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Novel CAG/CTG Repeat Expansion Mutations Do Not Contribute to the Genetic Risk for Most Cases of Bipolar Disorder or Schizophrenia

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The possible presence of anticipation in bipolar affective disorder and schizophrenia has led to the hypothesis that repeat expansion mutations could contribute to the genetic etiology of these diseases. Using the repeat expansion detection (RED) assay, we have systematically examined genomic DNA from 100 unrelated probands with schizophrenia and 68 unrelated probands with bipolar affective disorder for the presence of CAG/CTG repeat expansions. Our results show that 28% of the probands with schizophrenia and 30% of probands with bipolar disorder have a CAG/CTG repeat in the expanded range, but that each expansion could be explained by one of three nonpathogenic repeat expansions known to exist in the general population. We conclude that novel CAG/CTG repeat expansions are not a common genetic risk factor for bipolar disorder or schizophrenia.

KEY WORDS: trinucleotide; anticipation; triplet; psychosis; depression

INTRODUCTION

The discovery of repeat expansion mutations 10 years ago explained and biologically validated the genetic concept of anticipation (earlier onset of disease in later generations of a family) [Fu et al., 1991; La Spada et al., 1991; Mahadevan et al., 1992]. The existence of these unstable mutations has led to intense speculation that many diseases with anticipation may be caused by repeat expansions [Ross et al., 1993]. For some diseases the speculation has proven accurate [HD Collaborative Research Group, 1993; Orr et al., 1993], while for others the discovery of more common types of causal mutations suggests that anticipation was an artifact or a consequence of other factors [Bürger et al., 1996; Fink, 2002]. For bipolar affective disorder and schizophrenia, the situation has remained unclear. Most, but not all, of the more than 20 studies that have addressed the issue have found anticipation in one or both of the diseases [Margolis et al., 1999]. However, even if present, anticipation might be explainable by mechanisms other than the expansion of unstable repeats during vertical transmission [Goossens et al., 2001; Jones et al., 2002].

Nonetheless, the relative frequency with which expansion mutations result in CNS disease, the relative ease with which repeat expansions can be detected, and the limited success in finding conventional genetic risk factors for bipolar disorder and schizophrenia support a search for repeat expansions in these diseases. The focus thus far has been on CAG/CTG repeats, since this is the type of expansion most commonly associated with brain disorders [Margolis et al., 1999], CAG/CTG repeats may be disproportionately present in transcriptional regulators of development [Margolis et al., 1997], and CAG/CTG repeat expansions can be readily detected [Margolis and Ross, 1998]. Three general approaches have been used: (1) a search for repeat expansions in individual candidate genes that have obvious relevance to brain function or development, such as KCNN3,

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which encodes a calcium activated potassium channel [Chandy et al., 1998; McInnis et al., 1999] or Mab21L1, which encodes a protein regulating neuronal differentiation [Margolis et al., 1996]; (2) a search for repeat expansions in loci linked to a disease, such as the 18q site linked to bipolar disorder [Breschel et al., 1997]; and (3) screens of genomic DNA or protein from patients for evidence of expansion at unknown loci [Bowen et al., 1996; Jones et al., 1997].

This latter type of analysis has been greatly facilitated by the use of the repeat expansion detection (RED) assay [Schalling et al., 1993], which detects the presence of repeats expanded beyond the longest normal length repeat (varying by type of repeat, but about 40 triplets for CAG/CTG repeats) found in genomic DNA. The RED assay itself provides no information about flanking sequence or the location of the repeat; however, it has been used in conjunction with other molecular techniques to find the specific repeat expansion responsible for four different disorders [Koob et al., 1998, 1999; Holmes et al., 1999, 2001]. Intriguingly, at least two groups have reported that between 48% and 66% of CAG/CTG repeat expansions detected by RED in schizophrenia, bipolar, or control populations could not be explained by any of the repeats known to undergo expansion [Guy et al., 1999; Vincent et al., 1999; Bowen et al., 2000], suggesting that currently unidentified CAG/CTG repeat expansions could contribute to the risk of bipolar disorder or schizophrenia. To explore this possibility, we used RED to identify CAG/CTG repeat expansions in 100 unrelated probands with schizophrenia and 68 probands with bipolar affective disorder, all ascertained in the United States.

