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ORIGINAL RESEARCH ARTICLE

Association of CAG repeat loci on chromosome 22 with schizophrenia and bipolar disorder

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Chromosome 22 has been implicated in schizophrenia and bipolar disorder in a number of linkage, association and cytogenetic studies. Recent evidence has also implicated CAG repeat tract expansion in these diseases. In order to explore the involvement of CAG repeats on chromosome 22 in these diseases, we have created an integrated map of all CAG repeats ≥5 on this chromosome together with microsatellite markers associated with these diseases using the recently completed nucleotide sequence of chromosome 22. Of the 52 CAG repeat loci identified in this manner, four of the longest repeat stretches in regions previously implicated by linkage analyses were chosen for further study. Three of the four repeat containing loci, were found in the coding region with the CAG repeats coding for glutamine and were expressed in the brain. All the loci studied showed varying degrees of polymorphism with one of the loci exhibiting two alleles of 7 and 8 CAG repeats. The 8-repeat allele at this locus was significantly overrepresented in both schizophrenia and bipolar patient groups when compared to ethnically matched controls, while alleles at the other three loci did not show any such difference. The repeat lies within a gene which shows homology to an androgen receptor related apoptosis protein in rat. We have also identified other candidate genes in the vicinity of this locus. Our results suggest that the repeats within this gene or other genes in the vicinity of this locus are likely to be implicated in bipolar disorder and schizophrenia. *Molecular Psychiatry* (2001) **6,** 694–700.

Keywords: chromosome 22; bipolar disorder; schizophrenia; CAG repeats; anticipation; microsatellite markers

Introduction

Bipolar disorder and schizophrenia are severe behavioral disorders which have a complex inheritance pattern and a uniform worldwide prevalence rate of ~1%. A genetic component for these disorders has been established on the basis of family and twin studies and numerous strategies have been attempted to identify the genes responsible for these diseases. These include linkage analysis as well as candidate gene and association studies. Both these approaches have yielded results that have not been readily reproducible in different sample sets.[1–3]

A few loci however, have been repeatedly implicated in bipolar disorder and schizophrenia by independent studies. One of the most intensively studied regions amongst these include several loci on chromosome 22.[4]

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Initial genome wide scans for schizophrenia by different groups suggested possible linkage for markers on chromosome 22q although neither of the groups reported statistically significant results.[4–6] A combined transmission disequilibrium and linkage analysis of D22S278 in 574 families further strengthened the possibility of a susceptibility locus on chromosome 22q.[7] While a number of further reports have failed to reproduce these results, others have demonstrated positive linkage findings for markers spread throughout chromosome 22q using various methods of analyses.[3,4] In the case of bipolar disorder, parametric analysis yielded a maximum lod score of 3.8 at D22S278 providing evidence for significant linkage to this chromosome.[8]

A further line of evidence implicating chromosome 22 in schizophrenia has come from the study of patients with a congenital malformation called velo cardio facial syndrome (VCFS). VCFS is known to be caused by deletions in the region of 22q11.2–q11.23 and patients suffering from this disorder show a high prevalence of psychiatric illnesses including both bipolar disorder and schizophrenia.[9–11] A sampling of 100 schizophrenic patients identified the presence of

interstitial deletions ranging from 1.5–2 Mb in the region of chromosome 22q11 in two of the patients.[12] Although large-scale epidemiological studies have not been carried out, initial studies have suggested that the frequency of chromosome 22 deletions may be 80 times higher than the general population.[11–14] Linkage analyses have also suggested a possible locus for bipolar disorder in the VCFS region.[15] Taken together, these independent lines of evidence from cytogenetic studies and linkage analysis studies suggest that chromosome 22 might indeed harbour susceptibility loci for schizophrenia and bipolar disorder.

Apart from linkage studies an alternative approach which has evoked a great deal of interest in recent years has been the study of trinucleotide repeat expansions in bipolar disorder and schizophrenia.[16] To date, more than a dozen neurological disorders have been shown to be caused by trinucleotide repeat expansions with the CAG repeat being the most frequently involved triplet. When the CAG triplet occurs in the coding regions, expansion results in long polyglutamine stretches which are thought to mediate the disease process.[17–19] One of the most important features common to these diseases and bipolar disorder and schizophrenia is the phenomenon of anticipation.[16,20,21] This inheritance pattern refers to the increase in disease severity with a reduction in the age at onset when the disease is transmitted in successive generations. The molecular basis for anticipation was elucidated with the discovery of trinucleotide repeat expansions where the increase of repeat number during intergenerational transmission results in the increased disease severity and a reduced age at onset.[17,21]

