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Minigene analysis of intronic variants in common *SPINK1* haplotypes associated with chronic pancreatitis

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Abstract

BACKGROUND AND AIMS—Two common haplotypes of the serine protease inhibitor Kazal type 1 (*SPINK1*) gene have been shown to increase the risk for chronic pancreatitis. A haplotype comprising the c.101A>G (p.N34S) missense variant and four intronic alterations has been found worldwide, whereas a second haplotype consisting of the c. –215G>A promoter variant and the c. 194+2T>C intronic alteration has been observed frequently in Japan.

METHODS—In the present study we examined the functional significance of the intronic variants in the pathogenic *SPINK1* haplotypes by utilizing minigenes, which harbor individual introns placed in the appropriate context of the full-length *SPINK1* cDNA. Cells transfected with the *SPINK1* minigenes secrete active trypsin inhibitor, thereby allowing evaluation of mutational effects simultaneously on transcription, splicing, translation and secretion.

RESULTS—We found that the c.194+2T>C intronic alteration abolished *SPINK1* expression at the mRNA level, with consequent loss of inhibitor secretion, whereas the p.N34S associated intronic variants had no detectable functional effect.

CONCLUSIONS—Taken together with previous studies, the results indicate that all known variants within the p.N34S associated haplotype are functionally innocuous, suggesting that a yet unidentified variant within this haplotype is responsible for the pathogenic effect. The marked negative impact of the c.194+2T>C variant on *SPINK1* expression supports the notion that *SPINK1* variants increase the risk of chronic pancreatitis by diminishing protective trypsin inhibitor levels.

Keywords

chronic pancreatitis; pancreatic secretory trypsin inhibitor; genetic risk factor; complex genetic disease; intronic variants

Susceptibility to chronic pancreatitis in most cases is inherited in a complex manner which involves mutations in different genes conferring various degrees of risk [1]. Genetic variants of the serine proteinase inhibitor Kazal type 1 (*SPINK1*) gene (OMIM *167790) are among the most frequently identified alterations in subjects with chronic pancreatitis. The c.101A>G

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COMPETING INTERESTS

None to declare.

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(p.N34S) variant has been found worldwide in patients and healthy controls with an average allele frequency of 9.7% and 1 %, respectively [2–4]. This variant is part of a conserved haplotype which also includes four intronic alterations, c.56-37T>C in intron 1; c.87+268A>G in intron 2; and c.195-606G>A and c.195-66_65insTTTT in intron 3 (Figure 1A) [2]. The heterozygous p.N34S associated haplotype increases the risk for chronic pancreatitis 11-fold on average, with higher risk observed in idiopathic and tropical cases than in alcoholic chronic pancreatitis [4 and references therein]. A second common haplotype has been observed with an average allele frequency of 6.3% in Japanese subjects with idiopathic, familial and alcoholic chronic pancreatitis [5–9]. The haplotype contains the c.-215G>A promoter variant and the c.194+2T>C variant which affects the 5' splice site in intron 3 (Figure 1A). It was also found in association with tropical chronic pancreatitis in Bangladesh [10] and Thailand [11] and it was reported from Europe [2,12] and the US [3,13] as well. In contrast to the p.N34S haplotype, the c.194+2T>C haplotype has never been detected in healthy controls.

The *SPINK1* gene codes for the pancreatic secretory trypsin inhibitor, a 6.3 kDa protein, which is produced and secreted together with the digestive zymogens by the acinar cells. The physiological role of *SPINK1* is to protect the pancreas against premature, intrapancreatic trypsin activation [14]. It seems reasonable to assume, therefore, that common *SPINK1* haplotypes associated with chronic pancreatitis would compromise *SPINK1* expression, activity or both. To demonstrate the pathogenic defect in the p.N34S associated haplotype, studies so far focused on the effect of the exonic missense mutation. Several laboratories showed that the p.N34S variation *per se* had no effect on the secretion of *SPINK1* protein from transfected cells and the trypsin inhibitory activity of the mutant protein was also unchanged [15–17]. These observations suggested that one or more of the p.N34S associated intronic variants might be responsible for the pathogenic effect, possibly by interfering with correct pre-mRNA splicing. However, a recent study failed to demonstrate alternatively spliced mRNA forms in resected pancreatic tissue from carriers of the p.N34S associated haplotype [18]. In contrast, gastric biopsies from carriers of the c.194+2T>C associated haplotype revealed the predominant expression of a shorter *SPINK1* mRNA lacking exon 3 [19]. This aberrant mRNA was predicted to code for a non-functional trypsin inhibitor.

