Genetic characterization of large parathyroid adenomas

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Abstract

In this study, we genetically characterized parathyroid adenomas with large glandular weights, for which independent observations suggest pronounced clinical manifestations. Large parathyroid adenomas (LPTAs) were defined as the 5% largest sporadic parathyroid adenomas identified among the 590 cases operated in our institution during 2005–2009. The LPTA group showed a higher relative number of male cases and significantly higher levels of total plasma and ionized serum calcium ($P<0.001$). Further analysis of 21 LPTAs revealed low MIB1 proliferation index (0.1–1.5%), *MEN1* mutations in five cases, and one *HRPT2* (*CDC73*) mutation. Total or partial loss of parafibromin expression was observed in ten tumors, two of which also showed loss of APC expression. Using array CGH, we demonstrated recurrent copy number alterations most frequently involving loss in 1p (29%), gain in 5 (38%), and loss in 11q (33%). Totally, 21 minimal overlapping regions were defined for losses in 1p, 7q, 9p, 11, and 15q and gains in 3q, 5, 7p, 8p, 16q, 17p, and 19q. In addition, 12 tumors showed gross alterations of entire or almost entire chromosomes most frequently gain of 5 and loss of chromosome 11. While gain of 5 was the most frequent alteration observed in LPTAs, it was only detected in a small proportion (4/58 cases, 7%) of parathyroid adenomas. A significant positive correlation was observed between parathyroid hormone level and total copy number gain ($r=0.48$, $P=0.031$). These results support that LPTAs represent a group of patients with pronounced parathyroid hyperfunction and associated with specific genomic features.

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Introduction

Primary hyperparathyroidism (PHPT) is one of the most common endocrine disorders. Affected patients typically present with high levels of ionized serum calcium (S-Ca^{2+}) resulting from abnormally high production of parathyroid hormone (PTH). In about 85% of cases, PHPT is due to an adenoma, multiglandular disease accounts for about 15% of cases, and 1% of patients have parathyroid carcinoma (DeLellis *et al*. 2004). The disease is more common among postmenopausal women, and screening studies in the Swedish population have recognized PHPT in about 3.4% of this entity (Lundgren *et al*. 2002).

Previous studies have identified several causative genetic events in PHPT contributing to the overall understanding of the molecular pathogenesis of the disease. Constitutional mutations are most common not only in the tumor suppressor genes *MEN1* (11q13; Chandrasekharappa *et al*. 1997, Lemmens *et al*. 1997) and *HRPT2* (*CDC73*; 1q31.2; Carpten *et al*. 2002) but also occur in *CASR* (3q21.1; Carling *et al*. 2000, Simonds *et al*. 2002) and *CDKN1B*/p27 (12p13.1;

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Pellegata *et al*. 2006). Somatic mutations are commonly seen for *MEN1* in parathyroid adenomas (Heppner *et al*. 1997, Farnebo *et al*. 1998) and for *HRPT2* in parathyroid carcinomas (Howell *et al*. 2003, Shattuck *et al*. 2003). Chromosome 11 rearrangements between *PTH* and cyclin D1/*PRAD1* have been detected in a few cases of sporadic PHPT (Arnold *et al*. 1989). In addition, two cases of somatic translocations have been reported, including a t(1;5)(p22;q32; Örndal *et al*. 1990) and a t(4;13)(q21;q14; Sammarelli *et al*. 2007).

Screenings of PHPT adenomas for genomic alterations by comparative genomic hybridization (CGH; Agarwal *et al*. 1998, Palanisamy *et al*. 1998, Farnebo *et al*. 1999, Hemmer *et al*. 2001, Garcia *et al*. 2002), loss of heterozygosity (LOH; Cryns *et al*. 1995, Tahara *et al*. 1996, 1997, Williamson *et al*. 1997, Carling *et al*. 1998, Dwight *et al*. 2000, 2002, Correa *et al*. 2002), and array CGH (Yi *et al*. 2008) have identified recurrent losses in 1p, 3q, 6q, 9p, 11p, 11q, and 15q and gains of 16p and 19p.

While the risk groups of familial and malignant PHPT have been partly elucidated at the molecular level, less is known about sporadic parathyroid adenomas with pronounced clinical and tumor phenotypes. The terms Large or Giant adenoma have been applied to describe cases of parathyroid tumors with glandular weights far larger than the median weight (650 mg) of unselected parathyroid adenomas (Almquist *et al*. 2010). Publications describing cases of large parathyroid tumors are so far limited; however, male overrepresentation and increased biochemical symptoms have been observed in limited case series (Takeichi *et al*. 1983, Lalanne-Mistrih *et al*. 2002, Chiofalo *et al*. 2005, O'Neal *et al*. 2011). Several studies have compared adenoma weight with biochemical parameters. While some of these have reported positive correlations between parathyroid adenoma weight and levels of blood calcium (both total plasma and S-Ca^{2+}) and PTH, other studies did not reveal such correlations (Hedbäck *et al*. 1995, Bindlish *et al*. 2002, Randhawa *et al*. 2007). Furthermore, increased parathyroid adenoma weight was found to be associated with higher risk of death (Hedbäck *et al*. 1995). These circumstances imply that large parathyroid adenoma (LPTA) patients could have more aggressive clinical presentations. The aforementioned features of LPTA are also in line with observations that parathyroid carcinomas generally have a higher glandular weight than regular adenomas (Kebebew 2001, Shane 2001, Robert *et al*. 2005, Agarwal *et al*. 2006, Fernandez-Ranvier *et al*. 2007) and mostly have pronounced clinical symptoms. In studies of sporadic parathyroid adenomas, associations have been made between molecular features and tumor size. For example, one study reported differential microarray gene expression profiles in sporadic parathyroid adenomas with different glandular weights (Rosen *et al*. 2005).

The genetic background of LPTA has not been characterized so far. In this study, we hypothesized that large glandular weight and associated phenotypes may be attributed to distinct genetic abnormalities. To test our hypothesis, we characterized the mutation status of the *MEN1* and *HRPT2* genes and applied high-resolution array CGH to assess copy number alterations (CNAs) in a panel of such tumors representing the 5% largest sporadic parathyroid adenomas in our institution.

Materials and methods

LPTA cases

Cases with LPTA were identified from patients operated for PHPT in the Karolinska University Hospital, Stockholm, Sweden, during 2005–2009. A total of 590 patients were identified who were without a known genetic predisposition for the disease and had a parathyroid adenoma of a specified weight that had been examined at routine clinical histopathology (Table 1). From the distribution of tumor sizes, we identified a group of 31 patients representing the 5% largest tumors with glandular weight ≥ 4 g (Supplementary Figure 1, see section on supplementary data given at the end of this article), which were classified as LPTAs.

Nineteen of these 31 LPTA cases were included in genetic studies based on frozen tissue sample availability. Two additional LPTA tumors (cases 1 and 2) were identified from our previous studies (Juhlin *et al*. 2006). Details for the 21 cases investigated here, concerning demographic and biochemical parameters, are presented in Table 2. One patient had a known history of irradiation to the head and neck region (case 8), two had renal stones (cases 4 and 20), and one had renal failure (case 3). No clinical/family history suggestive of multiple endocrine neoplasia or familial PHPT syndrome could be elicited. Based on the clinical, surgical, and pathological findings, all patients were diagnosed with single gland disease classified as parathyroid adenoma or atypical adenoma according to the World Health Organization (WHO) criteria (DeLellis *et al*. 2004). The diagnosis of atypical adenoma was based on the histopathological features such as capsular engagement, trabecular formation, vascular invasion, fibrous bands, and increased MIB1

