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Gerald Stöber · Jobst Meyer · Indrajit Nanda · Thomas F. Wienker · Kathrin Saar · Susanne Jatzke Michael Schmid · Klaus-Peter Lesch · Helmut Beckmann

hKCNN3 which maps to chromosome 1q21 is not the causative gene in periodic catatonia, a familial subtype of schizophrenia

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Abstract The human calcium-activated potassium channel gene (*hKCNN3, hSKCa3)* contains two tandemly arranged, multiallelic CAG repeats located in exon 1 which result in short to moderate polyglutamine stretches of unknown functional significance. Case-control and family-based association studies suggested an association of *hKCNN3* repeats with susceptibility for schizophrenia. Twelve multiplex pedigrees with periodic catatonia, a schizophrenia subtype with major gene effect and patterns of anticipation, were genotyped using the multiallelic *hKCNN3* repeat polymorphism. Using a dominant model of inheritance with sex- and age-dependent penetrance classes, cumulative results showed exclusion of linkage of *hKCNN3* to periodic catatonia under the assumption of genetic homogeneity with lod score of –48.01 at zero recombination fraction. Our results provide evidence that *hKCNN3* is not the causative gene in the familial schizophrenia subtype of periodic catatonia. By fluorescent *in situ* hybridization we confirmed the assignment of *hKCNN3* to chromosome 1q21 near the heterochromatin region. Linkage mapping showed segregation with marker D1S498 ($\theta = 0.05$) and placed *hKCNN3* in the genetic linkage map in a cluster of genes near the centromeric region of chromosome 1.

G. Stöber, M. D. (✉) · J. Meyer · S. Jatzke · K.-P. Lesch
H. Beckmann
Department of Psychiatry and Psychotherapy, University of Würzburg, Füchsleinstr. 15, D-97080 Würzburg, Germany
E-mail: nerk105@rzkli.uni-wuerzburg.de

I. Nanda · M. Schmid
Institute of Human Genetics, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

T. F. Wienker
Institute of Medical Biometry, Informatics and Epidemiology, University of Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany

K. Saar
Max-Delbrück-Center for Molecular Medicine, Gene Mapping Center, Robert-Rössle-Str. 10, D-13092 Berlin, Germany

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Introduction

The human KCNN3 gene (*hKCNN3, hSKCa3*) is a member of small-conductance, calcium-activated potassium channel genes that play important roles in afterhyperpolarization of central neurons (Köhler et al. 1996). *hKCNN3* encodes a protein of 731 amino acids and contains two tandemly arranged CAG trinucleotide repeats separated by 74 basepairs in exon 1 (Chandy et al. 1998; Wittekindt et al. 1998). The first repeat seems to be biallelic, whereas the second is multiallelic with most CAG repeats within a range of 12 to 28 units which results in short to moderate polyglutamine stretches in the protein. While the functional relevance of the CAG repeat/polyglutamine array needs to be determined, the candidate role of *hKCNN3* in schizophrenia susceptibility has been proposed by two case-control studies that noted an overrepresentation of large alleles among schizophrenics (Chandy et al. 1998; Bowen et al. 1998). In a family-based association study we found short CAG repeats of *hKCNN3* associated with schizophrenia, particularly with familial cases (Stöber et al. 1998).

Periodic catatonia is a schizophrenic subtype with high familial aggregation of homogenous psychoses and pronounced vertical transmission indicating a major gene effect according to Leonhard and our own findings (Stöber et al. 1995; Beckmann et al. 1996; Leonhard 1999). In a family study we found an age-corrected morbidity risk of 33.7% for mothers, 15.4% for fathers and 24.4% for siblings. The age of onset distribution revealed patterns of anticipation, e. g., a significantly earlier age of onset and more severe course of the illness in the probands compared to parents (Stöber et al. 1995). The central syndrome in periodic catatonia consists of qualitative psychomotor disturbances in hyperkinesia or akinesia with a characteristic mixture of both poles during the acute psychotic attack: psychomotor excitement with iterations and stereotypes,

grimacing, akinetic stupor with negativism, rigid postures, and stiff shoving movements. The psychomotor disturbances continue in the residual state with inharmonious movements, apathy and lack of drive, and become more prominent with each successive attack (Leonhard 1999). Based on our previous findings of an association of short *hKCNN3* CAG repeats with familial cases of schizophrenia we hypothesized that *hKCNN3* variants segregate with periodic catatonia or, if the *hKCNN3* variants are not functionally relevant, a major disease gene may be in linkage disequilibrium with the *hKCNN3* polymorphism.