In the present study we extended these previous observations by utilizing a minigene approach to evaluate the functional effects of the intronic mutations present in the common pathogenic *SPINK1* haplotypes. We designed *SPINK1* minigenes which contain single introns placed in the appropriate context of the cDNA and give rise to full-length *SPINK1* mRNA upon transcription and splicing (Figure 1B). By measuring the secreted trypsin inhibitor levels, we could assess not only splicing anomalies but also splicing-dependent downstream effects on mRNA stability and translation.

MATERIALS AND METHODS

Nomenclature

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_003122.2). To describe intronic variants that lie closer to the 5' end of the intron; the cDNA number of the last nucleotide of the preceding exon is indicated, followed by a plus sign and the position of the variant counted from the first nucleotide of the intron. To denote intronic variants that are found closer to the 3' end of the intron; the cDNA number of the first nucleotide of the next exon is given, followed by a minus sign and the position of the variant counted backwards from the last nucleotide of the intron. In the published literature, *SPINK1* intronic variants c.56-37T>C, c.87+268A>G, c.195-606G>A, c.195-66_65insTTTT and c.194+2T>C used to be designated as IVS1-37T>C, IVS2+268A>G, IVS3-606G>A, IVS3-66_65insTTTT and IVS3+2T>C.

Construction of expression plasmids harboring SPINK1 minigenes

The pcDNA3.1(-)_SPINK1 expression plasmid containing the wild-type human *SPINK1* cDNA has been described previously [20]. All minigenes were created in this plasmid background. Details of construction are available upon request. Full sequences of the *SPINK1* minigenes are given in the online *Supplementary Material*. Minigenes 1 and 2 also contained a Glu-Glu affinity tag (EYMPME) at the C terminus of SPINK1.

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells and HeLa cells were cultured in 6-well tissue culture plates (10^6 and 5×10^5 cells per well, respectively) in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO₂. Transfections were performed using 1 µg minigene plasmid DNA and 1 µg empty pcDNA3.1(-) vector DNA (2 µg total plasmid DNA) and 10 µL Lipofectamine 2000 (Invitrogen) in 2 mL DMEM, unless indicated otherwise. After overnight incubation at 37 °C, cells were washed and the transfection medium was replaced with 2 mL OptiMEM. Time courses of expression were measured starting from this medium change and were followed for 48 h.

Determination of SPINK1 inhibitory activity

Aliquots (10–30 µL) of conditioned media were mixed with assay buffer (0.1 M Tris-HCl, pH 8.0; 1 mM CaCl₂, 0.05% Tween) and 80 nM recombinant human cationic trypsin (final concentration) in a final volume of 50 µL. After 5 min incubation at room temperature 150 µL N-CBZ-Gly-Pro-Arg-*p*-nitroanilide trypsin substrate was added to 0.14 mM final concentration. One-min rates of *p*-nitroaniline release were measured at 405 nm and compared to an uninhibited control sample. Inhibitor concentrations in the conditioned media were then calculated from the residual trypsin activities.

Reverse-transcriptase (RT)-PCR analysis

RNA was isolated from transfected cells with the RNAqueous kit (Ambion) and 1 µg RNA was reverse-transcribed with M-MLV reverse transcriptase (Ambion). Semi-quantitative measurement of *SPINK1* mRNA was performed by PCR using the following primers; SPINK1_RNA-S 5' – CCA TGA AGG TAA CAG GCA TCT TTC T – 3', SPINK1_RNA-AS 5' – GCG GTG ACC TGA TGG GAT TT – 3'. The 276 nt amplicons were visualized on ethidium bromide stained agarose gels. As an internal standard, a 261 nt fragment of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was also amplified using the following primers; GAPDH sense primer 5'- GTC CAC TGG CGT CTT CAC CA -3', GAPDH antisense primer 5'- GTG GCA GTG ATG GCA TGG AC -3'.

