Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1772 (2007) 96–102
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbadis

The *ITPA* c.94C>A and g.IVS2+21A>C sequence variants contribute to missplicing of the *ITPA* gene

Monica Arenas [a], John Duley [a,d], Satoshi Sumi [a,c], Jeremy Sanderson [b], Anthony Marinaki [a,*]

[a] *Purine Research Laboratory, Department of Chemical Pathology, Guy's Hospital, London, SE1 9RT, UK*
[b] *Department of Gastroenterology, Guy's and St. Thomas' Hospitals*
[c] *Department of Paediatrics, Neonatology and Congenital Disorders, Nagoya City University, Nagoya, Japan*
[d] *School of Pharmacy, University of Queensland, and Department of Pathology, Mater Hospital, Brisbane, Australia*

Received 8 August 2006; received in revised form 18 September 2006; accepted 2 October 2006
Available online 18 October 2006

Abstract

Inosine triphosphate pyrophosphatase (ITPase) catalyzes the conversion of inosine triphosphate (ITP) to the correspondent monophosphate. The *ITPA* c.94C>A and g.IVS2+21A>C allelic variants are associated with decreased red cell enzyme activity. The *ITPA* c.94C>A [P32T] sequence variant is associated with an increased risk of adverse drug reactions in patients treated with the thiopurine drug azathioprine. The aim of this study was to explore the molecular mechanisms of ITPase deficiency. *ITPA* mRNA was extracted from peripheral blood leukocytes (PBL), Epstein–Barr virus transformed lymphoblast cell cultures, reticulocytes, and cultured fibroblast from patients with known *ITPA* genotypes. *ITPA* mRNA was reversed transcribed, sequenced and the relative amounts of misspliced transcripts quantitated from three independent experiments. The *ITPA* g.IVS2+21A>C sequence variant resulted in missplicing of exon 3. The *ITPA* c.94C>A allelic variant resulted in missplicing of exons 2 and 3 representing, in PBL samples, 61% of the total mRNA expressed in *ITPA* c.94C>A homozygotes. We proposed that the *ITPA* c.94C>A allelic variant destroys an exonic splicing silencing (ESS) element in exon 2, resulting in the activation of two nearby upstream 5′ splice sites and missplicing of the exons 2 and 3 cassette causing structural changes to the enzyme and contributing to ITPase deficiency.

Keywords: *ITPA*; ITPase, Inosine triphosphate pyrophosphatase; Azathioprine; ADR, Adverse drug reaction; Pharmacogenetic; Cryptic 5′ splice site; Missplicing

1. Introduction

The cytosolic enzyme ITPase (EC 3.6.1.19) catalyses the hydrolysis of ITP to inosine monophosphate (IMP). ITPase deficiency is a benign red cell enzymopathy marked by the accumulation of ITP in red cells [1]. ITPase is ubiquitously expressed although activity differs according to cell type [2]. Its function is not clearly understood but possible roles for ITPase could be to prevent the accumulation of rogue nucleotides [3] which would be otherwise incorporated into DNA and RNA, or compete with nucleotides such as GTP in signalling processes. ITPase might also regulate intracellular ATP pools [4] through the IMP intermediate.

ITPase deficiency is polymorphic. An early population study showed that ~5% of a normal population have decreased ITPase activity resulting in a bimodal activity distribution [5]. Accumulation of high levels of endogenous ITP, in the red cells of completely ITPase deficient individuals, was first demonstrated by Vanderheiden in 1964 [6].

The *ITPA* gene, GenBank accession number AF026816, is located on the short arm of chromosome 20 (20p13) [7]. Lin et al. [8] isolated a cDNA clone encoding human ITPase, and subsequently, Sumi et al. [9] determined the genomic structure of the *ITPA* gene which consists of eight exons and is ~13 kb long. In a Caucasian population, two allelic variants in the *ITPA* gene were associated with decreased ITPase enzyme activity, *ITPA* c.94C>A [P32T] in exon 2, and *ITPA* g.IVS2+21A>C [9]. Homozygotes for the *ITPA* c.94C>A mutation had zero red cell enzyme activity, whereas enzyme activity in heterozygotes was 22.5% of the control

* Corresponding author. Tel.: +44 207 188 1265; fax: +44 207 188 1280.
E-mail address: tony.marinaki@kcl.ac.uk (A. Marinaki).

0925-4439/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2006.10.006

mean, a level consistent with impaired subunit assembly of a dimeric enzyme. Patients heterozygous for the *ITPA* g.IVS2+21A>C allelic variant had ITPase enzyme activity which averaged 60% of the control mean, while compound heterozygotes for the *ITPA* c.94C>A and g.IVS2+21 A>C allelic variants had 10% residual activity. Recently, two studies have identified different mutations at the same position in intron two of the *ITPA* gene, g.IVS2+68T>G [10] in a Japanese patient with decreased enzyme activity, and g.IVS2+68T>C [11].

