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Novel polymorphisms of the *AP-2* gene (6p24): Analysis of association with schizophrenia

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Abstract The transcription factor activator protein 2 (*AP-2*) gene is a possible candidate gene for schizophrenia, since it maps near D6S470, a marker on chromosome 6p24 that provided evidence of linkage to schizophrenia. In the present study we analyzed the promoter region and the whole coding region of the human *AP-2* gene in order to identify genetic variations that may lead to the modification of *AP-2* expression or the alteration of protein function, contributing to schizophrenia or particular schizophrenic phenotypes. Genomic DNA was isolated from the whole blood samples of 87 unrelated schizophrenics and 100 healthy controls. Polymerase chain reaction (PCR) was performed, using 15 primer sets that spanned the promoter region and the whole coding region, and amplified products were screened by single-strand conformational polymorphism (SSCP) analysis. Aberrant SSCP patterns were analyzed by direct sequencing. Three novel polymorphisms were found in the promoter region; two relatively common (−90G→C, −803G→T) and one rare (−1769G→A). Polymorphic status at both loci suggested strong linkage disequilibrium between the −90G and −803G alleles, and between the −90C and −803T alleles. Although no significant differences in genotypic and allelic frequencies at the −90 and −803 loci were found between patients and controls, significant differences in the distribution of genotypes at the −90 ($P = 0.008$) and −803 ($P = 0.037$) loci were observed in patients with an episodic course compared with controls. However, the difference for the −803 locus was not significant after Bonferroni correction for multiple comparisons. Our data provided no direct evidence of an association between schizophrenia and the polymorphisms of the *AP-2* gene, although the positive result at the −90 locus in schizophrenics with an episodic course is potentially interesting.

Key words Activator protein 2 · Association · Polymorphism · Schizophrenics · Linkage disequilibrium · Episodic course

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Introduction

Schizophrenia is thought to be a multifactorial disease. Twin, family, and adoption studies have consistently revealed that genetic factors are involved in this disease (Gottesman 1994). A number of recent linkage studies have suggested the presence of schizophrenia susceptibility loci on chromosome 6p24-21 (Antonarakis et al. 1995; Moises et al. 1995; Schwab et al. 1995; Straub et al. 1995; Wang et al. 1995; Schizophrenia Linkage Collaborative Group for Chromosomes 3, 6 and 8 1996). Integrated physical maps of this region have been constructed to facilitate the identification of candidate genes for schizophrenia, and a large number of genes have been mapped (Davies et al. 1995; Olavesen et al. 1995; Olavesen et al. 1997). Studies of the spinocerebellar ataxia type 1 (*SCA1*) gene, mapped to chromosome 6p23, were not consistent with regard to its association with schizophrenia (Wang et al. 1996; Morris-Rosendahl et al. 1997). As the chromosomal markers spanned a relatively wide region on the short arm of chromosome 6, further studies of this region are required to investigate other candidate genes involved in schizophrenia.

The transcription factor activator protein 2 (*AP-2*) gene may be a possible candidate gene for schizophrenia, as it mapped near the marker, D6S470, on chromosome 6p24 (Davies et al. 1995; Olavesen et al. 1997) that provided evidence of linkage to schizophrenia (Schwab et al. 1995; Straub et al. 1995). *AP-2* regulates the expression of genes required for the development of tissues of ectodermal origin, such as neural crest, brain, and skin (Mitchell et al. 1991; Williams and Tjian 1991a,b). AP-2 binds as a

homodimer to the consensus recognition sequence GCCNNNGGC, which is an important cis-regulatory element for a variety of genes, including proenkephalin (Hyman et al. 1989), presenilin-2 (Pennypacker et al. 1998), β_1-adrenergic receptor (Okawa et al. 1998), synapsin II (Petersohn et al. 1995), α3 nicotinic receptor subunit (Fornasari et al. 1997), dopamine D1 receptor (Minowa et al. 1992), dopamine β-hydroxylase (Kim et al. 1998), phenylethanolamine *N*-methyltransferase (Ebert et al. 1998), metallothionein II_A (Imagawa et al. 1987), keratin (Leask et al. 1991), and *AP-2* (Bauer et al. 1994).

