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Parathyroid Neoplasms: Immunohistochemical Characterization and Long Noncoding RNA (lncRNA) Expression

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Abstract

Parathyroid adenomas are slow growing benign neoplasms associated with hypercalcemia, while atypical parathyroid adenomas and parathyroid carcinomas are uncommon tumors and their histologic features may overlap with parathyroid adenomas. LncRNAs participate in transcription and in epigenetic or post-transcriptional regulation of gene expression, and probably contribute to carcinogenesis. We analyzed a group of normal, hyperplastic, and neoplastic parathyroid lesions to determine the best immunohistochemical markers to characterize these lesions and to determine the role of selected lncRNAs in tumor progression. A tissue microarray consisting of 111 cases of normal parathyroid ($n = 14$), primary hyperplasia ($n = 15$), secondary hyperplasia ($n = 10$), tertiary hyperplasia ($n = 11$), adenomas ($n = 50$), atypical adenomas ($n = 7$), and carcinomas ($n = 4$) was used. Immunohistochemical staining with antibodies against chromogranin A, synaptophysin, parathyroid hormone, and insulinoma-associated protein 1(INSM1) was used. Expression of lncRNAs including metastasis-associated lung adenocarcinoma transcript one (MALAT1), HOX transcript antisense intergenic RNA (HOTAIR), and long intergenic non-protein coding regulator of reprograming (Linc-ROR or ROR) was also analyzed by in situ hybridization and RT-PCR. All of the parathyroid tissues were positive for parathyroid hormone, while most cases were positive for chromogranin A (98%). Synaptophysin was expressed in only 12 cases (11%) and INMS1 was negative in all cases. ROR was significantly downregulated during progression from normal, hyperplastic, and adenomatous parathyroid to parathyroid carcinomas. These results show that parathyroid hormone and chromogranin A are useful markers for parathyroid neoplasms, while synaptophysin and INSM1 are not very sensitive broad-spectrum markers for these neoplasms. LincRNA ROR may function as a tumor suppressor during parathyroid tumor progression.

Keywords Parathyroid · In situ hybridization · INMS1 · lncRNA · Parafibromin

Introduction

Parathyroid carcinomas are difficult lesions for a pathologist to diagnose and to distinguish from parathyroid adenomas in some cases [1, 2]. Lesions with some of the histological features of parathyroid adenomas and carcinomas have been designated atypical parathyroid adenomas [1, 3]. In recent years, the application of immunohistochemical staining for Ki-67 [4] and other biomarkers such as retinoblastoma gene product [4], parafibromin [5], and fluorescent in situ hybridization (FISH) studies [6] have assisted in the distinction between parathyroid adenomas and carcinomas. However, significant challenges in making these distinctions remain [3].

Broad spectrum neuroendocrine markers such as chromogranin and synaptophysin have been used to characterize neuroendocrine tumors with secretory granules from many tissues and organs in the body [1]. These neuroendocrine biomarkers have proven to be very effective as general markers for the diagnosis of neuroendocrine tumors. In recent years, another broad spectrum neuroendocrine marker, insulinoma-associated one (INSM1), is a new marker for neuroendocrine cells and tumors [7–9]. Because INSM1 is a transcription factor, its localization in the nucleus of the cells provides some obvious advantages, such as low background staining, in the immunohistochemical characterization of neuroendocrine tumors [7]. A large series of parathyroid tissues and tumors have not been examined for INSM1 expression to date.

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Noncoding RNAs range from microRNAs which average around 22 nucleotides in length, to small nucleolar RNAs and Piwi-interacting RNAs [10]. Long noncoding RNAs (lncRNAs) are defined as transcripts that are 200 nucleotides or larger and do not contain a protein coding sequence. Many studies have shown that there are thousands of lncRNAs which may have many functions and may also contribute to carcinogenesis as oncogenes or tumor suppressor [11–13]. Although microRNAs have been examined in parathyroid tissues by several investigators, very few studies of lncRNAs in parathyroid tissues have been reported [14]. A recent study by Zhang et al. [14] reported expression of lncRNAs PNT1 and GLIS2-AS1 in parathyroid neoplasms, and they suggested that these molecules could be involved in parathyroid carcinoma tumorigenesis. We examined a series of normal, hyperplastic, and neoplastic parathyroid tissues using a tissue microarray (TMA) to characterize the immunohistochemical features and lncRNA expression patterns in benign and malignant parathyroid tissues.

Materials and Methods

Tissue Microarrays

A tissue microarray (TMA) was constructed and included normal parathyroid tissues ($N = 14$) primary hyperplasia ($n = 15$), secondary hyperplasia ($n = 10$), tertiary hyperplasia ($n = 11$), adenomas ($n = 50$), atypical adenomas ($n = 7$), and carcinomas ($n = 4$). One carcinoma consisted of three local recurrences and a lung metastasis. Cases were identified by searching the institutional electronic medical record system and confirmed by immunohistochemical analysis (details provided below). Each case was represented by duplicate 0.6-mm cores which were prepared using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). The study was approved by the Institutional Review Board at the University of Wisconsin-Madison.

Immunohistochemistry

Immunohistochemical analyses were performed on a Ventana BenchMark Ultra system (Ventana Medical Systems, Inc., Tucson, AZ) according to the manufacturer's protocols. Primary antibodies used included Ki-67 (1:50 dilution with Van Gogh Yellow; Biocare, Pacheco, CA), parathyroid hormone Clone MRQ-31, prediluted, Cell Marque, Rocklin,CA chromogranin A (clone LK2H10, prediluted; Ventana Medical Systems, Inc., Tucson, AZ), synaptophysin (polyclonal, prediluted; Cell Marque, Rocklin, CA), and insulinoma-associated protein one (INSM1; clone SC-271408 from Santa Cruz Biotechnology, Dallas, TX; 1:1000 dilution from Biocare, Pacheco, CA). In addition, manual immunohistochemistry staining was performed using anti-parafibromin from Santa Cruz Biotechnology and used at 1:8000. All immunolabeled markers were visualized with DAB. Positive and negative controls were prepared with each analysis. Staining was scored on the TMA based on the intensity of staining (0 negative, 1+ weak, 2+ moderate, and 3+ strong) and on the percentage of positive cells (negative 0%, focal 0–25%, and diffuse > 25%).

