

CDC73 mutations and parafibromin immunohistochemistry in parathyroid tumors: clinical correlations in a single-centre patient cohort

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Abstract

Objective To determine if molecular and immunohistochemical (IHC) features of the *HRPT2/CDC73* gene and its product, parafibromin, predict the natural history of parathyroid malignancy, particularly atypical adenoma, as seen in a single-centre patient cohort.

Methods Matched tumor and non-tumor tissues were obtained from 46 patients with parathyroid carcinoma (CA) ($n=15$), atypical adenoma (AA) ($n=14$) and typical adenoma (TA) ($n=17$), as defined by standardized histopathological criteria. Exons and exon-intron boundaries of the *CDC73* gene were sequenced to identify germline or

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somatic mutations. IHC staining for parafibromin was performed and scored as positive if nuclear staining was at least partially IHC-positive.

Results Mutations of *CDC73* were observed in 9/15 (60 %) CA, 2/14 (14 %) AA, and 1/17 (6 %) TA tumors. A recurrent two basepair mutation in exon 7 – c.679_680delAG – accounted for half of all identified mutations. Absence of parafibromin nuclear staining was noted in 8/12 (67 %) CA, 2/13 (15 %) AA, and 3/17 (18 %) TA tumors. Median follow up times were 88 months for CA, 76 months for AA, and 104 months for TA patients. One patient, a member of a previously reported multiplex family with a germline *CDC73* mutation was found to have a second adenoma after removal of an atypical adenoma.

Conclusions Molecular screening and IHC are both useful tools in the differential diagnosis of parathyroid tumors, but both have limited sensitivity and specificity. *CDC73* mutations and negative immunostaining were common in atypical adenomas, but no local recurrence was observed in any case with successful surgical removal after follow-up periods of 27 to 210 months.

Keywords *CDC73* · HRPT2 · Parafibromin · Parathyroid tumours · Atypical adenoma

1 Introduction

Primary hyperparathyroidism (PHPT) is caused by parathyroid adenoma in 85 %, hyperplasia in 15 %, and cancer in less

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than 1 % of cases [1, 2]. The biological behaviours of carcinoma and adenoma are clearly different but the timely pathological diagnosis of parathyroid carcinoma can be difficult. Indeed, malignancy is commonly diagnosed only after local recurrence or detection of distant metastases [3, 4].

The diagnostic criteria for parathyroid carcinoma (CA) were defined in 1973 [5], but have been recently revised to include a new class of tumour [6]. Parathyroid tumors lacking unequivocal evidence of invasion, but showing features suspicious of malignancy (i.e., fibrous bands, questionable capsular invasion, increased mitotic figures, and adherence to surrounding tissues) are now defined as ‘atypical adenomas’ [7]. Unlike parathyroid carcinoma, the natural history of the atypical adenoma (AA) is not well established, even though the recommendation is that such patients should be closely followed [7]. In order to follow these patients, improved diagnostic and predictive tools would be helpful.

Parathyroid carcinoma is a feature of the hyperparathyroidism-jaw tumor (HPT-JT) syndrome due to *HRPT2/CDC73* mutation [8, 9], and is implicated in sporadic carcinoma as well [10]. Identification of *CDC73* mutations or loss of expression of the *CDC73* gene product, parafibromin, may both be useful markers of malignancy in parathyroid tumours [3, 11, 12].

The prevalence of *CDC73* gene mutations has been reported to be 70 % of parathyroid carcinomas [3, 10, 13, 14], but only 1–2 % of typical adenomas (TA) [8, 13, 15, 16]. About 20 % of atypical adenoma (AA) cases have mutations [11, 12].

Loss of parafibromin nuclear expression, as assessed by immunohistochemical (IHC)-negativity, has been reported to vary from 68 to 100 % for parathyroid carcinoma [11, 12, 17, 18]. Different scoring systems used may no doubt contribute to variability in frequency among the various studies [7, 11, 12, 17–19]. While partially reduced expression of parafibromin has been described in 16 of 46 atypical adenomas [11, 12, 17–21], only 4 of 238 typical adenomas appear to be similarly affected [12, 17, 18, 20–22].

Strong concordance between *CDC73* mutation identification and IHC-negativity for nuclear parafibromin expression has been considered evidence in support of the suggestion that IHC can be the primary means of evaluating malignancy [12, 14, 17]. Immunohistochemistry is less expensive and time-consuming than genetic screening [12], and is theoretically more likely to pick up deleterious variants leading to complete loss of parafibromin expression. However, others have more recently reported a lower success rate for ‘IHC-negativity’ in malignancy [7, 20, 21]. Some studies have evaluated molecular screening and parafibromin expression simultaneously [11, 12, 22, 23] but data on atypical adenomas are sparse, despite the fact that this is the group for which optimal assessment might be most clinically beneficial [11, 12].

We therefore undertook to identify *CDC73* mutations along with IHC-assessment of nuclear parafibromin expression, in a large, single-centre cohort of patients with

histopathologically proven parathyroid tumors followed for up to 20 years.

2 Subjects and methods

2.1 Study inclusion criteria

All patients admitted to the Endocrinology Unit of the “Casa Sollievo della Sofferenza” Hospital in San Giovanni Rotondo (Foggia, Italy) with a working diagnosis of primary hyperparathyroidism were eligible for inclusion. All patients whose pathological diagnosis was parathyroid carcinoma or atypical adenoma were included, while 15 typical parathyroid adenomas were randomly selected from a large ($n > 150$) archival pool. Two subjects with a family history of typical parathyroid adenoma, but without mutations in calcium-sensing receptor (*CASR*) or multiple endocrine neoplasia type I (*MEN1*) genes [24] were included in the latter group. First contact with the patients occurred between calendar years 1990 and 2007 and outpatient clinic follow-up extended to 2010 (Table 1).

