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ORIGINAL RESEARCH ARTICLE

Association and linkage studies between bipolar affective disorder and the polymorphic CAG/CTG repeat loci *ERDA1*, *SEF2-1B*, *MAB21L* and *KCNN3*

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Several reports have suggested the presence of anticipation in bipolar affective disorder (BPAD). In addition, independent studies using the RED (repeat expansion detection) have shown association between BPAD and longer CAG/CTG repeats. Therefore loci with large CAG/CTG repeats are plausible candidates in the inheritance of BPAD. The present study assesses the length of the repeats in four loci: the *ERDA-1* locus which is known to account for most of the long CAG repeats detected by RED, the *SEF2-1b* locus which is placed in a region where positive linkage results have been reported and the loci *MAB21L* and *KCNN3* as functional candidate genes. A Brazilian case-control sample with 115 unrelated BPAD patients and 196 healthy control subjects and 14 multiply affected bipolar families was investigated. With the case-control design the distribution of alleles between the two groups did not approach statistical significance. The extended transmission disequilibrium test (ETDT) performed in our families did not show evidence for linkage disequilibrium. Parametric and non-parametric linkage analysis also did not provide support for linkage between any of the four loci and BPAD. Our data do not support the hypothesis that variation at the polymorphic CAG/CTG repeat loci *ERDA-1*, *SEF2-1b*, *MAB21L* or *KCNN3* influence susceptibility to BPAD in our sample. *Molecular Psychiatry* (2001) **6,** 565–569.

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Introduction

Although a hereditary basis for bipolar disorder (BPAD) has been consistently supported by genetic epidemiology studies,[1] it has proved difficult to localise predisposing genes. The attempts to identify susceptibility genes may be summarised in two approaches: either through the search for a genetic linkage of the phenotype with genetic markers on the chromosomes or through the demonstration of an association between the disease phenotype and polymorphism in candidate genes, chosen on the basis of a pathophysiologic hypothesis.

A new perspective arose in the early '90s with the discovery of the 'dynamic mutation', a novel class of genetic mutation related with trinucleotide repeats expansion (TRE). A growing number of diseases has been found resulting from the expansion of reiterated sequences of trinucleotides such as Huntington's disease, myotonic dystrophy, fragile X syndrome, Friedreich's ataxia and spinocerebellar ataxias. Almost all of the TRE disorders affect the central nervous system, display variable phenotypic expressions and increase the disease severity or decrease the age of onset throughout successive generations of a family, a phenomenon that is termed anticipation.[2,3]

A large number of reports has suggested the presence of anticipation in BPAD,[4–7] although these data should be interpreted with caution due to some ascertainment biases that can not be ruled out (such as recall bias, cohort effect, decreased fertility, sensitisation to illness).[8] In addition, many aspects of inheritance of bipolar disorder could be explained by TRE (such as variable phenotypic expression, skipped generations or twin discordance).[9]

The RED (repeat expansion detection), a strategy developed to detect repeat expansions on genomic DNA without prior knowledge of its location,[10] has shown association between BPAD and longer trinucleotide CAG/CTG repeats,[11–15] although not all studies found a significant association.[16–18]

In the light of these data it has been postulated that

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loci with CAG/CTG repeat expansions could be candidate genes for the genetic inheritance of bipolar disorder. The present study investigated four CAG/CTG loci using a Brazilian case-control sample and BPAD multiply affected families. The four loci were:

The ERDA1 (MIM 603279) also called Dir-I, a repeat localised on 17q21.3, highly polymorphic (10–92 repeats) and whose flanking sequences showed no homology with any reported gene,[19] although Northern blot analyses indicate that it is expressed in the brain and other tissues.[20] This repeat is known to account for most of the long CAG repeats detected by RED.[21,22]

The highly polymorphic CAG/CTG repeat (typically 10–37 repeats) which is placed within the third intron of transcription factor gene SEF2-1b (MIM 602272). This locus maps to chromosome 18q21.1,[23] a region where positive linkage results have been reported for BPAD families.[24,25]

The polymorphic CAG/CTG repeat locus (6–31 copies) identified within the 5′ untranslated region of the gene MAB21L (MIM 601280). This gene is localised on 13q13 and encodes a protein regulator of neuronal development.[26]

The fourth, a polymorphic repeat coding for a polyglutamine stretch in the exon 1 of the KCNN3 (MIM 602983) formerly named hSKCa3.[27] This CAG repeat localised on 1q21 is an exception from the previous ones, because it showed no sufficiently large alleles to explain the findings of RED studies in BPAD-affected patients and no allelic instability has been reported during parental transmission.[3] Since this gene encodes a calcium gated potassium channel, these CAG repeat arrays are of interest because of the function of gene in which they reside.[2]

