however, they showed good social and occupational functioning and reported themselves with no history of psychiatric disorders. The subjects for the case-control study and the TDT analysis were not overlapping. The subjects for the case-control analysis consisted of 265 patients with

schizophrenia (142 males and 123 females; mean age, 45.2 [SD 14.9] years) and 424 controls (217 males and 207 females: mean age, 38.3 [SD 10.5] years). The mean age at onset and duration of illness of patients were 23.7 (SD 7.9) and 21.3 (SD 12.7) years, respectively. Thirteen patients met criteria for paranoid, 113 for disorganized, four for catatonic, 132 for undifferentiated, three for residual subtype of schizophrenia. The patients with family history of schizophrenia within their first degree relatives were 111 (41.9%). The TDT analysis included 83 trios, made up of patients and their both parents. The patients consisted of 44 men and 39 women, with mean age 29.9 (SD 9.5) years. The mean age at onset and duration of illness were 21.1 (SD 5.1) and 8.7 (SD 7.6) years, respectively. Eight patients met criteria for paranoid, 31 for disorganized, four for catatonic, 40 for undifferentiated subtype of schizophrenia. Eight out of 83 (9.6%) probands in the TDT analysis had family history of schizophrenia within their first degree relatives. The Ethics Committee on Genetics of Niigata University School of Medicine approved the present study, and written informed consent was obtained from all participants.

2.2 Genotyping

Genomic DNA was extracted from peripheral blood by standard phenol/chloroform method. We genotyped four single nucleotide polymorphisms (SNPs), -T1031C (rs1799964), -C857T (rs1799724), -G308A (rs1800629), and -G238A (rs361525), in promoter region of the *TNF-α* gene by the TaqMan 5'-exonuclease assay. Table 1 summarizes primer and probe sets, which were designed and synthesized by Applied Biosystems (Foster City, CA). We carried out polymerase chain reaction amplification using TaqMan 2× Universal Master Mix, No AmpErase UNG (Applied Biosystems), 5 ng of DNA, 0.9 μM of each primer and 200 nM of each probe in total volume of 5 μl. Each 96-well plate contained 94 samples and 2 no-DNA template controls. Thermal cycler conditions were 95°C for 10 min, 40 cycles of 92°C for 15 sec and 60°C for 1 min. Fluorescence and allelic discrimination were measured with an ABI PRISM 7900HT Sequence Detection System using SDS 2.0 software (Applied Biosystems).

2.3 Statistical analysis

Deviation of Hardy-Weinberg equilibrium (HWE) was tested by using the exact test for

goodness of fit. Allele and genotype frequencies between cases and controls were compared using the Fisher's exact test. Haplotype frequencies were estimated using the expectation maximization algorithm with SNPAlyse (DYNACOM, Yokohama, Japan). Pair-wise linkage disequilibrium (LD) indices, D' and r^2, were calculated in control subjects, since we consider that controls represent population, but patients do not. Case-control haplotype analysis was performed by the permutation test. In this analysis, very rare haplotypes with frequencies less than 1% were not assessed, because the majority of the Japanese patients with schizophrenia are unrelated to these haplotypes, even if there are significant associations between these haplotypes and schizophrenia. In the TDT analysis, the McNemar test was conducted to test the transmission distortion for SNPs. Haplotype transmission was analyzed by the TRANSMIT program v2.5.4 (Clayton and Jones, 1999; Clayton, 1999). We did not assessed very rare haplotypes with frequencies less than 1% in this analysis. A probability level of $P < 0.05$ was considered to be statistically significant.

3. Results

Table 2 shows genotype and allele frequencies of four SNPs in promoter region of the *TNF-α* gene among patients and controls. Genotype frequencies of all SNPs in both groups were not significantly different from the values expected from HWE ($P > 0.05$). Genotype and allele frequencies in cases did not differ from those in controls ($P > 0.05$). Also, there was no significant difference in genotype and allele frequencies when comparering controls with patients with or without family history of schizohorenia within their first degree relatives ($P > 0.05$). The value of absolute D' and r^2 for controls are presented in Table 3. There was statistically significant evidence of LD between -G238A and -T1031C, and -C857T and -T1031C ($P < 0.05$). Then, we performed haplotype analysis of the case-control sample. There was no significant difference in haplotype frequencies between cases and controls (global permutation $P =$ 0.485; Table 4). In the TDT analysis, our genotype data were consistent with mendelian inheritance in 83 trios, we therefore consider genotyping errors were unlikely. We did not observe transmission distortion in four SNPs ($P > 0.05$; Table 5). We analyzed transmission of multilocus haplotypes and did not find significant excess transmission in any four-SNPs haplotypes (Global test: $\chi^2 = 4.90$, df = 4, $P = 0.30$;

Table 6) or any two- or three SNPs haplotypes (data not shown).

