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SERPING1 exon 3 splicing variants using alternative acceptor splice sites

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ABSTRACT

Mutations in the C1 inhibitor (C1INH) encoding gene, *SERPING1*, are associated with hereditary angioedema (HAE) which manifests as recurrent submucosal and subcutaneous edema episodes. The major C1INH function is the complement system inhibition, preventing its spontaneous activation. The presented study is focused on *SERPING1* exon 3, an alternative and extraordinarily long exon (499 bp). Endogenous expression analysis performed in the HepG2, human liver, and human peripheral blood cells revealed several exon 3 splicing variants alongside exon inclusion: a highly prevalent exon skipping variant and less frequent + 38 and -15 variants with alternative 3′ splice sites (ss) located 38 and 15 nucleotides downstream and upstream from the authentic 3′ ss, respectively. An exon skipping variant introducing a premature stop codon, represented nearly one third of all splicing variants and surprisingly appeared not to be degraded by NMD. The alternative -15 3′ ss was used to a small extent, although predicted to be extremely weak. Its use was shown to be independent of its strength and highly sensitive to any changes in the surrounding sequence. -15 3′ ss seems to be co-regulated with the authentic 3′ ss, whose use is dependent mainly on its strength and less on the presence of intronic regulatory motifs. Subtle *SERPING1* exon 3 splicing regulation can contribute to overall C1INH plasma levels and HAE pathogenesis.

1. Introduction

C1 inhibitor (C1INH), a member of the serine protease inhibitor (serpin) family, represents a key regulator in several immune and inflammatory pathways. Its major function is to prevent inappropriate or excessive activation of the classical complement pathway by binding to and inactivating C1r and C1s proteases (Sim and Porter, 1976). It also inhibits the lectin complement pathway by inactivating mannan-binding lectin serine proteases (MASP) and alternative complement pathway by binding to C3b (Dobó et al., 2011; Hansen et al., 2015; Jiang et al., 2001). C1INH is also a contact system proteases, factor XII, kallikrein and factor XI inhibitor (Pixley et al., 1985; Schapira et al., 1982; van der Graaf et al., 1983; Wuillemin et al., 1995).

C1INH is a heavily glycosylated single chain protein of 500 amino acids, including a 22 amino acid long signal sequence. It is composed of two distinct domains: C-terminal domain, similar to other serpins with conserved nine α-helices and three β-sheets, and a unique N-terminal domain, not conserved among other serpin proteins (Beinrohr et al., 2007; Bock et al., 1986; Odermatt et al., 1981). The C-terminal domain is responsible for C1INH inhibitory function (Coutinho et al., 1994), and the N-terminal domain (encoded by exon 3) contains important cysteine residues forming disulphide bridges with cysteines in serpin domain, stabilizing the active conformation of C1INH and most of the glycosylation sites that probably play a role in C1INH clearance and leukocyte migration inhibition (Bos et al., 2002; Cai and Davis, 2003).

SERPING1, a gene encoding C1INH, (OMIM no. 606860; GenBank ID710) extends over a 17,159-bp genomic region located on chromosome 11q12-q13.1. It is comprised of eight exons and seven introns, particularly rich in repetitive Alu sequences (Bock et al., 1986; Carter et al., 1991). Over 450 *SERPING1* mutations related to hereditary angioedema (HAE), the most common human disease due to a genetic complement protein defect, have been reported up to now. HAE is an autosomal dominant disorder manifesting as recurrent submucosal and subcutaneous edema episodes. HAE type I, which accounts for 85% of cases, is caused by mutations occurring throughout the whole gene and leading to truncated or misfolded dysfunctional protein that is not secreted efficiently, thus the C1INH plasma level is decreased. HAE type II, which accounts for 15% of cases, is usually caused by mutations in a reactive center loop coding part of exon 8, leading to a mutant protein that is secreted but dysfunctional (Cugno et al., 2009; Rosen et al., 1971). In both HAE types, kallikrein-kinin system activation is dysregulated, which leads to an excessive generation of bradykinin, the

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major swelling mediator in HAE (Joseph et al., 2008; Nussberger et al., 1998).

SERPING1 is a naturally alternatively spliced gene. Duponchel et al. (2006) reported *SERPING1* exon 3 skipping in monocytes. However, this isoform's functional role remains unclear. Although it seems that the abundance of exon 3 skipping is highly variable (de la Cruz et al., 2012), there is a possibility that the balance of both full-length and exon 3 skipped splicing isoforms is subtly regulated and contributes to the overall C1INH level. It is additionally supported by some exon 3 mutations manifested by the dysregulated proportion of exon 3 skipping (de la Cruz et al., 2012; Grodecká et al., 2017). Up to now, no study has elucidated *SERPING1* exon 3 skipping's role in immune pathways or HAE pathogenesis. The aim of this study was to characterize *SERPING1* splicing regulation in healthy individuals and to identify elements regulating exon 3 inclusion. First, we analysed exon 3 splicing variants in the HepG2, human liver and human peripheral blood cells. Next, using nonsense-mediated decay (NMD) inhibition and PCR mutagenesis followed by splicing minigene assay, we investigated two of the detected variants in detail: a frameshift exon 3 skipping variant which introduced a premature stop codon, surprisingly representing up to approximately one third of all splicing variants; and an in-frame -15 variant with an extremely weak alternative acceptor splice site (3′ ss) located 15 nt upstream from the authentic one, a slightly changing signal peptide C-terminal part and prolonging the C1INH bysix amino acids (AA).

