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Using a Novel Diagnostic Nomogram to Differentiate Malignant from Benign Parathyroid Neoplasms

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Abstract

We sought to develop an immunohistochemical (IHC) tool to support the diagnosis of parathyroid carcinoma (PC) and help differentiate it from atypical parathyroid neoplasms (atypical) and benign adenomas. Distinguishing PC from benign parathyroid neoplasms can be challenging. Many cases of PC are histopathologically borderline for definitive malignancy. Recently, individual IHC biomarkers have been evaluated to aid in discrimination between parathyroid neoplasms. PC, atypical parathyroid neoplasms, and parathyroid adenomas treated at our institution from 1997 to 2014 were studied retrospectively. IHC analysis was performed to evaluate parafibromin, retinoblastoma (RB), protein gene product 9.5 (PGP9.5), Ki67, galectin-3, and E-cadherin expression. Receiver operating characteristic (ROC) analysis and multivariable logistic regression model for combinations of biomarkers were evaluated to classify patients as PC or atypical/adenoma. A diagnostic nomogram using 5 biomarkers was created for PC. Sixty-three patients were evaluated. The percent staining of parafibromin ($p < 0.0001$), RB ($p = 0.04$), Ki67 ($p = 0.02$), PGP9.5 ($p = 0.04$), and Galectin-3 ($p = 0.01$) differed significantly in the three diagnostic groups. ROC analysis demonstrated that parafibromin had the best performance in discriminating PC from atypical/adenoma; area under the curve (AUC) was 81% (cutoff, 92.5%; sensitivity rate, 64%; specificity rate, 87%). We created a diagnostic nomogram using a combination of biomarkers; AUC was 84.9% (95% confidence interval, 73.4-96.4%). The optimism-adjusted AUC for this model was 80.5% (mean absolute error, 0.043). A diagnostic nomogram utilizing an immunoexpression, a combination of immunohistochemical biomarkers, can be used to help differentiate PC from other parathyroid neoplasms, thus potentially improving diagnostic classification.

Keywords Parathyroid cancer · Parathyroid neoplasms · Nomograms · Biomarkers

Introduction

Parathyroid neoplasms are a heterogeneous group of tumors affecting 0.1–5.0% of the population [1, 2]. Parathyroid adenoma is the most common cause of primary hyperparathyroidism (PHPT) (80–85%). Microscopically, a parathyroid adenoma is composed of varying proportions of chief, clear, and transitional and oncocytic cells [3, 4]. An atypical parathyroid neoplasm (atypical) has some cytologic features of a parathyroid carcinoma (PC) but without

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definitive histopathological evidence of invasion [5–7]. Histopathologically, PC is definitively defined by the presence of lymphovascular invasion and/or invasion into soft tissue, thyroid parenchyma, or muscle, often with secondary cytologic features including identifiable mitoses, atypia/pleomorphism, and/or a trabeculated or sheet-like growth pattern.

In general, these different categories of parathyroid neoplasms all have the same or overlapping clinical symptoms and preoperative biochemical findings making their preoperative differentiation often impossible [2, 3]. Furthermore, they frequently demonstrate similar histopathological features making their exact classification and prognosis of future behavior challenging.

Clinically, the diagnosis of PC is confirmed if regional and/or distant metastases are present. However, standard histopathological methods to distinguish between PC and atypical neoplasms are limited to cytological (hematoxylin and eosin) slide review, without uniformly defined ancillary studies to further define borderline cases of parathyroid neoplasms which remain concerning for PC [2, 8–10]. Furthermore, distinguishing between these two entities is sometimes possible only in clinical follow-up with the detection of regional or distant metastasis [7].

Recently, the use of immunohistochemical (IHC) biomarkers to increase the accuracy of the diagnosis of these parathyroid neoplasms has become a point of interest, because the use of a panel of multiples biomarkers is more likely to give us greater diagnostic utility in difficult parathyroid neoplasms [11]. Parafibromin has been the most widely used biomarker in the last decade, typically in combination with other biomarkers and as a unique biomarker in the identification of PC, as it exhibits an outstanding specificity, but with low prevalence [1, 12–17]. Even recently, it has been suggested that parafibromin IHC provides information with higher predictive values than all the histopathological criteria used to evaluate parathyroid neoplasms [17]. Although parafibromin is highly specific for parathyroid carcinoma, additional immunomarkers are essential to improve the sensitivity for parathyroid carcinoma. Some of the biomarkers that have stood out in recent reports, which increase the diagnostic sensitivity of parafibromin, are galectin-3 [1, 13, 15, 18, 19] and protein gene product 9.5 (PGP9.5) [15, 16, 20]. Recently, Kumari et al. evaluated the combination of galectin-3 positivity, PGP9.5 positivity, and parafibromin loss, demonstrating a 50% sensitivity, 98% specificity, and a 95% predictive accuracy for distinguishing PC [21].

