Table 1 Primers used for the multiplex PCR assay of SMN

Gene	*Sense primer*	*Antisense primer*	*Size of the amplicon (bp)*
SMN	5' AGACTATCAACTTAATTTCTGATCA 3'*†	5' CCTTCCTTCTTTTTGATTTTGTTT 3'‡	188
MLH1	5' GTAGTCTGTGATCTCCGTTT 3'	5' ATGTATGAGGTCCTGTCCTA 3'*	244
BRCA1	5' TGATTTGAACACCACTGAGA 3'*	5' CCGCCTATCATTACATGTTT 3'	265

*5' (6-FAM) labelled.
†R111 primer.[5]
‡X7-Dra primer.[17]

(*SMN2*) generates alternatively spliced variants lacking the C-terminal sequence.[5 7] The *SMN* region contains low copy repeats triggering homologous recombination events. Indeed, approximately 95% of SMA patients lack both *SMN1* genes owing to either deletion or gene conversion.[4] In SMA patients who lack only one *SMN1* gene, allelic intragenic mutations have been identified, confirming the involvement of *SMN1* in the pathogenesis of SMA.[5 8–10]

The heterozygote frequency has been estimated to be 1/40. However, the duplication of the SMA locus makes the detection of SMA carriers in the general population difficult, and this has hampered genetic counselling in affected families. Initial attempts to estimate the *SMN* copy number were based on the measurement of the *SMN1*/*SMN2* ratios,[11–13] but the broad variability of *SMN2* copy number hinders reliable quantification. For this reason, subsequent studies have included two internal standards in the PCR reaction, corresponding to the modified *SMN1* and *CFTR* sequences, respectively.[10 14 15] In these methods, the quantification of *SMN* copies is based on the ratio between the PCR amplification of the specific genomic DNA and that of an internal standard for each subject tested. The results are normalised to the mean of control samples. Although these methods can efficiently detect heterozygous *SMN1* deletions,[10 14 15] overlaps between carriers and non-carriers have been observed.[10]

In the present study, we describe a novel method which allows easy detection of heterozygous *SMN1* deletions in SMA carriers and SMA patients without homozygous *SMN1* deletions. We devised a multiplex PCR assay of fluorescent fragments based on the approach that we initially developed for the detection of mismatch repair gene rearrangements in hereditary non-polyposis colorectal cancer.[16] We simultaneously amplified exon 7 of the *SMN1* and *SMN2* genes using a mismatch primer X7-Dra, which introduced a *Dra*I restriction site into amplified *SMN1* exon 7,[17] *BRCA1* exon 11, and *MLH1* exon 18, which contains a natural internal *Dra*I restriction site (table 1). The PCR reaction was performed in a final volume of 50 µl, using 0.75 µmol/l *SMN* primers, 0.5 µmol/l *BRCA1* primers, 0.35 µmol/l *MLH1* primers, 0.2 mmol/l dNTP, 1.5 mmol/l $MgCl_2$, 1 unit of *Taq* polymerase (Eurobio, Les Ulis, France), and 100 ng of genomic DNA. The PCR consisted of 20 cycles of 94°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds, preceded by an initial denaturation step of five minutes at 94°C and followed by a final extension of five minutes at 72°C. The entire PCR reaction was then digested using 4 units of *Dra*I (New England Biolabs) in a total volume of 150 µl for at least four hours. After purification using the Qiagen Gel Extraction Kit, PCR products were resuspended in a mix containing 2.5 µl of deionised formamide, 0.5 µl of GeneScan-500 Rox (PE Applied Biosystems, Perkin Elmer), and 1 µl of loading buffer. After denaturation for two minutes at 90°C, 2 µl of each sample was loaded onto a 4.25% denaturing polyacrylamide gel (Sequagel). Electrophoresis was performed for three hours on an Applied Biosystems model 377 automated sequencer (PE Applied Biosystems, Perkin Elmer). Data were analysed using the Gene Scanner Model 672 Fluorescent Fragment Analyser (PE Applied Biosystems, Perkin Elmer) and electropherograms generated from different samples were superimposed.

Each multiplex PCR yielded a pattern composed of four fluorescent peaks corresponding to exonic fragments of *BRCA1*, *MLH1, SMN1*, and *SMN2* respectively and the patterns generated from two control samples could be easily superimposed (fig 1A). For validation, we studied the *SMN1* and *SMN2* copy numbers (fig 1) in a SMA family in which linkage analysis, using the C212 and C272 microsatellite markers,[18] and analysis of the *SMN1* and *SMN2* genes by PCR digestion had previously shown a homozygous *SMN1* gene deletion in the affected child and a homozygous *SMN2* gene deletion in an unaffected sib, which was suggestive of a large deletion encompassing both *SMN1* and *SMN2* on the paternal allele (fig 2). The relatives of this family were therefore predicted to harbour a variable number of *SMN1* and *SMN2* copies. Fig 1 shows that the multiplex PCR, using as a control a subject predicted to carry two copies of *SMN1* and two of *SMN2,* easily detected no, one, or two copies of *SMN1* or *SMN2* within this family. This technique confirmed the large paternal deletion and showed a gene conversion event on the mutant maternal allele. We then tested 86 parents of SMA patients carrying a homozygous *SMN1* deletion (50 parents of SMA type I, 28 parents of SMA type II, two parents of SMA type III, and six parents of SMA patients of undetermined type). An approximate 0.5 reduction of the *SMN1* peak area, indicative of a heterozygous deletion, was clearly observed in 80 parents (93%). Two *SMN1* copies were detected in six putative carriers. In four out of these six families, linkage analysis with the C212 and C272 microsatellite markers and quantification of *SMN1* in relatives allowed us to show the existence of two de novo deletions and two *SMN1* duplications.

In contrast to the previously reported methods,[10 14 15] the estimation of *SMN1* copy number in this assay is based on the comparison of the fluorescence levels between the *SMN1* peak generated from different samples rather than between the different peaks generated from the same sample. In order to keep PCR amplification within an exponential range, we tested various numbers of cycles (18, 20, 22, and 24) and found that 20 cycles, with shorter times of annealing and extension than those previously described, were optimal.[10 14 15] The simultaneous amplification of two other fragments (*BRCA1* and *MLH1*) allowed an accurate comparison of electropherograms generated from different samples. The absence of the 244 bp *MLH1* PCR product and the appearance of a 209 bp peak (fig 1), expected from *Dra*I digestion, indicated that the enzymatic digestion was complete, a feature which is essential to distinguish between *SMN1* and *SMN2* amplified fragments.

