The effect of disease-associated *HRPT2* mutations on splicing

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Abstract

Mutations in the tumour suppressor *HRPT2* occur in patients with parathyroid carcinoma, kidney tumours and Hyperparathyroidism–Jaw Tumour syndrome. Disruption of exonic splicing through mutation of donor/acceptor splice sites or exonic splice enhancer (ESE) sites leads to loss of function of a number of major tumour suppressors including *BRCA1*, *APC* and *MLH1*. Given that the effect of *HRPT2* mutations on splicing has not been widely studied, we used an *in vitro* splicing assay to determine whether 17 *HRPT2* mutations located in hot-spot and other exons predicted to disrupt ESE consensus sites led to aberrant splicing. Using two independent web-based prediction programs, the majority of these mutations were predicted to disrupt ESE consensus sites; however, aberrant splicing of *HRPT2* transcripts was not

observed. Canonical donor or acceptor splice site mutations were also investigated using this splicing assay and transcripts assessed from tumour tissue. Splice site mutations were shown to lead to either exon skipping or retention of intronic sequences through the use of cryptic splice sites comprised of non-classical splicing signals. Aberrant splicing caused by disruption of ESE sites does not appear to have a major role in *HRPT2*-associated disease; however, premature truncation of parafibromin as the result of canonical donor or acceptor splice site mutations is associated with pathogenicity. Functional splicing assays must be undertaken in order to confirm web-based software predictions of the modification of putative ESE sites by disease-associated mutations.

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Introduction

HRPT2, also known as *CDC73* or *C1orf28*, is a ubiquitously expressed tumour suppressor that contains 17 exons that encode the 531 amino acid protein parafibromin (Carpten *et al.* 2002). Linkage analysis was used to map this gene to 1q25–q31; however, recent mapping data available through Ensembl suggests a more discreet location for *HRPT2* at 1q31.2. Parafibromin is a member of the PAF1 complex that is associated with RNA polymerase II-related transcriptional initiation and elongation (Rozenblatt-Rozen *et al.* 2005, Yart *et al.* 2005). To date, over 60 mutations have been reported, both in the germline of patients with Hyperparathyroidism–Jaw Tumour (HPT–JT) syndrome (Carpten *et al.* 2002, Marsh *et al.* 2007) and Familial Isolated Hyperparathyroidism (FIHP; Simonds *et al.* 2004), as well as somatic mutations in sporadic parathyroid carcinoma (Howell *et al.* 2003, Shattuck *et al.* 2003) and kidney tumours (Zhao *et al.* 2007). Approximately 80% of mutations occur in exons 1, 2 or 7 (Marsh *et al.* 2007). Loss of the wild-type *HRPT2* allele, as well as ‘second-hit’ mutations have been reported (Howell *et al.* 2003, Bradley *et al.* 2006, Kelly *et al.* 2006, Cetani *et al.* 2007). The vast majority of *HRPT2* mutations are predicted to prematurely truncate parafibromin; however, seven missense mutations have also been reported (reviewed in Marsh *et al.* 2007).

Accurate demarcation of intron/exon boundaries is required for correct pre-mRNA splicing and is achieved by critical sequences including 5' and 3' splice sites as well as exonic splice enhancer (ESE) sequences. ESE motifs are distinct but degenerate six to eight nucleotide sequences that bind the conserved family of splicing factors known as serine/arginine-rich proteins (SR proteins) including SF2/ASF, SC35, SRp40 and SRp55. SR proteins bind to ESE consensus motifs through their RNA-binding domain and recruit spliceosomal components that function to define exons.

Recently, some mutations in the coding regions of genes thought to function by premature introduction of a stop codon or alteration of single amino acids have been shown to affect pre-mRNA splicing by disruption of ESE sequences leading to exon skipping (Cartegni *et al.* 2002, Blencowe 2006). This has been reported for a number of tumour suppressor genes including *BRCA1* (Mazoyer *et al.* 1998), *BRCA2* (Fackenthal *et al.* 2002), *APC* (Montera *et al.* 2001, Aretz *et al.* 2004), *MLH1* (Nystrom-Lahti *et al.* 1999) and *NF1* (Colapietro *et al.* 2003).