Subject AA7 and his family have been reported previously [25, 26]. In the other probands bearing a germline mutation, specific mutation testing was offered to all available first-degree relatives, and performed on consenting subjects (Table 2).

2.2 Clinical follow-up and surveillance

All patients with parathyroid carcinoma and atypical adenoma were followed up with yearly measurement of serum calcium, creatinine and PTH plus ultrasonography of neck in order to detect the presence of early recurrence of the disease. In probands and their relatives bearing a germline mutation of the *CDC73* gene, our protocol includes annual clinical examination, biochemical profile (i.e., serum ionized calcium, creatinine, PTH) and imaging (i.e., neck, total abdominal ultrasonography) with jaw pantomograms every 5 years.

2.3 Histopathologic diagnosis

For histopathological analysis at diagnosis, tissue samples were excised and fixed in 10 % buffered formalin followed by conventional processing and paraffin embedding. Four micron thick sections were cut and stained with haematoxylin and eosin for standard histopathological assessment. Parathyroid tumors were carefully re-evaluated and classified according to current WHO guidelines [6, 27]. Where discordances in IHC-negativity and molecular studies arose subsequently, the histopathological classification was independently re-evaluated in a blinded fashion. However, no discrepancies in routine histopathological classification were found.

All patients, or immediate relatives if the subject was deceased, gave informed consent. The study protocol was approved by the ethics committee of the hospital.

2.4 Molecular studies

For all subjects, whole blood and matching tumor tissues available in paraffin blocks were processed for the analysis of germline and somatic mutations, respectively. In the case of a deceased individual, non-tumor tissue was collected. DNA from peripheral leukocytes was isolated by phenol-chloroform extraction, and DNA from tissues was extracted as described previously [28].

Molecular screening of the entire *CDC73* coding sequence (17 exons including exon-intron boundaries) (Fig. 1) was performed on germline DNA, and somatic DNA from all tumor samples. Amplifications [8], were carried out in a 25 μ L reaction volume containing 2.5 μ L 10X PCR Buffer (Eppendorf), 0.25 nM dNTPs, 20 pmol of each primer, 1 U HotMaster Taq (Eppendorf) and 100 ng of DNA. Cycling conditions consisted of initial 5 min denaturation step at 95 °C, followed by 35 cycles of 95 °C for 30 s annealing for 30 s, and extension at 72 °C for 30 s, with final extension at 72 °C for 7 min. PCR products were visualised by ethidium bromide staining on 2 % agarose gels. PCR products were purified using the GFX™ PCR and Band Purification Kit (GE Healthcare, Buckinghamshire UK) and then sequenced using the BigDye Terminator Cycle Sequencing Kit v. 1.1 (Applied Biosystems, Foster City CA). Sequencing reactions were loaded on an ABI 3100 capillary sequencer (Applied Biosystems, Foster City CA) and analysed using the Sequencing Analysis software v.3.7.

PCR controls included reactions without DNA. Mutations were confirmed by sequencing in both directions with forward and reverse primers on the original amplicon, and on another PCR performed on DNA re-extracted from the same tumor. Germline mutations were confirmed on the corresponding somatic tissue sample(s).

2.5 Parafibromin immunohistochemistry (IHC)

Heat-induced antigenic retrieval for detection of parafibromin was performed by placing deparaffinized 4 μ m-thick sections in 10 mM citrate buffer (pH 6.0) and incubating in a 360 W microwave oven for 3 cycles of 15 min each. Tissue sections were then incubated with a primary monoclonal antibody to parafibromin (1:200 dilution; clone sc-33638 from Santa Cruz Biotechnology, Santa Cruz, CA), raised against a peptide comprising amino acids 87 to 100 in the molecule. Immunostaining was performed using an Envision™ Kit on a Dako automatic stainer (Dako, CA, USA), according to the manufacturer's instructions. Adjacent endothelial cells served as the internal positive controls, while

Table 1 Clinical, biochemical and molecular features of parathyroid tumors by group