Although ITPase deficiency has not been associated with any disease or clinical condition, it was hypothesized that deleterious effects might be observed only under certain conditions of metabolic stress. It was also suggested that ITPase deficient patients treated with purine drug analogues such as thiopurines, would be at risk of suffering from side effects [1]. ITPase has a broad substrate affinity for both purines (ITP, dITP, XTP) and pyrimidines (UTP) [8]. This locus is therefore of particular interest from a pharmacogenetic point of view. In a retrospective study of inflammatory bowel disease (IBD) patients treated with azathioprine (AZA), we reported a significant association between the *ITPA* c.94C>A allelic variant and adverse drug reactions (ADR) such as flu-like symptoms, rash and pancreatitis [12]. Recently, a prospective clinical trial of IBD patients treated with AZA, showed that patients with the *ITPA* c.94C>A allelic variant were significantly more likely to be withdrawn from therapy due to ADR [13]. Another study has shown that AZA-related leucopenia observed in IBD patients on AZA was significantly associated with the *ITPA* c.94C>A allelic variant [14]. However, others studies have shown no association between variant *ITPA* genotypes and adverse reactions to AZA therapy [15,16].

The mechanism behind the loss of enzyme activity due to the *ITPA* c.94C>A allelic variant is not known. We previously proposed that the amino acid substitution P32T may affect enzyme function by impairing dimer formation between wild-type and variant subunits. It was also proposed that the *ITPA* g.IVS2+21A>C allelic variant altered a branch point resulting in abnormal mRNA splicing [9].

Pre-mRNA splicing is an essential step that leads to the removal of introns from RNA. Spliceosome assembly and splicing efficiency are guided by the presence of conserved sequences in the pre-mRNA, such as the 5′ and 3′ splice sites, and the branch site. The 5′ splice site (5′ss) conserved sequence is recognized by the U1 small nuclear (snRNA) 5′ terminus [17], guiding the first step in spliceosome assembly. In addition to these conserved sequences, introns and exons have sequence elements, exonic/intronic splicing enhancers (ESE/ISE) and exonic/intronic splicing silencers (ESS/ISS), which are binding sites for Serine Rich proteins (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP). The binding of these proteins will enhance or inhibit splicing.

At least 15% of disease-associated point mutations result in abnormal pre-mRNA splicing [18]. Furthermore, 74% of human genes have been reported to be alternatively spliced [19]. Alternative pre-mRNA splicing is a way for eukaryotic cells to regulate gene expression and generate a wide array of protein isoforms, affecting protein activity and localization in the cell [20].

The aim of this study was to further investigate the effect of *ITPA* gene allelic variants on ITPase mRNA splicing.

2. Materials and methods

Ethical approval was received from Guy's and St. Thomas' ethics committee. EDTA blood was drawn by veno-puncture after informed consent. In order to investigate *ITPA* mRNA missplicing, total RNA was rapidly extracted using Total RNA Isolation Reagent (Invitrogen Ltd., Paisley, UK) from peripheral blood leucocytes (PBL) (n=9), Epstein–Barr virus (EBV) transformed lymphoblasts (n=2), reticulocytes (n=2), and fibroblasts (n=2), with known *ITPA* genotypes (Table 1) and single strand cDNA synthesized using an oligo dT_{15} primer and Moloney murine leukaemia virus (MMLV) reverse transcriptase (Invitrogen Ltd., Paisley, UK). The *ITPA* cDNA coding region was amplified in two rounds of PCR using outer 34forward-CCAGCCGGAAGTTTTCTGTCAC and 814reverse-CAGACAGGCCGGTGAGGCTAC primers, and nested 49forward-CTGTCACTGGACGCCAAGGAG and 779reverse-AAAGGGGAAACCCTGAAGGAAG primers. Thermocycler conditions were 40× (94 °C 1 min, 54 °C 30 sec, 72 °C 1 min) and 30× (94 °C 1 min, 54 °C 30 sec, 72 °C 1 min) for outer and nested primers, respectively. PCR products were gel purified and sequenced on an ABI 377 Sequencer using the ABI PRISM®BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems, Foster City, USA). To quantitate *ITPA* mRNA misspliced transcripts, *ITPA* cDNA was amplified from the outer PCR product with 49forward and 386reverse-TGGTGGAGACCTTCAGGCTTTAAC-TET nested primers. Thermocycler conditions were as follows 30× (94 °C 1 min, 55 °C 30 s, 72 °C 45 s). Relative quantification of the amount of *ITPA* mRNA misspliced transcripts was carried out on an ABI 377 sequencer using GeneScan fragment analysis software v2.0.2. This technique allowed misspliced ITPA mRNA to be sized to 1 bp relative to a PRISM™-GeneScan-500 TAMRA internal standard (Applied Biosystems, Foster City, USA) as previously described [21], and quantified. The fluorescent label on the reverse primer allowed accurate quantitation of the cDNA transcripts by peak area as described and validated by others [22]. Duplicate determinations were carried out in three independent experiments. Peak areas for each *ITPA* cDNA transcript were then expressed as a percentage of the total *ITPA* transcripts. One way analysis of variance (ANOVA), Bonferroni, and Dunn's multiple comparison tests were used to test for significant differences between genotypes.