MATERIALS AND METHODS

Subjects

One hundred probands with schizophrenia (including schizoaffective disorder) and a first degree family member with schizophrenia were selected from among a large group of cases collected in the United States for genetic analyses [DeLisi et al., 2002]. After obtaining informed consent, DSM-III-R [American Psychiatric Association, 1987] criteria were used to diagnose schizophrenia on the basis of structured interviews, review of medical records, and information provided by an informant. DNA used in this study was derived from cell lines. DNA from 74 bipolar probands was collected and isolated by the Johns Hopkins Bipolar study. As previously described, all probands provided informed consent, had at least two affected first degree relatives, and were assigned a diagnosis based on Research Diagnostic Criteria using the SADS-L (Schedule for Affective Disorders and Schizophrenia, Lifetime version) semi-structured interview [Simpson et al., 1992]. Six bipolar probands had previously been determined to have CTG18.1 repeat expansions [Breschel et al., 1997]. The probability of detecting an unknown expansion in our samples was calculated using an equation of binomial probability: $P = (1\text{-}(1\text{-}f)^N)*100$, in which P = probability, f = frequency of the mutation in affected individuals, and N = sample size.

Genotyping

The RED assay was performed using 5 μg (bipolar) or 2 μg (schizophrenia) of genomic DNA added to 2 μl 10× Ampligase® DNA Ligase Buffer (Epicentre) and 100 ng $(CTG)_{10}$ oligonucleotide. After denaturation at 95°C for 10 min, 10 U Ampligase™ DNA Ligase (Epicentre) was added, and the mixture was subjected to 495 cycles of 95°C for 45 sec and 80°C for 45 sec. Products were denatured at 95°C for 10 min, subjected to electrophoresis through a 6% polyacryl-urea gel, blotted onto nitrocellulose, and probed with a 3′ P^{32}-labeled (CAG)10 oligomer. After successive washes with 1× SSC/0.1% SDS at room temperature and then with 0.1 × SSC/0.1% SDS at 60°C, images were obtained from a Molecular Dynamics Phosphoimager after an overnight exposure. All RED assays were performed using DNA from an individual with a Huntington's disease expansion of a known length as a positive control. Assay conditions were adjusted to facilitate the use of small quantities of DNA (2–5 μg) such that DNA from individuals without repeat expansions always yielded bands reflecting a maximal repeat length of 30–40 CAG/CTG triplets, consistent with the longest known unexpanded CAG/CTG repeats in the human genome.

PCR of Dir-1 was performed using standard methods as previously described [Ikeuchi et al., 1998]. To confirm results visualized on agarose gel, in DNA from the schizophrenia probands, the forward primer was fluorescently labeled and automated genotyping was performed using ABI capillary electrophoresis and the software application Genotyper. There were no discrepancies with the agarose gel data. CTG18.1 and SCA8 genotyping was performed by Southern blotting. Ten micrograms of genomic DNA was digested (EcoR1 for SEF2-1, HincII for SCA8), subjected to electrophoresis on a 1% agarose gel, and transferred onto a Hybond-N+ membrane (Amersham). Probes consisted of random labeled genomic fragments flanking the repeats (1.3 kb for SEF2-1, generated as previously described [Breschel et al., 1997], and 571 bp for SCA8, generated by PCR using primers 5′-ACCACTGCTCCTACTTTTAGTG-CAG-3′ and 5′-TCCTCACAAGCCTTTCTCAAACAC-3′). After washes in 1×SSC/0.1% SDS at room temperature and 0.1×SSC/0.1% SDS at 60°C, images were obtained from a Molecular Dynamics Phosphoimager after an overnight exposure.

RESULTS

The sample size in the study provided >80% probability of detecting an unknown expansion present in 2% of cases of schizophrenia and 3% of cases of bipolar disorder (Binomial Probability equation). The results of the RED analysis revealed that 28 of 100 schizophrenia probands (28%) and 16 of 68 of bipolar probands (23.5%; 29.7% including the six probands with known CTG18.1 expansions) had repeat expansions (Fig. 1A, Table I).

We next determined if these expansions could be accounted for by expansions of the Dir-1/ERDA locus on chromosome 17q21.3 [Nakamoto et al., 1997; Ikeuchi et al., 1998]. Expansions at this locus range from just