In Situ Hybridization

TMAs were probed for ROR, MALAT1, and HOTAIR expression using the RNAscope 2.5 HD-Brown Manual Assay (Advanced Cell Diagnostics, Newark, CA) as per manufacturer's recommendations with the following modifications: antigen retrieval was performed in a Biocare Decloaker for 3 min, protease digestion for 30 min, and probe incubation overnight at 40°C. The probes used included hs-ROR (315401), hs-MALAT1 (400811), hs-HOTAIR (312341), hs-PPIB (positive control, 313901), and dapB (negative control, 310043) (Advanced Cell Diagnostics). Expression levels of all probes were visualized with DAB as previously reported [15].

Automated Multispectral Image Quantitation

The hybridized TMA slides were visualized with the Vectra slide scanner (PerkinElmer, Waltham, MA). InForm 1.4.0 software (PerkinElmer, Waltham, MA) was used to segment each tissue core into architectural compartments (tumor cells versus stroma) and subcellular compartments (nucleus versus cytoplasm). Expression levels of ROR, MALAT1, and HOTAIR were quantitated for each tissue core as average nuclear optical density (OD) as previously described [15]. Results from duplicate cores were averaged for each case to obtain a representative score.

Real-time PCR

Total RNA was extracted from samples with TRIzol reagent (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions, and RNA quality and concentrations were assessed with a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA). One microgram of total RNA was reverse-transcribed using the All-in-One miRNA RT-qPCR detection kit (GeneCopoeia, Rockville, MD). RT-qPCR was performed on a CFX96 PCR detection system (Bio-Rad Laboratories, Hercules, CA) using Bullseye EvaGreen qPCR master mix (MIDSCI, St. Louis, MO), normalized to 18S rRNA; relative fold change was determined by the ΔΔ CT method as previously reported [15]. The PCR primers used for, ROR, HOTAIR, MALAT1, and 18S are as follows: ROR; Forward 5′-CTGGCTTTCTGGTTTGACG-3′ and Reverse 5′-CAGGAGGTTACTGGACTTGGAG-3′,

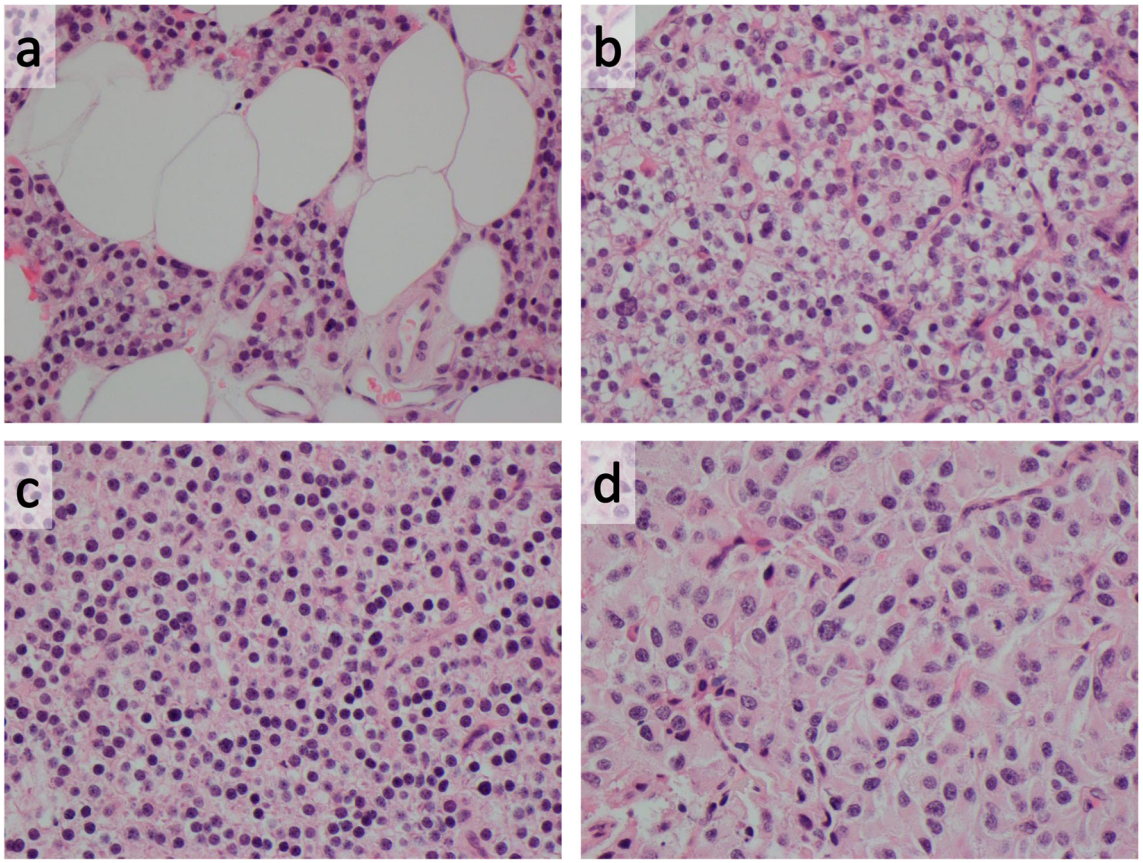

Fig. 1 Representative H&E sections of normal parathyroid (**a**), parathyroid adenoma (**b**), atypical parathyroid adenoma (**c**), and parathyroid carcinoma (**d**). The atypical adenomas and carcinomas had larger nuclei than the normal parathyroid and adenomas

HOTAIR; Forward 5′-CAGTGGGGAACTCTGACTCG-3′ and Reverse 5′-GTGCCTGGTGCTCTCTTACC-3′, MALAT1; Forward 5′-GACGGAGGTTGAGATGAAGC-3′ and Reverse 5′-ATTCGGGGCTCTGTAGTCCT-3′, and 18S; Forward 5′-GTAACCCGTTGAACCCCATT-3′ and Reverse 5′-CCATCCAATCGGTAGTAGCG-3′.

