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Heterogeneous expression of SNARE proteins SNAP-23, SNAP-25, Syntaxin1 and VAMP in human parathyroid tissue

Ming Lu^{1#}, Lars Forsberg^{1#}, Anders Höög², Christofer C. Juhlin^{1,2}, Vladana Vukojević³, Catharina Larsson¹, Arthur D. Conigrave⁴, Leigh Delbridge⁵, Anthony Gill⁶, Christina Bark¹, Lars-Ove Farnebo¹ and Robert Bränström^{1§}

From the ¹Department of Molecular Medicine and Surgery, ²Department of Oncology-Pathology, ³Department of Clinical Neuroscience, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden; ⁴School of Molecular and Microbial Biosciences and ⁵Endocrine Surgical Unit, University of Sydney, Sydney, NSW, Australia; ⁶Department of Pathology, Royal North Shore Hospital, St Leonards, NSW, Australia.

Key words: SNAP-23, SNAP-25, Syntaxin1, VAMP, SNARE, parathyroid and human

Contributed equally to this study.

§ Author to whom correspondence should be addressed:

Dr. Robert Bränström
Karolinska Institutet
Department of Molecular Medicine and Surgery
Section of Surgery
Karolinska University Hospital, L5:02
SE-171 76 Stockholm, Sweden
Telephone: +46-8-517 792 13
E-mail: robert.branstrom@ki.se

Abstract

In regulated exocytosis synaptosomal-associated protein of 25 kDa (SNAP-25) is one of the key-players in the formation of SNARE (soluble N-ethylmaleimide-sensitive fusion attachment protein receptor) complex and membrane fusion. SNARE proteins are essentially expressed in neurons, neuroendocrine and endocrine cells. Whether parathyroid cells express these proteins is not known. In this study, we have examined the expression of the SNARE protein SNAP-25 and its cellular homologue SNAP-23, as well as syntaxin1 and VAMP (vesicle-associated membrane protein) in samples of normal parathyroid tissue, chief cell adenoma, and parathyroid carcinoma, using immunohistochemistry and Western blot analysis. SNAP-23 and VAMP were evenly expressed in all studied parathyroid tissues using immunohistochemistry and/or Western blot analysis. SNAP-25 (and Syntaxin1) was not expressed in normal parathyroid tissue, but in approximately 20% of chief cell adenomas, and in ~ 45% of parathyroid carcinoma samples. It is likely that the SNARE proteins SNAP-23 and VAMP play a role in the stimulus-secretion coupling and exocytosis of parathyroid hormone as these proteins were expressed in all of the parathyroid samples we studied. In particular, preferential expression of SNAP-23 rather than SNAP-25 provides an explanation of the high level of PTH secretion that occurs under conditions of low cytoplasmic free Ca^{2+} concentration (around 0.1 $\mu\text{mol/l}$). SNAP-25 (and Syntaxin1) appears to be a tumour-specific protein(s) in parathyroid tissues since its expression was restricted to pathological tissues.

1. Introduction

Intracellular membrane trafficking and fusion is essential for many cellular processes and requires a wide range of proteins. In regulated exocytosis, secretion vesicles fuse with the plasma membrane after appropriate stimuli and release their contents into the extracellular space. Central to the process of membrane fusion are soluble N-ethylmaleimide-sensitive fusion attachment protein receptors (SNAREs) localized in various intracellular organelles and membranes (Chen and Scheller, 2001). Specific SNARE proteins are present both on membraneous vesicles (v-SNARE, such as vesicle-associated membrane protein (VAMP) and Synaptotagmin) and the target membranes (t-SNARE, such as Syntaxin and synaptosomal-associated proteins (SNAP)), and the interaction forms a highly stable complex which is tightly coupled to membrane fusion. SNARE proteins have been studied extensively in neurons and neuroendocrine tissues, but, in contrast, relatively little information is currently available on the expression and roles of SNAREs in endocrine tissues (Wollheim and Maechler 2002). In most endocrine cells elevated intracellular free Ca^{2+} concentration (Ca^{2+}_i) stimulates hormone secretion. In parathyroid cells, however, elevated levels of Ca^{2+}_i occur under conditions in which PTH secretion is markedly suppressed (Nemeth and Scarpa, 1986), which could indicate a different arrangement of exocytotic proteins. We have therefore focused on the SNARE proteins well known to be involved in Ca^{2+}_i -regulated exocytosis, namely SNAP, Syntaxin and VAMP. For example, parathyroid cells might express the Ca^{2+} -independent SNARE, SNAP-23 rather than SNAP-25 which works at higher Ca^{2+}_i levels (Chiergatti et al., 2004). In this study we have characterized the expression of SNAP-23, SNAP-25, Syntaxin1, and VAMP in human normal, adenomatous and carcinoma parathyroid tissues.

2. Materials and Methods

2.1. Clinical cases

The study material included 51 parathyroid glands (Tables 1 and 2), all classified according to the World Health Organization classification (DeLellis et al., 2004). All samples except 7 of 9 carcinoma cases were collected at the Karolinska University Hospital, Stockholm, Sweden, the Mater Private Hospital, North Sydney, NSW, Australia or the Royal North Shore Hospital, St Leonards, NSW, Australia. Fifteen of the glands (Case No. 28-42) were diagnosed as normal parathyroid tissue (obtained from patients undergoing major thyroid surgery and the normal parathyroid being localized under the fibrous capsule of the removed thyroid), 27 as chief cell adenomas (No. 1-27), and two as parathyroid carcinomas (No. 44-45). In addition, a pooled sample from biopsies of three different normal parathyroid glands was used in Western blot analysis. The informed consent of each patient was obtained and the local ethics committees of the Karolinska University Hospital, St Vincents Hospital, Darlinghurst, NSW, Australia (covering the Mater Private Hospital, North Sydney) and Northern Sydney Area Health Service covering the Royal North Shore Hospital approved the study. Seven additional samples of parathyroid carcinomas (No. 43, 46-51) had been previously collected world wide for a molecular genetics study (Bondeson et al., 1993). The patients had previously consented to an investigation of the causes of carcinogenesis and the present study was considered to be an extension of the original study.

