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## **Molecular analysis of the HPJ-JT syndrome and sporadic parathyroid carcinogenesis**

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## *Chapter 4*

HRPT2 mutations are associated with malignancy in sporadic parathyroid tumours.

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## ORIGINAL ARTICLE

## HRPT2 mutations are associated with malignancy in sporadic parathyroid tumours

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**Background:** Hyperparathyroidism is a common endocrinopathy characterised by the formation of parathyroid tumours. In this study, we determine the role of the recently identified gene, *HRPT2*, in parathyroid tumorigenesis.

**Methods:** Mutation analysis of *HRPT2* was undertaken in 60 parathyroid tumours: five HPT-JT, three FIHP, three MEN 1, one MEN 2A, 25 sporadic adenomas, 17 hyperplastic glands, two lithium associated tumours, and four sporadic carcinomas. Loss of heterozygosity at 1q24-32 was performed on a subset of these tumours.

**Results:** *HRPT2* somatic mutations were detected in four of four sporadic parathyroid carcinoma samples, and germline mutations were found in five of five HPT-JT parathyroid tumours (two families) and two parathyroid tumours from one FIHP family. One HPT-JT tumour with germline mutation also harboured a somatic mutation. In total, seven novel and one previously reported mutation were identified. "Two-hits" (double mutations or one mutation and loss of heterozygosity at 1q24-32) affecting *HRPT2* were found in two sporadic carcinomas, two HPT-JT-related and two FIHP related tumours.

**Conclusions:** The results in this study support the role of *HRPT2* as a tumour suppressor gene in sporadic parathyroid carcinoma, and provide further evidence for *HRPT2* as the causative gene in HPT-JT, and a subset of FIHP. In light of the strong association between mutations of *HRPT2* and sporadic parathyroid carcinoma demonstrated in this study, it is hypothesised that *HRPT2* mutation is an early event that may lead to parathyroid malignancy and suggest intragenic mutation of *HRPT2* as a marker of malignant potential in both familial and sporadic parathyroid tumours.

Hyperparathyroidism (HPT) is one of the most common endocrinopathies, believed to affect approximately three individuals per 1000 adults.<sup>1</sup> HPT is characterised by the formation of parathyroid tumours and, if left untreated, patients develop bone disease, renal stones, and neuromuscular dysfunction. The majority of tumours are sporadic, but approximately 5% are associated with the autosomal dominant hereditary cancer syndromes multiple endocrine neoplasia type 1 and 2A (MEN 1 and 2A), familial isolated hyperparathyroidism (FIHP), and hyperparathyroidism-jaw tumour syndrome (HPT-JT).<sup>2</sup>

Sporadic HPT may occur as primary, secondary, or tertiary disease. Primary HPT can be attributed to a single adenoma in 80-85% of cases, multiglandular hyperplasia in 15-20% of cases, and carcinoma in less than 1% of cases.<sup>3</sup> Secondary HPT arises in response to 1,25-dihydroxyvitamin D<sub>3</sub> deficiency, hyperphosphatemia, or hypocalcemia, due to renal failure, and presents as multiglandular hyperplasia. Tertiary HPT is defined as autonomous parathyroid hyperfunction in a patient with a previously well documented history of secondary HPT. Its presentation has been described as nodular hyperplasia or multiple adenoma. HPT may also arise in response to lithium treatment as a therapy for bipolar disorder.<sup>4</sup>

Our understanding of the molecular basis of parathyroid tumorigenesis has increased over the past 10 years. It has been clearly established that increased expression of the oncogene *cyclin D1* (formerly named *PRAD1*) is associated with the formation of parathyroid hyperplastic glands, adenomas and carcinomas.<sup>5,7</sup> The MEN 1 gene, *MEN1*, located at 11q13,<sup>8,9</sup> has also been shown to play a major role

in both familial<sup>10-12</sup> and sporadic<sup>13-16</sup> parathyroid tumorigenesis, functioning as a tumour suppressor. However the genetic basis of many sporadic and familial parathyroid tumours is still unknown. Parathyroid carcinoma, for example, is not a feature of MEN 1, and neither loss of heterozygosity (LOH) at 11q13, nor mutations in *MEN1* have been reported.<sup>17,18</sup> The tumour suppressor gene, *Retinoblastoma (Rb1)*, located at 13q14, was suggested to have a primary role in parathyroid carcinogenesis following the finding of allelic loss of *Rb1* in the majority of parathyroid carcinomas but rarely in benign parathyroid tumours.<sup>19,20</sup> However, subsequent immunohistochemical studies found decreased *Rb1* expression to be unreliable as a marker of malignancy.<sup>21-23</sup>