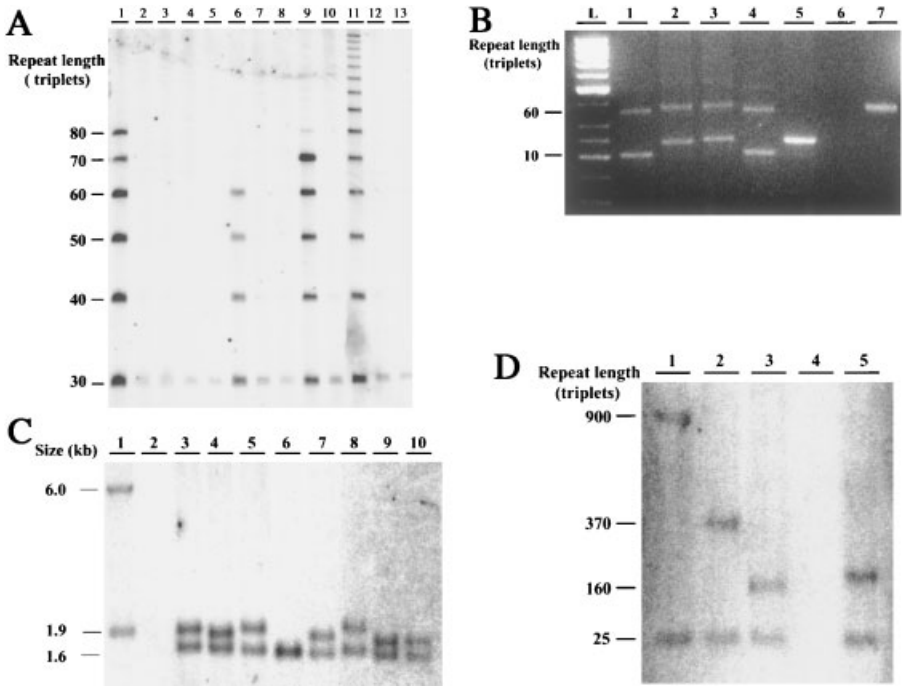

Fig. 1. RED and PCR assays. **A**: RED result. CAG/CTG expansions in schizophrenia probands are present in **lanes 1** (80 triplets), **6** (60 triplets), **9** (70 triplets), and **11** (≫110 triplets). **B**: Dir-1 expansions. Expansions in Dir-1, detected by PCR and visualized on a 1% agarose gel, in four schizophrenia probands (lanes 1–**4**), along with a case with no expansion (**lane 5**), negative control (**lane 6**), and a control sample from a known Dir-1 expansion homozygote (**lane 7**). **C**: CTG18.1 expansions. Southern blot of an EcoR1 digest of genomic DNA from the eight individuals with schizophrenia who were RED positive but DIR1 negative. On this gel, 1.6 kb fragments correspond to repeat lengths of approximate 10 triplets, 1.9 kb corresponds to about 100 triplets, and 6.0 kb corresponds to about 1500 triplets. Lane 1: Control with known SEF2-1 expansions. Lane 2: Negative control (no DNA). Lanes 3–10: Schizophrenia probands with CAG/CTG expansions by RED and no expansion of Dir-1. Lanes 3–5 and 7–10 demonstrate moderate expansions, while lane 6 demonstrates an apparent homozygote with no repeat expansion. **D**: SCA8 expansion. Southern blot of a HincII digest of DNA from the schizophrenia proband with a CAG/CTG expansion by RED and no Dir-1 or CTG18.1 expansion. Lanes 1–3: Positive controls with different known lengths of the SCA8 repeat expansion. Lane 4: Negative control (no DNA). Lane 5: Schizophrenia proband with SCA8 expansion of about 180–200 triplets.

over 40 to 90 triplets and occur in 6 to 28% of the population, varying by ethnic group, and have not been associated with a phenotype [Nakamoto et al., 1997; Ikeuchi et al., 1998; Vincent et al., 1999; Meira-Lima et al., 2001; Del Favero et al., 2002]. Using PCR (Fig. 1B), we found that all 16 bipolar probands and 20 of 28 schizophrenia probands with repeat expansions by RED also had DIR-1 expansions, with lengths corresponding to the RED expansion lengths predicted by RED.

We then examined repeat length of the CTG18.1 locus on chromosome 18q21.1 in the eight cases of schizophrenia with RED expansions not accounted for by Dir-1. CTG18.1 was originally identified because of its

TABLE I. Summary of CAG/CTG RED Results

Repeat length (RED estimate)	Schizophrenia probands	Specific expansions detected	Length of detected expansions	Bipolar probands	Specific expansions detected	Length of detected expansions
30	51	—	—	44	—	—
40	21	—	—	8	—	—
50	6	5 Dir1	55–61	1	1 Dir1	60
		1 SEF2-1	60			
60	15	12 Dir1	62–78	14	14 Dir1	65–77
		3 SEF2-1	70–80			
70	1	1 Dir1	85	1	1 Dir1	N/A
80	4	2 Dir1	75, 80	—		
		2 SEF2-1	80, 100			
110	1	1 SEF2-1	120	—		
≫110	1	1 SCA8	200	—		