The simplicity of this assay should facilitate its development in molecular diagnostic laboratories and hopefully aid in genetic counselling in SMA families. However, one must keep in mind the existence of (1) small intragenic mutations within the *SMN1* gene, (2) *SMN1* duplications in cis (on one chromosome) masking a heterozygous deletion on the other chromosome, (3) de novo deletions, and

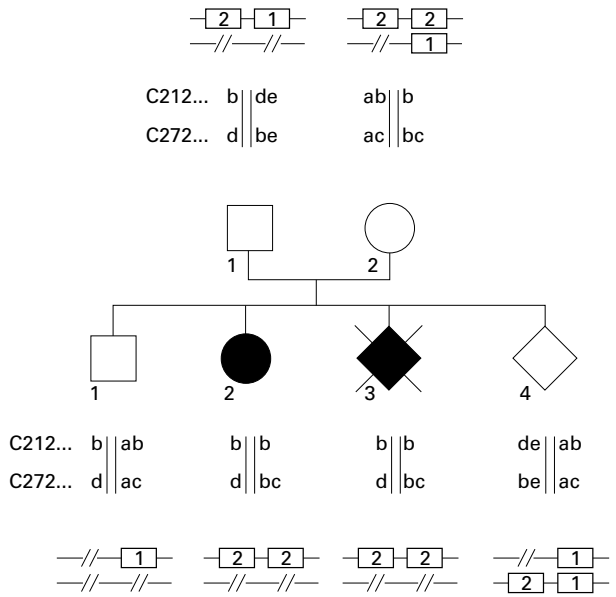

Figure 2 Pedigree of the SMA family used to validate the multiplex PCR. Filled symbols, affected subjects; open symbols, asymptomatic subjects. For each subject, the haplotype analysis using the C212 and C272 microsatellite markers[18] and the schematic representation of the SMN locus (1: SMN1, 2: SMN2) are indicated.

(4) germline mosaicism. Small intragenic *SMN1* mutations account for 1.3-3.4% of the mutant *SMN1* alleles and have been identified in SMA patients carrying heterozygous *SMN1* deletions.[5 8–10] On the other hand, de novo *SMN1* deletions have been shown to be involved in approximately 2% of SMA cases.[15 19] In order to estimate the error risk resulting from duplication or de novo deletion, we counted *SMN1* copies in 86 parents of SMA children carrying a homozygous *SMN1* deletion and found that six out of 86 putative carriers (7%) had more than one *SMN1* copy. These data are in complete agreement with the results of Chen *et al*,[15] who detected 5/60 putative SMA carriers with two copies of *SMN1* (8.3% including one carrier with a small intragenic *SMN1* mutation, two putative carriers with a de novo deletion, and two carriers with a *SMN1* duplication). Finally, germline mosaicism has to be considered.[20] Despite this error risk (less than 10%), the determination of *SMN1* copy number in relatives of SMA patients, harbouring homozygous *SMN1* deletions, will make genetic counselling easier and hopefully limit prenatal screening. For example, for a couple with an a priori risk of 1/320 of having an affected child (corresponding to the situation of the index case's uncle or aunt), detection of two *SMN1* copies in both the relative and his/her spouse will reduce the probability of having an affected child to 1/32 000 ($[1/2 \times 1/10] \times [1/40 \times 1/10] \times 1/4$), which is lower than the risk of the general population. Detection of one copy in the relative and two copies in his/her spouse will decrease the risk to 1/1600 ($1 \times [1/40 \times 1/10] \times 1/4$). This assay will also facilitate the detection of heterozygous *SMN1* deletion in SMA patients without a homozygous *SMN1* deletion who must be screened for small *SMN1* mutations on the other allele, as previously shown by Wirth *et al*.[10] Finally, this assay will allow the study of the influence of *SMN2* copy numbers on the SMA phenotype for research purposes, a feature previously suggested by both the observation of an increased number of *SMN2* copies in patients with a milder phenotype[5 12 14 15 21 22] and by the effect of the expression of human *SMN2* in *Smn*$^{-/-}$ mice.[23 24]

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Low prevalence of *SPINK1* gene mutations in adult patients with chronic idiopathic pancreatitis

EDITOR—Chronic idiopathic pancreatitis is a genetically heterogeneous disease.[1–3] Mutations of the cationic trypsinogen (*CT*) gene underlie some cases of juvenile pancreatitis,[3–5] and mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene have been associated with chronic pancreatitis in adults.[6–8] However, these genes account for only a relatively small proportion of cases. More recently, the serine proteinase inhibitor Kazal type 1 (*SPINK1*) gene, also called *PSTI*, has attracted attention as a possible cause for chronic pancreatitis.[9 10] One study by Chen *et al*[9] did not find disease causing mutations of *SPINK1* among 14 families with hereditary and 30 patients with sporadic pancreatitis, apart from two rare amino acid substitutions which were observed at a comparable frequency to the general population. By contrast, another study by Witt *et al*[10] reported 23 out of 68 children and adolescents with chronic pancreatitis whose disease was associated with the occurrence of *SPINK1* mutations in the heterozygous or homozygous state. In particular, one founder mutation, N34S, was identified in 18/68 German patients but only in 1/279 controls.[10] In the work presented here, we have addressed the role of *SPINK1* mutations in a series of 20 adult German pancreatitis patients, a cohort that we had previously analysed for mutations in the *CT* and *CFTR* genes.[8]

The mean age of patients in our series was 32 years (range 19-46 years). All of them presented with either recurrent pancreatitis characterised by at least three episodes of pancreatitis at least 12 months apart or with chronic idiopathic pancreatitis.[8] Genomic DNA was extracted from white blood cells and the four exons of the *SPINK1* gene were amplified by PCR using published primers[9 10] and scanned for mutations by single strand conformation polymorphism (SSCP) analysis and direct sequencing.