Recently, germline mutations in a newly identified gene, *HRPT2* (AF312865, Hs.5722), previously identified as *Chromosome 1 open reading frame 28*<sup>24</sup> have been identified in 14 of 24 HPT-JT kindreds and one of two FIHP kindreds.<sup>25</sup> *HRPT2* maps to 1q25, consists of 17 exons, containing 1596 nucleotides and encodes a 531 amino acid protein termed parafibromin with unknown function.<sup>25</sup> Patients with HPT-JT present in childhood or early adulthood with HPT, and are also at risk of developing fibro-osseous jaw tumours and renal lesions.<sup>26,27</sup> While sporadic parathyroid carcinoma is rare, the incidence of parathyroid carcinoma is reported to be

**Abbreviations:** FIHP, familial isolated hyperparathyroidism; HPT, hyperparathyroidism; HPT-JT, hyperparathyroidism-jaw tumour syndrome; LOH, loss of heterozygosity; MEN, multiple endocrine neoplasia

15% in HPT-JT.<sup>2</sup> The majority of parathyroid tumours in these patients are aggressive, occasionally recurrent adenomas, notable also for their cystic histology.<sup>28</sup> LOH studies at 1q24-32 have identified allelic loss in some, but not all, HPT-JT associated tumours, suggestive of a tumour suppressor role for *HRPT2*.<sup>29,30</sup> Similar loss of the *HRPT2* locus in tumours from members of a FIHP family has been reported.<sup>31</sup> Studies in sporadic parathyroid tumours report that 9–13% of adenomas have LOH at 1q24-32.<sup>32,33</sup> In addition to germline *HRPT2* mutations in HPT-JT and FIHP kindreds, Carpten *et al* also detected somatic *HRPT2* mutations in two of 47 sporadic cystic parathyroid adenomas.<sup>28</sup> One of these was also found to have LOH at 1q24-32.<sup>33</sup> LOH at this locus has not been identified in lithium-associated parathyroid tumours<sup>34</sup> or secondary HPT.<sup>35</sup>

To determine the role of *HRPT2* in parathyroid tumourigenesis, mutation analysis was undertaken in both sporadic and familial parathyroid neoplasms. A subset of tumours was also assessed for LOH at the *HRPT2* locus, 1q24-32.

## MATERIALS AND METHODS

### Subjects and samples

Sixty parathyroid samples were obtained from subjects (31 female and 29 male) who underwent parathyroidectomy for hyperparathyroidism at Royal North Shore Hospital, Sydney, Australia; Leiden University Medical Centre, Leiden, Netherlands; or Martin Luther University, Halle-Wittenberg, Germany. Peripheral blood samples were also available from 42 of these patients. Patients gave informed consent according to protocols approved by each centre's human ethics committee.

The tumours were classified as familial or sporadic, including lithium-associated. Further classification of adenoma, hyperplasia (secondary or tertiary), or carcinoma was established according to detailed WHO guidelines.<sup>36</sup> Twelve familial tumour specimens were collected. Three were from two FIHP families, five from two HPT-JT families, three from three MEN 1 families and one from a MEN 2A patient. Orthopantomography of the jaw and renal ultrasound were performed on all affected members of the FIHP families. No case of jaw or renal tumour was found. Twenty-five sporadic adenomas, two lithium-associated tumours, 11 secondary and six tertiary hyperplastic glands, as well as four parathyroid carcinomas were also collected. The percentage of neoplastic tissue in each sample was assessed histologically from a paraffin embedded representative piece of tissue. All samples were composed of at least 70% neoplastic cells.