Another biomarker that has been extensively studied in parathyroid tumors is the Ki67 proliferation index, which has shown higher levels of proliferative activity in PC than other parathyroid neoplasms [1, 15, 22, 23]. Therefore, it has been included in most of the different IHC panels that are available in the literature for PC, even demonstrating that its association with galectin-3 positivity its specificity reached 100% [13]. Another biomarker potentially useful in the PC recognition is retinoblastoma (RB), due to its abnormal expression or total loss of expression demonstrated in PC [24]. RB has been included in two IHC panels in the last decade, demonstrating an overall accuracy of 84% in distinguish PC [1], which suggests a utility in the assessment of parathyroid neoplasms [25], but none of these panels included PGP9.5. Moreover, the last biomarker to consider, which has only been evaluated alone, is E-cadherin. Fendrich et al. showed that in all PC studied showed loss of E-cadherin membranous expression, unlike hyperplasia and parathyroid adenomas, suggesting that loss of E-cadherin in PC could be related to a critical role of epithelial and mesenchymal transitions in the tumorigenesis of PC [26].

Studies to date have used different comparison groups to determine sensitivity and specificity between parathyroid adenomas and carcinomas; in addition, the heterogeneity of data regarding differentiation of atypical neoplasms from PC remains contradictory [27]. In this retrospective study, we sought to develop a diagnostic tool based on the combination of biomarkers for parathyroid neoplasm that can increase the confidence in diagnosis of PC and differentiate it from atypical and adenoma. To achieve this, we investigated and compared the expression of six IHC biomarkers in patients with PC, atypical parathyroid neoplasm, or parathyroid adenoma to determine whether combinations of these biomarkers would align with a diagnosis of PC, thereby aiding in its classification. We hypothesized that a combination of these biomarkers would lead to increase useful additional diagnostic information.

Materials and Methods

Patients and Specimens

All patients with PC and atypical neoplasms surgically treated at MD Anderson from September 1997 to December 2014 were identified in the Department of Surgical Oncology research parathyroid database from patients with sporadic primary hyperparathyroidism, and all PC cases from the Hospital Universitari de Bellvitge in Barcelona, Spain. All primary PC and atypical neoplasms with available tissue blocks were included in the study. For the parathyroid adenomas, 20 cases were selected using a random integer generator. Patients with parathyroid hyperplasia were excluded from the study. The study was approved by the Institutional Review Board at MD Anderson and from the collaborating Institutions that shared their samples, with a waiver of informed consent due this is a retrospective sample analysis without diagnostic or therapeutic intervention with no direct patient contact.

All histopathology was reviewed by an experienced head and neck/endocrine pathologist (MDW), who examined

hematoxylin- and eosin-stained slides to confirm the diagnostic classification based on WHO criteria [4]. The cases were categorized based on a combination of histopathology and clinical criteria as PC, atypical, or adenoma. PC was defined by the histopathological presence of lymphovascular invasion and/or invasion of soft tissue, muscle, or any surrounding structures (thyroid, trachea, esophagus, and jugular vein), and/or documented regional or distant metastasis. Atypical neoplasm was defined by the presence of two or more worrisome histologic features including mitotic figures, broad intratumoral fibrous bands, necrosis, trabecular growth, and diffuse cellular atypia/pleomorphism and/or adherence to adjacent structures in the absence of the absolute criteria for PC described above.

Immunohistochemical evaluation was performed on coded tissue samples. Correlative clinical data including demographics, biochemical, and outcome data were recorded.

Immunohistochemistry

Immunohistochemical analysis of paraffin-embedded tissue sections for RB, parafibromin, Ki67, galectin-3, and E-cadherin was performed using existing CLIA setting clinical protocols utilizing an automated IHC system (Leiobiosystems, Inc, BOND III) System, Buffalo Grove, IL). PGP9.5 was analyzed under research conditions as this antibody is not currently available in a CLIA environment; the antibody characteristics, dilutions and protein cellular localization used in this study are listed in Table 1.

Biomarkers selected and outlined below for the current investigation were chosen based on their potential role in parathyroid neoplasia characterization, development, and/or progression.