Western blotting

SPINK1 expressed from minigene 3 was detected with a rabbit polyclonal antiserum against human SPINK1 (a kind gift from Walter Halangk and Thomas Wartmann, University of Magdeburg) used at a dilution of 1:5,000 followed by a horseradish peroxidase (HRP) conjugated goat polyclonal anti-rabbit IgG (Pierce, #31460) at a dilution of 1:20,000. Glu-Glu-tagged SPINK1 expressed from minigenes 1 and 2 was detected with an HRP-conjugated goat polyclonal antibody against the Glu-Glu tag (Abcam, #ab1267) used at a dilution of 1:10,000.

RESULTS

Construction of SPINK1 minigenes harboring single introns

The human *SPINK1* gene on chromosome 5 is 7.1 kb in length and comprises 4 exons and 3 introns (Figure 1A). The *SPINK1* minigenes used in this study were designed to contain the

full-length cDNA and single introns, appropriately positioned within the cDNA sequence between their flanking exons (Figure 1B; see *Supplementary Material* for complete minigene sequences). Upon pre-mRNA splicing, the minigenes give rise to full-length *SPINK1* mRNA, which is translated into functional SPINK1 protein. The SPINK1 trypsin inhibitor is a secretory protein and SPINK1 concentrations can be readily measured in the conditioned medium of transfected cells by trypsin inhibition assays or by western blots. In addition to the three minigenes harboring wild type sequences, three minigenes containing the p.N34S variant and the associated intronic variants were also generated, as listed in Table 1. Finally, a minigene with intron 3 and the c.194+2T>C variant was constructed.

Expression of SPINK1 from minigenes

HEK 293T cells transiently transfected with the wild-type minigene constructs secreted SPINK1 to the growth medium. Remarkably, compared to cells transfected with the intronless *SPINK1* cDNA construct, cells expressing minigenes 1 and 3 secreted the trypsin inhibitor at 5-fold higher rates, whereas cells transfected with minigene 2 secreted SPINK1 comparably to the cDNA control (Figure 2A). RT-PCR analysis revealed that cells expressing minigenes 1 and 3 contained notably higher levels of *SPINK1* mRNA than cells expressing minigene 2 or *SPINK1* cDNA (Figure 2B). These observations are in agreement with published accounts documenting that the presence of introns can markedly boost gene expression. This phenomenon is due to the regulatory action of exon junction complexes, proteins deposited to mRNAs as a result of pre-mRNA splicing [21, 22].

Secretion of SPINK1 was directly proportional to the amount of minigene plasmid used for transfection, at least in the 0.5 – 2 µg range. This linear relationship was confirmed for all three minigene constructs (Supplementary Figure S1). To test the effects of intronic mutations with optimal sensitivity, we chose to use 1 µg minigene plasmid DNA per transfection, which yielded high enough levels of SPINK1 secretion but was well within the linear response range of our system.

Effect of pancreatitis-associated intronic variants on SPINK1 expression

Secretion of SPINK1 from cells harboring minigenes with the p.N34S missense variant and associated intronic variants was unchanged relative to SPINK1 secretion from cells expressing wild-type minigenes (Figure 3). In contrast, no SPINK1 protein could be detected in the conditioned medium of cells transfected with minigene 3 carrying the c.194+2T>C variant either by activity assays (Figure 4A) or by western blot (Figure 4B). RT-PCR analysis revealed only traces of correctly spliced *SPINK1* mRNA in these cells (Figure 4C).

To confirm the observations in a second cell line, we have transfected HeLa cells with the minigenes. The results were essentially identical to those observed in HEK 293T cells. Thus, the c.194+2T>C variant abolished SPINK1 secretion, whereas the p.N34S associated intronic variants had no effect (Supplementary Figures S2 and S3).

DISCUSSION

There are two important findings presented in this paper; (1) all four intronic variants associated with the p.N34S haplotype are functionally harmless, and (2) the c.194+2T>C variant abolishes *SPINK1* expression at the mRNA level, which results in diminished trypsin inhibitor production. The c.194+2T>C variant alters the 5' splice site in intron 3 and is predicted to impair pre-mRNA splicing, which in our minigene analysis we detected as loss of normal mRNA expression. This observation is in complete agreement with a recent report by Kume et al. (2005) demonstrating that expression of full-length *SPINK1* mRNA is markedly reduced in gastric biopsy samples from carriers of the c.194+2T>C variant [18]. Instead, these subjects

expressed a non-functional, shortened *SPINK1* mRNA, which lacked exon 3, suggesting that the main splicing defect caused by the c.194+2T>C variant *in vivo* was exon-skipping.