2. Materials and methods

2.1. Biological material

Peripheral blood samples were obtained from two healthy donors, liver samples were obtained from two liver transplantation patients from the Centre for Cardiovascular Surgery and Transplantation in Brno. The study was approved by the Medical Ethics Committee of the Centre for Cardiovascular Surgery and Transplantation, Brno. Informed consent was obtained from all the participants before being included in the study.

2.2. Minigene construction and PCR mutagenesis

The *SERPING1* minigene construct was prepared by amplifying *SERPING1* wild-type genomic fragment, containing exon 3 (499 bp) and parts of flanking introns 2 and 3 (209 and 235 bp respectively) using Platinum Pfx DNA Polymerase (ThermoFisher Scientific) and C1Ie3_clonF and C1Ie3_clonR primers (Supplementary Tab. S1), and by following cloning to Exontrap Cloning Vector pET01 (MoBiTec), using ApaI and SmaI restriction enzymes (ThermoFisher Scientific). PCR mutagenesis was performed using Platinum Pfx DNA Polymerase (LifeTechnologies) and primers listed in Supplementary Tab. S1. The detailed protocol is summarized in Supplementary Tab. S2.

2.3. Cell culture and transfection

HepG2 cell lines (Sigma-Aldrich) were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and incubated at 37 °C in a 5% CO2 atmosphere. For splicing minigene assay, HepG2 cells were seeded into a 12-well plate one day prior to the transfection to achieve 30% confluency. Cells were transfected with 2.4 µl XtremeGENE 9 Transfection Reagent (Roche) and 800 ng plasmid DNA in RPMI 1640 medium (Roche), according to the manufacturer's instructions and incubated for 24 h. For NMD inhibition assay, cells were cultured in T25 cell culture flask and transfected with 32.5 µl lipofectamine (ThermoFisher Scientific) and 16 µM siRNA targeting *UPF1* (ThermoFisher Scientific) in Opti-MEM I Reduced Serum Medium (ThermoFisher Scientific), according to the manufacturer's instructions and incubated for 48 h.

2.4. RNA extraction and RT-qPCR

Total RNA was isolated from: cultivated HepG2 cells using an innuPREP RNA Micro Kit (Analytik Jena), human liver samples using an *mir*Vana miRNA Isolation Kit (ThermoFisher Scientific), peripheral blood samples using an RiboPure-Blood Kit (Thermo Fisher Scientific), according to the manufacturers' instructions. For nuclear and cytoplasmic fractionation, a series of lysis and centrifugation steps were carried out (lysis buffer composition is reported in Supplementary Tab. S3; detailed protocol available on request) before standard RNA isolation. Reverse transcription was carried out using anchored dT18 primer (Supplementary Tab. S1) and Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer's instructions. Real-time PCR was performed to determine the exponential phase of PCR amplification using *Taq* DNA Polymerase (ThermoFisher Scientific), EvaGreen (Biotium) and the following primers: C1Ie2_F and C1Ie4_R for endogenous expression analysis; pET_F and pET_R for splicing minigene assay; GAPDH_F and GAPDH_R, UPF1_F and UPF1_R for NMD inhibition evaluation (Supplementary Tab. S1). To determine the NMD inhibition efficiency, *UPF1* expression levels normalized to *GAPDH* expression levels of inhibited and non-inhibited samples were compared.

2.5. Capillary electrophoresis

Capillary electrophoresis was used to quantify particular *SERPING1* exon 3 splicing isoforms. cDNA samples were diluted to unify the specific cDNA concentration. Thus, PCR of the compared samples could have been run for the same number of cycles, preserving the exponential phase of PCR, and amplified using *Taq* DNA Polymerase (ThermoFisher Scientific) and the following primers (one of each pair was FAM-labeled): C1Ie2_F_FAM and C1Ie4_R; pET_F_FAM and pET_R. 1 µl of each amplicon was mixed with 12.5 µl of HiDiformamide and 0.5 µl GeneScan 1000 ROX Size Standard(ThermoFisher Scientific), denatured and frozen. The capillary electrophoresis was performed on an ABI 3500 Genetic Analyzer (ThermoFisher Scientific) and the obtained data were analysed using GeneMapper 4.1 (ThermoFisher Scientific). The exemplary results from capillary electrophoresis are appended in Supplementary Fig. S1. The peaks with a size which did not correspond to any previously described splicing variants were further identified. The novel splicing variant estimation was based on the product length and splice site predictions according to Human Splicing Finder 3.1 (http://www.umd.be/HSF3/), which helps us to identify weaker 3′ss at the correct distance from the authentic one. To prove the use of predicted transcripts with alternative 3′ ss, specific primers C1Ie3_-61_F, C1Ie3_-51_F, C1Ie3_+38endo_F, and C1Ie3_+38_R were designed to overlap the junction of pET exon1 and *SERPING1* exon 3 in predicted transcripts and used for PCR amplification with pET_R and pET_F primers. Amplicons were then separated on 2% agarose gel stained with MIDORI Green Advance (Elisabeth Pharmacon), purified using QIAquick Gel Extraction Kit (Qiagen) and sequenced using ABI 3500 Genetic Analyzer (ThermoFisher Scientific).

3. Results and discussion

3.1. Alternative splicing variants of SERPING1 exon 3

The presented study was focused on *SERPING1* exon 3, which is known to be alternatively spliced. As it is an extraordinarily long exon (499 bp), a complex system of splicing regulatory factors is supposed to be implicated.

