



The somatic *FAH* C.1061C>A change counteracts the frequent *FAH* c.1062+5G>A mutation and permits U1snRNA-based splicing correction

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ABSTRACT

In tyrosinaemia type 1 (HT1), a mosaic pattern of fumarylacetoacetase (*FAH*) immunopositive or immunonegative nodules in liver tissue has been reported in many patients. This aspect is generally explained by a spontaneous reversion of the mutation into a normal genotype. In one HT1 patient carrying the frequent *FAH* c.1062+5G>A mutation, a second somatic change (c.1061C>A) has been reported in the same allele, and found in immunopositive nodules. Here, we demonstrated that the c.1062+5G>A prevents usage of the exon 12 5' splice site (ss), even when forced by an engineered U1snRNA specifically designed on the *FAH* 5'ss to strengthen its recognition. Noticeably the new somatic c.1061C>A change, in linkage with the c.1062+5G>A mutation, partially rescues the defective 5'ss and is associated to trace level (~5%) of correct transcripts. Interestingly, this combined genetic condition strongly favored the rescue by the engineered U1snRNA, with correct transcripts reaching up to 60%. Altogether, these findings elucidate the molecular basis of HT1 caused by the frequent *FAH* c.1062+5G>A mutation, and demonstrate the compensatory effect of the c.1061C>A change in promoting exon definition, thus unraveling a rare mechanism leading to *FAH* immune-reactive mosaicism.

Introduction

Hereditary tyrosinemia type I (HT1) (OMIM 276700) is an autosomal recessive disorder caused by genetic defects in the fumarylacetoacetate hydrolase (*FAH*), the last enzyme in the catabolic pathway of tyrosine [1]. The accumulation of toxic metabolites in liver is believed to cause liver failure, cirrhosis, hepatocellular carcinoma, and death.

Several patients (28% and up to 100% in the Saguenay-Lac-Saint-Jean region of Quebec) [2] carry the *FAH* c.1062+5G>A mutation at the exon 12 5' splice site (ss). Investigations in patients [3] and with minigenes [4] demonstrated that this change induces exon 12 and exons 12-13 skipping as well as partial intron 12 inclusion with usage of an

intronic cryptic 5'ss, thus explaining the *FAH* immune-negativity of liver sections from homozygotes. Differently, the presence of the new c.1061C>A (c.P354Q) somatic change with the c.1062+5G>A mutation was found to be associated with hepatic *FAH* immune-positivity [5]. Here we demonstrated the compensatory effect of the c.1061C>A change in promoting *FAH* immune-positivity, which also renders the defective *FAH* 5'ss highly responsive to correction by a compensatory U1snRNA.

Material and methods

To create the pFAHwt minigenes, the 804-bp genomic fragment spanning *FAH* intron 11 (from position c.960-357) through intron 12 (until position c.1062+327) was amplified from genomic DNA of a normal subject using high-fidelity PfuI DNA-Polymerase (Transgenomic, Glasgow, UK) with primers 5'-CATATGGACTGGAGGGTGTCCCA-3' (forward) and 5'-CATATGCCACCTCATCCTGGGAGGGT-3' (reverse), and cloned in the pTB expression vector by exploiting the *NdeI* restriction site within primers (underlined) [6]. The mutant pFAH constructs were generated by site-directed mutagenesis (QuickChange II XL Site-directed