^a ID	Age at first operation (yr)	Gender (f/m)	Serum calcium (mg/dL)	Serum PTH (pg/mL)	Tumor size (cm)	Length of follow up (months)	Clinical progression at follow-up (months)	Germline mutation	Somatic mutation	^b IHC
Parathyroid carcinoma group (CA, n=15)										
CA1	22	f	13.8	746	3.5	91	n/p	n/f	n/f	n/d
CA2	59	f	12.6	387	2.5	245	LR (165), BM (219)	n/f	n/f	neg
CA3	41	f	13.4	337	1.3	214	n/p	n/f	c.13C>T	pos
CA4	37	f	11.2	845	3	139	n/p	n/f	n/f	pos
CA5	57	f	15	240	2	195	n/p	n/f	c.679_680delAG	neg
CA6	70	f	11.6	563	2	88 ^b	n/p	n/f	c.679_680delAG	neg
CA7	23	m	16.5	353	3	160	n/p	n/f	c.94insTA	neg
CA8	54	m	12.9	253	3	107	n/p	n/f	n/f	pos
CA9	47	f	11.7	642	6	72	n/p	n/f	n/f	pos
CA10	52	f	12.7	367	3	43	LR (43) Deceased	c.415C>T	n/f	neg
CA11	35	f	13.0	660	6	54	n/p	c.520_523delTCTG	n/f	neg
CA12	49	f	20.2	940	4	2	CH (2) Deceased	n/f	c.679_680delAG	neg
CA13	44	m	14.0	325	4	86	LR (37 and 46), LM (81) Deceased	c.679_680delAG	c.42delG	neg
CA14	56	m	10.6	60	3	84	n/p	n/f	n/f	n/d
CA15	44	m	10.6	163	2	85	n/p	n/f	c.679_680delAG	n/d
Summary data	47 (22–70) ^c	10/15 (67 %) ^d	12.9 (10.6–20.2) ^{ee}	367 (60–940) ^{ee}	3 (1.3–6) ^{ee}	88 (2–245) ^e		3/15 (20 %) ^d	7/15 (47 %) ^d	8/12 (67 %) ^d
Atypical parathyroid adenoma group (AA, n=14)										
AA1	53	m	11.6	154	1.5	134	n/p	n/f	n/f	pos
AA2	55	f	11.1	108	2	101	n/p	n/f	n/f	pos
AA3	42	f	15.8	1633	2	98	n/p	n/f	n/f	pos
AA4	58	m	13.3	1154	2.3	27 ^b	n/p	c.679_680delAG	c.231C>G	pos
AA5	86	m	11.9	542	4	72	n/p	n/f	n/f	pos
AA6	44	f	14.4	276	2.5	208 ^b	n/p	n/f	n/f	n/d
f ^a AA7	30	m	11.5	160	2	210	AA (144)	c.685_688delAGAG	n/f	neg
AA8	53	f	11.4	300	4	79	n/p	n/f	n/f	pos
AA9	71	m	13.8	568	3.3	47	n/p	n/f	n/f	pos
AA10	23	m	12.2	200	2.3	48	n/p	n/f	n/f	pos
AA11	66	f	11.6	231	1.5	68	n/p	n/f	n/f	pos
AA12	71	m	11.7	756	2.5	100	n/p	n/f	n/f	pos
AA13	59	f	11.7	139	1.5	58	Deceased due to unrelated disease	n/f	n/f	pos
AA14	46	f	12.9	1335	2.5	45	n/p	n/f	n/f	neg
Summary data	54 (23–86) ^c	7/14 (50 %) ^d	11.8 (11.1–15.8) ^{ee}	288 (108–1633) ^{ee}	2.3 (1.5–4) ^e	76 (27–210) ^e		2/14 (14 %) ^d	1/14 (7 %) ^d	2/13 (15 %) ^d

Table 1 (continued)

^a ID	Age at first operation (yr)	Gender (f/m)	Serum calcium (mg/dL)	Serum PTH (pg/mL)	Tumor size (cm)	Length of follow up (months)	Clinical progression at follow-up (months)	Germline mutation	Somatic mutation	^a IHC
Typical parathyroid adenoma group (TA, n=17)										
TA1	66	f	10.5	84	1.5	100	n/p	n/f	n/f	pos
TA2	41	m	11.8	254	2.8	183	n/p	n/f	n/f	neg
TA3	59	f	11.5	132	1.5	197	n/p	n/f	n/f	pos
TA4	61	f	10.9	162	3	129	n/p	n/f	n/f	pos
TA5	49	m	12.3	273	2.5	104	n/p	n/f	n/f	pos
TA6	65	f	10.5	187	0.4	102	n/p	n/f	n/f	pos
TA7	64	f	10.1	55	2	189	n/p	n/f	n/f	pos
TA8	56	m	10.2	423	2	182	n/p	n/f	c.679_680delAG	neg
TA9	31	f	11.0	72	2	173	n/p	n/f	n/f	pos
TA10	63	f	10.7	209	2	102	n/p	n/f	n/f	pos
TA11	51	f	11.3	97	2.2	125	n/p	n/f	n/f	pos
TA12	56	f	12.0	376	5	47	n/p	n/f	n/f	pos
TA13	66	m	12.8	262	3.5	46	n/p	n/f	n/f	pos
TA14	53	f	10.2	81	0.8	122	n/p	n/f	n/f	pos
TA15	58	f	11.6	156	1.9	56 ^b	n/p	n/f	n/f	pos
TA16 ^h	62	f	11.2	76	1	97	n/p	n/f	n/f	neg
TA17 ^h	42	f	10.8	155	1	95	n/p	n/f	n/f	pos
Summary data	58 (31–66) ^c	13/17 (76 %) ^d	11.0 (10.1–12.8) ^e	156 (55–423) ^c	2.0 (0.4–5) ^c	104 (46–197) ^c		0/17 (0 %) ^d	1/17 (6 %) ^d	3/17 (18 %) ^d
All groups summary	54 (22–86) ^c	30/46 (65 %) ^d	11.7 (10.1–20.2) ^e	258 (55–1633) ^c	2.3 (0.4–6) ^c	99 (2–245) ^c		5/46 (11 %) ^d	9/46 (20 %) ^d	13/42 (31 %) ^d

LR local recurrence; LM lung metastasis; BM brain metastasis; AA atypical adenoma; CH cholangiocarcinoma; (in parentheses, months since the first operation)

^aID identification code; IHC immunohistochemistry—parafibrin immunostaining; n/d not determined; pos positive at nuclear immunostaining of parafibrin; neg negative at nuclear immunostaining of parafibrin