Endogenous expression analysis performed in the HepG2, human liver, and human peripheral blood cells, revealed several *SERPING1* exon 3 splicing variants alongside exon inclusion: very common exon skipping, and the less frequent +38 variant with alternative 3′ ss 38 nt downstream from the authentic one, and a -15 variant with an alternative 3′ ss 15 nt upstream from the authentic one (Fig. 1). For further

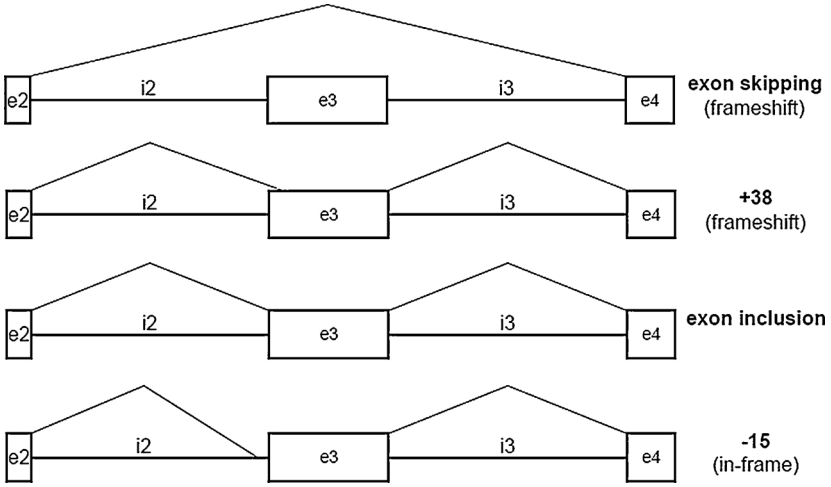

Fig. 1. Scheme of *SERPING1* splicing variants detected in minigene, HepG2 cells, human liver and human peripheral blood (e – exon, i – intron).

Table 1
Frequencies of *SERPING1* splicing variants detected in minigene, HepG2, human liver and human peripheral blood cells are represented as average value ± SD.

splicing variant	minigene (%)	HepG2 (%)	liver (%)	peripheral blood (%)
exon skipping	24 ± 6	36 ± 7	32 ± 4	35 ± 6
+38	4 ± 1	7 ± 0	5 ± 0.4	6 ± 3
exon inclusion	62 ± 7	56 ± 10	62 ± 4	57 ± 10
−15	10 ± 4	1 ± 0	1 ± 0.03	2 ± 0.2

studies of exon 3 splicing variants, a minigene splicing assay was used. In addition to the abovementioned variants, two more minor (both less than 1%) variants were detected in the minigene: -51 and -61 with alternative 3′ ss located 51 and 61 nt upstream from the authentic one, respectively. The variant proportions are presented in Table 1. It is evident that the splicing minigene assay provided data consistent with the results from endogenous expression analysis (with the exception of -15 3′ ss, which is overestimated to the detriment of the exon skipping variant in the minigene) (Table 1), so it can be considered a useful and reliable tool for splicing and its regulation investigation.

Transcripts corresponding to exon skipping and -15 variants have already been deposited in databases (ENST00000531133.5 and ENST00000378323.8). Exon 3 skipping variant has already been reported to be expressed in human monocytes, but not in hepatocytes (Duponchel et al., 2006). To our knowledge, we first described its expression in the human liver. +38 is a novel variant first described in this study.

3.2. Are alternative splicing variants degraded by nonsense-mediated decay?

As some of the detected exon 3 splicing variants cause a frameshift and introduce a premature stop codon (Fig. 1), they are supposed to be degraded by nonsense-mediated decay (NMD) (Behm-Ansmant et al., 2007). To test this assumption, an evaluation of variants proportions in nucleus and cytoplasm of HepG2 cells was first performed (Fig. 2). Our results showed that the exon skipping variant was more abundant in the cytoplasm (16% vs. 32%), contrary to the +38 variant, which was more abundant in the nucleus (21% vs. 10%). The exon inclusion variant was present equally in the nucleus and cytoplasm (62% vs. 58%). As degradation steps of the NMD pathway occur predominantly in the cytoplasm (Chang et al., 2007), these data suggest that the +38 variant could be subjected to degradation, contrary to the exon skipping variant, which surprisingly seems not to be degraded by NMD.

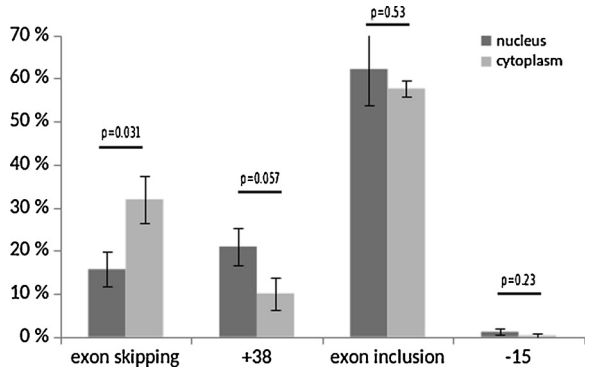

Fig. 2. *SERPING1* splicing variants in the nucleus and cytoplasm in HepG2 cells (% of total nuclear/cytoplasmic *SERPING1* transcripts). Error bars represent SD. The significance of changed frequencies was evaluated using t-test.