Only two patients were found to carry sequence alterations of the *SPINK1* gene (table 1). One 26 year old patient was homozygous for the previously reported missense mutation N34S.[9 10] His parents were both heterozygotes and did not show any signs of pancreatitis, consistent with an autosomal recessive mode of inheritance. This patient had not been found to carry a *CFTR* gene alteration in our previous study[8] (table 1). The second patient, a 35 year old male, was heterozygous for a new *SPINK1* mutation, a G→A transition at nucleotide 194, that is, the last nucleotide of exon 3 (fig 1A). The 194G→A transition could be confirmed by restriction enzyme analysis as it abolishes a recognition site for *Hph*I and creates a new site for *Tsp*RI (fig 1B). This substitution does not seem to affect splicing of *SPINK1* mRNA, as assessed by nested RT-PCR from a rectal biopsy of the patient (not shown). However, it leads to a missense mutation R65Q at an amino acid position that is conserved in rat and mouse, although some variability exists in cattle.[11] Interestingly, the same patient had also been found to be heterozygous for a nonsense mutation of the *CFTR* gene, Y1092X in exon 17b[8] (table 1). To elucidate the role of this double heterozygosity further, we performed a segregation analysis among the healthy family members of the patient. Both the *SPINK1* and *CFTR* mutations were also found in the patient's

Table 1 Summary of genotypes of 20 German adults with chronic idiopathic pancreatitis analysed for mutations of the SPINK1, CFTR, and CT genes (this study).[8] CFTR and SPINK1 gene alterations were identified in six patients as listed in columns 1 and 2, respectively. Mutations of unknown significance are shown in italics. No mutations have been found in the CT gene[8]

	CFTR mutations		*SPINK1 mutations*	
Patient	*Allele 1*	*Allele 2*	*Allele 1*	*Allele 2*
1	*R75Q*	—	—	—
5	I336K	*R75Q*	—	—
8	—	—	N34S	N34S
11	IVS8-5T	—	—	—
12	Y1092X	—	*R65Q*	—
20	ΔF508	—	—	—

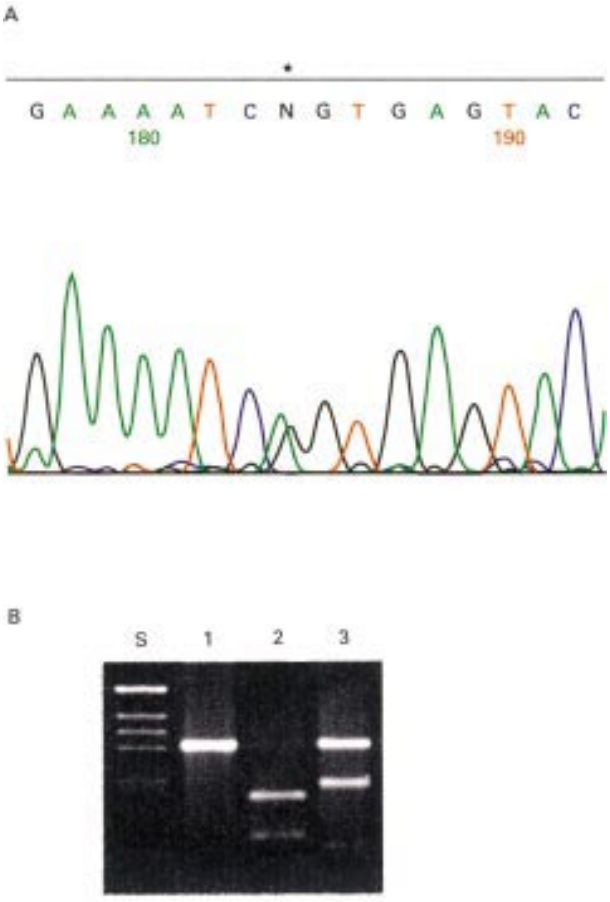

Figure 1 (A) Direct sequencing of exon 3 of the SPINK1 gene showing heterozygosity for the R65Q substitution (asterisk). (B) Screening for missense mutations N34S and R65Q by restriction enzyme analysis. Exon 3 PCR products were amplified using primers from Witt et al[10] and were digested with TspRI and separated on a 2% agarose gel. Lane 1: size marker (kb ladder, BRL), lane 2: wild type control, lane 3: homozygous N34S, lane 4: heterozygous R65Q. Note the distinct patterns for mutations N34S and R65Q in this assay.

mother and sister who did not show any signs of pancreatitis. These observations indicate that double heterozygosity for the *SPINK1* R65Q and the *CFTR* Y1092X mutations is not sufficient to cause chronic pancreatitis or at least not fully penetrant.

In summary, the frequency of *SPINK1* mutations is low in our cohort of adult patients with chronic idiopathic pancreatitis. This is in agreement with the previous report from Brittany,[9] but seems to contrast with the study of German patients by Witt *et al*.[10] One possible reason for the discrepancy may lie in a sampling bias. Similar to CT mutations, *SPINK1* gene mutations might be predominantly responsible for juvenile early onset pancreatitis, a condition not preferentially selected for in our study. In total, mutations of the *CT*, *CFTR*, and *SPINK1* genes constituted less than one third of our cases with adult pancreatitis suggesting that additional genes may be involved in the aetiology of this disorder. A pathogenic role of double heterozygosity for *SPINK1* and *CFTR* gene variations could not be confirmed in the single family identified here, but such a possibility may deserve further investigation in larger cohorts.

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Interaction of coding region mutations and the Gilbert-type promoter abnormality of the *UGT1A1* gene causes moderate degrees of unconjugated hyperbilirubinaemia and may lead to neonatal kernicterus

EDITOR—Crigler-Najjar syndrome types 1 and 2 (CN1 and CN2) are inherited as autosomal recessive conditions and are characterised by severe non-haemolytic unconjugated hyperbilirubinaemia. CN1 is the most severe form, in which a virtual absence of hepatic bilirubin-uridinediphosphoglucuronate glucuronosyltransferase (UGT1A1) (PIR Accession A31340) activity results in serum bilirubin levels of 340-685 μmol/l or higher (normal 1.7-17.1 μmol/l).[1] A milder variant of this disorder, termed CN2, is associated with intermediate levels of hyperbilirubinaemia (120-340 μmol/l) as a result of an incomplete deficiency of hepatic UGT1A1 activity. Usually, the residual UGT1A1 activity can be induced by phenobarbital administration, resulting in partial amelioration of jaundice.[2] A third type of inherited unconjugated hyperbilirubinaemia, termed Gilbert's syndrome, is associated with mild, fluctuating hyperbilirubinaemia, ranging from normal levels to up to 85 μmol/l.[3] Hepatic UGT1A1 activity is reduced to approximately 30% of normal in Gilbert's syndrome. In all three forms of inherited hyperbilirubinaemia, other hepatic functions are unaffected and the liver is morphologically normal.[4] Before the institution of routine phototherapy, CN1 used to be uniformly lethal because of bilirubin encephalopathy (kernicterus).[5 6] Although phototherapy has prolonged survival, it becomes progressively ineffective around puberty.[6 7] Hepatocyte transplantation has resulted in partial amelioration of jaundice,[8 9] but at present liver transplantation remains the only definitive therapy.[10 11] Kernicterus is much less common in CN2, and does not occur in Gilbert's syndrome.