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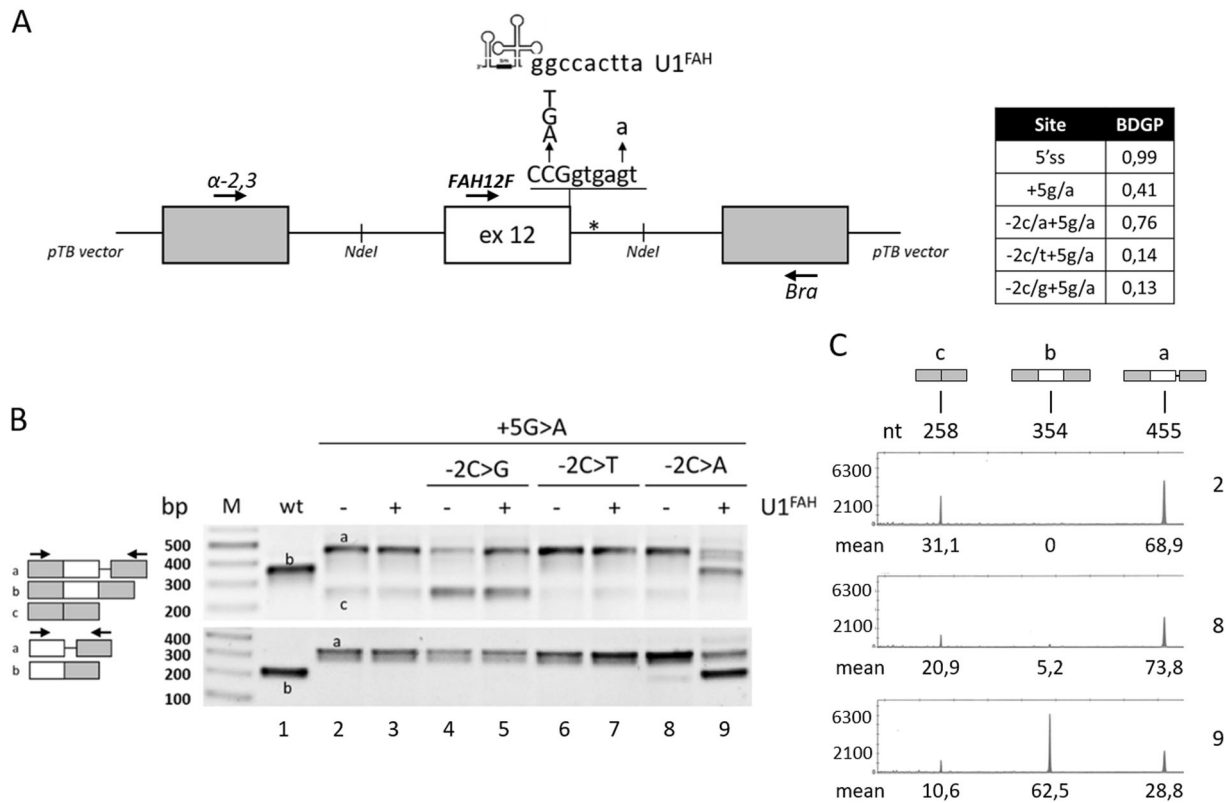


Fig. 1 Splicing patterns of FAH minigenes in HepG2. **a** Schematic representation of the FAH minigene, cloned within the *NdeI* sites (indicated) in the pTB vector, with the sequence of the exon 12 5'ss and of the investigated changes as well as of the 5' tail of the compensatory U1^{FAH}. The asterisk indicates the cryptic 5'ss. The table reports the predicted score of the wild type or mutated 5'ss (http://www.fruitfly.org/seq_tools/splice.html). **b** FAH splicing patterns in HepG2 cells transiently transfected with pFAH minigenes (1 μ g) alone or with a molar excess (1.5 \times) of pU1FAH. RT-PCR was

conducted with primers α -2,3 and Bra (upper panel) or primers FAH12F and Bra (lower panel), and amplicons were separated on 2% agarose gel. The scheme of amplicons is reported on the left together with primers used (arrows). **c** FAH splicing patterns as in the upper panel of section b (lanes 2, 8, and 9) evaluated by fluorescently labeled RT-PCR with primers α -2,3 and Bra^{FAM}, followed by denaturing capillary electrophoresis. The scheme of amplicons and the length (nt) are reported on top. Numbers below peaks indicate the relative amount (%; mean from three independent experiments) of each transcript

Mutagenesis Kit; Agilent Technologies, Santa Clara, CA, USA) using primers 5'-CATCAGCGGGCCGGTGAA-TATCTGGCTGCACTGAG-3' and 5'-CTCAGTGCAGC-CAGATATTCACCGGCCCGCTGATG-3', to introduce the c.1062+5G>A change, and primers 5'-CCAT-CAGCGGGCDGGTGAGTATCTG-3' and 5'-CAGAT-TACTCACCHGCCCGCTGATGG-3', to introduce the c.1061C>A/T/G changes. The pU1^{FAH} expression vector was created as previously described [7].

