Parafibromin immunoreactivity: its use as an additional diagnostic marker for parathyroid tumor classification

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Abstract

Parafibromin is a protein product derived from the hyperparathyroidism 2 (*HRPT2*) tumor suppressor gene and its inactivation has been coupled to familial and sporadic forms of parathyroid malignancy. In this study, we have conducted immunohistochemistry on 33 parathyroid carcinomas (22 unequivocal and 11 equivocal) using four parafibromin antibodies directed to different parts of the protein. Furthermore, for a fraction of cases, the immunohistochemical results were compared with known *HRPT2* mutational status. Our findings show that 68% (15 out of 22) of the unequivocal carcinomas exhibited reduced expression of parafibromin while the 25 sporadic adenomas used as controls were entirely positive for parafibromin expression. Additionally, three out of the six carcinomas with known *HRPT2* mutations showed reduced expression of parafibromin. Using all four antibodies, comparable results were obtained on the cellular level in individual tumors suggesting that there exists no epitope of choice in parafibromin immunohistochemistry. The results agree with the demonstration of a ~60 kDa product preferentially in the nuclear fraction by western blot analysis. We conclude that parafibromin immunohistochemistry could be used as an additional marker for parathyroid tumor classification, where positive samples have low risk of malignancy, whereas samples with reduced expression could be either carcinomas or rare cases of adenomas likely carrying an *HRPT2* mutation.

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Introduction

Parathyroid carcinoma is an uncommon but potentially life-threatening form of primary hyperparathyroidism (PHPT; Grimelius & Johansson 1997, Shane 2001, Rodgers & Perrier 2006). The patients can develop a palpable mass in the neck, concomitant kidney, and bone disease, and frequently high parathyroid hormone (PTH) levels along with persistent hypercalcemia in the case of locally invasive or metastatic disease (Mittendorf & McHenry 2005). Early detection and *en bloc* resection of all tumor tissue is necessary for a favorable outcome (Rodgers & Perrier 2006). However, parathyroid carcinoma often presents a diagnostic challenge both concerning presurgical distinction between a highly active adenoma and the malignant counterpart, as well

as histopathological identification of carcinoma in the absence of invasive growth pattern (Sandelin *et al.* 1992, DeLellis 2005). While unequivocal parathyroid carcinoma shows indisputable evidence of malignancy such as invasion, metastasis, or recurrence (DeLellis *et al.* 2004), equivocal cancers only present characteristics associated with malignant behavior at histopathology (Bondeson *et al.* 1993). The difficulties in parathyroid tumor diagnosis are further exemplified by the histological subgroup ‘atypical adenoma’ which is considered of uncertain malignant potential (Levin *et al.* 1987). In the search of a marker for parathyroid malignancy, analysis of DNA content, and immunohistochemical expression of pRb, bcl-2, Ki-67, cyclin D1, p21, and p53 have been evaluated, but not found to be specific enough

(Bondeson *et al.* 1993, Cryns *et al.* 1994, Erickson *et al.* 1999, Farnebo *et al.* 1999, Vasef *et al.* 1999, Stojadinovic *et al.* 2003).

The hyperparathyroidism 2 (*HRPT2*) gene is the disease gene for hyperparathyroidism–jaw tumor syndrome (HPT–JT), characterized by benign and malignant PHPT in combination with tumors of the jaws, kidney, and uterus (Carpén *et al.* 2002, Bradley *et al.* 2005, Gimm *et al.* 2006). Its encoded protein parafibromin is a member of the polymerase-associated factor 1 (PAF1) complex associated with RNA polymerase II which regulates transcription elongation and histone modification (Carpén *et al.* 2002, Rozenblatt-Rosen *et al.* 2005, Yart *et al.* 2005). *In vitro* studies have revealed dominant negative tumor suppressor properties (Zhang *et al.* 2006), a functional nuclear localization signal (NLS; Hahn & Marsh 2005, Woodard *et al.* 2005, Zhang *et al.* 2006, Bradley *et al.* 2007), and coupling to the wntless type (Wnt) signaling pathway (Mosimann *et al.* 2006).