Studies of anonymous CAG repeats using the repeat expansion detection (RED) technique have demonstrated expanded repeats in schizophrenia and bipolar disorder with considerable overlap between patients and controls.[22–24] A great deal of effort has focused on the identification of loci containing trinucleotide repeats as candidate genes for these diseases. The candidate gene approach however, has been unable to demonstrate large expansions of trinucleotide repeats in the range of those seen in the diseases caused by triplet repeat expansions in patients suffering from schizophrenia and bipolar disorder.[16] The failure to observe large expansions has led to suggestions that it might be worthwhile studying moderate trinucleotide repeat expansions in patients suffering from these diseases.[25] We have also earlier proposed that a difference in allele sizes or 'allele span' at such polymorphic trinucleotide repeat loci may also be implicated in bipolar disorder and schizophrenia.[26,27]

As chromosome 22 has been repeatedly implicated in bipolar disorder and schizophrenia, we sought to test the hypothesis that susceptibility loci on this chromosome might contain expanded CAG repeats involved in the pathogenesis of these disorders. Taking advantage of the recent availability of the complete sequence of chromosome 22[28] we have mapped all CAG repeats ≥ 5 on this chromosome and studied the longest of these repeat tracts in regions previously implicated in bipolar disorder and schizophrenia and coding for genes expressed in the brain.

Materials and methods

Selection of patients and controls

A total of 108 bipolar patients (59 males and 49 females, mean age: 31.8 ± 12.1 years, age at onset: 21.8 ± 7.8 years) and 108 schizophrenia patients (58 males and 50 females, mean age: 30.8 ± 10.2 years, mean age at onset: 23.0 ± 10.2 years) were used in the study. Depending on the loci analyzed, the number of subjects ranged from 100–107 patients with bipolar disorder and 103–106 patients with schizophrenia (Table 1). All patients were of Indian origin and were recruited from the clinical services of the National Institute of Mental Health and Neuroscience, Bangalore, India. Diagnoses were made using OPCRIT ver. 3.3[29] after a personal interview and examination of case notes. All patients fulfilled the ICD-10 criteria for bipolar disorder or schizophrenia. All bipolar patients had a history of at least one previous manic episode, and thus could be seen as bipolar type 1 phenotype. The number of normal individuals ranged from 106–120 taken from a total 129 normal subjects (85 males and 44 females, mean age: 34.5 ± 16.7 years). Controls were recruited from a voluntary blood donation camp and were all of South Indian origin as were the patients in order to reduce any effect of genetic heterogeneity. Normal subjects were interviewed to exclude any history of psychiatric illness. Informed consent was obtained from patients and controls before carrying out the study. Some of the patients and controls have been used in earlier published studies.[26,27] The number of patient and control samples used for the individual loci are given in Table 1.

Identification and selection of CAG repeats on chromosome 22

The complete nucleotide sequence of chromosome 22 was obtained through ftp from The Sanger Centre (ftp://sanger.ac.uk) and the file used for the present analysis was Chr_22_01-12-1999.fa.gz which contained the full chromosome 22 nucleotide sequence. Custom PERL (Practical Extraction and Reporting Language) scripts were written to identify sequences containing five or more continuous CAG or CTG repeats. The sequences were then extracted along with 400 bp of unique sequence upstream and downstream of the repeat region. The coding status of the repeat containing regions was determined from the existing gene annotation and in case the repeats were found in the coding region, they were searched for in the EST database to check whether they were expressed in the brain. After obtaining CAG repeats and their positions along chromosome 22, a map was constructed superimposing these CAG repeats with microsatellite markers and genes already implicated in schizophrenia and bipolar disorder. The CAG repeat sizes on this map correspond to the repeat number obtained from Sanger centre sequence data. The list of markers implicated in

Table 1 Details of samples and PCR conditions for selected CAG repeat loci on chromosome 22

