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Three CAG Trinucleotide Repeats on Chromosome 6 (D6S1014, D6S1015, and D6S1058) Are Not Expanded in 30 Families with Schizophrenia

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Since 1991, more than five neuro-genetic disorders have been recognized to be caused by trinucleotide repeat expansions, and the list of such diseases should grow. The diseases are characterized clinically by the phenomenon of anticipation, i.e., worsening of the disease phenotype in successive generations with increasing trinucleotide repeat expansion. The presence of anticipation in familial schizophrenia has been suggested. Several studies have provided supportive evidence that the suceptability locus for schizophrenia is on chromosome 6. Therefore, we analyzed three CAG trinucleotide repeat clones D6S1014, D6S1015, and D6S1058 on chromosome 6, which are polymolphic in 30 families with schizophrenia. No unusually, long alleles that would suggest abnormal expansion of more than 35 trinucleotide repeats were observed for these genes. Also, no statistically significant differences were found between the offspring and parental generations of affected subjects or between the affected and unaffected subjects in families with schizophrenia.
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KEY WORDS: *Schizophrenia, Trinucleotide repeat, Chromosome 6, Anticipation, Polymorphism*

It has been recognized since 1991 that the expansion of trinucleotide repeats comprises the pathogenesis of several inherited neurotic diseases, i.e., fragile X syndromes, myotonic dystrophy (DM), X-linked spinal and bulbar muscular atrophy (SBMA), Huntington's disease (HD), spinocerebellar ataxia type 1 (SCA1), dentatorubral-pallidolusian atrophy (DRPLA), Machado-Joseph disease (MJD), and Friedreich's ataxia (Paulson and Fischbeck 1996; Warren 1996).

These diseases are characterized by genetic anticipation, i.e., a decrease in the age of onset and/or an increase in the severity of the disease in subsequent generations. Although the presence of anticipation in psychoses had been proposed by Morel (1857) and Mott (1910, 1911), the idea was dismissed due to various ascertainment biases (Penrose 1948, 1971). However, the recent discovery of trinucleotide repeat expansion diseases has reawakened interest in the phenomenon of anticipation in psychiatric diseases (McInnis et al. 1993; Bassett and Honer 1994; Ohara et al. in press). These re-

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sults suggest the possibility of trinucleotide repeat expansion in the pathogenesis of schizophrenia.

There have been several investigations on trinucleotide expansions in schizophrenics. O'Donovan et al. (1995), and Morris et al. (1995) found, using the method of Repeat Expansion Detection, that the number of CAG repeats in schizophrenics was larger than that in healthy control subjects. We studied five novel trinucleotide repeats, i.e. clones CTG (GAG) - A4, B1, B43 and B45d, and CCG (CGG) - A3, identified by Li et al. (1993) in a human brain cDNA library in subjects from 17 families with schizophrenia. None of the trinucleotide repeats was abnormally expanded (Ohara et al. in press). Sasaki et al. (1996) showed that the trinucleotide repeats B1, B33, and B37, and the N-cadherin gene were not expanded in unrelated schizophrenics (n = 53 to 74).

Recently, a region of the short arm (p) of chromosome 6 has received much attention in schizophrenia. Wang et al. (1995) performed linkage analysis in 186 multiplex families with schizophrenia using a nonparametric affected pedigree member test, and provided supportive evidence for a susceptibility locus for schizophrenia on distal 6p (Wang et al. 1995). Subsequent studies supported their findings (Straub et al. 1995; Moises et al. 1995). Gastier et al. (1996) identified a (CAG/CTG)n repeat in 479 unique sequences, including 15 clones containing the repeat on chromosome 6, on the screening of a human genomic cosmid library. They also showed that only repeats of eight or more units tended to be polymorphic (Gastier et al. 1995).

These results prompted us to analyze the three CAG trinucleotide repeat clones, D6S1014, D6S1015, and D6S1058 on chromosome 6 reported by Gastier et al. (1996), which comprise eight or more CAG trinucleotide repeats and exhibit polymorphism, in 30 families with schizophrenia.