UGT1A1 is one of the several UGT isoforms that are expressed from the *UGT1A* gene locus,[12] located in human chromosome 2q37.[13] The locus contains four exons at the 3' end (exons 2, 3, 4, and 5), which are used in several UGT1A isoform mRNAs and encode the identical carboxy-terminal half of these enzymes. Upstream to these common region exons are a series of unique exons (exon 1A1 to 1A12), only one of which is used in a given UGT1A isoform mRNA. Each unique exon encodes the variable N-terminal region of one UGT1A isoform, which imparts its substrate specificity. The individual isoforms of the UGT1A subfamily are named according to the unique exon used. For example, UGT1A1 is encoded by exons 1A1, 2, 3, 4, and 5, and the gene is termed *UGT1A1* (GenBank accession No AF 180372, OMIM 191740). Following the initial report in 1992,[14] studies from several laboratories have shown that CN1 and CN2 are caused by genetic lesions of any of the five exons of the *UGT1A1* gene, or their flanking splice junctions. These mutations have been reviewed recently.[15 16] The severity of the functional deficiency of UGT1A1 is determined by the nature of the genetic lesion.[17] CN1 results from genetic lesions that cause premature truncation of UGT1A1 or substitution of critical amino acid residues, whereas CN2 is caused by substitution of single amino acid residues that markedly reduce, but do not abolish the catalytic activity of the enzyme. Although the incidence of Crigler-Najjar syndromes is not known precisely, these disorders probably occur in less than one in a million live births in the western

hemisphere.[11] Thus, the gene frequency can be estimated to be 1:1000 or less. Because the expression of one structurally normal allele is sufficient to keep serum bilirubin levels within normal limits, heterozygous carriers are not expected to have hyperbilirubinaemia.

In contrast to CN1 and CN2, Gilbert's syndrome is one of the most common inherited disorders in humans. In western populations, this condition is associated with a TA insertion within a dinucleotide repeat in the TATAA element, upstream of the first exon of *UGT1A1*. The normal TATAA element sequence is $A(TA)_6TAA$, whereas in Gilbert syndrome the sequence is $A(TA)_7TAA$.[18] The variant TATAA element reduces the expression of the structurally normal enzyme.[18] Two additional polymorphisms of the TATAA element, a shorter ($A(TA)_5TAA$) and a longer ($A(TA)_8TAA$) variant, have been described in people of African origin.[19] An inverse relationship between the length of the TATAA element and UGT1A1 expression has been reported. The common Gilbert-type TATAA element ($A(TA)_7TAA$) is present in homozygous state in approximately 9% of the European and North American population, the gene frequency being 30%.[18 20–22] Homozygosity for the variant TATAA element is required, but not sufficient for the manifestation of the Gilbert phenotype, which is found in 4-7% of the general population.[18] Other factors contribute to the serum bilirubin levels. For example, the Gilbert phenotype is seen less commonly in women, probably because of lower daily bilirubin production.[23] In addition to the promoter abnormality, Japanese investigators have reported that some structural mutations of *UGT1A1* can result in mild hyperbilirubinaemia, compatible with the diagnosis of Gilbert's syndrome.[24–26] These mutations have not been reported in white, black, south Asian, or Middle Eastern populations.

Interestingly, intermediate levels of hyperbilirubinaemia are observed commonly in families of patients with CN1 or CN2, which had given rise to conflicting opinions regarding the mode of inheritance of CN2 in the past.[2 7 27] Because the gene frequency of the Gilbert-type promoter is 30% in the western population, a significant percentage of heterozygous carriers of CN-type mutations of the structural region of *UGT1A1* would be expected also to carry a Gilbert-type promoter on one or both alleles. Sporadic cases of coexistence of a Gilbert-type promoter and a structural mutation of *UGT1A1* have been reported.[28–30] We postulated that such compound heterozygosity should be a more common cause of intermediate levels of jaundice than homozygosity for a structural mutation. We have examined this postulate in a systematic study over the course of the last three years by performing routine genetic analysis of a large number of referred patients with various levels of unconjugated hyperbilirubinaemia and their families. Here we report eight patients from four families, who had intermediate levels of unconjugated hyperbilirubinaemia because of compound heterozygosity of a Gilbert-type promoter and a structural region mutation of *UGT1A1*. Three of the structural mutations are being reported here for the first time. In one of these families, a pair of dizygotic twins, who also had ABO incompatibility with the mother, developed severe neonatal hyperbilirubinaemia, leading to chronic encephalopathy.

Four families, designated families A to D, were studied. The probands in all the families showed clinical jaundice with predominantly unconjugated hyperbilirubinaemia. In families A, B, and D the children were the probands, while in family C the mother was the primary subject of the study. In all probands and their family members, the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase levels were within normal limits and there was no evidence of any other liver disease or chronic haemolysis. Therefore, an inherited disorder of bilirubin glucuronidation was suspected.