Parafibromin is a nuclear protein encoded by CDC73, a tumor suppressor gene (previously known as HPRT2) located on chromosome 1q31. Germ-line mutations in CDC73 define hyperparathyroidism-jaw tumor (HPT JT) syndrome where the risk of PC is ~ 15% [28–33]. Additionally, CDC73 mutations have been identified in the majority of sporadic parathyroid cancers [15, 31]. Parafibromin is the most studied biomarker of PC, with sensitivity rates ranging from 29 to 100% and specificity rates ranging from 61 to 100% [1, 13–17, 20, 34].

The retinoblastoma (RB) gene is a negative regulator of cell-cycle progression where by bialleric inactivation or loss is critical in the initiation and/or progression of a significant number of human cancers [35–37]. Loss of Rb expression and function is a hallmark of malignancy [38]. The inactivation of the RB gene and allelic loss have been implicated in PC pathogenesis [24, 39]; therefore, it has been studied in IHC panels in PC but with variable RB losses [1, 25].

E-cadherin is a single-pass transmembrane glycoprotein encoded by the cadherin 1 gene located at 16q22.1. E-cadherin plays a key role in cell-to-cell adhesion in epithelial tissue, and its loss may promote invasive growth and metastatic behavior of several epithelial tumors [40]. A single report demonstrated that atypical neoplasms, as well as, benign adenomas have membranous patterns of E-cadherin expression, compared to PC [41].

Ki67 is a cell cycle associated protein located at 10q26.2. This protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis) but absent from G0 phase [42]. Ki67 is considered an appropriate indicator related to the growth fraction of a cellular population. Prior studies show parathyroid adenoma differs from PC; in parathyroid neoplasms, a Ki67 labeling index greater than 4% is frequently present in malignancy [1, 43–45].

Protein gene product 9.5 (PGP9.5) is a neuron-specific protein encoded by the ubiquitin C-terminal hydrolase L1 gene located at 4p13 [46]. The PGP9.5 protein is involved in the processing of ubiquitin precursors and ubiquinated proteins via proteolysis by ubiquitin C-terminal hydrolase, which is potentially a critical mechanism that regulates genes during cell-cycle progression [47]. In 3 studies in the literature, investigators used this biomarker for PC, obtaining varying results regarding the incidence of PGP9.5-positive staining of PC samples [15, 21, 48].

Galectin-3 is a carbohydrate-binding protein encoded by its gene located at 14q22.3. Galectin-3 acts as a transcriptional regulator, interacting with transcription factors to induce

Table 1 Characteristics of the antibodies used in our immunohistochemical panel

Antibody	Cellular location	Species/antibody specificity	Source	Catalog no.	Clone no.	Dilution
Parafibromin	Nuclear	Mouse/monoclonal	Santa Cruz Biotechnology	sc-33638	ZH1	1:250
RB	Nuclear	Mouse/monoclonal	Calbiochem	OP66	LM95.1	1:30
E-cadherin	Cytoplasmic and extracellular domains	Mouse/monoclonal	Life Technologies (Invitrogen)	13-1700	HECD-1	1:7000
Ki67	Nuclear	Mouse/monoclonal	Dako	M7240	MIB-1	1:100
PGP9.5	Cytoplasmic, membranous, paranuclear or nuclear reaction	Rabbit/polyclonal	Dako	Z511601-2	Polyclonal	1:1500
Galectin-3	Nuclear	Mouse/monoclonal	Santa Cruz Biotechnology	sc-32790	B2C10	1:400

upregulation of its transcriptional activity, as well as a cytoplasmic antiapoptotic factor favoring and angiogenic effector promoting cellular endothelial migration. Through these mechanisms, galectin-3 plays a role in numerous cellular functions and contributes to the proliferation and progression of cancer [49]. Researchers have detected galectin-3 overexpression in the majority of PC cases compared to benign, with sensitivity rates ranging from 45.4 to 93.3% and specificity rates ranging from 73.7 to 100% [13, 15, 20, 22, 50].

Interpretation of Immunohistochemistry

Each IHC stain was reviewed by a board-certified pathologist with expertise in head and neck/endocrine pathology (MDW). Each biomarker was evaluated for the proportion of parathyroid cells with staining across the entire tissue section and recorded in percentages. The percentages of positive cells were recorded as continuous data (0% [no expression] to 100% [complete expression]). RB, parafibromin, PGP9.5, and Ki67 were evaluated for nuclear staining, galectin-3 for cytoplasmic staining, and E-cadherin for membranous staining (complete versus partial loss). Each biomarker was evaluated as a continuous variable, and also categorized and defined as positive or negative according to previously established cutoff values (Table 2).