MATERIALS AND METHODS

Family Samples

Thirty Japanese and Chinese extended non-consanguineous families containing two or more live individuals affected by schizophrenia in two generations were identified in Hamamatsu, Japan, and Shanghai, China. Each subject (or his or her legal guardian) gave written informed consent for participation in this study, after the procedures had been fully explained. The ages of the subjects studied were 16 years or above. The families were identified by designated psychiatrists, and local hospitals which were searched for schizophrenic patients who had at least one living first-degree relative with schizophrenia. Family-history information was obtained for each subject from two or more family members by means of the Family History-Research Diagnostic Criteria (FH-RDC) method (Andreasen et al. 1977). Subsequently, the diagnoses were made for all the living subjects, using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (American Psychiatric Association 1994) criteria for schizophrenia, by means of a structured diagnostic interview with the Schedule for Affective Disorders and Schizophrenia (SADS) (Endicott and Spitzer 1978), by trained psychiatrists (KoO for the Japanese, and H-DX and D-SX for the Chinese subjects). Bilinear families with evidence, in the family, or collateral history, of schizophrenia or other non-affective psychotic disorders on both sides were excluded. The subjects from 17 of the families with schizophrenia had been previously studied using the trinucleotide repeats, B43, B45d, A3, A4 and B1, (Li et al. 1993), but no disease specific expansions was found (Ohara et al. in press). The numbers of subjects examined per family were; five subjects, two families; four subjects, 17 families; three subjects, nine families; and two subjects, two families. The details are given in Table 1. Each family comprised two or three affected individuals in two generations. One family included three generations, while the other 29 families included two generations. The affected subjects were classified according to the DSM-IV criteria; disorganized type schizophrenia, 7, 2, 0; paranoid type schizophrenia, 9, 8, 0; catatonic type schizophrenia, 3, 3, 1; undifferentiated type schizophrenia, 13, 14, 0; residual type schizophrenia, 0, 2, 0; and schizotypal personality disorder, 0, 2, 0, of the offspring, parental, and grandparental generations, respectively. The linkage to chromosome 6 in schizophrenia was not analyzed in these families.

Table 1. Demographic and Clinical Characteristics of the Subjects in 30 Families with Schizophrenia

	Offspring Generation				Parental Generation				Grandparental Generation			
Variable	**T**	**S**	**P**	**U**	**T**	**S**	**P**	**U**	**T**	**S**	**P**	**U**
Number	49	32	0	17	57	29	2	26	1	1	0	0
Male/female	33/16	25/7		8/9	29/28	11/18	2/0	16/10	0/1	0/1		
Age (SD) (yrs)	31.2 (1.07)				56.3 (1.40)				70			
Age at onset (SD) (yrs)	21.3 (6.06)				35.2 (11.4)				67			

T, total subjects; S, schizophrenia; P, personality disorder; U, unaffected subjects.

Amplification of the Trinucleotide Repeats by Polymerease Chain Reaction (PCR)

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood. The primer sequences used for the amplification of the three (CAG)n trinucleotide repeats localized on chromosome 6, i.e., D6S1014, D6S1015, and D6S1058, were described by Gastier et al. (1996) in the electronic information source of the Whitehead/MIT Center for Genome Research WWW site (http://www genome.wi.mit.edu). The sizes and CAG repeat numbers of the major PCR products were: 139 bp and 11 repeats for D6S1014, 178 bp and 11 repeats for D6S1015, and 160 bp and eight repeats for D6S1058, respectively. PCR for D6S1014 and D6S1058 was performed in tubes containing 100 ng of genomic DNA, 100 ng of each primer, 200 μmol of each dNTP, and 2.5 U of Gene Taq polymerase (Wake Co.), in a final volume of 25 μl Gene Amp buffer. As D6S1015 could not be amplified with Gene Taq polymerase, Pwo polymerase (Boehringer Mannheim), in addition to TaqStart antibody (Clonetech), was used, in a final volume of 50 μl. Amplification was carried out for 30 cycles: each cycle consisted of: incubations for 60 sec at 96°C for denaturation; 60 sec at 62°C and 60°C for the annealing of D6S1014 and D6S1058, and D6S1015, respectively; and 60 sec at 72°C for primer extension. At the beginning of the first cycle, the DNA was denatured at 95°C for 3 min; and following the last cycle, the samples were incubated at 72°C for 4 min. The samples were stored at 4°C.

Electrophoresis and Southern Blot Hybridization

The amplified DNA samples were analyzed by gel electrophoresis on a 7.5% polyacrylamide gel in 1 × TGE buffer at 200 V for 30 minutes, at room temperature. The gel was stained with ethidium bromide, with visualization by UV transillumination. The gel was transferred to a 3MM filter paper, followed by DNA electrotransfer to a Hybond N+ nylon membrane (Amersham) at 1A for 2 hours in 1 × TBE. Following UV immobilization, the membrane was hybridized for 16 hours at 58°C to digoxigenin tail-labeled (GAG)10. Subsequently, a luminescence detection reaction was performed according to the manufacturer's protocol (Boehringer Mannheim), followed by autoradiography for 2 hours with Kodak X-ray film. The CAG repeat numbers were determined from the product sizes. See Figure 1.