We have used the web-based resources ESEfinder (Cartegni *et al.* 2003) and Rescue-ESE (Fairbrother *et al.* 2002) to predict whether 17 reported missense, non-sense and small insertion or deletion *HRPT2* mutations alter ESE sites. Using an *in vitro* splicing assay, we have assessed whether these mutations, as well as reported mutations at canonical donor/acceptor splice sites, cause aberrant splicing.

Materials and Methods

Prediction of ESE site disruption

The web-based prediction softwares ESEfinder (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese finder.cgi?process=home>) and Rescue-ESE (<http://genes.mit.edu/burgelab/rescue-ese/>) were used to determine whether 17 mutations in *HRPT2* hot-spot exons 2 and 7, as well as 4 and 5, previously identified in patients with HPT-JT or parathyroid carcinoma were likely to disrupt ESE sites.

Hybrid minigene construction

Three hybrid minigene cassettes were constructed to investigate putative splicing variants induced by mutations in *HRPT2* exons 2, 4/5 and 7 respectively pTBNde-HR2, pTBNde-HR4/5 and pTBNde-HR7 (Fig. 1). All sequences were compared with NCBI Genbank Accession number AL390863. Given the small size of IVS4 (83 nucleotides), exons 4 and 5 were able to be studied in a single construct. Wild-type *HRPT2* consisting of the 3' 279 nucleotides of intron 1, exon 2 (106 bp) and the 5' 246 nucleotides of intron 2 was PCR amplified from genomic DNA (Promega) using the primers 5'-TAATCATATGTGGTAAAGCCCA-GAGCCTAAT-3' and 5'-TGCTCATATGACAGCAA-GACCCCTCATC-3'. Wild-type *HRPT2* consisting of the 3' 399 nucleotides of intron 3, exon 4 (63 bp), intron 4 (83 bp), exon 5 (53 bp) and the 5' 184 nucleotides of intron 5 was amplified using 5'-TAATCATATGTGCATGTT-TAGGGTGAATGC-3' and 5'-TGCTCATATGGCTTG-CATGTGAGAGAGCTA-3'. Similarly wild-type *HRPT2* consisting of the last 294 nucleotides of intron 6, exon 7 (217 bp) and the first 244 nucleotides of intron 7 was

Figure 1 Schematic representation of the α -globin (black boxes)/fibronectin (grey boxes) minigene containing *HRPT2* exons 2, 4/5 or 7 (intronic sequences shown as dashed lines). The minimal α -globin promoter and SV enhancer are marked with an arrow head and the polyadenylation signal with a solid circle. Three different fragments of *HRPT2* containing either exon 2 (pTBNde-HR2), exons 4/5 (pTBNde-HR4/5) or exon 7 (pTBNde-HR7) and flanking intronic sequences were cloned into the *NdeI* site of the hybrid minigene. The position of primers used to analyse transcripts expressed from this minigene are indicated by solid arrows.

amplified using 5'-TAATCATATGCCTATATTAAT-GAGTCCTGCTT-3' and 5'-TGCTCATATGTGTGAAG-GAGCTTGCATTTT-3'. The *NdeI* site within each primer (underlined) was used to clone each PCR product into the unique *NdeI* site of the mammalian expression vector pTBNde(min) (kind gift of Francisco E Baralle, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy). This vector contains a hybrid minigene composed of the first three introns/exons of the α -globulin gene into which a small portion of the fibronectin gene has been inserted (Fig. 1). This hybrid minigene has been used in numerous studies to investigate the splicing of a number of different genes including the neurofibromatosis type 1 gene *NF1* and the cystic fibrosis gene *CFTR* (Pagani *et al.* 2000, 2003, Baralle *et al.* 2003). Each *HRPT2* minigene construct was verified by sequence analysis (Supamac, The University of Sydney, NSW, Australia).