The modification of AP-2 expression or the alteration of protein function due to mutations of the *AP-2* gene may contribute to susceptibility to schizophrenia. In this study, we investigated genetic variations in the promoter region and the coding region of the human *AP-2* gene. We also conducted a case-control study to explore the association between polymorphisms in the *AP-2* gene and schizophrenia, including the subtypes and course specifiers in the *Diagnostic and Statistical Manual of Mental Disorders* (*DSM*)-*IV* (American Psychiatric Association 1994).

Materials and methods

DNA samples

Written informed consent, according to research protocols approved by the Ethics Committee of Tsukuba University, was obtained from 87 unrelated schizophrenics consisting of 53 men (mean age, 46.3 ± 12.7 years; mean age at onset, 25.7 ± 7.6 years) and 34 women (mean age, 47.3 ± 16.3 years; mean age at onset, 27.7 ± 10.7 years) who met the DSM-IV criteria for schizophrenia (American Psychiatric Association, 1994). The subtype and course specifier for each patient had been determined with the DSM-IV before the genomic DNA analysis in this study was performed.

The controls 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 area as the patients. All patients and controls were ethnically Japanese. Genomic DNA was prepared from whole blood samples collected in disodium ethylenediaminetetraacetic acid (EDTA; 3 mg/l). Genomic DNA was extracted by the sodium iodide method (DNA Extractor WB Kit; Wako Pure Chemical Industries, Tokyo, Japan).

Polymerase chain reaction (PCR) conditions

The nucleotide sequence and the genomic structure of the human *AP-2* gene have been reported previously, and the nucleotide sequence numbering used in this study was based on that in a previous report (Bauer et al. 1994). The mature *AP-2* mRNA is spliced from seven exons distributed over a region of 18 kb of genomic DNA, and an inhibitory AP-2 protein, designated AP-2B, is generated by alternative usage after the fifth exon (Bauer et al. 1994). The promoter of the *AP-2* gene contains three AP-2 binding sites (nucleotides −622 to −614, −425 to −417, −385 to −378), an NF-1 binding site (nucleotides −365 to −353), an octamer binding site (nucleotides −339 to −330), a CT-rich repetitive element (nucleotides −240 to −103) (Bauer et al. 1994; Creaser et al. 1996), and a potential binding site for transcription factor NF-κB (nucleotides −1827 to −1818) (Baeuerle 1991). Fifteen sets of PCR primers were prepared to produce fragments covering these motifs and the CT-rich repetitive element in the promoter region, and the whole coding region, including the AP-2B-specific region, and all of the exon-intron boundaries of the *AP-2* gene (Table 1). The 5′-terminus of each primer, except for the AP2STRPR primer, was labeled with indodicarbocyanine fluorescent dye (Pharmacia Biotech, Uppsala, Sweden) to perform fluorescence-based single-strand conformational polymorphism (SSCP) analysis. PCR was performed as reported previously (Kawanishi et al. 1998), with a minor modification: demethyl sulfoxide (final concentration, 5%) was added to the PCR reaction mixture for two primers sets; AP2PMF and AP2PMR, and AP2EX4PF and AP2EX4PR primers.

Single-strand conformational polymorphism (SSCP) analysis

Fluorescence-based SSCP analysis was performed as reported previously (Kawanishi et al. 1998), with slight modifications. Polyacrylamide gels (PAG) used were 7% (49:1, acrylamide: bisacrylamide ratio) or 10% (99:1, acrylamide: bisacrylamide ratio) containing 0.5× Tris-Borate-EDTA buffer, or 7% (49:1, acrylamide: bisacrylamide ratio) containing 0.5× Tris-Borate-EDTA buffer and 10% glycerol.

PCR product sequencing

PCR products showing altered band patterns by SSCP analysis were purified by centrifugation, using Microcon tubes (Amicon, Danver, MA, USA). DNA sequences of PCR products were directly determined from both directions, using a cycle sequencing system (Dye Terminator Cycle Sequencing Ready Reaction and ABI PRISM 310 Genetic Analyzer; Perkin-Elmer, Norwalk, Conn, USA).

Restriction enzyme assay

Restriction fragment-length polymorphism (RFLP) analysis was performed, using a commercially available restriction enzyme according to the manufacturer's recommendations (NIPPON GENE, Tokyo, Japan). Digested products were visualized by electrophoresis in 2% agarose gels after ethidium bromide staining.

Statistical analysis

Deviation of the genotype counts from Hardy-Weinberg equilibrium was tested using χ^2 test for goodness of fit.