Table 1
The effect of different *ITPA* genotypes on mRNA transcript level in different cell types shown as a percentage of total *ITPA* mRNA expression

ITPA genotype c.94C>A/g.IVS2+21A>C	Cell type	Transcript Type 2 exons 2 and 3 out (%)	Transcript Type 3 exon 3 out (%)	Transcript Type 1 full length (%)
hom/wt	PBL	69%	0%	31%
hom/wt	PBL	56%	1%	43%
hom/wt	PBL	58%	0%	42%
het/wt	PBL	32%	1%	67%
het/het	PBL	44%	4%	52%
wt/hom	PBL	12%	16%	72%
wt/het	PBL	15%	5%	80%
wt/wt	PBL	25%	5%	70%
wt/wt	PBL	14%	4%	82%
wt/het	Reticulocytes	25%	5%	70%
wt/wt	Reticulocytes	8%	0%	92%
het/wt	Lymphoblasts	36%	1%	63%
het/wt	Lymphoblasts	41%	0%	59%
wt/wt	Fibroblasts	1%	2%	97%
wt/wt	Fibroblasts	1%	5%	94%

wt=wild-type, het=heterozygous, hom=homozygous, PBL=peripheral blood leukocytes.

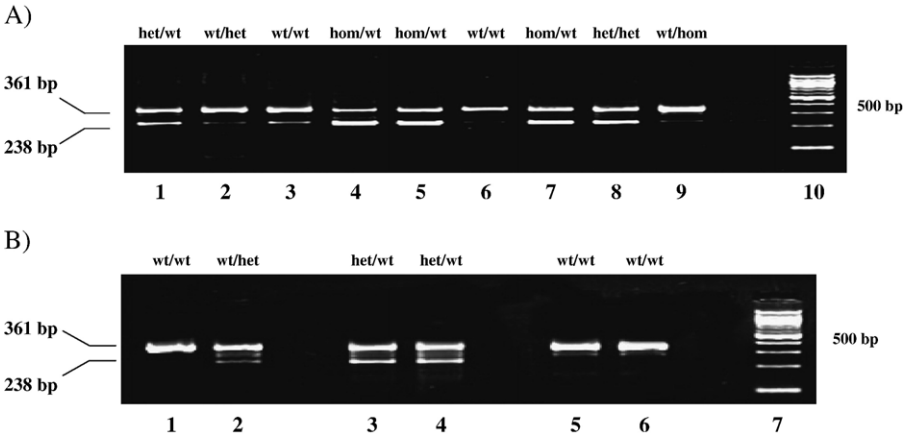

Fig. 1. (A) *ITPA* genotype and *ITPA* mRNA missplicing in peripheral blood leukocytes, (B) *ITPA* genotype and mRNA missplicing in fibroblasts, reticulocytes and cultured lymphoblasts. *ITPA* cDNA amplification gave fragments of 361 bp (full length mRNA transcript Type 1) and 238 bp (exons 2 and 3 spliced out, transcript Type 2) in length. The *ITPA* c.94C>A/g.IVS2+21A>C genotypes are shown above each lane. (A) Lanes 1–9: peripheral blood leukocytes. Lane 10: 100 bp DNA Ladder marker. (B) Lanes 1 and 2: reticulocytes. Lanes 3 and 4: cultured lymphoblasts. Lanes 5 and 6: fibroblasts. Lane 7: 100 bp DNA Ladder marker. Wt=wild-type, het=heterozygous, hom=homozygous.

The electronic databases http://rulai.cshl.edu/cgi-bin/tools/ESE, http://www.ebi.ac.uk/asd-srv/wb.cgi, and http://cubweb.biology.columbia.edu/pesx/ were used to search for exonic splicing enhancer/silencer (ESE/ESS) and intronic splicing enhancer/silencer (ISE/ISS) sequence motifs. http://www.ncbi.nlm.nih.gov/dbEST/ was used to search for alternative spliced forms of *ITPA* mRNA clones.

3. Results

ITPA cDNA from patients with various *ITPA* genotypes were analyzed (Table 1). PCR products resulting from the amplification of the full length *ITPA* mRNA with nested primers yielded two different fragments, a high molecular weight (HMW) band, which corresponded in size to the predicted *ITPA* mRNA full length transcript of 751 bp with exons 1–8, and an unexpected low molecular weight (LMW) band of ~600 bp. Both bands were gel purified and sequenced. The HMW band corresponded in sequence to the full length transcript (Type 1). Sequencing of the LMW band showed a cDNA transcript with a deletion of exons 2 and 3 (Type 2) (Fig. 2).

To quantify the relative amounts of *ITPA* Type 1 and Type 2 transcripts, the outer primer (34forward and 814reverse) PCR products, were amplified with the nested primers (49forward and 386reverse), with the reverse primer located in exon 4. Two bands were observed on the gel, a HMW band corresponding to the Type 1 transcript with a predicted size of 361 bp, and a LMW band corresponding to the Type 2 transcript with a predicted size of 238 bp. It was observed that the intensity of the band corresponding to the Type 2 transcript varied with genotype, with the highest proportion seen in *ITPA* c.94C>A homozygous genotypes (Fig. 1). However, low levels of the Type 2 transcript were also observed in wild-type PBL. The 386reverse primer was synthesized with a fluorescent label