Inactivating *HRPT2* mutations are frequently detected on the somatic and sometimes germ line levels in apparently sporadic parathyroid carcinoma (Howell *et al.* 2003, Shattuck *et al.* 2003, Cetani *et al.* 2004), as opposed to a small minority of adenomas (Carpén *et al.* 2002, Howell *et al.* 2003, Krebs *et al.* 2005, Bradley *et al.* 2006, Juhlin *et al.* 2006). In immunohistochemical studies of unequivocal and HPT–JT-related carcinomas, complete loss, or reduced nuclear immunoreactivity was found in the majority of cases. By contrast, 98–100% of unselected sporadic adenomas stained completely positive (Tan *et al.* 2004, Gill *et al.* 2006). In a subset of cystic adenomas, loss of parafibromin has been associated with *HRPT2* mutations (Juhlin *et al.* 2006).

Here, we have evaluated immunohistochemical expression of parafibromin in parathyroid carcinomas using antibodies directed to different epitopes of the protein and further related the findings to western blot analyses and genetic information.

Materials and methods

Parathyroid carcinoma patients and tissue samples

This study includes 33 tumor samples collected worldwide from 32 patients (Table 1). Twenty-four samples are part of a previously published historical material (Bondeson *et al.* 1993, Farnebo *et al.* 1999). Six cases carry inactivating *HRPT2* mutations, five of which have been previously reported (Shattuck *et al.* 2003). Three additional samples were newly collected

with informed consent and ethical approval from two patients undergoing surgery at Karolinska University Hospital in Stockholm.

Histopathological classifications of tumor specimens were according to the WHO criteria. Twenty-two patients had definite (unequivocal) cancer with vascular invasion, and/or local invasion of surrounding organs, and/or distant metastasis, and/or evidence of recurrence after parathyroid surgery. They also exhibited tumor characteristics associated with parathyroid cancer such as fibrous bands, mitotic figures, focal necrosis, macronucleoli, and distinct cellular atypia (T1–T22, Table 1). The 11 cases classified as equivocal carcinomas exhibited at least one histopathological indication of malignancy mentioned above, but were without evidence of recurrence, invasion, or metastases (T23–T33, Table 1). Sample T13 was originally classified as an adenoma; however, this tumor relapsed 4 years after primary surgery thereby establishing the diagnosis as unequivocal carcinoma in this patient.

Control samples

The following positive controls were used: HeLa cells transfected with plasmid DNA containing full-length *HRPT2* cDNA and two parathyroid carcinomas (T4 and T23) for western blot and immunohistochemistry (Juhlin *et al.* 2006), four paraffin-embedded adenomas on the same slide for immunohistochemistry including one with a rim of normal parathyroid (control adenoma 1) and 21 sporadic adenomas (the majority with a normal rim) as normal reference for immunohistochemistry. For sub-cellular fractionation and western blot analysis, frozen samples of two cystic adenomas with or without *HRPT2* gene mutation and parafibromin inactivation (corresponding to T20 and T23 in Juhlin *et al.* 2006), one secondary HPT gland, and a regular adenoma (control adenoma 2) were used. All samples were obtained with informed consent and local ethical approval.

Antibodies and blocking peptides

A rabbit polyclonal antibody TNYV was raised with ethical approval against aa 39–58 by the authors through AgriSera Co. (Umeå, Sweden) using published methodology (Polak & Van Noorden 1986), affinity purified and eluted at pH 7.0. The mouse monoclonal antibody 2H1 targets aa 87–100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). BL648 is an affinity-purified rabbit polyclonal antibody directed against a 24 aa sequence within aa 250–300 (Bethyl Laboratories, Montgomery, TX, USA). APVF, an

Table 1 Clinical details of the 33 unequivocal and equivocal parathyroid carcinoma cases in the study