2.2. Antibodies and Controls

Following primary antibodies were used: anti-SNAP-25:SMI 81, a monoclonal antibody (mAb) directed against the C-terminal of the protein (Cat. No. SMI-81R, Sternberger Monoclonals, USA), anti-SNAP-25 polyclonal antibody (Cat. No. 111 002, Synaptic System, Germany), anti-SNAP-23, a polyclonal antibody against the N-terminal (Cat. No. 111 203,

Synaptic System, Germany), anti-Syntaxin1 (mAb) (Prod No. S0664, Sigma-Aldrich, USA), anti-PTH (mAb) (Cat. No. NCL-PTH-488, Novocastra, UK) and anti-VAMP (Cat No. 104 001, Synaptic System, Germany) which is a monoclonal antibody directed against the N-terminal and binds to VAMP-1, VAMP-2 and VAMP-3 (synonymous with Cellubrevin). In fluorescent immunocytochemistry experiments, the following secondary antibodies were used: tetramethylrhodamine isothiocyanate (TRITC)-conjugated Donkey anti-mouse IgG (Code No. 715-025-150, Jackson ImmunoResearch Incorporated Inc, PA, USA) and cyanine 3 (Cy3)-conjugated goat anti-rabbit IgG (Code No. 111-165-045, Jackson ImmunoResearch)

Protein extracts from human Uterus and mouse Brain (C57BL/6NCrI) served as positive control in Western blot. Mouse brain was homogenized in (mM): 20 HEPES, 1 MgCl₂, 250 D-sucrose, 2 EDTA with a protease inhibitor cocktail (Roche Diagnostics GmbH) (pH 7.4). The animal experiments in this study were approved by the local ethics committee for animal experimentation. Omission of the primary antibody served as a negative control in immunohistochemistry experiments, and human brain and/or pheochromocytoma tissue sections were used as positive controls for the expression of all SNARE proteins investigated (data not shown).

2.3. *Western blot*

Total protein samples were extracted from frozen human parathyroid adenomas and a pool of normal parathyroid biopsies according to standard procedures and were quantified using a dye-binding assay (Bradford, 1976). Before loading the protein samples (75 µg/lane), the protein suspensions were preheated at 70°C for 10 min in Novex-Tricine SDS sample buffer (Invitrogen, Cat no: LC 1676) with 5% (v/v) beta 2-mercaptoethanol. Samples were subsequently size-fractionated by electrophoresis in 10% Tricine gels (Invitrogen, Carlsbad,

CA, USA) at 125 V for 1.5 h, followed by blotting onto 0.2 μm nitrocellulose membranes (Invitrogen) at 25 V for 2 h. Membranes were stained with Ponceau red to confirm equivalent protein loading between lanes and successful protein transfer in Western blots. The membranes were then blocked in milk for 1 h, incubated overnight at 4°C with the respective primary antibody (with dilutions in parenthesis): anti-SNAP-25:SMI 81 (1:2x10⁶), anti-SNAP-23 (1:1,000), anti-Syntaxin1 (1:5,000) or anti-VAMP (1:800). Incubation with appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit 1:25,000 or goat anti-mouse 1:25,000) then followed for 1.5 h at room temperature. The signals were subsequently revealed using enhanced chemiluminescence (ECL, Amersham Biotech, Little Chalfont, UK) for 1 min and exposure to Hyperfilm (Amersham Biotech). The western standard used was MagicMark™Xp (Cat no. LC5602, Invitrogen).

2.4. Immunohistochemistry

Paraffin sections of parathyroid tissues and control samples were all cut at 4 μm , deparaffinized, rehydrated and immersed in preheated citrate buffer, pH 6 (Dako, Glostrup, Denmark) at 95°C for 20 min in order to unmask the antigens. The sections were incubated in 0.5% hydrogen peroxide for 30 min, blocked in 1% BSA with Na-azide for 1 h and subsequently incubated overnight with primary antibody diluted in 1% BSA. The following primary antibodies were applied: anti-SNAP-25:SMI 81 (dilution 1:750), mouse anti-PTH (1:200), anti-SNAP-23 (1:200). The slides were then incubated with appropriate biotinylated secondary antibody (1:200) for 45 min at room temperature. The antigen-antibody complex was visualized using the avidin-biotin-peroxidase complex (ABC) method (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 45 min. Diaminobenzidine tetrahydrochloride was used as a chromogen, followed by counterstaining for 3 min in

haematoxylin. Sections were washed five times in Tris-buffered saline (pH 7.6) between all steps.

2.5. Fluorescent immunocytochemistry

Paraffin sections of parathyroid tissues were deparaffinized, rehydrated and retrieved as described in immunohistochemistry section above. The slides were incubated with mouse anti-SNAP-25 SMI 81 (dilution 1:750) or rabbit anti-SNAP-23(1:200) overnight at 4 °C and thereafter with tetramethylrhodamine isothiocyanate (TRITC)-conjugated Donkey anti-mouse IgG (1:200) or cyanine 3 (Cy3)-conjugated goat anti-rabbit IgG (1:800) for 1-h at room temperature. Prior to the last wash the sections were incubated with DAPI (4,6-Diamidino-2-Phenylindole) in order to detect cell nuclei. The slides were then mounted with Glycerol-gelatin and examined in Confocal Laser Scanning Microscopy (CLSM). CLSM images were obtained using the Axiovert 200 M inverted microscope equipped with Ar/ArKr (458, 477, 488 and 514 nm), HeNe 543 nm and HeNe 633 nm lasers, and the scanning module LSM 510 META (Carl Zeiss, Jena, Germany). Images were recorded at a 512×512 pixels resolution. The C-Apochromat 40x, NA = 1.2, water immersion UV-VIS-IR objective was used. Further magnification was obtained electronically, by decreasing the scanned area. Fluorescent dyes, Cy3 and TRITC were excited using the HeNe 543 nm laser. Images were recorded by averaging more than eight scans of each image line (line average); scanning rate 6.4 μs/pixel; main dichroic beam splitter HFT 488/543/633 and long pass 560 nm collecting filter. TRITC and Cy3 fluorescence was visualized using green and red pseudo-colors, respectively.