Materials and methods

Subjects

Families Fourteen probands had families available for a linkage analysis. DNA was obtained from 96 members of these families who were interviewed personally by a psychiatrist using the Schedule for Affective Disorder and Schizophrenia—lifetime version (SADS-L). Forty-two individuals fulfilled the DSM-III-R criteria for BPAD, 16 had spectrum disorders (major depression, schizoaffective), and 38 were currently unaffected. Individuals with spectrum disorders were considered affected in linkage analysis.

BPAD patients A hundred and fifteen unrelated patients with BPAD (62% females), aged between 18–80 years (mean age 43, SD ± 12) were recruited from public psychiatric assistance services in the São Paulo City metropolitan area. All patients fulfilled the DSM-III-R criteria for BPAD after psychiatric interviews and analysis of medical notes. Venous blood samples were collected after written informed consent was obtained.

Controls A hundred and ninety-six ethnically matched healthy control subjects (50% females) aged between 18–60 years (mean age 33, SD ± 9) were selected from unrelated subjects admitted to the Blood Donation Center of the Fundação Pró-Sangue/São Paulo University Medical School. An attempt to match both groups according to ethnic origin was based on the phenotype (pigmentation of the abdomen, hair colour and type and conformation of the nose and lips) and family history assessed by two different physicians.[28] The ethnic distribution of both groups was as follows: 45% Caucasian, 45% mulatto and 10% black. Venous blood was obtained for genomic DNA extraction after consent had been given.

Genotyping

The polymorphic CAG/CTG repeats of the studied loci were amplified by PCR using the following primer pairs, previously described:[20,23,26,27] for *ERDA1* f:(5′ACCAATAAGACTATCAATGGC3′), b:(5′TTATAACCCTGTCCAGTCAGTG3′); for *SEF21b* f:(5′AATCCAAACCGCCTTCCAAGT3′), b:(5′CCAAAACTTCCGAAAGCCATTTCT3′); for *MAB21L* f:(5′GATAAAAGGAAGGGAAAA3′), b:(5′CAGAAATGGATCAAAAAT3′) and for *KCNN3* f:(5′AAGTGCCCCTGTCCATCCTCT3′), b:(5′GCCAAGCAAGTGGTCATTGAG3′). Amplification conditions for thermal cycling (PTC-200, MJ Research, Watertown, MA, USA) of ERDA1 and SEF2–1b consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 50 s, annealing at 56°C for 100 s, extension at 72°C for 60 s and finally an elongation step at 72°C for 6 min. For MAB21L we used an initial denaturation at 96°C for 2 min, followed by 35 cycles of 96°C for 30 s, 52°C for 30 s, 72°C for 60 s and final extension at 72°C for 7 min. For KCNN3 a 5′ primary denaturation at 95°C was followed by 30 cycles of 96°C for 45 s, 59°C for 45 s, 72°C for 45 s and finally an extension at 72°C for 7 min.

PCR was performed in a total volume of 25 μl containing 40–100 ng of genomic DNA, 12.5 pM of each primer, buffer with 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris, 125 μM of each dNTP, 0.1% Triton, 1.25 μl of the DMSO 5% and 0.5 U of *Taq* Polymerase.

The PCR products were electrophoresed on denaturing 7% polyacrylamide gels and visualised after staining with SYBR Gold (Molecular Probes, Eugene, OR, USA) in direct fluorescence apparatus (Storm System, Molecular Dynamics, Sunnyvale, CA, USA). Initially, the size of the bands was determined by comparison to a known sequence ladder and the number of repeats was estimated based on the allele lengths. Later, the alleles of modal frequency of each loci were sequenced on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Because expanded alleles could be resistant to PCR amplification, all apparent homozygous and PCR 'dropouts' of loci ERDA1 and SEF2-1b ('super' alleles have been reported in both) were reamplified with PCR conditions optimised to amplify large DNA fragments. These conditions consisted of mixed reactions containing the same components described above, but with the use of 1 U of eLONGase enzyme (Gibco-BRL, Rockville, MD, USA) instead of Taq DNA polymerase. In addition,