^b Patients lost to follow-up ; n/p no progression; n/f not found

^c Median (range)

^d Occurrences (frequency)

^e CA vs TA, p<0.05 (ANOVA with Bonferroni post hoc analysis)

^f See text and ref. [25]

^g AA vs TA, p<0.05 (ANOVA with Bonferroni post hoc analysis)

^h Typical parathyroid adenoma in patient belonging to familial primary hyperparathyroidism

Table 2 *CDC73* mutations in probands and carriers

Mutation	Exon	Predicted protein	No. of mutations (somatic / germline)	No. of carriers	Carrier ^a F/U (patient-yrs)	Previous reports
c.13C>T	1	L5F	1 / 0	n/a		None
c.42delG	1	Q14fsX20	1 / 0	n/a		None
c.94insTA	1	Y32fsX37	1 / 0	n/a		None
c.231C>G ()	2	R77P	1 / 0	n/a		None
c.415C>T	5	R139X	0 / 1	^b 6	12	[16]
c.520_523delTCTG	7	K174fsX200	0 / 1	^c 1	3	None
c.679_680delAG	7	R227fsX263	2 / 2	^d 0	0	[13], [36]
c.685_688delAGAG	7	R227fsX255	0 / 1	^e 9	^e 49	[25], [26]

^a F/U follow-up; n/a not applicable

^b Family members who consented to testing (ages given in parentheses) and identified as carriers were: the proband's sister (62 years), the sister's daughter and son (niece 42 years old and nephew 38 years old, respectively) and the niece's three children (2, 7 and 15 years old). Two first-degree relatives of the proband who declined testing

^c Two of the proband's brothers and one sister consented to testing. One brother (40 years old) was identified as a carrier while the other brother (44 years old) and the sister (30 years old) were negative

^d There were no first-degree relatives for either proband (CA13 and AA4) available for counselling or testing

^e Family previously reported [25]

omission of the primary antibody served as a negative control. Slides were lightly counterstained with haematoxylin, dehydrated, and mounted. The stained sections from all cases were examined by one of us (MB), and the absence or presence of nuclear immunoreactivity was recorded as IHC-negative or IHC-positive, respectively (Table 1). At variance with some studies [11, 17, 19], but concordant with others [14, 18, 21], we considered samples IHC-negative only if they did not reveal any nuclear staining at all.

3 Results

3.1 Clinical characteristics

Our cohort consisted of 46 subjects, all of Italian heritage (Table 1). There were more women ($n=30$) than men ($n=16$), but the difference did not reach significance and there was no difference in their median age. The proportion of women was higher in the TA (13 of 17) and CA (10 of 15) groups, compared to the AA group (7 of 14) but the differences were not significant.

The median age at the time of operation was 54 years (range: 22 to 86). When analyzed by group, median age at first operation was 47 years for CA, 54 years for AA, and 58 years for TA subjects ($p = \text{NS}$). Preoperative serum total calcium (all-group median 11.7 mg/dL) was significantly higher in CA [12.9 mg/dl (range 10.6–20.2)] and AA [11.8 mg/dL (range 11.1–15.8)] groups compared to the TA [11.0 mg/dL (range 10.1–12.8)] group (ANOVA with Bonferroni post hoc analysis, $p<0.05$). Preoperative PTH level (all-groups median 258 pg/mL) was also significantly higher in CA [367 pg/mL (range 60–940)] and AA [288 pg/mL (range 108–1,633)] groups vs the TA [156 pg/mL (range 55–423)] group ($p<0.05$). Tumor size (all-groups median 2.3 cm) was significantly higher in the CA [3 cm (range 1.3–6 cm)] than in the TA [2.0 (range 0.4–5 cm)] group ($p<0.05$). Median tumor size for the AA group was 2.3 cm (range 1.5–4 cm).

3.2 Clinical follow-up

Overall, the follow-up interval ranged from 2 months (0.2 years) to 245 months (20.4 years) with a median of 99 months (8.3 years). Four patients were lost to follow up—

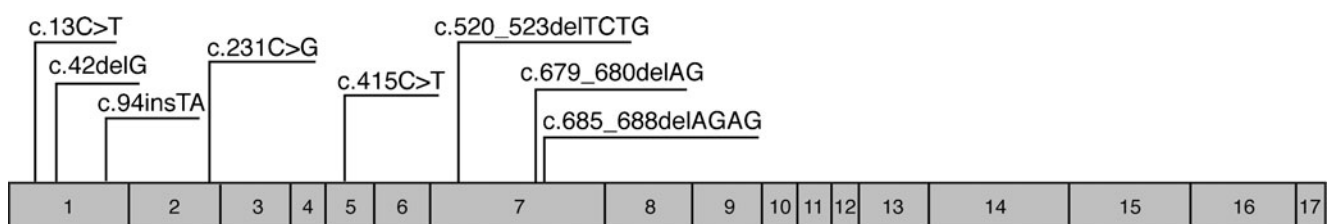


Fig. 1 Schema showing the numbered *CDC73* exons and the specific mutations identified in the present study

one with carcinoma at 88 months, two with atypical adenoma at 27 and 208 months and one with typical adenoma at 56 months.