CAG trinucleotide Repeat Analyses

The purified DNAs were re-amplified by PCR and ligated into plasmid pCR™II, according to the manufacturer's protocol (TA cloning kit, Invitrogen). The numbers of CAG trinucleotide repeats in the main amplified samples of 140 bp for D6S1014, 180 bp for D6S1015, and 160 bp for D6S1058 were confirmed by the dideoxy chain termination method (Sanger et al. 1977). The numbers of CAG trinucleotide repeats in the rest of the amplified samples were determined by size comparison with the main band, respectively.

RESULTS

No unusually large alleles for the three genes tested, which might reflect abnormal expansion of the trinucleotide repeat, were observed in any of the individuals in the 30 families with schizophrenia.

The allele counts are summarized in Table 2. The allele was measured by Southern blot analysis, and the number of repeats determined.

The PCR product for D6S1014 ranged from about 140 bp to 200 bp, which represents 11 to 31 CAG trinucleotide repeats. Other than one or two major bands, sometimes a few less intense bands were observed for the D6S1014 PCR amplified product (Figure 1). There was no difference in allele frequency between the affected and unaffected subjects ($df = 5$, $\chi^2 = 2.38$, $p = 0.795$), or between the affected subjects of the offspring and parental generations ($df = 5$, $\chi^2 = 6.41$, $p = 0.268$).

D6S1015 has two polymorphisms. The allele frequencies were not different between the affected and unaffected subjects ($df = 1$, $\chi^2 = 0.24$, $p = 0.622$), or between the affected subjects of the offspring and parental generations ($df = 1$, $\chi^2 = 0.003$, $p = 0.954$).

The sizes of the PCR products at D6S1058 are about 150 bp, 155 bp, and 160 bp. There was no difference in allele frequency between the affected and unaffected subjects ($df = 2$, $\chi^2 = 0.085$, $p = 0.969$), or between the affected subjects of the offspring and parental generations ($df = 2$, $\chi^2 = 2.40$, $p = 0.302$).

DISCUSSION

Trinucleotide repeat expansion diseases are classified into CAG expanded and non-CAG expanded types. The CAG expanded type comprises five progressive neuro-degenerative disorders, i.e., SBMA, HD, SCA1, DRPLA, and MJD. These disorders involve constrained CAG repeat expansions that encode polyglutamine tracts in the disease gene protein. The premutation alleles cause little or no disease in individuals, but give rise to significantly amplified repeats in affected progeny. The numbers of CAG trinucleotide repeats in the corresponding pathogenesis genes range from 6 to 39 in a normal population, and 36 to 121 in an affected population (Paulson and Fischbeck 1996).

The numbers of the three trinucleotide repeats studied here were all below 34, and no large expanded trinucleotide repeats were found in the subjects in the