											HRPT2 mutation status		
Case no.	Sex	Age at op	Tissue analyzed	S-Ca (mmol/l)	Initial diagnosis	Dead from disease	Recurrent disease	Time to recurrence	Final diagnosis	Weight (mg) or size (∅ in cm)	HRPT2 sequence	Mutation type	Predicted effect
Unequivocal cases													
T1	M	61 years	Lung metastasis	3.5	Carcinoma	Yes	Lung met	30 m	Carcinoma	3400 mg	226C>T ex2	Somatic	Stop at aa 76
T2	F	–	Lung metastasis	–	Carcinoma	–	Lung met	–	Carcinoma	–	664C>T ex7	Germ line	Stop aa 222
T3	M	25 years	Neck metastasis	–	Carcinoma	–	–	–	Carcinoma	–	–	–	–
T4	F	61 years	Neck metastasis	–	Carcinoma	Yes	–	–	Carcinoma	–	–	–	–
T5	F	35 years	Local rec	4.8	Carcinoma	–	Local rec, lung met	36 m	Carcinoma	–	–	–	–
T6	M	54 years	Local rec	3.8	Carcinoma	Yes	Local rec, lung met	13 m	Carcinoma	2500 mg	70G>T ex1, 746delT ex8	Somatic ×2	Stop at aa 24 and 256 respectively
T7	F	60 years	Local rec	–	Carcinoma	Yes	Local rec	5 m	Carcinoma	–	–	–	–
T8	M	52 years	Local rec	5.5	Carcinoma	Yes	Local rec	16 m	Carcinoma	–	–	–	–
T9	F	78 years	Local rec	5.0	Carcinoma	Yes	Local rec	–	Carcinoma	6600 mg	–	–	–
T10	–	–	Local rec	4.0	Carcinoma	–	Local rec	24 m	Carcinoma	–	–	–	–
T11	F	64 years	Primary tumor	3.0	Carcinoma	Yes	Local rec	72 m	Carcinoma	25 000 mg	–	–	–
T12	F	58 years	Primary tumor	3.3	Carcinoma	–	Local rec	24 m	Carcinoma	2 cm	–	–	–
T13	M	57 years	Primary tumor	3.3	Adenoma	–	Local rec	48 m	Carcinoma	2.7 cm	–	–	–
T14	F	32 years	Primary tumor	3.9	Carcinoma	–	–	–	Carcinoma	3100 mg	–	–	–
T15	F	63 years	Primary tumor	3.2	Carcinoma	–	–	–	Carcinoma	1.3 cm	–	–	–
T16	M	74 years	Primary tumor	3.2	Carcinoma	–	–	–	Carcinoma	3 cm ^a	–	–	–
T17	M	78 years	Primary tumor	3.1	Carcinoma	–	–	–	Carcinoma	5 cm	–	–	–
T18	F	85 years	Primary tumor	Elevated	Carcinoma	–	–	–	Carcinoma	3 cm	–	–	–
T19	M	78 years	Primary tumor	4.5	Carcinoma	–	–	–	Carcinoma	3.1 cm	–	–	–
T20	F	61 years	Primary tumor	4.9	Carcinoma	Yes	Lung met	14 m	Carcinoma	3100 mg	82del4 ex1, 732delT ex8	Somatic ×2	Stop at aa 35 and 256 respectively
(Continued)													

(Continued)

Table 1 (continued)

