

## ONLINE MUTATION REPORT

# Utilisation of a cryptic non-canonical donor splice site of the gene encoding PARAFIBROMIN is associated with familial isolated primary hyperparathyroidism

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More than 99% of all splice sites conform to consensus sequences that usually include the invariant dinucleotides gt and ag at the 5' and 3' ends of the introns, respectively. We report on the utilisation of a non-consensus (non-canonical) donor splice site within exon 1 of the *HRPT2* gene in familial isolated primary hyperparathyroidism (FIHP). *HRPT2* mutations are more frequently associated with the hyperparathyroidism-jaw tumour syndrome (HPT-JT). Patients with FIHP were identified to have a donor splice site mutation, IVS1+1 g→a, and the consequences of this for RNA processing were investigated. The mutant mRNA lacked 30 bp and DNA sequence analysis revealed this to result from utilisation of an alternative cryptic non-canonical donor splice site (gaatgt) in exon 1 together with the normally occurring acceptor splice site in intron 1. Translation of this mutant mRNA predicted the in-frame loss of 10 amino acids in the encoded protein, termed PARAFIBROMIN. Thus, these FIHP patients are utilising a ga-ag splice site pair, which until recently was considered to be incompatible with splicing but is now known to occur as a rare (<0.02%) normal splicing variant.

It is well established that nearly all splice sites conform to consensus sequences,<sup>1,2</sup> which usually include invariant dinucleotides at each end of the intron. Thus, the dinucleotides gt and ag are invariably found at the 5' and 3' ends of introns, respectively,<sup>1,2</sup> and most gene finding software will find only introns that begin with a gt and end with an ag. However, non-consensus splice sites have been described,<sup>1,2</sup> and we report on the utilisation of such a non-canonical donor splice site in patients with familial isolated primary hyperparathyroidism (FIHP; OMIM 145000).

Primary hyperparathyroidism (HPT), which may result from parathyroid adenomas, hyperplasia, or carcinoma, has an estimated prevalence of 3 per 1000 in the general population and is most frequently encountered as a non-familial disorder.<sup>3,4</sup> However, approximately 10% of patients with primary HPT will have a hereditary form, which may occur as an isolated endocrinopathy or as part of a complex tumour syndrome such as multiple endocrine neoplasia (MEN) or the hereditary hyperparathyroidism-jaw tumour syndrome (HPT-JT; OMIM 145001).<sup>4</sup> The MEN syndromes are autosomal dominant disorders characterised by the combined occurrence of tumours in two or more endocrine tissues.<sup>5</sup> Thus, in MEN type 1 (MEN1; OMIM 131100), the occurrence of tumours of the parathyroids, pancreas, and pituitary are associated with inactivating germline mutations of the *MEN1* gene on chromosome 11q13,<sup>5</sup> while in MEN type 2 (MEN2; OMIM 171400), activating mutations of the *RET* proto-oncogene located on chromosome 10q11 cause

medullary thyroid carcinoma, pheochromocytomas, and parathyroid tumours.<sup>6</sup> HPT is usually the first manifestation of MEN1 and occurs in >95% of patients,<sup>7</sup> while it is less common in MEN2.<sup>5,6</sup> HPT-JT is an autosomal dominant disorder characterised by parathyroid tumours, which may be adenomas or carcinomas, fibro-osseous tumours of the jaw bones, uterine tumours, Wilms' tumours, renal cysts, or hamartomas.<sup>8-12</sup> The gene responsible for HPT-JT, a putative tumour suppressor gene termed *HRPT2*, is located on chromosome 1q31.2 and encodes a 531 amino acid protein, referred to as PARAFIBROMIN.<sup>13</sup> In addition, hereditary HPT occurring without the association of other tumours has also been described as a distinct clinical entity in more than 100 families, and this is referred to as FIHP.<sup>4,14</sup> The distinction between FIHP and the other hereditary hyperparathyroid disorders may at times be difficult, particularly as FIHP in some families is associated with MEN1 mutations.<sup>14-17</sup> Furthermore, FIHP has also been demonstrated to be due to *HRPT2* mutations, thereby indicating that FIHP in some families may be an allelic variant of HPT-JT.<sup>11,18,19</sup> We ascertained a previously unreported FIHP family and undertook studies to identify the underlying genetic abnormality. These studies revealed a germline donor splice site mutation of the *HRPT2* gene that resulted in the utilisation of an alternate, cryptic, non-canonical donor splice site.