To delineate further a role of *hKCNN3* in psychiatric disorders, the aim of the present study was, first, to give information on genetic order and distance of *hKCNN3* in the context of other markers and genes on chromosome 1 and, second, to investigate whether *hKCNN3* alleles segregate in multiplex families of the schizophrenic subtype of periodic catatonia.

Individuals and methods

Families

Pedigrees were identified from our initial family study on chronic DSM III-R catatonic schizophrenia (Stöber et al. 1995; Beckmann et al. 1996). The nuclear family was extended, when second and third degree relatives with periodic catatonia were available. Probands and cooperative relatives were personally interviewed using a semistructured clinical interview and diagnosed according to the highly operationalized classification of Leonhard (1999). For diagnosis, all available information was used. The detailed diagnostic procedure in periodic catatonia and its delimitation from other schizophrenic subgroups is described elsewhere (Stöber et al. 1995; Beckmann et al. 1996; Leonhard 1999). Twelve multiplex families of periodic catatonia comprising 135 individuals were included in the present study. Mean age of the individuals at assessment was 51.1 ± 17.1 years (mean ± standard deviation; range 18–88). Average family size was 11.2 ± 5.8 probands (range 5–22). The pedigrees contained 57 affected individuals with an age of onset of 29.4 ± 12.3 years and 45.9 ± 15.4 years at study. All families were of Caucasian origin. Approval of the study was obtained by the Ethics Committee of the University of Würzburg and by the data protection officials of the Bavarian State Ministry of Culture and Science. All probands actively participated in the study after giving their informed consent.

Genotyping and linkage analysis of *hKCNN3*

To generate fragments containing both *hKCNN3* $(CAG)_n$ repeat sets in exon 1, primers corresponded to nucleotide (nt) position 339–361 (5'-ACCCCAAGTGCCCCTGTCC-ATCC–3') and 700–674 (5'-GCCAAGCAAGTGGTCATTGAG-3') according to the published sequence (Chandy et al. 1998; EMBL/GenBank accession no. AF031815). PCR (45 s at 95 ^{0}C, 60 s at 60 ^{0}C, and 60 s at 72 ^{0}C for 32 cycles) was performed as previously described in a final volume of 25 µl containing 80 ng genomic DNA, 20 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.025 % Tween 20, 0.025 mg/ml BSA, 1.5 mM $MgCl_2$, 6 % DMSO, 0.5 U Taq DNA polymerase, 200 µl M of each dNTP, and 0.04 pmol/µl α-^{32}P-dCTP (3000 Ci/mmol) (Stöber et al. 1998). PCR products were separated by electrophoresis through a 6 % denaturing polyacrylamide gel.

Based on the results of our previous clinico-genetic family study on periodic catatonia we applied an autosomal dominant inheritance model with 17 age- and sex-dependent penetrance classes. Liability classes included autosomal dominant inheritance with different penetrance (0.99/0.90/0.80), six liability classes for siblings, and four liability classes each for mothers and fathers according to their lifetime morbidity risk. We assumed a disease allele frequency of 0.0001 and a phenocopy rate of 0.001. Linkage simulation (1000 replicates) were performed for the whole panel and for each pedigree separately, resulting in average maximum expected lod scores based on quadratic interpolation (Ott 1989). The families reached a maximum expected lod score (e-lod) of 7.48 ± 2.69. The power in the presence of genetic homogeneity was > 95 % to detect lod scores ≥ 2.0 (5 marker alleles, uniformly distributed; θ = 0.05) indicating that we had sufficient power to detect linkage in the pedigrees with this genetic model. Lod score analysis was performed using the LINKAGE software package, version 5.1 (Lathrop et al. 1984) and tests of heterogeneity using the HOMOG program, version 3.33 (Ott 1991). For linkage mapping we carried out two-point linkage analysis against 27 markers of chromosome 1 using the MAKE SCAN program (TF Wienker, unpublished). The families were genotyped using the following genetic markers of chromosome 1: D1S243, D1S468; D1S214, D1S244, D1S228, D1S436, D1S193, D1S200, D1S209, D1S207, D1S435, D1S221, D1S252, D1S514, D1S498, D1S431, D1S305, D1S2640, D1S422, D1S2655, D1S2891, D1S419, D1S251, D1S235, D1S2670, D1S423, D1S2682 (Gyapay et al. 1994; Dib et al. 1996).