Case no.	Sex	Age at op	Tissue analyzed	S-Ca (mmol/l)	Initial diagnosis	Dead from disease	Recurrent disease	Time to recurrence	Final diagnosis	Weight (mg) or size (Ø in cm)	HRPT2 mutation status		
											HRPT2 sequence	Mutation type	Predicted effect
T21	M	–	Primary tumor	–	Carcinoma	–	–	–	Carcinoma	–	23TGCG>GTG ex1	Somatic	Stop at aa 20
T22	M	59 years	Primary tumor	4.0	Carcinoma	–	No	–	Carcinoma	5 cm	–	–	–
Equivocal cases													
T23	M	16 years	Primary tumor	4.0	Carcinoma	–	–	–	Carcinoma	1300 mg	c127insC ex1	Germ line	Stop at aa 65
T24	M	62 years	Primary tumor	–	Carcinoma	–	–	–	Carcinoma	2.5 cm	–	–	–
T25	F	66 years	Primary tumor	3.6	Carcinoma	–	–	–	Carcinoma	3 cm	–	–	–
T26	F	29 years	Primary tumor	3.7	Carcinoma	–	–	–	Carcinoma	19 000 mg	–	–	–
T27	F	58 years	Primary tumor	3.5	Carcinoma	–	–	–	Carcinoma	3 cm	–	–	–
T28	M	46 years	Primary tumor	3.5	Carcinoma	–	–	–	Carcinoma	3100 mg	–	–	–
T29	M	70 years	Primary tumor	Elevated	Carcinoma	–	–	–	Carcinoma	1.5 cm	–	–	–
T30	F	51 years	Primary tumor	3.0	Carcinoma	–	–	–	Carcinoma	–	–	–	–
T31	M	53 years	Primary tumor	3.3	Carcinoma	–	–	–	Carcinoma	1050 mg	–	–	–
T32	M	34 years	Primary tumor	4.3	Carcinoma	–	–	–	Carcinoma	10 800 mg	–	–	–
T33	F	28 years	Primary tumor	–	Carcinoma	–	–	–	Carcinoma	2 cm ^a	–	–	–

M, male; F, female; Local rec, local recurrence; Lung met, lung metastasis; –, not known, not determined; aa, amino acid; elevated, above normal range, no known numerical value.

^aCase T16 and T33 was originally described as 'walnut' and 'hazelnut' respectively.

affinity-purified rabbit polyclonal antibody raised against aa 509–531, have been previously published (Juhlin *et al.* 2006).

Blocking peptides used as controls were synthesized by the authors through AgriSera Co. or commercially available (BP648, Bethyl Laboratories). Dot blot experiments with immobilized peptide antigen confirmed that all four parafibromin antibodies bind to their respective immunization peptide (data not shown).

Western blot analyses

Total proteins were extracted according to standard procedures, quantified using a dye-binding assay (Bradford 1976), and used for western blot analyses using previously described methodology (Juhlin *et al.* 2006). The sub-cellular fractionation and verification with anti Lamin A/C and anti Prohibitin was performed as previously described or has been reported before (Forsberg *et al.* 2006). After electrophoresis and blotting, presence of protein was confirmed by staining with Ponceau Red solution (Sigma–Aldrich), and the membranes were incubated with the respective parafibromin antibody: 1.7 µg/ml TNYV (1:300), 4 µg/ml 2H1 (1:50), 0.2 µg/ml BL648 (1:5000), 1.7 µg/ml APVF (1:300), followed by the respective secondary HRP-conjugated antibody (goat anti-rabbit 1:12 500, goat anti-mouse 1:10 000 Bio-Rad Laboratories). In control experiments, the antibodies were preincubated with peptide antigen for 1 h at room temperature and 2 h at 4 °C.

Immunohistochemistry

Paraffin sections of parathyroid carcinomas and control samples were cut at 4 µm, deparaffinized, rehydrated, and immersed in preheated citrate buffer pH 6 (Dako, Glostrup, Denmark) at 95 °C for 20 min. In our experience, antigen retrieval by heating in citrate buffer was necessary to obtain a distinct nuclear signal without interfering background, in agreement with previous publications (Tan *et al.* 2004, Gill *et al.* 2006). For the monoclonal antibody 2H1, the following parameters were tested: antibody dilution (1:20, 1:100, and 1:200), antigen retrieval time (10, 20, 30, and 50 min) as well as incubation time (1 h and overnight). These parameters were studied in four adenomas on the same slide and in carcinomas T4, T14, T15, and T23. In our laboratory settings, we obtained expected positive immunoreactivity in the adenomas and the western blot verified carcinomas using citrate heating for 20 min, antibody dilution of 1:20, and overnight incubation. Positive signals were not obtained using

shorter antigen retrieval time, increased antibody dilution, or shorter incubation time.