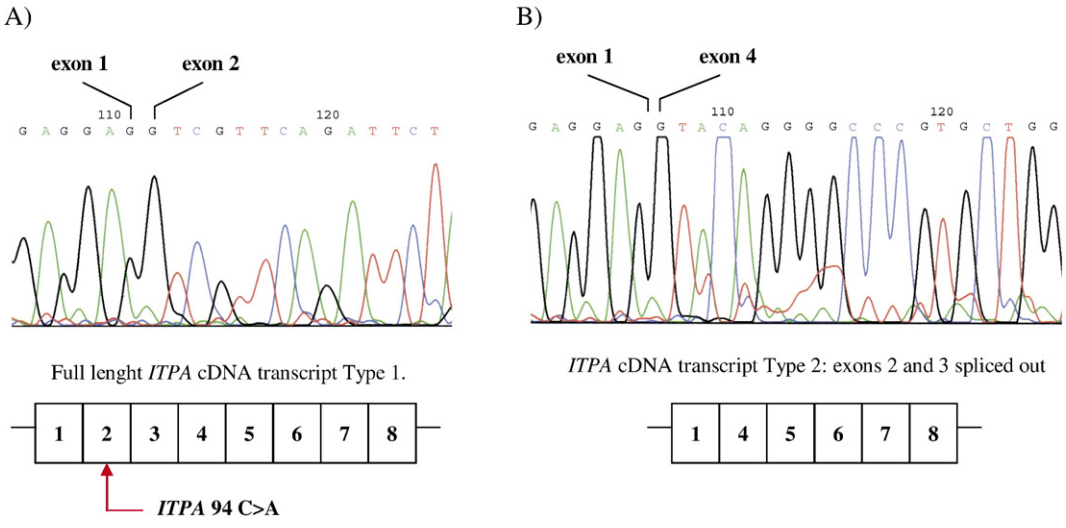

Fig. 2. Detail of *ITPA* cDNA sequences. (A) *ITPA* cDNA sequence of the high molecular weight band (361 bp) corresponding to the normally spliced *ITPA* mRNA transcript (Type 1). (B) *ITPA* cDNA sequence of the low molecular weight band (238 bp) corresponding to the ITPA mRNA transcript with exons 2 and 3 spliced out (Type 2).

allowing the relative amounts of misspliced *ITPA* mRNA to be detected and quantified. Three fragments were detected, a full length fragment (361 bp) and two other fragments of 238 bp and 296 bp. The 238 bp fragment corresponded in size to the Type 2 transcript with exons 2 and 3 spliced out, whereas the 296 bp fragment had the predicted size of a transcript lacking exon 3 only, and was designated a Type 3 transcript. The relative amounts of misspliced transcripts as a percentage of total *ITPA* transcription in various *ITPA* genotypes are summarized in Table 1.

The highest percentage of Type 2 transcripts was observed in *ITPA* c.94C>A homozygotes (Table 1). In PBL this represented on average 61% (61.3±8.2, mean±SD) of the total *ITPA* mRNA expressed, which was significantly different ($P<0.001$) from the 39% (39±7.9) observed in heterozygotes and the 17% (16.9±8.6) in wild-types ($P<0.001$). Both of the lymphoblast samples analyzed were heterozygous for the *ITPA* c.94C>A alteration with 38% of the misspliced Type 2 transcript, similar to the result obtained for PBL samples with a heterozygous genotype. Reticulocyte samples were wild-type for the *ITPA* c.94C>A mutation, and had 16% of the misspliced Type 2 transcript, a result in accordance with that obtained for the *ITPA* c.94 C>A wild-type homozygous genotype in PBL. Wild-type fibroblasts showed the most striking difference when compared to the other wild-type cell types, only 1% of misspliced Type 2 transcript was observed (Fig. 2).

The Type 3 transcript was present at low levels (1–5%) in all patients with a wild-type genotype for *ITPA* c.94C>A and g.IVS2+21A>C. The proportion of this transcript increased to 16% in one patient homozygous for the g.IVS2+21A>C mutation, which was significantly different from the wild-type genotype ($P<0.05$, Dunn's multiple comparison test), suggesting that this allelic variant causes missplicing of exon 3.

4. Discussion

At least 15% of human genetic diseases are due to mutations in consensus splice site sequences such as the 5′ss (C/A)AG|GURAGU, the 3′ splice site (3′ss) consensus sequence YAG|G, the branch point sequence, YNYURAY and mutations present in ESE/ISE, and ESS/ISS elements [18,20,23]. ESE/ESS and ISE/ISS are highly variable sequences in the pre-mRNA that enhance or reduce the efficiency of splicing. ESE and ESS sequences are binding sites for SR and hnRNP proteins, respectively, which are essential components of the splicing machinery. The balance between these elements determines the strength of exons and introns.

This study has shown that the putative branch site mutation *ITPA* g.IVS2+21A>C results in missplicing of exon 3 only. Furthermore, the results reported in this study show that although the *ITPA* c.94C>A alteration is located in the coding region of the *ITPA* gene, and results in the amino acid substitution P32T, the mutation also results in *ITPA* mRNA missplicing of exons 2 and 3. The *ITPA* Type 2 transcript (exons 2 and 3 spliced out) constituted 17% on average of total mRNA transcripts in *ITPA* c.94C>A wild-type PBL, levels increased to 39% for *ITPA* c.94C>A heterozygotes, and 61% for variant homozygotes.