Statistical Analysis

The Kruskal-Wallis test was used to compare the distribution of continuous variables among the three patient groups. Fisher's exact test was used to compare the distribution of categorical variables among the groups. Receiver operating characteristic (ROC) analysis was performed to assess the performance of each biomarker in classifying patients into the following groups: PC versus atypical/adenoma. Logistic regression models were fitted using combinations of biomarkers; the resulting predictions of the different groups were also assessed using a ROC curve. Nomograms were created for visual representation of this model. Also, a calibration plot for the fitted logistic regression model was generated. All statistical analyses were performed using the R computing language (version 3.3.1). All statistical tests used a significance level of 5%.

Results

Patients and Specimens

Twenty-one patients with PC treated at The University of Texas MD Anderson Cancer Center (MDACC) with available blocks of their primary tumors were available for IHC analysis. We also included 2 PC cases treated at Hospital Universitari de Bellvitge in Barcelona, Spain. Of the 32 patients who underwent surgery for atypical parathyroid neoplasms at MDACC, 20 had tissue blocks that were available for analysis. Additionally, 1206 parathyroidectomies were performed in patients with PHPT from 1997 to 2014 at MDACC. We excluded those with parathyroid hyperplasia (i.e., we excluded any patient with multi-gland disease) (n = 181), leaving a total of 1025 adenoma cases. A random integer generator was used to select 20 adenoma cases for comparison. Thus, in our final analysis, we studied 63 cases of uniglandular parathyroid neoplasms that were reviewed individually according to the World Health Organization criteria for PC, atypical neoplasm, and adenoma [4].

The demographic and clinical characteristics of each group are listed in Table 3. Per the clinical records, none of the 63 cases were subjected to prior parathyroid fine needle aspiration (FNA), and none of the 20 benign adenomas exhibits a medical history indicative of familial isolated HPT or HPT JT syndrome. And one atypical neoplasm patient had a

Table 2 Biomarker evaluation and thresholds for categorization

Biomarker	Category	Cutoff in literature % staining	Location of staining	References
Parafibromin	Positive (intact) Negative (loss)*	> 10% ≤ 10%	Nuclear	[12, 17, 21]
RB	Positive (intact) Negative (loss)*	> 10% ≤ 10%	Nuclear	[1]
E-cadherin*	Positive (intact) Negative (loss)*	>90% complete staining ≥ 10%	Membranous	[26, 41]
Ki67	High proliferative* Low proliferative	> 4% ≤ 4%	Nuclear	[15] [20, 21]
PGP9.5s	Positive* Negative	> 50% ≤ 50%	Cytoplastmic/nuclear	
Galectin-3	Positive* Negative	> 30% ≤ 30%	Nuclear	[21]

*Abnormal expression

Table 3 Demographic and clinical characteristics of the study patients with parathyroid neoplasms

Demographic feature at diagnosis	Carcinoma (n = 23)	Atypical (n = 20)	Adenoma (n = 20)	p value*
Sex				0.030
Male, n (%)	13 (57)	4 (20)	5 (25)	
Female, n (%)	10 (43)	16 (80)	15 (75)	
Age at diagnosis, median years (range)	54 (13–79)	63 (12–74)	56 (16–74)	0.440
Clinical feature at diagnosis				
Median serum parathyroid hormone level, pg/ml (range)	530 (67–2203)	158 (75–322)	112 (59–195)	< 0.00010
Highest median serum calcium level, mg/dl (range)	13 (9–21)	11 (10–14)	11 (10–13)	0.00038
Histopathological feature at diagnosis				
Parathyroid size, median, cm (range)	2.5 (1.1–5.0)	2.1 (0.8–4.8)	1.7 (0.8–4.0)	0.160
Parathyroid weight, median, g (range)	3.5 (1.8–12.9)	1.7 (0.1–23.0)	0.7 (0.2–2.0)	0.00750
Adjacent tissue invasion, n (%)	20 (87)	0	0	0.00050
Vascular invasion, n (%)	11 (48)	0	0	0.00050
Necrosis, n (%)	4 (17)	0	0	0.04000
Fibrosis, n (%)	17 (74)	14 (70)	0	
Pleomorphism, n (%)	16 (70)	9 (45)	0	
Disease progression				
Locoregional recurrence, n (%)	7 (30)	0	0	0.00100
Distant metastasis, n (%)	5 (22)	0	0	0.00300
Death of disease, n (%)	5 (22)	0	0	0.00550
Follow-up median [range (years)]	3.3 (0.6–20.0)	1.3 (0.5–.6)	1.2 (0.02–18.7)	0.02000