CAG repeat locus	*No. of samples used*			*PCR primers used*	*Annealing temp (°C)*	*Expected size (bp)*
	BP	SZ	NC			
22CH4	100	106	106	FP 5′ ACC GAG TGC TGC TTG TTC TGC CT 3′ RP 5′ GCG GCT GGT GGT GCT GTG 3′	62	420 (13 repeats)
22CH1	105	106	111	FP 5′ CTG GGC TGC TGG GCT GCT GAG 3′ RP 5′ GTC GCT GCT AAC CGG TGA GAA TCC 3′	60	438 (13 repeats)
22CH2	107	103	107	FP 5′ GGC GGC GGA TGC GGA CCT GA 3′ RP 5′ ATG CAC AAC GGC GCT CTG GAT AAT 3′	55	488 (9 repeats)
22CH3	100	103	120	FP 5′ AAC CGG GCA CAG TCT TTT C 3′ RP 5′ AGG CCT GGC TCT GCT ATT TA 3′	55	470 (8 repeats)

BP, bipolar disorder; SZ, schizophrenia; NC, normal controls.

both the disorders was obtained from the Chromosome 22 workshop Report, 1999.[4] The primer sequences which amplify these microsatellite loci were used to identify the positions of these markers along chromosome 22 using the chromosome 22 specific BLAST program available at http://sanger.ac.uk. In some cases we were unable to identify the primer sequences using BLAST and these markers were not included in the map. The order of the markers identified in this manner was found to be accurate to the limits of genetic mapping. BLAST homology searches for unknown ORFs were performed using web-based programs available at http://www.ncbi.nlm.nih.gov.

CAG repeat size estimation

Ten millilitres of blood were obtained from patients and controls and DNA extracted using a modification of the salting out method.[30] PCR primers were designed for the loci containing CAG repeats using the Primer Select Program (DNASTAR, Madison, WA, USA). The PCR products were fluorescently labeled either using fluorescently labeled primers or fluorescent dUTP. All PCR reactions were carried out with an initial denaturation of 94°C for 3 min followed by 35 cycles of denaturation and extension steps of 94°C and 72°C, respectively, for 30 s. The annealing step was carried out for 30 s and temperature varied with the locus used. The reaction was completed with extension at 72°C for 10 min. The details of the primers used for each locus, the annealing temperatures and expected size of the PCR products are given in Table 1. PCR products for each locus were diluted and mixed together and loaded in the same lane. Sizing was carried out using the Genescan software on an ABI Prism 377 Automated DNA sequencer (Perkin Elmer, Foster City, USA) with a TAMRA labeled marker (CBT 550) run in each lane.

Heterozygosity indices were calculated using the formula $1-\Sigma\ p_i^2$ where p_i represents the frequency of the i^{th} allele. Chi-square tests were used to determine significance of association between alleles in patients and controls at the various loci studied with correction applied for multiple testing. ANOVA tests with a post hoc Newman–Keuls comparison were also used to compare patient and control populations.

Results

Fifty-two loci were identified on chromosome 22 which contained five or more continuous CAG repeats with 13 repeats being the longest stretch found. Five repeats was the most common length accounting for 55.7% of the loci identified followed by six repeats (21.1%), seven repeats (9.6%), eight repeats (5.8%), nine repeats (3.8%) and 13 repeats (3.8%). The map of the CAG repeats together with microsatellite markers and genes previously implicated in schizophrenia and bipolar disorder are shown in Figure 1. Of the 52, four of the longest loci were chosen for further study. The details of the four loci are given in Table 2. We have chosen the longest CAG repeat tracts as it is well known that the longer tracts are more likely to be polymorphic. Thus, the loci 22CH1 and 22CH4 which contain 13 CAG repeats as the reported size were chosen because they were the longest tracts on chromosome 22 and were also in the proximity of markers previously implicated in bipolar disorder and schizophrenia. 22CH1 lies in a non coding region while the CAG repeats at 22CH4 lie within an ORF encoding 733 amino acids, with the CAG repeats coding for glutamine. This protein shows partial identity to a human Transcriptional Adaptor protein isolated from placenta and bone marrow (GenBank Acession No. 7706225). The mRNA for this repeat-containing region was also identified in a study designed to pick up CAG repeat-containing genes expressing in the brain.[31] The loci, 22CH2 and 22CH3 containing nine and eight repeats, respectively, were chosen over 22CH5 (eight CAG repeats) and 22CH6 (nine CAG repeats) because both of the former were in the vicinity of previously implicated markers. Furthermore, both 22CH2 and 22CH3 were found in the coding regions and were expressed in the brain, with the repeats coding for glutamine. The CAG repeat at 22CH2 is part of a gene MNI (GenBank Accession No. 4505222) which is 1343 amino acids long and is involved in the pathogenesis of meningioma brain tumors (OMIM No. 156100). The repeats at 22CH3 are part of a predicted gene encoding 1203 amino acids. A BLAST analysis revealed that the C terminal 248 amino acids of this protein show a 97%