Table 1 Characteristics of the sporadic PHPT adenoma population 2005–2009

Parameter number of informative	LPTA ≥4 g (n=31)	*P* ♂–♀	Adenomas <4 g (n=559)	*P* ♂–♀	*P* <4 to ≥4 g	All adenomas (n=590)	*P* ♂–♀	LPTA 1–21 ≥4 g (n=21)	*P* ♂–♀
Weight (g)	n=31		n=559			n=590		n=21	
Male									
Median (min–max)	7.10 (4.07–12.30)		0.54 (0.12–3.91)			0.60 (0.05–27.80)		8.88 (4.07–12.30)	
Mean±S.E.M.	7.56±0.90		0.76±0.07			1.34±0.02		8.54±1.02	
		0.86		<0.001			<0.001		0.77
Female									
Median (min–max)	6.59 (4.12–27.80)		0.37 (0.05–3.80)			0.39 (0.05–27.80)		7.50 (4.23–27.80)	
Mean±S.E.M.	7.82±1.31		0.60±0.03			0.90±0.09		9.67±2.44	
Gender	n=31		n=559			n=590		n=21	
Male									
Number (%)	11 (35%)		121 (22%)		0.037	132 (22%)		8 (38%)	
Female									
Number (%)	20 (65%)		438 (78%)			458 (78%)		13 (62%)	
Age at diagnosis (years)	n=31		n=559			n=590		n=21	
Male									
Median (min–max)	66.0 (37–86)		59.0 (17–85)			59.0 (17–86)		66.0 (37.84)	
Mean±S.E.M.	63.2±4.7		56.5±1.5			57.0±1.4		64.4±5.5	
		0.66		0.054	0.54		0.087		0.41
Female									
Median (min–max)	61.0 (29–90)		60.0 (19–92)			60 (19–92)		62.0 (38–83)	
Mean±S.E.M.	60.7±3.7		60.1±0.6			60.1±0.6		62.3±3.9	
Total plasma calcium (mmol/l)	n=29		n=547			n=576		n=21	
Male									
Median (min–max)	3.03 (1.72–3.53)		2.80 (2.48–3.94)			2.81 (2.48–3.94)		3.10 (2.73–3.53)	
Mean±S.E.M.	2.94±0.14		2.82±0.03			2.83±0.03		3.1±0.09	
		0.74		0.001	<0.001		<0.001		0.46
Female									
Median (min–max)	3.02 (2.72–3.49)		2.75 (2.46–3.57)			2.75 (2.46–3.57)		3.00 (2.72–3.49)	
Mean±S.E.M.	2.94±0.16		2.76±0.01			2.77±0.01		3.07±0.07	
Ionized serum calcium (mmol/l)	n=28		n=550			n=578		n=18	
Male									
Median (min–max)	1.59 (1.43–1.85)		1.47 (1.30–2.06)			1.48 (1.30–2.06)		1.64 (1.43–1.85)	
Mean±S.E.M.	1.55±0.07		1.50±0.01			1.50±0.01		1.64±0.04	
		0.84		0.001	<0.001		<0.001		0.54
Female									
Median (min–max)	1.59 (1.43–1.86)		1.44 (1.29–1.87)			1.44 (1.29–1.87)		1.59 (1.43–1.86)	
Mean±S.E.M.	1.63±0.03		1.45±0.01			1.47±0.01		1.63±0.04	

n, number; min, minimum; max, maximum. Reference values: glandular weight 0.04–0.06 g; total plasma calcium 2.15–2.55 mmol/l; ionized serum calcium 1.15–1.33 mmol/l. *P* values <0.05 were considered as statistically significant.

Table 2 Clinical and histopathological details of the 21 LPTA cases

Case no.	Age (years)	Sex (M/F)	Tumor weight (g)	Calcium (mmol/l) Total	Calcium (mmol/l) Ionized	P-PTH (ng/l)	Original diagnosis	Dominating cell type	Histopathological features of atypical adenoma
1	75	F	6.54	2.85	–	711	Atypical adenoma	Chief cell	Trabecular
2	36	F	4.86	3.08	–	83	Adenoma	Chief cell	No
3	62	F	5.07	3.00	1.55	424	Adenoma	Chief cell	No
4	41	M	4.28	2.82	–	149	Adenoma	Chief cell	Trabecular
5	57	F	8.50	2.97	1.59	358	Adenoma	Chief cell	No
6	58	F	7.60	3.19	1.86	351	Adenoma	Chief cell	No
7	66	M	5.51	3.12	1.74	256	Adenoma	Chief cell	No
8	74	F	6.06	3.16	1.78	298	Adenoma	Chief cell	No
9	83	F	4.67	2.56	1.48	–	Adenoma	Oxyphilic	No
10	65	F	7.50	2.92	1.41	147	Adenoma	Chief cell	Pleomorphism
11	72	M	12.30	3.00	1.63	1070	Adenoma	Mixed	Trabecular
12	59	M	11.60	3.12	1.85	1280	Atypical adenoma	Chief cell	Capsular engagement
13	69	M	10.00	2.66	1.62	1400	Atypical adenoma	Mixed	Pleomorphism
14	54	F	13.50	3.19	1.85	245	Adenoma	Chief cell	Trabecular
15	38	F	7.00	2.81	1.58	248	Adenoma	Mixed	No
16	78	F	4.12	2.72	1.55	142	Adenoma	Chief cell	No
17	64	F	4.50	2.95	1.61	356	Adenoma	Oxyphilic	No
18	83	M	7.10	2.92	1.68	200	Adenoma	Chief cell	No
19	37	M	4.07	2.60	1.43	123	Adenoma	Chief cell	No
20	51	F	27.80	3.13	1.67	513	Adenoma	Chief cell	Trabecular
21	66	M	9.75	3.10	1.72	506	Adenoma	Chief cell	Fibrous bands

Age refers to age at diagnosis; M, male; F, female; –, not available; P-PTH, plasma parathyroid hormone. Reference values: glandular weight 0.04–0.06 g; total plasma calcium 2.15–2.55 mmol/l; ionized serum calcium 1.15–1.33 mmol/l; serum-PTH 10–65 ng/l.

proliferation index. None of the tumors presented a diagnostic feature of parathyroid carcinoma, i.e. local invasion, lymph node, or distant metastasis. For the purpose of this study, routine histopathological reports were reviewed, and routine slides were histopathologically reexamined for LPTA cases 1–21 (Table 2).

Tissue samples

All samples have been collected with oral informed consent from the patients and documented in the patients' medical files. Ethical approval was granted from the local ethics committee. Fresh frozen parathyroid tissue samples stored at −80 °C were obtained from the tissue biobank at the Karolinska University Hospital. Representative sections from all tumor samples studied were examined microscopically by a histopathologist and shown to contain at least 70% tumor cells. Frozen tissue samples were obtained from the 21 LPTA tumors and from three histopathologically confirmed nontumorous parathyroid tissues (N1, N2, and N3) used as references for the gene expression study. One of these (N1) was obtained from the normal rim of a parathyroid adenoma, while the other two (N2 and N3) were removed inadvertently during thyroid surgery. Paraffin-embedded tumor tissue sections of LPTAs 3–21 were obtained for immunohistochemistry, together with anonymous sections of nontumor colon tissue and colon cancer used as controls. In addition, 58 previously published (Forsberg *et al.* 2005, Haglund *et al.* 2010) DNA samples from parathyroid adenomas were used for qPCR of chromosome 5 loci. Adenomas with a known weight $\geq$4 g were not included.

DNA and RNA extraction

Total genomic DNA and total RNA were extracted using DNeasy Blood and Tissue DNA isolation kit (Qiagen AB) and *mir*Vana miRNA Isolation Kit (Ambion, Life Technologies, Applied Biosystems), respectively, following the manufacturer's protocols. DNA and the RNA samples were quantified and checked for quality using NanoDrop A100 (Thermo Scientific, Waltham, MA, USA) and stored at −20 °C until use.

Immunohistochemistry

Protein expression of parafibromin, APC, and Ki-67 was determined by immunohistochemistry on slides cut at 4 μm thickness from paraffin-embedded blocks of LPTAs 3–21 and controls using previously published methodologies (Juhlin *et al*. 2006). The following antibodies were used: APC rabbit monoclonal (EP701Y, cat no. ab40778; Abcam, Cambridge, UK) at a dilution of 1:100, parafibromin mouse monoclonal (2H1, cat no. sc-33638; Santa Cruz Biotechnology, CA, USA) at a dilution of 1:20, and monoclonal MIB1 (Ki67, clone MIB1; Dako, Hamburg, Germany, M7240) at a dilution of 1:200. Stained slides were scanned with a virtual slide scanner (NanoZoomer 2.0-HT, Hamamatsu, Japan) and viewed using NDP viewer (NanoZoomer Digital Pathology). Exclusion of the primary antibody as well as staining of normal colon tissue were used as negative controls for all antibodies. Colon cancer tissue was used as positive control of Ki-67 expression, as well as parathyroid tissues with known parafibromin and APC expressions.

The result was evaluated by two or more of the authors. MIB1 index was determined from Ki-67-stained slides using both automated scoring of scanned slides and counting of more than 2000 cells from 5 or more randomly selected high power fields (×40) in the same tumor. Parafibromin staining was scored as positive according to the previous reports, total loss or partial loss of nuclear expression, and in addition, nucleolar expression was evaluated (Juhlin *et al*. 2006, 2010). APC was scored as positive or negative as described in Juhlin *et al*. (2010).