Site-directed mutagenesis

HRPT2 mutations previously identified in patients with HPT-JT or parathyroid carcinoma (reviewed in Marsh *et al.* 2007) that were predicted to disrupt putative ESE sites, two reported splice site mutations (IVS2+1G>C and IVS6-1delG) as well as four synthetic splicing mutations at donor or acceptor splice sites (IVS1-2A>G, IVS4-2A>G, IVS5+1G>T and IVS7+1G>C) were introduced into the *HRPT2* minigene cassettes using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Synthetic mutants were used as positive controls to test the efficacy of the *in vitro* splicing assay, a strategy employed in previous studies (Anczukow *et al.* 2008). Each mutation and the primers used for mutagenesis, as well as the disease associations of these mutations, are listed in Table 1. All mutagenesis was verified by sequence analysis (Supamac).

Cell culture

Human embryonic kidney (HEK293) and immature osteoblastic (MG63; American Type Culture Collection, Manassas, VA, USA) cells were maintained in DMEM (Gibco, Invitrogen Australia Pty Ltd), supplemented with 10% FCS (Trace Scientific Ltd, Noble Park, VIC, Australia) and incubated at 37 °C in a humidified 5% CO₂ atmosphere.

Analysis of minigene expression

HEK293 cells (2×10^5 /well) were seeded into six-well culture dishes. After 24 h, cells were transfected with 1 μ g of each wild-type or mutant minigene construct using Eugene 6 according to the manufacturer's instructions (Roche Diagnostics Australia Pty Ltd).

Seventy-two hours after transfection, cytoplasmic RNA was extracted (RNeasy, Qiagen Pty Ltd) and reverse transcribed using SuperScript III First-Strand Synthesis