Table 1. PCR primers for amplification of the promoter region and whole coding region of the human *AP-2* gene

Primer name	Primer sequence	Primer position	Region	PCR product (bp)	Annealing temperature
AP2PUF	5′-GAATTGTGCTCAGTTCAGGTAG	−1,959–−1,938	Promoter	327	58°C
AP2PUR	5′-TCATCTGGGGCTTGTTTCTCG	−1,633–−1,653			
AP2PMF	5′-CACCCAGGAAACCTTAGCCTG	−1,052–−1,032	Promoter	313	66°C
AP2PMR	5′-AGACCGGCGAAGTCACTCCAG	−740–−760			
AP2PO-PF	5′-CAGCTCGGATCGTGGTAGCAG	−669–−649	Promoter	261	58°C
AP2PO-PR	5′-CTCTCTACGCCGCGAACTTGC	−409–−429			
AP2PD-PF	5′-GAGCTCGAGGAAGGTTTTATC	−478–−458	Promoter	213	58°C
AP2PD-PR	5′-GAGATCTCCCTCTAATGGTAG	−266–−286			
AP2STRPF	5′-CTACCATTAGAGGGAGATCTC	−286–−266	Promoter and	322	58°C
AP2STRPR	5′-CTCGTACTTGATATTATCCGTC	36–15	partial exon 1		
AP2STRPF	5′-CTACCATTAGAGGGAGATCTC	−286–−266	Exon 1	366	58°C
AP2EX1PR	5′-TGACCGCA+CGGATGATCGAG	80–61			
AP2EX2UF	5′-CAACGGGAACGGGCCATTTCC	4,438–4,458	Upstream region	324	64°C
AP2EX2UR	5′-TGCAGGGGGTTCAGGCTGTAG	4,761–4,741	of exon 2		
AP2EX2DF	5′-GTCGCAAGATCCTTACTCCCAC	4,708–4,729	Downstream	311	62°C
AP2EX2DR	5′-CTGTGTTCCCTCGGCTGGTTG	5,018–4,998	region of exon 2		
AP2EX3PF	5′-CACTTACATCCATGTGTATC	8,011–8,030	Exon 3	216	58°C
AP2EX3PR	5′-TGCCTATCTATTTGCTAATTC	8,226–8,206			
AP2EX4PF	5′-GACGCCCAACACGCGGCCTC	10,098–10,117	Exon 4	348	71°C
AP2EX4PR	5′-GTTTCGGTCGCCGCCACCGC	10,445–10,426			
AP2EX5PF	5′-GTGGTGCAGAGAACCCAATG	12,273–12,292	Exon 5	212	58°C
AP2EX5PR	5′-GAAGTTCCTTCTAGTTAGCAAG	12,484–12,463			
AP2BEX5F	5′-CGTTACCCTGCTCACATCAC	12,421–12,440	AP-2B-specific	307	58°C
AP2BEX5R	5′-GGAGAATGTGCAGTTCTTAAAC	12,727–12,706	region		
AP2EX6PF	5′-CACTCTTCATTCTCTCGCAC	14,259–14,278	Exon 6	251	58°C
AP2EX6PR	5′-TTTGGTTTCTCTTTCTCTTGAC	14,509–14,481			
AP2EX7UF	5′-CTACTAGTGCTGCCCATAGTG	16,041–16,061	Upstream region	278	62°C
AP2EX7UR	5′-TGAGGTACATTTTGTCCATGGC	16,318–16,297	of exon 7		
AP2EX7DF	5′-TCACGGCCCTGCAGAACTATC	16,259–16,279	Downstream	215	62°C
AP2EX7DR	5′-GCTGATCCCGGAGCTGTCAC	16,473–16,454	region of exon 7		

PCR, Polymerase chain reaction

Significant differences in genotypic frequencies between patients and controls were analyzed using the χ^2 test (2×3 table) for independence at a significance level of 0.05 (two-tailed). Significant differences in allelic frequencies between patients and controls were analyzed by Fisher's exact test (2×2 table). Bonferroni correction for multiple comparisons was carried out to exclude type I error. Analysis of linkage disequilibrium was performed with an Associate program (version 2.32) in the Linkage Utility programs (Terwilliger and Ott 1994).

