***CDC73/HRPT2* CpG island hypermethylation and mutation of 5'-untranslated sequence are uncommon mechanisms of silencing parafibromin in parathyroid tumors**

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Abstract

The tumor suppressor *HRPT2/CDC73* is mutated in constitutive DNA from patients with the familial disorder hyperparathyroidism–jaw tumor syndrome and in ~70% of all parathyroid carcinomas. In a number of *HRPT2* mutant tumors however, expression of the encoded protein parafibromin is lost in the absence of a clear second event such as *HRPT2* allelic loss or the presence of a second mutation in this tumor suppressor gene. We sought to determine whether hypermethylation of a 713 bp CpG island extending 648 nucleotides upstream of the *HRPT2* translational start site and 65 nucleotides into exon 1 might be a mechanism contributing to the loss of expression of parafibromin in parathyroid tumors. Furthermore, we asked whether mutations might be present in the 5'-untranslated region (5'-UTR) of *HRPT2*. We investigated a pool of tissue from 3 normal parathyroid glands, as well as 15 individual parathyroid tumor samples including 6 tumors with known *HRPT2* mutations, for hypermethylation of the *HRPT2* CpG island. Methylation was not identified in any specimens despite complete loss of parafibromin expression in two parathyroid carcinomas with a single detectable *HRPT2* mutation and retention of the wild-type *HRPT2* allele. Furthermore, no mutations of a likely pathogenic nature were identified in the 5'-UTR of *HRPT2*. These data strongly suggest that alternative mechanisms such as mutation in *HRPT2* intronic regions, additional epigenetic regulation such as histone modifications, or other regulatory inactivation mechanisms such as targeting by microRNAs may play a role in the loss of parafibromin expression.

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Introduction

The tumor suppressor *HRPT2*, also known as *CDC73*, maps to 1q31.2 (Ensembl release 55), and has 17 exons encoding the 531 amino acid protein parafibromin. Mutation of *HRPT2* has been strongly associated with parathyroid tumorigenesis with mutations reported in patients with the familial disorder hyperparathyroidism–jaw tumor (HPT–JT) syndrome (OMIM #145001; Carpten *et al.* 2002, reviewed in Marsh *et al.* (2007))

and in a small subset of patients classified as familial isolated HPT (FIHP) (OMIM #145001; reviewed in Marsh *et al.* (2007)). Inactivating *HRPT2* mutations are found in ~70% of parathyroid carcinomas but only rarely in non-HPT–JT benign parathyroid tumors (Howell *et al.* 2003, Shattuck *et al.* 2003, Cetani *et al.* 2004, Krebs *et al.* 2005, Gill *et al.* 2006). Mutations in *HRPT2* have also been identified in sporadic kidney tumors (Zhao *et al.* 2007). Approximately, 80% of mutations occur in exons 1, 2, or 7,

Table 1 *HRPT2* mutation, putative *HRPT2* promoter variant, and allelic loss in parathyroid tumor specimens

Sample	Tumor type	<i>HRPT2</i> allelic loss	– 466C > A	<i>HRPT2</i> mutation
1	Sporadic adenoma	LOH	A	nmd
2	Sporadic adenoma	R	CA	nmd
3	Sporadic adenoma	R	AA	nmd
4	Sporadic adenoma	R	CA	nmd
5	Sporadic adenoma	R	CA	nmd
6	Sporadic adenoma	LOH	A	nmd
7	Primary hyperplasia	R	AA	nmd
8	Primary hyperplasia	R	CA	nmd
9	Primary hyperplasia	R	AA	nmd
10	HPT–JT adenoma	LOH	A	c.191C > T (L64P)
11	HPT–JT carcinoma	LOH	– ^a	c.76delA
12	HPT–JT carcinoma	R	– ^a	c.76delA, c.686delGAGT
13	Sporadic carcinoma	R	– ^a	c.76delA
14	Sporadic carcinoma	R	– ^a	IVS6-1delG, c.165C > A (Y55X)
15	Sporadic carcinoma	R	– ^a	c.162C > G (Y54X)

All mutations have been previously reported (3, 12). Genotype is recorded for a variant identified in the putative *HRPT2* promoter 466 nucleotides upstream of the *HRPT2* translational start site. nmd, no mutation detected; LOH, loss of heterozygosity; R, retention of heterozygosity.