2.6. Statistics

Where appropriate, the data are expressed as means +/- SDs. Statistical comparison of tumour weight, PTH levels and serum Ca²⁺ concentrations was performed using Student's t-test (two

tailed). Distribution rates were compared using the χ^2 test. P-value less than 0.05 was considered to be statistically significant.

3. Results

This study was undertaken to examine the putative expression of SNARE proteins (SNAP-23, SNAP-25, Syntaxin1 and VAMP) in a panel of parathyroid tissues. In total, 51 tissue sections were investigated (Tables 1 and 2). Concordant results were obtained at immunohistochemistry and Western blot analysis. In addition, the subcellular localization of SNAP-23 and SNAP-25 in the parathyroid tissue was detected by fluorescent immunocytochemistry and is illustrated in figure 4.

3.1. Western blot results

The expression of different SNARE proteins was first investigated using Western blot analysis (Table 1). Protein extracts from a total of 20 chief cell adenomas (cases 8-27) and a pooled sample from 3 different normal parathyroid biopsies (labelled Nx in Table 1 and Figure 1) were used for the analysis. The SNAP-25 antibody (SMI 81), directed against the C-terminal, detected a single 25 kDa band in 5 out of 20 adenoma samples (Figure 1). However, no SNAP-25 expression was detected in the pooled normal parathyroid sample. SNAP-25 expression was verified by an identical immunoblotting result using an alternative polyclonal anti-SNAP-25 antibody (data not shown). A SNAP-23 reactive product of 23 kDa was detected in all 21 samples analysed, including the pooled parathyroid normal tissue sample. The level of expression appeared to be even among the adenomas and in the normal parathyroid pool. Since there were several bands/lane we verified the specificity of our findings by performing a blocking experiment with the corresponding SNAP-23 peptide antigen, which resulted in a total block of the signal at 23 kDa. The Syntaxin1 antibody

detected a protein product at 35 kDa in the same five adenomas positive for SNAP-25. However, the signals obtained from the Syntaxin1 antibody showed considerable variability (Table 1). The VAMP antibody detects the VAMP1-3 protein products with predicted size of 11-13 kDa. A detectable but weak signal was obtained in all 21 samples analysed (20 adenoma samples and in the pooled normal sample).

3.2. Immunohistochemistry and Fluorescent Immunocytochemistry

In addition to the 21 samples analysed by Western blotting, another 31 parathyroid sections were evaluated by immunohistochemistry by staining with anti-SNAP-23, anti-SNAP-25 and anti-PTH antibodies. The results are summarized in Tables 1 and 2. PTH immunoreactivity confirmed the parathyroid origin of all tissue samples investigated. The immunohistochemical investigation of SNAP-23 demonstrated an even expression in all samples tested, i.e. normal, adenoma, and carcinoma (Figures 2 and 3). The protein signals were distributed in the cytoplasm and in the plasma membrane region as well (Figure 4A).

SNAP-25 immunoreactivity was not detected in any of the 15 normal parathyroid sections studied. Of the 27 chief cell adenomas analysed, however, samples from five adenomas showed strong SNAP-25-positive immunoreactivity (>95% of parathyroid cells were positive, Table 1). In addition, three chief cell adenomas were considered as partially positive (1–10% of all cells were positive), and six other adenomas had single positive cells amounting to less than 1% of the total cell count. SNAP-25 was also expressed in 4 out of 9 parathyroid carcinomas (> 95% of cells were positive). The protein signal was weak in the cytoplasm and strong in the region of the plasma membrane (Figure 4B). In the immunocytochemistry experiment, the sections were double stained for DAPI and nuclei were clearly detectable by the confocal microscope but are not shown in the figure. Considering the results from the adenomas and carcinomas in combination, 9 out of 36 samples were positive

for SNAP-25, a significantly higher rate of distribution compared to normal parathyroid tissue ($P < 0.05$).

Despite the differences in SNAP-25 expression between different adenoma samples, no significant differences in serum PTH levels (162 ± 86 vs 241 ± 208 ng/l), serum total calcium concentration (2.78 ± 0.15 vs 2.80 ± 0.21 mmol/l) or tumour weight (1.21 ± 0.71 vs 2.38 ± 2.48 g) were observed between SNAP-25 positive and SNAP-25 negative chief cell adenomas, respectively.

Interestingly, case 23 displayed strong SNAP-25 immunoreactivity that was restricted to a distinct region of the parathyroid section (Fig 2B). Upon analysis of a consecutive section, no detectable variation in either PTH or SNAP-23 expression was observed between SNAP-25-positive and SNAP-25-negative regions.

4. Discussion

In the vast majority of neurons and neuroendocrine cells, increased Ca^{2+}_i stimulates neurotransmitter and hormonal release via the formation of SNARE complexes (Stojilkovic, 2005). In contrast, the secretion of parathyroid hormone is high under conditions in which Ca^{2+}_i is low (around $0.1 \mu\text{mol/l}$) and inversely related to stimuli that increase Ca^{2+}_i , most notably, elevated extracellular Ca^{2+} concentration (Nemeth and Scarpa, 1986; Sherwood et al., 1970). Could this inversed stimulation-secretion coupling be explained by a distinct arrangement of SNARE proteins? To address this question, we have studied the presence of four different SNARE proteins (SNAP-23, SNAP-25, Syntaxin1 and VAMP) in normal parathyroid tissues, chief cell parathyroid adenomas and parathyroid carcinomas.