The functional deficit associated with the c.194+2T>C variant argues strongly that pathogenic *SPINK1* variants are loss-of-function mutations [2,3], which increase the risk for chronic pancreatitis by diminishing protective trypsin inhibitor levels in the pancreas. This notion is also supported by recent studies on rare *SPINK1* mutations. Thus, mutations p.L14R and p.L14P, which affect the hydrophobic core of the *SPINK1* signal sequence, block secretion of the inhibitor, in all likelihood due to impaired targeting to the endoplasmic reticulum [20]. Mutations p.G48E, p.D50E, p.Y54H, and p.R67C were also shown to cause reduced *SPINK1* secretion [16,17] because of intracellular retention and degradation [16]. Furthermore, several other published *SPINK1* variants can be predicted with certainty to cause diminished *SPINK1* secretion. These include mutation of the initiator methionine (c.2T>C) [2], frame shift mutations (c.27delC and c.98insA) [23,24], a splice site mutation (c.87+1G>A) [23], and partial or complete deletions of the *SPINK1* gene [25,26].

The mechanism of action of the p.N34S associated haplotype remains one of the most intriguing, unsolved questions of pancreas genetics. Previous studies convincingly showed that the p.N34S variant *per se* had no detectable effect on *SPINK1* expression or inhibitory activity [15–17]. The results presented here extend these observations by demonstrating that none of the four intronic variants associated with p.N34S affect *SPINK1* expression or function. Therefore, we propose that the true pathogenic variant within the p.N34S haplotype is yet unidentified and it is most likely found in the so far uncharacterized flanking regions of the *SPINK1* gene. On the basis of the common loss-of-function phenotype exhibited by other *SPINK1* variants, one might speculate that the functional defect caused by the p.N34S haplotype would involve a reduction in *SPINK1* expression. A recent study, however, found normal serum levels of *SPINK1* both in healthy and affected carriers of the p.N34S haplotype, whereas *SPINK1* levels were decreased in subjects with the c.194+2T>C haplotype [9].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Common pathogenic *SPINK1* gene haplotypes (A) and *SPINK1* minigenes used in this study (B). See text for further details.

Figure 2.

Expression of SPINK1 from minigenes. **A.** HEK 293T cells were transfected with minigenes or *SPINK1* cDNA and levels of secreted SPINK1 were measured from the conditioned medium at the indicated times. **B.** Alternatively, total RNA was prepared from transfected cells after 48 h and *SPINK1* mRNA levels were measured by RT-PCR, as described in *Materials and Methods*. Control RNA was prepared from untransfected cells. Expression of *GAPDH* was measured as an internal standard.

Figure 3.

Effect of p.N34S and associated intronic variants on the expression of SPINK1 from minigenes. HEK 293T cells were transfected with minigenes and secreted SPINK1 was measured from the conditioned medium at the indicated times by activity assay and western blots, as described in *Materials and Methods*.

Figure 4.

Effect of the c.194+2T>C intronic variant on the expression of SPINK1 from minigene 3. HEK 293T cells were transfected with minigenes and secreted SPINK1 was measured from the conditioned medium at the indicated times by activity assay (**A**) and western blot (**B**), as described in *Materials and Methods*. **C**. Total RNA was prepared from transfected cells after 48 h and *SPINK1* mRNA levels were measured by RT-PCR, as described in *Materials and Methods*. Expression of *GAPDH* was measured as an internal standard.

Table 1

SPINK1 minigene constructs used in the present study.

<i>SPINK1</i> minigene	Alterations in minigene
minigene 1 (wt)	wild type cDNA with intron 1
minigene 2 (wt)	wild type cDNA with intron 2
minigene 3 (wt)	wild type cDNA with intron 3
minigene 1 (p.N34S)	p.N34S <u>and</u> c.56–37T>C
minigene 2 (p.N34S)	p.N34S <u>and</u> c.87+268A>G
minigene 3 (p.N34S)	p.N34S <u>and</u> c.195–606G>A <u>and</u> c.195–66_65insTTTT
minigene 3 (c.194+2T>C)	c.194+2T>C