*p value between carcinoma versus atypical versus adenoma

hyperparathyroidism 2 gene (HRPT2) germ-line mutation; five PC patients had TP53 somatic mutation, and one PC patient had a multiple endocrine neoplasia 1 (MEN1) germ-line mutation. At the time of diagnosis (preoperatively), the PC group had parathyroid hormone and serum calcium values that were demonstrably higher, on average, than those in the atypical neoplasm and adenoma groups ($p < 0.00010$ and $p = 0.00038$, respectively). Seven patients in the PC group had locoregional recurrence: 4 who had locoregional recurrence alone and 3 who had locoregional recurrence together with distant metastasis to the lung and/or bone. In addition, two patients developed distant metastasis to the lung without locoregional recurrence. Of the 5 patients who died of PC, 4 had metastatic disease, and 1 had persistent hypercalcemia despite a second surgery. None of the atypical neoplasm patients had persistent or recurrent hyperparathyroidism. One patient with atypical neoplasm died of recurrent well-differentiated liposarcoma of the peritoneum, and two patients with adenoma died of pancreatic adenocarcinoma and advanced colorectal cancer, respectively.

Immunohistochemistry Results

We performed IHC analysis of E-cadherin, PGP9.5, and galectin-3 expression in tumor samples obtained from all 63 patients (Ki67 and RB were not evaluable in one PC sample, and parafibromin was not in one adenoma sample). The levels of expression of each biomarker in the three groups are shown in Table 4. We found strong evidence that the percent staining for parafibromin ($p < 0.0001$), RB ($p = 0.04$), Ki67 ($p = 0.02$), PGP9.5 ($p = 0.04$), and galectin-3 ($p = 0.01$) differed significantly among the three groups, a representative immunostaining are shown in Fig. 1. For each of these biomarkers, the mean expression levels were similar in the atypical neoplasm and adenoma groups, and these levels differed from those in the PC group.

The negativity and positivity for the biomarkers in the parathyroid groups are listed in Table 5. In particular, galectin-3 expression differed considerably among the three groups. Specifically, approximately one-third of the PC patients had overexpression of galectin-3 compared with none of the atypical neoplasm patients and only one of the adenoma patients ($p = 0.0015$). Of the 8 PC patients who had galectin-3 overexpression, 5 had locoregional recurrence and/or distant metastasis.

Four PC patients had Ki67 greater than 4%, and 6 PC patients had overexpression of PGP9.5 ($p = 0.08$). All 5 PC patients with p53 mutations had abnormal expression of E-cadherin (mean, 30% expression [range, 10–80%]).

Table 4 Biomarker expression levels (continuous) in the three parathyroid patient groups

Biomarker	q1*	Carcinoma mean ± SD	q3*	q1*	Atypical mean ± SD	q3*	q1*	Adenoma mean ± SD	q3*	*p* value
Parafibromin	0.6	0.67 ± 0.32	0.9	0.9	0.88 ± 0.21	1.0	0.9	0.87 ± 0.22	1.0	< 0.0001
RB	0.1	0.57 ± 0.38	0.9	0.9	0.79 ± 0.31	1.0	0.9	0.78 ± 0.22	1.0	0.0400
E-cadherin	0.1	0.41 ± 0.34	0.8	0.3	0.40 ± 0.21	0.5	0.1	0.17 ± 0.08	0.2	0.1400
Ki67	0.6	2.26 ± 3.03	2.6	0.2	0.73 ± 0.70	1.2	0.7	0.89 ± 0.96	0.9	0.0200
PGP9.5	0.0	0.25 ± 0.40	0.5	0.0	0.05 ± 0.16	0.1	0.0	0.05 ± 0.18	0.0	0.0400
Galectin-3	0.0	0.31 ± 0.42	0.8	0.0	0.04 ± 0.07	0.1	0.0	0.03 ± 0.13	0.0	0.0100

**q1*, first quartile, *q3* third quartile, *SD* standard deviation

Receiver Operating Characteristic Analysis of Individual Biomarkers

We performed receiver operating characteristic (ROC) analysis of the 6 biomarkers individually with regards to classifying patients as having PC versus atypical/adenoma. Parafibromin expression was the best biomarker at discriminating PC from atypical neoplasm/adenoma, with an area under the curve (AUC) of 81% (cutoff, 92.5%; sensitivity rate, 64%; specificity rate, 87%). The other AUCs were 69% for RB, 65% for E-cadherin, 71% for Ki67, 65% for PGP9.5, and 65% for galectin-3.