4. Discussion

In the present study, we found no association between the *TNF-α* gene promoter polymorphisms (-G238A, -G308A, -C857T, and –T1031C) and schizophrenia in our Japanese sample. Among these polymorphisms, -G308A has been the most extensively investigated. Boin et al. (2001) found that the frequency of the A allele was significantly increased in Italian patients with schizophrenia as compared to controls, while inverse association was reported in Chinese Singaporean (Tan et al., 2003), Brazilian (Meira-Lima et al., 2003), and German (Schwab et al., 2003). Recently, Saviouk et al. (2005) demonstrated that a specific haplotype (-238G, -308A) was associated with schizophrenia. However, other studies failed to find an association in German (Riedel et al., 2002), populations in the Asian-Pacific region (Handoko et al., 2003), Korean (Pae et al., 2003; Pae et al., 2006), Han Chinese (Tsai et al., 2003; Duan et al., 2004), Japanese (Hashimoto et al., 2004), and Finnish (Hänninen et al., 2005; Kampman et al., 2005). A meta-analysis showed an association between

-G308A and schizophrenia in pooled Caucasian sample, but not in pooled Asian sample (Duan et al., 2004). When we pooled our case-control study sample (265 cases and424 controls) with Hashimoto et al.'s Japanese sample (297 cases and 458 controls), the results were also negative ($P = 0.30$ for genotype and $P = 0.74$ for allele). These findings may indicate that there is an ethnic heterogeneity in the –G308A polymorphism of the *TNF-α* gene in schizophrenia.

We investigated other polymorphisms (-G238A, -C857T, and –T1031C) in promoter region of the *TNF-α* gene. It is noteworthy that the *TNF-α* gene promoter polymorphisms (-C857T, -C863A, and –T1031C) are related to differences in levels of TNF-α production in immune response to stimulus (Higuchi et al., 1998), suggesting that these polymorphisms might be functional. We found no significant association between -G238A, -C857T, or –T1031C and schizophrenia in our Japanese sample. These results are in line with the negative findings came from other Asian populations (Handoko et al., 2003; Tan et al., 2003; Duan et al., 2004; Zhang et al., 2005; Pae et al., 2006). However, there is the possibility that these polymorphisms could be implicated in vulnerability to schizophrenia in non-Asian populations. Thus, future studies of

these polymorphisms to evaluate across other ethnic populations are needed to draw any conclusion.

In the present study, the diagnosis of schizophrenia was made by all available information and subsequently reassessed by an experienced psychiatrist, and controls showed good social and occupational functioning and reported themselves with no history of psychiatric disorders, although any structured interview was not conducted. We therefore consider that the quality of the sample in terms of the diagnosis is sufficient. Our case-control sample has a power of 0.17 for -G238A, 0.20 for -G308A, 0.84 for -C857T, and 0.77 for -T1031C to detect the genotypic relative risk for homozygote of 2.0 and heterozygote of 1.5 with α of 0.05, using the Genetic Power Calculation (Purcell et al., 2003). Thus, our sample size can be considered large enough for -C857T and -T1031C but not for -G238A and -G308A. However, even if there is a significant association between -G238A or -G308A and schizophrenia, the majority of the Japanese patients with schizophrenia are unrelated to this polymorphism, since the minor alleles (-238A and -308A) were very rare (1.5% and 1.8%, respectively). Because case-control studies have potential problems of population stratification, it is

important to use a family based analysis with internal control as in the present study to eliminate population stratification. In the TDT analysis, we also did not find significant excess transmissions in the promoter polymorphisms of the *TNF-α* gene in the single marker and haplotype transmission analysis.

We conclude that the *TNF-α* gene promoter polymorphisms do not play a major role in conferring susceptibility to schizophrenia in a Japanese population. However, several studies indicate that TNF-α might be implicated in the etiology or pathology of schizophrenia. Our results do not imply the exclusion of the contribution of other regions such as coding-regions and exon-intron boundaries of the *TNF-α* gene to the pathogenesis of schizophrenia. Further studies for other critical polymorphisms in extended region of the *TNF-α* gene should be performed in several ethnic populations.

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