We show for the first time that the SNARE proteins SNAP-23, SNAP-25, Syntaxin1 and VAMP are expressed in human parathyroid tissues. SNAP-23 (a cellular homologue of SNAP-25 with 59% identity at the amino acid level) displays an almost

ubiquitous expression pattern (Ravichandran et al., 1996). In most cells, it serves as the t-SNARE protein taking part in the formation of SNARE complexes which precedes membrane fusion and exocytosis (Wang et al., 1997; Sorensen et al., 2003). Compared to SNAP-25, which forms a Ca^{2+}_i -sensitive complex with synaptotagmin 1 at Ca^{2+}_i in the micromolar range (Chiergatti et al., 2002), SNAP-23 accomplishes secretory vesicle fusion with the plasma membrane at low/resting Ca^{2+}_i (0.1 $\mu\text{mol/l}$) through interactions with different SNARE members (Chiergatti et al., 2004). SNAP-23 and VAMP were evenly expressed in all our samples (normal and pathological), although SNAP-23 generally showed a notably higher level of expression compared to VAMP. The expression of SNAP-23 and VAMP suggests their involvement in the exocytotic machinery of the parathyroid cell, and may explain, at least partly, how the cells are able to sustain high rates of PTH secretion at low Ca^{2+}_i . The use of silencing technique, i.e. siRNA, directed against various SNARE proteins could potentially define their role in the exocytotic machinery.

Immunocytochemistry analysis shows that SNAP-25 protein signal was weak in the cytoplasm and strong in the plasma membrane, in agreement with the pattern described previously for neurons (Oyler et al., 1989). SNAP-23, on the other hand, was distributed in the cytoplasm and in the plasma membrane region. The SNAP-23 distribution pattern fits well what has been published in other cell types (Wong et al., 1997).

In addition to forming a Ca^{2+}_i -sensitive complex with synaptotagmin 1, SNAP-25 forms SNARE complexes with Syntaxin1 (Ravichandran et al., 1996). In the current study, SNAP-25 and syntaxin1 were expressed in a subpopulation (~20%) of chief cell adenomas and were notably absent in normal parathyroid tissue. Although neither was generally expressed in parathyroid adenomas, Syntaxin1 was co-expressed in all of the SNAP-25 positive samples. Our results talk in favour for that the genes of SNAP-25 and Syntaxin1 (localized on chromosome 20p and 7q11) are regulated in a coordinate fashion. Thus, SNAP-

25 and Syntaxin1 may indicate tumour differentiation, as previously reported for human undifferentiated carcinoma of the colon and rectum (Grabowski et al., 2002) and small cell carcinomas of the lung (Graff et al., 2001). This is corroborated by the finding that SNAP-25 was expressed in four out of nine parathyroid carcinoma samples tested (~45%). One may hypothesize that the detected over expression of the SNAP-25 protein in a subpopulation of the parathyroid tumors is a result of gene copy amplification. The chromosome locus that harbours the SNAP-25 gene (20p11) has, to our knowledge, not been implicated in parathyroid carcinogenesis. In extensive cytogenetic study, designed to explore gene dosage alteration in sporadic parathyroid adenomas (Palanisamy et al., 1998), none out of 53 cases showed amplification of the chromosome 20p. In a similar cytogenetic study of 29 parathyroid carcinomas (Kytölä et al., 2000), only one case showed amplification of chromosome arm 20p. This data strongly suggests that an amplification of the SNAP-25 gene is not the most likely explanation of the detected over expression.

In case 23, strong focal SNAP-25 staining was detected in a specific region of the adenomatous sample (Figure 2B). It is tempting to suggest that an additional genetic hit has occurred in one cell within this region followed by clonal expansion of SNAP-25-positive cells. Whether such a clonal expansion exists in all SNAP-25-expressing tumours remains to be demonstrated. On analysing immunoreactivity from the very same area on a consecutive section, no variation of PTH expression was seen between the focal SNAP-25-positive area and the adjacent SNAP-25-negative area. It has been reported that increased SNAP-25 immunoreactivity reflects secretory activity in pituitary adenomas (Nishioka and Haraoka, 2005). In the present study, however, no significant correlation was detected between parathyroid adenomas positive for SNAP-25 and serum-PTH, Ca^{2+} levels or tumour weight. Further analysis with an expanded number of SNAP-25 positive tumours may be informative.

To summarize; it is likely that the Ca^{2+} -independent SNARE proteins SNAP-23 and VAMP play a role in the stimulus-secretion coupling and exocytosis of parathyroid hormone as these proteins were expressed in all parathyroid samples studied including, notably, normal parathyroid tissue. SNAP-25 (and Syntaxin1), however, appears to be a tumour-specific protein(s) in parathyroid tissues since its expression was restricted to pathological tissues.