Oligonucleotide probes and libraries

Flanking the *hKCNN3* $(CAG)_n$ repeats, PCR fragments of 316 bp and 377 bp were generated by the use of sets of primers ranging from nt position 45 to 64 (5'-ACCCCCTCTTCTTTCTCCAA-3') and 360 to 343 (5'-GATGGACAGGGGCAC-TTG-3') and from 542 to 559 (5'-TCTCAGCTCGCCCAACTC-3') and 918 to 901 (5'-AAAAGCTG-GAGGGGTTGG-3'). PCR (45 s at 94 ^{0}C, 60 s at 58 ^{0}C, and 60 s at 72 ^{0}C for 35 cycles) was carried out in a final volume of 50 µl containing 80 ng genomic DNA, 20 pmol of each primer, 200 µl M of each dNTP, 1.5 mM $MgCl_2$, 2.0 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0), 0.01 % Tween–2.0 and 1.5 U of Taq DNA polymerase. Clone RPCIP 704A0214Q25 was verified by hybridization and PCR typing following screening under high-stringency hybridization conditions of human PAC library 704 (Resource Center/Primary Database of the Human Genome Project, Berlin) with the pooled α-^{32}P-labeled PCR probes. PAC DNA was prepared by the alkaline-lysis method (Sambrook et al. 1989).

Fluorescence *in situ* hybridization

Metaphase chromosomes were prepared from phytohemagglutinin stimulated lymphocyte cultures of human peripheral blood cells obtained from normal individuals using standard protocol of hypotonic treatment and methanol : acetic acid (3:1) fixation. Prior to hybridization, slides were pretreated with RNAse A and pepsin followed by a brief fixation in formaldehyde (Köhler and Vogt 1994). DNA from P1-phage genomic clone containing *hKCNN3* was labeled by nicktranslation in the presence of biotin–16-dUTP. Approximately 150 ng of the labeled probe was precipitated with 10 µg of sonicated salmon sperm DNA and 10 µg of human Cot1 DNA, then resuspended in a hybridization mixture (2 × SSC, 10 % dextran sulfate and 50 % formamide). The probe mixture was denatured at 75 ^{0}C for 10 min, allowed to reanneal for 30 min at 37 ^{0}C, then applied to the denatured slides. After overnight hybridization at 37 ^{0}C, the slides were washed 2 × 5 min in 50 % formamide, 2 × SSC at 42 ^{0}C and in 1 × SSC at 60 ^{0}C for 10 min. Hybridization sites were detected with fluorescein-labeled avidin and amplified by the addition of anti-avidin antibody and a second layer of fluorescein-labeled avidin (Vector Laboratories, Burlingame, USA). Chromosome preparations were counterstained with both DAPI (diamidino–2-phenylindole) and propidium iodide and viewed using a Zeiss Axiophot microscope equipped for epifluorescence. The precise location of hybridization signals was achieved by relating the dull Q-bands observed after DAPI staining of more than 30 metaphases to standard G-band ideogram of human chromosomes (Francke 1994).