Blood samples were obtained from all the subjects studied with appropriate informed consent. Genomic DNA was extracted from whole anticoagulated blood using the Qiagen kit (Qiagen, Valenica, CA), according to the manufacturer's instructions, and was used as template for amplification by polymerase chain reaction (PCR). A segment of the *UGT1A1* upstream untranslated region, the five exons, and the flanking intronic sequences were amplified in three pieces using specific amplimers. A 104 bp segment upstream of the translation initiation codon (ATG), exon 1, and a short segment of intron 1 were amplified as a single amplicon. Exons 2, 3, and 4 and introns 2, 3, and a short segment of intron 4 were amplified as a second amplicon. Exon 5 and its flanking intronic sequences were amplified as the third amplicon. The amplicons generated by the first PCR were isolated by electrophoresis on low melting agarose gels and reamplified. The sequences and the locations of the primers used for the PCR reactions are as follows. Exon 1 (including 29 bp untranslated region + 85 bp upstream genomic sequence: sense: 5' GTCACGTGACACAGT CAAAC 3' (85 nt upstream of 5' end of exon 1), antisense: 5' GCTTGCTCAGCATATATCTGG 3' (74 bp downstream of the 3' end of exon 1); exons 2, 3, and 4, intron 2 and 3 and flanking DNA sequences: sense: 5' CTCTATCT CAAACACGCATGCC 3' (105 nt upstream of 5' end of exon 2), antisense: 5' TTATCATGAATGCCATGACC 3' (29 nt downstream of 3' end of exon 4); exon 5 and flanking DNA sequences: sense: 5' GAGGATTGTTCATAC CACAGG 3' (35 nt upstream of 5' end of exon 5), antisense: 5' TGAATTTAACACTGATTCTGTT 3' (nt 1666 to nt 1687). The DNA template for PCR was first denatured for five minutes at 94°C and then cycled 35 times using the following cycling parameters: 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 60 seconds. Finally, a five minute extension cycle at 72°C was performed. After the second amplification, the PCR products were purified using Qiagen PCR purification kit (Qiagen). The nucleotide sequence of the purified amplicons was determined using the sequitherm excel DNA sequencing kit, (Epicenter Technologies, Madison, WI 53713). Nesting primers end labelled with radioactive ^{32}P (Amersham Pharmacia Biotech Inc, NJ) were used for sequence analysis. The primers used for sequence determination were as follows. TATAA element of the promoter region: antisense, 5' GGACACCACT GGGCCCAGCACA 3' (+59 to +81); exon 1: sense, 5' AGGAGCAAAGGCGCCATGGCT 3' (−15 to +6), sense, 5' GCGTGTCATCAAAACATACAA 3' (+321 to +341), antisense, 5' GCTTGCTCAGCATATATCTGG 3' (intronic primer, 74 nt downstream of 3' end of exon 1); exon 2: sense, 5' GTATGTAGTCATCAAAGAATATG 3' (intronic primer 63 nt upstream of 5' end of exon 2); exon 3: sense, 5' TAGTTAGTATAGCAGA 3' (intronic primer 59 nt upstream of 5' end of exon 3); exon 4: sense, 5' GTCCAGCTGTGAAACTCAGA 3' (intronic primer, 39 nt upstream of 5' end of exon 4); exon 5: sense, 5' GAGGATTGTTCATACCACAGG 3' (intronic primer 36 nt upstream of 5' end of exon 5); antisense, 5' TGAATT TAACACTGATTCTGTT 3' (+1666 to +1687). Nucleotide sequences were determined by the dideoxy chain termination method[31] using a cycle sequencing procedure as follows: denaturation, 94°C for 60 seconds, followed by 20 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 60 seconds, and 10 cycles of 94°C for 30 seconds, and 72°C for 60 seconds. Sequence abnormality in each sample was confirmed by repeat analysis of an amplicon generated by a second independent PCR.

To determine the consequence of the two newly observed point mutations that predicted single amino acid

substitutions (the 1490T>A and 1452G>A mutations, found in families B and C, respectively) on the catalytic activity of UGT1A1, site directed mutagenesis was performed on a plasmid, pSVK3, which contains the entire UGT1A1 coding region.[17] The mutagenised UGT1A1 cDNA was excised by partial digestion with *Eco*RI and complete digestion with *Xho*I and subcloned into the pcDNA3.1/Zeo(+) (Invitrogen) expression vector and the nucleotide sequence was determined to confirm the introduction of the point mutation. COS-7 cells, grown in 100 mm dishes and at 50-60% confluency, were transfected with the pcDNA3.1/Zeo(+) containing normal or mutagenised UGT1A1 sequences, using DEAE dextran (Amersham Pharmacia Biotech) as previously described.[17] Total protein in the cell lysates was quantitated[32] and an equal amount of protein from lysates of cells transfected with the different plasmids was subjected to western blot analysis using a monoclonal antibody against UGT1A1 (mab WP1). The immunoreactive bands were detected using a chemiluminescent substrate (Pierce, Rockford, IL).

Expressed UGT1A1 in each cell lysate was quantitated by a sandwich ELISA as previously described.[17] Briefly, ELISA plates were coated overnight with WP1 (a monoclonal antibody against UGT1A isoforms), blocked with a solution containing 3% bovine serum albumin and 5% fetal calf serum, and then overlaid with lysates of the transfected cells. A rabbit antibody raised against a synthetic peptide corresponding to a unique region of UGT1A1 (Pab136) was applied. The detection system consisted of a goat anti-rabbit IgG, conjugated with horseradish peroxidase. Based on ELISA quantitation, equal amounts of UGT1A1 from the various cell lysates were assayed for bilirubin-UGT activity in the presence of 80 μmol/l bilirubin and 4.4 mmol/l UDP-glucuronic acid as previously described.[17] At the end of incubation for 60 to 120 minutes, the reaction was stopped by adding 0.4 mol/l HCl-glycine buffer (pH 1.8) and pigments were extracted in chloroform/ethanol (1:1 v/v).[17] The solvents were evaporated under a stream of nitrogen, the pigments were dissolved in dimethyl sulphoxide and methanol, and analysed by reverse phase high pressure liquid chromatography using a Waters C_{18} μBondapak column (Waters, Milford, MA) as previously described.[33] Formation of bilirubin glucuronides was calculated from the electronically integrated areas under the bilirubin glucuronide peaks.

Brief clinical histories of the four families follow. The genotypes of the TATAA element and coding region of *UGT1A1* are listed in table 1.

Family A. Eighteen month old twin girls were brought to the paediatrician for evaluation of jaundice. Both girls had severe neonatal hyperbilirubinaemia, with peak serum bilirubin levels of 456.5 μmol/l and 410.4 μmol/l (>90% "indirect" by van den Bergh reaction) on the fifth postnatal day. Initially the babies were Coomb's test positive and the exaggerated neonatal hyperbilirubinaemia had been attributed to ABO incompatibility. However, the jaundice persisted after their blood samples became Coomb's nonreactive, and a diagnosis of Crigler-Najjar syndrome was suspected after excluding other causes of hyperbilirubinaemia. The twins were placed on phenobarbital and phototherapy. This resulted in a reduction of serum bilirubin concentrations to the current level of 136-171 μmol/l. Unfortunately, the infants had already sustained brain damage, presumably from the high neonatal bilirubin levels. At the age of 18 months, neurological examination of the twins showed spastic cerebral palsy, chorioathetoid movements, and signs of retarded mental development. The affected infants were compound heterozygotes for the Gilbert-type TATAA element and the deletion of the "A" at nt 1223. The frameshift caused by this deletion introduces a premature stop codon at codon 497, predicting the truncation of the protein by 122 amino acids and complete catalytic inactivation of UGT1A1. The father was heterozygous for the structural region mutation but had a normal TATAA sequence. The mother carried a Gilbert-type TATAA element on one allele, but the entire coding region had normal nucleic acid sequence.