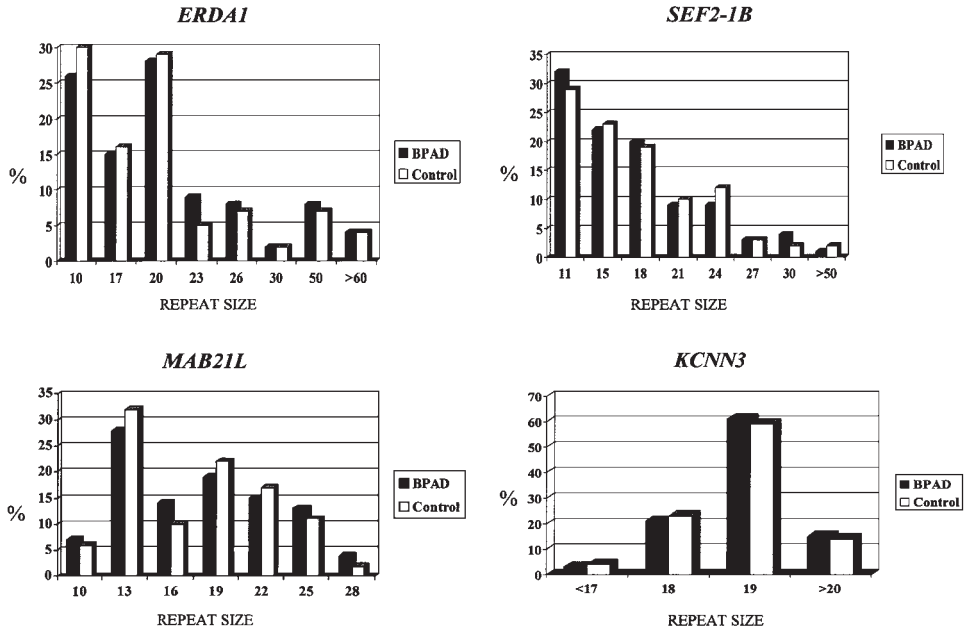

Figure 1 Allele frequency at each locus in BPAD patients and control subjects.

a program for touchdown PCR was performed as follow: initial denaturation at 94°C for 5 min; 15 cycles of 91°C for 50 s, 57°C for 100 s, 72°C for 60 s; 20 cycles of 94°C for 50 s, 56°C for 100 s, 72°C for 60 s and a final extension at 72°C for 9 min.

Statistical analysis

The distribution of alleles at each locus was compared in the patients and controls using the Mann–Whitney U test (two-tailed) implemented in the statistical package SPSS for windows version 7.0. We also used the CLUMP program, which is useful to test association between disease and alleles at highly polymorphic loci.[29] This program obtains empirical *P*-values by a permutation test procedure.

The test for non-random transmission of alleles on affected individuals from our 14 families was performed using the Extended Transmission Disequilibrium Test (ETDT)[30] and linkage analysis was performed using the computer program GENEHUNTER version 1.3.

Results

The alleles obtained in the ERDA1 locus showed a repeat number of 10–90 copies with heterozygosity of 83% for BPAD patients and 80% for controls. In the SEF2-1b locus alleles estimated at 11–60 repeats were found with heterozygosity of 82% for BPAD and 84% for controls. The MAB21L displayed alleles of 10–28 repeats with heterozygosity indices for BPAD at 91% and for controls at 89%. Finally, the KCNN3 showed alleles of 16–22 repeats with heterozygosity of 63% on the BPAD and 68% on the controls.

The distribution of the allele frequency at each locus in 115 patients and 196 controls is represented in Figure 1. The alleles with rare frequency were grouped together with the closest adjacent alleles.

No significant differences were observed in the allele distribution between BPAD patients and control subjects by Mann–Whitney U test at either the *ERDA1* locus (U = 42299.5, W = 119327.5, $z = -1.320$, $P = 0.187$), *SEF2–1b* (U = 43659.5, W = 70224.5, $z = -0.673$, $P = 0.501$), *MAB21L* (U = 43967.5, W = 120995.5, $z = -0.526$, $P = 0.599$) or *KCNN3* (U = 43408.5, W = 120436.5, $z = -0.878$, $P = 0.380$). The comparison of the allelic frequency between the groups using the CLUMP program also did not demonstrate significant differences (*ERDA1* $\chi^2 = 4.81$, 7 df, $P = 0.68$; *SEF2–1B* $\chi^2 = 4.05$, 7 df, $P = 0.78$; *MAB21L* $\chi^2 = 6.18$, 6 df, $P = 0.40$; *KCNN3* $\chi^2 = 0.94$, 3 df, $P = 0.81$)