It was postulated that the *ITPA* g.IVS2+21A>C mutation in intron 2 altered the conserved adenine of a putative branch site causing missplicing of exon 3 [9] resulting in 60% residual activity in the heterozygous genotype. A study reported by Heller et al. [24] concluded that the *ITPA* g.IVS2+21A>C allelic variant had a lower splicing efficiency compared to the wild-type allele confirming that missplicing was the molecular basis underlying the low enzyme activity associated with this mutation. Heller et al. argued that the long distance between the g.IVS2+21A>C mutation and the putative down stream splice acceptor site provided an unlikely mechanism for a branch point effect [9], and proposed instead that the mutation altered an extended conserved splice site consensus region next to the exon–intron junction. But, although branch points are normally located 20–40 nucleotides upstream of the 3′ ss, some studies have reported distances >150 nucleotides upstream. For example, the branch point of intron 2 of the alpha-tropomyosin gene is located at an unusual position 177 nucleotides upstream of the 3′ acceptor splice site, only 42 nucleotides from the exon 2 donor splice site [25]. It was also shown that splicing of exons 5 to 7 in the rat tropomyosin 1 gene was associated with the use of one of three adenine residues located at positions 144, 147 and 153 upstream of the 3′ splice acceptor site [26]. Therefore, the branch point does not exclusively need to be close to the 3′ splice acceptor site. Other putative branch sites are present along the intron. Consequently, it is likely that the *ITPA* g.IVS2+21A>C alteration destroys a preferred adenine branch point inhibiting lariat formation and intron 2 splicing.

How the *ITPA* c.94C>A mutation causes skipping of exons 2 and 3 was not clear. We studied the possibility that this mutation affects splicing by altering ESE or ESS sequence motifs recognized by SR or hnRNP proteins. This was analyzed using different motif finders available on the web. The first finder searched http://www.ebi.ac.uk/asd-srv/wb.cgi/, gives information about ESE/ESS, and ISE/ISS sequence elements. A search using the *ITPA* c.94C>A wild-type sequence showed that the mutation is located in an exonic enhancer element recognized by SC35 (AGTTT**C**CA). When the same search was performed with the variant sequence, the site was recognized by two SR proteins, SRp40 (T**A**CATG) and SRp55 (TT**A**CATG-CACTTT) which are also exonic splicing enhancers. The second finder, http://rulai.cshl.edu/cgi-bin/tools/ESE/esefinder.cgi/, focuses only on exonic splicing enhancer elements. With this finder, no enhancer elements were identified which included the *ITPA* c.94C>A wild-type sequence, although the variant sequence was recognized by SRp40 (TT**A**CATG) and SRp55 (T**A**CATG).The third finder, http://cubweb.biology.columbia.edu/pesx/, identifies putative ESE/ESS and ISE/ISS sequence motifs recognizable by SR and hnRNP proteins. This finder showed that two ESS elements were destroyed when the mutation was present (Fig. 3).

Exon 2 of the *ITPA* gene has two cryptic 5′ss at positions −35 and −19 relative to the authentic 5′ss at position +1 in intron 2 (Fig. 4). None of the *ITPA* exon 2 cryptic 5′ss, nor the authentic splice site, share complete homology with the 5′ss consensus sequence as shown in Fig. 4., therefore, the three 5′ss can be considered as weak sites needing upstream enhancer

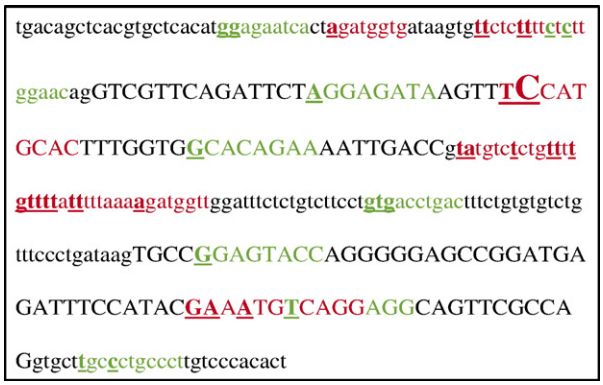

Fig. 3. Location of ESE/ESS, and ISE/ISS sequence motifs on exon 2, intron 2 and exon 3 of the *ITPA* wild-type gene. Exons are represented in uppercase. Green sequences denote ESE/ISE. Red sequences denote ESS/ISS. The *ITPA* c.94C>A mutation is shown in a larger uppercase font. The first bases of ESE/ISE and ESS/ISS are underlined. The *ITPA* 94C wild-type sequence is a binding site for a silencer. When the *ITPA* 94A mutation is present, the silencer is destroyed. Data obtained from http://cubweb.biology.columbia.edu/pesx/. ESE/ISE=exonic/intronic splicing enhancers, ESS/ISS=exonic/intronic splicing silencers.

elements to be activated [27]. For the *ITPA* c.94C>A wild-type sequence, the choice of the authentic 5′ss in preference to the cryptic sites may be explained by the presence of a silencing element which inactivates the two cryptic 5′ss at −35 and −19. When the *ITPA* c.94C>A mutation is present, the ESS is destroyed and one or both of the cryptic 5′ss may be activated. It is known that when U1 snRNP does not bind stably to any splice site, all the weak sites compete with each other [28]. Furthermore, the mutation introduces a sequence element recognized by two SR proteins (SRp40, SRp55), and the presence of these elements may increase the chances of cryptic splice site utilization. In addition, exon 2 of the *ITPA* gene is small (58 bp). The close proximity of splice sites on either side of small exons is hypothesized to introduce stearic interference between bound splicing factors [20] and has been related to inefficient splicing of some small exons [29]. The use of any of the cryptic 5′ss is likely to decrease even further the length of exon 2 and the splicing efficiency of this exon. Interference between splice sites may also explain the presence of the Type 2 transcript in wild-type patients.