Of the CA group, three (20 %) died of their disease at 2, 43, and 86 months after diagnosis. The rest of the group ($n=12$) were monitored annually for a median interval of 99 months (range 54–245), but only one subject (CA2) presented with local recurrence and brain metastasis 219 months after diagnosis. She was still well at 245 months after radiotherapy. The AA group of 14 subjects was followed up for a median interval of 76 months. The patient (AA7) belonging to a 4-generation family with multiple affected members carrying a germline truncating mutation, reported by Guarnieri et al. 2006 [25], had a recurrence after 144 months. The 17 subjects from the TA group did not show any biochemical or ultrasonographic recurrence after a median follow-up of 104 months.

3.3 Molecular findings

In the CA group, 10 *CDC73* mutations were found in 9 out of 15 subjects (60 %); three of these were germline (Table 1). A smaller proportion of the AA group (2/14 or 14 %) were mutation positive (2 germline mutations). In the TA group the number of mutation-positive subjects was still smaller (1/17 or 6 %) and there were no germline mutations. The trend across groups was statistically significant ($p=0.007$, Fisher exact test).

Sites of mutation are shown in Fig. 1. In our cohort, the recurrent c.679_680delAG dinucleotide deletion accounted for 2 of the 5 germline and 5 of 9 somatic mutations (Table 2). This frequency (7/14 or 50 %) is higher ($p<.001$, Fisher exact test) than suggested by Masi et al. 2008 [29] in their most recent review [cf. 7/93 (8 %) independent mutations], but consistent with the view that it is a hot spot. The other seven exonic mutations were observed only once in our cohort but, of the seven, the three that were germline in our series have been reported before as germline mutations by others (Table 2). One of the three is a nonsense mutation in exon 5, while the other two are frameshift mutations in exon 7, consistent with the excess of similar germline mutations in that region. Two of the four novel exonic mutations, all somatic, were frameshift, but the other two are relatively non-conservative missense changes (c.13C>T or p.L5F, c.231C>G or p.R77P). There was no indication that missense and nonsense mutations were less common in the CA group (2 of 10 mutations) than in the other two groups (1 of 4 mutations). Both missense mutations were predicted to be damaging according to the majority (2 of 3) of the bioinformatic predictive programs used for in silico evaluation (Polyphen; L5F, benign; R77P, possibly damaging; SIFT; L5F, not tolerated; R77P, tolerated; SNPs3D; L5F, deleterious; R77P, deleterious).

Of the 14 *CDC73* mutations, 7 were somatic only. Overall, somatic mutations were more frequent in the CA group (7 of

15 subjects) than in AA (1 in 14 subjects) or TA groups (1 in 17 subjects). Double hits (2 somatic or somatic+germline), consistent with the Knudson two-hit hypothesis, were found in one CA and one AA subject. In a parallel study, allelic imbalance (loss of heterozygosity = LOH) [30] was evaluated for its contribution to genetic risk (see [Supplementary Material](#)).

First-degree relatives with positive carrier status were identified in families of three probands with germline mutations (Table 2). One family has been reported in detail [25]. In the other two, all seven healthy carriers have been followed for a total of 15 patient years without identifying any evidence of disease.

3.4 Immunohistochemistry

Immunostaining of parafibromin showed loss of nuclear immunoreactivity (IHC-negativity) in 8 out of 12 CA, 2 out of 13 AA, and 3 out of 17 TA tissue samples (Table 1). Examples of tumors that are either parafibromin IHC-positive or IHC-negative, respectively, are shown for carcinomas (Fig. 2), atypical adenomas (Fig. 3) and typical adenomas (Fig. 4). The prevalence of IHC-negativity is higher in our sample of typical sporadic adenomas than reported by others [12, 17–22] despite a more conservative definition of IHC loss. Interestingly, in one of the two TA subjects without any nuclear staining for parafibromin (Fig. 4c and d) and without a positive family history (TA#8), there was a double hit, consisting of the recurrent somatic mutation, c.679_680delAG, and LOH (See Suppl Fig. 1). In the other subject (TA#2), LOH was also observed and review of the slides showed small cysts in the excised gland.

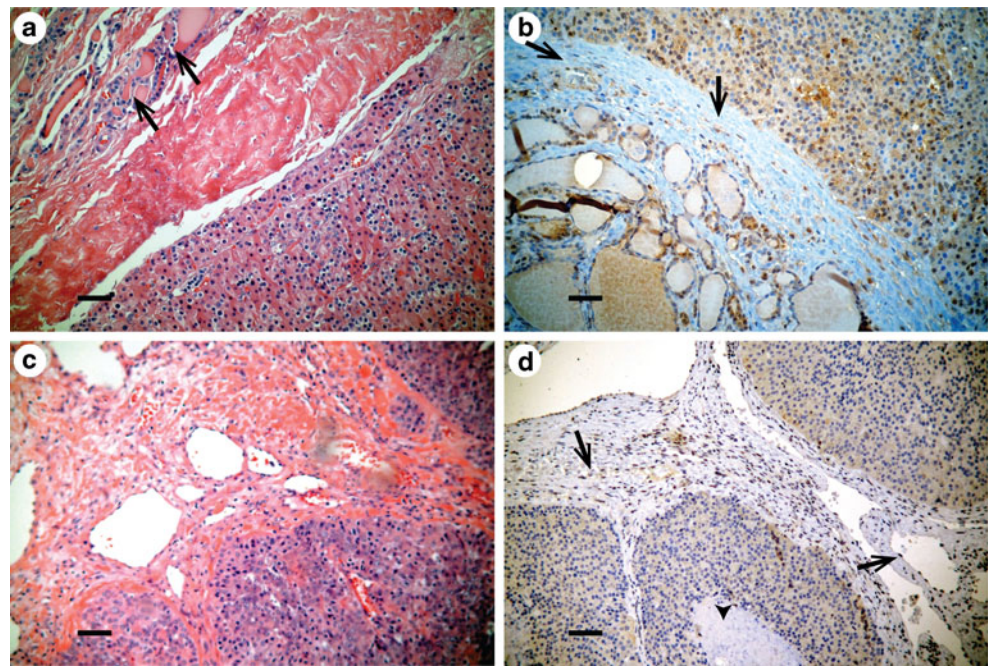
3.5 Sensitivity and specificity of molecular and IHC results