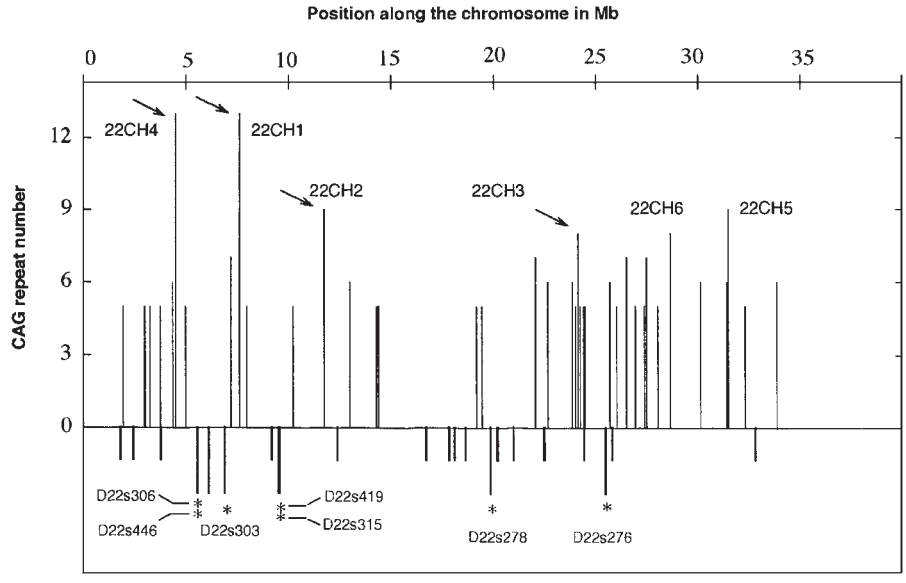

Figure 1 Distribution of all CAG repeats ≥5 on chromosome 22q together with microsatellite markers implicated in schizophrenia or bipolar disorder. Arrows indicate CAG repeat loci chosen for study. The bars below the *x* axis represent microsatellite markers, with the longer bars marked with asterisks indicating markers having lod scores or NPL values >2 or *P* values ≤0.01. The *x* axis represents the distance in Mb along the length of chromosome 22q with the 0 position corresponding to the centromere. The sequenced portion of Chr22q extends to a length of 33.4 Mb. The *y* axis represents length of the CAG repeat tract as reported in the Sanger centre database.

Table 2 Details of the four chosen CAG repeat loci on chromosome 22

CAG repeat locus	*Location along chromosome 22 (bp)*	*Coding status*	*Size range of alleles in CAG repeat units (No. of alleles)*	*Heterozygosity index*
22CH4	4 497 628	Coding (CAG codes for glutamine)	12–17 (5)	0.39
22CH1	7 608 653	Non-coding (intergenic)	8–20 (6)	0.48
22CH2	11 734 903	Coding (CAG codes for glutamine)	2–19 (8)	0.20
22CH3	24 119 007	Coding (CAG codes for glutamine)	7–8 (2)	0.45

identity to an androgen-related apoptosis protein in rat (GenBank Accession No. 9295519). The transcript encoding this gene has been found to be expressed in the human brain.[32]

The CAG repeat lengths at these four loci were estimated in normal controls, bipolar and schizophrenic patients (Figure 2). All the loci studied were polymorphic; the number of alleles and heterozygosity indices of these loci are given in Table 2. Except for 22CH1 where 12 repeats rather than the 13 repeats was the modal allele, all the loci had modal alleles corresponding to the repeat number in the database sequence. Both 22CH1 and 22CH4 showed a near bimodal distribution with 12 and 13 repeats in the case of 22CH1 and 13 and 14 repeats in the case of 22CH4 accounting for the majority of alleles. In the case of 22CH2, an almost unimodal distribution was observed with the majority of alleles corresponding to nine repeats. However, a schizophrenia patient having a size corresponding to two repeats and a normal individual with 19 repeats were observed at this locus. Unlike the other loci, 22CH3 was unique in that it was purely bi-alleleic exhibiting alleles of seven and eight repeats. The allele frequencies and genotype frequencies for this locus are given in Table 3. The genotype frequencies at this locus were in Hardy–Weinberg equilibrium in both groups of patients and controls.