Mutational analysis

The coding exons 2–10 of the *MEN1* gene and all the 1–17 exons of the *HRPT2* gene were sequenced in both directions in LPTAs 1–21 using previously published primer sequences and experimental conditions with some modifications (Villablanca *et al*. 2004, Juhlin *et al*. 2006). PCR was performed in 25 μl reactions with 50 ng DNA, 1× PCR buffer, 200 μM of each dNTPs, 2 mM $MgSO_4$, 0.2 μM of each primers, and 1 unit TaqDNA polymerase (Invitrogen Life Technologies). PCR cycling conditions included 94 °C for 30 s as initial denaturation followed by 30 cycles (*MEN1*) or 35 cycles (*HRPT2*) of 30 s of denaturation at 95 °C, annealing for 30 s at 52–58 °C, and extension for 30 s at 72 °C. All PCR products were verified in 2% agarose gels, quantified, and purified using ExoSAP-IT (USB-Affymetrix, Cleveland, OH, USA). Sequencing was done using BigDye terminators v. 1.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) in ABI 3730 PRISM DNA Analyzer (Applied Biosystems) following the protocol of the manufacturer, and the sequencing data were analyzed for mutations using SeqScape version 2.5.0 (Applied Biosystems).

Array CGH and data analysis

Array CGH was performed for LPTA cases 1–21 using human BAC 38K arrays generated at the SCIBLU Genomics Centre at Lund University, Sweden (www.lu.se/sciblu). These arrays contain 38 000 BAC clones (CHORI BACPAC resources; http://bacpac.chori.org/genomicRearray.php) arranged in a tiling fashion resulting in a final resolution of about 100–150 kb. Laboratory procedures and data analyses were carried out according to the previously established protocols (www.lu.se/sciblu; Hashemi *et al*. 2011). Array CGH slides were scanned in an Axon Scanner 4000A (Axon Instruments, Burlingame, CA, USA) and the GenePix Pro 6.0 software (Axon Instruments) was used for spot identification and image analysis. The resulting data were imported into the BioArray Software Environment (BASE; http://base.thep.lu.se/) version 2 for further analysis. CNAs were classified according to $\log_2$ ratios applying the following thresholds: +0.25 for gain, −0.25 for loss, −1 for homozygous loss, and +1 for amplification. Recurrent aberrations were defined as aberrations identified in four or more of the samples. Gross chromosomal changes were defined as changes that involved an entire or almost entire chromosome. Minimal overlapping regions (MORs) were defined as the smallest recurrent CNAs according to the definition by Rouveirol *et al*. (2006). Array CGH data for all the 21 cases have been submitted in NCBI's Gene Expression Omnibus through GEO Series accession number GSE36511 (http://www.ncbi.nlm.nih.gov/geo/quary/acc.cgi).

Unsupervised hierarchical clustering was performed using Pearson's correlation distance matrix (R software v2.12.2). Briefly, this algorithm scans through the entire array CGH dataset (sex chromosome clones were excluded), calculates all pair-wise distances (degree of similarity regarding the copy number changes), estimates the correlation efficiency for each single clone spotted on the slide, and joins the closest pair of samples. Then, it calculates the pair's distance to all other samples in the same pair-wise approach to find the two other closest pairs. This process will be repeated until the distances for all the samples are calculated and based on that, samples will be reordered into a hierarchical structure represented by a dendrogram tree (Quackenbush 2006).

Genomic quantitative real-time PCR (qPCR)

LPTAs 1–21 as well as 58 additional parathyroid adenomas were analyzed by qPCR for copy number detection at individual loci in regions of frequent losses or gains in chromosomes 1, 5, and 11. Two separate TaqMan Copy Number assays were used for each of the loci *RNF11* (Hs_cn 02893920 in intron 1/exon 2 and Hs_cn 02226491 in exon 3), *TERT* (3078158 in exon 2 and 2818605 in exon 16), *CARD6* (3056294 in exon 1 and 888745 in exon 3), *PIK3R1* (2532703 in exon 6/intron 6 and 2558647 in exon 17), *APC* (02966112 in intron 3/exon 4), *LMNB1* (1652643 in exon 1 and 2496887 in exon 11), and *MEN1* (02189358 in exon 1 and 00270415 in exon 3). Analyses were carried out according to the protocol recommended by the manufacturer (Applied Biosystems). Samples were amplified in a 7500 FAST machine (Applied Biosystems), using a 96-well format and the cycling conditions: 95 °C for 10 min, and 40 cycles of (95 °C for 15 s and 60 °C for 1 min). Each sample was amplified in triplicate in two different experiments. Amplification data were analyzed in the Sequence Detection Software SDS 2.2 (Applied Biosystems) followed by the CopyCaller software V1.0 (Applied Biosystems) for prediction of copy numbers. Data were normalized to *RNaseP* (Hs_cn 4403326), which was amplified in parallel, and calibrated to pooled normal DNA (Promega). Negative controls constituted of amplifications without sample DNA.

Quantitative reverse transcription PCR (qRT-PCR)

Expression of the *CARD6* gene was quantified in LPTAs 1–21 and the three reference parathyroids N1–3 by qRT-PCR. In brief, 500 ng total RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. qRT-PCR was performed in triplicate applying standard amplification conditions and a 96-well plate format in a StepOnePlus real-time PCR machine (Applied Biosystems). Normalization was done against the housekeeping gene *36B4*, which is known to be ubiquitously expressed in parathyroid tissue (Forsberg *et al*. 2005, Juhlin *et al*. 2006). Commercially available assays were used for *CARD6* (Hs00261581_m1; Applied Biosystems) and *36B4* (Hs99999902_m1). The expression of *CARD6* was then quantified using the Delta–Delta C_t method ($2^{-\Delta\Delta C_t}$).

Statistical analysis

All statistical calculations were performed using the SPSS software (Statistical Software Package for Windows, V.17; SPSS Inc., Chicago, IL, USA). The nonparametric Mann–Whitney *U* test was used to compare the demographic characteristics between LPTAs and adenomas <4 g, while χ^2 test was used to investigate the differences in gender distribution. Relationships between variables were assessed with Spearman's rank correlation test. *P* values below 0.05 were considered as statistically significant.

Results

General characteristics for 590 PHPT cases operated for a sporadic parathyroid adenoma during 2005–2009 at the Karolinska University Hospital, Stockholm, Sweden, are summarized in Table 1. In the entire material, the median adenoma weight, total plasma calcium, and S-Ca^{2+} levels were significantly higher in male patients (0.60 vs 0.39 g; 2.81 vs 2.75, and 1.48 vs 1.44 mmol/l, respectively, $P<0.001$). However, no statistically significant difference in age at diagnosis was observed. Thirty-one of the 590 cases (5%) presented as LPTA weighing ≥ 4 g, while 559 cases (95%) had an adenoma weight <4 g (Supplementary Figure 1). Comparison of demographic variables revealed a higher relative number of male cases among LPTAs (11 males/20 females, 35%) compared with tumors <4 g (121 males/438 females, 22%; $P=0.037$). Furthermore, preoperative levels of calcium (both total plasma and S-Ca^{2+}) were significantly higher for LPTA cases than adenomas <4 g (median 3.03 and 1.59 vs 2.80 and 1.47 mmol/l, respectively, $P<0.001$; Table 1).

The 21 cases entered into the molecular characterization also tended to include relatively more male cases ($P=0.075$) and exhibited significantly higher S-Ca^{2+}levels (median 1.64 vs 1.47 mmol/l, $P<0.001$; Tables 1 and 2). Eighteen of these had been classified as adenoma and three as atypical adenoma at the original routine histopathological work-up. However, review of all cases showed that some histopathological features associated with atypical adenoma were also present in six tumors classified as adenoma originally (LPTAs 4, 10, 11, 14, 20, and 21, Table 2). These included trabecular growth pattern, pleomorphism, and fibrous bands. However, the limited extent of these features did not motivate a classification as atypical adenoma. In addition, automated and manual scoring of Ki-67 immunoreactivity revealed low MIB1 proliferation index of 0.1–1.5 and 0.1–0.6%,

respectively, in all cases analyzed. The hypercalcemia was normalized in all cases and no recurrences were detected at the follow-up.

Mutation screening of the *MEN1* and *HRPT2* genes

Detected mutations are illustrated in Supplementary Figures 2 and 3, see section on supplementary data given at the end of this article and detailed in Table 3. Five *MEN1* mutations were observed, which were all predicted to lead to the introduction of a premature stop codon and shorter protein. In the *HRPT2* gene, one missense mutation was revealed. In addition, intronic base substitutions were detected in *HRPT2* (c1067-85C>T intron 12 of case 1, c729+50delAG in intron 7 of case 19, and c1418-17C>G in intron 15 of case 8).

Expression of parafibromin and APC

Expression of the APC and parafibromin proteins was evaluated by immunohistochemistry in LPTAs 3–21 for which slides were available (Table 3). The findings are illustrated in Fig. 1. Nine cases showed positive parafibromin expression, while five cases (7, 12, 13, 17, and 19) were negative and five cases (8, 10, 11, 14, and 16) had partial loss of nuclear staining. For APC, 17/19 tumors were positive, while two tumors (cases 13 and 14) were negative for both nuclear and cytoplasmic immunoreactivity.