Table 1 Primers used for mutagenesis of *HRPT2* minigenes

	Mutation type	Disease association	
Mutation			
Primers used to generate exon 2 mutants			
IVS1-2A>G ^a	Splice Site	None	5'-CTTGCCTTAATTATTTCTGGGACTGGAAAGGAAGGC-3' 5'-GCCTTCCTTTCCAGTCCCGAAATAATTAAGGCAAG-3'
c.162C>G (Y54X) (Howell <i>et al.</i> 2003)	Nonsense	Sp. cancer	5'-GCCAAGCCAGAGAGTA G TACACATTGGATTCC-3' 5'-GGAATCCAATGTGTACTACTCTCTGGGTTGGC-3'
c.165delC (Howell <i>et al.</i> 2003)	Frameshift	Sp. cancer	5'-GCCAAGCCAGAGAGTACTA A CATTGGATTCCATT-3' 5'-AATGGAATCCAATGTTAGTACTCTCTGGGTTGGC-3'
c.165C>A (Y55X) (Howell <i>et al.</i> 2003)	Nonsense	Sp. cancer	5'-GCCAAGCCAGAGAGTACTA A ACATTGGATTCCATT-3' 5'-AATGGAATCCAATGT T TAGTACTCTCTGGGTTGGC-3'
c.165C>G (Y55X) (Carpten <i>et al.</i> 2002)	Nonsense	HPT-JT	5'-GCCAAGCCAGAGAGTACTA G ACATTGGATTCCATT-3' 5'-AATGGAATCCAATGTCTAGTACTCTCTGGGTTGGC-3'
c.191T>C (L64P) (Carpten <i>et al.</i> 2002)	Missense	FIHP	5'-CCATTTTATTTCTAC C TATAACGTGCACC-3' 5'-GGTGCACGTTATTAGTAGAAATAAAATGG-3'
c.195insA (Cetani <i>et al.</i> 2004)	Frameshift	Sp. cancer	5'-CATTTTATTTCTACTT A ATAACGTGCACCTTCTCATCC-3' 5'-GGATGAGAAAGGTGCA C GTTTATTAAGTAGAAATAAAATG-3'
c.195insT (Cetani <i>et al.</i> 2004)	Frameshift	Sp. cancer	5'-CATTTTATTTCTACTTA A CTGTCACCTTCTCATCC-3' 5'-GGATGAGAAAGGTGCA C GTTTATTAAGTAGAAATAAAATG-3'
c.226C>T (R76X) (Shattuck <i>et al.</i> 2003)	Nonsense	Sp. cancer	5'-CATCCTGTTTATGTCTGACGT G CAGCTGTAAG-3' 5'-CTTACAGCTGCACGT C AGACATAAACAGGATG-3'
IVS2+1G>C ^b (Villablanca <i>et al.</i> 2004)	Splice Site	FIHP	5'-GTCCGACGTGCAGCT C TAAAGTAGAATTCATT-3' 5'-AATGAATTCTACTTAG A GCTGCACGTCCGAC-3'
Primers used to generate exon 4 and 5 mutants			
IVS4-2A>G ^a	Splice Site	None	5'-CTTCTCTTTCTTTTAT G GTCAAACGAGCTGCA-3' 5'-CTGCAGCTCGTTTGAC C CATAAAAGAAAGAGAAG-3'
c.356delA (Carpten <i>et al.</i> 2002)	Frameshift	HPT-JT	5'-AGAAATAGGTCTTC_G C GATCTACTCAAG-3' 5'-CTTGAGTAGATCGC_GAAGACCTATTCT-3'
c.373insA (Shattuck <i>et al.</i> 2003)	Frameshift	FIHP	5'-CTCTTTCTTTATAGTCA A AAACGAGCTGCAGATGAAG-3' 5'-CTTCATCTGCAGCTCGTTT G ACTATAAAAGAAAGAG-3'
c.406A>T (K136X) (Carpten <i>et al.</i> 2002)	Nonsense	HPT-JT	5'-GTTTTAGCAGAAGCATAG A ACCACGAATTG-3' 5'-CAATTCGTGGTTTCT A TGCTTCTGCTAAAC-3'
IVS5+1G>T ^a	Splice Site	None	5'-GAAACCACGAATTG A GTTAAAGAACTGTATT-3' 5'-AAATACAGTTTCTTTA A CTCAATTCGTGGTTTC-3'
Primers used to generate exon 7 mutants			
IVS6-1delG ^b (Howell <i>et al.</i> 2003)	Splice Site	Sp. cancer	5'-AAGAAATTGCTTTTA_GTCTTTGTCTGAAGC-3' 5'-GCTTCAGACAAAGAC_TAAAAGCAATTTCTT-3'
c.636delT (Carpten <i>et al.</i> 2002)	Frameshift	HPT-JT	5'-CCTTAAACAGAGGAG_TTTGTGGATGCTGAG-3' 5'-CTCAGCATCCACAAA_CTCCTCTGTTAAGG-3'
c.664C>T (R222X) (Shattuck <i>et al.</i> 2003)	Nonsense	FIHP	5'-GAGGTAGATGTGACCTGAGATATTGTCAGCA-3' 5'-TGCTGACAATATCTC A GTCACATCTACCT-3'
c.679insAG (Carpten <i>et al.</i> 2002)	Frameshift	HPT-JT	5'-CGAGATATTGTCAG C AGAGAGAGAGATATGGAGG-3' 5'-CTCCATACTCTCTCT C TGCTGACAATATCTCG-3'
c.679delAG (Howell <i>et al.</i> 2003)	Frameshift	HPT-JT	5'-CGAGATATTGTCAGC_ A GAGAGAGATATGGAG-3' 5'-CTCCATACTCTCTCT_GCTGACAATATCTCG-3'
c.686delGAGT (Howell <i>et al.</i> 2003)	Frameshift	HPT-JT	5'-ATATTGTCAGCAGAGAGA_ A TGGAGGACGAAACAAC-3' 5'-GTTGTTCTGTGTCCTCAT_ T CTCTCTGACAAAT-3'
c.700C>T (R234X) (Shattuck <i>et al.</i> 2003)	Nonsense	Sp. cancer	5'-GAGTATGGAGGACATGAACAACATCTTAC-3' 5'-GTAAGATAGTTGTT A TGCTCTCCATACTC-3'
IVS7+1G>C ^a	Splice Site	None	5'-ATCTTACAAAGCACAG G AAAGCTAATTAATAATTTACTCAT-3' 5'-ATGAGTAAATATTTTAATTA G CTTCTCTGTGCTTTGTAAGAT-3'

^aSynthetic positive control donor or acceptor splice site mutations to determine efficacy of the *in vitro* splicing assay.^bNaturally occurring donor or acceptor splice site mutations.