Taking the clinical diagnosis as the gold standard, *CDC73* mutations had diagnostic sensitivity and specificity of 60 % and 94 %, respectively. Positive and negative predictive values were 90 % and 73 %, respectively, for differentiating carcinoma from typical adenoma. With immunostaining, sensitivity and specificity were 67 % and 82 %; while positive and negative predictive values were 73 % and 78 %, respectively. When combined, there was improvement in sensitivity (75 %) with specificity of 82 %. The overall accuracy for the combined tests (80 %) was modestly higher than either IHC or molecular alone (76 % and 78 %).

4 Discussion

The distinction between benign and malignant parathyroid tumors can be difficult in the absence of obvious malignant characteristics, such as local recurrence or distant

Fig. 2 Histochemistry in carcinomas. **a, c** HE stain; **b, d** parafibromin IHC. **a, b** IHC-positive carcinoma from #CA9; **a** tumor shows a diffuse proliferation pattern with invasion of adjacent thyroid tissue (*arrows*) (magnification $\times 100$; bar $52\ \mu\text{m}$); **b** nuclei of tumor cells along with endothelial cells and fibroblasts (*arrows*) are positive for parafibromin staining (magnification $\times 100$; bar $52\ \mu\text{m}$). **c, d** IHC-negative carcinoma from #CA11; **c** diffuse follicular proliferation pattern (magnification $\times 100$; bar $52\ \mu\text{m}$); **d** tumor cell nuclei are immunonegative for parafibromin while endothelial cell and fibroblast nuclei (*arrows*) are immunopositive. Areas of necrosis (*arrowhead*) are also seen (magnification $\times 100$; bar $52\ \mu\text{m}$)



metastasis. Indeed, in a large series of metastatic CA cases, nearly half were initially classified as adenomas [31]. The high prevalence of *CDC73* mutations in CA [10, 13, 14] led to suggestions that genetic screening may be an important clinical tool for improving diagnostic accuracy.

It has also been suggested that immunostaining of parafibromin should be considered a criterion for malignancy. The more common mutations of *CDC73* gene are truncating and are most often accompanied by loss of parafibromin immunostaining. Indeed, several studies [11, 12, 14, 18, 21] indicate a high specificity for loss of parafibromin staining

as a criterion in diagnosing carcinoma, since only one of some hundreds of unselected parathyroid adenomas was similarly IHC-negative. Consequently, loss of parafibromin staining has been advanced as a key index of malignant behaviour [12].

Our carcinoma series confirms a high prevalence (73 %) of *CDC73* mutations in apparently sporadic tumors, with the expected range of clinical outcomes. Of the 15 cases, 11 were still alive after a median follow-up of 83 months (range 30–221 months). For the three whose cause of death was known, all had metastatic disease, all had mutations, and all

Fig. 3 Histochemistry in atypical adenomas. **a, c** HE stain; **b, d** parafibromin IHC. **a, b** IHC-positive atypical adenoma from #AA2; **a** tumor cells show trabecular proliferation pattern, and apparent capsular invasion (*arrows*) (magnification $\times 200$; bar $20\ \mu\text{m}$); **b** nuclei of tumor cells as well as endothelial cells and fibroblasts (*arrows*) are uniformly and strongly immunopositive (magnification $\times 200$; bar $20\ \mu\text{m}$). **c, d** IHC-negative atypical adenoma from #AA14; **c** follicular proliferation pattern (magnification $\times 200$; bar $20\ \mu\text{m}$); **d** nuclei of tumor cells are immunonegative, while nuclei of fibroblasts and endothelial cells (*arrows*) are immunopositive (magnification $\times 200$; bar $20\ \mu\text{m}$)