Results

Three variants in the promoter region of the *AP-2* gene were identified by SSCP analysis, followed by sequencing. The first variant that showed as an altered SSCP banding pattern was characterized by a single base pair substitution (G→C) at nucleotide position −90 by sequencing. The presence of a −90G→C nucleotide substitution was confirmed by RFLP analysis using *Msp* I. Figure 1 shows the SSCP analysis, sequencing, and RFLP analysis for the −90G→C variant. The second variant was characterized by a single base pair substitution (G→T) at nucleotide position −803 (Fig. 2). No restriction enzyme was commercially available to allow RFLP analysis of the −803G→T variant. The third variant was characterized by a single base pair substitution (G→A) at nucleotide position −1769, which was found as a heterozygote in a single control sample. However, no variant was identified in the whole coding region, or in any of the exon-intron boundaries of the *AP-2* gene.

Contrary to previously published sequence data (Bauer et al. 1994), inconsistencies, i.e., the C nucleotide insertion between nucleotides −89 and −88 (see Fig. 1), the G nucleotide insertion between nucleotides −807 and −806, and the G instead of C at nucleotide position −808 (see Fig. 2) were observed in all samples tested.

Table 2 indicates polymorphic status between the −90 and −803 loci in schizophrenics and controls. The polymorphic status of each sample at these two loci was almost identical, and suggested that strong, but not complete, linkage disequilibrium had occurred between the −90G and −803G alleles, and between the −90C and −803T alleles.

Table 3 shows the genotypic and allelic frequencies at the AP-2 −90 and −803 loci in schizophrenics, including subtypes and course specifiers, and controls. Deviation from Hardy-Weinberg equilibrium was tested for each of the groups. Evidence of significant deviation was obtained in schizophrenics with a continuous course ($\chi^2 = 4.05$; degree of freedom [df] = 1; $P = 0.044$). This probably results from the relatively small population samples. No significant deviation was observed in other groups. No significant differences in genotypic and allelic frequencies at the −90 and