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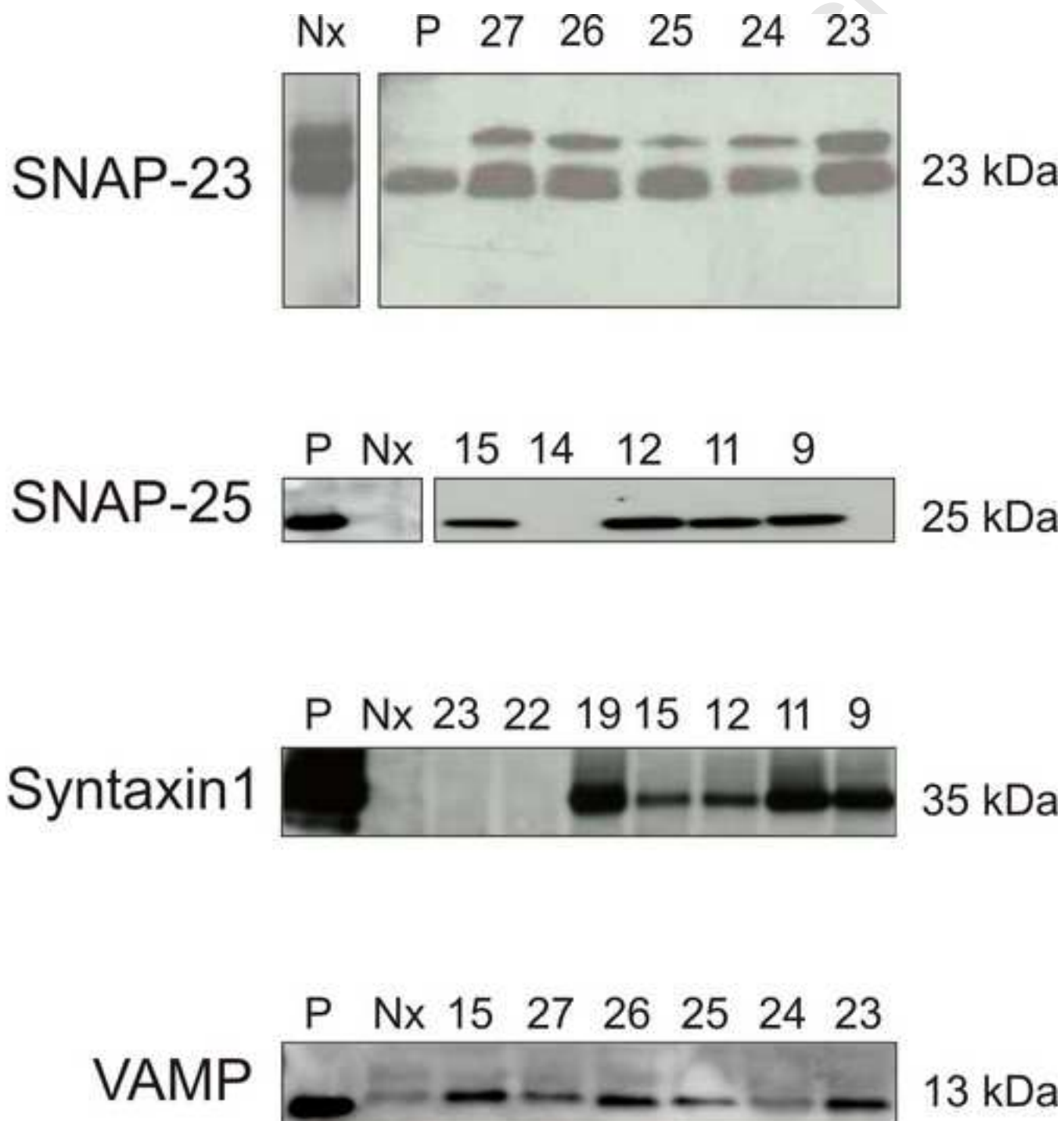
FIGURE LEGENDS

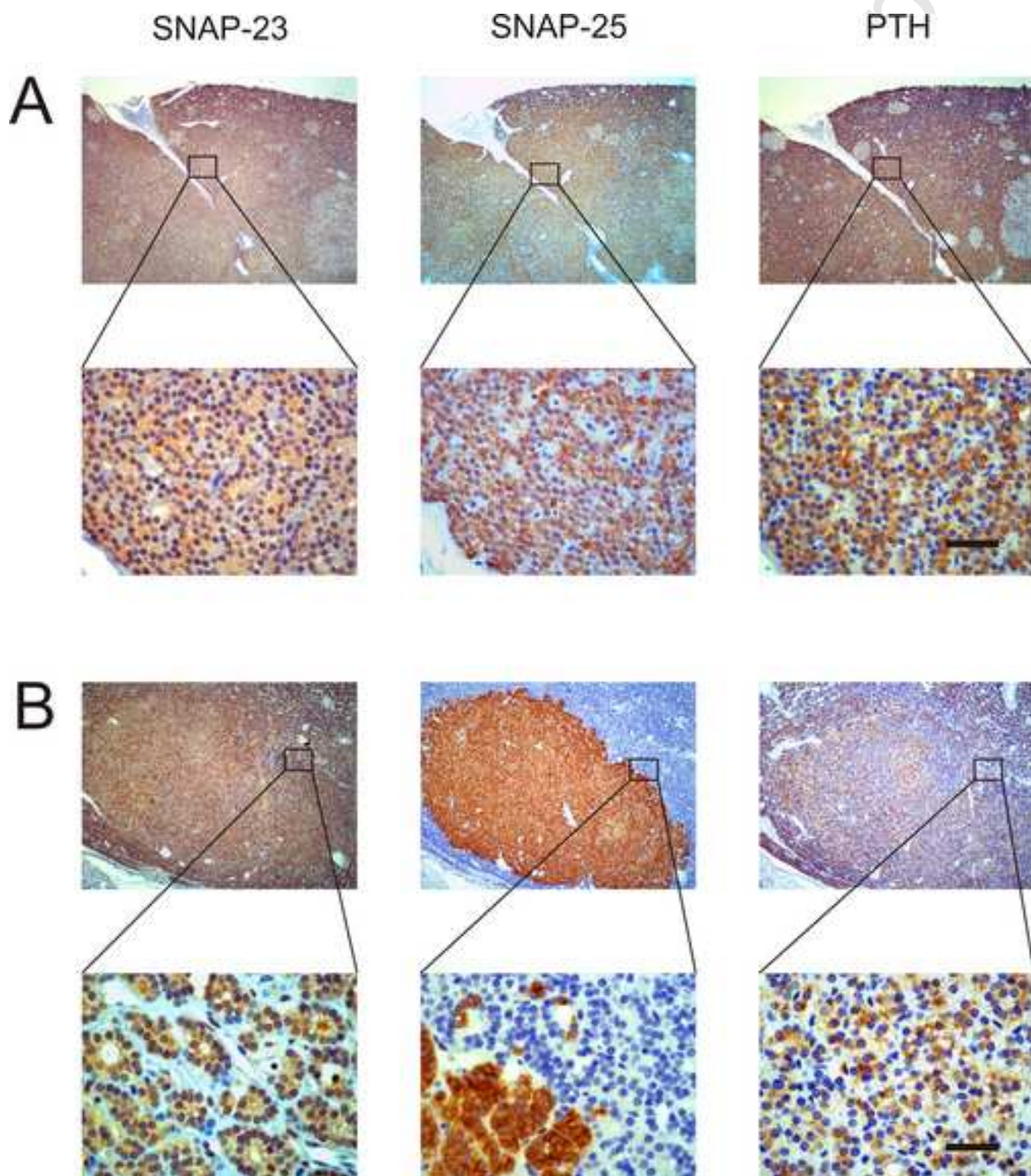
Fig. 1. Western blot analysis of SNARE protein expression in parathyroid tissues. The expression was demonstrated by using antibodies against the SNAP-23, SNAP-25, Syntaxin1 and VAMP proteins, respectively. Total brain extracts from mouse served as a positive control (P) for SNAP-25, Syntaxin and VAMP, and human uterus for SNAP-23. A pooled sample from 3 different normal parathyroid biopsies (Nx) and total cell extracts from 20 chief cell adenomas were examined. Case numbers are indicated above each lane.