The family study using the ETDT did not show preference for transmission of any allele or genotype in each locus to affected members, ie the number of transmitted alleles to affected individuals was not different from the number of nontransmitted alleles. The results were: ERDA1 allele-wise $\chi^2 = 11.1$, 7 df, $P = 0.14$; genotype-wise $\chi^2 = 17.4$, 14 df, $P = 0.23$; SEF2-1b allele-wise $\chi^2 = 11.6$, 10 df, $P = 0.32$; genotype-wise $\chi^2 = 4.4$, 6 df, $P = 0.62$; *MAB21L* allele-wise $\chi^2 = 5.8$, 6 df, $P = 0.45$; genotype-wise $\chi^2 = 12.6$, 11 df, $P = 0.32$ and *KCNN3* allele-wise $\chi^2 = 0.9$, 4 df, $P = 0.93$; genotype-wise $\chi^2 = 0.9$, 4 df, $P = 0.93$.

The highest Lod score obtained in a parametric linkage analysis was –2.014 at *KCNN3* locus under a dominant model, indicating nonlinkage of the studied loci with BPAD. Nonparametric linkage analysis also showed no statistical significance for sharing alleles identical by descent for *ERDA1* ($P = 0.27$), *SEF2-1b* ($P = 0.6$), *MAB21L* ($P = 0.83$), and *KCNN3* ($P = 0.59$).

Discussion

Early investigations with these repeats displayed interesting findings: at least two studies reported that 90% of larger CAG repeats detected by RED could be explained by expansions on the loci *ERDA-1* and *SEF2-1B*.[21,22] In the *ERDA-1* locus, Vincent *et al* documented the inheritance of a very large allele which expanded the number of repeats (±115 repeats) through the transmission in a family with childhood onset depression[31] and Verheyen *et al* found a modest association ($P = 0.032$) between larger alleles (≥40 repeats) in this locus and BPAD.[32] Lindblad *et al* reported that the larger alleles of *SEF2-1b* (≥40 repeats) are over-represented in patients with familial affective disorder ($P < 0.04$), conferring an odds ratio of a 2.6–2.8 fold higher risk factor for this disorder.[22] A large CAG repeat at the *MAB21L* locus (46 repeats) was identified in a subject with a bipolar disorder type II phenotype.[26] Finally, Chandy *et al* have shown a trend for association between larger repeats of CAG in the *KCNN3* locus (≥20 repeats) and BPAD.[27]

The results of our study, however, did not reveal differences in the distribution of the alleles between 115 Brazilian unrelated BPAD patients and the 196 healthy controls. Furthermore, it did not demonstrate cosegregation of larger alleles with the affected phenotype in the members of the 14 pedigrees.

As in other similar studies, the statistical power is an important issue in the interpretation of the findings. Power statistical analysis performed on EpiInfo software v. 6.0 showed that, assuming a frequency of 0.05 for the larger alleles, our sample had approximately 83–89% power to detect larger alleles as a susceptibility factor associated with an odds ratio of 2.6–2.8. as related by Lindblad *et al*.[22]

The mean age of the control sample is lower than the bipolar group, and it is possible that some individuals from the control group may eventually develop BPDA when they get older. However, since the population prevalence of BPAD is low (<1%), the potential bias added by this difference is very small. In addition, our results were similar in both population and family-based approaches.

One methodological issue regarding the PCR analysis of these loci might be that preferential amplification of small repeats leads to a failure of larger alleles to be amplified and therefore to the misclassification of the heterozygous by not showing one very large repeat as homozygous. We tried to avoid this apparent error, reamplifying the homozygous individuals with PCR conditions optimised for larger alleles, although we can not be sure that the optimisation was sufficient to identify this issue. However, the heterozygosity indices were determined for each loci studied in both groups and no differences were observed. Thus, it is unlikely that missing alleles influenced the final result.

In conclusion, the data obtained in this investigation did not support the hypothesis that larger CAG/CTG repeats at the loci *ERDA-1*, *SEF2-1B*, *MAB21L* and *KCNN3* play a role in the susceptibility for BPAD in our Brazilian patients. Some authors suggested that the total number of potential candidate genes for bipolar disorder could reach as much as 20 000.[33] However, if the research is focused only on CAG/CTG repeats, the total number of loci can be restricted to approximately 1500,[34] making the investigation easier to approach. We explored only a very small fraction of these types of loci. Thus, it is important to stress that our results do not reject the hypothesis that CAG/CTG repeats participate in the genesis of bipolar disorder. Therefore, we consider that the investigation of loci with CAG/CTG repeats continues to be an attractive strategy.

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