The activation of cryptic 5′ss has been addressed in several studies. Thalassemia-associated point mutations in intron 1 position +1 and +5 activate three cryptic neighboring 5′ss at positions −38, −16 (within the exon) and +13 (within the intron) [28]. More recently, a novel mutation in the E1α PDH (pyruvate dehydrogenase) gene located downstream of exon 7 has been shown to activate a cryptic splice donor site leading to retention of intronic sequences through increased binding of SC35 to the mutated sequence [27].

ITPA mRNA missplicing of exons 2 and 3 (transcript Type 2) does not generate a frameshift, however skipping of exon 2 or 3 alone is predicted to result in a frameshift raising the possibility of nonsense mediated decay (NMD). A fragment corresponding to a deletion of exon 3 only (transcript Type 3) was observed in *ITPA* g.IVS2+21A>C variant genotypes at a low percentage by the sensitive fluorescent system. Levels of Type 3 transcripts in *ITPA* g.IVS2+21A>C heterozygotes did not correlate with the 40% reduction in enzyme activity phenotype. The low percentage of misspliced mRNA lacking exon 3 is consistent with the NMD of a frameshifted transcript.

No *ITPA* mRNA corresponding to a deletion of exon 2 only was seen in the *ITPA* c.94C>A variant, implying that splicing of exon 3 is dependent on the splicing of exon 2. Hence factors affecting splicing of exon 2 will lead to the generation of transcripts lacking both exons 2 and 3. By contrast, missplicing of exon 3 associated with the *ITPA* g.IVS2+21A>C mutation did not influence splicing of other exons.

Due to the high levels of Type 2 transcripts observed in wild-type samples (17%), the GenBank Expressed Sequence Tags data base (dbEST) (http://www.ncbi.nlm.nih.gov/dbEST/) was searched for alternative spliced forms of *ITPA* mRNA clones. Of the 206 ESTs listed in the dbEST, 95% corresponded to the full length transcript, 0.33% corresponded to transcript Type 2 and 1.7% to Type 3. The low levels of *ITPA* Type 2 mRNA transcript observed in the dbEST were in agreement with the 1% observed for fibroblasts (Table 1). Most of the clones are

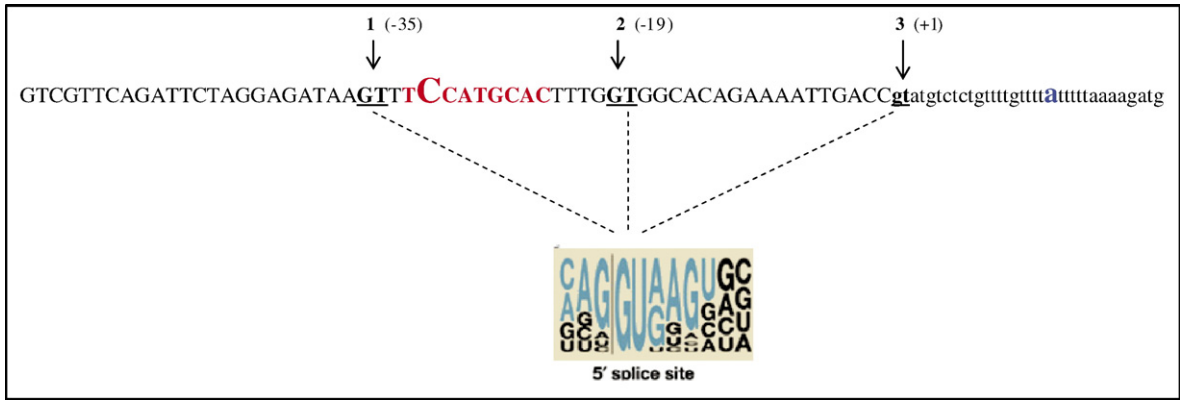

Fig. 4. Portion of the *ITPA* gene showing cryptic 5′ splice sites (5′ss). Exon 2 is represented by uppercase letters and intron 2 in lowercase. The exonic splicing silencer (ESS) element is shown in red. The *ITPA* c.94C>A allelic variant is shown in a large uppercase font and the *ITPA* g.IVS2+21A>C allelic variant in blue. 5′ss are underlined. Arrow number 3 shows the authentic 5′ss, arrows 1 and 2 point to two cryptic 5′ss. The 5′ ss consensus sequence cartoon has been obtained from Cartegni et al. [32]. The size of each base represents its frequency.

derived from tissue such as brain, lung, and tumor. In contrast, the majority of the samples analyzed in this study came from hematopoietic tissue; reticulocytes, peripheral blood leucocytes and EBV transformed lymphoblasts, suggesting that alternative splicing of exons 2 and 3 is tissue or cell-type dependent. Further studies are needed to determine levels of alternatively spliced transcripts in various tissues from patients with different *ITPA* genotypes.

In this study, fifteen individuals with six *ITPA* genotypes were studied. Although three patients were homozygous for the *ITPA* c.94C>A allelic variant, fewer patients with other variant genotypes were available for study. Therefore, it would be interesting to expand the study to gain a view of the extent of inter-individual variation in *ITPA* splicing in both wild-type and variant genotypes.