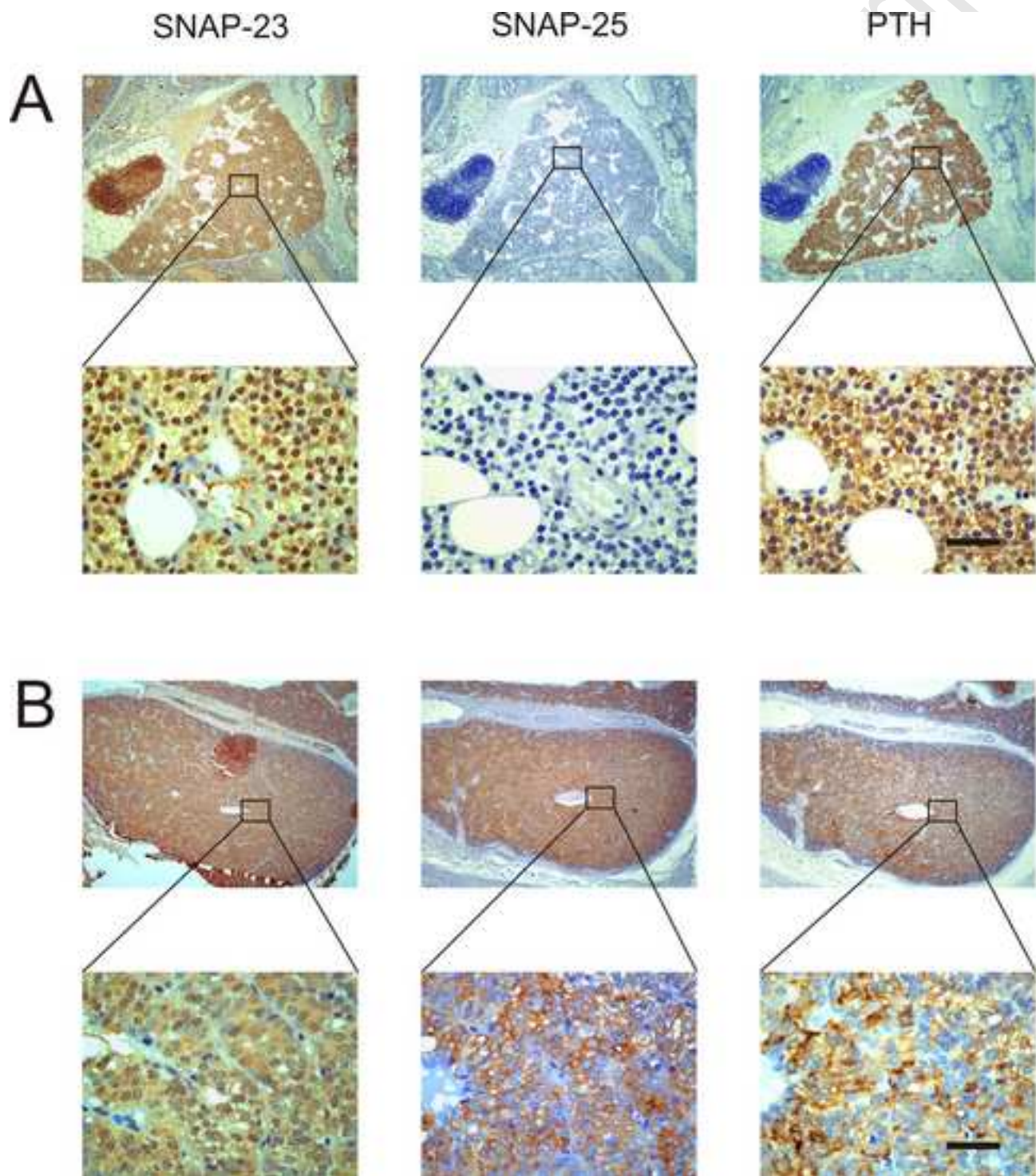
Fig. 2. Photomicrographs of chief cell adenomas. Consecutive sections are shown after immunohistochemical staining with antibodies against SNAP-23, SNAP-25 and PTH proteins (A). In case 23 (B) there is a nodule that is immunoreactive for SNAP-25, whereas the surrounding tissue is negative for SNAP-25, but positive for SNAP-23 and PTH. The scale bar is 400 μm and 40 μm in the top and bottom photomicrographs, respectively.

Fig. 3. Photomicrograph of consecutive sections from normal parathyroid tissue (A) and parathyroid carcinoma (B). Notably that the normal parathyroid tissue is imbedded in thyroid tissue. As in Figure 2, consecutive sections are shown after immunohistochemical staining with antibodies against SNAP-23, SNAP-25 and PTH proteins. The scale bar is 400 μm and 40 μm in the top and bottom photomicrographs, respectively.

Fig. 4. Representative immunocytochemistry images of two cases of chief cell adenomas. Immunofluorescent stains with antibodies against SNAP-23 (A, red) and SNAP-25 (B, green), respectively.