## METHODS

### Patients

The proband (patient 1) who was a previously healthy female, developed HPT at the age of 21 years. She presented with a 5 month history of a tender swelling that was 3 cm in size on the left side of her neck. The swelling was associated with discomfort and dysphagia, and moved with swallowing. She was also noted to suffer from tiredness, polydipsia, polyuria, aches in her limbs, and dyspepsia. Investigations revealed her to have hypercalcaemia (corrected calcium: 3.38 mmol/l, normal: 2.20–2.65 mmol/l), hypophosphataemia (phosphate: 0.77 mmol/l; normal 0.80–1.50 mmol/l), and an elevated serum parathyroid hormone (PTH) concentration of 136 ng/l (normal: 10–64 ng/l). All other serum biochemical investigations were normal. She underwent a neck exploration which revealed an egg sized cystic lesion situated at the left upper pole of the thyroid, and another cystic lesion representing the left lower parathyroid located in the cervical thymus. The right lower parathyroid, which was a firm nodule, was also located in the cervical thymus and the

**Abbreviations:** ARMS, amplification refractory mutation system; EBV, Epstein-Barr virus; FIHP, familial isolated primary hyperparathyroidism; HPT, hyperparathyroidism; HPT-JT, hyperparathyroidism-jaw tumour syndrome; m, mutant; MEN, multiple endocrine neoplasia; PTH, parathyroid hormone; RT-PCR, reverse transcriptase PCR; SpGVs, splicing affecting genomic variants; WT, wild type

right upper parathyroid was found to be slightly enlarged. A 3.5 gland parathyroidectomy together with a left thyroid lobectomy was performed. Histology revealed multi-gland disease with features of atypical parathyroid adenomas containing cysts and frequent mitoses. These findings were consistent with parathyroid carcinoma although the findings of local invasion or distant metastases that are required for an unequivocal diagnosis of parathyroid carcinoma<sup>20</sup> were absent. Post-operatively, the patient required treatment with oral vitamin D supplements to maintain normocalcaemia. During a 9 year follow up period, there has not been a recurrence of the HPT. In addition, there have been no clinical or biochemical abnormalities to indicate the occurrence of anterior pituitary or pancreatic islet cell tumours that would be consistent with MEN1.<sup>7</sup> Radiology of the maxilla and mandible did not reveal any lesions that would indicate the occurrence of ossifying fibromas.<sup>12</sup> A family history revealed that her mother, maternal grandmother, a maternal uncle, and his daughter had suffered from HPT. However, only the proband's mother (patient 2) who had mild hypercalcaemia (corrected calcium: 2.79 mmol/l) and an elevated serum PTH but had not undergone parathyroidectomy, was available for study. Informed oral consent was obtained from the individuals, using guidelines approved by the local ethics committee at the Hammersmith and Oxford Radcliffe Hospitals.

#### DNA sequence analysis of the *MEN1* and *HRPT2* genes

Leukocyte DNA was extracted using previously reported methods.<sup>21</sup> Fifteen pairs of primers were used for PCR amplifications of the nine coding exons of the *MEN1* gene and their corresponding 16 exon-intron boundaries, utilising conditions described previously.<sup>22</sup> Seventeen pairs of primers were used for PCR amplification of the 17 coding exons of the *HRPT2* gene and their corresponding 32 exon-intron boundaries utilising conditions previously described.<sup>8–13</sup> The DNA sequences of gel purified PCR products were determined by the use of Taq polymerase cycle sequencing<sup>22–23</sup> and a semi-automated detection system (ABI 377 sequencer; PE Applied Biosystems, Foster City, CA).

#### Amplification refractory mutation system PCR (ARMS PCR)

The DNA sequence abnormality was confirmed by the use of a modified ARMS PCR,<sup>24</sup> which utilised reverse (R2WT and R2m) primers specific for the 3' nucleotide sequence for either the wild type (WT) or mutant (m) allele in combination with a forward (F) primer. A control reverse (R1) primer was also incorporated into the reaction. The primer details are as follows: F, 5' TGCTGCTGTCGTAGGCGAGG 3' (140 bp to 159 bp upstream of initiation codon); R1, 5' CGAACACCCGTTTATCC 3' (+100 bp to +117 bp in intron 1); R2WT, 5' CAGCCATGCCGACTTAC 3' (+1 bp to +18 bp in intron 1); and R2m, 5' CAGCCATGCCGACTTAT 3' (+1 bp to +18 bp in intron 1). The DNA sequence abnormality was demonstrated to cosegregate with the disorder and to be absent as a common polymorphism in DNA obtained from 55 unrelated normal individuals.