Overall array CGH findings

All 21 LPTAs investigated in this study exhibited CNAs including gains and losses. However, no homozygous deletions (<-1) or high-level gains ($>+1$) indicating amplifications were observed. First, we investigated CNAs at the genomic loci of genes

Table 3 Results from immunohistochemistry, mutation screening, and array CGH in LPTAs 1–21

					Mutation screening[a]			Total change by array CGH (Mb)		
Case no.	IHC parafibromin	IHC APC	CNA at *MEN1*	CNA at *HRPT2*	*MEN1*	*HRPT2*	Predicted protein effect	Losses	Gains	Total
1	–	–	No	No	Wt	Wt	–	187.6	100.2	287.8
2	–	–	No	Loss	c.1715del6[b]	Wt	Stop codon at nt 1825	798.5	308.6	1107.1
3	Pos	Pos	No	No	Wt	Wt	–	0.0	45.8	45.8
4	Pos	Pos	No	No	Wt	c214C>T	p.Pro71Ser	10.4	1.0	11.4
5	Pos	Pos	Loss	No	c.1125del4	Wt	Stop codon at nt 1327	165.0	46.4	211.4
6	Pos	Pos	No	No	Wt	Wt	–	144.9	16.8	161.7
7	Total loss	Pos	No	No	Wt	Wt	–	1.9	0.3	2.2
8	Partial loss	Pos	No	No	Wt	Wt	–	3.8	14.1	17.9
9	Pos	Pos	No	No	Wt	Wt	–	127.7	78.7	206.4
10	Partial loss	Pos	Loss	No	Wt	Wt	–	171.4	2.8	174.2
11	Partial loss	Pos	Loss	No	Wt	Wt	–	267.8	445.9	713.7
12	Total loss	Pos	No	No	Wt	Wt	–	4.2	614.1	618.3
13	Total loss	Neg	No	No	Wt	Wt	–	49.8	785.4	835.2
14	Partial loss	Neg	No	No	Wt	Wt	–	19.9	3.6	23.4
15	Pos	Pos	No	No	Wt	Wt	–	38.0	6.3	44.3
16	Partial loss	Pos	No	No	Wt	Wt	–	207.9	508.4	716.3
17	Total loss	Pos	Loss	Loss	c.113del11	Wt	Stop codon at nt 334	526.1	389.8	915.9
18	Pos	Pos	No	No	c.398insT	Wt	Stop codon at nt 537	123.7	3.0	126.7
19	Total loss	Pos	No	No	c.207del14	Wt	Stop codon at nt 333	1.8	1.5	3.2
20	Pos	Pos	Loss	No	Wt	Wt	–	176.2	2.3	178.5
21	Pos	Pos	No	No	Wt	Wt	–	210.9	175.6	386.5
Total								3237.4	3550.4	6787.8

IHC, immunohistochemistry; APC, adenomatous polyposis coli; Pos, positive; Neg, negative; Wt, wild type; CNA, copy number aberration.
[a]Numbering was according to ensemble for the *MEN1* (ENST00000312049) and *HRPT2* (ENST00000367435) genes.
[b]The mutation has been previously reported in Juhlin *et al.* (2006).

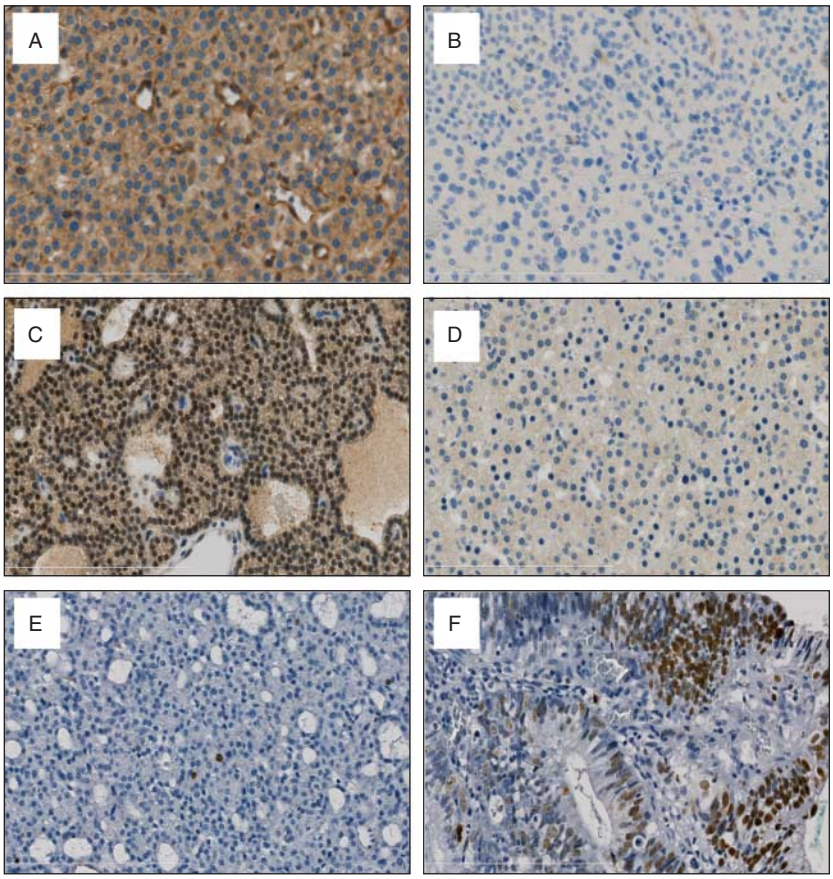

Figure 1 Photomicrographs showing examples of immunohistochemistry analysis of LPTAs. Positive (A) and negative (B) cytoplasmic immunoreactivity in parathyroid adenomas for APC. Positive (C) and negative (D) staining of parathyroid adenomas for nuclear parafibromin. MIB1 proliferation index determined by Ki-67 immunostaining of a parathyroid adenoma (E) and a colon cancer (F) used as positive control. All images are shown at ×40 power.

known to be implicated in parathyroid tumors including *MEN1*, *HRPT2*, *CASR*, *APC*, *CCND1*, and *RB1*. The *MEN1* locus at 11q13 was deleted in 19% of the cases, the *HRPT2* locus at 1q31.2 in 9%, and the *CCND1* locus at 11q13.3 in 14%. The *CASR* locus at 3q21 showed normal array CGH profiles in all cases. The *APC* locus at 5q22.2 was gained in 22.2% of the cases and the *RB1* locus in 13q14.2 was gained in 5% and lost in 14% of LPTAs (Table 3).

We subsequently investigated CNAs in the entire genome which revealed that all chromosomes were involved and changes restricted to a small part of the chromosome were more common than gross chromosomal aberrations involving an entire or almost entire chromosome (Fig. 2). Recurrent losses were detected on 1p, 7q, 11, 15q, and 21q, and gains were observed on 3q, 5, 7p, 8p, 16q, 17p, and 19q with the longest combined interval CNAs detected on chromosomes 1, 5, and 11 (Fig. 2). The total amplitude of CNAs detected in a single tumor ranged from about 2.2 Mb (case 7) to 1107.1 Mb (case 2) with a median of 178.5 Mb (Table 3).

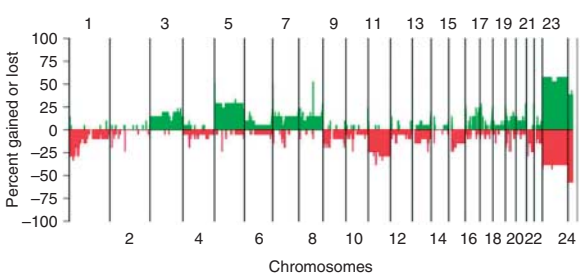

Figure 2 Overview of copy number alterations detected by array CGH in 21 cases of LPTA. The whole-genome frequency plot shows the proportion (in %) of gains (green) and losses (red) detected in each individual chromosome from pter (left) to qter (right). Individual chromosomes are shown in numbering order from 1 (left) to 22 (right). Sex chromosomes (23 and 24) were not included in the analyses.

