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In vitro and *in silico* evidence against a significant effect of the *SPINK1* c.194G>A variant on pre-mRNA splicing

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We read with interest the recent publication of Beer and Sahin-Tóth reporting that exonic variants affecting pre-mRNA splicing contribute to the genetic burden in chronic pancreatitis.[1] One particular variant, affecting the last nucleotide of exon 3 of the *SPINK1* gene, c.194G>A, was found to cause an ~80% reduction in *SPINK1* mRNA expression as compared to the wild-type in a minigene assay performed in human embryonic kidney 293T (HEK293T) cells. The *SPINK1* sequence inserted into the minigene expression vector however comprised only exon 1, exon 2, exon 3, intron 3 and exon 4 of the four-exon gene.[1] Note that the potential effect of c.194G>A as a missense mutation (p.Arg65Gln) on protein function had previously been analysed; engineered expression of the full-length mutant coding sequence in Chinese hamster ovary cells and HEK293T cells showed consistently a 70-80% reduction in protein secretion as compared to the wild-type.[2,3]

We recently analysed the functional consequences of 24 *SPINK1* intronic variants in relation to their associated mRNA splicing phenotypes[4,5] by means of a full gene splicing assay in which the full-length 7 kb *SPINK1* genomic sequence (including all four exons plus all three introns of the gene) was cloned into the pcDNA3.1/V5-His-TOPO vector.[6] This full-length gene expression system has already proved itself in practice by accurately representing the *in vivo* situation in the context of the observed splicing patterns of the *SPINK1* wild-type gene and two pathogenic splice site variants, c.87+1G>A and c.194+2T>C.[4] Naturally, a full-length gene construct corresponds more closely to the *in vivo* chromosomal contexts of the studied genes than a minigene construct.

We attempted to replicate the above-mentioned finding with respect to the *SPINK1* c.194G>A variant[1] using our full-length splicing assay, essentially as previously described.[4,5] We introduced the c.194G>A variant into the wild-type full-length *SPINK1* expression vector by directed mutagenesis; reverse transcription-polymerase chain reaction (RT-PCR) analyses of mRNAs from subsequently transfected HEK293T cells revealed a single transcript of

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similar size to the wild-type (figure 1A); sequencing of the RT-PCR products revealed that the variant transcript was correctly spliced as per the wild-type. We then performed quantitative RT-PCR analyses of the wild-type and c.194G>A variant mRNA expressions as previously described,[7] except that the primers used for amplifying the full-length target gene transcripts was changed to the primer pair Q1 as described in ref. 6. However, we failed to observe any significant difference between the c.194G>A variant and the wild-type in terms of mRNA expression (figure 1B), contrary to the results of the previous authors.[1] To validate the performance of our quantitative RT-PCR analysis, we did two additional experiments. First, we demonstrated that mRNA expression of the 500 ng pcDNA3.1 plasmid harbouring the full-length *SPINK1* genomic sequence used for quantitative RT-PCR analysis, was within the linear range of a transfection dosage-gene expression curve (see online supplementary figure S1). Second, we demonstrated that a known *SPINK1* variant, c.27delC (p.Ser10fsX5) located in the middle of exon 1 coding sequence,[8] caused a significant decreased mRNA expression that could be reversed by cycloheximide, a known inhibitor of nonsense-mediated mRNA decay[7] (figure S2).

We further assessed the likely impact of the *SPINK1* c.194G>A variant in terms of the disruption of known splice sites and/or creation of new splice sites using the five splicing prediction algorithms viz. SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer and Human Splicing Finder included within the Alamut® Visual software suite (version 2.7.1; Interactive Biosoftware, Rouen, France). The c.194G>A variant was predicted to have only slightly reduced scores as compared to the wild-type intron 3 splice donor site (see figure S3A). These slightly reduced scores are not suggestive of a significant impact on splicing based upon our current knowledge of *in silico* analyses.[9] Moreover, the creation of a potential splice acceptor site was predicted by only three of the five programs; and the predicted scores were invariably lower than 80% of the three programs' corresponding maximal scores (i.e.,

70.9 of 100 for SpliceSiteFinder-like, 79.2 of 100 for Human Splice Finder and 3.7 of 16 for MaxEntScan; figure S3A). Further, the Alamut software suite predicted that the c.194G>A variant would neither affect branch-point properties (figure S3A) nor create or disrupt splicing regulatory elements such as exonic/intronic splicing enhancers/silencers (figure S3B). Taken together, the *in silico* analyses did not support a significant impact of the *SPINK1* c.194 variant on splicing.

In summary, our *in vitro* and *in silico* analytical data contradict the previous report that the *SPINK1* c.194G>A variant significantly affects pre-mRNA splicing. Consequently, we propose instead that the known pathogenicity of this variant is likely to be attributable to its impact as a missense mutation that significantly decreases protein secretion.[2,3] This notwithstanding, it should be kept in mind that findings obtained from a model system may not accurately reflect *in vivo* situations. It is thus desirable to investigate the mRNA expression in the pancreatic tissue of the c.194G>A carrier.

Contributors JMC, ZL, ZSL and CF designed and directed the study. HW and AB performed functional analysis. JMC drafted the manuscript. DNC critically revised the manuscript. All authors analysed the data and approved the final manuscript.

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Figure 1 Functional analyses of the potential impact of the *SPINK1* c.194G>A variant on splicing using a previously described full-length gene expression assay.[4,5] (**A**) RT-PCR analyses of HEK293T cells transfected with full-length gene expression constructs harbouring respectively the *SPINK1* wild-type and c.194G>A variant sequences. (**B**) Relative mRNA expression level of the *SPINK1* c.194G>A variant compared to that of the wild-type as determined by quantitative RT-PCR analysis of HEK293T cells transfected with the corresponding full-length gene expression constructs. Results are given as the mean ± SD from three independent transfection experiments.

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