Statistical Analysis

Student's *t* test was used to analyze the expression of digitally quantified expression levels of ROR, HOTAIR, and MALAT1 data collected from RNA ISH and real-time PCR. Two-tailed *P* values of < 0.05 were considered to be statistically

Table 1 Summary of immunohistochemical analysis of parafibromin in parathyroid carcinomas, atypical adenomas, and in normal and hyperplastic parathyroid tissues

Case		No. of cases	Parafibromin loss
Carcinoma 1	First recurrence	1	1
	Second recurrence		1
	Third recurrence		1
	Lung metastasis		1
Carcinoma 2–4		3	0
Atypical adenoma		7	1
Adenoma		50	0
Hyperplasia	Primary	15	0
	Secondary	10	0
	Tertiary	11	0
Normal parathyroid		14	0

Parafibromin nuclear staining was analyzed on TMA slides. Loss of greater than 90% nuclear staining was considered parafibromin loss

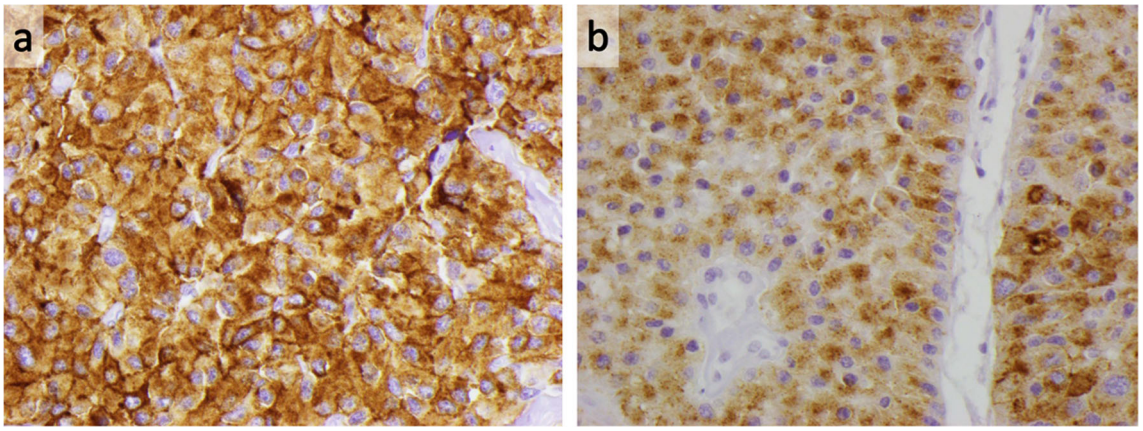

Fig. 2 Patient with parathyroid carcinoma recurrence (**a**) and lung metastasis (**b**) showed positive staining for parathyroid hormone. Both of these sections and all of the cases showed diffuse cytoplasmic staining for parathyroid hormone

significant. Data are expressed as means ± standard error of the mean (SEM).

Results

Clinico-pathological Findings

Patients with parathyroid carcinomas ranged in age from 47 to 66 (mean age 62). All four cases were females. Serum calcium before surgery ranged from 11 to 15.3 mg/dL and serum parathyroid hormone levels ranged from 120 to 584 ng/L. One patient with a parathyroid carcinoma had three recurrences and a pulmonary metastasis. The primary tumor from this patient was not available for review or analysis. Follow up of the four patients with parathyroid carcinomas showed that one was alive with disease, two were alive without disease, and one patient died of pneumonia without any evidence of recurrent parathyroid carcinoma. The follow-up period ranged from 13 to 110 months (mean of 57 months). Among the seven patients with atypical adenomas, the age range was 36 to 75 (mean age 57). All seven patients were men. Serum calcium before surgery ranged from 11 to 16 mg/dL and parathyroid hormone ranged from 280 to 1514 ng/L. The follow-up time was from 0.5 to 68 months (mean of 17 months).

Histopathological Features

The normal parathyroids consisted mainly of chief cells, but some glands also had variable amounts of oxyphilic cells (Fig. 1). The cell to fat ratio ranged from 30:70 to 50:50. Primary and secondary hyperplasia consisted of nodular proliferation of chief cells with variable numbers of oxyphilic cells. Glands with tertiary hyperplasia usually had a dominant nodule with proliferating chief cells. All neoplasms were hypercellular. Adenomas showed diffuse hyperplasia while fibrous bands were present in atypical adenomas and carcinomas (not shown). In the atypical adenomas, the neoplastic cells were confined to the parathyroid while carcinomas had cells extending into the capsule and sometimes into the adjacent adipose tissues. Mitotic figures were readily seen in