A panel of 65 anonymised peripheral blood samples collected from healthy volunteers constituted the normal germline DNA panel.

### DNA/RNA preparation

Parathyroid tissue was frozen in liquid nitrogen immediately after surgical removal and stored at -70°C or below. Peripheral blood was collected into EDTA anti-coagulant tubes and stored at -70°C. DNA was extracted from the frozen tissue and peripheral blood leucocytes according to standard procedures. RNA was extracted from frozen tissue using TRI Reagent (Sigma-Aldrich Corporation, St Louis, MO) according to the manufacturer's protocol.

### LOH studies

Where DNA was available from matched tumour and blood samples, allelic deletion of the chromosome 1q24-32 region flanking *HRPT2* was assessed using a selection of the following microsatellite markers: centromeric-D1S218-D1S238-D1S422-D1S2625-D1S081-HRPT2D1S533-D1S2757-D1S2794-D1S477-telomeric.<sup>37,38</sup> PCR was performed in a 7.5 µl reaction volume containing 0.17 µM each of

HEX-labelled forward and unlabelled reverse primer (Invitrogen, Life Technologies, Carlsbad, CA), 4 mM MgCl<sub>2</sub>, 0.3 units AmpliTaq Gold polymerase and 1 × Buffer II (Applied Biosystems, Foster City, CA), 250 µM dNTPs (Invitrogen, Life Technologies), and 15 ng of genomic DNA. Amplification was performed in a DNA Engine Tetrad (MJ Research, Incline Village, NV) with an initial denaturation of 95°C for 10 min, followed by 10 cycles of 94°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds, and 20 cycles of 89°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 min. The resulting microsatellite PCR products for each specimen were then pooled, and 1 µl was added to 10 µl of Hi-Di formamide (Applied Biosystems) and 0.2 µl of ROX 400HD size standard (Applied Biosystems), denatured at 95°C for 5 min and loaded into an ABI Prism 3700 Genetic Analyser (Applied Biosystems). Assessment of LOH was performed using Genescan v. 3.7 and Genotyper v. 3.7 software (Applied Biosystems). LOH was defined according to the following formula: LOH index = (T2/T1)/(N2/N1), where T was the tumour sample, N was the matched normal sample, 1 and 2 were the intensities of smaller and larger alleles, respectively. If the ratio was <0.5 or >1.5, the result was determined to be LOH.<sup>39</sup>

### HRPT2 mutation analysis

Initially, normal thyroid tissue RNA was sequenced in 4 overlapping segments between 5'UTR and 3'UTR to clarify the intron/exon boundaries and confirm the published sequence of the recently identified *HRPT2* gene.<sup>25</sup> cDNA was synthesized using the SUPERScript First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies) and amplified in a 20 µl reaction containing 200 ng of cDNA, 200 µM dNTPs (Invitrogen, Life Technologies), 1.5–4 mM MgCl<sub>2</sub>, 1 µM of each primer, 1 unit of Platinum Taq DNA Polymerase and 1 × PCR buffer (Invitrogen, Life Technologies). Following an initial denaturation at 94°C for 5 minutes, 35 cycles of 15 second steps at 94°C, 55–60°C (depending on the primer set) and 72°C were performed in a DNA Engine Dyad (MJ Research), finishing with an extension at 72°C for 10 minutes. The PCR products were purified using the QIAquick PCR Purification kit (QIAGEN Pty Ltd, Clifton Hill, Vic, Australia), cycle sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and sequenced on an ABI PRISM 3700 Genetic Analyser, according to the manufacturer's recommended protocol. The organisation of the gene is detailed in table 1 and primer sequences are available on request.