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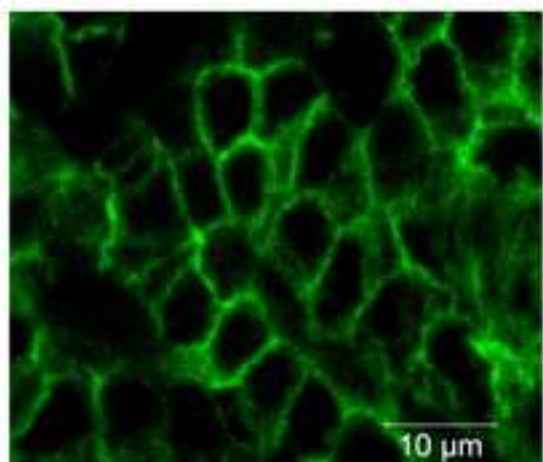
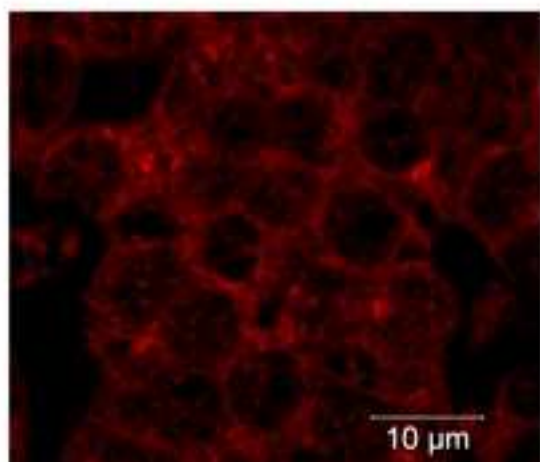
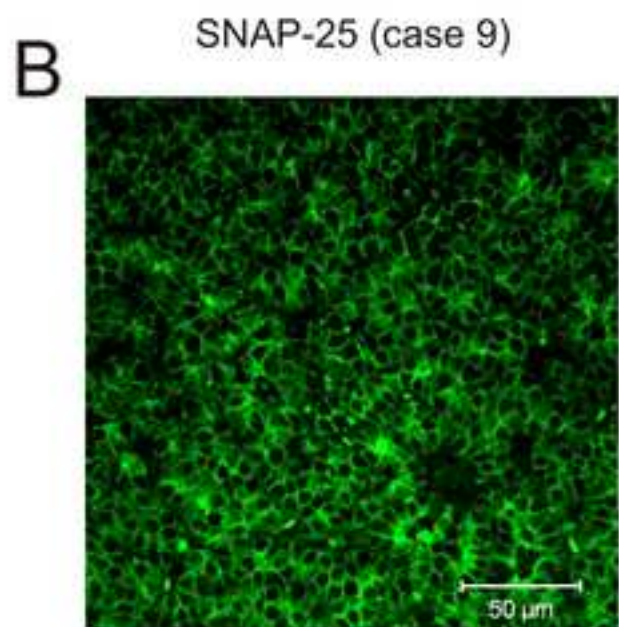
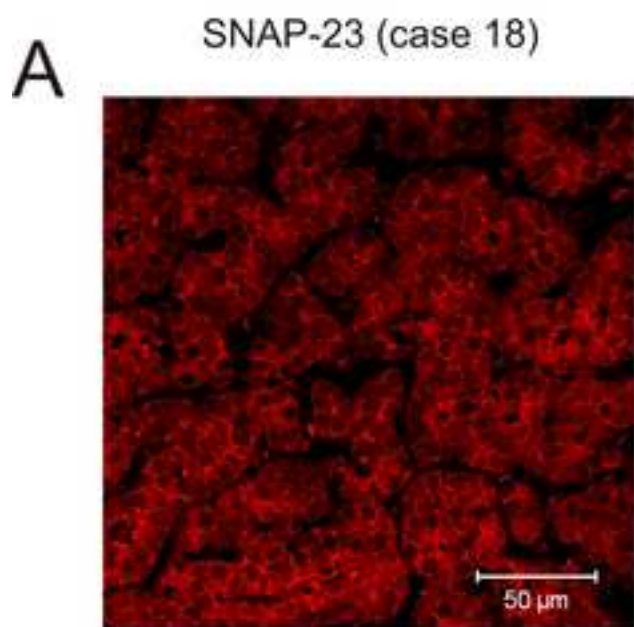


Table 1. Clinical characteristics, Western blot and immunohistochemistry data on the 27 patients with chief cell adenomas.