#### Reverse transcriptase PCR (RT-PCR) studies

RT-PCR was utilised to investigate mRNA splicing abnormalities, using total RNA extracted from Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines from the two patients and an unrelated normal individual, as previously described.<sup>25</sup> The PCR primer pair consisted of a forward primer, 5' GGAGACGAAGTGATCTTCGG 3' (nucleotides +64 to +83 in exon 1) and a reverse primer, 5' GCACGTCCGACATAAACAGG 3' (nucleotides +214 to +233 in exon 2). The DNA sequences of the purified RT-PCR

products were then determined using methods previously reported.<sup>26</sup>

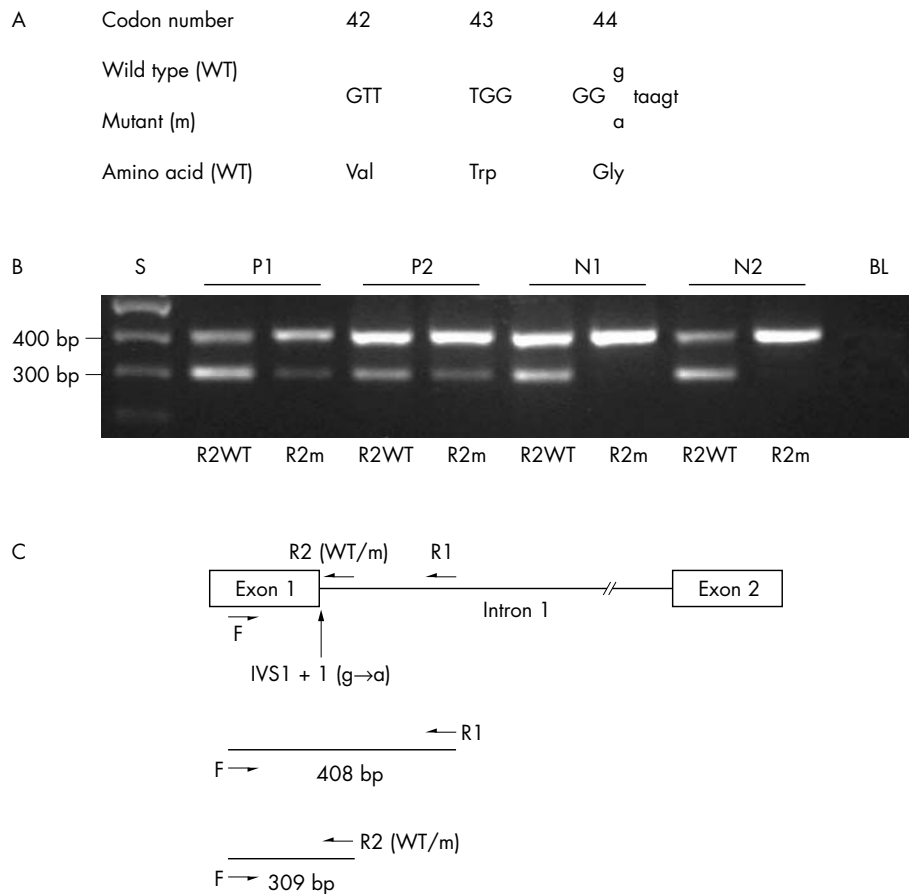
#### Database analysis

A web based splice site prediction programme was utilised to predict alternative donor splice sites (<http://www.fruitfly.org>).

#### RESULTS

We initially analysed the *MEN1* gene for mutations as more FIHP families have been reported to harbour *MEN1* mutations than *HRPT2* mutations.<sup>17</sup> DNA sequence analysis of the entire 2.79 kb coding region and exon-intron boundaries of the *MEN1* gene did not reveal any abnormalities. However, DNA sequence analysis of the entire 1593 bp coding region and 32 exon-intron boundaries of the *HRPT2* gene revealed the presence of a germline mutation in patient 1 (fig 1). This consisted of a g→a transition at position 1 of the donor splice site of intron 1. This DNA sequence change, which altered the nearly invariant gt dinucleotide of the donor splice consensus site, was confirmed by the use of ARMS PCR (fig 1) and was also confirmed to be present in the affected mother (patient 2; fig 1). In addition, an analysis of the DNA from 55 unrelated individuals, confirmed the absence of this g→a transition at position +1 of intron 1 in 110 alleles, thereby indicating that it was not a common polymorphism that would be expected to occur in over 1% of the population.