3.3. Exon skipping variant

As the exon skipping variant represented approximately one third of all splicing variants, the next experiment was focused on it. NMD in HepG2 cells was inhibited using siRNA targeting *UPF1*, coded for one of the main components in the NMD pathway. It resulted in the *UPF1* mRNA level decreasing to 25% compared to the non-inhibited sample. Nevertheless, the exon skipping variant frequency remained unchanged (Fig. 3), which further supported this variant not being subject to NMD.

Exon 3 encodes the last five amino acids (AA) of the signal peptide (Supplementary Fig. S1), the entire N-terminal domain and part of the serpin domain of C1INH. The N-terminal domain is unique for C1INH, not conserved among other serpins. It contains important cysteine

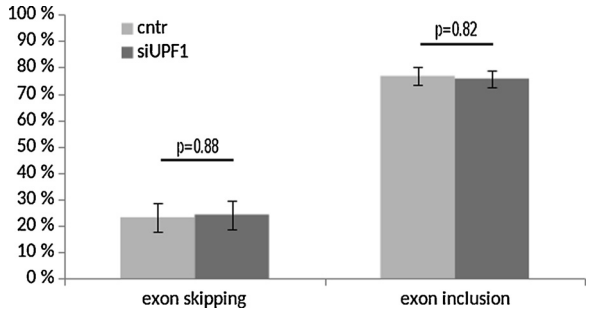

Fig. 3. The effect of NMD inhibition using siRNA targeting *UPF1* on the ratios of exon 3 inclusion and exon 3 skipping splicing variants in HepG2 cells. Cntr – nontreated HepG2 cells. Error bars represent SD.

residues forming disulphide bridges with cysteines in the serpin domain, stabilizing the active conformation of C1INH (Bos et al., 2002). Therefore, exon 3 mutations either in the N-terminal or the serpin domain could be disease-causing and many of them were actually reported in HAE patients (Andrejević et al., 2015; Bafunno et al., 2014; Bowen et al., 2001; Cumming et al., 2003). It is even more surprising that frameshift splicing variant lacking a whole exon 3, giving rise to transcript coding for only 43 amino acid peptide, is expressed to such an extent. Mutations causing premature stop codon inclusion in *SERPING1* typically result in mRNA degradation via NMD (Colobran et al., 2014; Roche et al., 2005, Hujová et al. not yet published), which is not the case of naturally occurring exon skipping variant. Therefore, we hypothesize that a specific mechanism helps to bypass the NMD to allow this variant's biological function. Among possible explanations for NMD bypassing, translation reinitiation can play an important role. Especially in the case of short upstream ORFs, a ribosome can resume RNA scanning for a downstream start codon and initiate translation (Kozak, 1999). In the exon skipping variant, next two AUGs are not in frame, contrary to the third consecutive one. We used the NetStart 1.0 Prediction tool (Pedersen and Nielsen, 1997) to predict translation start in the exon skipping variant and abovementioned AUG displayed a high score above the cut-off limit 0.5 (0.746). If the translation is re-initiated, a new 191 amino acid long protein (identical with corresponding part of C1 inhibitor) is produced. As no peptide corresponding to the transcript lacking exon 3 was detected, neither in the human plasma nor in a medium from HepG2 cell cultivation using mass spectrometry (data not shown), its role at the transcript rather than protein level is suggested. Distinguishing these two possibilities will require additional experiments. Anyway, it is clear that an exon skipping variant cannot compensate for the full-length transcript function and has to operate at a different level.

3.4. -15 variant

3.4.1. In silico predictions of -15 variant properties

The -15 variant preserves a reading frame, changes and prolongs a protein sequence. To predict the presence of signal peptide and AA composition of the secreted protein derived from the -15 transcript variant, SignalP-4.1 was used (Petersen et al., 2011). While in a normal splicing variant, a cleavage site should be between positions 22 and 23, this site is shifted one AA upstream in the -15 variant, which results in protein prolonged by six AA (QDRASS) at its N-terminus (Supplementary Fig. S2), providing two potential glycosylation sites (Van den Steen et al., 1998). C1 inhibitor is the most heavily glycosylated plasma protein and most of the glycosylation sites are located in the N-terminal domain (Bock et al., 1986). Besides contributing to C1INH clearance, glycosyl residues can participate in the leukocyte rolling down-regulation during inflammatory reactions via binding to endothelial selectins. C1INH molecules binding to the endothelial surface may also serve to localize and concentrate the C1INH at inflammatory sites, thus resulting in more efficient regulation of complement and contact system activation (Cai and Davis, 2003). Two additional glycosylation sites in protein arising from -15 transcript may emphasize both this type of regulation and the C1 inhibitor's anti-inflammatory activity. As we observed increased levels of neutrophil activation markers in HAE patients (even in a symptom-free period) in our previous study (submitted manuscript), it would be interesting to compare -15 splicing variant expression in healthy donors and HAE patients.

3.4.2. Determination of -15 3′ ss usage

Using an extremely weak alternative 3′ ss located only 15 nt upstream from the authentic one and with no preceding polypyrimidine tract (PPT), this splicing variant is also interesting from a mechanistic point of view. To decipher the regulatory mechanism of -15 3′ ss recognition, a range of mutations between -15 and the authentic 3′ ss were performed (Fig. 4).

Predictions of mutations' influence on splice site strength according to MaxEntScan (Yeo and Burge, 2004), and their potential effect on splicing regulatory elements (SRE) according to SpliceAid2 (Piva et al., 2012) are summarized in Table 2. The results of the splicing minigene assay are presented as a ratio of each splicing variant's abundance (mutant/wild-type; Fig. 5; Supplementary Tab. S4).