The sections were incubated in 0.3% hydrogen peroxide in water for 30 min, blocked in 1% BSA with 0.01% sodium azide for 45 min, and incubated with primary antibody diluted in 1% BSA overnight using concentrations determined from dilution trials with positive controls: TNYV 2 µg/ml (1:250), 2H1 10 µg/ml (1:20), BL648 10 µg/ml (1:100), and APVF 2 µg/ml (1:250). The slides were then incubated with biotinylated secondary antibody (7.5 µg/ml (1:100) of goat anti-rabbit BA-1000 and horse anti-mouse B-200; Vector Laboratories, Burlingame, CA, USA) for 45 min, and the antigen–antibody complex was visualized using the avidin–biotin–peroxidase complex method (Vectastain Elite Kit, Vector Laboratories) for 45 min, followed by diaminobenzidine tetrahydrochloride for 6 min, and counterstaining in hematoxylin for 3 min. Sections were washed in five cycles of Tris-buffered saline (TBS, pH 7.6) between each step. Peptide neutralization tests for all four parafibromin antibodies were performed on all cases as negative controls.

Parafibromin expression was independently scored by two of the authors (C J and A H), whereby the levels of expression and the sub-cellular localization were evaluated. Each specimen was classified as having either ‘negative’ (staining of ≤10% tumor nuclei), ‘partial loss’ (staining of 11–89% of tumor nuclei), or ‘positive’ (staining of ≥90% of tumor nuclei) parafibromin expression. The partial loss group was further studied by scoring at least 1500 cells from a total of five randomly selected grid areas of the sections in high power magnification (40×). The cells were classified as having either positive or negative nuclear parafibromin expression. The negative controls were independently evaluated by three of the authors (C J, F H, and A H), including an author blinded of the primary positive findings. All slides were blinded of their diagnosis during the entire microscopic validation process.

Results

Antibody characterization and western blot analysis

The four parafibromin antibodies gave similar results with high affinity for a ~60 kDa band representing the predicted size of parafibromin (Figs 1 and 2). Furthermore blocking experiments with corresponding immunizing peptides confirmed the specificity of our findings. Strong expression was revealed in *HRPT2*-transfected HeLa cells, when compared with the weaker endogenous signal observed in untransfected

Figure 1 Schematic of the *HRPT2* gene, parafibromin, and the antibodies studied. (A) Genomic structure of *HRPT2*, its 17 coding exons and the gene product parafibromin. ATG represent the initiation codon. Functionally important regions are marked: the nuclear localization signal (NLS) is encoded from exon 5, the β -catenin interaction domain (CID) by exons 7 and 8, and the evolutionary conserved PAF1 complex-binding domain (Cdc73 core) constitutes exons 12–17. The targets of the four antibodies are shown below the schematic of parafibromin. (B) Details of the four parafibromin antibodies.

HeLa cell controls (Fig. 2). The secondary HPT gland showed positive parafibromin expression in the total and nuclear extracts, while in the cytosolic extract parafibromin was absent or showed weak expression (Fig. 2). Similar results were obtained at analysis of nuclear and cytosolic extracts from control adenoma 2

(Fig. 3B). In the cystic adenoma with wild-type *HRPT2* (Juhlin et al. 2006), all four antibodies detected a ~60 kDa product (Fig. 2), while the cystic adenoma with known *HRPT2* mutation (Juhlin et al. 2006) was negative for parafibromin by means of all four antibodies used (Fig. 2).

Figure 2 Western blot analyses of parafibromin expression. Parafibromin protein is demonstrated as a ~60 kDa protein in various parathyroid lesions using the antibodies TNYV, 2H1, BL648, and APVF. The expressions were visually classified as strong +, weak (+), or undetectable (—).