In vitro splicing studies are needed to confirm and further explore the mechanism by which mutation in the *ITPA* gene influences splicing. Expression studies are necessary to see whether the protein encoded by the transcript lacking exons 2 and 3 has enzyme activity, is able to form functional homo- or hetero-dimers, or is targeted to other cellular compartments.

ITPA is a candidate pharmacogenetic locus, the *ITPA* c.94C>A allelic variant has been associated with adverse drug reactions to thiopurine drug therapy [12–14]. *ITPA* expression is reportedly increased in tumor cells compared to normal tissues [30]. *ITPA* is over-expressed and significantly correlated to the expression of the MLL gene, over-represented in acute myeloid leukemia and myelodysplastic syndrome [31]. *ITPA* allelic variants are thus potentially relevant in carcinogenesis, and worthy of further study in this context.

In conclusion, the two mutations associated with ITPase deficiency both cause mRNA missplicing. The *ITPA* g.IVS2+21A>C intron mutation alters a preferred branch site resulting in missplicing of exon 3. It is proposed that the *ITPA* c.94C>A mutation causes missplicing of both exons 2 and 3 by a novel mechanism involving the destruction of an ESS and the activation of two cryptic 5′ss.

References

[1] J.A. Duley, H.A. Simmonds, D.A. Hopkinson, R.J. Levinsky, Inosine triphosphate pyrophosphohydrolase deficiency in a kindred with adenosine deaminase deficiency, Clin. Chim. Acta 188 (1990) 243–252.

[2] S.L. Holmes, B.M. Turner, K. Hirschhorn, Human inosine triphosphatase: catalytic properties and population studies, Clin. Chim. Acta 97 (1979) 143–153.

[3] M.Y. Galperin, O.V. Moroz, K.S. Wilson, A.G. Murzin, House cleaning, a part of good housekeeping, Mol. Microbiol. 59 (2006) 5–19.

[4] B.S. Vanderheiden, ITP pyrophosphohydrolase and IDP phosphohydrolase in rat tissue, J. Cell. Physiol. 86 (1975) 167–175.

[5] J.H. Fraser, H. Meyers, J.F. Henderson, L.W. Brox, E.E. McCoy, Individual variation in inosine triphosphate accumulation in human erythrocytes, Clin. Biochem. 8 (1975) 353–364.

[6] B.S. Vanderheiden, Inosine triphosphate in human erythrocytes: a genetic trait, Proc. 10th Congress Int. Soc. Blood Transf., 1964, Karger AG, Basel, pp. 540–548 (Stockholm).

[7] T. Mohandas, R.S. Sparkes, M.B. Passage, M.C. Sparkes, J.H. Miles, M.M. Kaback, Regional mapping of ADA and ITP on human chromosome 20: cytogenetic and somatic cell studies in an X/20 translocation, Cytogenet. Cell Genet. 26 (1980) 28–35.

[8] S. Lin, A.G. McLennan, K. Ying, Z. Wang, S. Gu, H. Jin, C. Wu, W. Liu, Y. Yuan, R. Tang, Y. Xie, Y. Mao, Cloning, expression, and characterization of a human inosine triphosphate pyrophosphatase encoded by the ITPA gene, J. Biol. Chem. (2001) 18695–18701.

[9] S. Sumi, A.M. Marinaki, M. Arenas, L. Fairbanks, M. Shobowale-Bakre, D.C. Rees, S.L. Thein, A. Ansari, J. Sanderson, R.A. De Abreu, H.A. Simmonds, J.A. Duley, Genetic basis of inosine triphosphate pyrophosphohydrolase deficiency, Hum. Genet. 111 (2002) 360–367.

[10] T. Maeda, S. Sumi, A. Ueta, Y. Ohkubo, T. Ito, A.M. Marinaki, Y. Kurono, S. Hasegawa, H. Togari, Genetic basis of inosine triphosphate pyrophosphohydrolase deficiency in the Japanese population, Mol. Genet. Metab. 85 (2005) 271–279.

[11] M. Shipkova, K. Lorenz, M. Oellerich, E. Wieland, N. von Ahsen, Measurement of erythrocyte inosine triphosphate pyrophosphohydrolase (ITPA) activity by HPLC and correlation of ITPA genotype–phenotype in a Caucasian population, Clin. Chem. 52 (2006) 240–247.

[12] A.M. Marinaki, A. Ansari, J.A. Duley, M. Arenas, S. Sumi, C.M. Lewis, M. Shobowale-Bakre el, E. Escuredo, L.D. Fairbanks, J.D. Sanderson, Adverse drug reactions to azathioprine therapy are associated with polymorphism in the gene encoding inosine triphosphate pyrophosphatase (ITPase), Pharmacogenetics 14 (2004) 181–187.

[13] N. von Ahsen, V.W. Armstrong, C. Behrens, C. von Tirpitz, A. Stallmach, H. Herfarth, J. Stein, P. Bias, G. Adler, M. Shipkova, M. Oellerich, W. Kruis, M. Reinshagen, Association of inosine triphosphatase 94C>A and thiopurine s-methyltransferase deficiency with adverse events and study drop-outs under azathioprine therapy in a prospective Crohn disease study, Clin. Chem. 51 (2005) 2282–2288.