Sequencing of *HRPT2* was then performed for all tumour samples. All 17 exons and the intron-exon boundaries were amplified in 15 separate reactions for each sample. PCR was performed in 40 µl reactions containing 75 ng of template DNA, 200 µM dNTPs, 1.5 mM MgCl<sub>2</sub>, 0.5 µM each primer, 1.5 units of Taq DNA Polymerase, and 1 × reaction buffer (Invitrogen, Life Technologies). Following an initial denaturation at 94°C for 5 minutes, 35 cycles of 30 second steps at 94°C, 55°C, and 45 seconds at 72°C were performed, finishing with an extension at 72°C for 10 minutes in a DNA Engine Tetrad. Primer sequences are available on request. The PCR products were purified through a Multiscreen PCR Filter plate (Millipore Corp., Billerica, MA) and sequenced as above.

Where matched blood was available, the status of mutations identified in the tumours were determined as either somatic or germline by sequencing of the germline DNA.

RNA was available from three tumours to test whether there were aberrant splicing products of the gene. cDNA was synthesised, amplified and sequenced as above.

**Table 1** HRPT2 (Unigene cluster HS.5722) exon/intron organisation

Exon						Intron		
No	Bp	Nucleotides	Amino acids	Genomic sequence no*	3' splice acceptor region	5' splice donor region	No	Kb
1	131	1-131	1-44	2583-2713	agggggggggaag/ATG	TGGGG/gtaag	1	2.8
2	106	132-237	45-79	5494-5599	atttcag/GACT	AGCT/gtaag	2	5.0
3	70	238-307	80-102	10,556-10,625	gttttag/ACTG	GCCT/gtaag	3	5.1
4	63	308-370	103-123	15,773-15,835	cttcag/CAAC	CAAG/gtaag	4	0.1
5	53	371-423	124-141	15,919-15,971	ctttatag/TCAA	ATTGAG/gtaag	5	2.5
6	89	424-512	142-170	18,467-18,555	atttcag/GATG	ATTAG/gtaag	6	3.7
7	217	513-729	171-243	22,232-22,448	cttttag/GTCT	GGAAAG/gtaag	7	5.8
8	99	730-828	244-276	28,249-28,347	atttcag/AATTTT	TGTG/gtaag	8	2.4
9	79	829-907	277-302	30,686-30,764	atttcag/GATC	GAAG/gtaag	9	2.0
10	65	908-972	303-324	32,762-32,826	cttttcag/AAAC	AACG/gtaag	10	51.3
11	58	973-1030	325-343	84,177-84,234	tttaag/GAGGTGc	CCAG/gtaag	11	8.2
12	36	1031-1066	344-355	92,447-92,482	tcacag/TTTCT	GAAAG/gtaag	12	0.3
13	88	1067-1154	356-385	92,772-92,859	cttttag/GATC	TGAA/gtaag	13	20.5
14	162	1155-1316	385-438	113,375-113,536	gttttcaag/ATTT	ACTG/gtaag	14	3.1
15	101	1317-1417	439-472	116,638-116,738	cttttag/GGAC	TAAAA/gtaag	15	13.4†
16	142	1418-1559	473-519	92,488-93,899	tatag/TTAA	ACAG/gtaag	16	0.8
17	37	1560-1596	520-531	10,194-10,230	atttcag/GTAC	CTGA/atttt	3'UTR	0.7

\*The genomic sequence no. is taken from clones RP11-239J11 (AL390863/AF312865) and RP11-185C19 (AL39133/AF312865).

†Position of overlap of these clones.

‡Base found to be discrepant with the published sequence is in bold.

### Polymorphism scanning of the normal DNA panel

All amplifications were performed with 1 µM of each primer (Invitrogen, Life Technologies), 200 µM dNTPs (Invitrogen, Life Technologies), 2 units of AmpliTaq Gold polymerase (Applied Biosystems), 1 × Buffer II (Applied Biosystems), 1.5–4 mM MgCl<sub>2</sub>, and cycling conditions as stated for PCR of cDNA above, with an annealing temperature of 55°C, unless otherwise stated. Primer sequences are available on request.