No large expansions of the range observed in the trinucleotide repeat disorders were seen in the patient group in any of the loci studied. Of the four loci, we could find a significant difference between the patients and control groups only in the case of 22CH3. At this locus, the allele frequencies varied significantly between both the patient groups and controls (bipolar *vs* control: $\chi^2 = 5.39$, df = 1, $P < 0.02$; schizophrenia *vs* control: $\chi^2 = 5.19$, df = 1, $P < 0.02$). These results were sustained after correcting for multiple testing. An ANOVA test at this locus with a post hoc Newman–Keuls comparison indicated that both patient groups did not differ from each other but were significantly different from the control groups ($P = 0.018$). The genotype frequencies were significantly different in bipolar

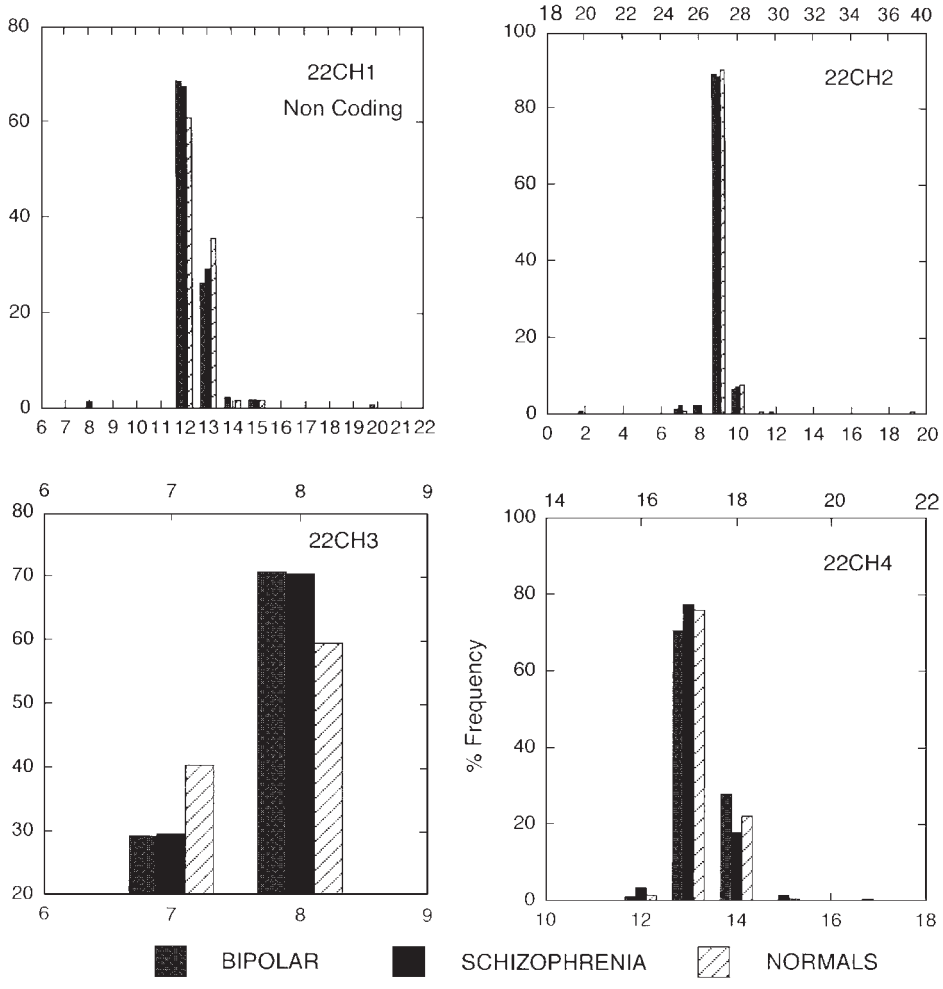

Figure 2 Distribution of CAG repeats at four selected loci in normal individuals, bipolar and schizophrenia patients. The bottom *x* axis represents CAG repeat number while the top *x* axis represents number of glutamine residues. The *y* axis represents % frequency of alleles.

Table 3 Allele and genotype frequencies for the locus 22CH3

	Allele frequencies		*Genotype frequencies*		
	7 repeat	*8 repeat*	*7/7*	*7/8*	*8/8*
Schizophrenia	0.30	0.70	0.09	0.42	0.49
Bipolar	0.29	0.71	0.10	0.38	0.52
Normal	0.40	0.60	0.16	0.49	0.35

patients as compared to controls but in patients with schizophrenia this difference did not reach statistical significance at the 5% level (genotype frequencies: bipolar *vs* control $\chi^2 = 6.25$, df = 2, $P = 0.04$; schizophrenia *vs* control $\chi^2 = 5.69$, df = 2, $P = 0.06$). The 8-repeat allele and the 8/8 genotype were found to be overrepresented in the patient group as compared to the controls.