^aReported in Table 2.

with the majority predicted to inactivate parafibromin by prematurely truncating this protein (reviewed in Marsh *et al.* (2007)).

Loss of heterozygosity at the *HRPT2* locus and ‘second-hit’ mutations in the coding region of *HRPT2* have been reported and are consistent with Knudson’s ‘two-hit’ hypothesis for inactivation of tumor suppressor genes (Howell *et al.* 2003, Bradley *et al.* 2006, Kelly *et al.* 2006, Cetani *et al.* 2007). However, allelic loss at the *HRPT2* locus is not always observed in parathyroid tumors with either a known or suspected *HRPT2* mutation (Carpten *et al.* 2002, Howell *et al.* 2003, Shattuck *et al.* 2003). Therefore, it would appear that despite the strong association with parathyroid tumorigenesis, mutation within the coding region of *HRPT2* or the loss of heterozygosity at 1q31.2 does not account for all reported cases of HPT–JT or sporadic parathyroid tumors. Mutation in non-coding regions of *HRPT2* or the involvement of other genes cannot be discounted. For example, mutation of the *PTEN* promoter has been reported in a number of patients with Cowden syndrome (Zhou *et al.* 2003). Furthermore, hypermethylation of an *HRPT2* CpG island may

play a role in silencing the expression of parafibromin in some parathyroid tumors.

The majority of cancer-associated CpG islands are located in the 5′-untranslated region (5′-UTR) and the first exon of tumor suppressor genes (Esteller 2007). In these islands, clusters of cytosines preceding guanines, CpGs, can undergo the addition of a methyl group to the 5 position of the cytosine ring. This process is mediated by DNA methyltransferases and leads to aberrant methylation resulting in down-regulation of gene expression. Well-known tumor suppressor genes associated with endocrine neoplasia syndromes where hypermethylation occurs in familial and sporadic counterpart tumors include *VHL* and *PTEN* (Esteller 2007). Here, we report the results of investigations into the methylation status of a CpG island within the 5′-UTR and first exon of *HRPT2* in normal parathyroid tissue, as well as benign and malignant parathyroid tumors. Furthermore, we report sequence analyses of an almost 2 kb segment of the 5′-UTR region of *HRPT2*. Data are presented in conjunction with *HRPT2* mutation status, allelic status at the *HRPT2* locus, and expression of parafibromin.

Table 2 Genotype of variants identified in the 5′-UTR of *HRPT2* in parathyroid carcinoma

Parathyroid carcinoma	– 1850 T > C (rs10754042 ^a)	– 1849 G > T (rs72731058 ^a)	– 1504 T > G (rs10754043 ^a)	– 1302 T > C	– 466 C > A (rs10737621 ^a)
11	C	T	G	T	C
12	TC	GT	TG	TT	CC
13	TC	GT	TG	TT	CC
14	TC	GG	TG	TC	CA
15	TC	GG	GG	TC	CA

^aReported in the NCBI human SNP database.

Materials and methods

CpG island identification

The web-based resource CpG plot (<http://www.ebi.ac.uk/emboss/cpgplot>) was used to analyse over 2500 nucleotides upstream of the *HRPT2* translational start site, as well as the first exon of *HRPT2*, for the presence of regions rich in CpGs (clone RP11-239J11, human DNA sequence from chromosome 1 containing the 5' end of the *HRPT2* gene, NCBI Accession No. AL390863).

Tumor samples

A single pool of tissue from 3 normal parathyroid glands and 15 individual parathyroid tumor samples consisting of 6 adenomas, 3 primary hyperplasias, and 6 tumors harboring known *HRPT2* mutations were studied (Tables 1 and 2). Samples were obtained from Royal North Shore Hospital, Sydney, NSW, Australia and the Institute of Pathology, University of Halle, Halle, Germany, according to protocols in place for each centres' human ethics committee. DNA was extracted from tissue stored at -80°C according to standard procedures. The *HRPT2* mutation status and allelic loss in the *HRPT2* region have been previously reported for the majority of these specimens (Howell *et al.* 2003, Haven *et al.* 2004). For all samples, the entire coding and flanking intronic regions were sequenced.

Immunohistochemical analyses for the expression of parafibromin were carried out as previously reported (Gill *et al.* 2006).