Figure 3 (A) Immunohistochemistry demonstrating mainly nuclear expression of parafibromin in control adenoma 1 as well as *HRPT2*-transfected HeLa cells using antibodies TNYV, 2H1, BL648, and APVF. Inserts are parathyroid cells from the normal glandular rim. The specificity is verified by peptide neutralization tests. All images are magnified 140 $\times$, while the insert are magnified 260 $\times$. (B) Western blot analysis after sub-cellular fractionation, showing parafibromin expression in the nuclear fraction of control adenoma 2. Prohibitin is a mitochondrial antigen.

Immunohistochemical analysis of parafibromin expression

In control experiments, *HRPT2*-transfected HeLa cells, the four regular parathyroid adenomas and normal parathyroid tissue (control adenoma 1) showed strong nuclear expression of parafibromin for each of the parafibromin antibodies, whereas the cytoplasm was only weakly positive in these samples (Fig. 3A). In addition, 21 sporadic adenomas analyzed with 2H1 showed positive expression in all cases (data not shown).

Examples of tumors scored as having negative, partial loss, or positive parafibromin expression are shown in Fig. 4. Fourteen of the 22 unequivocal carcinoma samples (64%) demonstrated partial loss of parafibromin expression with 25–83% positive nuclei, and one sample (4%) was completely negative for parafibromin immunoreactivity (T5, Table 2, Fig. 4). The remaining seven samples (32%) were positive, exhibiting parafibromin expression in almost 100% of the tumor cell nuclei. Among the equivocal carcinomas, 5 out of 11 cases (45%) exhibited partial loss, ranging from 52 to 80% positive nuclei (Table 2), while six samples (55%) were completely positive for parafibromin nuclear expression. Samples T4 and T7, representing two relapses within 1-year time in the same patient, were both completely positive.

In general, positive samples demonstrated nuclear staining, while surrounding fibrous tissue was negative. Accompanying low levels of cytoplasmic staining were often observed at varying levels between tumors, especially with the BL648 antibody. In support of the specificity of the nuclear and cytoplasmic signals, the positive expression was completely eliminated in the subsequent blocking experiments.

The immunohistochemical findings were also supported by western blot analysis of two positive carcinomas (Fig. 2). Sample T4 represents a relapsing unequivocal carcinoma growing into the larynx, esophagus, and blood vessels, and sample T23 is an equivocal carcinoma from a 16-year-old boy. Both these tumors expressed the ~60 kDa product at western blot analyses.

Discussion

The concordant results obtained at immunohistochemistry and western blot analyses suggest that the four antibodies are adequate in detecting parafibromin regarding both sensitivity and specificity. Moreover, the predominant nuclear localization observed at immunohistochemistry and sub-cellular fractionation is in accordance with previous reports (Hahn & Marsh 2005, Woodard *et al.* 2005, Bradley *et al.* 2007, Zhang

Figure 4 Photomicrographs showing immunohistochemical analyses of parafibromin expression in parathyroid carcinomas T5, T24, and T23 using antibodies TNYV, 2H1, BL648, and APVF. The unequivocal carcinoma T5 (negative) exhibits a total loss of parafibromin immunoreactivity with all four antibodies. In the equivocal carcinoma T24 (partial loss), the staining pattern represents ~80% positive tumor cells, while the equivocal cancer T23 (positive) demonstrates clear nuclear staining in 100% of the tumor cells. All images are magnified 160 $\times$, while the inserts are magnified 280 $\times$. To fully evaluate samples with partial loss, high power magnification is required as demonstrated in T24.

et al. 2006). The specificity of the cytoplasmic signal was supported by peptide neutralization experiments (antibody preincubated with immunizing peptide) and detection of a ~60 kDa band in the cytosolic fraction of the secondary HPT gland at western blot analysis.