Case number	Reason for surgery	Age at operation years	Sex M/F	Total Ca ²⁺ mmol·L ⁻¹	PTH ng·L ⁻¹	Histopathology	Gland weight mg	SNAP-23		SNAP-25		Syntaxin-1A	VAMP	PTH
								IHC	Western blot	IHC	Western blot	Western blot	Western blot	IHC
1	pHPT	67	F	2.67	128	Chief cell adenoma	180	>95%	<i>n.d.</i>	0%	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	>95%
2	pHPT	75	F	2.61	44	Chief cell adenoma	430	>95%	<i>n.d.</i>	>95%	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	>95%
3	pHPT	64	F	2.64	138	Chief cell adenoma	190	>95%	<i>n.d.</i>	<1%	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	>95%
4	pHPT	66	F	2.57	81	Chief cell adenoma	390	>95%	<i>n.d.</i>	<10%	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	>95%
5	pHPT	64	F	2.70	165	Chief cell adenoma	310	>95%	<i>n.d.</i>	0%	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	>95%
6	pHPT	61	M	2.56	100	Chief cell adenoma	370	>95%	<i>n.d.</i>	<1%	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	>95%
7	pHPT	71	F	2.81	98	Chief cell adenoma	180	>95%	<i>n.d.</i>	<1%	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	>95%
8	pHPT	61	M	2.72	156	Chief cell adenoma	840	>95%	4	0%	0	0	1	>95%
9	pHPT	59	F	2.66	241	Chief cell adenoma	1,920	>95%	4	>95%	3	3	1	>95%
10	pHPT	50	F	2.90	211	Chief cell adenoma	3,070	>95%	4	0%	0	0	1	>95%
11	pHPT	67	F	2.78	104	Chief cell adenoma	470	>95%	4	>95%	3	3	2	>95%
12	pHPT	57	F	2.86	185	Chief cell adenoma	1,520	>95%	4	>95%	3	1	1	>95%
13	pHPT	52	F	2.60	68	Chief cell adenoma	560	>95%	4	<1%	0	0	2	>95%
14	pHPT	59	F	2.79	114	Chief cell adenoma	1,060	>95%	4	0%	0	0	1	>95%
15	pHPT	22	M	2.67	104	Chief cell adenoma	1,800	>95%	4	<10%	2	1	2	>95%
16	pHPT	63	F	3.19	351	Chief cell adenoma	7,600	>95%	4	0%	0	0	1	>95%
17	pHPT	55	F	2.88		Chief cell adenoma	2,100	>95%	4	<1%	0	0	1	>95%
18	pHPT	62	M	1.59*	83	Chief cell adenoma	1,780	>95%	4	0%	0	0	1	>95%
19	pHPT	67	F	2.98	234	Chief cell adenoma	1,700	>95%	4	>95%	4	3	1	>95%
20	pHPT	59	F	2.97	358	Chief cell adenoma	8,500	>95%	4	0%	0	0	1	>95%
21	pHPT	75	M	2.80	879	Chief cell adenoma	1,100	>95%	4	0%	0	0	3	>95%
22	pHPT	76	M	2.42	125	Chief cell adenoma	3,150	>95%	4	0%	0	0	1	>95%
23	pHPT	63	F	2.86	244	Chief cell adenoma	1,540	>95%	4	<10%	0	0	2	>95%
24	pHPT	43	M	2.82	149	Chief cell adenoma	4,280	>95%	3	0%	0	0	1	>95%
25	pHPT	63	F	3.07	424	Chief cell adenoma	5,070	>95%	4	0%	0	0	1	>95%
26	pHPT	85	F	2.96	596	Chief cell adenoma	1,920	>95%	4	<1%	0	0	2	>95%
27	pHPT	91	F	3.26	485	Chief cell adenoma	6,280	>95%	4	0%	0	0	1	>95%
Nx	Thyroid					Normal parathyroid		<i>n.d.</i>	4	<i>n.d.</i>	0	0	1	<i>n.d.</i>

The observed relative variation in Western blot expression between samples was distinguished in a 0-graded scale with 4 as the strongest. The percentage of IHC represents the ratio of cells that are positively stained. Abbreviations are: M, male; F, female; IHC, immunohistochemistry; pHPT, primary hyperparathyroidism; *n.d.*, not done; Nx, Normal pool of 3 small samples parathyroid glands removed at thyroid surgery. * free Ca²⁺

Table 2. Clinical characteristics and immunohistochemistry data on 15 cases with normal parathyroid tissues and 9 patients with parathyroid carcinomas.

Case number	Reason for surgery	Age at operation years	Sex M/F	Total Ca ²⁺ mmol·L ⁻¹	PTH ng·L ⁻¹	Histopathology	Gland weight mg	SNAP-23 IHC	SNAP-25 IHC	PTH IHC
28	Thyroid	30	F	2.33		Normal parathyroid tissue		>95%	0%	>95%
29	Thyroid	41	F	2.39		Normal parathyroid tissue		>95%	0%	>95%
30	Thyroid	49	M	2.66	600	Normal parathyroid tissue* ¹	190	>95%	0%	>95%
31	Thyroid	36	F	2.52		Normal parathyroid tissue		>95%	0%	>95%
32	Thyroid	57	F	2.41		Normal parathyroid tissue		>95%	0%	>95%
33	Thyroid	64	F	2.30		Normal parathyroid tissue		>95%	0%	>95%
34	Thyroid	35	F	2.30		Normal parathyroid tissue		>95%	0%	>95%
35	Thyroid	67	F	2.67	128	Normal parathyroid tissue* ²	50	>95%	0%	>95%
36	Thyroid	24	F	2.37		Normal parathyroid tissue		>95%	0%	>95%
37	Thyroid	57	F	2.29		Normal parathyroid tissue		>95%	0%	>95%
38	Thyroid	78	F	2.20		Normal parathyroid tissue		>95%	0%	>95%
39	Thyroid					Normal parathyroid tissue		>95%	0%	>95%
40	Thyroid					Normal parathyroid tissue		>95%	0%	>95%
41	Thyroid					Normal parathyroid tissue		>95%	0%	>95%
42	Thyroid					Normal parathyroid tissue		>95%	0%	>95%
43	pHPT	32	F	3.9		Parathyroid carcinoma	3100	>95%	>95%	>95%
44	pHPT	16	F	4.0		Parathyroid carcinoma	1300	>95%	>95%	>95%
45	pHPT	61	F			Parathyroid carcinoma		>95%	>95%	>95%
46	pHPT	59	M	4.0		Parathyroid carcinoma		>95%	0%	>95%
47	pHPT	51	F	3		Parathyroid carcinoma		>95%	0%	>95%
48	pHPT	64	F	3		Parathyroid carcinoma	25000	>95%	0%	>95%
49	pHPT	46	M	3.5		Parathyroid carcinoma	3100	>95%	0%	>95%
50	pHPT	74	M	3.2		Parathyroid carcinoma		>95%	>95%	>85%
51	pHPT	63	F	3.2		Parathyroid carcinoma		<i>n.d.</i>	0%	<i>n.d.</i>

The percentage of IHC represents the ratio of cells that are positively stained. Abbreviations are: M, male; F, female; IHC, immunohistochemistry; pHPT, primary hyperparathyroidism; sHPT, secondary hyperparathyroidism; n.d., not done. *1 first removed parathyroid gland for suspected hyperplasia; *2 suppressed normal parathyroid from patients with pHPT.