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Pancreatic Cancer Cell Lines Heterozygous for the *SPINK1* p.N34S Haplotype Exhibit Diminished Expression of the Variant Allele

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To the Editor

The c.101A>G (p.N34S) variant and its associated haplotype in the serine protease inhibitor Kazal-type 1 (*SPINK1*) gene is one of the clinically most significant risk factors for chronic pancreatitis [1, 2]. Although a loss-of-function mechanism has been proposed for the increased pancreatitis risk conferred by this haplotype, experimental evidence is lacking, since neither the p.N34S variant nor any of the four associated intronic variants affect *SPINK1* expression or trypsin inhibitory function [3–8]. Recently, two pancreatic adenocarcinoma cell lines, PaCa44 and PancTu-I were reported to carry a heterozygous p.N34S variant [9]. The aim of the present study was to take advantage of the unique opportunity these cell lines offer and investigate whether the p.N34S haplotype affects mRNA expression of *SPINK1*.

PaCa44 and PancTu-I cells [10] were kind gifts from Matthias Löhr (Karolinska Institutet) and Ralf Jesenofsky (University of Heidelberg). Both cell lines expressed *SPINK1* mRNA detectable by reverse transcription (RT) PCR. Heterozygosity for the p.N34S variant was confirmed by sequencing both genomic DNA and cDNA (Figure 1A). Relative expression levels of the two *SPINK1* alleles were determined using RT-PCR followed by allele-specific digestion with the restriction endonuclease Hpy166II and quantitation of the digestion products (Figure 1BC). Remarkably, in this experiment the p.N34S allele showed reduced expression, which was 6.7-fold lower than the expression of the wild-type allele (Figure 1D). Although not shown, additional experiments using different primer sets showed 3–4-fold reduced expression of the variant allele. The observations suggest that the p.N34S haplotype may comprise a negative regulatory variant likely located in the 5' region upstream of the gene. To search for potential candidates, we determined the genomic DNA

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sequence for the entire *SPINK1* gene (6.9 kb) and the flanking 5' (6.2 kb) and 3' (1.2 kb) regions. We identified 22 variations relative to the reference sequence, which were, unexpectedly, identical in the two cell lines (Table 1). The 5' region contained six single-nucleotide variants. When subjects homozygous (n=3) or heterozygous (n=3) for the p.N34S haplotype and non-carrier controls (n=9) were genotyped for these six 5' variants, only variant c.-4141G>T was consistently found in p.N34S carriers while absent in controls indicating that this variant is part of the p.N34S haplotype.

In conclusion, the findings indicate that the p.N34S allele may cause reduced *SPINK1* expression. Further studies are needed to verify whether such a negative effect is also manifested in human pancreatic acinar cells. The identification of the c.-4141G>T variant extends the number of published variants within the p.N34S associated haplotype and represents a potential candidate for the pathogenic variant responsible for reduced *SPINK1* expression and pancreatitis risk. Finally, the apparent genetic identity of the PaCa44 and PancTu-I cell lines suggests a common origin [10].

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Figure 1.

Effect of the p.N34S associated haplotype on the expression of *SPINK1* mRNA in the PaCa44 and PancTu-I pancreatic cancer cell lines. (A) Electropherograms of genomic DNA and cDNA sequences of *SPINK1* from PancTu-I showing the c.101A>G (p.N34S) variant. Note the smaller G signal in the cDNA sequence indicating reduced expression of the p.N34S allele. (B) Total RNA (n=3 from each cell line) was reverse transcribed to cDNA and a 300 bp *SPINK1* fragment was PCR amplified. The amplicon was digested with restriction endonuclease Hpy166II which cleaves the p.N34S variant allele only, generating

two bands of 195 bp and 105 bp size (Experimental digestions). To quantitate the relative expression levels of the two alleles, a calibration curve was generated using defined mixtures of plasmids carrying the wild-type and p.N34S *SPINK1* cDNA as templates (Calibration digestions). The bands were resolved on agarose gels, stained with ethidium bromide, band intensities were measured by densitometry and the ratio of the 195 bp and 300 bp bands was calculated. **(C)** The intensity ratio in the calibration digestions was plotted as a function of the p.N34S allele concentration and levels of the p.N34S allele in the experimental digestions were determined from this calibration curve (arrows). **(D)** The bar graph shows the pooled average ($\pm$ S.D, n=6) mRNA expression levels for the p.N34S allele relative to the wild-type allele in the two cell lines.

Table 1

Nucleotide variants in the *SPINK1* gene of the PaCa44 and PancTu-I cell lines compared to the GenBank NC_000005.10 chromosome 5 primary assembly reference sequence. The two cell lines harbored identical variants.

Location	Variation	dbSNP identifier
5' region	c.-6136AAAG[44] ¹	
5' region	c.-5444C>T	rs77400277
5' region	c.-4754AC[19];[20] ²	
5' region	c.-4568G>A	rs77171680
5' region	c.-4141G>T	rs142703147
5' region	c.-2471A>G	rs80298935
5' region	c.-2156A>G	rs77135112
5' region	c.-2090A>G	rs148276928
Intron 1	c.56-37T>C	rs17107318
Intron 2	c.87+268A>G	rs17107316
Intron 2	c.87+703delA	rs34409032
Intron 2	c.88-352A>G (hm)	rs6580502
Exon 3	c.101A>G (p.N34S)	rs17107315
Intron 3	c.194+1159C>G	rs1897577
Intron 3	c.195-1414A>T (hm)	rs2436411
Intron 3	c.195-714delA	
Intron 3	c.195-606G>A	rs149882377
Intron 3	c.195-66_65insTTTT	rs554919880
3' region	c.*32C>T	rs11319
3' region	c.*318A>T (hm)	rs3777126
3' region	c.*407C>G (hm)	rs3777125
3' region	c.*1191C>T (hm)	rs17107298

Hm, homozygous; all other variants were heterozygous. The p.N34S associated haplotype is highlighted in bold.

¹Variable short sequence repeat. The cell lines carry 44 repeats of AAAG; the reference sequence contains 16 repeats.

²Variable short sequence repeat. The cell lines carry 19 and 20 repeats of AC on the two alleles, the reference sequence contains 17 repeats.