Disease association is indicated for naturally occurring mutations. Sp. cancer indicates sporadic parathyroid carcinoma. Mutations are bolded and underlined.

SuperMix according to the manufacturer's instructions (Invitrogen Australia Pty Ltd). The minigene α -globulin/fibronectin gene rearrangement within pTBNde (min) does not occur normally, and the use of a reverse primer that spans the 3' fibronectin/ α -globulin exon boundary allows for the

analysis of transcripts expressed only from the minigene cassette. cDNA was subjected to PCR (AmpliTaQ Gold, Applied Biosystems), using the oligonucleotides 5'-CTCCTAAGCCACTGCCTGCTGGTGA-3' and 5'-GGTCACCAGGAAGTTGGTTAAATCA-3' and the

following PCR cycles: 35 cycles of 95 °C for 30 s; 55 °C for 30 s; and 72 °C for 30 s. PCR products were separated on 2% agarose and visualised using ethidium bromide staining. PCR products were either purified immediately after PCR (Wizard PCR Preps DNA Purification System, Promega) or were separated on 2% agarose and then gel purified (UltraClean GelSpin DNA Purification Kit, MO BIO Laboratories, Inc., Carlsbad, CA, USA) as per the manufacturer's instructions. The sequence of purified PCR products was verified by commercial sequencing (Supamac).

Amplification of cDNA from parathyroid tissue

PCR amplification of *HRPT2*-spanning exons 4–8 was carried out using cDNA generated from tumour tissue of a patient with sporadic parathyroid carcinoma and a previously identified *HRPT2* mutation (IVS6–1delG; Howell *et al.* 2003). Informed consent was obtained according to human ethics protocols. No additional fresh frozen or paraffin-embedded tissue was available from this carcinoma. cDNA was subjected to PCR as described above using the primers Ex4F 5'-GCAAGTATAGACAGAAGCGC-3' and Ex8R 5'-CACAGGTGCTGCATTTGG-3'. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega) and cloned into the pGEM® – T Easy Vector (Promega) as per the manufacturer's instructions. Constructs were verified by sequencing.

Results

ESE site analysis of 17 endogenously occurring HRPT2 mutations

Seventeen *HRPT2* mutations occurring in exons 2, 4, 5 or 7, which had previously been identified in patients with HPT-JT or parathyroid carcinoma, were analysed by ESEfinder and Rescue-ESE. Out of the 17 *HRPT2* mutations analysed, 14 were predicted by ESEfinder to alter ESE sites. Ten out of these mutations resulted in the loss of one or more ESE sites, three mutations induced gain of an ESE site, while one resulted in prediction of a less efficient ESE site, but still above the matrix score threshold level (Fig. 2). Although a number of different ESE sites were predicted by Rescue-ESE to alter as the result of *HRPT2* mutation, 11 out of these overlapped with those predicted by ESEfinder (Fig. 2).

Transcripts expressed from wild-type or mutant HRPT2 exon 2 minigene constructs

In HEK293 cells transfected with an empty vector or wild-type *HRPT2* exon 2 minigene, a single PCR product of ~300 or 400 bp respectively was detected. The synthetic IVS1–2A>G mutation resulted in transcripts in which exon 2 had been skipped or a portion of exon 2 had been deleted (Fig. 3A). The donor splice site mutation IVS2+1G>C that

Figure 2 Out of the 17 reported *HRPT2* mutations, 14 were predicted by ESEfinder to disrupt ESE sites of one or more of four different SR proteins (SF2/ASF (red), SC35 (blue), SRp40 (green), SRp55 (yellow)). Predicted ESE sites within wild-type *HRPT2* exons 2 (A), 4 (B), 5 (C) and 7 (D) are shown in the top panels. Coloured bars represent individual ESE sites with the matrix score (higher score represents a stronger prediction) plotted on the y-axis and the position of the ESE within each *HRPT2* exon represented on the x-axis. Matrix score changes induced by each mutation are represented in the lower panels. If matrix scores for a particular SR protein fell below threshold levels as predicted by ESEfinder (SF2/ASF=1.956, SC35=2.383, SRp40=2.67, SRp55=2.676), the coloured bar representing an ESE site was lost. In the case of c.165C>G, the matrix score for SRp40 was reduced but remained above threshold level. Mutations marked with an * were predicted by both ESEfinder and Rescue-ESE to disrupt ESE binding sites.