All lengths are in number of triplets. N/A = quantitative results not available. Note that six bipolar probands with previously identified CTG18.1 expansions are not included (RED assay not performed).

location near a bipolar linkage region [Breschel et al., 1997]. The repeat is within an intron of SEF2-1, a widely expressed transcription factor. Expansions as long as thousands of repeats are present in about 3% of the population. Most [Guy et al., 1999; Vincent et al., 1999; Bowen et al., 2000; McInnis et al., 2000; Meira-Lima et al., 2001], though not all [Del Favero et al., 2002], evidence suggests that CTG18.1 expansion is not associated with a psychiatric phenotype. Southern blots of EcoR1 digested DNA using a probe flanking the repeat demonstrated that CTG 18.1 could account for 7 of the 8 Dir-1 negative RED expansions in our sample of schizophrenia patients (Fig. 1C). The frequency of CTG18.1 expansions in our schizophrenia sample (7%) was similar to the frequency identified previously in our bipolar sample (6 of 74, 8%).

We next examined the SCA8 CTG repeat, located on a nontranslated gene on chromosome 13q21, in the remaining RED positive case. Repeat expansions of about 100–150 triplets have been associated with the rare neurodegenerative disorder spinocerebellar ataxia type 8 [Koob et al., 1999], and have also been detected (with lengths of 45 to >1000 triplets) in some samples of the neurologically well general population [e.g., Vincent et al., 2000a]. The penetrance of the mutation, if it is indeed causally related to the SCA8 phenotype, is therefore probably low; an individual with the expansion is more likely to be unaffected than affected. Southern blots of HincII digests of genomic DNA from the single DIR-1 and CTG18.1 negative schizophrenia proband revealed an expansion similar in length to that predicted by RED (Fig. 1D). This rather atypical individual was first noted to be unusually agitated and aggressive by age 3. He was initially treated for ADHD, and attended special education classes. Symptoms have included depression and irritability, with multiple paranoid delusions beginning at age 13. Diagnosis at time of study entry at age 15 was schizoaffective disorder with ADHD. A sibling has a very similar presentation, the mother has a history of depression, and other maternal relatives reportedly have schizophrenia. There is no known history of ataxia in the proband or in other family members.

DISCUSSION

Our results indicate that unexplained CAG/CTG repeat expansions probably do not contribute to the genetic risk for most cases of bipolar disorder or schizophrenia, and are probably quite rare. The discrepancy in the percentage of unexplained RED positive individuals between our findings and that of previous studies is quite marked. It seems unlikely that differences in ethnic composition (American vs. U.K.) or the sensitivity and specificity for detecting Dir-1, CTG18.1, or SCA8 expansions (performed by fairly standard PCR and Southern assays) could account for such a large difference in unexplained RED positives. The most likely explanation is a difference in RED technique and interpretation, as minor technical changes in the protocol can lead to detection of artifactually long repeats.

While this study provides strong evidence against the hypothesis that CAG/CTG repeat expansions contribute to the genetic risk for most cases of bipolar disorder or schizophrenia, we cannot exclude the possibility that rare cases of bipolar disorder or schizophrenia may be associated with a CAG/CTG repeat expansion. In addition, our methods do not exclude the possibility that some of the RED positive individuals had both one of the known repeat expansions and a second, unidentified repeat expansion (these can occasionally be detected by variations of RED or subtle variations in the RED ladder). Furthermore, our methods would not have detected very short expansions, such as the expansion of the CAG repeat in the CACNA1A gene to 20–30 triplets that causes spinocerebellar ataxia type 6 [Zhuchenko et al., 1997]. Though our study was not designed to address the association between one of the known repeat expansions and bipolar disorder or schizophrenia, it is of potential interest that 7% of schizophrenia subjects and 8% of bipolar subjects had an CTG18.1 expansion, somewhat higher than in the general population and similar to the percentage detected in the two studies that concluded that CTG18.1 may be a risk factor for bipolar disorder [Lindblad et al., 1998; Del Favero et al., 2002]. It has also been suggested that SCA8 expansions, detected in one proband with schizoaffective disorder in this study, could be a genetic risk factor for psychiatric disorders [Vincent et al., 2000b]. Finally, and most importantly, our study addresses only CAG/CTG repeat expansions. Other types of repeats, including GAA/TTC, CGG/CCG, ATTCT, and CCTG/GGAC, expand to cause disease, and remain possible etiologies for psychiatric disorders [Margolis et al., 1999; Matsuura et al., 2000; Liquori et al., 2001].

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