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

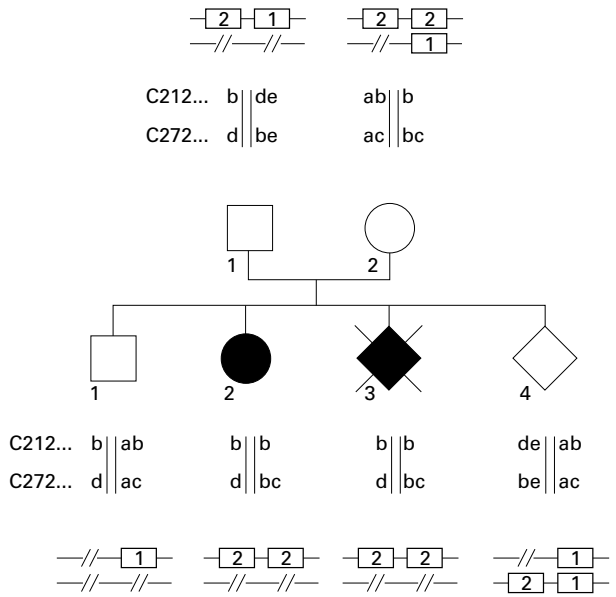

Figure 2 Pedigree of the SMA family used to validate the multiplex PCR. Filled symbols, affected subjects; open symbols, asymptomatic subjects. For each subject, the haplotype analysis using the C212 and C272 microsatellite markers[18] and the schematic representation of the SMN locus (1: SMN1, 2: SMN2) 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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PASCALE SAUGIER-VEBER*
NATHALIE DROUOT*
SUZIE LEFEBVRE†
FRANÇOISE CHARBONNIER*
ELODIE VIAL†
ARNOLD MUNNICH†
THIERRY FRÉBOURG*

**INSERM EMI 9906, Faculté de Médecine et de Pharmacie, 76183 Rouen, IFRMP, 76821 Mont-Saint-Aignan and Service de Génétique, CHU de Rouen, 76031 Rouen, France*

†INSERM U393 and Département de Génétique, Hôpital Necker Enfants-Malades, 75743 Paris, France

Correspondence to: Dr Saugier-Veber, Pascale.Saugier-Veber@chu-rouen.fr.

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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	—	—	—

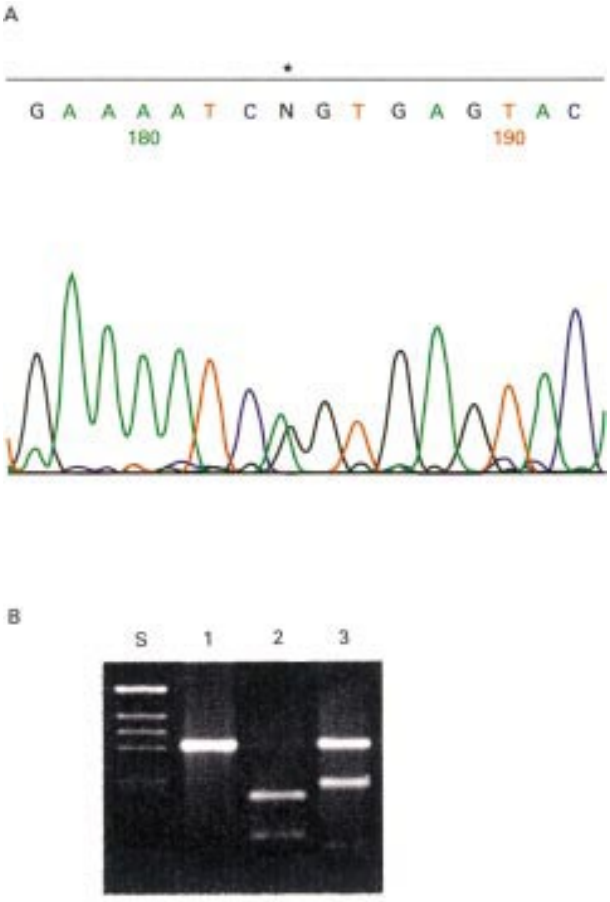

Figure 1 (A) Direct sequencing of exon 3 of the SPINK1 gene showing heterozygosity for the R65Q substitution (asterisk). (B) Screening for missense mutations N34S and R65Q by restriction enzyme analysis. Exon 3 PCR products were amplified using primers from Witt et al[10] and were digested with TspRI 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.