Table 4 Most common recurrent MORs detected by array CGH in 21 LPTA cases

Cytoband	Start clone	End clone	Position (Mb)	Size (Mb)	No. of clones	Frequency %	Selected for qPCR
Losses							
1p36.31–p35.3	CTD-2280K14	RP13-485J14	6.1–28.7	22.60	281	29	
1p35.2–p35.1	RP11-51C12	RP11-125D21	30.8–34.1	3.16	36	29	
1p32.3	RP11-183G22	RP11-12C19	50.4–55.2	4.71	53	29	*RNF11*
7q11.22–q11.23	RP11-746H3	RP11-379D10	71.1–75.3	4.31	73	19	
9p21.3–p13.3	RP11-560J9	RP11-475O13	32.9–34.4	1.43	15	19	
11p15.5–p11.2	RP11-371C18	RP11-56E13	1.3–48.1	46.80	666	24	
11q13.1–q13.2	RP11-472L10	RP11-259D17	63.3–68.2	4.88	55	19	*MEN1*
11q14.1–q14.2	RP11-726H9	RP11-317J19	85.1–86.1	1.00	10	33	
11q22.1–q24.3	RP11-463H5	CTD-2120F5	99.8–130.2	30.40	363	29	
15q11.1–q11.2	RP11-207G6	RP11-59F20	18.2–20.3	2.01	39	28	
15q15.1–q21.1	RP11-451G20	RP11-226C3	38.5–42.9	4.32	58	19	
Gains							
3q22.3–q26.33	RP11-809A16	RP11-139K3	140.1–183.8	43.70	542	19	
3q27.1	RP11-778D9	RP11-328G15	185.1–186.0	0.63	11	24	
5p13.1	RP11-94C22	RP11-19F12	40.8–41.4	0.62	6	29	*CARD6*
5q12.3–q13.1	RP11-475K24	RP11-417A16	65.9–68.3	2.50	48	29	*PIK3R1*
5q23.2	RP11-772E11	RP11-368F9	125.9–126.1	0.20	4	33	*LMNB1*
7p14.1	RP11-556H1	RP11-170L15	37.6–38.1	0.51	4	19	
8p23.1	RP11-231L4	RP11-672P7	6.8–7.7	0.87	9	23	
16q24.2–24.3	RP11-483N11	RP11-180K1	86.7–87.9	0.13	9	19	
17p13.1	RP11-558E15	RP13-696M16	6.9–8.0	1.02	16	23	
19q13.2–q13.31	RP11-108I20	RP11-427D11	47.1–48.3	1.14	17	19	*DEDD2*

Mb, mega base pairs; MOR, minimal overlapping region.

Subchromosomal alterations

In order to identify chromosomal regions that may harbor genes involved in the tumorigenesis of LPTAs, we compared CNA patterns between tumors and estimated MORs. MORs that largely or completely overlapped with known normal copy number polymorphisms according to the Database of Genomic Variants (http://projects.tcag.ca/variation/) were excluded. In addition, we identified 11 MORs of loss and ten MORs of gain that were supported by recurrent CNAs in four or more LPTAs (Table 4).

Copy number losses involving the short arm of chromosome 1 was a frequent finding and detected in six cases (29%; Fig. 3). However, loss of 1q was infrequent and chromosome 1 rarely showed any gains. Three MORs of loss were identified on the short arm of chromosome 1. A 3.2 Mb region in 1p35.1–2, a 4.7 Mb interval in 1p32.3, and a 22.6 Mb region in 1p36.31–35.3 were each involved in 6/6 cases with loss in 1p (Fig. 3). The 1p32.3 region harbors several genes, such as the tumor suppressor genes *CDKN2C* (*P18*) and *RNF11*.

Chromosome 5 displayed gains in eight LPTAs (38%), which in six cases encompassed almost the entire chromosome (Fig. 4). Three MORs of gains were detected in chromosome 5. One MOR was defined at 5p13.1 (0.6 Mb) and the other two MORs at 5q23.2 (0.2 Mb) and 5q12.1–13.1 (2.5 Mb). The MOR located at 5q23.2 was gained in 7/8 cases with gain of chromosome 5 and overlapped few genes most importantly *LMNB1*, while the other MOR of gain identified at 5q12.1–13.1 was observed in six LPTAs (29%) and overlapped several genes among others, *PIK3R1*.

Chromosome 11 displayed copy number losses in seven of the LPTAs (33%) involving the q-arm in all the seven cases and the p-arm in five cases (Fig. 5). Gains of chromosome 11 were detected infrequently. Four MORs of losses were identified. The MOR in 11p15.5–p11.2 including almost the entire p-arm was deleted in 5/7 cases with the loss of chromosome 11. The three MORs of losses in 11q were defined at 11q13.1–2 (4.9 Mb), 11q14.1–2 (1.0 Mb), and 11q22.1–24.3 (30.4 Mb; Table 4). The 11q14.1–2 interval including *PICALM* was involved in 7/7 cases with 11q loss, while the 11q13.1–2 region that encompasses the *MEN1* gene was deleted in four cases.

Large-scale chromosomal alterations

CNAs corresponding to large-scale chromosomal changes were noted in a subset of the 21 LPTAs screened with array CGH and revealed as losses or

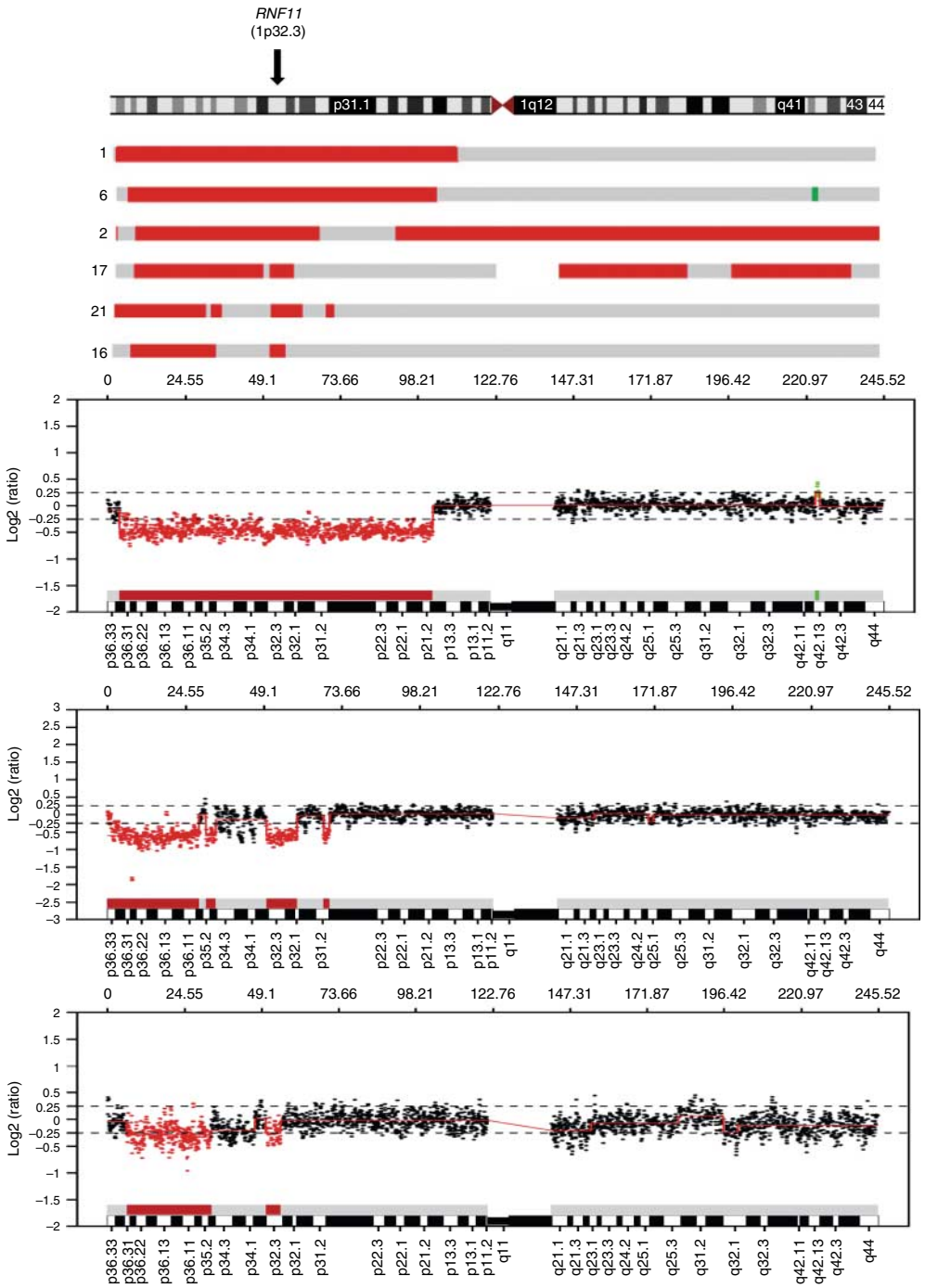

Figure 3 Mapping of copy number losses by array CGH in chromosome 1. At the top is shown an ideogram of chromosome 1 with the location of the qPCR assay *RNF11* used for verification indicated by arrow. Each horizontal bar represents one tumor with a detected loss in chromosome 1 indicated in red. Corresponding array CGH profiles of chromosome 1 are shown below for LPTA cases 6 (top), 21 (middle), and 16 (bottom).

gains that involved an entire or almost entire chromosome. A total of 53 such large-scale changes were detected in 12 cases, ranging from 1 to 10 aberrations per tumor. By contrast, in the remaining nine tumors, no large-scale changes were detected. The most common large-scale aberrations were gain of chromosome 5 (29%), 3 (19%), 7 (14%), and 8 (14%) and loss of chromosome 11 (24%),