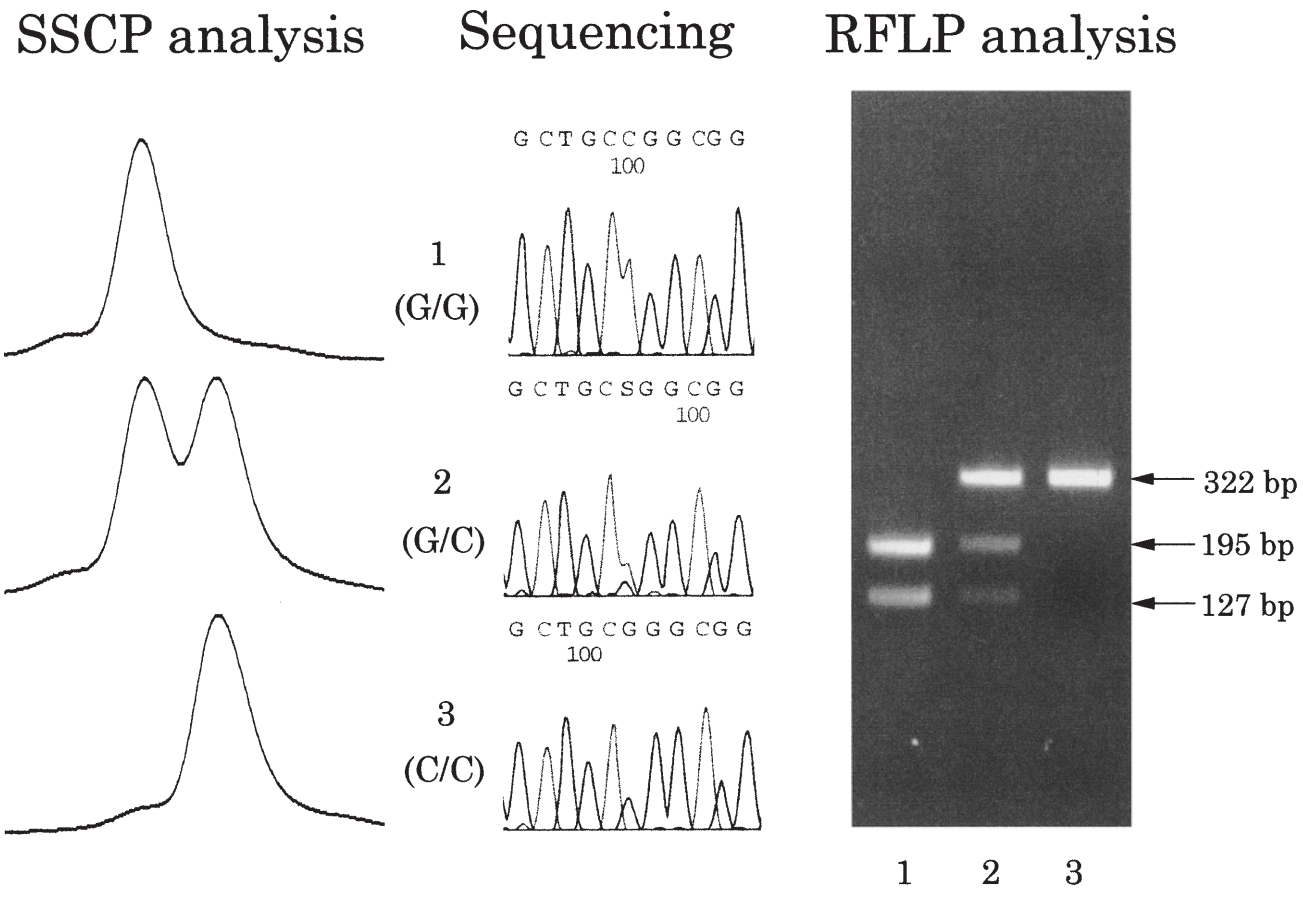

Fig. 1. Single-strand conformational polymorphism (*SSCP*) analysis, direct sequencing, and restriction fragment-length polymorphism (*RFLP*) analysis of polymerase chain reaction (PCR) products generated using the AP2STRPF and AP2STRPR primer sets. Shown here is reverse sequencing. A single base pair substitution (G→C) at nucleotide position −90 was detected by sequencing (S = C or G). A C nucleotide insertion between nucleotides −89 and −88 was observed in all samples, contrary to previously published sequence data. In the absence of the mutated site, the 322-bp PCR product was cut by *Msp* I, producing fragments of 195 and 127 bp. *Samples 1, 2, and 3* indicate homozygous (G/G), heterozygous (G/C), and homozygous (C/C) −90 locus genotypes, respectively

Table 2. Polymorphic status between the −90 and −803 loci in **a** schizophrenics and **b** controls

a

−803 \ −90	GG	GC	CC	Total
GG	35	1	0	36
GT	0	38	1	39
TT	0	0	12	12
Total	35	39	13	87

Delta value = 0.2268; χ^2 = 157.259; degree of freedom (df) = 1; $P < 0.000001$

b

−803 \ −90	GG	GC	CC	Total
GG	43	1	0	44
GT	0	49	0	49
TT	0	1	6	7
Total	43	51	6	100

Delta value = 0.2107; χ^2 = 159.198; df = 1; $P < 0.000001$

−803 loci were found between patients and controls. The limited size of the patient sample presented a problem for the statistical analysis. Because the frequency of the expected values smaller than 5 was more than 20%, a complete anaysis of each of the individual subtypes and course specifiers within the patient group was not possible. For the purpose of the analysis, therefore the catatonic subtype and the undifferentiated subtype were grouped into a single category. The same was done for the patients in the single episode and other or unspecified pattern. No association was observed between the genotypic and allelic distributions, and the subtypes of schizophrenia. In regard to course specifiers, there was a significant difference in the distribution of genotypes at the −90 locus between patients with an episodic course and controls (χ^2 = 9.56; df = 2; P = 0.008; and P = 0.016 after Bonferroni correction). There was also a significant difference in the distribution of genotypes at the −803 locus between patients with the episodic course and controls (χ^2 = 6.60; df = 2; P = 0.037), but the observed P value was 0.074 after Bonferroni correction. No significant differences in allelic frequencies at the loci were found between the groups.