Methylation analysis

Bisulfite treatment of extracted DNA was performed using the MethylEasy DNA Bisulfite Modification Kit (Human Genetic Signatures, Sydney, NSW, Australia) according to the manufacturer's instructions. The amount of starting DNA varied between 100 and 1000 ng per sample, and DNA was resuspended in 20 μl H_2O after bisulfite treatment. PCR was performed using 1 μl bisulfite-treated DNA. CpGenome Universal Methylated and Unmethylated DNA (Chemicon Australia, Boronia, VIC, Australia) were used as controls for these assays.

Primers were designed to amplify fully converted (C $\rightarrow$ T) bisulfite-treated DNA. The primers did not contain any CpGs and therefore amplified both methylated and unmethylated DNA. A semi-nested approach was used in which a first round PCR was performed using the primers 5'-GTTATTATTTAAAGAAATGAAGAGGGAGGATTT-3' (primer 1, Fig. 1) and 5'-ACAATCTCCTTCTTCTAAATATTATAC-TATC-3' (primer 3, Fig. 1). One microliter from the first round reaction was used as template in a second round PCR using the nested primer 5'-GTTGGG-GATGGAAGTGTGATTATTGTTT-3' (primer 2, Fig. 1) and the same reverse primer (primer 3) as previously. PCR was performed using the PCRx Enhancer System (Invitrogen Australia Pty Ltd) and the following cycling conditions: 95°C for 3 min, followed by 30 cycles at 95°C for 1 min, 50°C for 2 min, and 72°C for 2 min followed by a final extension at 72°C for 10 min.

Figure 1 *HRPT2* 5'-UTR. (A) Schematic representation of the conserved sequence alignment and conservation scoring of ~2000 nucleotides of the *HRPT2* 5'-UTR across 17 different vertebrate species. Degree of sequence conservation is indicated by shading, with darker regions representing stronger conservation (UCSC Genome Browser with VISTA tracks, <http://enhancer.lbl.gov/>). The region represented by -284 to +1 contains the strongest areas of conservation within the 5'-UTR (area sequenced extends to -1953) and constitutes part of the CpG island. (B) *HRPT2* contains a 713 nucleotide CpG island harboring 78 CpGs extending 648 nucleotides upstream of the translation start site and 65 nucleotides into exon 1. Individual CpGs are marked by solid circles. The positions of primers used for amplification of the CpG island are indicated.

PCR products were purified using the Wizard PCR Preps Purification System (Promega Australia) and directly sequenced using primer 3 (SUPAMAC, The University of Sydney, NSW, Australia).

Analysis of the 5'-UTR of *HRPT2*

Primer pairs were designed to amplify two overlapping regions of the 5'-UTR and first exon of *HRPT2*; –2002 to –652 relative to the *HRPT2* translational start site, 5'-TCTGTAGACACAGGGTCTAG-3' and 5'-GACCTCTAGGACTTAGTGATCC-3', and –1135 to +150, 5'-CACTGAGACTTCTGAGTACC-3' and 5'-ACAGCCATGCCGGAAGTAA-3'. PCR amplification was performed using Platinum Taq (Invitrogen Australia Pty Ltd) and the following cycling conditions: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min followed by a final extension at 72 °C for 10 min. Fragments were gel purified (UltraClean GelSpin DNA Purification Kit, MO BIO Laboratories, Inc., Carlsbad, CA, USA) and sequenced using respectively 5'-TCTGTAGACACAGGGTCTAG-3' and 5'-GTACTGAGTGCTCTGACACAGG-3', and 5'-ACAGCATGCCGGAAGTAA-3' and 5'-CGCACGTACGGGGAAGTTTG-3'. A fifth internal primer was also used for sequencing 5'-TTAGATCGGACCCAGGCC-TAGC-3'. These primer combinations facilitated the interrogation of 1953 nucleotides of the *HRPT2* 5'-UTR in tumor samples studied.

Results

Identification of a CpG island in the 5'-UTR of *HRPT2*

Using CpG plot, a 713 bp CpG island containing 78 CpGs was identified beginning 648 nucleotides upstream of the *HRPT2* translational start site and extending 65 nucleotides into exon 1 of *HRPT2* (Fig. 1). Although the promoter region of *HRPT2* remains to be experimentally determined, this CpG island contains the most highly evolutionarily conserved regions of the *HRPT2* 5'-UTR predicted by sequence alignment of 17 vertebrate species (UCSC Genome Browser with VISTA tracks, <http://enhancer.lbl.gov/>; Fig. 1A). This computational prediction would suggest that this region, and in particular regions within the segment between –284 and +1, may play a significant role in regulating *HRPT2* transcription.