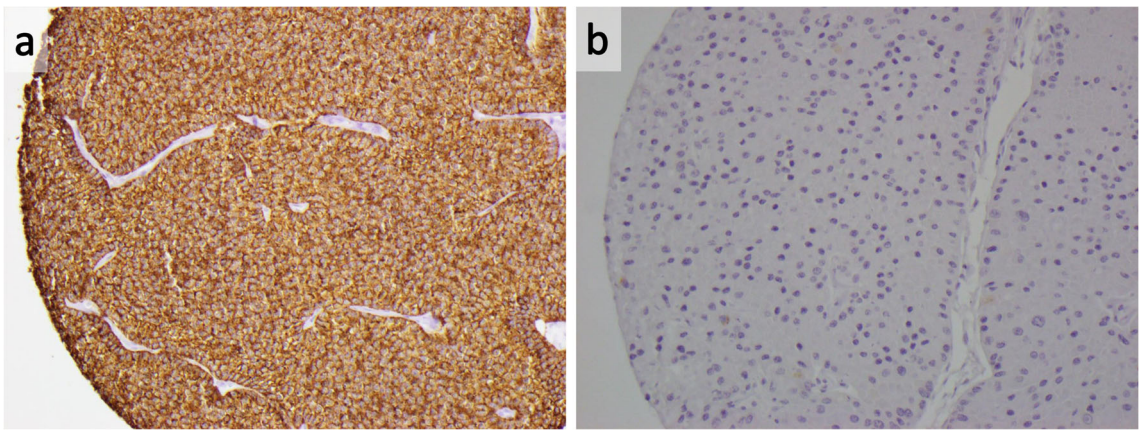

Fig. 3 A parathyroid carcinoma showing diffuse cytoplasmic staining for chromogranin A (**a**). Chromogranin A staining was negative in the three recurrence and lung metastasis in another patient with recurrent disease (**b**)

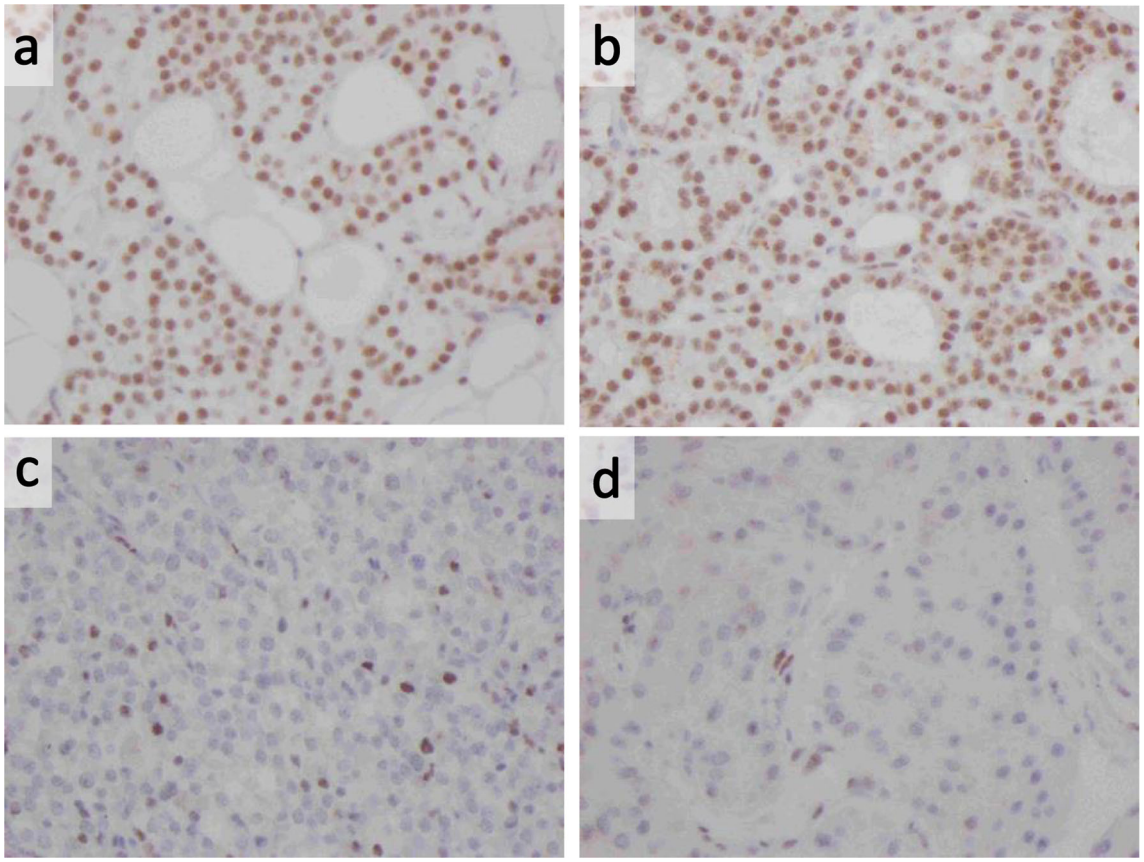

Fig. 4 Nuclear staining for parafibromin in normal parathyroid tissue (**a**) and parathyroid adenomas (**b**). One atypical adenoma (**c**) and one parathyroid carcinoma with recurrences and lung metastasis (**d**) showed loss of nuclear parafibromin

atypical adenomas and carcinomas. Vascular and perineural invasion was not identified in the four carcinomas.

Immunohistochemical Staining

All of the parathyroid tissues were positive for parathyroid hormone including the lung metastasis (Table 1 and Fig. 2). Chromogranin A was positive in most cases except for one parathyroid carcinoma, one atypical adenoma, and one adenoma (Fig. 3). Synaptophysin was positive in only 11% of parathyroid tissues including one atypical adenoma and a carcinoma (Table 1). INSM1 was negative in all parathyroid tissues, while the positive control section for INSM1 was positive. IHC for parafibromin showed nuclear loss of parafibromin staining in one of four carcinomas (Table 1 and Fig. 4). This negative case was the carcinoma with multiple recurrent tumors and a lung metastasis (Table 1). One atypical adenoma showed loss of parafibromin with loss of staining in more than 90% of the tumor cells. The cases of hyperplasia and normal parathyroid all showed positive nuclear staining for parafibromin. Ki-67 proliferative index was much higher in atypical parathyroid adenomas and carcinomas compared with normal and hyperplastic parathyroid tissues (Table 2 and Fig. 5).