Results

Linkage analysis of *hKCNN3* and periodic catatonia

To determine whether the polymorphic CAG repeat/polyglutamine stretches in *hKCNN3* are the causative factors in periodic catatonia, we performed a linkage analysis by genotyping twelve pedigrees with periodic catatonia comprising 135 individuals. Allele sizes of both *hKCNN3* CAG repeats ranged from 13 to 21 repeat units. No intergenerational expansions of the repeats were detected (Fig. 1). Using 17 liability classes, overall lod scores resulted in exclusion of linkage of *hKCNN3* and periodic catatonia under the assumption of genetic homogeneity (Table 1). At zero recombination fraction (θ), the lod score was –48.01 and at θ = 0.05 –8.20. Eight families unambiguously showed negative lod scores at the *hKCNN3* locus. One small family (pedigree 5) was not informative. Three families showed no exclusion of linkage with lod scores of 0.51 (pedigree 13), 0.14 (pedigree 19), and 0.24 (pedigree 24) at θ zero. Since there is no reason to classify the pedigrees a priori, one has to use the admixture test on genetic heterogeneity (HOMOG test: Ott 1991). However, we did not find even a hint of indicating a possible linkage relationship in any of the pedigrees and, thus, this test lacks the basis for a conclusive answer. In fact, estimating two family types, one with linkage, one without, linkage heterogeneity or homogeneity at max. lnL of 0.106 and theta 0.45 revealed the following inconclusive results: heterogeneity vs homogeneity (χ^2 = 0.0; df = 1; L-ratio = 1.00), homogeneity vs no linkage (χ^2 = 0.21; df = 1; L-ratio= 1.11), and heterogeneity vs no linkage (χ^2 = 0.21; df = 2; L-ratio = 1.11). In summary, there is no evidence for linkage nor is there evidence for genetic heterogeneity.

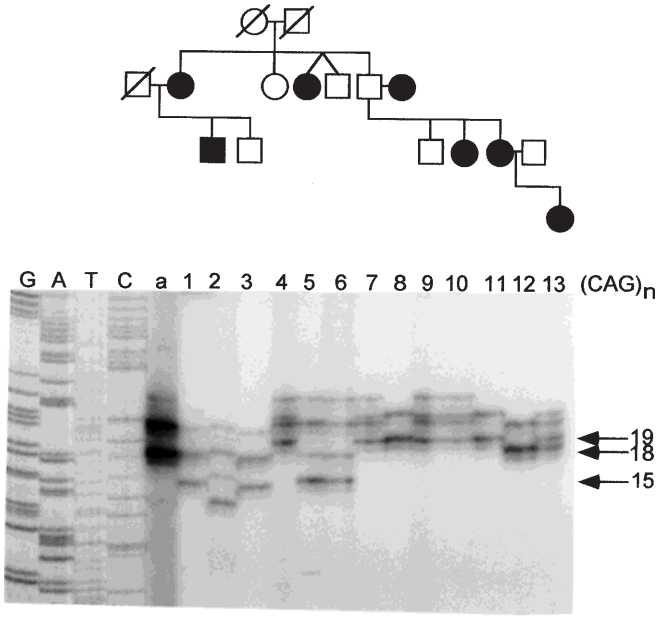

Fig. 1 Analysis of the PCR-amplified products generated with primers flanking the CAG repeat in the hSKCa3 gene in pedigree 23 (squares indicate males; circles represent females; full shaded symbols indicate affection with periodic catatonia and unshaded symbols diagnosis unknown; a slash through a symbol indicates that the person is deceased and was not genotyped): Lanes GATC: standard sequencing reaction of pGEM® T-vector with M13 primer. Lane a: Genotyping of a sequenced PCR product containing 18 and 21 repeat units as internal standard. Lane 1–3: Genotyping of *hKCNN3* CAG repeats pedigree 23: 1: 15/18, 2: 13/18, 3: 15/18, 4: 19/21; 5: 15/21; 6: 15/21, 7: 19/21; 8: 19/19; 9: 19/21; 10: 19/21; 11: 19/19; 12: 18/18; 13: 18/19.