At first, we introduced a -5tc mutation, supposed to disrupt two overlapping splicing enhancers and slightly strengthen the authentic 3′ ss, which led to -51 and -61 splicing variant upregulation. Exon inclusion and -15 variant representation did not significantly change compared to wild-type, suggesting that the 3′ ss strength is more important for the authentic and -15 3′ ss use than the presence of an enhancer.

-14aa was designed to disrupt the predicted enhancer binding T-cell-restricted intracellular antigen-1 (TIA-1)/TIA-1-related protein (TIAR) and the overlapping predicted silencer binding hnRNP-I, alongside weakening both the authentic and -15 3′ ss. The effect of this mutation was so strong that -51 and -61 ss use was markedly preferred. To distinguish between the influence of the enhancer and 3′ ss strength, -14cc mutation, supposed not to significantly influence the authentic and -15 ss strength, was tested. Except the +38 variant, the transcript profile was similar to -14aa, suggesting the role of enhancer disruption. Nevertheless, as the proportions of -51 and -61 variants were lower compared to -14aa mutation, the effect of the 3′ ss strength could also be implicated, despite the MaxEntScan predictions that might not cover the effect of polypyrimidine sequence change (thymidines are preferred to cytidines) in this specific case (Coolidge et al., 1997).

To evaluate the role of the authentic 3′ ss sequence through its incremental strength, a -9tt mutation was designed. Although the -15, -51 and -61 variants were downregulated, the proportion of the exon inclusion variant did not differ from the wild-type variant. These results suggest that the authentic ss strength either enhances its use only to a certain extent, or there is another factor implicated, for example a predicted splicing regulatory element binding Sam68, possibly acting as a silencer in this case (Lin et al., 1997; Pedrotti et al., 2010).

Regarding -9tt-14aa mutation, -9tt, strengthening the authentic 3′ ss and weakening the -15 3′ ss should compensate for splicing enhancer disruption caused by -14aa. According to the results, the compensation seems to be successful – it positively affected both the authentic and -15 ss usage, further indicating that the authentic ss strength is more important for the use of both investigated ss than the presence of the enhancer.

The last mutation, -16_-15 in..g, was designed to evaluate the role of -15 ss strength, predicted to be increased by this mutation. Although the authentic 3′ ss was weakened, according to predictions the change of the authentic 3′ ss MaxEnt score was negligible compared to the -15 3′ ss score change. This mutation preserved the splicing enhancer binding TIA-1/TIAR sequence, but it also created an overlapping splicing silencer binding, ZRANB2. Exon inclusion and also surprisingly the -15 variants were downregulated, whereas the +38 and -61 variants were upregulated. The result (together with the -14aa result, where the -15 ss was weakened) indicated that -15 ss use was not dependent on its strength. Both exon inclusion and -15 variants' downregulation suggest a possible role for the created silencer binding ZRANB2 (Loughlin et al., 2009).

Altogether, the results of mutagenesis analysis suggest that -15 3′ ss use is not dependent on its strength and is very sensitive to any changes in the surrounding sequence. It seems to be co-regulated with the authentic 3′ ss, the use of which is conversely dependent mainly on its strength, and less on the presence of the enhancer predicted to bind TIA-1 or TIAR. TIA-1/TIAR binding examination should be a subject of future experiments. TIA-1 is known to bind U-rich motifs downstream of 5′ ss, interact with U1C protein, stabilize U1 snRNP recruitment and thus facilitate 5′ ss recognition (Del Gatto-Konczak et al., 2000; Förch et al., 2002, 2000). Regarding the role of TIA-1 binding to the SRE in 3′ ss recognition, current knowledge is limited. The recent study investigating EPB41 exon 16 described TIA-1 (but not TIAR) binding to

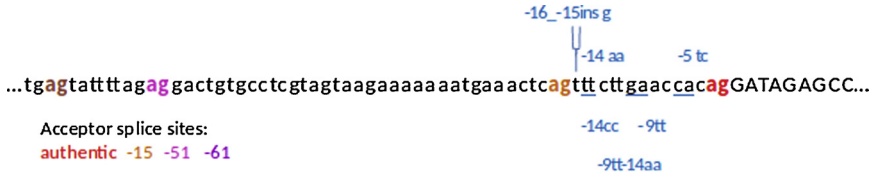

Fig. 4. Scheme of mutations performed between -15 and the main 3′ ss (upper cases – exon, lower cases – intron, bold "ag" – 3′ ss, mutated nt underlined, ins – insertion). All used 3′ss are depicted.

Table 2
Predictions of acceptor splice site (ss) strength (MaxEntScan) and splicing regulatory elements (SpliceAid2) in mutants (m) compared to wild-type (wt). (ISE – intronic splicing enhancers (underlined), ISS – intronic splicing silencers (in italics), = – potential change cannot be evaluated by MaxEntScan).

mutation	authentic 3' ss strength	−15 3' ss strength	ISE and ISS wt/m
−5tc	4.95 > 5.23	=	SF2, SRp30c/ -
−14aa	4.95 > 2.42	−4.08 > -5.38	TIA-1, *hnRNPI*/ -
−14cc	4.95 > 4.83	−4.08 > -4.24	TIA-1, *hnRNPI*/ *hnRNPK*
−9tt	4.95 > 7.1	=	SF2/ TIA-1, *Sam68*
−9tt-14aa	4.95 > 5.38	−4.08 > -5.38	TIA-1, SF2, *hnRNPI*/ TIA-1, *Sam68*
−16_-15 in..g	4.95 > 4.45	−4.08 > -2.19	TIA-1/ TIA-1, *ZRANB2*