The g nucleotide at position +1 of the donor splice consensus sequence is nearly invariant in eukaryotic sequences<sup>1–27</sup> and mutations of this nucleotide have been previously reported in patients with many disorders<sup>1–2</sup> including *MEN1*<sup>28</sup> and autosomal recessive hypoparathyroidism.<sup>25</sup> These and other studies<sup>1–2–25–29</sup> have revealed that mutations in the donor splice site region may be associated with an accumulation of unspliced precursor mRNA, retention of incompletely spliced precursors, complete absence of transcripts, or the appearance of aberrantly processed mRNA from the use of alternative normally occurring splice sites or cryptic splice sites. We initially used a splice site prediction programme (<http://www.fruitfly.org>) to search for alternate donor splice sites within the wild type *HRPT2* sequence. This revealed that a very strong candidate alternate donor splice site consensus sequence (gtaagg), with a predictive score of 1, was located at +162 bp to +167 bp in intron 1. Utilisation of this alternate donor splice site would result in a mutant mRNA that was 161 bp larger than the wild type and, if translated, this would lead to a truncated PARAFIBROMIN protein of 44 amino acids. To investigate this and other possibilities, we investigated *HRPT2* mRNA processing by detection of its transcription in EBV transformed lymphoblastoid cell lines (fig 2). This revealed the presence of an aberrantly processed mRNA that was smaller than normal. DNA sequence analysis of the mutant *HRPT2* product revealed that it lacked nucleotides +102 to +131 of the wild type exon 1 sequence. The loss of this 30 bp of exon 1 sequence is predicted to result in an in-frame deletion of 10 amino acids (codons 35–44). These results demonstrate that as a result of the g→a mutation of the donor splice site at +1 to +6 bp of intron 1, an alternate cryptic donor splice site at nucleotides +102 to +107 of exon 1 is being utilised. This naturally occurring, but not normally utilised, donor splice site consists of a non-canonical donor splice site sequence (gaatgt). Use of this non-canonical donor splice site with the normally occurring acceptor splice site in intron 1 results in the mutant *HRPT2* mRNA that lacks 30 bp and is predicted to result in an in-frame loss of 10 amino acids from PARAFIBROMIN.