Optimal methodological conditions for parafibromin immunohistochemistry are crucial if the results ought to be used for diagnostic purposes. We found that the results were influenced by the intensity of antigen retrieval, antibody dilution, and incubation time. The combination of immunohistochemistry and western blot analysis was, as illustrated in case T4 and T23, used to adjust the immunohistochemical methodology to positive findings in these parathyroid carcinomas evidently expressing parafibromin demonstrated by western blot analysis.

Partial loss of parafibromin immunoreactivity was frequently detected, while negative staining was only observed in a single carcinoma. Similar results were obtained for the four antibodies used with comparable

proportions of positive and negative cells in individual cases. The concordant findings with the different antibodies would suggest that full-length parafibromin is present in the positive nuclei found. Furthermore, the positive findings with BL648 and APVF would suggest that the functionally important β -catenin interaction domain (CID) and complex-binding domain (Cdc73) domains are physically present in the positive nuclei found.

Two tumors with double somatic *HRPT2* mutations exhibited positive and partial loss of parafibromin expression respectively. This situation is not generally expected in the case of tumor suppressor gene inactivation, where loss of the normal function often is seen. However, a previous study has demonstrated *in vitro* expression of truncated parafibromin protein resulting from site-specific *HRPT2* mutations introduced in a cell line, suggesting that *HRPT2* mutated cells can still propel the production of parafibromin,

Table 2 Results from the immunohistochemical staining of the 33 parathyroid carcinomas

Sample	HRPT2 mutation	Parafibromin immunohistochemistry (% positive cells)				Average %	Staining pattern	
		TNYV (aa 39–58)	2H1 (aa 87–100)	BL648 (aa 250–300)	APVF (aa 509–531)			
Unequivocal cases								
T1	Somatic	100	100	100	100	100	Positive	
T2	Somatic	81	76	–	64	74	Partial loss	
T3	–	96	100	–	100	99	Positive	
T4	–	100	100	100	100	100	Positive	
T5	–	0	0	0	0	0	Negative	
T6	Somatic×2	88	10	57	63	55	Partial loss	
T7	–	100	100	100	100	100	Positive	
T8	–	35	28	60	29	38	Partial loss	
T9	–	48	35	90	20	48	Partial loss	
T10	–	79	69	–	74	74	Partial loss	
T11	–	44	92	80	70	72	Partial loss	
T12	–	67	64	45	65	60	Partial loss	
T13	–	21	33	22	23	25	Partial loss	
T14	–	60	65	59	70	64	Partial loss	
T15	–	58	62	70	57	62	Partial loss	
T16	–	52	34	64	29	45	Partial loss	
T17	–	100	100	90	100	98	Positive	
T18	–	49	64	64	39	54	Partial loss	
T19	–	100	100	100	100	100	Positive	
T20	Somatic×2	100	100	100	100	100	Positive	
T21	Somatic	60	63	–	95	73	Partial loss	
T22	–	76	84	77	94	83	Partial loss	
Summary							Positive (n=7)	32%
							Partial loss (n=14)	64%
							Negative (n=1)	4%
Equivocal cases								
T23	Germ line	100	100	100	100	100	Positive	
T24	–	55	87	88	89	80	Partial loss	
T25	–	60	81	53	84	70	Partial loss	
T26	–	48	80	43	36	52	Partial loss	
T27	–	100	100	100	100	100	Positive	
T28	–	81	100	90	100	93	Positive	
T29	–	51	79	68	72	68	Partial loss	

(Continued)

Table 2 (continued)

Sample	HRP2 mutation	Parafibromin immunohistochemistry (% positive cells)					Average %	Staining pattern
		TNYV (aa 39–58)	2H1 (aa 87–100)	BL648 (aa 250–300)	APVF (aa 509–531)			
T30	–	100	100	100	100	100	Positive	
T31	–	61	72	68	80	70	Partial loss	
T32	–	100	100	100	100	100	Positive	
T33	–	100	100	100	100	100	Positive	
Summary							Positive (n=6)	
							Partial loss (n=5)	
							Negative (n=0)	
							55%	
							45%	
							0%	