ROC Analysis, Multivariable Logistic Regression Model, Nomogram and Calibration Plot for Fitted Logistic Regression Model Using a Combination of Biomarkers To Classify as PC versus Atypical/Adenoma

We created and used a logistic regression model to classify patients as having PC or atypicals/adenomas. A ROC curve for, calibration plot for, and visual representation of this model in a nomogram are shown in Fig. 2. The visual representation shows that expression of PGP9.5 may have been correlated with one or more of the other biomarkers. The calibration plot

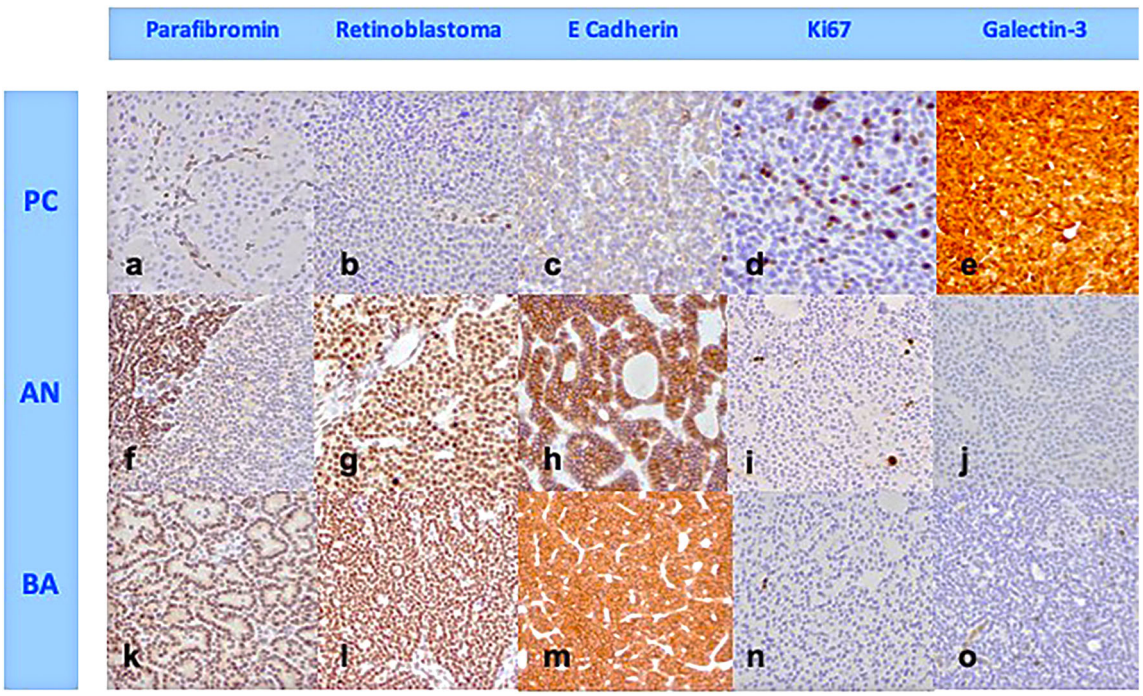

Fig. 1 Representative immunostaining of parathyroid carcinoma (PC), atypical parathyroid neoplasm (AN), and benign parathyroid adenoma (BA) for parafibromin, retinoblastoma, E cadherin, Ki67, and galectin-3 are shown. parathyroid carcinoma showing **a**, **b** loss parafibromin and retinoblastoma staining, **c** decreased E cadherin membranous staining, **d** high proliferative Ki67 index (> 4%), **e** positive galectin-3 nuclear, and cytoplasm staining. **f**–**k** Positive (retained) parafibromin staining in an AN and BA. **g**–**l** Positive (retained) retinoblastoma staining in an AN and BA. **h**–**m** Positive (retained) E cadherin membranous staining in an AN and BA. **i**–**n** Low proliferative Ki67 index in an AN and BA. **j**–**o** Negative nuclear galectin-3 staining in a AN and BA.