Table 2 Immunohistochemical staining for Ki-67 in parathyroid neoplasms

Case		No. of cases	Ki-67(%)
Carcinoma #1	First recurrence	1	10
	Second recurrence		20
	Third recurrence		17
	Lung metastasis		13
Carcinoma #2–4		3	8.7 (5–11)
Atypical adenoma		7	23.3 (5–32)

The analysis of Ki-67 for normal parathyroid, hyperplastic parathyroid, and parathyroid adenomas was estimated from the TMA. Whole tissue sections were used to analyze the atypical parathyroid adenomas and carcinomas. A total of 500 cells were enumerated in the hot spot areas to estimate the proliferative index. For normal parathyroid, hyperplastic parathyroid, and parathyroid adenomas, the Ki-67 index was less than 2% for each category

In Situ Hybridization Analysis

ISH showed predominantly nuclear localization of all three lncRNAs in the parathyroid tissues (Fig. 6). The positive control probe (PPIB) was positive in all of the

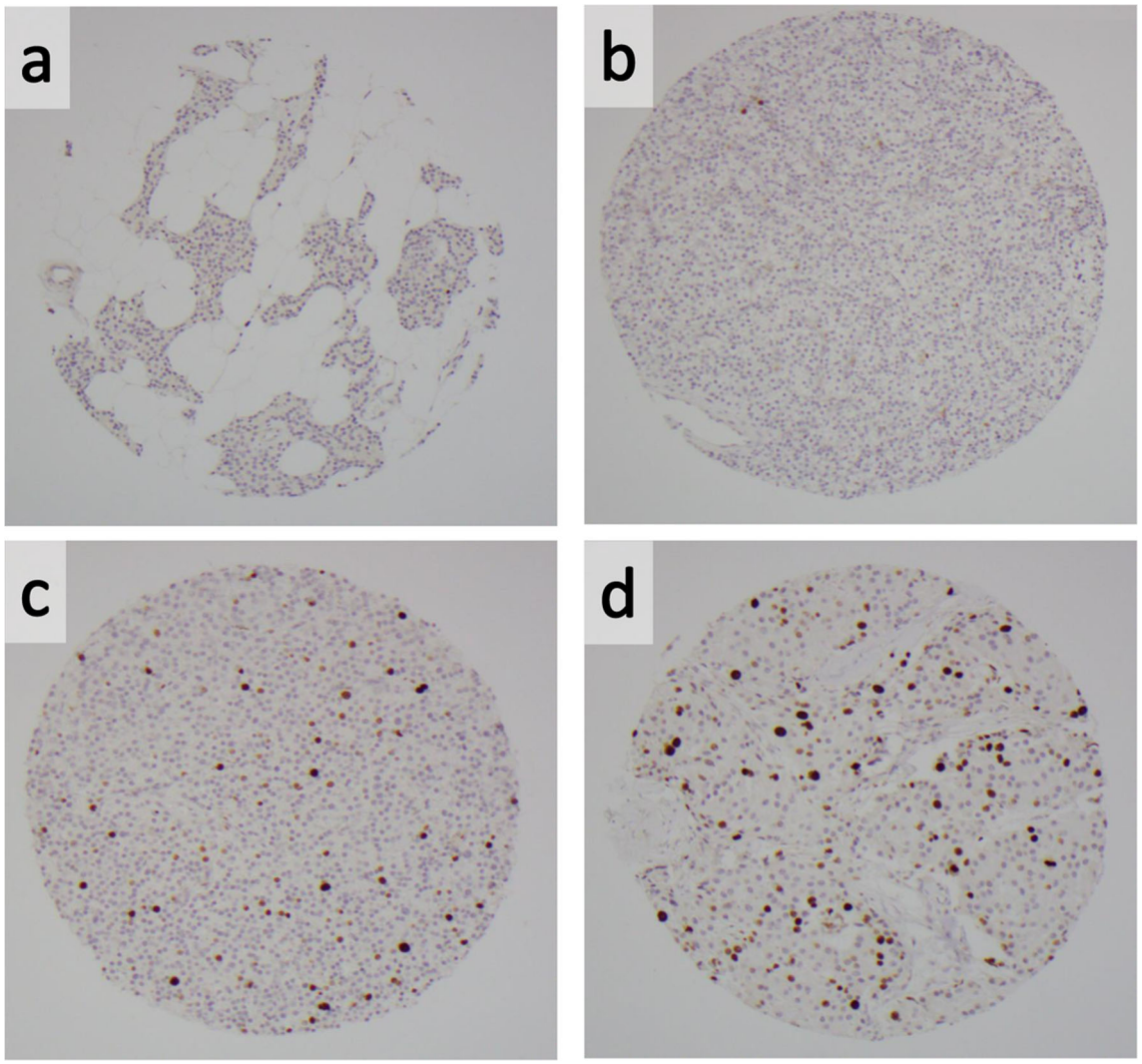

Fig. 5 Representative immunohistochemical staining for Ki-67 in normal parathyroid (**a**), parathyroid adenoma (**b**), an atypical parathyroid adenoma (**c**), and a parathyroid carcinoma (**d**). The atypical adenomas and carcinomas all had a Ki-67 labeling index > 5%

tissues, while the negative control resulted in no staining. Vectra image analysis technology and quantification with Nuance and inForm software showed a significant decrease in the expression of Linc-ROR in parathyroid carcinoma (Fig. 7) but not in HOTAIR or MALAT1 lncRNAs in parathyroid tissues (data not shown).

qRT-PCR

qRT-PCR analysis showed higher levels of expression of Linc-ROR in normal parathyroid and parathyroid adenomas compared with atypical adenomas and parathyroid carcinomas (Fig. 8). However, these differences were not statistically significant. HOTAIR and MALAT1 analysis did not show significant differences between the various groups (data not shown).