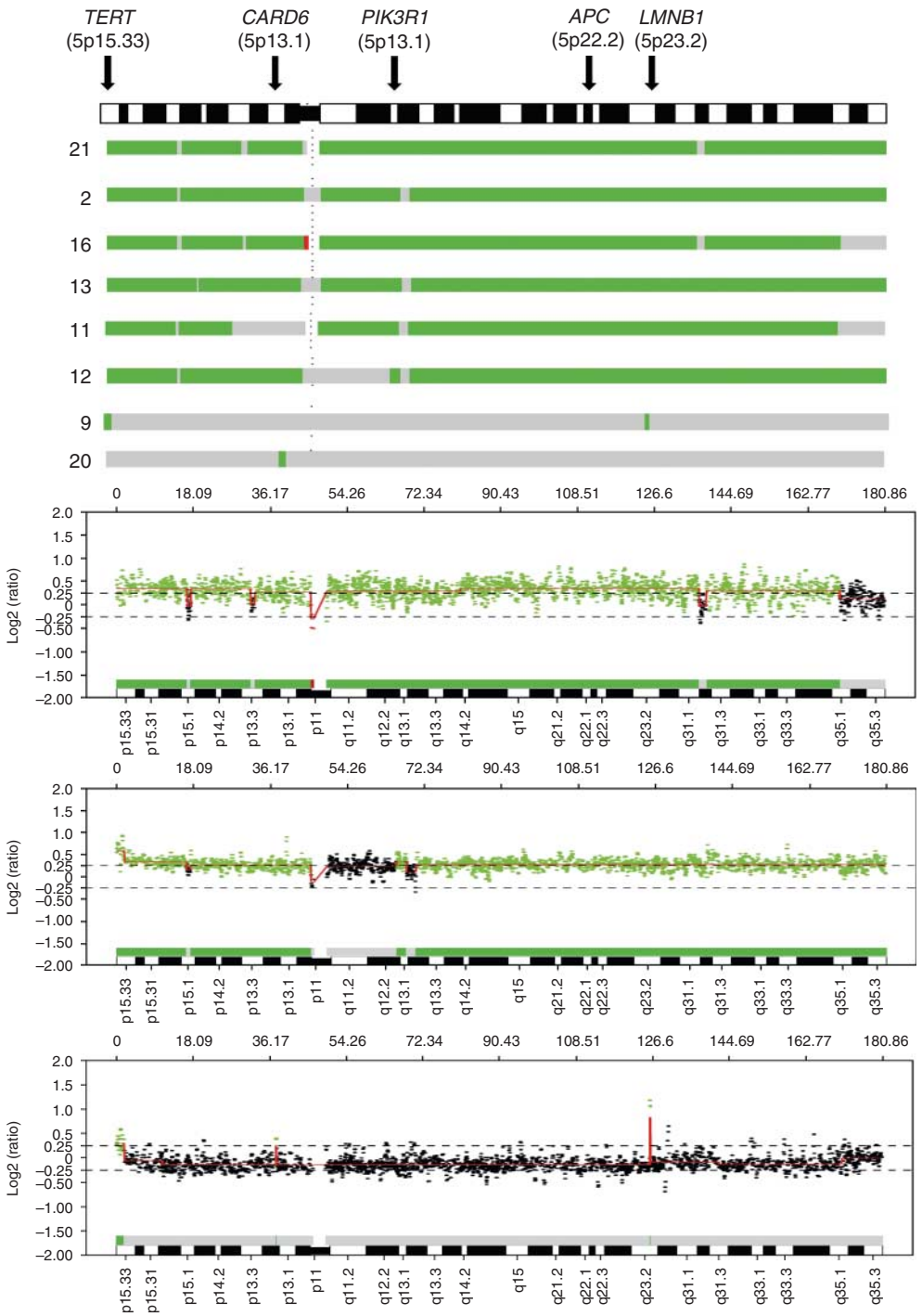

Figure 4 Mapping of copy number gains by array CGH in chromosome 5. An ideogram of chromosome 5 is shown together with the location of qPCR assays (arrows) above and horizontal bars representing tumors with detected gain (green) below. Individual array CGH profiles are shown for LPTA cases 16, 12, and 9.

21 (24%), 13 (14%), and 15 (14%; Fig. 6A). Chromosomes 2, 14, and 16 did not display any large-scale aberrations. Seven tumors showed one or more such gains which in 6/7 cases included chromosome 5. Ten tumors showed losses which in five cases involved chromosomes 11, but only one of these tumors overlapped with gain of chromosome 5 (case 11).

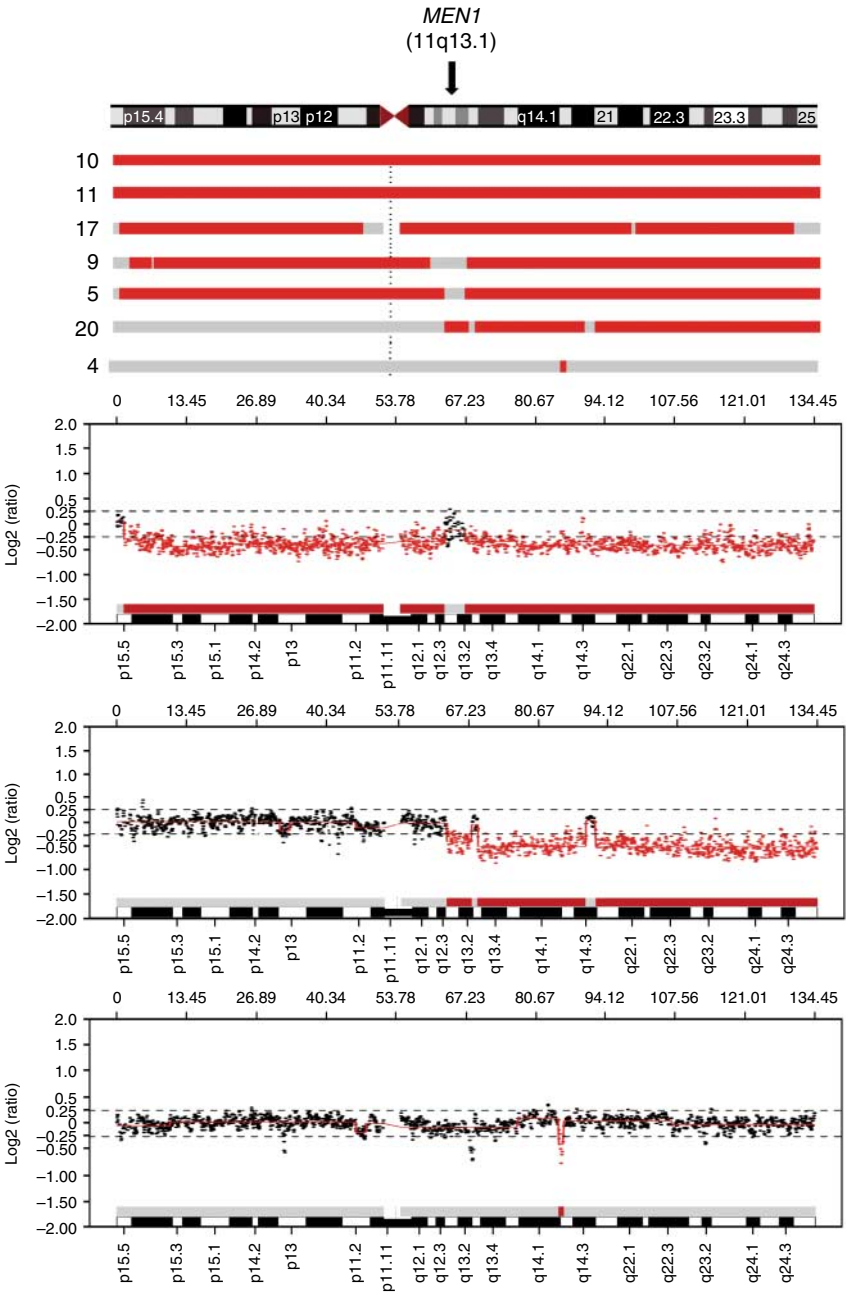

Figure 5 Mapping of copy number losses by array CGH in chromosome 11. Horizontal bars represent seven LPTAs with chromosome 11 loss (red). The location of the *MEN1* qPCR assay is indicated by arrow and individual profiles are shown for cases 9, 20, and 4.

Clustering and comparison of CNAs with clinical parameters

Unsupervised hierarchical clustering for all array CGH data resulted in two main clusters (Fig. 6B). Cluster 1 included six cases that were exclusively females. Four of the six cases in Cluster 1 showed gross chromosome 11 loss without gross chromosome 5 gain. Cluster 2 included 15 cases of which eight were male. While only one case (case 11) from Cluster 2 had gross chromosome 11 loss, all the six cases with entire chromosome 5 gain fell into this cluster.