[14] Z. Zelinkova, L.J. Derijks, P.C. Stokkers, E.W. Vogels, A.H. van Kampen, W.L. Curvers, D. Cohn, S.J. van Deventer, D.W. Hommes, Inosine triphosphate pyrophosphatase and thiopurine s-methyltransferase genotypes relationship to azathioprine-induced myelosuppression, Clin. Gastroenterol. Hepatol. 4 (2006) 44–49.

[15] L. De Ridder, J.M. Van Dieren, H.J. Van Deventer, P.C. Stokkers, J.C. Van der Woude, A.J. Van Vuuren, M.A. Benninga, J.C. Escher, D.W. Hommes, Pharmacogenetics of thiopurine therapy in paediatric IBD patients, Aliment. Pharmacol. Ther. 23 (2006) 1137–1141.

[16] R.B. Gearry, R.L. Roberts, M.L. Barclay, M.A. Kennedy, Lack of association between the ITPA 94C>A polymorphism and adverse effects from azathioprine, Pharmacogenetics 14 (2004) 779–781.

[17] D.S. Horowitz, A.R. Krainer, Mechanisms for selecting 5′ splice sites in mammalian pre-mRNA splicing, Trends. Genet. 10 (1994) 100–106.

[18] M. Krawczak, J. Reiss, D.N. Cooper, The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences, Hum. Genet. 90 (1992) 41–54.

[19] J.M. Johnson, J. Castle, P. Garrett-Engele, Z. Kan, P.M. Loerch, C.D. Armour, R. Santos, E.E. Schadt, R. Stoughton, D.D. Shoemaker, Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays, Science 302 (2003) 2141–2144.

[20] C.W. Smith, J.G. Patton, B. Nadal-Ginard, Alternative splicing in the control of gene expression, Annu. Rev. Genet. 23 (1989) 527–577.

[21] A.M. Marinaki, M. Arenas, Z.H. Khan, C.M. Lewis, M. Shobowale-Bakre el, E. Escuredo, L.D. Fairbanks, J.F. Mayberry, A.C. Wicks, A. Ansari, J. Sanderson, J.A. Duley, Genetic determinants of the thiopurine methyltransferase intermediate activity phenotype in British Asians and Caucasians, Pharmacogenetics 13 (2003) 97–105.

[22] S.E. Roberts, N.S. Thomas, A quantitative polymerase chain reaction method for determining copy number within the Prader–Willi/Angelman syndrome critical region, Clin. Genet. 64 (2003) 76–78.

[23] A.J. Matlin, F. Clark, C.W. Smith, Understanding alternative splicing: towards a cellular code, Nat. Rev., Mol. Cell Biol. 6 (2005) 386–398.

[24] T. Heller, M. Oellerich, V.W. Armstrong, N. von Ahsen, Rapid detection of ITPA 94C>A and IVS2+21A>C gene mutations by real-time fluorescence PCR and in vitro demonstration of effect of ITPA IVS2+21A>C polymorphism on splicing efficiency, Clin. Chem. 50 (2004) 2182–2184.

[25] C.W. Smith, B. Nadal-Ginard, Mutually exclusive splicing of alpha-tropomyosin exons enforced by an unusual lariat branch point location: implications for constitutive splicing, Cell 56 (1989) 749–758.

[26] D.M. Helfman, W.M. Ricci, Branch point selection in alternative splicing of tropomyosin pre-mRNAs, Nucleic Acids Res. 17 (1989) 5633–5650.
[27] M. Gabut, M. Mine, C. Marsac, M. Brivet, J. Tazi, J. Soret, The SR protein SC35 is responsible for aberrant splicing of the E1alpha pyruvate dehydrogenase mRNA in a case of mental retardation with lactic acidosis, Mol. Cell. Biol. 25 (2005) 3286–3294.
[28] K.K. Nelson, M.R. Green, Mechanism for cryptic splice site activation during pre-mRNA splicing, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 6253–6257.
[29] G.R. Adami, G.G. Carmichael, The length but not the sequence of the polyoma virus late leader exon is important for both late RNA splicing and stability, Nucleic Acids Res. 15 (1987) 2593–2610.
[30] S. Shichijo, K. Azuma, N. Komatsu, N. Kawamoto, H. Takedatsu, H. Shomura, H. Sawamizu, Y. Maeda, M. Ito, K. Itoh, Identification of two novel tumor-associated antigens recognized by HLA-B46-restricted cytotoxic T lymphocytes, Int. J. Mol. Med. 12 (2003) 895–902.
[31] B. Poppe, J. Vandesompele, C. Schoch, C. Lindvall, K. Mrozek, C.D. Bloomfield, H.B. Beverloo, L. Michaux, N. Dastugue, C. Herens, N. Yigit, A. De Paepe, A. Hagemeijer, F. Speleman, Expression analyses identify MLL as a prominent target of 11q23 amplification and support an etiologic role for MLL gain of function in myeloid malignancies, Blood 103 (2004) 229–235.
[32] L. Cartegni, S.L. Chew, A.R. Krainer, Listening to silence and understanding nonsense: exonic mutations that affect splicing, Nat. Rev., Genet. 3 (2002) 285–298.

Database

[1] ITPA – OMIM: 147520, GenBank Accession AF026816; http://www.ncbi.nlm.nih.gov/dbEST/ (NCBI's dbEST), http://rulai.cshl.edu/cgi-bin/tools/ESE; http://www.ebi.ac.uk/asd-srv/wb.cgi; http://cubweb.biology.columbia.edu/pesx/.