Direct sequencing of bisulfite-converted DNA

We employed direct sequencing of PCR products amplified from bisulfite-converted DNA. A semi-nested PCR approach amplified a 563 bp fragment of the *HRPT2* CpG island (Fig. 1). Sequence data are presented using the reverse primer that allowed interrogation of 67 of the 78 CpGs within this island (Fig. 2). An example of a sequence chromatogram obtained from bisulfite-treated DNA from a parathyroid carcinoma harboring a single *HRPT2*

Figure 2 Sequence chromatograms, reversed and complemented, of bisulfite-treated DNA. (A) Complete sequence obtained from a PCR product amplified from bisulfite-converted DNA from a sporadic parathyroid carcinoma tissue sample (sample 15). CpGs within the island identified by CpG plot are numbered. The 5'-UTR variant –466C>A is indicated with *, and the translation start site is marked with an arrow. The region depicted in panel B is indicated by a black bar. (B) Representative sequence obtained from PCR products amplified from positive (methylated) and negative control (unmethylated) bisulfite-converted DNA (top two panels) and bisulfite-converted DNA obtained from a pooled normal, sporadic carcinoma, adenoma, and hyperplastic parathyroid tissue showing no methylation. CpGs 65–74 are shaded grey.

mutation, Y54X, and displaying retention of heterozygosity (sample 15, Table 2) shows no methylation, i.e. complete conversion of all cytosines to thymines (Fig. 2A).

Hypermethylation of the *HRPT2* CpG island is not present in normal parathyroid tissue and parathyroid tumors

Sequence data obtained from PCR products of bisulfite-treated DNA from all 15 parathyroid tumors and pooled normal parathyroid tissue showed no methylation. This was consistent with analyses of unmethylated negative control DNA (Fig. 2B). In contrast, sequencing of the methylated positive control DNA revealed methylation at each CpG, i.e. retention of the cytosine, and complete conversion of cytosines to thymines at all non-CpG sites. Representative sequence chromatograms spanning CpGs 65–74 for both the methylated positive and unmethylated negative control samples as well as an example of each type of parathyroid tumor examined are shown in Fig. 2B.

Two-hit mechanisms of *HRPT2*-associated parathyroid tumorigenesis

From our previous studies, six tumors were shown to harbor *HRPT2* mutations (Tables 1 and 2; Howell *et al.* 2003). Two tumors had both a single *HRPT2* mutation and *HRPT2* allelic loss (samples 10 and 11). An additional two tumors had two *HRPT2* mutations and retention of heterozygosity at the *HRPT2* locus (samples 12 and 14). Previously reported immunohistochemical analyses for these tumors showed weak or complete absence of nuclear parafibromin (Gill *et al.* 2006) consistent with biallelic inactivation of *HRPT2*. These results suggest that either double mutation or mutation combined with allelic loss functions as two-hit mechanisms to silence parafibromin. Two additional carcinoma specimens (samples 13 and 15) harbored only single *HRPT2* mutations and retention of heterozygosity; however, immunohistochemical analyses showed complete loss of expression of parafibromin in both cases (Fig. 3D and F).

Mutational analysis of the *HRPT2* 5'-UTR in five carcinoma samples

Mutation in the 5'-UTR of *HRPT2* was investigated as a possible second-hit mechanism that may result in the loss of parafibromin staining. In carcinoma samples 11–15, 1953 nucleotides upstream of the *HRPT2* translational start site were sequenced. Five single

nucleotide alterations were identified, four of which are reported in the NCBI single nucleotide polymorphism (SNP) database (<http://www.ncbi.nlm.nih.gov/snp>): –1850T>C (rs10754042), –1849G>T (rs72731058), –1504T>G (rs10754043), and –466C>A (rs10737621). The last of these SNPs, –466C>A, was present in the fragment sequenced to assess *HRPT2* methylation; therefore, data are available for all samples (Tables 1 and 2; Fig. 2A). A fifth variant, –1302 T>C, was not present in the NCBI SNP database. None of the genotypes showed specific association with carcinoma samples harboring either one or two hits (Tables 1 and 2), suggesting that these are likely polymorphisms not associated with disease and do not act as 'second-hits' to inactivate *HRPT2*. A stretch of 13 Ts, –810 to –798, was unable to be reliably assessed by these analyses; however, variants in this region have also been reported in the NCBI SNP database.