Table 5 Biomarker based on defined thresholds (categorical) according to parathyroid patient group

Biomarker		Carcinoma	Atypical	Adenoma	*p* value*
Parafibromin	Negative (≤ 10%), *n* (%)	3 (13)	1 (5)	1 (5)	0.6100
	Positive (> 10%), *n* (%)	20 (87)	19 (95)	18 (95)**	
RB	Negative (≤ 10%), *n* (%)	7 (32)	2 (10)	3 (15)	0.2100
	Positive (> 10%), *n* (%)	15 (68)	18 (90)	17 (85)	
E-cadherin	Negative, *n* (%)	14 (61)	10 (50)	8 (40)	0.4100
	Positive, *n* (%)	9 (39)	10 (50)	12 (60)	
Ki67	≤ 1%	9 (41)	14 (70)	15 (75)	0.0800
	1–4%	9 (41)	6 (30)	4 (20)	
	> 4%	4 (18)	0	1 (5)	
PGP9.5	Negative (≤ 50%), *n* (%)	17 (74)	19 (95)	19 (95)	0.0800
	Positive (> 50%), *n* (%)	6 (26)	1 (5)	1 (5)	
Galectin-3	Negative (≤ 30%), *n* (%)	15 (65)	20 (100)	19 (95)	0.0015
	Positive (> 30%), *n* (%)	8 (35)	0	1 (5)	

*p value between carcinoma versus atypical versus adenoma; **One adenoma failed internal control testing for parafibromin

demonstrated an optimism-adjusted AUC for absolute error of 78.4% (mean absolute error, 0.058). In order to limit the contradictory results of its performance that increased the error rate for PC in our diagnostic model, we excluded it from the panel.

To increase the actual diagnostic probability in this logistic regression model, we fit it using five of the biomarkers (excluding PGP9.5). A ROC curve for, calibration plot for, and visual representation of this fitted model in a nomogram are shown in Fig. 3. The AUC for this model was 84.9% (95% confidence interval, 73.4–96.4%). The optimism-adjusted AUC for this model was 80.5% (mean absolute error, 0.043).

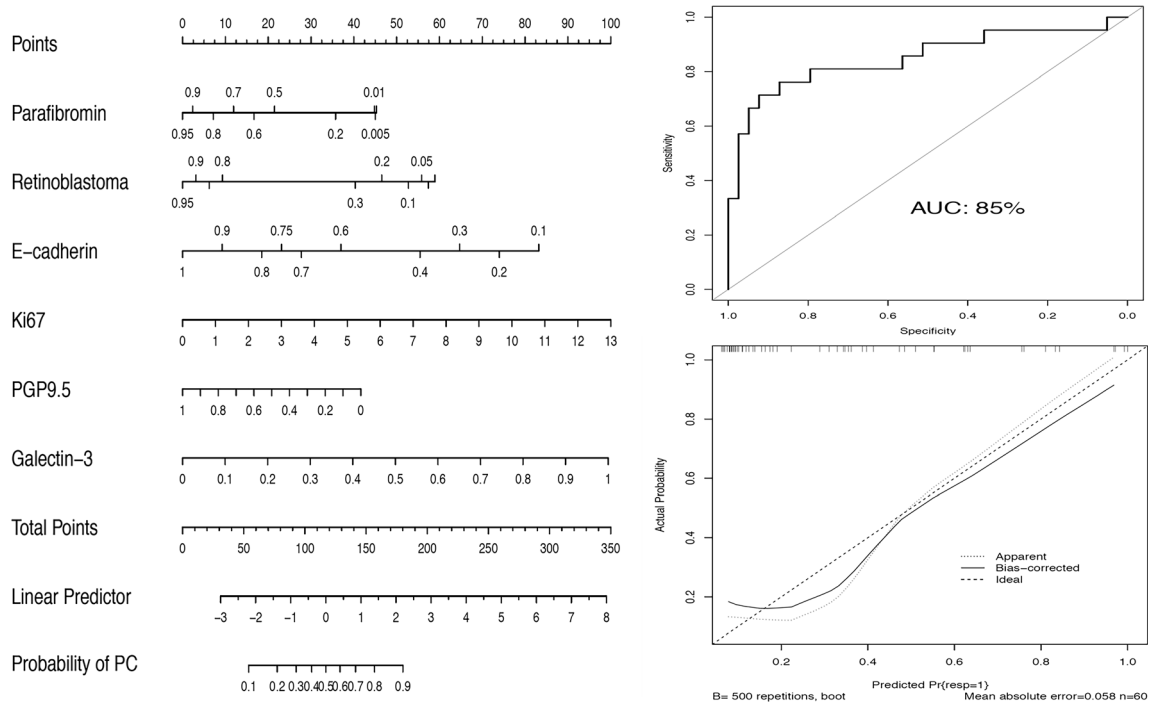

Fig. 2 Nomogram using all six biomarkers to classify patients as having PC or atypical/adenoma along with a ROC curve and calibration plot for the logistic regression model used to classify the patients. Each immunohistochemical marker value is the percent staining as viewed as a fraction from 0–1(100%), i.e., 10% staining = 0.1 for determining the corresponding points for that marker. *Parafibromin, retinoblastoma, E-cadherin, PGP9.5, and galectin-3 values represented from 0 to 1, equivalent to 0 to 100%. Ki-67 represented from 0 to 13, equivalent to 0 to 13%.