Family B. The proband, a male, had persistent scleral icterus, first brought to the attention of the physician at the age of 10 years. The peak serum bilirubin level was 205 μmol/l (>90% "indirect" reacting). An 18 year old sister of the proband was also noted to have milder hyperbilirubinaemia (85.5 μmol/l, >85% "indirect" reacting). The proband and his sister were compound heterozygotes for the Gilbert-type TATAA element and a point mutation 1490T>A in exon 5. The father had a normal TATAA sequence but was heterozygous for the point mutation 1490T>A in exon 5. The mother was heterozygous for the Gilbert-type TATAA element and had normal coding region sequence. The premature termination codon introduced by this mutation is predicted to truncate the carboxy-terminal end of the protein by 37 amino acids. This was confirmed by immunoblot analysis of lysates of COS cells transfected with the mutagenised expression plasmid, pcDNA3.1/zeo(+)- hUGT1A1 (fig 1). The expressed truncated UGT1A1 was catalytically inactive.

Family C. The proband was a female infant with persistent hyperbilirubinaemia since birth with a peak level of 376.2 μmol/l (predominantly "indirect" reacting). Phenobarbital treatment was not effective in reducing the serum bilirubin

Table 1 Promoter and structural mutations in the four families

Subjects (gender)	*TATAA element*	*Structural mutation*	*Effect on protein sequence*	*Serum bilirubin*
Family A				
Infant A1 (F)	$(TA)_6TAA/(TA)_7TAA$	1223delA/normal	Truncated/normal	456 μmol/l*
Infant A2 (F)	$(TA)_6TAA/(TA)_7TAA$	1223delA/normal	Truncated/normal	410 μmol/l*
Mother	$TA)_6TAA/(TA)_7TAA$	Normal/normal	Normal/normal	10 μmol/l
Father	$(TA)_6TAA/(TA)_6TAA$	1223delA/normal	Truncated/normal	15 μmol/l
Family B				
Proband (M)	$(TA)_6TAA/(TA)_7TAA$	1490T>A/normal	L497X/normal	205 μmol/l
Sib (F)	$(TA)_6TAA/(TA)_7TAA$	1490T>A/normal	L497X/normal	85 μmol/l
Mother	$(TA)_6TAA/(TA)_7TAA$	Normal/normal	Normal/normal	7 μmol/l
Father	$(TA)_6TAA/(TA)_6TAA$	1490T>A/normal	L497X/normal	9 μmol/l
Family C				
Child C (F)	$(TA)_6TAA/(TA)_6TAA$	1452G>A/1452G>A	W484X/W484X	359 μmol/l*
Mother	$(TA)_6TAA/(TA)_7TAA$	1452G>A/normal	W484X/normal	51 μmol/l
Father	$(TA)_6TAA/(TA)_6TAA$	1452G>A/normal	W484X/normal	10 μmol/l
Family D				
Case D1 (F)	$(TA)_7TAA/(TA)_7TAA$	524T>A/524T>A	L175Q/L175Q	282 μmol/l
Case D2 (F)	$(TA)_7TAA/(TA)_7TAA$	524T>A/524T>A	L175Q/L175Q	203 μmol/l
Father	$(TA)_7TAA/(TA)_7TAA$	524T>A/normal	L175Q/normal	60 μmol/l

*Represents peak serum bilirubin levels.

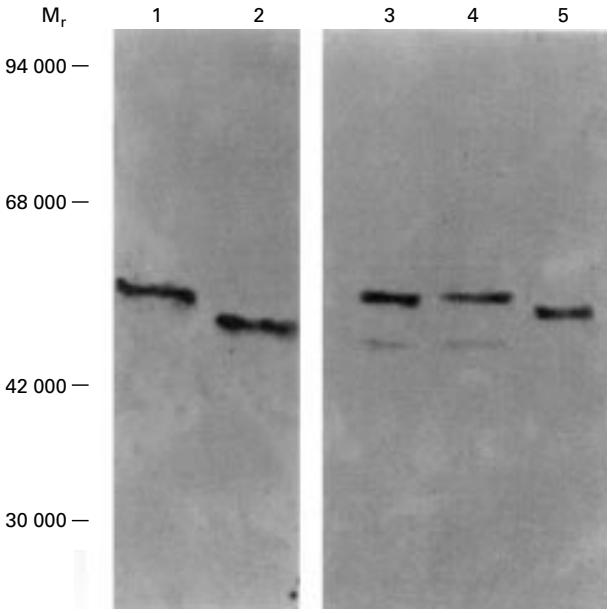

Figure 1 Western blot analysis. The mutations detected in two of the four families, 1490T>A (family B) and 1452G>A (family C), were introduced in the plasmid pSVK3-UGT1A1 (see text). The mutated and normal UGT1A1 cDNAs were subcloned into pcDNA3.1/Zeo(+) (see text) and transfected into COS cells. The lysate of the cells were checked for the synthesis of UGT1A1 by western blotting as described in the text. Lanes 1, 3, and 4 represent normal UGT1A1 (53 kDa). Lanes 2 and 5 are the mutated forms of the protein synthesised from UGT1A1 cDNAs carrying the mutations 1452G>A and 1490T>A, respectively. In both cases, 1452G>A and 1490T>A, the protein is truncated at its C-terminal end by 52 and 37 amino acids respectively. These mutant forms of the protein migrated faster than their normal counterpart corresponding to their respective truncations.

levels and the patient is currently being treated with phototherapy. A diagnosis of CN1 was made. The parents of the infant are first cousins and the father has normal bilirubin levels. The mother had a history of prolonged neonatal hyperbilirubinaemia. Her recent serum bilirubin level was 51.3 μmol/l. Several other relatives of the proband have noticed intermittent yellow discolouration of the sclera, but these samples were not available for genetic analysis. The proband was homozygous for a 1452G>A mutation in exon 5, which predicts the truncation of UGT1A1 at Trp484. As for family B, this was confirmed by Western blot analysis of the expressed mutagenised UGT1A1 (fig 1). The expressed truncated UGT1A1 was catalytically inactive. The father, who was heterozygous for the structural mutation, but had normal *UGT1A1* TATAA elements, had normal serum bilirubin levels. In contrast, the mother, who was also heterozygous for the 1452G>A mutation, but carried a Gilbert-type TATAA element on the structurally normal allele, was hyperbilirubinaemic (51 μmol/l).