Figure 3 *In vitro* splicing analyses of wild-type and mutant *HRPT2* exon 2 and flanking sequence. (A) PCR products expressed from the wild-type or mutated *HRPT2* minigene. Minigenes containing each of the *HRPT2* exon 2 mutations were spliced identically to wild-type. Canonical splice site mutations (in bold) led to aberrant splicing products. (B) Schematic representation of the splicing outcomes for the splice site mutation IVS2+1G>C. The IVS2+1G>C mutation is underlined and newly generated splice signals are shown in bold. Hatched boxes represent fibronectin exons belonging to the pTBNde(min) mammalian expression vector.

has been reported in a family with FIHP (Villablanca *et al.* 2004) caused either exon 2 skipping resulting in premature truncation of parafibromin at residue 73, or retention of four nucleotides at the 5' end of intron 2 by way of use of the cryptic donor-splicing signal AAGT (Fig. 3B). This sequence deviates from classical consensus motifs for donor splice sites (Cartegni *et al.* 2002). The aberrant splice product generated by retention of intronic sequence is predicted to lead to premature truncation of parafibromin at residue 82 following two non-parafibromin amino acids.

By contrast, cells transfected with the *HRPT2* exon 2 minigene harbouring exonic mutations generated a single PCR product similar in size to the wild-type *HRPT2*

product, indicating that none of these changes altered exon 2 splicing (Fig. 3A). Direct sequencing of all transcripts verified these observations.

Nine exonic mutations in exon 4/5 or 7 of HRPT2 do not disrupt splicing

In HEK293 cells transfected with the empty vector, wild-type, or mutated *HRPT2* exon 4/5, a single PCR product was generated (Fig. 4). The two synthetic mutations at donor or acceptor splice sites used as positive controls to test the efficacy of the *in vitro* splicing assay led to transcripts in which exon 5 had been skipped (IVS5+1G>T), or a portion of

Figure 4 *In vitro* splicing analyses of wild-type and mutant *HRPT2* exons 4/5 and flanking sequence. PCR products expressed from the wild-type or mutated exon 4/5 *HRPT2* minigene. Canonical splice site mutations (in bold) led to aberrant splicing products. Schematic shows that minigenes containing each of the *HRPT2* exon 4/5 mutations were spliced identically to wild-type. Hatched boxes represent fibronectin exons belonging to the pTBNde(min) mammalian expression vector.

exon 5 had been deleted (IVS4–2A>G). The three exonic *HRPT2* exon 4 or 5 mutations did not alter the splicing of these exons and their flanking intronic sequences (Fig. 4).

Similarly, to assess *HRPT2* exon 7 splicing, two donor or acceptor splice site mutations were analysed (Fig. 5). The IVS7+1G>C synthetic mutation resulted in transcripts with either loss of some exon 7 sequence or exon 7 skipping. The IVS6–1delG mutant, reported in a case of sporadic parathyroid carcinoma (Howell *et al.* 2003), led to two aberrant transcripts. The first retained the last 40 nucleotides of intron 6 as a direct result of use of the cryptic acceptor-splicing signal AGGA (Fig. 5B). Translation of this mutant transcript suggests that parafibromin would be prematurely truncated at amino acid 188 after a string of 16 non-parafibromin amino acids. The second aberrant transcript was missing the first G of exon 7 as a direct result of loss of the G at position –1 of IVS6 constituting part of the canonical acceptor splice site AGGT. This loss resulted in use of the A at position –2 of IVS6 and the first three nucleotides of exon 7 (GTC) as the new splice recognition signal AGTC. This sequence deviates from classical consensus motifs for acceptor splice sites (Cartegni *et al.* 2002). Translation of this mutant transcript suggests that parafibromin would be prematurely truncated at residue 201 after a string of 30 non-parafibromin amino acids. By contrast, six *HRPT2* mutations in exon 7 did not alter splicing (Fig. 5A). Direct sequencing confirmed these results.