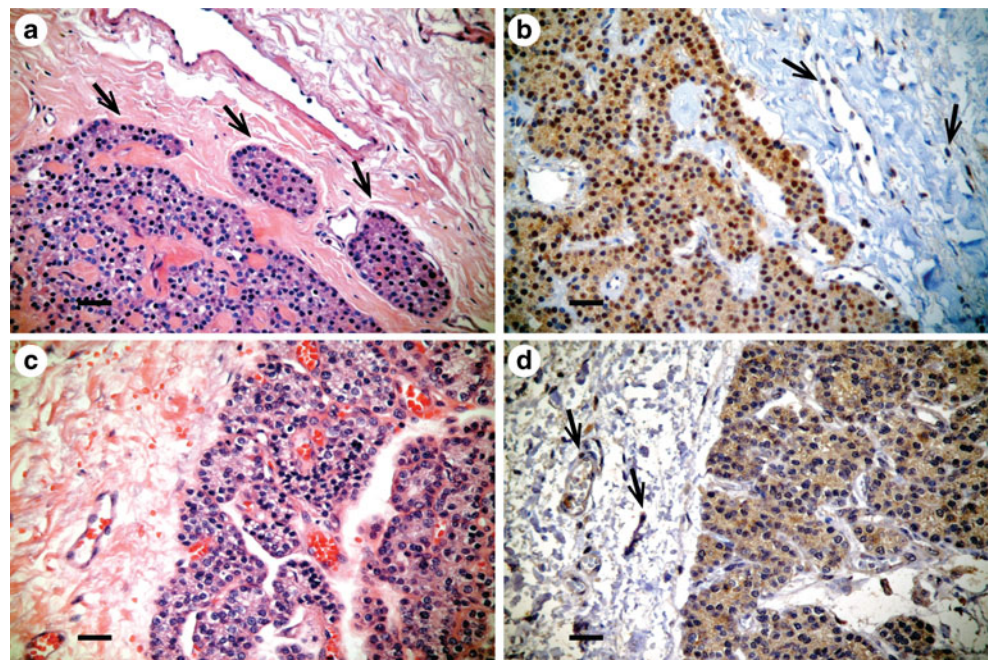
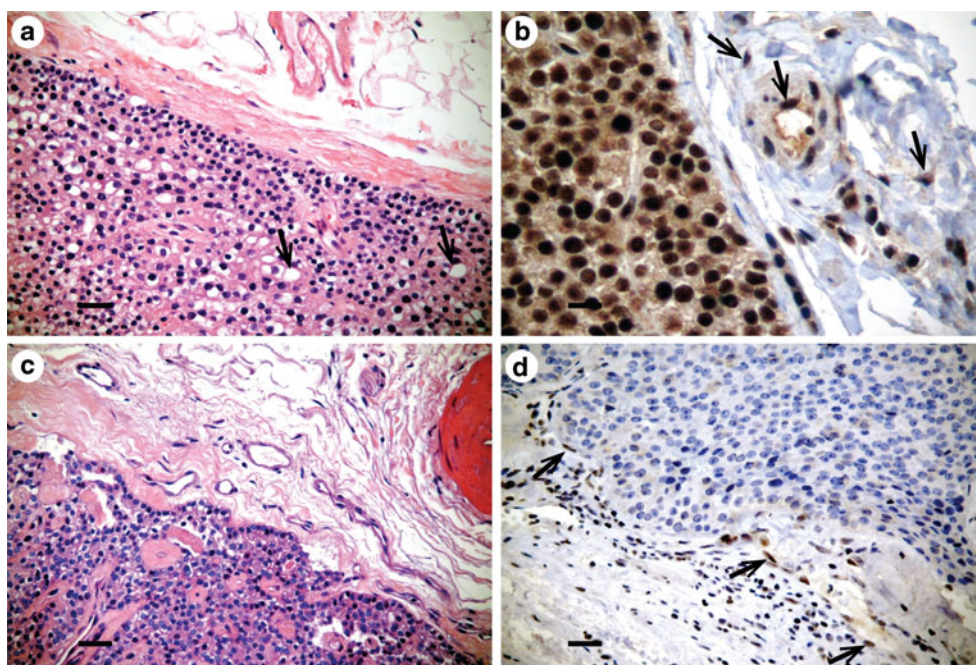


Fig. 4 Histochemistry in typical adenomas. **a, c** HE stain; **b, d** parafibromin IHC. **a, b** IHC-positive typical adenoma from #TA11; **a** diffuse proliferation pattern and small cysts (*arrows*) (magnification $\times 200$; bar 20 μm); **b** nuclei of tumor cells along with endothelial cells and fibroblasts (*arrows*) are diffusely and strongly immunopositive (magnification $\times 400$; bar 13 μm). **c, d** IHC-negative typical adenoma from #TA8; **c** diffuse proliferation pattern (magnification $\times 200$; bar 20 μm); **d** absence of immunostaining for parafibromin in tumor cells but nuclei of fibroblasts and endothelial cells (*arrows*) are immunopositive (magnification $\times 200$; bar 20 μm)



were IHC-negative. In the additional single living patient with metastasis, she had a *CDC73* mutation and her tumor was also IHC-negative.

In patients whose disease was locally confined, the genetic and IHC findings were more heterogeneous. Of the 11 subjects so classified, 6 had a mutation and 4 were IHC-negative (see Table 1). The number of IHC-positive carcinomas (4 of total 12) is in line with the recent observations of DeLellis [7]. The fact that all IHC patterns showing some significant degree of immunopositivity were classified as immunopositive, likely contributes to the overall rate, but the size of this effect is not known, and the assessment subjective. A consensus-driven IHC scoring system would be welcome.

Among the histologically typical adenomas, we identified *CDC73* gene lesions in 18%. It is not surprising that we did not identify constitutional mutations, but the same cannot be said of IHC negativity. It has been suggested that sporadic adenoma is never essentially “negative” at immunostaining, but we identified three cases that we considered unequivocally IHC-negative (Fig. 4c and d). In one case, a relative was affected by PHPT so we speculate that the *CDC73* mutation was not identified, and further analysis is required. The other two with immunonegativity (#TA2 and #TA8) are more difficult to explain but the fact that they both showed deleterious somatic *CDC73* mutations suggests that the tumor phenotype can vary significantly in response to mutation and that immunostaining will indeed be negative in a small but significant non-zero proportion of classical adenomas.