HepG2 cells were transfected with Lipofectamine 2000 (ThermoFisher SCIENTIFIC, Carlsbad, CA, USA) in 12-well plate with pFAH minigenes (1 μ g) alone or with a molar excess (1.5 \times) of pU1FAH. Total RNA extraction was performed 24 h post-transfection using TRIreagent (ThermoFisher SCIENTIFIC) and reverse transcribed using the M-MLV (ThermoFisher SCIENTIFIC). The primer couples α -2,3 (5'-CAACTTCAAGCTCCTAA GCCA CTGC-3') and Bra (5'-TAGGATCCGGTCCAC-CAGGAAGTTGGTTAAATCA-3') in the neighboring exons of the hybrid minigene, or FAH12F in exon 12

(5'-ACATGTACTGGACG ATGCTGCA-3') and Bra, indicated by arrows in Fig. 1, were used for the RT-PCR that was run for 40 cycles at the following conditions: 95 $^{\circ}$ C for 30 s, 56 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 40 s. The densitometric analysis of bands was performed using ImageJ software.

For the denaturing capillary electrophoresis [8] on the automated ABI-3100 instrument, the RT-PCR has been performed with primer α -2,3 and Bra, the latter labeled with FAM dye (Bra^{FAM}).

Results and discussion

To dissect the mechanisms triggered by the HT1-causing FAH c.1062+5G>A mutation alone or in combination with the somatic FAH c.1061C>A change, we performed expression studies with FAH minigenes (Fig. 1a). As the splicing process is cell specific, the experiments were conducted into human hepatoma cell lines (HepG2), being liver the major physiologic site of FAH synthesis.

Consistently with previous data [3–5], the c.1062+5G>A mutation, as revealed by both densitometric analysis of bands or fluorescent RT-PCR followed by denaturing capillary electrophoresis, induced exon 12 skipping (~31% of total transcripts) and partial intron retention (~69%), with no traces of correct transcripts (Fig. 1b, upper panel and Fig. 1c), a splicing profile that validated our experimental approach. Interestingly, the introduction of the c.1061C>A substitution in the c.1062+5G>A background partially rescued the defective 5' splice site recognition and led to residual levels of correct transcripts ($5.2 \pm 0.9\%$) (Fig. 1c), which was further demonstrated by a RT-PCR focused on exon 12 (Fig. 1b, lower panel).

Computational analysis of 5' splice site scores (Fig. 1a, inset table), an estimate of the complementarity between the 5' splice site sequence and the 5' tail of the key spliceosomal U1 small nuclear RNA (U1snRNA) [9], predicts that the c.1061C>A change, but not the other c.1061C>T or c.1061C>G substitutions, strengthen the affected 5' splice site. This would favor the utilization of the mutated 5' splice site and the production of correct transcripts. Consistently, the impact of the other changes (c.1061C>T or c.1061C>G) on the splicing profile of the c.1062 + 5G>A mutation was negligible (Fig. 1b).

Altogether these observations demonstrated that in the c.1062+5G>A background the c.1061C>A somatic change accounts for trace levels of correct transcripts. In turn, this would explain the residual FAH protein expression and immune-positivity in HT1 liver nodules with the above mentioned genetic profile, thus providing an additional mechanism leading to FAH immune-reactive mosaicism. It is worth noting that the FAH immune-reactive mosaicism has been so far attributed to the reversion of the mutated allele into the wild type [10], as also reported in other diseases [11]. Both mechanisms would be favored by the high mutation rate of HT1 hepatocytes, [11] followed by positive selection of FAH-expressing hepatocytes, which would lead to the formation of FAH-immunopositive nodules.

Splicing mutations, relatively frequent in metabolic disorders (<http://www.hgmd.cf.ac.uk>) as well as in others human diseases [12], represent potential targets for RNA-based therapies. Several studies have indicated that splicing mutations can be counteracted by engineered U1snRNAs with increased complementarity with the 5' splice site of the defective exon [13–15]. We therefore created a U1snRNA variant specifically designed on the FAH exon 12 5' splice site (U1^{FAH}). However, in co-expression experiment, the compensatory U1^{FAH} was ineffective on the c.1062+5G>A mutation, which demonstrated the severe impairment of the 5' splice site recognition. Notably, and consistently with a slightly improved 5' splice site, the combination of the HT1-causative mutation with the c.1061C>A change resulted in remarkable responsiveness to the U1^{FAH} that promoted exon 12

inclusion, as witnessed by the robust synthesis of correct transcripts ($62.5 \pm 1.2\%$) (Fig. 1b, c).

Conclusion

Altogether these findings elucidate the molecular basis of HT1 caused by the frequent FAH c.1062+5G>A mutation, and demonstrate the compensatory effect of the c.1061C>A change in promoting exon definition, thus unraveling a rare mechanism leading to FAH immune-reactive mosaicism.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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