HRPT2 IVS6–1delG splicing in parathyroid carcinoma

Parathyroid tumour tissue was available from a patient with sporadic parathyroid carcinoma and the somatic *HRPT2* mutation IVS6–1delG. As observed in the *in vitro* splicing assay, the cryptic splice signal AGGA in intron 6 was used resulting in retention of the last 40 nucleotides of intron 6.

Wild-type transcript was also observed, most likely transcribed from the wild-type allele we had previously shown was retained in this tumour (Howell *et al.* 2003; Fig. 6). The other transcript observed in the *in vitro* splicing assay was not seen in tumour tissue. We believe the likely explanation is that wild-type *HRPT2* transcript expressed from the retained allele was in greater abundance than this alternative transcript in tumour tissue. Sequencing of a large number of clones would likely have revealed this additional transcript in tumour tissue.

Discussion

We have developed an *in vitro* assay to determine the effect on splicing of *HRPT2* mutations occurring at canonical donor/acceptor splice sites, as well as those predicted to disrupt putative ESE sites. Mutations occurring at splice sites led to aberrant splicing including exon skipping or retention of intronic sequence; however, only wild-type splicing patterns were observed for all exonic *HRPT2* mutations predicted to disrupt ESE sites.

Four point mutations resulting in nucleotide substitutions at canonical GT splice donor or AG splice acceptor sites have been reported to date in *HRPT2* (Howell *et al.* 2003, Villablanca *et al.* 2004, Bradley *et al.* 2005, Moon *et al.* 2005). While the splicing effects of two of these mutations have been previously reported (Bradley *et al.* 2005, Moon *et al.* 2005, Table 2), we have used our splicing assay to determine the effects of the remaining two. IVS2+1G>C identified in members of an FIHP family (Villablanca *et al.* 2004) led to exon 2 skipping, as well as retention of four nucleotides at the 5' end of intron 2 through use of the cryptic donor-splicing signal AAGT. While this signal contains the nearly invariant GT, the exonic nucleotides making up this signal do not constitute previously described classical donor splice signal consensus motifs (Cartegni *et al.* 2002).

The IVS6–1delG mutation previously reported in a sporadic parathyroid carcinoma (Howell *et al.* 2003) was analysed in both the splicing assay and in tumour tissue from an affected patient. This mutant resulted in retention of the last 40 nucleotides of intron 6 through use of the cryptic acceptor splice signal AGGA. A second transcript was generated by use of the cryptic acceptor splice signal AGTC as the consequence of the loss of a G. While this signal contains the nearly invariant AG, the exonic nucleotides making up this sequence do not constitute previously described classical acceptor splice signal consensus motifs (Cartegni *et al.* 2002). Analysis of *HRPT2* transcripts from tumour tissue from the patient with the sporadic parathyroid carcinoma was consistent with results from the splicing assay.

Previous studies have also identified discrepancies between predictive and functional analyses of ESE sites. Anczuków *et al.* (2008) used an *in vitro* splicing assay to determine whether 108 variants of unknown significance in *BRCA1*,

Figure 5 *In vitro* splicing analyses of wild-type and mutant *HRPT2* exon 7 and flanking sequence. (A) PCR products expressed from the wild-type or mutated *HRPT2* minigene. Minigenes containing each of the *HRPT2* exon 7 mutations were spliced identically to wild-type. Canonical splice site mutations (in bold) led to aberrant splicing products. (B) Schematic representation of the splicing outcomes for the IVS6-1delG mutation. The IVS6-1delG mutation is underlined and newly generated splice signals are shown in bold. Hatched boxes represent fibronectin exons belonging to the pTBNde(min) mammalian expression vector.

including missense mutations, silent variants and small deletions and insertions might cause aberrant splicing. Approximately 70% of mutations were predicted to either abolish or decrease the threshold score of a putative ESE site. While they were able to identify previously reported wild-type alternative transcripts, only one mutant was found to have an effect on splicing. This was not confirmed in a human lymphoblastoid cell line from a patient carrying this mutation. While the possibility that splicing may have been tissue specific in this case could not be excluded, this mutation was later shown not to segregate with all cases of early bilateral breast cancer patients in this family (Anczuków *et al.* 2008). Additionally, Lastella *et al.* (2004) used an *in vitro* splicing assay to analyse mutants in exon 12 of *hMLH1* predicted to disrupt ESE sites, and found that only 4 out of 16 led to aberrant splicing of exon 12.