We also note that LOH at the *CDC73* locus was observed in 10/46 cases (see Supplementary Material). The frequency

was similar in the TA group (3/17) as in AA or CA subjects (3/14 and 4/15, respectively). In no instance did the LOH region span the entire range of markers (D1S215 to D1S413, ~ 20 megabases). Somatic point mutations, consistent with the Knudson 2-hit hypothesis, were found in 3 of the LOH positive cases, but our methods would only detect those somatic mutations that led to small sequence changes in *CDC73* exons or intron-exon boundaries that were amplifiable by standard PCR techniques and Sanger sequencing. Furthermore, deletions that are too big to be detected by PCR but still too small to be detected by LOH using our panel of markers would also be missed, although such mutations are uncommon [32]. Another explanation for the absence of matching second hits is the possibility that large-scale hemizygous deletions by themselves may be tumorigenic [33]. Evidence is accumulating that deletion-derived haploinsufficiency at chromosomal loci with an excess of those genes down-regulating cell proliferation results in clonal selection of cells that proliferate faster, thereby creating a cell population predisposed to downstream tumorigenic events other than mutation or large-scale deletion, but resulting in homozygous inactivation of the locus. Better understanding of deletions in the *CDC73* region (1q31.2) would be an important first step in the exploration of this hypothesis.

Similarly, LOH was not detected in 4 of 9 cases with somatic mutations. Two of those cases are readily explained by the presence of a germline point mutation, but the others may be attributable to the methodological limitations noted above. LOH, using the standard marker panels, did not appear to contribute to the diagnosis or management of our patients. However, more detailed studies using methods such as comparative genomic hybridization and exomic

sequencing may provide important new clues to unanswered questions about the phenotype associated with *CDC73* mutations [32].

In our series, parafibromin immunostaining showed a sensitivity of 67 % and a specificity of 82 % in diagnosing carcinoma, both somewhat lower than others have reported [16, 18]. Since typical adenomas are much more frequent than carcinomas, immunostaining can be regarded as only one tool for assessing malignancy.

One of the purposes of this study was to clarify the clinical, genetic and immunohistochemical characteristics distinguishing atypical adenoma from typical adenoma on one hand and parathyroid carcinoma on the other. While the hypothesis that atypical adenoma may be a precursor to carcinoma remains an attractive one, there is little direct evidence for this progression. As an intermediate form of parathyroid tumor in terms of typical metaplastic features (mitotic index, partial capsular invasion), the large group of atypical adenomas we studied here was also intermediate with respect to frequency of *CDC73* mutation and IHC negativity. Clinical progression was surprisingly low. However, it was not much different from the experience of DeLellis [7], who found no recurrence in 24 AA subjects after a mean of 8 years follow-up. Our one exception was an individual belonging to a 4-generation family with multiple affected members carrying a germline truncating mutation. The details of that family have been reported previously [25].

Looking at all three types of parathyroid tumors in our cohort, we suggest that careful clinical follow-up, accurate histopathologic classification, reliable immunohistochemical staining for parafibromin and a comprehensive search for somatic and germline *CDC73* mutations may all be important requisites for appropriate management of HRPT2 related tumors. In the case of typical adenomas, clinical assessment and routine histopathology are almost always sufficient for diagnosis and appropriate disposition. The key exception remains a positive family history, or the presence of other tumors suggestive of HPT-JT syndrome.

In contrast, genetic analysis (tumor and germline DNA) is probably warranted in most atypical adenoma cases, but the role of immunohistochemistry is less certain. Indeed the presence of somatic mutations could suggest the diagnosis of carcinoma and/or a progression of an AA in a carcinoma; however there are no data demonstrating that the presence of a *CDC73* mutation is predictive for a more aggressive behavior.

Longer follow-up is needed to assess whether this tumor type progresses to frank neoplasia. In suspected parathyroid carcinoma, the usual clinical characteristics (tumor dimension, stony consistency and progressive hypercalcemia) are hardly sufficient for diagnosis and specific histopathologic characteristics (total capsular invasion and/or vessel invasion and/or distant metastasis and/or local recurrence) should be present to establish a diagnosis.

Since most parathyroid carcinomas are the result of parafibromin deficiency, genetic analysis of genomic DNA and search for somatic mutation of *CDC73* gene are recommended. The importance of such a genetic analysis lies in the expectation that screening of asymptomatic carriers in the affected family will lead to effective early intervention [10, 25, 34]. This also applies to the proband in relation to the risk of recurrence, and to the increased risk to carriers of other *CDC73*-related tumors [26].

Finally the recommendation regarding the diagnostic properties of immunostaining alone needs to be reconsidered. Although initially stated to be highly specific, IHC-negativity may be observed in typical adenomas (as we show here), and in atypical adenoma [16]. It seems likely that some combination of specific immunohistochemistries [14, 19, 35] may improve sensitivity and specificity. In the interim, IHC may be considered most useful for special situations where classification is uncertain.

Our study confirms that truncating constitutional mutations of *CDC73* are common in sporadic carcinoma. Moreover, we observed a recurrent mutation in exon 7, which may be a genetic hot spot, since it is seen in both germline and somatic DNA.

In conclusion our data suggest that parafibromin immunostaining cannot replace molecular analysis as a means of materially improving the accuracy of a diagnosis of parathyroid malignancy. In our cohort, *CDC73* gene lesions or loss of nuclear immunostaining of parafibromin was not associated with a malignant behaviour of parathyroid AA followed up, on average, for 6 years. Combined molecular and immunohistochemistry testing appears to be the most effective approach to the differential diagnosis of parathyroid tumors.

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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