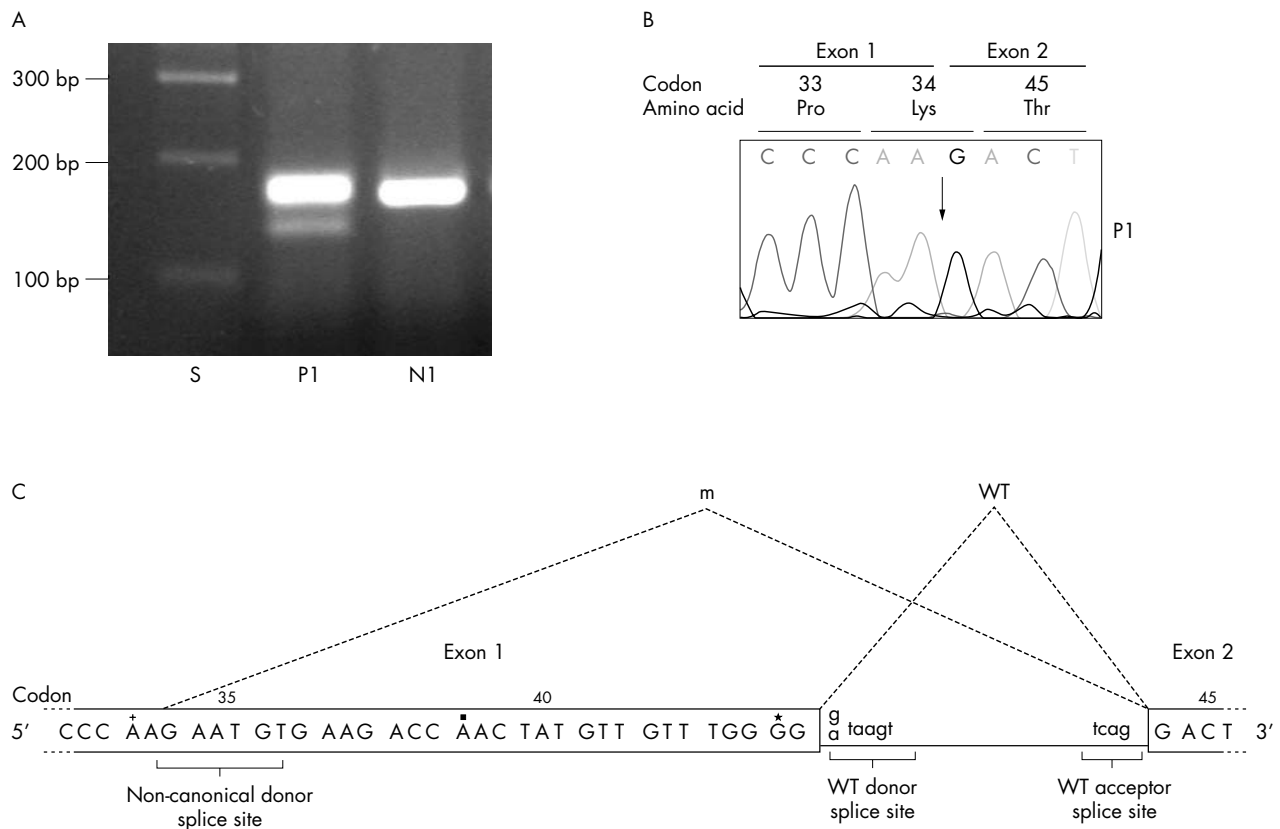
**Figure 1** Detection of donor splice site mutation in intron 1 by ARMS PCR. DNA sequence analysis of patient 1 (P1) revealed a g→a transition of the nearly invariant gt dinucleotide of the donor splice site consensus sequence of intron 1 (panel A). The mutation was confirmed in the proband (P1) and in her affected mother (P2) by the use of ARMS PCR (panels B and C). Control primers, forward (F) and reverse (R1), were used to amplify a 408 bp segment, which was detected in all individuals. Primer F was used in combination with one of two reverse (R2) primers designed to differ only at the extreme 3' nucleotide and thus be specific for either the wild type (R2WT) or mutant (R2m) sequence. Following PCR using these primers, wild type (WT) products of 408 and 309 bp were obtained from 55 normal individuals (N1 and N2, shown) and the two affected individuals (P1 and P2). However, mutant products (R2m) were obtained only from the affected individuals (P1 and P2). Thus, the affected individuals are heterozygous (WT/m) and the unaffected (that is, normal) individuals are homozygous (WT/WT). The positions of the size markers (S, 100 bp ladder) at 300 and 400 bp are shown. BL indicates the control water blank. Exon and intron sequences are shown by upper and lower case letters, respectively.

## DISCUSSION

Our study demonstrates that utilisation of a cryptic non-canonical donor splice site within exon 1 of the *HRPT2* gene is associated with FIHP (fig 2). This non-canonical donor splice site is not normally used, but is utilised as a result of a germline mutation that disrupts the nearly invariant gt dinucleotide of the normally occurring donor splice site in FIHP patients (fig 1). Thus, these results represent an example of splicing affecting genomic variants (SpaGVs), which are increasingly being recognised as a cause of human disease phenotypes.<sup>2</sup> The non-canonical donor splice site consensus sequence (gaatgt) within exon 1 of the *HRPT2* gene is a very unusual splice site variant, and likely represents <0.02% of all splice sites.<sup>1 2 30</sup> It is well established that nearly all splice sites conform to consensus sequences and that these sequences possess nearly invariant dinucleotides, which are gt at the donor splice site and ag at the acceptor splice site.<sup>1</sup> An extensive review of all known mammalian splice site sequences has revealed that 99.24% contain so called canonical gt-ag splice site pairs, whereas 0.69% have non-canonical gc-ag splice site pairs.<sup>1 31</sup> The remaining non-canonical alternative splice site consensus sequences are extremely rare with at-ac introns found at a frequency of 0.05% and with no other single pairing occurring at a frequency of >0.02%.<sup>1 30 31</sup> The ga-ag splice