Discussion

We have identified a 713 bp CpG island in *HRPT2* that extends 648 nucleotides upstream of the translation start site and 65 nucleotides into exon 1. Using direct sequencing of PCR products amplified from bisulfite-treated DNA, we have shown that the *HRPT2* CpG island is not methylated in normal parathyroid tissue or benign or malignant parathyroid tumors. Two sporadic carcinomas harboring a single *HRPT2* mutation showed complete absence of parafibromin. These tumors did not have a detectable second-hit event that in other *HRPT2* mutant samples was either loss of the wild-type allele or biallelic inactivation due to a second *HRPT2* mutation. While these tumors did harbor a number of variants in the 5'-UTR region, most have either been reported in the NCBI SNP database or were found in tumors where a two-hit mechanism had already been established. While this study does not exclude the possibility that these SNPs represent low-risk variants for the development of parathyroid carcinoma, we provide no evidence that they participate in a two-hit mechanism leading to the loss of parafibromin. Therefore, in the tumors studied, mutations in the 5'-UTR of *HRPT2* or hypermethylation of the *HRPT2* CpG island are not alternative second-hit mechanisms that might explain complete silencing of parafibromin.

The lack of *HRPT2* hypermethylation found in this study is consistent with two previous case reports. CpG hypermethylation was not identified in DNA extracted from five parathyroid tumors and four matched peripheral leucocyte specimens from members of a

Figure 3 Sequential H&E (A, C, and E) and parafibromin-stained (B, D, and F) sections of normal parathyroid tissue (A and B) and two parathyroid carcinomas (sample 13 – C and D, sample 15 – E and F) showing the loss of parafibromin in carcinoma specimens. Non-neoplastic cells in sample 15 adjacent to the tumor field shown (panel D) stained positive for parafibromin (not shown).

single FIHP kindred harboring the germline *HRPT2* mutation c.518–521del (Mizusawa et al. 2006). Two of these five tumors demonstrated biallelic inactivation with the identification of the somatic mutations c.70–73del and c.95–102del. All five specimens exhibited retention of heterozygosity; however, immunohistochemistry for parafibromin was not reported; therefore, it is unknown whether parafibromin was silenced in these tumors. In a second study of a HPT–JT kindred harboring the germline *HRPT2* mutation c.433–442delinsAGA, two parathyroid tumors without additional somatic mutations as well as matched leucocyte DNA did not exhibit hypermethylation of the *HRPT2* CpG island (Masi et al. 2008). While allelic loss was not described in this study, the loss of parafibromin expression was shown by immunohistochemistry in these tumors.

When combining these reports with the present study, lack of *HRPT2* CpG island hypermethylation has now been described in seven parathyroid tumors with an apparently single *HRPT2* mutation as well as in non-*HRPT2*-associated benign parathyroid tumors. These findings differ from a single study reporting *HRPT2* CpG island methylation in 2 of 11 parathyroid carcinomas and 1 of 6 adenomas from 2 patients with HPT–JT (Hewitt et al. 2007). As that study did not investigate *HRPT2* mutation status, loss of heterozygosity at the *HRPT2* locus, or the expression of parafibromin, it is difficult to interpret these data in the context of hypermethylation being an important mechanism leading to the loss of parafibromin expression. Consistent with the present study that showed no evidence of *HRPT2* methylation in benign non-HPT–JT tumors, hypermethylation of the

Table 3 Tumor suppressor genes implicated in endocrine neoplasia syndromes – frequency of methylation in counterpart tumors