Discussion

We have analyzed the possible association of CAG repeat polymorphisms on chromosome 22 with schizophrenia and bipolar disorder. We were able to identify 52 loci containing five or more continuous CAG repeats. Of the 52, four of the longest repeat tracts in the vicinity of markers previously implicated in these diseases were selected for further study. In three of these four loci the CAG tracts were part of genes expressed in the brain and coding for glutamine residues. This makes them ideal candidate genes for schizophrenia and bipolar disorder as expanded glutamine repeats have been associated with neuro-degenerative disorders and novel proteins with expanded polyglutamine tracts have been identified in patients with schizophrenia.[16,33]

All the loci we have studied were polymorphic although the heterozygosity is not as high as that seen in most of the diseases associated with unstable trinucleotide repeats.[34] We were unable to observe any large-scale expansion in the patients as compared to

the normals. This does not, however rule out these loci as candidates for trinucleotide repeat expansions. In the case of SCA2, expansions are observed in diseased individuals in spite of the low heterozygosity observed for the normal alleles at this locus.[34] The nine pure CAG repeats at 22CH2 are part of a tract of 27 glutamine residues in the MN1 gene coded for by additional stretches of short CAG repeats interrupted by CAA codons. In one of the normal samples, this pure CAG repeat stretch was expanded to 19 repeats corresponding to a total of 37 glutamine residues. This range is at the threshold of polyglutamine repeats above which disease onset is observed in the majority of the disorders caused by polyglutamine expansions.[17] The 22CH2 locus might therefore be a putative candidate in the other diseases where unstable trinucleotide repeats have been implicated.

Except in the case of 22CH3 there was no significant difference in allele frequencies between patients and ethnically matched controls at the loci studied. At the 22CH3 locus only two alleles of seven and eight repeats were observed in both patient and control groups. The 8-repeat allele and the 8/8 genotype were significantly overrepresented in both bipolar and schizophrenia patients when compared to normal controls. The repeat lies at a position of 24 119 007 bp along the length of chromosome 22 in the vicinity of the markers D22s279 (24 443 698 bp) and D22s276 (25 500 500 bp) which have been previously implicated in schizophrenia. This is part of a region around the markers D22s278 (19 857 258 bp) to D22s279 where a large number of the positive findings on schizophrenia are concentrated and also includes a locus for bipolar disorder.[4] It is interesting that our findings of a positive association for both disorders at the same locus mirror these previous reports. This is one of the few chromosomal locations where there is an overlap of loci for bipolar disorder and schizophrenia and raises the possibility that these diseases may share some common susceptibility genes.[35]

The functional significance of the repeat variation at 22CH3 is difficult to ascertain as the CAG repeats are part of a novel protein whose function is yet unknown. We find that this protein shows partial identity to an androgen-related apoptosis protein in rat. It is therefore only possible to speculate whether this gene is itself involved in the pathogenesis of schizophrenia or whether the repeat variation is merely a marker implicating other genes in the vicinity. Chromosome 22 is particularly gene dense and a 2 Mb region surrounding the 22CH3 CAG repeat contains 36 genes and four pseudo genes,[28] a number of which might be putative candidates for bipolar disorder and schizophrenia. This includes a gene for a voltage-dependent alpha II subunit of a calcium channel. Calcium channels have been previously implicated in bipolar disorder and schizophrenia.[27] The gene which encodes synaptogyrin 1, a protein which is involved in presynaptic vesicle formation in neurons,[36] is also found within 2 Mb of the 22CH3 locus. While this manuscript was under review, Mirnics *et al* reported altered levels of synaptogyrin 1 in the prefrontal cortex of some of the patients suffering from schizophrenia using microarray analysis.[37] Neuro-developmental and synaptic regulatory mechanisms are important to our understanding of schizophrenia and thus synaptogyrin 1 appears to be an attractive candidate for schizophrenia susceptibility and needs to be investigated further. Also lying adjacent to the CAG repeat-containing gene at the 22CH3 locus is the adenylosuccinate lyase gene which has been shown to be involved in autism and psychomotor delay in children.[38]

In conclusion, our results indicate a possible association of schizophrenia and bipolar disorder with a CAG repeat-containing locus on chromosome 22. This region contains a number of interesting candidate genes and their involvement in these disorders needs to be evaluated further.

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