Discussion

This study provides new information about the immunohistochemical and in situ hybridization characterization of parathyroid tumors and other parathyroid tissues. While parathyroid hormone and chromogranin A are reliable broad-spectrum biomarkers for the immunohistochemical characterization of parathyroid tumors, our study shows that synaptophysin is not a very sensitive marker for parathyroid tissues and tumors, since only 11% of the cases were positive. In early publications about synaptophysin immunoreactivity, Gould and coworkers reported synaptophysin immunoreactivity in about 50% of their parathyroid adenomas [16]. This is in contrast with a smaller percentage of synaptophysin immunoreactivity in a large series of parathyroid tissues in this study.

In this study, the four parathyroid carcinomas were all negative for synaptophysin including the case with a lung

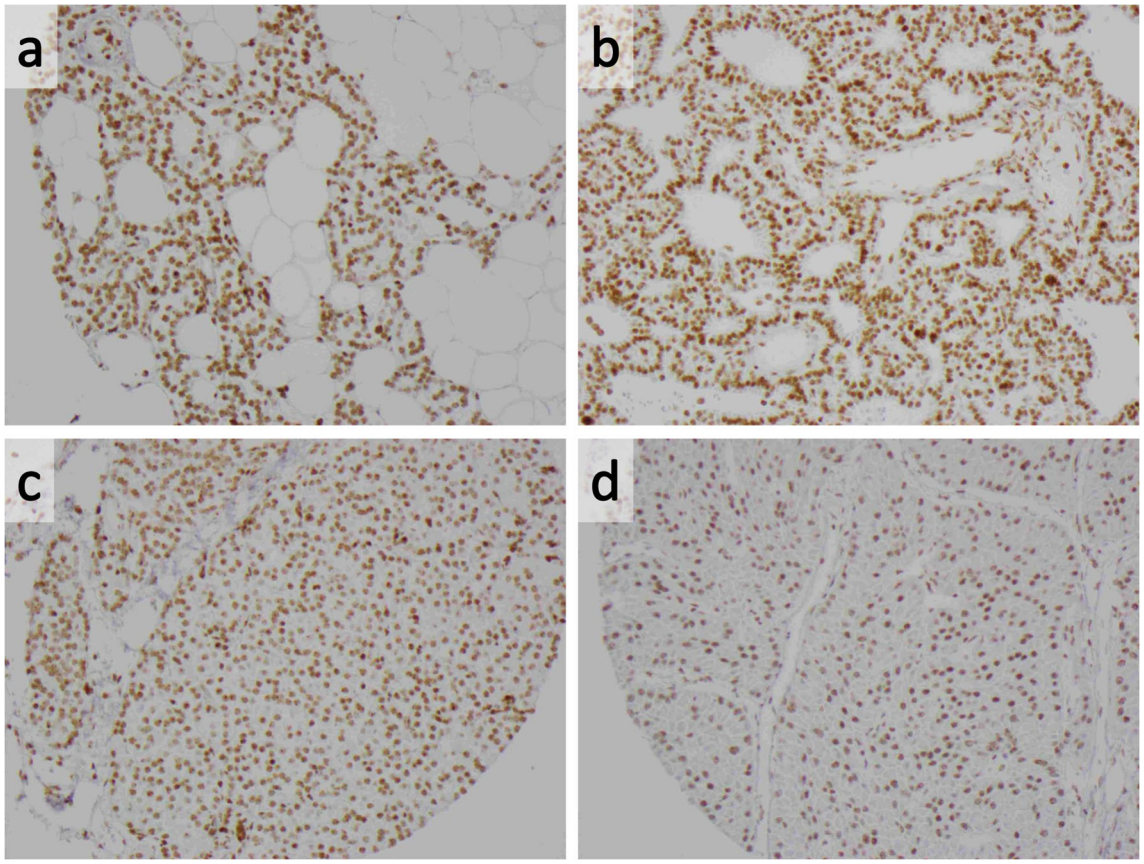

Fig. 6 Representative nuclear expression of Linc-ROR in normal parathyroid (**a**), parathyroid adenoma (**b**), atypical parathyroid adenoma (**c**), and parathyroid carcinoma (**d**). There was decrease nuclear expression of ROR in atypical parathyroid adenomas and carcinomas

metastasis. This indicates that the use of synaptophysin without other broad-spectrum markers would not be a reliable biomarker for metastatic parathyroid tumors.

INSM1 is a relatively new and sensitive transcription factor that is a useful marker for most neuroendocrine tumors [7–9]. However, surprisingly, all cases of normal, hyperplastic, and neoplastic parathyroid tissues were negative for this biomarker. In the original study by Rosenbaum et al., the four cases of parathyroid tumors studied were negative for INMS1 [7]. The current study establishes in a more definitive manner that parathyroid tissues do not express this transcription factor. Since parathyroid tissues express several members of the chromogranin family [17], secrete peptide hormones, and contain dense core secretory granules, it is difficult to explain the absence of staining for INSM1 and the paucity of staining for synaptophysin in these tissues and in tumors derived from parathyroid cells. It is possible that some endocrine tissues that do not have neuroendocrine features such as thyroid follicular cells and steroid-producing adrenal cortical cells, form a subset of endocrine tissues. Parathyroid tissues may be a subset of neuroendocrine cells and tumors with different immunohistochemical features than classical neuroendocrine cells and tumors.

Nuclear parafibromin loss was present in only one case of parathyroid carcinoma and in one atypical adenoma in this study. In spite of the small number of carcinomas and atypical adenomas in this study, this finding indicates that the use of this biomarker may not be as sensitive as a biomarker for parathyroid carcinoma and atypical adenomas and must be used with other biomarkers such as antibodies to adenomatous polyposis coli in characterizing parathyroid carcinomas [2, 5]. In an earlier study of 73 parathyroid adenomas and 21 carcinomas, loss of parafibromin was reported in 33% of carcinomas and 1% of adenomas [18]. Parafibromin loss was not found in any of the three atypical adenomas in this previous study [18]. In a recent analysis of a large series of cases, Juhlin et al. [2] found loss of nuclear parafibromin in 5% of adenomas, 59% of atypical adenomas, and in 70% of parathyroid carcinomas. In the same study, adenomatous polyposis coli loss had a sensitivity and specificity for the recognition of parathyroid carcinoma versus atypical adenomas and adenomas of 70 and 91%, respectively [2].