site pair utilised as a consequence of the donor splice site mutation in the family with FIHP (fig 2) is very unusual and, indeed, until recently was thought to be incompatible with splicing.<sup>32</sup> However, it has been shown to exist very rarely (<0.02%) as a normal splicing variant and examples are in the mouse fibroblast growth factor receptor 2,<sup>33</sup> human heparanase,<sup>34</sup> and human fibroblast growth factor receptor genes.<sup>32 35</sup> Activation of cryptic splice sites represents a common disease causing mechanism that occurs in approximately one third of splicing mutations,<sup>36</sup> and hence the recognition of such SpaGVs is important. However, many studies do not pursue investigation of SpaGVs, such that one recent review stated “that many researchers still focus only on GV [genomic variants] that change the protein sequence and do not consider follow up studies on SpaGVs that are shown to vary between cases and controls and that might be of equal or greater importance because of their effect on splicing”.<sup>2</sup> This statement is well supported by our study as the g→a donor splice site mutation at position +1 of intron 1 of the *HRPT2* gene was also recently reported in an unrelated FIHP family, but its consequences were not explored.<sup>37</sup> Furthermore, most gene finding software programmes do not identify non-canonical splice sites, as illustrated by our study.

The manner whereby this *HRPT2* mutation, which predicts a mutant PARAFIBROMIN protein of 521 amino acids that



**Figure 2** Abnormal mRNA splicing due to intron 1 donor splice site mutation. The g→a transition at nucleotide +1 of intron 1 (fig 1) leads to a loss of the consensus donor splice site (gtaagt). The possible mRNA splicing abnormalities were investigated by the use of RT-PCR using total RNA obtained from EBV transformed lymphoblastoids from patients 1 and 2 (data from P1 shown), and a control normal individual (N1). Only one RT-PCR product (170 bp) was obtained from the normal individual, but two products (170 and 140 bp) were obtained from the patient (panel A). The positions of the size markers (S, 100 bp ladder) at 100, 200, and 300 bp are shown. DNA sequence analysis of these RT-PCR products revealed that the normal (wild type) product consisted of exon 1 spliced to exon 2 (data not shown), but that the mutant product lacked the last 30 nucleotides of exon 1 with a resultant loss of 10 amino acid residues (codon 35–44) (deletion site indicated by arrow head; panel B). An examination of the DNA sequence of codons 34–36 revealed it to consist of a non-canonical donor splice site sequence (gaagt) that is normally not utilised (panel C). However, the loss of the WT canonical donor splice site due to the g→a transition involving nucleotide +1 of intron 1 (fig 1) has led to utilisation of this cryptic, non-canonical donor splice site. Thus, the mutant product has resulted from the combined use of this normally occurring, but redundant, non-canonical donor splice site and the normal acceptor splice site. The position of every fifth codon (that is, 35, 40, and 45) is indicated and the locations of nucleotides +100, +115, and +130 are indicated by the plus symbol, the filled square, and the asterisk, respectively.

lacks residues 35–44, leads to FIHP remains to be elucidated. To date, four different germline *HRPT2* mutations have been identified in seven FIHP families. These comprise: one donor splice site mutation at +1g→a of intron 1 in two unrelated families, one of which is reported in this study and the other of which has been previously reported;<sup>37</sup> one donor splice site mutation at +1g→c of intron 2 in one family;<sup>19</sup> one missense mutation (L64P) in two unrelated families;<sup>19 38</sup> and one frameshift mutation (679insAG) in two unrelated kindreds.<sup>8 39</sup> Thus, it appears that more than 50% of FIHP families have *HRPT2* mutations that do not lead to a truncated and hence inactivated PARAFIBROMIN protein. This contrasts with the situation in HPT-JT families, in whom >95% of *HRPT2* mutations are predicted to result in truncated and inactivated forms of PARAFIBROMIN.<sup>8</sup> One possibility is that the missense and intron 1 donor splice site *HRPT2* mutations result in the production of either reduced levels or dysfunctional forms of PARAFIBROMIN and that this may be associated with a less severe form, that is, forme fruste, of the disorder. This may then be analogous to the situation that occurs in non-classical cystic fibrosis, whereby some SpaGVs result in a reduced level of normal transcript and a less severe form of the disease.<sup>2</sup> Indeed, it is interesting to note that the presentation of HPT in the proband (patient 1) could represent a forme fruste of HPT-JT. Thus, patient 1

had the unusual co-occurrence of two cystic parathyroid tumours, consistent with HPT-JT, yet had an absence of maxillary or mandibular tumours, which does not support a diagnosis of HPT-JT. These features also illustrate the variable penetrance of the mutant *HRPT2* alleles. Additional studies in FIHP and HPT-JT families that aim to fully characterise the consequences of the *HRPT2* SpaGVs and mutations, as illustrated by our study, are required to explore these possibilities.

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## ELECTRONIC-DATABASE INFORMATION



The a splice site prediction programme mentioned in this study can be found at <http://www.fruitfly.org>.

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