Table 1 Relationship between periodic catatonia and *hKCNN3* by using linkage analysis assuming autosomal dominant inheritance

Pedigree No.	*LOD score values at each recombination fraction (theta)* 0.0	0.01	0.05	0.1	0.2	0.3	0.4
2	–4.09	–1.24	–0.59	–0.34	–0.15	–0.06	–0.02
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9	–9.66	–3.30	–1.74	–1.07	–0.49	–0.22	–0.08
11	–7.97	–3.60	–1.62	–0.86	–0.25	–0.04	0.01
13	0.51	0.49	0.43	0.36	0.22	0.10	0.02
15	–0.12	–0.11	–0.09	–0.07	–0.04	–0.02	–0.00
17	–3.78	–1.13	–0.48	–0.24	–0.06	–0.01	–0.00
18	–3.37	–1.32	–0.54	–0.19	0.09	0.16	0.12
19	0.14	0.13	0.12	0.09	0.06	0.03	0.01
21	–10.42	–4.21	–2.18	–1.36	–0.62	–0.27	–0.08
23	–9.48	–3.21	–1.76	–1.11	–0.50	–0.20	–0.05
24	0.24	0.24	0.24	0.23	0.20	0.15	0.08
Total	–48.01	–17.26	–8.20	–4.56	–1.54	–0.38	–0.02

Physical localization and genetic linkage mapping

To obtain information on genetic order and distance of *hKCNN3*, we performed fluorescence *in situ* hybridization and genetic linkage mapping with 27 genetic markers on chromosome 1 in twelve families with periodic catatonia. Hybridization of PAC clone (RPCIP 704A0214Q25) to metaphase spreads revealed specific signals on human chromosome 1 (Fig. 2). Fluorescent spots were detected in 90% (38 of 40) of the metaphases analyzed. No specific signals were observed at other chromosomal sites. The hybridization signals were found to be located in the region adjacent to the heterochromatin specific, prominent DAPI stained region of the long arm (1q-). Since this heterochromatic region extends up to 1q12, the position of the locus in the long arm region could be determined to 1q21 (Fig. 2). Only those chromosomes with signals present on both chromatids at the same band position were taken into consideration. Genotyping of 24 selected markers of chromosome 1 in our sample of twelve multiplex pedigrees with periodic catatonia allowed us to perform linkage mapping. Two-point analysis placed *hKCNN3* near the marker D1S498 with a lod score of 18.56 at θ = 0.05 (Table 2).