An increase proliferative index was present in both atypical adenomas and parathyroid carcinomas compared with parathyroid adenomas and hyperplastic parathyroid tissues. The range of expression of Ki-67 was quite wide

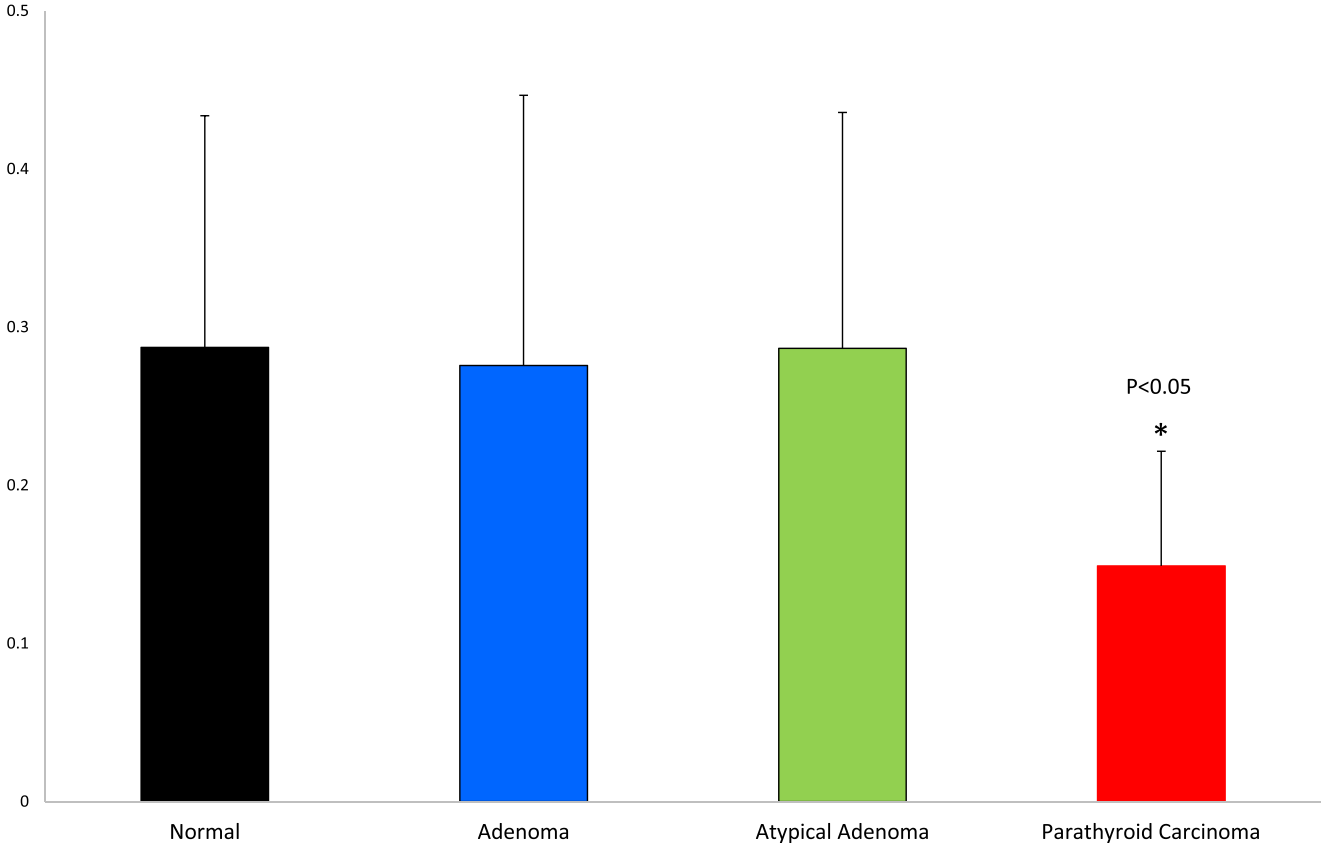

Fig. 7 Quantitative analysis of In situ hybridization results in normal parathyroid tissues, parathyroid adenomas, atypical parathyroid adenomas, and parathyroid carcinomas. There was a significant decrease of Linc-ROR nuclear expression in the parathyroid carcinomas. Error bars showing SEM; *P <0.05

for both atypical adenomas and carcinomas, so a clear cut demarcation of a proliferative index to separating lesions with these two diagnoses was not possible. However, the higher proliferative index was useful in separating typical parathyroid adenomas from atypical neoplasm. In a recent study, Mohammed et al. [18] reported that Ki-67 (MIB1) was elevated above 5% in most of their atypical adenomas and carcinomas [18]. In the current study, conventional parathyroid adenomas have a proliferative index of less than 2%, while atypical adenomas and carcinomas had a proliferative index of at least 5%. The use of both Ki-67 and p27 has been previously proposed as a useful approach to assist in separating parathyroid adenomas and carcinomas [19]. Erickson and Mete recently recommended using a wide spectrum of biomarkers, including Ki-67 and TERT promoter mutations, when trying to separate benign and malignant parathyroid lesions [20].