Family D. Two sisters and their father were studied from this family. One of the sisters had been included in the original study in 1962 describing CN2 as an entity distinct from CN1.[2] This patient has had predominantly unconjugated hyperbilirubinaemia since birth, averaging 282 μmol/l. Serum bilirubin concentrations were reduced by 80% after administration of phenobarbital. The sister of this case, who was also studied, had a serum unconjugated bilirubin level of 203.5 μmol/l. The father was also hyperbilirubinaemic with serum bilirubin levels of 60 μmol/l. Both sisters were homozygous for the Gilbert-type promoter and also for a 524T>A mutation that predicted the substitution of leucine by glutamine at amino acid residue 175. The father was homozygous for the Gilbert-type promoter and heterozygous for the 524T>A mutation. We had previously determined the effect of the 524T>A mutation (L175Q, family D), which reduced the UGT1A1 activity towards bilirubin to approximately 38% of normal.[17]

The findings in the four families described here are examples of the effect of coexistence of a structural mutation of *UGT1A1* with a Gilbert-type promoter abnormality. In families A and B, the fathers of the probands had a normal TATAA element in the promoter on both alleles of *UGT1A1* and were heterozygous for a structural mutation that predicted (in family A) or was shown (in family B) to truncate and completely inactivate UGT1A1. Consistent with the recessive inheritance of CN1, these heterozygous carriers did not have hyperbilirubinaemia. The mothers in both these families were heterozygous for the Gilbert-type promoter, but the *UGT1A1* coding regions were structurally normal. In these women also, serum bilirubin levels were normal, which is consistent with recessive inheritance of Gilbert's syndrome. In contrast, the twin infants in family A and the two children in family B who inherited the Gilbert-type promoter abnormality from their mothers and the structural lesions from their fathers were hyperbilirubinaemic. The presence of the Gilbert-type promoter on the structurally normal *UGT1A1* allele resulted in hyperbilirubinaemia in the children. In family C, the mother of the affected child with CN1 was heterozygous for the 1452G>A mutation (which was shown to truncate and inactivate UGT1A1) and carried a Gilbert-type TATAA element on the structurally normal allele. Since heterozygosity for the variant promoter or the CN1 type mutation does not cause hyperbilirubinaemia by itself, the increased serum bilirubin levels in the mother again illustrates the effect of interaction of the Gilbert-type promoter and coding region lesions.

In family D, the two sisters studied were homozygous for both the Gilbert-type TATAA element and the structural region abnormality (L175Q), which reduces the UGT1A1 catalytic activity to 38%.[17] This level of residual UGT1A1 activity is similar to that seen in subjects with Gilbert syndrome and, therefore, does not explain the relatively high level of hyperbilirubinaemia seen in these sisters. Because the observed bilirubin levels cannot be accounted for by either the structural mutation or the Gilbert-type promoter alone, these cases show that the reduced expression of the mutated *UGT1A1* owing to the presence of a Gilbert-type promoter results in a more severe phenotype, where the expressed enzyme has some residual catalytic activity. In the father, the Gilbert-type promoter on both alleles and a heterozygous 524T>A mutation caused mild hyperbilirubinaemia (serum bilirubin 60 μmol/l). Such mild hyperbilirubinaemia would be compatible with the promoter defect alone, but might have been accentuated by the coexistence of the structural mutation.

Promoter reporter studies indicate that the Gilbert-type promoter reduces the expression of *UGT1A1* to 20-30% of normal.[18] In cases where one allele produces nonfunctional UGT1A1 and the expression of the only structurally normal allele is reduced because of the presence of the variant promoter, the residual UGT1A1 activity can be calculated to be only 10-15% of normal. The resulting hyperbilirubinaemia is expected to be more severe than that found in Gilbert's syndrome, but less severe than that seen in Crigler-Najjar syndrome type 1. These considerations and our observed results differ from the conclusion of Ciotti *et al*[29] that the presence of a Gilbert-type promoter on one allele and a CN1 type mutation on the other allele leads to a CN1 phenotype.

Depending on the nature and location of a mutation, amino acid substitutions can lead to a wide range of serum bilirubin concentrations. At one end of the spectrum, there may be only a small reduction of the catalytic activity of UGT1A1, resulting in unconjugated hyperbilirubinaemia which is mild enough to be compatible with the diagnosis of

Gilbert syndrome. At the other end are those that cause the most severe forms of CN2 and CN1. In any case, the coexistence of a Gilbert-type promoter, either on the allele bearing the CN-type mutation or on the structurally normal allele (in the case of a heterozygous mutation), can further aggravate the UGT1A1 deficiency. It should be noted, however, that serum bilirubin levels depend not only on the residual UGT1A1 activity, but also on other factors, such as the rate of bilirubin production. This may explain the strong sex difference in the phenotypic expression of Gilbert's syndrome. The majority of females who are homozygous for the Gilbert-type TATAA element do not have clinical hyperbilirubinaemia, probably because of a lower bilirubin load.[23] Consistent with this, the mother in family C had a relatively mild hyperbilirubinaemia (51.3 μmol/l), despite the presence of an inactivating genetic lesion on one allele and a Gilbert-type promoter on the other. The gender effect is also apparent from the data on family B. Although the proband and his sister had identical compound heterozygosity, the sister had lower serum bilirubin levels (table 1). In addition to gender, other known or unknown variables, superimposed on the UGT1A1 genotype, can affect serum bilirubin concentrations. For example, reduced UGT1A1 activity, in combination with a high bilirubin load, can result in higher levels of hyperbilirubinaemia. In the twins in family A, the increased bilirubin load because of ABO incompatibility and the resulting haemolysis led to marked exaggeration of neonatal hyperbilirubinaemia (~428 μmol/l). After cessation of haemolysis and initiation of phenobarbital therapy serum bilirubin levels stabilised at 136-171 μmol/l. Unfortunately, however, permanent brain damage had already occurred in the affected infants. This case exemplifies the importance of compounding of heterozygosity for a structural mutation with the Gilbert-type TATAA element in the promoter of the structurally normal allele. Because of the very high incidence of Gilbert's syndrome in the general population, the possibility of compound heterozygosity for a structural mutation and the Gilbert-type of TATAA element in the promoter should be considered even if only one of the parents has a family history of inherited jaundice.