### RsaI digest for detection of c.33C→T (Y11Y)

DNA was amplified in a 20 µL volume with exon 1 primers and 1 unit of AmpliTaq Gold polymerase. Four microlitres of the resulting 228 bp amplicon was digested with 1 unit of restriction endonuclease RsaI (Fermentas AB, Vilnius, Lithuania) in 1 × Buffer Y+Tango (Fermentas AB) with a final volume of 8 µL, and incubated at 37°C for 8 hours. One microlitre of the resulting digest was mixed with 1 µL of loading dye (5 g/L Bromophenol Blue, 12.5 mM EDTA, 50% formamide) and electrophoresed in a Corbett Research Gel-Scan 2000 Real-time Electrophoresis System (Corbett Research, Mortlake, NSW, Australia) through a 5% polyacrylamide, 0.6 × TBE gel under suggested non-denaturing conditions. Wild type amplicons were distinguished by the presence of bands at 109 bp and 119 bp. Heterozygotes contained an additional band at 228 bp.

### dHPLC detection of polymorphisms

DNA was amplified in a 50 µL volume using exon 2, 7, or 13 primers and following amplification the resulting products underwent enhancement of heteroduplex formation. This involved a denaturation at 94°C for 5 min immediately followed by a slow ramp over 35 cycles, starting at 94°C and decreasing 2°C/1 min cycle, finishing with 1 min incubation at 25°C. Five microlitres of amplicon was then injected into the flowpath of a DNASEP-MD cartridge in the WAVE-MD Mutation Detection System Model 2000 denaturing HPLC (Transgenomic, Omaha, NE) under WAVE-MD Standard Method gradient conditions and WAVEMAKER (Transgenomic) predicted temperatures for each amplicon. Eluted fragments were detected by the system's UV detector and analysed as chromatograms. Wild type amplicons eluted as a single homoduplex peak whereas heterozygous samples presented with up to four peaks representing homo- and heteroduplexes. For the

IVS2+28C→T variant, homozygotes for either the C or T allele were mixed with an equal aliquot of an exon 2 amplicon homozygous for the C allele, denatured, slowly reannealed and re-injected into the dHPLC to determine which allele was present.

### Statistical analysis

The  $\chi^2$  contingency test was performed to compare the occurrence of mutations between four cases of sporadic carcinomas and 44 cases of sporadic non-carcinomas. A *p* value less than 0.05 is considered as significant.

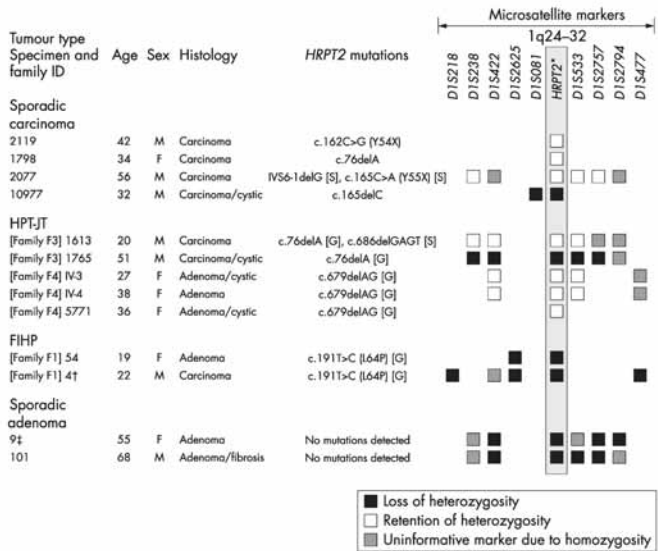
## RESULTS

### HRPT2 mutation analysis

HRPT2 mutations were detected in the DNA from four of four sporadic parathyroid carcinoma samples, five of five HPT-JT parathyroid tumours (two families) and parathyroid tumours from one of two FHP families (fig 1). No mutations were detected in any of the other 49 tumours sequenced. Eight different HRPT2 mutations were detected. With the exception of L64P, that has been previously published,<sup>20</sup> all the mutations identified were novel (fig 2). One of the mutations (c.76delA) was detected both in a sporadic carcinoma and in a HPT-JT family. Where available, matched constitutive DNA was analysed to determine the germline versus somatic nature of these mutations (fig 1). HPT-JT tumour #1613 harboured both a germline and a somatic HRPT2 mutation, and two somatic HRPT2 mutations were detected in carcinoma #2077.