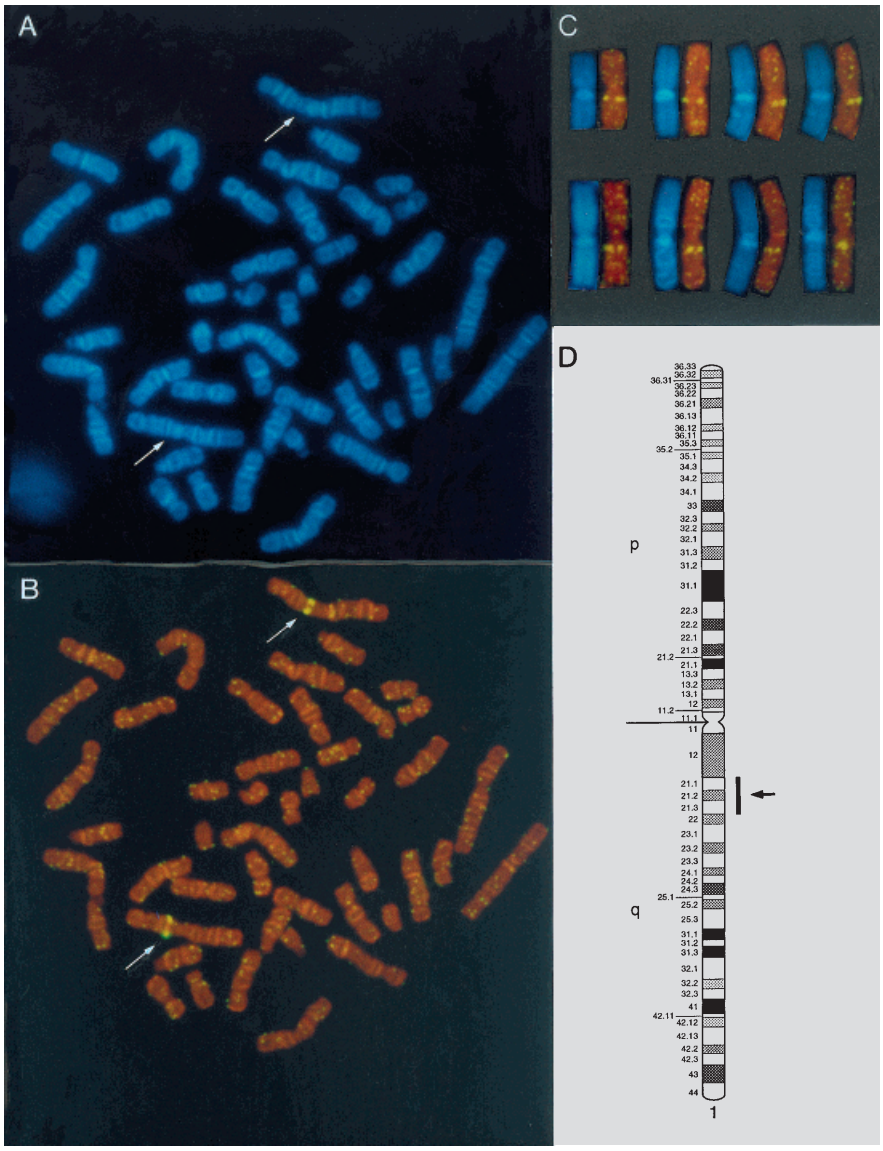

Fig. 2 Localization of *hKCNN3* by fluorescent in situ hybridization: male metaphase with DAPI counterstaining (**A**) and propidium iodide staining (**B**) showing the in situ hybridization signal of the probe RPCIP 704A0214Q25 (arrows). **C** Selected examples of chromosome 1 showing consistent, specific location of the FISH signals. **D** Diagram of human chromosome 1 illustrating the position of *hKCNN3* (vertical bar).

Discussion

In the present study we provide physical and linkage mapping of *hKCNN3* (alias *hSKCa3*) to chromosome 1q21 near the marker D1S498 and excluded linkage of *hKCNN3* to the schizophrenic subtype of periodic catatonia. We investigated twelve multiply affected families with periodic catatonia, a schizophrenia subtype with high aggregation of homogenous psychoses (Beckmann et al. 1996; Leonhard 1999). Periodic catatonia exhibits an excess of homogenous psychoses with an age-corrected morbidity risk of 26.9 % in first-degree relatives (Stöber et al. 1995; Beckmann et al. 1996). The pronounced vertical inheritance pattern with homogenous psychoses indicates a major gene effect and renders periodic catatonia a suitable candidate for evaluation of linkage to *hKCNN3*. Preliminary reports of case-control studies pointed to an association of moderately large *hKCNN3* CAG repeats with schizophrenia (Chandy et al. 1998; Bowen et al. 1998). In contrast to these initial studies, our family-based study found short (≤19) CAG repeats within the hKCNN3 gene associated with DSM IV schizophrenia, in particular in familial cases (Stöber et al. 1998); negative results of the multiallelic transmission-disequilibriumtest and no evidence of parent-of-origin effects challenged the hypothesis of an etiological importance of *hKCNN3* in schizophrenia. At the time, other case-controlled and family-based association studies failed to confirm an etiological role of *hKCNN3* in schizophrenia (Bonnet-Brilhault et al. 1999; Wittekindt et al. 1999). In the present study, cumulative results exclude the involvement of *hKCNN3* or a nearby locus in the etiology of periodic catatonia. Two-point lod score analysis gave –48.01 at zero recombination fraction under an autosomal dominant inheritance model with sex- and age-dependent penetrance classes. We used this genetic model according to the lifetime morbidity risk of first degree relatives, i. e., mothers, fathers, and siblings, obtained in a previous family study

Table 2 Two-point lod scores between *hKCNN3* and selected chromosome 1 markers in twelve pedigrees with periodic catatonia (in total analysis of 24 genetic markers on chromosome 1)