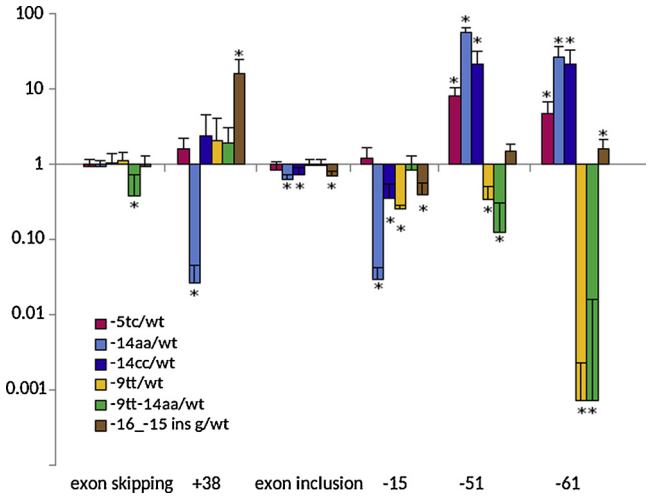

Fig. 5. The influence of chosen mutations on splicing pattern. Abundances of particular transcripts are expressed as fold changes compared to wild-type. The values are depicted on a logarithmic scale (wt – wild-type, ins. – insertion). Error bars represent SD. *significant results according to t-test; $p < 0.05$.

the intronic splicing enhancer situated at bp -15 to -24 upstream of the 3′ ss, and thus promoting U2 snRNP binding to a weak branch point. This requires interaction with PCBP-1 and RBM39 and a following association with U2AF65 and SF3B155 proteins (Huang et al., 2017). As there is no poly-C motif adjacent to the TIA-1 binding site, co-operation with PCBP-1 cannot be expected in our case and a different mechanism might be exerted.

All these results show that *SERPING1* exon 3 splicing is subtly regulated, and very small changes in acceptor splice site sequence, exonic sequence (de la Cruz et al., 2012; Grodecká et al., 2017), or splicing factor effects can shift the balance of all splicing isoforms and subsequently functional C1INH production. As HAE I patients typically display C1INH plasma levels lower than expected 50% (Pappalardo et al., 2004), a mechanism downregulating wild type *SERPING1* allele expression has to be involved, and RNA splicing may participate in this regulation. We hypothesize that monocytes and macrophages, as the principal C1INH producing blood cells (Yeung Laiwah et al., 1985), can play an important role in this process. *SERPING1* expression can be stimulated by interferon-gamma (Lappin et al., 1992), an agent that forces the polarization of macrophages to M1 cells (Mantovani et al., 2004). Concurrently, these stimuli can change the splicing pattern (de Baey et al., 1997; Herová et al., 2015), which could also affect functional *SERPING1* expression. In our current work (Grymová et al., submitted manuscript), we showed that a certain proportion of HAE patients display a shifted expression pattern in some inflammation-related genes in neutrophils. If monocytes/macrophages behave in a similar manner, the heterogeneous exon 3 skipping variant abundance (de la Cruz et al., 2012) can arise from the "activation state" of these cells. It is not very well known how *SERPING1* expression regulation and subsequent monocyte C1INH production contributes to edema episode outbreaks. In subsequent studies, HAE severity should be correlated not only with current C1INH plasma levels, but also with monocyte activation state and *SERPING1* splicing patterns.

4. Conclusions

The study presents a comprehensive description of *SERPING1* exon 3 splicing variants with a focus on two variants using alternative acceptor splice sites: exon skipping variant and -15 variant. A frameshift exon 3 skipping variant was surprisingly shown not to be degraded by NMD and to represent nearly one third of all transcripts, suggesting its biological importance. An in-frame -15 variant uses an extremely weak 3′ ss, which is probably recognized in a non-classical manner and co-regulated with the authentic one. To our knowledge, such co-regulation between close acceptor splice sites has not yet been described. A transcript arising from an in-frame -15 splicing variant presumably leads to the production of protein prolonged by six AA (QDRASS) at its N-terminus, providing two potential glycosylation sites. It is present in small amounts and its function is unclear. Further studies are needed to better understand *SERPING1* splicing regulation and alternative transcripts' significance.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.01.007.

References

Andrejević, S., Korošec, P., Šilar, M., Košnik, M., Mijanović, R., Bonači-Nikolić, B., Rijavec, M., 2015. Hereditary angioedema due to C1 inhibitor deficiency in Serbia: two novel mutations and evidence of genotype-phenotype association. PLoS One 10, e0142174.

Bafunno, V., Bova, M., Loffredo, S., Divella, C., Petraroli, A., Marone, G., Montinaro, V., Margaglione, M., Triggiani, M., 2014. Mutational spectrum of the c1 inhibitor gene in a cohort of Italian patients with hereditary angioedema: description of nine novel mutations. Ann. Hum. Genet. 78, 73–82.

Behm-Ansmant, I., Kashima, I., Rehwinkel, J., Saulière, J., Wittkopp, N., Izaurralde, E., 2007. mRNA quality control: an ancient machinery recognizes and degrades mRNAs with nonsense codons. FEBS Lett. 581, 2845–2853.

Beinrohr, L., Harmat, V., Dobó, J., Lörincz, Z., Gál, P., Závodszky, P., 2007. C1 inhibitor serpin domain structure reveals the likely mechanism of heparin potentiation and conformational disease. J. Biol. Chem. 282, 21100–21109.