### LOH studies

LOH analysis of 1q24-32 was performed on 42 of the 60 tumours. All samples tested were informative for at least one of the markers (data not shown). LOH was detected in six samples: one sporadic carcinoma (#10977), one HPT-JT tumour (#1765), two FHP tumours (#4 and #54) and two sporadic adenomas (#9 and #101) (fig 1). LOH at 1q24-32 has been previously reported in two of these samples (#9<sup>21</sup> and #4<sup>22</sup>). LOH was confirmed in sporadic carcinoma sample #10977, HPT-JT sample #1765 and FHP samples #4 and #54 by the presence of only the mutant allele in tumour DNA sequence. Retention of heterozygosity was demonstrated in the remaining 18 tumours not assessed for LOH by the



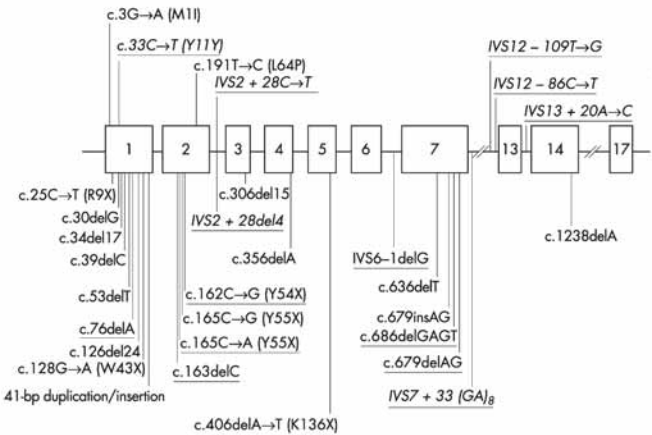
**Figure 1** Mutations detected within *HRPT2*, and LOH analysis of flanking markers. "Age" is the age in years at the time of parathyroidectomy. *HRPT2* mutations detected are listed as nucleotide changes and amino acid changes where relevant. Where two mutations were detected in the same patient, both are listed. Germline mutations are denoted "[G]", and somatic mutations are denoted "[S]". For the microsatellite markers, see key in figure. Blank spaces indicate LOH testing not performed (germline DNA not available). \*The approximate location of *HRPT2* within the 1q24-32 region. The results in this shaded column represent retention or loss of heterozygosity at the *HRPT2* locus by combined microsatellite and intragenic mutation results. †D1S218 and D1S477 have been reported previously.<sup>31</sup> ‡Previously reported as having LOH at 1q and 1p and CGH loss at 1q31-qter.<sup>34</sup>

presence of a heterozygous mutation or polymorphism in *HRPT2*.

**Polymorphisms**

Five intronic and one exonic sequence variants, five of which were also identified in a screen of 65 normal subjects, were identified in parathyroid lesions, and where available,

matched constitutive DNA (table 2). An additional variant, not detected in the tumours, was found in the normal panel (fig 3). Twenty six of the 54 parathyroid tumour samples not displaying LOH were heterozygous for the common polymorphism, IVS2+28C>T, confirming retention of heterozygosity for *HRPT2*. A dinucleotide repeat variant in intron 7, IVS7+33(GA)<sub>8</sub> (wild type IVS7+33 (GA)<sub>9</sub>) was found in five



**Figure 2** Distribution of *HRPT2* mutations and polymorphisms. Exons 8-12 inclusive, 15, and 16, have not been shown for clarity. Underlined variants are from this study, others are from Carpten *et al*.<sup>25</sup> Variants predicted to be polymorphisms are italicised.