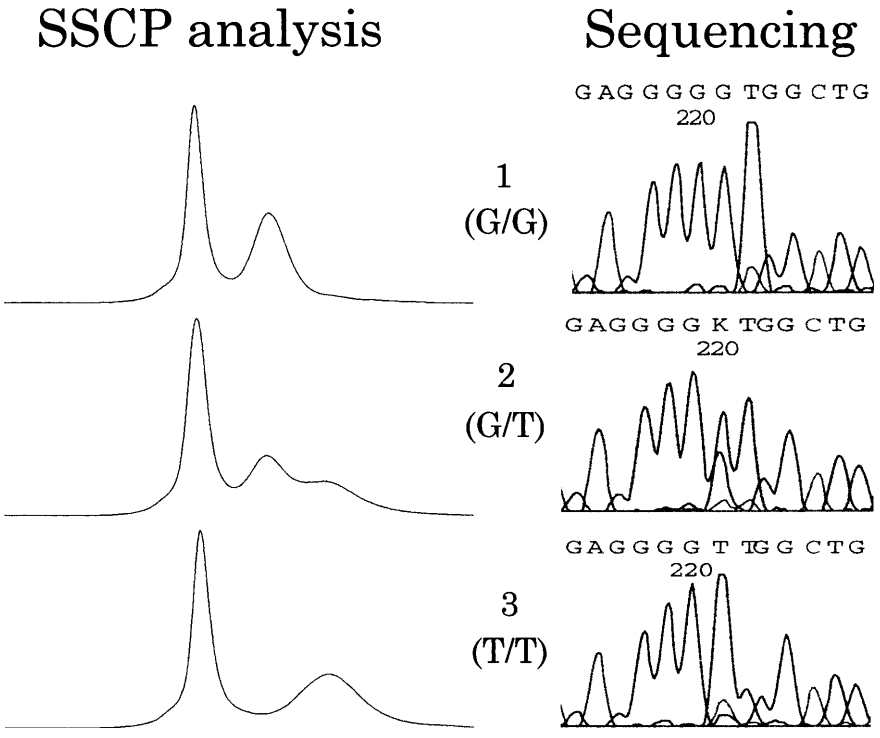

Fig. 2. SSCP analysis and direct sequencing of PCR products generated using the AP2PMF and AP2PMR primer sets. A single base pair substitution (G→T) at nucleotide position −803 was detected by sequencing (K = G or T). *Samples 1, 2, and 3* indicate homozygous (G/G), heterozygous (G/T), and homozygous (T/T) −803 locus genotypes, respectively. A G nucleotide insertion between nucleotides −807 and −806, and a G instead of C at nucleotide position −808 were observed in all samples, contrary to previously published sequence data

Table 3. Genotypic and allelic frequencies at the AP-2 −90 and −803 loci in schizophrenics, including subtypes and course specifiers (American Psychiatric Association 1994), and controls

		−90 locus					−803 locus				
		Genotypes			Alleles		Genotypes			Alleles	
	Subjects (*n*)	GG (%)	GC (%)	CC (%)	G (%)	C (%)	GG (%)	GT (%)	TT (%)	G (%)	T (%)
Schizophrenics (87)		35 (40)	39 (45)	13 (15)	109 (63)	65 (37)	36 (41)	39 (45)	12 (14)	111 (64)	63 (36)
		$\chi^2 = 4.12$, $P = 0.128$			$P = 0.275$		$\chi^2 = 2.36$, $P = 0.307$			$P = 0.381$	
	Paranoid type (18)	11 (61)	5 (28)	2 (11)	27 (75)	9 (25)	11 (61)	5 (28)	2 (11)	27 (75)	9 (25)
		$\chi^2 = 3.41$, $P = 0.181$			$P = 0.556$		$\chi^2 = 2.80$, $P = 0.247$			$P = 0.556$	
	Disorganized type (28)	7 (25)	16 (57)	5 (18)	30 (54)	26 (46)	7 (25)	16 (57)	5 (18)	30 (54)	26 (46)
Subtypes		$\chi^2 = 5.55$, $P = 0.062$			$P = 0.056$		$\chi^2 = 5.02$, $P = 0.081$			$P = 0.056$	
	Catatonic type and undifferentiated type (13)	5 (39)	6 (46)	2 (15)	16 (62)	10 (38)	6 (46)	5 (39)	2 (15)	17 (65)	9 (35)
		$\chi^2 = 1.54$, $P = 0.463$			$P = 0.507$		$\chi^2 = 1.30$, $P = 0.523$			$P = 0.824$	
	Residual type (28)	12 (43)	12 (43)	4 (14)	36 (64)	20 (36)	12 (43)	13 (46)	3 (11)	37 (66)	19 (34)
		$\chi^2 = 2.22$, $P = 0.330$			$P = 0.628$		$\chi^2 = 0.423$, $P = 0.810$			$P = 0.748$	
	Episodic (53)	21 (39)	20 (38)	12 (23)	62 (58)	44 (42)	22 (41)	20 (38)	11 (21)	64 (60)	42 (40)
		$\chi^2 = 9.56$, $P = 0.008$*			$P = 0.101$		$\chi^2 = 6.60$, $P = 0.037$**			$P = 0.166$	
Course	Continuous (19)	7 (37)	12 (63)	0 (0)	26 (68)	12 (32)	7 (37)	12 (63)	0 (0)	26 (68)	12 (32)
		$\chi^2 = 1.73$, $P = 0.421$			$P = 1.000$		$\chi^2 = 2.15$, $P = 0.342$			$P = 1.000$	
	Single episode and other or unspecified pattern (15)	7 (47)	7 (47)	1 (6)	21 (70)	9 (30)	7 (47)	7 (47)	1 (6)	21 (70)	9 (30)
		$\chi^2 = 0.098$, $P = 0.952$			$P = 1.000$		$\chi^2 = 0.038$, $P = 0.981$			$P = 1.000$	
Controls (100)		43 (43)	51 (51)	6 (6)	137 (69)	63 (31)	44 (44)	49 (49)	7 (7)	137 (69)	63 (31)