SR proteins are known to be differentially expressed in different cell types and tissues. For example, expression of SF2/ASF and its agonist hnRNP varies greatly in rat tissues including heart, ovaries, liver and pancreas (Hanamura *et al.* 1998). A study of SR proteins in calf tissues including kidney, skeletal muscle, liver, heart, lung, thymus and brain also shows considerable variability of expression (Zahler *et al.* 1993). We are unable to exclude the likelihood that we did not see aberrant splicing from mutations at putative ESE sites in our HEK293 cell model due to lack of tissue-specific expression of elements of the necessary splicing machinery. However, this is a less likely explanation, given that identical results were seen for a number of mutations analysed in the osteosarcoma cell line MG63 transfected with our splice assay constructs (data not shown).

Figure 6 Splicing outcomes obtained from HEK293 cells in which wild-type *HRPT2* is expressed or cDNA obtained from a parathyroid carcinoma harbouring IVS6–1delG. The IVS6–1delG mutation is underlined and newly generated splice signals are shown in bold.

This study has elucidated aberrant transcripts and the mechanisms by which they have been generated for canonical donor/acceptor splice site mutations in *HRPT2*. It is unlikely that additional splicing aberrations caused by disruption of ESE sites play a role in the pathogenicity

of exonic *HRPT2* mutations. While predictive programs may be useful in giving a preliminary indication of the effect of sequence variants on splicing, functional assays are required to determine the true effects, if any, on splicing.

Table 2 Splicing mutants reported in Hyperparathyroidism–Jaw Tumour (HPT-JT), Familial Isolated Hyperparathyroidism (FIHP) or sporadic carcinoma

	Effect on transcript	Cryptic donor splice site used	Cryptic acceptor splice site used	Predicted effect on parafibromin	Phenotype
Mutation					
IVS1 + 1G > A	Deletion of 30 nts in exon 1 (+102 to +131)	GAAT GT ^a	–	In frame loss of residues 35–44	FIHP; Bradley <i>et al.</i> (2005)
IVS2 – 1G > A	Exon 3 skipping Deletion of first 23 nts of exon 3	–	– AGAA ^a	Truncation at residue 85 Truncation at residue 82	HPT-JT; Moon <i>et al.</i> (2005)
IVS2 + 1G > C ^b	Exon 2 skipping Retention of 4 nts at 5' end of IVS2	– AAGT ^a	–	Truncation at residue 73 Truncation at residue 82	FIHP; Villablanca <i>et al.</i> (2004)
IVS6 – 1delG ^c	Retention of 40 nts at 5' end of IVS6 c.513delG	–	AGGA AGTC ^a	Truncation at residue 188 Truncation at residue 201	Sporadic parathyroid carcinoma; Howell <i>et al.</i> (2003)

nts, nucleotides.
^aCryptic donor or acceptor splice sites used that are comprised of non-classical splicing consensus motifs (Cartegni *et al.* 2002).
^bEffect of mutation reported in this study in an *in vitro* splicing assay.
^cEffect of mutation reported in this study both *in vitro* and *in vivo*.
Splicing motifs (Cartegni *et al.* 2002) are indicated with the nearly invariant GT and AG dinucleotides either at the 5' or 3' end of the intron bolded and underlined.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Dr Hahn supervised Ms McDonnell to conduct this project and was involved in all aspects of experimental design and data analysis. He co-wrote the paper with Dr Marsh.

Ms McDonnell functioned as a research assistant on this project and conducted much of the cloning, transfections and sequencing described under the supervision of Drs Hahn and Marsh.

Dr Marsh was responsible for conceiving this project and obtaining grant funding to conduct it. She was responsible for supervision of research staff carrying out this project. She was involved in all aspects of experimental design and data analysis and co-wrote the paper with Dr Hahn.

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