LncRNAs and other noncoding RNAs are being studied by many laboratories for their potential diagnostic and prognostic usages in pathology and oncology [10, 11, 13, 14]. A great deal of information has accumulated about microRNAs in parathyroid tumors [21], but very little is known about lncRNA in parathyroid tumors. Zhang et al. [14] recently reported that there were differences in lncRNA expression between parathyroid adenomas and carcinomas. They reported that lncRNA PVT1 was more highly expressed in parathyroid carcinomas while GLIS2-AS1 was more highly expressed in parathyroid adenomas. Our study with ROR, HOTAIR, and MALAT1 found expression of all three of these lncRNAs in normal, hyperplastic, and neoplastic parathyroid tissues. Interestingly, ROR showed decreasing expression during progression from parathyroid adenomas to parathyroid carcinomas. There were no significant differences in the expression of HOTAIR or MALAT1 lncRNAs in the non-neoplastic and neoplastic parathyroid tissues. The findings with ROR suggest that this lncRNA may be functioning as a tumor suppressor in parathyroid tumors. Similar findings with MALAT1 have recently been reported by our group in neuroendocrine tumors from the pancreas and gastrointestinal tract [22]. This new observation suggests that some lncRNAs may function as tumor suppressors in some neuroendocrine tumors as has been reported for MALAT1 and other lncRNAs in breast and brain tumors [23–26]. Recent studies have also suggested that ROR may function as a tumor suppressor in gliomas [27] and nasopharyngeal carcinomas [28].

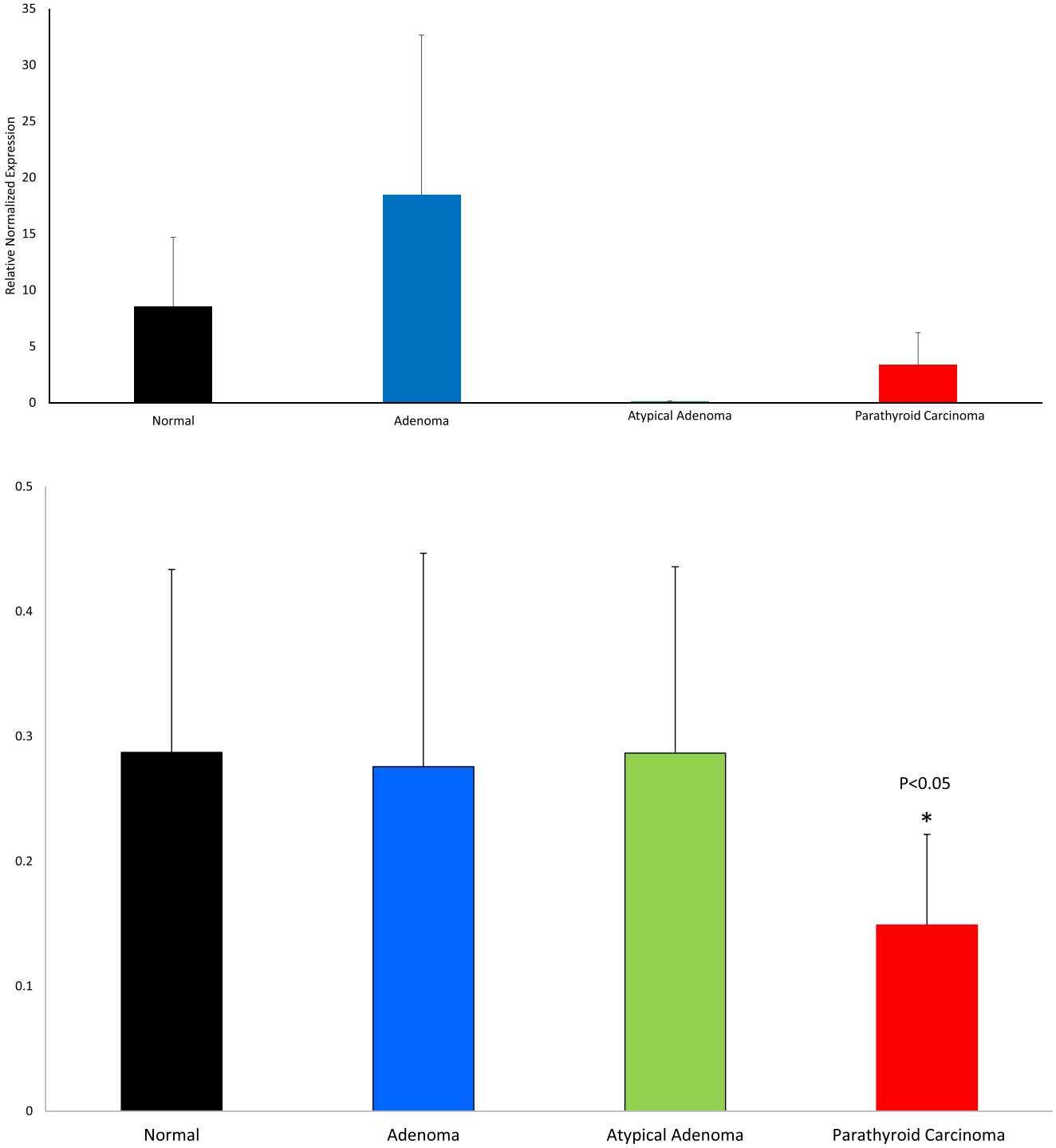

Fig. 8 qRT-PCR analysis of normal parathyroid tissue, parathyroid adenomas, atypical parathyroid adenomas, and parathyroid carcinomas showing decreased expression of Linc-ROR in atypical parathyroid adenomas and parathyroid carcinomas compared with normal parathyroid and parathyroid adenomas. The number of cases analyzed included normal parathyroid [7], adenomas [10], atypical parathyroid adenomas [7], and parathyroid carcinomas [4]. Error bars showing SEM

In summary, our study shows that while parathyroid hormone and chromogranin A are useful biomarkers for normal and neoplastic parathyroid tissues, other biomarkers such as synaptophysin and the newly described sensitive neuroendocrine transcription factor INSM1 are not reliable broad-spectrum biomarkers for the diagnosis of parathyroid tumors. This study also shows that several lncRNAs are expressed in parathyroid tissues and that ROR may be functioning as a tumor suppressor in parathyroid tumors.

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Compliance with Ethical Standards

The study was approved by the Institutional Review Board at the University of Wisconsin-Madison.

Conflict of Interest All authors declare that they have no conflict of interest.

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