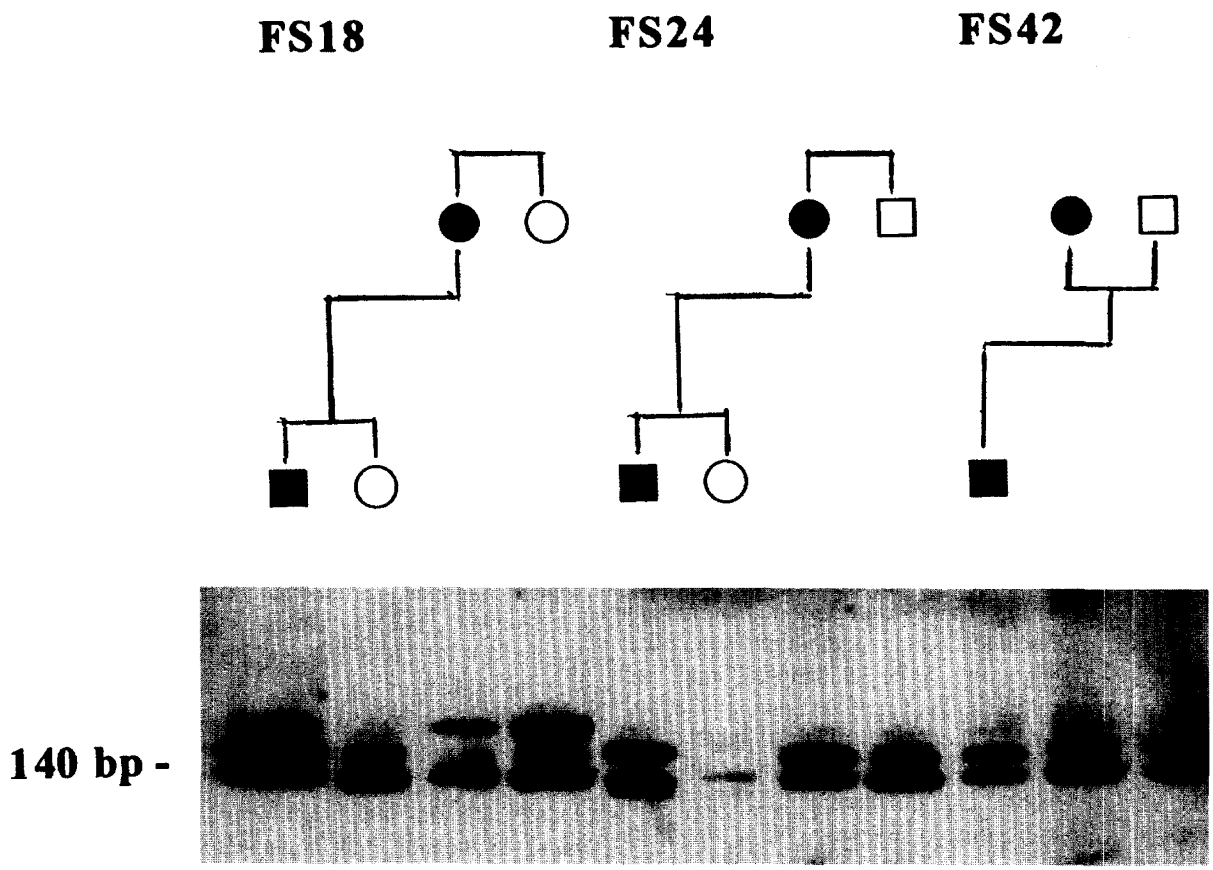

Figure 1. Southern blot analyses of D6S1014 in the subjects from three families. PCR amplified samples were electrophoresed on a polyacrylamide gel, followed by subsequent electro-transfer to a nylon membrane. The DNAs were detected with digoxigenin tail-labeled (CAG)10. See also Materials and Methods. ■, □, male; ●, ○, female; ■, ●, affected subject; □, ○, unaffected subject.

families with schizophrenia. Our results suggest that clones D6S1014, D6S1015, and D6S1058 are not part of the pathogenesis of schizophrenia. However, the pathogenesis of schizophrenia should be heterogeneous, and the number of families with schizophrenia examined here was small, so the results may not rule out the expansion of trinucleotide repeats in a large number of schizophrenics. Risch and Merikangas (1996) proposed that 100 to 1000 families were needed for association studies on complex human diseases. The possibility should also be considered that the PCR may have not efficiently amplified the larger expansions of CAG, and thus expansions may have been missed.

A few less-intense bands, in addition to the most intense major band(s) of the D6S1014 PCR products, were present to some extent. Although some corrections might be required if contributions from such PCR artifacts are to be avoided, correction for the PCR products derived from genomic DNA containing multiple copies with different CAG: repeat lengths could be complex. The presence of several PCR products may reflect the length heterogeneity of CAG repeats in somatic cells. Alternatively,

Table 2. Allele Frequencies of D6S1014, D6S1015 and D6S1058

	D6S1014						D6S1015		D6S1058		
Approximate size (bp)	140	150	160	170	180	200	170	180	150	155	160
Estimated repeats numbers	11	12–15	16–18	19–21	22–25	26–31	8–10	11	5	6, 7	8
Offspring generation											
Affected subjects	35	3	9	3	2	2	6	58	33	0	31
Unaffected subjects	19	0	11	0	1	3	3	31	15	1	18
Parental generation											
Affected subjects	33	3	21	0	5	0	6	56	28	2	32
Unaffected subjects	28	3	16	1	3	1	7	45	26	0	26

the alleles could have been derived from a separate polymorphic system located on another chromosome, as was observed for clone CTG-B33 (Li et al. 1993).

Gastier et al. (1996) reported 15 clones of CAG trinucleotide repeats on chromosome 6. We did not study the remaining 12 clones, because 10 clones (GCT4A11, GCT5A01, GCT5A02, GCT8G05, GCT10C05, GCT11EO1, GCT12B05, GCT12B12, GCT12G04, and GCT16F02) are not polymorphic according to their Table 2 and we could not obtain enough information on GCT16B08 (repeat number, 8; allele, 2) or GCT16D06 (repeat number, 7; allele, 2) from the electronic information source of the Whitehead/MIT Center for Genome Research WWW site.

Non-CAG expanded type diseases have been reported, i.e., Fragile X syndromes (CGG/GCC), DM (CTG in the coding strand), and Friedreich's ataxia (GAA). These types of trinucleotide repeats should also be studied in regards to schizophrenia.

We should also mention that not all trinucleotide repeat expansions are disease-related. Schalling et al. (1993) found an expanded trinucleotide repeat on chromosome 18, segregating at the Centre d'Etude du Polymorphism Humain, in families unaffected by either neuro-psychiatric or neuro-degenerative disorders.

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