Bock, S.C., Skriver, K., Nielsen, E., Thøgersen, H.C., Wiman, B., Donaldson, V.H., Eddy, R.L., Marrinan, J., Radziejewska, E., Huber, R., 1986. Human C1 inhibitor: primary structure, cDNA cloning, and chromosomal localization. Biochemistry 25, 4292–4301.

Bos, I.G.A., Hack, C.E., Abrahams, J.P., 2002. Structural and functional aspects of C1-inhibitor. Immunobiology 205, 518–533.

Bowen, B., Hawk, J.J., Sibunka, S., Hovick, S., Weiler, J.M., 2001. A review of the reported defects in the human C1 esterase inhibitor gene producing hereditary angioedema including four new mutations. Clin. Immunol. 98, 157–163.

Cai, S., Davis, A.E., 2003. Complement regulatory protein C1 inhibitor binds to selectins and interferes with endothelial-leukocyte adhesion. J. Immunol. 171, 4786–4791.

Carter, P.E., Duponchel, C., Tosi, M., Fothergill, J.E., 1991. Complete nucleotide sequence of the gene for human C1 inhibitor with an unusually high density of Alu elements. Eur. J. Biochem. 197, 301–308.

Chang, Y.-F., Imam, J.S., Wilkinson, M.F., 2007. The nonsense-mediated decay RNA surveillance pathway. Annu. Rev. Biochem. 76, 51–74.

Colobran, R., Pujol-Borrell, R., Hernández-González, M., Guilarte, M., 2014. A novel splice site mutation in the SERPING1 gene leads to haploinsufficiency by complete degradation of the mutant allele mRNA in a case of familial hereditary angioedema. J. Clin. Immunol. 34, 521–523.

Coolidge, C.J., Seely, R.J., Patton, J.G., 1997. Functional analysis of the polypyrimidine tract in pre-mRNA splicing. Nucleic Acids Res. 25, 888–896.

Coutinho, M., Aulak, K.S., Davis, A.E., 1994. Functional analysis of the serpin domain of C1 inhibitor. J. Immunol. 153, 3648–3654.

Cugno, M., Zanichelli, A., Foieni, F., Caccia, S., Cicardi, M., 2009. C1-inhibitor deficiency and angioedema: molecular mechanisms and clinical progress. Trends Mol. Med. 15, 69–78.

Cumming, S.-A., Halsall, D.J., Ewan, P.W., Lomas, D.A., 2003. The effect of sequence variations within the coding region of the C1 inhibitor gene on disease expression and protein function in families with hereditary angio-oedema. J. Med. Genet. 40, e114.

de Baey, A., Fellerhoff, B., Maier, S., Martinozzi, S., Weidle, U., Weiss, E.H., 1997. Complex expression pattern of the TNF region gene LST1 through differential regulation, initiation, and alternative splicing. Genomics 45, 591–600.

de la Cruz, R.M., López-Lera, A., López-Trascasa, M., 2012. Analysis of SERPING1 expression on hereditary angioedema patients: quantitative analysis of full-length and exon 3 splicing variants. Immunol. Lett. 141, 158–164.

Del Gatto-Konczak, F., Bourgeois, C.F., Le Guiner, C., Kister, L., Gesnel, M.C., Stévenin, J., Breathnach, R., 2000. The RNA-binding protein TIA-1 is a novel mammalian splicing regulator acting through intron sequences adjacent to a 5' splice site. Mol. Cell. Biol. 20, 6287–6299.

Dobó, J., Major, B., Kékesi, K.A., Szabó, I., Megyeri, M., Hajela, K., Juhász, G., Závodszky, P., Gál, P., 2011. Cleavage of kininogen and subsequent bradykinin release by the complement component: mannose-binding lectin-associated serine protease (MASP)-1. PLoS One 6, e20036.

Duponchel, C., Djenouhat, K., Frémeaux-Bacchi, V., Monnier, N., Drouet, C., Tosi, M., 2006. Functional analysis of splicing mutations and of an exon 2 polymorphic variant of SERPING1/C1NH. Hum. Mutat. 27, 295–296.

Förch, P., Puig, O., Kedersha, N., Martínez, C., Granneman, S., Séraphin, B., Anderson, P., Valcárcel, J., 2000. The apoptosis-promoting factor TIA-1 is a regulator of alternative pre-mRNA splicing. Mol. Cell 6, 1089–1098.

Förch, P., Puig, O., Martínez, C., Séraphin, B., Valcárcel, J., 2002. The splicing regulator TIA-1 interacts with U1-C to promote U1 snRNP recruitment to 5' splice sites. EMBO J. 21, 6882–6892.

Grodecká, L., Hujová, P., Kramárek, M., Kršjaková, T., Kováčová, T., Vondrášková, K., Ravčuková, B., Hrnčířová, K., Souček, P., Freiberger, T., 2017. Systematic analysis of splicing defects in selected primary immunodeficiencies-related genes. Clin. Immunol. 180, 33–44.

Hansen, C.B., Csuka, D., Munthe-Fog, L., Varga, L., Farkas, H., Hansen, K.M., Koch, C., Skjødt, K., Garred, P., Skjoedt, M.-O., 2015. The levels of the lectin pathway serine protease MASP-1 and its complex formation with C1 inhibitor are linked to the severity of hereditary angioedema. J. Immunol. 195, 3596–3604.

Herová, M., Schmid, M., Gemperle, C., Hersberger, M., 2015. ChemR23, the receptor for Chemerin and resolvin E1, is expressed and functional on M1 but not on M2 macrophages. J. Immunol. 194, 2330–2337.