The detected CNAs were compared with clinical characteristics for the 21 LPTA cases concerning amplitude of changes, cluster groups, subchromosomal changes, and large-scale aberrations. A statistically significant positive correlation between PTH levels and

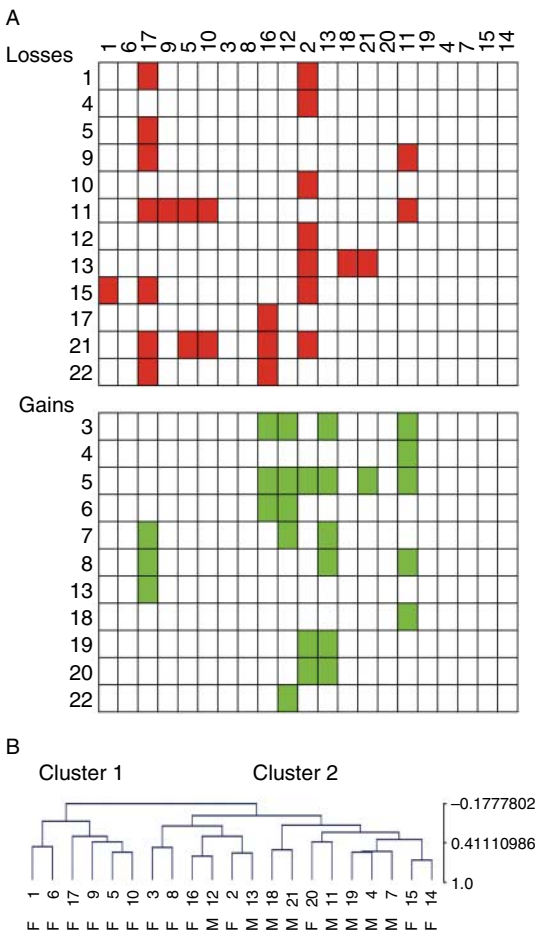

Figure 6 Distribution of copy number alterations by array CGH in the 21 LPTAs. (A) Graphical representation of large-scale alterations that involve the entire or almost the entire chromosome. Each colored block represents one chromosomal alteration in one tumor with gains marked in green and losses in red. Case numbers are indicated at the top and chromosome numbers to the left. (B) Unsupervised clustering of LPTAs 1–21 based on all array CGH data using Pearson correlation matrix distance. Information of male (M) or female (F) gender is indicated next to the case numbers.

total amplitude of copy number gains was observed ($r=0.48$, $P=0.031$; Supplementary Figure 4, see section on supplementary data given at the end of this article). No statistically significant differences in biochemical or histopathological characteristics were observed between the cases in Clusters 1 and 2.

Validation by qPCR

qPCR-based copy number assays were used to validate selected regions identified as recurrently lost or gained by array CGH in the set of 21 LPTAs (Table 5). For the 4.7 Mb MOR of loss in 1p32.3, we selected the *RNF11* gene for validation. Loss of *RNF11* showed a high concordance with array CGH findings and could be confirmed in 4/6 (67%) cases with 1p32.3 loss. To validate the loss of 11q, we selected only a well-known gene, *MEN1*, located in the 4.8 Mb MOR of loss in 11q13.1–2. Correlation between CNAs detected by array CGH and qPCR was revealed in 4/4 (100%) cases with loss at this specific locus. For chromosome 5, we selected five gene loci including *TERT* and *CARD6* on the p-arm and *PIK3R1*, *APC*, and *LMNB1* on the q-arm (Table 5). The gains observed by array CGH were also demonstrated by qPCR in 4/6 (67%) cases for *TERT*, 3/6 (50%) for *CARD6*, 5/6 (83%) for *PIK3R1*, 5/6 (83%) for *APC*, and 4/7 (57%) for *LMNB1*.

We also selected chromosome 5 for further analysis in an extended series of parathyroid adenoma cases. This was based on the finding of chromosome 5 gain as the most common aberration detected in this group of LPTA tumors and which has not been previously reported as a frequent alteration in parathyroid adenomas (Palanisamy *et al*. 1998, Garcia *et al*. 2002). Out of 58 cases, only four cases showed copy number gains for at least one chromosome 5 assay (Table 5).

CARD6 gene expression

Given the frequent gain of chromosome 5, expression of the *CARD6* gene was quantified in LPTAs 1–21 using qRT-PCR. Five LPTAs (cases 3, 4, 18, 20, and 21) displayed more than twofold increased expression compared with the reference parathyroid tissues (Fig. 7).

Discussion

The clinical entity of LPTA has not been well defined, and in previous studies, different cutoff points for glandular sizes have been applied to define abnormally LPTAs (Lalanne-Mistrih *et al*. 2002, Rosen *et al*. 2005). The smallest cutoff used in a recently published paper was 2 g (O'Neal *et al*. 2011). In our study, we defined 4 g as the cutoff based on the distribution of glandular weights for parathyroid adenomas operated in our institution during the period 2005–2009. This group of cases, representing the 5% largest PHPT adenomas, exhibited significantly increased calcium levels and a relatively higher male to female ratio. These observations are well in agreement with the previous studies on large-sized PHPT adenomas (Takeichi *et al*. 1983, Lalanne-Mistrih *et al*. 2002, Chiofalo *et al*. 2005, O'Neal *et al*. 2011).

As part of the genetic characterization of LPTAs, we screened the *MEN1* and *HRPT2* genes for mutations as these are known to be frequently mutated in

Table 5 Details and results for qPCR analyses

	Location		Alteration detected		
Gene locus	**Mb**	**Cytoband**	**Type**	**LPTA cases 1–21**	**Adenomas** ($n=58$)
Chromosome 1					
RNF11	51.7	1p32.3	Loss	7/21 cases (33%)	ND
Chromosome 5					
TERT	1.2	5p15.33	Gain	5/21 cases (24%)	ND
CARD6	40.8	5p13.1	Gain	4/21 cases (19%)	0/58 cases (0%)
PIK3R1	67.5	5q13.1	Gain	11/21 cases (52%)	2/58 cases (3%)
APC	112.0	5q22.2	Gain	6/21 cases (29%)	2/58 cases (3%)
LMNB1	126.1	5q23.2	Gain	4/21 cases (19%)	0/58 cases (0%)
Chromosome 11					
MEN1	64.6	11q13.1	Loss	6/21 cases (29%)	ND
Reference gene					
RNaseP	19.9	14q11.2			

Reported alterations were observed by one or both assays used for each gene. ND, not determined.

parathyroid tumors (Heppner *et al*. 1997, Farnebo *et al*. 1998). This revealed five cases with an *MEN1* mutation predicted to give a truncated menin protein and one case with a missense *HRPT2* mutation in agreement with the previous reports of frequent *MEN1* mutations and rare *HRPT2* mutations in parathyroid adenomas (Carpten *et al*. 2002, Howell *et al*. 2003, Krebs *et al*. 2005, Bradley *et al*. 2006, Juhlin *et al*. 2006). None of the patients for which a *MEN1* or *HRPT2* mutation was detected presented clinical indications or a family history indicating a familial form of the disease, which would suggest that the mutations are somatic. However, as constitutional DNA was not sequenced, the presence of constitutive mutations cannot be determined. Hence, the potential risk of heritable disease should be considered, especially in the *HRPT2* mutated case for which unexpected mutation carriers are known to occur. Two *MEN1* mutated cases (cases 5 and 17) exhibited CNA at the *MEN1* locus by qPCR (Table 3) and/or array CGH (Fig. 5) together with presence of sequencing traces for the wild-type allele (Supplementary Figure 2). The wild-type sequencing trace could result from one or more factors such as contaminating normal tissue, variable amplification efficiency of the different alleles, subclonal copy number loss in the tumor cells, or the tumors are hyperdiploid so that a copy number loss still leaves more than one allele in some or all tumor cells.

Using array CGH, we demonstrate recurrent CNAs corresponding to gross chromosomal and/or subchromosomal alterations in sporadic LPTAs. This approach is widely used to identify net CNAs targeting cancer-related genes but does not identify copy number neutral alterations that could be approached by screening for LOH using, e.g. SNP arrays. In our study, CNAs were detected in all cases with frequent involvement of chromosomes 1, 5, and 11. Among these, gain of chromosome 5 was the most common and unique alteration detected in 38% of the 21 LPTAs analyzed. The array CGH profiles detected confirmed several findings from the previous reports of chromosomal imbalances by LOH or metaphase CGH and in addition identified chromosome 5 gain as a recurrent aberration in the LPTA entity. Aberrations restricted to small chromosomal regions were also frequently observed, which may be attributed to the increased resolution of array CGH compared with LOH and metaphase CGH. While the only published array CGH analysis of parathyroid tumors has focused on chromosome 11 (Yi *et al*. 2008), comparison to published array CGH data for other chromosomes cannot be readily performed.