P values after the Bonferroni correction were * $P = 0.016$; ** $P = 0.074$

Discussion

Three polymorphisms were found in the promoter region, two relatively common (−90G→C, −803G→T) and one rare (−1769G→A). Polymorphic status at both loci suggested strong linkage disequilibrium between the −90 and −803 loci. This may be the first report of nucleotide sequence variants of the human *AP-2* gene. However, none of the three nucleotide variants reported in this study corresponded to the motifs relating to transcriptional regulation of the *AP-2* gene. This suggests that the variants are not likely to have functional influence on the transcriptional activity of the *AP-2* gene.

The whole coding region containing the AP-2B specific region, and all of the exon-intron boundaries of the *AP-2* gene were analyzed in order to identify genetic variations that may lead to the alteration of protein function. However, no variant was detected in these coding regions. This suggests that the sequence in the coding region of the *AP-2* gene may be well conserved, or that a severe phenotype may be expected from the alteration of protein function due to mutations of the *AP-2* gene, as is seen for the CREB binding protein (CBP) in Rubinstein-Taybi syndrome (Petrij et al. 1995). However, it is possible that we missed a variant, as the sensitivity of SSCP analysis is not believed to be 100% (Hayashi 1992; Jordanova et al. 1997).

No significant differences in genotypic and allelic frequencies at the −90 and −803 loci were found between patients and controls. Significant differences in the distribution of genotypes at the −90 and −803 loci were observed in patients with an episodic course compared with controls, although no significant differences in allelic frequencies were observed between the groups. The observed *P* value for the distribution of genotypes in patients with an episodic course showed siginificance with regard to the –90 locus after the Bonferroni correction for multiple comparisons. In contrast, a type I error may be involved in the positive result of the –803 locus, since the observed P value showed no significance after the Bonferroni correction. Our small number of samples may have led to a discrepancy between the results of statistical analysis of the –90 and –803 loci with strong linkage disequilibrium and to a discrepancy between the results of statistical analysis of genotypic and allelic frequencies at the −90 locus. A substantially larger sample size may clarify these inconsistencies.

Recent studies have suggested that functional polymorphisms in the promoter regions of genes expressed in the brain are involved in the susceptibility to neuropsychiatric disorders, including schizophrenia, affective disorder, panic disorder, and Alzheimer's disease (Collier et al. 1996; Arinami et al. 1997; Artiga et al. 1998; Ohara et al. 1998; Wang et al. 1998). Considering the many genes that contain AP-2 binding sites, the widespread roles of AP-2, and the profound developmental abnormalities observed in AP-2 null mice (Schorle et al. 1996; Zhang et al. 1996), it is possible that variants located not in the coding region but in the promoter region of the *AP-2* gene, which may exert a subtle effect on AP-2 protein expression, could be involved in the development of particular schizophrenic phenotypes.

In conclusion, our data provided no direct evidence of an association between schizophrenia and the polymorphisms of the *AP-2* gene, although the positive result at the −90 locus in schizophrenics with an episodic course is potentially interesting. Therefore, a follow-up study with a large sample size will be necessary to determine whether there is significant statistical evidence for involvement of the *AP-2* gene in susceptibility to schizophrenia. The polymorphic site did not correspond to any putative consensus sequences of transcriptional factors reported previously, but we can not exclude the possibility that this polymorphic site may be involved in the binding cis-elements of unknown transcriptional factors. Further detailed analysis will be necessary to support the notion that the transversion from −90 G to C changes the transcriptional activity of the *AP-2* gene. These analyses are being carried out in our laboratory.

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