Huang, S.-C., Zhang, H.S., Yu, B., McMahon, E., Nguyen, D.T., Yu, F.H., Ou, A.C., Ou, J.P., Benz, E.J., 2017. Protein 4.1R Exon 16 3' splice site activation requires coordination among TIA1, Pcbp1, and RBM39 during terminal erythropoiesis. Mol. Cell. Biol. 37.

Jiang, H., Wagner, E., Zhang, H., Frank, M.M., 2001. Complement 1 inhibitor is a regulator of the alternative complement pathway. J. Exp. Med. 194, 1609–1616.

Joseph, K., Tuscano, T.B., Kaplan, A.P., 2008. Studies of the mechanisms of bradykinin generation in hereditary angioedema plasma. Ann. Allergy Asthma Immunol. 101, 279–286.

Kozak, M., 1999. Initiation of translation in prokaryotes and eukaryotes. Gene 234, 187–208.

Lappin, D.F., Guc, D., Hill, A., McShane, T., Whaley, K., 1992. Effect of interferon-gamma on complement gene expression in different cell types. Biochem. J. 281 (Pt 2), 437–442.

Lin, Q., Taylor, S.J., Shalloway, D., 1997. Specificity and determinants of Sam68 RNA binding. Implications for the biological function of K homology domains. J. Biol. Chem. 272, 27274–27280.

Loughlin, F.E., Mansfield, R.E., Vaz, P.M., McGrath, A.P., Setiyaputra, S., Gamsjaeger, R., Chen, E.S., Morris, B.J., Guss, J.M., Mackay, J.P., 2009. The zinc fingers of the SR-like protein ZRANB2 are single-stranded RNA-binding domains that recognize 5' splice site-like sequences. Proc. Natl. Acad. Sci. U. S. A. 106, 5581–5586.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M., 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25, 677–686.

Nussberger, J., Cugno, M., Amstutz, C., Cicardi, M., Pellacani, A., Agostoni, A., 1998. Plasma bradykinin in angio-oedema. Lancet 351, 1693–1697.

Odermatt, E., Berger, H., Sano, Y., 1981. Size and shape of human C1-inhibitor. FEBS Lett. 131, 283–285.

Pappalardo, E., Zingale, L.C., Cicardi, M., 2004. C1 inhibitor gene expression in patients with hereditary angioedema: quantitative evaluation by means of real-time RT-PCR. J. Allergy Clin. Immunol. 114, 638–644.

Pedersen, A.G., Nielsen, H., 1997. Neural network prediction of translation initiation sites in eukaryotes: perspectives for EST and genome analysis. Proc. Int. Conf. Intell. Syst. Mol. Biol. 5, 226–233.

Pedrotti, S., Bielli, P., Paronetto, M.P., Ciccosanti, F., Fimia, G.M., Stamm, S., Manley, J.L., Sette, C., 2010. The splicing regulator Sam68 binds to a novel exonic splicing silencer and functions in SMN2 alternative splicing in spinal muscular atrophy. EMBO J. 29, 1235–1247.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786.

Piva, F., Giulietti, M., Burini, A.B., Principato, G., 2012. SpliceAid 2: a database of human splicing factors expression data and RNA target motifs. Hum. Mutat. 33, 81–85.

Pixley, R.A., Schapira, M., Colman, R.W., 1985. The regulation of human factor XIIa by plasma proteinase inhibitors. J. Biol. Chem. 260, 1723–1729.

Roche, O., Blanch, A., Duponchel, C., Fontán, G., Tosi, M., López-Trascasa, M., 2005. Hereditary angioedema: the mutation spectrum of SERPING1/C1NH in a large Spanish cohort. Hum. Mutat. 26, 135–144.

Rosen, F.S., Alper, C.A., Pensky, J., Klemperer, M.R., Donaldson, V.H., 1971. Genetically determined heterogeneity of the C1 esterase inhibitor in patients with hereditary angioneurotic edema. J. Clin. Invest. 50, 2143–2149.

Schapira, M., Scott, C.F., Colman, R.W., 1982. Contribution of plasma protease inhibitors to the inactivation of kallikrein in plasma. J. Clin. Invest. 69, 462–468.

Sim, R.B., Porter, R.R., 1976. Isolation and comparison of the proenzyme and activated forms of the human serum complement subcomponents C1r and C1s. Biochem. Soc. Trans. 4, 127–129.

Van den Steen, P., Rudd, P.M., Dwek, R.A., Opdenakker, G., 1998. Concepts and principles of O-linked glycosylation. Crit. Rev. Biochem. Mol. Biol. 33, 151–208.

van der Graaf, F., Koedam, J.A., Bouma, B.N., 1983. Inactivation of kallikrein in human plasma. J. Clin. Invest. 71, 149–158.

Wuillemin, W.A., Minnema, M., Meijers, J.C., Roem, D., Eerenberg, A.J., Nuijens, J.H., ten Cate, H., Hack, C.E., 1995. Inactivation of factor XIa in human plasma assessed by measuring factor XIa-protease inhibitor complexes: major role for C1-inhibitor. Blood 85, 1517–1526.

Yeo, G., Burge, C.B., 2004. Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. J. Comput. Biol. 11, 377–394.

Yeung Laiwah, A.C., Jones, L., Hamilton, A.O., Whaley, K., 1985. Complement-subcomponent-C1-inhibitor synthesis by human monocytes. Biochem. J. 226, 199–205.