Interestingly, gain of chromosome 5 was observed as the most frequent (38%) aberration and commonly involved the entire or large parts of the chromosome. By contrast, this aberration has not been reported in high frequency in previous studies of regular parathyroid adenomas. For example, studies based on

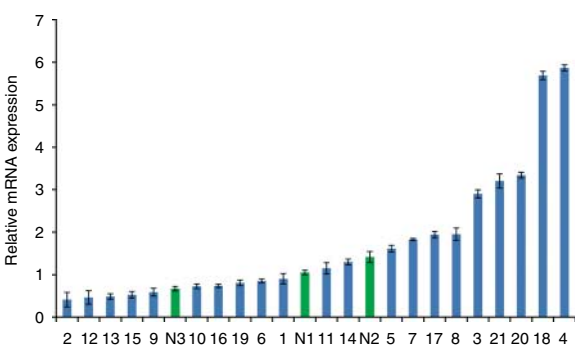

Figure 7 mRNA expression of the *CARD6* gene in LPTAs 1–21 and reference parathyroids (N1–3).

metaphase CGH analysis have reported gain of chromosome 5 in <14% of parathyroid adenomas (Palanisamy *et al*. 1998, Garcia *et al*. 2002). However, in parathyroid carcinomas, this abnormality was revealed in varying frequencies from <10% (Agarwal *et al*. 1998, Kytölä *et al*. 2000). The frequent occurrence of chromosome 5 gain in LPTAs compared with PHPT adenomas is unlikely to result from different resolutions of the applied techniques but may rather be regarded as a nonrandom chromosomal change characteristic of LPTAs or subsets among LPTAs. Copy number gains of chromosome 5 have also been associated with several other tumors such as low-grade renal cell carcinoma (Reutzel *et al*. 2001), adrenocortical tumors (Kjellman *et al*. 1996), as well as bronchial and gastrointestinal carcinoids (Voortman *et al*. 2010). Although the target gene(s) for chromosome 5 gain are presently unknown, several genes with known or potential involvement in human cancer are located on this chromosome. For example, the MOR at 5q23.2 that was gained in 33% of cases included the *LMNB1* gene encoding the lamin B1 protein expressed in tumors and plasma from hepatocellular carcinoma patients (Sun *et al*. 2010). Furthermore, the MOR at 5p13.1 includes the *CARD6* gene, which has been found overexpressed on the protein level in other cancers such as esophageal, gastric, and colorectal cancers (Kim *et al*. 2010). CARD6 plays an important role in the activation of NF-κB signaling, involved in proliferation, differentiation, and apoptosis (Aggarwal 2004) and deregulated in many tumor types including parathyroid tumors (Corbetta *et al*. 2005). The present observations of *CARD6* overexpression as well as copy number gains suggest that evaluation of the protein expression could be valuable to further study the possible involvement of this gene in parathyroid tumors.

Copy number loss in the short arm of chromosome 1 was recurrently observed in the LPTAs studied (29%) in agreement with most previous CGH and LOH studies of parathyroid adenomas (Cryns *et al*. 1995, Agarwal *et al*. 1998, Farnebo *et al*. 1999). Similar to observations in regular adenomas, losses in 1p mainly affected the distal part, in contrast to parathyroid carcinomas where deletions cluster in the central part of 1p (Kytölä *et al*. 2000, Välimäki *et al*. 2002). Loss in the short arm of chromosome 1 has been reported for many different cancer types, among others by LOH in the neuroendocrine tumors such as phaeochromocytoma and medullary thyroid carcinoma (Mathew *et al*. 1987, Khosla *et al*. 1991) as well as neuroblastoma (Fong *et al*. 1989). The genes *LCK*, *EPS15*, *RNF11*, and *CDKN2C* (*P18*) are potential candidates located in 1p, of which *CDKN2C* (*P18*) has been screened for point mutations with negative results (Tahara *et al*. 1997). The failure to detect frequently mutated target genes in 1p in parathyroid adenoma and other cancers may suggest the involvement of multiple target genes and/or possibly other inactivating mechanisms such as chromosomal rearrangements or inactivation by epigenetic mechanisms.

The LPTAs demonstrated frequent loss in chromosome 11, with preferential involvement of the long arm, comparable to the previously published findings in parathyroid adenomas based on the metaphase CGH, array CGH, and LOH analyses (Friedman *et al*. 1992, Cryns *et al*. 1995, Agarwal *et al*. 1998, Palanisamy *et al*. 1998, Farnebo *et al*. 1999, Dwight *et al*. 2000, Correa *et al*. 2002, Yi *et al*. 2008). Interestingly, LOH studies on parathyroid adenomas have demonstrated larger parathyroid adenoma weights in cases with 11q LOH (Friedman *et al*. 1992, Välimäki *et al*. 2002). The *MEN1* gene is known to be mutated in the majority of parathyroid adenomas with loss within the 11q13 region. In the LPTAs, the *MEN1* locus was lost in the majority of cases with 11q loss; however, loss was more frequently observed within distal chromosome 11 at 11q14 and 11q22.1–q24.3 (Fig. 5). Similar observations suggesting an additional locus on chromosome 11 outside the *MEN1* gene were reported using array CGH in parathyroid adenomas (Yi *et al*. 2008).

Based on the similarity in glandular size between LPTAs and parathyroid carcinomas, it has been speculated whether large parathyroid tumors could also be associated with a malignant potential (Hundahl *et al*. 1999). None of the LPTAs studied exhibited histopathological features of parathyroid cancer and, although the follow-up time is limited, no recurrences have been detected at the follow-up. To further approach this question, we determined MIB1 proliferation index as well as expression of the parafibromin and APC proteins, each of which have been associated with parathyroid malignancy. The proliferation index was found to be low, below 1.5%, in all LPTAs studied. This observation would suggest a benign behavior of the LPTAs based on the previous reports of low MIB1 index in parathyroid adenomas compared with carcinomas (Lloyd *et al*. 1995). Total or partial loss of parafibromin reactivity was observed in ten tumors, two of which also showed loss of APC immunoreactivity. However, this was not accompanied by *HRPT2* mutations, supporting the previous reports that loss of parafibromin immunoreactivity does not necessarily reflect an *HRPT2* mutation (Gill *et al*. 2006, Zhang *et al*. 2006, Juhlin *et al*. 2007, 2010).

Out of the three cases with an established diagnosis of atypical adenoma, two were available for IHC analyses (cases 12 and 13). Both these cases exhibited total loss of parafibromin expression, and in case 13, this feature was accompanied by loss of APC expression. These findings are fully in line with the previous reports regarding atypical adenomas and parafibromin and APC immunoreactivity (Juhlin *et al*. 2010). As loss of APC and parafibromin expression are associated with parathyroid carcinoma and atypical adenoma (Juhlin *et al*. 2007, 2010), our observations suggest that a subset of LPTAs share molecular characteristics with these entities.

Comparison of the genomic profiles, *MEN1* and *HRPT2* mutations, and APC and parafibromin expression of LPTAs in this study with published studies of regular parathyroid adenomas and parathyroid carcinomas suggest the presence of overlapping as well as distinguishing features. Hence, LPTAs were similar to adenomas concerning frequent *MEN1* mutations, infrequent *HRTP2* mutations, and losses of distal 1p and 11, and more like carcinomas concerning loss of APC and parafibromin expression and loss of chromosome 13. However, gain of chromosome 5 was common between LPTA and carcinoma. Taken together, these observations suggest that LPTAs exhibit partly overlapping and partly distinguishing genomic features compared with adenomas and carcinomas.

The 21 LPTAs fell into two main clusters based on the array CGH data, which in large corresponded to the presence/absence of large-scale chromosomal aberrations. This observation may suggest that gain of chromosome 5 reflect a genetic pathway for a subset of LPTAs that is independent of chromosome 11 loss. It can also be noted that all male cases fell into Cluster 2 characterized by chromosome 5 gain. Interestingly, a positive correlation was also observed between PTH level and total amplitude of copy number gain. However, gender-related differences and statistical associations to PTH levels for array CGH abnormalities were based on a limited sample set and should therefore be substantiated or rejected in a larger panel of LPTAs.

In summary, we conclude that LPTAs could constitute a subset of parathyroid lesions associated with pronounced PHPT features and specific genomic aberrations.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0140.

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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Author contribution statement

L Sulaiman, I-L Nilsson, C C Juhlin, C Larsson, and J Hashemi conceived and designed the study; L Sulaiman performed the experiments. I-L Nilsson, F Haglund, C C Juhlin, A Höög, and C Larsson contributed to the tumor and patient characterization. All authors contributed to the interpretation of results and have revised and approved the final version of the manuscript.

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