Gene	Tumor	Methylation	Methylation detection method	Study
<i>HRPT2</i>	Parathyroid		Sequencing	This study
	Adenoma (non-HPT–JT)	0/6 (0%)		
	Primary hyperplasia (non-HPT–JT)	0/3 (0%)		
	Adenoma (HPT–JT, <i>HRPT2</i> mutant)	0/1 (0%)		
	Carcinoma (HPT–JT, <i>HRPT2</i> mutant)	0/2 (0%)		
	Carcinoma (sporadic, <i>HRPT2</i> mutant)	0/3 (0%)		
	Parathyroid (FIHP-associated)	0/5 (0%)	Sequencing ^a	Mizusawa <i>et al.</i> (2006)
	Parathyroid (HPT–JT-associated)	0/2 (0%)	Sequencing ^a	Masi <i>et al.</i> (2008)
	Parathyroid		MSP and sequencing	Hewitt <i>et al.</i> (2007)
	Adenoma (non-HPT–JT)	0/37 (0%)		
<i>VHL</i>	Adenoma (HPT–JT-associated)	1/6 (17%)		
	Carcinoma	2/11 (18%)		
	Pheochromocytoma	0/53 (0%)	Methylation-sensitive restriction endonuclease digestion	Bender <i>et al.</i> (2000)
		0/35 (0%)	MSP and sequencing	Cascon <i>et al.</i> (2004)
		0/25 (0%)	MSP	Dammann <i>et al.</i> (2005)
	Renal cell carcinoma	5/26 (19%)	Methylation-sensitive restriction endonuclease digestion	Herman <i>et al.</i> (1994)
		11/99 (11%)	Methylation-sensitive restriction endonuclease digestion	Clifford <i>et al.</i> (1998)
		13/77 (17%)	Methylation-sensitive restriction endonuclease digestion	Brauch <i>et al.</i> (2000)
		11/202 (5%)	MSP	Kondo <i>et al.</i> (2002)
		19/93 (20%)	MSP	Banks <i>et al.</i> (2006)
		16/185 (9%)	MSP	Smits <i>et al.</i> (2008)
		17/205 (8%)	Sequencing	Nickerson <i>et al.</i> (2008)
	Hemangioblastoma	0/9 (0%)	Methylation-sensitive restriction endonuclease digestion	Tse <i>et al.</i> (1997)
		0/42 (0%)	Methylation-sensitive restriction endonuclease digestion	Glasker <i>et al.</i> (2001)
<i>SDHB</i>	Pheochromocytoma	9/28 (32%)	Sequencing ^a	Astuti <i>et al.</i> (2004)
		0/35 (0%)	MSP and sequencing	Cascon <i>et al.</i> (2004)
<i>SDHD</i>	Pheochromocytoma	0/35 (0%)	MSP and sequencing	Cascon <i>et al.</i> (2004)
<i>PTEN</i> ^b	Thyroid		MSP	Alvarez-Nunez <i>et al.</i> (2006)
	Papillary thyroid carcinoma	21/46 (46%)		
	Follicular thyroid carcinoma	6/7 (86%)		
	Follicular thyroid adenoma	5/6 (83%)		
	Thyroid		MSP	Schagdarsurengin <i>et al.</i> (2006)
	Medullary thyroid carcinoma	0/6 (0%)		
	Follicular thyroid carcinoma	0/10 (0%)		
	Papillary thyroid carcinoma	2/12 (17%)		
	Undifferentiated thyroid cancer	1/7 (14%)		
	Follicular thyroid adenoma	2/9 (22%)		
	Endometrial carcinoma	26/138 (19%)	Methylation-sensitive restriction endonuclease digestion and MSP	Salvesen <i>et al.</i> (2001)
	Breast	43/90 (48%)	MSP and sequencing	Garcia <i>et al.</i> (2004)
		15/44 (34%)	MSP	Khan <i>et al.</i> (2004)
<i>MEN1</i>	Pancreatic ductal adenocarcinoma	5/24 (21%)	MSP	Cavallari <i>et al.</i> (2009)

^aRefers to sequencing of cloned PCR products from bisulfite-treated DNA. Sequencing otherwise refers to sequencing of a PCR product amplified directly from bisulfite-treated genomic DNA. MSP refers to 'methylation-specific PCR' where primers have been designed to amplify either methylated or unmethylated bisulfite-treated genomic DNA. Methylation-sensitive restriction endonuclease digestion involves digestion of genomic DNA with a methylation-sensitive enzyme followed by either PCR amplification where fragments will only amplify in the absence of digestion, or Southern blotting that recognizes a methylation-specific digest pattern.

^bCare must be taken when interpreting *PTEN* hypermethylation data due to 5'-UTR sequence similarities between *PTEN* and the *PTEN* pseudogene (Zysman *et al.* 2002).

HRPT2 CpG island was not reported in 37 sporadic parathyroid adenomas (Hewitt et al. 2007).