Based on a gene frequency of 0.3 for the Gilbert-type promoter, as found in several studies in Europe and the USA,[18 20–22] 9% of the general population would be homozygous and 42% would be heterozygous for the variant promoter. Thus, approximately 51% of the general population carry a Gilbert-type promoter on at least one allele. Therefore, heterozygous carriers of a structural mutation of *UGT1A1* (CN1 or CN2 type) have a relatively high probability of carrying a Gilbert-type promoter on the normal allele. This explains the long standing observation that members of families of patients with CN often exhibit intermediate grades of hyperbilirubinaemia. Although the exact incidence of CN is not known, it appears to be less than one in a million, indicating that the gene frequency is less than 1:1000. Based on the gene frequency for the Gilbert-type promoter and that of the structural mutations, compound heterozygosity for a Gilbert-type promoter and CN-type genetic lesion can be calculated to be over 300 times more common than is homozygosity for a structural region mutation. Consistent with this, in our analysis of over 100 cases of intermediate levels of unconjugated hyperbilirubinaemia (persistently between 51 μmol/l and 308 μmol/l) in whom the clinical history was clearly known, we identified the eight cases reported here, in whom an interaction between the Gilbert-type promoter and the structural region mutation was thought to be the cause of jaundice. In only one other case (not included in this paper) was homozygosity for a structural mutation, in the absence of a promoter mutation, the basis of the intermediate level hyperbilirubinaemia. These findings are different from those reported in a Japanese population by Yamamoto *et al*.[30] In that report, homozygosity or compound heterozygosity for structural region mutations was the cause of jaundice in six cases and in only one case hyperbilirubinaemia was caused by the interaction of a Gilbert-type promoter and a structural region mutation. One possible explanation for the difference in results is that the incidence of the longer TATAA element (A(TA)7TAA) is much lower (gene frequency ~0.11) in Japan than in the white or black populations (gene frequency ~0.30).[24–26] Furthermore, structural mutations causing mild hyperbilirubinaemia appear to be much more common in Japan.[25 34 35] In the American population, however, coexistence of a Gilbert-type promoter and a mutation in the structural region of the *UGT1A1* gene appears to be a much more common cause of intermediate grades of hyperbilirubinaemia than homozygosity for a mutation in the coding sequences of the gene. The routine use of intense phototherapy has permitted an increasing number of patients with CN1 or CN2 to survive to ages where pregnancy becomes an option. These patients ask for genetic counselling regarding the probability of inherited jaundice in their offspring and the risk of kernicterus in the newborn period. Our results indicate that both the structural mutation and the promoter genotype of the parents should be taken into account in providing the genetic counselling.

Electronic database Information: GenBank-http://www.ncbi.nlm.nih.gov/genbank, Online Mendelian Inheritance in man (OMIM)-http://www.ncbi.nlm.nih.gov/omim, Protein sequence link-http://www-nbrf.georgetown.edu/

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Identification of two novel mutations in the *CACNA1A* gene responsible for episodic ataxia type 2

EDITOR—Episodic ataxia type 2 (EA-2) (OMIM 108500) is an autosomal dominant neurological disorder. Affected subjects experience discrete episodes of cerebellar ataxia usually associated with migraine symptoms, interictal nystagmus, as well as residual mild and, in some cases, a progressive cerebellar incoordination. These attacks usually begin in childhood or adolescence, last a few hours, may be precipitated by stress, exercise, or fatigue, and respond to acetazolamide.[1–3]

As with other acetazolamide responsive diseases, EA-2 is a channelopathy.[4] It was first linked to chromosome 19p13[3 5–7] and subsequently shown to be allelic to familial hemiplegic migraine (FHM) when mutations for both disorders were identified in the P/Q type calcium channel α_{1A} subunit gene, *CACNA1A*.[8] Shortly thereafter, an intragenic expansion of a CAG repeat within *CACNA1A* was shown to cause spinocerebellar ataxia type 6 (SCA6).[9] To date, mutations causing EA-2 all appear to disrupt the translational reading frame of the α_{1A} subunit gene,[8 10–12] while those causing FHM all seem to be missense mutations.[8 13–15] A single missense mutation, however, in the *CACNA1A* gene has also been shown to cause severe progressive cerebellar ataxia.[16]

The α_{1A} subunit has been shown to be the pore forming unit of the P/Q type calcium channel[17 18] which is involved in controlling neurotransmitter release[19] and is expressed throughout the brain with abundant expression in the cerebellum.[20–22] This high voltage activated calcium channel consists of five subunits, α_{1A}, β_4, α_2, δ, and γ. The α_{1A} subunit is subdivided into four homologous domains (DI-DIV) that each contain six putative transmembrane regions (S1-S6) (fig 1).[19] The fourth transmembrane domain functions as the voltage sensor while the four loops between transmembrane domains S5-S6 compose the pore forming unit. Thus, the α_{1A} subunit of the P/Q type calcium channel is responsible for directing channel activity, while the other subunits appear to act as auxiliary regulators of the channel.[19 23]

Here, we describe two novel mutations in the *CACNA1A* gene that cause EA-2: a guanine insertion after nucleotide 3091 (insG3091) that is the first mutation identified to occur in an intracellular loop and a guanine deletion at nucleotide 5123 (delG5123) representing the most 3' mutation reported to date. Similar to previously reported EA-2 mutations, these nucleotide changes disrupt the *CACNA1A* translational reading frame and are predicted to result in proteins which prematurely truncate after domain I.

Blood samples were obtained with informed consent from 81 subjects: an apparently sporadic case of EA-2 and her sib, 29 members of a family segregating EA-2, and 50 unrelated, healthy controls. Genomic DNA was extracted from the blood samples using standard techniques. Probands were assessed to have EA-2 by a clinical neurologist and were referred for study.

Single stranded conformational polymorphism (SSCP) analysis was used to screen polymerase chain reaction (PCR) products of exons in the *CACNA1A* gene for molecular variants.[24 25] Published primers[8 16] and redesigned primers (table 1) were used to amplify all 47 exons from the intronic sequences flanking each exon. PCR amplification conditions were optimised for each primer pair and the products were labelled by incorporation of [α-^{32}P]dCTP into the amplification reaction. The labelled