–, not determined. Staining patterns: positive ≥90% positive nuclei, partial loss 11–89% positive nuclei, negative ≤10% positive nuclei.

albeit structurally defective (Zhang et al. 2006). In our two cases with double mutations, this finding offers a possible explanation for the positive results obtained with the three most N-terminally located parafibromin antibodies. Furthermore, both tumors with double *HRPT2* mutations carry one deleterious mutation in exon 8 (Table 1). The resulting frameshifts fortuitously creates new amino acid sequences prior to truncation that have partial homology with the APVF immunizing peptide; thus cross-binding with the APVF polyclonal antibody (although affinity purified) cannot be excluded. In general, the complexity of parafibromin immunostaining might be aggravated by other factors. For example, increased proliferation can result from co-expression of mutant and wild-type parafibromin (Zhang et al. 2006). Furthermore, parathyroid carcinomas reveal frequent gain of 1q, including the *HRPT2* gene locus (Kytölä et al. 2000), in contrast to the frequent observations of copy number losses at other tumor suppressor gene loci such as the *MEN1* locus in parathyroid adenomas.

The present study agrees with previous reports in which normal parathyroid tissue and parathyroid adenomas show positive parafibromin expression in all cells, giving a high specificity for the method. Since adenomas in turn are much more frequent than carcinomas, the negative predictive value (i.e. the chance that an adenoma is correctly identified by positive parafibromin staining) would end up as high, although probably not 100% since reduced expression of parafibromin has been reported in small subsets of parathyroid adenomas (Gill et al. 2006, Juhlin et al. 2006). Hence, in parathyroid tumors with positive parafibromin expression, the risk of malignancy is very low, however, cannot be fully excluded. In the group with partial loss or negative staining, a putative positive predictive value (i.e. the chance that a carcinoma is correctly identified by negative parafibromin staining) would end up as low, indicating that a negative staining is not automatically consistent with parathyroid carcinoma. Cases with partial loss or negative staining could either be parathyroid carcinomas or adenomas likely carrying an *HRPT2* mutation. In this group, mutation screening of tumor and blood is well motivated especially for detection of familial disease.

The question arises whether tumors diagnosed as parathyroid adenomas with partial loss or negative parafibromin staining could have a more aggressive course of disease. One tumor in our series exemplifies this situation; sample T13 was originally classified as an adenoma; however, the advent of recurrent disease 4 years after primary surgery ascertained this tumor as carcinoma. Careful histopathological reexamination of

the primary lesion found no evidence for malignant disease, although our ensuing parafibromin staining of this specimen was consistent with partial loss. Further analysis of similar cases will establish whether parafibromin immunostaining could detect malignant potential before the occurrence of required histopathological characteristics.

The initial findings of frequent *HRPT2* mutations in parathyroid carcinomas have inspired the development of parafibromin antibodies for immunohistochemical applications. The presently available data suggest that this approach has additive value in parathyroid diagnostics. However, loss of parafibromin immunoreactivity cannot be used as a diagnostic marker alone since the sensitivity is limited. Mutation screening of *HRPT2* is expected to increase the sensitivity as illustrated in this study where three of the six cases with known *HRPT2* mutations had positive expression (100% positive nuclei). Additional biomarkers for identification of parathyroid malignancy before the development of spread disease could be sought among markers for high proliferation and within the parafibromin pathways.

To summarize, parafibromin immunohistochemistry could be applied as an additional diagnostic marker. Positive expression would indicate benign disease, however, does not fully exclude a cancer diagnosis or the presence of an *HRPT2* mutation. Negative or partial loss staining of parafibromin should motivate genetic analysis of the *HRPT2* gene in blood and tumor tissue from the affected patient. However, our results suggest that parafibromin immunostaining cannot replace genetic testing or be used as a sole discriminator to separate adenoma from carcinoma. Our findings furthermore suggest that there exists no 'epitope of choice' for parafibromin detection related to malignant behavior of parathyroid tumors.

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