Marker locus	Lod score	Recombination fraction (theta)	Position (centiMorgan)
D1S436	–78.97	0.00	39.9
D1S193	–63.36	0.00	73.8
D1S200	–61.24	0.00	84.3
D1S209	–64.66	0.00	95.9
D1S207	–64.56	0.00	117.6
D1S435	–33.24	0.00	128.9
D1S221	4.08	0.05	146.7
D1S252	10.03	0.05	155.0
D1S498	**18.56**	**0.05**	**160.7**
D1S431	–30.29	0.00	187.2
D1S2640	–47.44	0.00	199.7
D1S422	–57.32	0.00	209.4
D1S2655	–88.79	0.00	221.1
D1S2891	–79.04	0.00	228.7
D1S419	–77.17	0.00	237.2
D1S251	–63.74	0.00	249.0

(Beckmann et al. 1996). Simulation studies of the twelve pedigrees segregating for periodic catatonia gave a statistical power of > 95 % to detect a lod score ≥2.0 with this genetic model. Misspecification of the mode of transmission may have deflated the lod score. However, negative linkage results were recently reported in other familial schizophrenias using parametric and nonparametric analysis (Antonorakis et al. 1999). In addition, studies on trinucleotide repeat expansions in brain-expressed loci failed to find evidence for involvement in schizophrenia (Lesch et al. 1994; Cardno et al. 1996; Bengel et al. 1998).

Using fluorescence *in situ* hybridization (FISH) we assigned *hKCNN3* to chromosome 1q21 near the centromeric region, confirming recent cytogenetic assignment to 1q21.3 (Wittekindt et al. 1998). This locates *hKCNN3* close to the D5 dopamine receptor pseudogene 2, spectrin gene, gene for C-reactive protein, Duffy blood group antigen (Fya-b+) gene and apolipoprotein A-II precursor gene. Linkage mapping placed *hKCNN3* near the marker D1S498 in the genetic linkage map and confirmed the linkage mapping of Meissner et al. (1999) to between D1S2624 and D1S1600 with θ zero. Assuming a major gene effect in periodic catatonia our linkage study excludes genes in the vicinity of the hKCNN3 locus to be of etiological relevance, e. g., 3-β-hydroxy–5-ene-dehydrogenase type I/II, arylhydrocarbon receptor nuclear translocator, dimethylalanine monooxygenase, binding regulatory factor, calpactin I, cathepsin K precursor, cornifin B or other yet unidentified transcripts (www.ncbi.nlm.nih.gov/genemap). Furthermore, based on homology mapping we found no other genes with structural homology to *hKCNN3* on 1q21, and physical mapping found no evidence of pseudogenes or homologous loci on other chromosomes.

Our negative linkage results are consistent with recent genome scans on schizophrenia pedigrees that found no support of linkage to markers related to chromosome 1q21 or other centromeric regions (Moises et al. 1995; Faraone et al. 1998; Levinson et al. 1998). Heterochromatic region variants (1qH) and Duffy blood group alleles may occur in weak association in some cases of schizophrenia (Kosower et al. 1995); candidate regions for psychotic disorders, however, seem to be located at telomeric regions of chromosome 1: at 1q32.3 and 1q43 as suggested by balanced chromosomal translocations (Bassett 1992), or at 1q32.2–41 as proposed by a genome scan in Finnish schizophrenia pedigrees (Hovatta et al. 1999). While etiologic involvement of *hKCNN3* in periodic catatonia is excluded and multiallelic tests in family based studies assume no association of individual *hKCNN3* CAG repeats with schizophrenia (Stöber et al. 1998), *hKCNN3* seems to be a considerable candidate in families of hemiplegic migraine which links to chromosome 1q21 (Ducros et al. 1997) and to other yet unmapped hereditary ataxias. As previously demonstrated, point mutations in a brain specific calcium-activated potassium channel gene (*hCACNA1A*) are causative factors in familial hemiplegic migraine type 1 and episodic ataxia type 2, whereas small expansions of a C-terminal CAG repeat are associated with progressive spinocerebellar ataxia type 6 (SCA-6) (Ophoff et al. 1996; Jodice et al. 1997).

Our linkage results, exclude *hKCNN3* from being the causative gene in periodic catatonia, a familial subtype of schizophrenia, and we confirmed the physical mapping of *hKCNN3* to chromosome 1q21.

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