**Table 2** HRPT2 predicted polymorphisms found in this study

Polymorphism	Heterozygote frequency in tumour DNA* (allele frequency)	Classification of tumours harbouring a polymorphism	Heterozygote frequency in germline DNA from a normal panel (allele frequency)
Y11Y (c.33C→T)	1 in 56 [0.01]	1 sporadic adenoma [G]	0 in 65
IVS2+28delCCTA	2 in 56 [0.02]	1 secondary hyperplasia [G] 1 tertiary hyperplasia [?]	5 in 65 [0.04]
IVS2+28C→T	26 in 56 (Heterozygous) (0.30)	all tumour types [G]	33 in 65 (Heterozygous) (0.32)
IVS7+33(GA) <sub>10</sub>	4 in 56 (Homozygous for T) 4 in 56 [0.04]	1 lithium [G] 1 secondary hyperplasia [G] 1 MEN 1 [?] 1 FIHP† (2 affected family members [G])	4 in 65 (Homozygous for T) 1 in 65 [0.01]
IVS12-86C→T	3 in 56 [0.03]	1 sporadic adenoma [?] 2 secondary hyperplasia [?]	3 in 65 [0.02]
IVS12-109T→G	5 in 56 [0.05]	1 secondary hyperplasia [G] 1 tertiary hyperplasia [?] 3 sporadic adenomas [?]	6 in 65 [0.05]
IVS13+20A→C	0 in 56		1 in 65 [0.01]

\*Only 1 sample from each family was included in the heterozygote frequency estimation.

[G]Present also in germline.

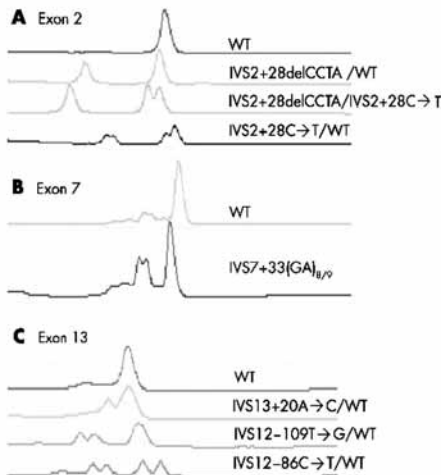
[?]Germline status not ascertained.

†The variant IVS7+33(GA)<sub>10</sub> was found in tumour and in germline in two affected members of the same family. Both tumours demonstrated LOH at 1q24-32 and the polymorphism was found on the mutated allele in tumour DNA.

tumours. We were unable to detect aberrant splicing in transcripts generated from three of these tumours (#4, #54, and #76).

#### Statistical analysis

A  $\chi^2$  contingency test showed that the occurrence of mutation in sporadic parathyroid carcinomas is highly significant compared with sporadic non-carcinomas ( $\chi^2 = 48$ ;  $p < 0.001$ ).



**Figure 3** Denaturing HPLC chromatograms of HRPT2 polymorphisms. The chromatograms plot the absorbance at 260 nm in mVolts v elution time in minutes for the various amplicons and show the different profiles that distinguish between the wild type (WT) and various polymorphisms for each amplicon. Wild type amplicons elute as a single homoduplex peak, whereas samples heterozygous for a polymorphism present with up to four peaks, representing homo- and heteroduplexes. Samples harbouring both polymorphisms in intron 2 (A) are also distinguishable from those heterozygous for a single intron 2 polymorphism.

#### DISCUSSION

Here we report the first finding of HRPT2 mutations in 100% (four of four) of sporadic parathyroid carcinomas studied (fig 1). All are predicted to prematurely truncate the parafibromin protein. No HRPT2 mutations were found in any of the 44 sporadic, non-carcinoma samples in this study. These results demonstrate a strong association between intragenic mutation of HRPT2 and malignancy in parathyroid tumours ( $p < 0.001$ ). Furthermore, our findings of two somatic mutations as well as one mutation with LOH at 1q24-32 in two of four carcinomas are consistent with Knudson's "two-hit" hypothesis, and suggest the role for HRPT2 as a tumour suppressor gene in sporadic parathyroid tumorigenesis.