Hypermethylation of growth suppressing genes has not been extensively studied in parathyroid tumors. The Rb-interacting zinc finger gene *RIZ1* is however hypermethylated in 36% of non-familial, predominantly benign parathyroid tumors, occurring in conjunction with allelic loss (Carling et al. 2003). Interestingly, *RIZ1* is also hypermethylated in malignant pheochromocytoma (Carling et al. 2003). Hypermethylation of the multidrug resistance 1 (*MDR1*) gene associated with silencing of the encoded ABC-transported protein has also been reported in benign parathyroid tumors (Takeuchi et al. 2007).

HPT-JT and FIHP are part of the familial endocrine neoplasia syndromes with known germline mutations in tumor suppressor genes that include Cowden syndrome (OMIM #158350; *PTEN*), von Hippel-Lindau syndrome (OMIM #193300; *VHL*), familial pheochromocytoma and paraganglioma syndrome (OMIM #115310; *SDHB*, *SDHC*), Carney complex (OMIM #160980; *PRKARIA*), and multiple endocrine neoplasia type 1 (OMIM #131100; *MEN1*; reviewed in Marsh & Zori (2002)). A sixth endocrine neoplasia syndrome, *MEN2* (OMIM #171400), is caused by activating mutations of the *RET* proto-oncogene, excluding a role for gene silencing (Mulligan et al. 1993). With the exception of *PRKARIA* that is yet to be investigated for methylation, hypermethylation of these tumor suppressors has been studied in a number of familial and sporadic counterpart tumors of these syndromes (Table 3). *PTEN* appears to be the most frequently hypermethylated gene with methylation reported in sporadic Cowden syndrome component tumors thyroid (Alvarez-Nunez et al. 2006, Schagdarsurengin et al. 2006), endometrial (Salvesen et al. 2001), and breast (Garcia et al. 2004, Khan et al. 2004). Of interest, *PTEN* methylation appears to be correlated with more aggressive breast tumors (Garcia et al. 2004, Khan et al. 2004) and with metastatic disease in endometrial carcinoma (Salvesen et al. 2001). Hypermethylation of *PTEN* is also seen in sporadic tumors that are not observed in Cowden syndrome such as colorectal tumors (Goel et al. 2004).

While *VHL* and *SDHD* do not appear to be methylated in pheochromocytoma (Bender et al. 2000, Cascon et al. 2004, Dammann et al. 2005), one study does show approximately one-third of sporadic pheochromocytomas with methylation of the *SDHB* promoter (Astuti et al. 2004). *VHL* is hypermethylated in between 5 and 20% of renal cell carcinomas

(Herman et al. 1994, Clifford et al. 1998, Brauch et al. 2000, Kondo et al. 2002, Banks et al. 2006, Nickerson et al. 2008, Smits et al. 2008), but not in hemangioblastoma (Tse et al. 1997, Glasker et al. 2001). Limited studies have been conducted regarding a role for CpG island hypermethylation in the regulation of *MEN1*; however, a recent study in pancreatic ductal adenocarcinoma tumors, distinct from the endocrine pancreatic tumors present in *MEN1*, has shown methylation in 21% of these tumors (Cavallari et al. 2009). In a number of studies of CpG island methylation of these tumor suppressor genes in familial and sporadic counterpart tumors, hypermethylation has been shown to function as a second-hit event important for biallelic inactivation (Table 3).

If hypermethylation does play a role in silencing parafibromin in parathyroid tumors, it would appear to be infrequent and confined to a small number of parathyroid carcinomas or HPT-JT-associated adenomas that likely harbor a mutation in *HRPT2* and have been previously described as being of increased 'malignant potential' (Howell et al. 2003, Hewitt et al. 2007). Like *VHL* that is not hypermethylated in all tumor types seen in *VHL*, it is possible that hypermethylation of the *HRPT2* CpG island may be seen at greater frequencies in kidney and jaw tumors that are also component tumors of HPT-JT.

Data presented in the current study suggest that parafibromin can be inactivated by as yet undescribed mechanisms. These may include mutation in intronic regions of *HRPT2*, alternative epigenetic mechanisms such as histone modifications (reviewed in Esteller (2007)), aberrant expression of genes that regulate parafibromin, or still other mechanisms of regulatory inactivation such as targeting by micro-RNAs (Ross et al. 2007, Corbetta et al. 2009).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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