Our finding of germline mutations in two of two HPT-JT families and confirmation of a germline mutation in one of two FIHP families support the conclusions of Carpten *et al.*,<sup>23</sup> that HRPT2 is the causative gene in HPT-JT and in a subset of FIHP (fig 1). Of additional significance was our finding of the "second-hit" in four of the seven samples with germline mutations. Three familial tumour samples demonstrated loss of the wild-type allele, and one tumour contained a somatic frameshift mutation (fig 1). These results provide evidence of a role for HRPT2 as a tumour suppressor gene in familial as well as sporadic parathyroid tumorigenesis.

The two germline mutations in the HPT-JT families are, like those found in the sporadic carcinomas, predicted to prematurely truncate the protein, whereas the FIHP mutation is a missense mutation of unknown consequence. Whether there is a phenotype/genotype correlation between mutation type and the presence of parathyroid disease alone, or in conjunction with jaw tumours or renal lesions (that is, FIHP v HPT-JT) will require a larger cohort of FIHP families with HRPT2 mutations to establish. Similarly, it is uncertain whether the severity or number of "hits" affecting HRPT2 determines the presentation of familial tumours as benign, cystic, or malignant. An alternative scenario may be that intragenic mutation of HRPT2 is an early event in a subset of patients with parathyroid lesions, and that additional, yet unknown events are required for the progression to malignancy. It is noteworthy that HRPT2 mutations have been previously detected in two of 47 (4%) sporadic cystic adenomas, and one (2%) had additional 1q24-32 LOH.<sup>23</sup> Given the strong association demonstrated between



intragenic mutation of *HRPT2* and malignancy in the current study, such a finding in a benign tumour might be considered a marker of malignant potential and long term monitoring is suggested for these patients.

Twenty four different *HRPT2* mutations have now been reported,<sup>29</sup> including seven in this paper (fig 2). Over 80% (20 of 24) of *HRPT2* mutations are located in exons 1, 2, or 7 or flanking intronic sequences. Exon 1 contains 42% (10 of 24) of all mutations detected to date, and exon 2 appears to contain a mutation hot spot at c.165C. Exon 7 is by far the largest exon, hinting at an important role for this exon, and to date, has been shown to harbour 21% (five of 24) of all *HRPT2* mutations detected. This exon contains a repeat element (AG)<sub>n</sub>, and three different frameshift mutations altering this motif have been detected. This study also detected seven apparent polymorphisms. We found these, in particular, IVS2+28C→T, to be useful markers for exclusion of LOH at the *HRPT2* locus.

The consistent detection of LOH at 1q24-32 in 9–13% of sporadic adenomas<sup>14–32–33</sup> is interesting. Our finding of LOH in two of 25 (12.5%) sporadic adenomas is in agreement with these previous reports, as is our finding of no LOH in 17 hyperplasia or two lithium-associated tumours.<sup>33–34</sup> We have previously shown that extensive loss of both arms of chromosome 1 occurs in approximately 10% of sporadic adenomas,<sup>14–32</sup> including adenoma #9<sup>34</sup> from this study. We did not find a *HRPT2* mutation on the remaining allele of either of the sporadic adenomas with LOH at 1q24-32 in this study and suggest that the LOH at 1q24-32 in the majority of sporadic adenomas may in fact be targeted at a tumour suppressor gene elsewhere on chromosome 1. To date, there is no evidence that LOH at 1q24-32 alone confers a higher likelihood of malignancy.

In conclusion, the results in this study support a role for *HRPT2* as a tumour suppressor gene in agreement with Knudson's "two-hit" hypothesis in sporadic parathyroid carcinoma. These results also provide further evidence for *HRPT2* as the causative gene in HPT-JT, and a subset of FIHP. Efficient detection of *HRPT2* mutations should begin with exons 1, 2, and 7, which together harbour 80% of mutations found to date. In light of the strong association between mutations of *HRPT2* and sporadic parathyroid carcinoma demonstrated in this study, we hypothesise that *HRPT2* mutation is an early event that may lead to parathyroid malignancy and therefore suggest intragenic mutation of *HRPT2* as a marker of parathyroid malignant potential in both familial and sporadic tumours. Further studies will clarify the exact nature of the relationship between intragenic mutation of *HRPT2* and parathyroid malignancy.

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