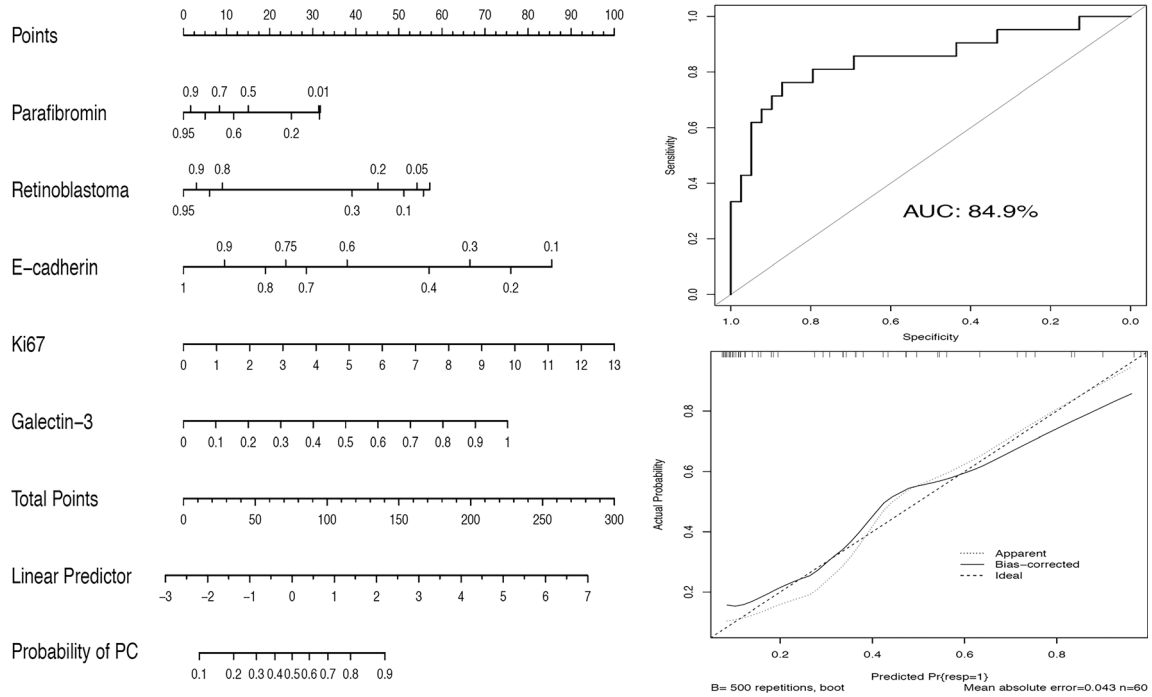

Fig. 3 Nomogram using five biomarkers to classify patients as having PC or atypical/adenoma along with a ROC curve and calibration plot of the logistic regression model used to classify the patients. Utilizing this nomogram, the total points is calculated from the five biomarkers. A total point value of 140 or more corresponds to a probability of parathyroid carcinoma of > 90%. *Parafibromin, retinoblastoma, E-cadherin, and galectin-3 values represented from 0 to 1, equivalent to 0 to 100%. Ki-67 represented from 0 to 13, equivalent to 0 to 13%. To determine the point value, draw a vertical line from the immunohistochemical value to the top point scale. (example, Ki67 of 4 (4%) = 30 points). Using the total points row, draw a vertical line from the total score of the 5 markers down through the linear predictor and probability of PC.

Discussion

PC is rare and remains challenging to diagnose preoperatively, and the histopathologic features to differentiate it from other parathyroid neoplasms, including atypical neoplasms, overlap. This study demonstrated that an IHC biomarker panel consisting of parafibromin, RB, E-cadherin, Ki67, and galectin-3 had good diagnostic performance, with an AUC of 84.5% (95% confidence interval, 73.4–96.4%; mean absolute error, 0.043), which was collectively better than any individual biomarker in determining whether a parathyroid tumor was benign or malignant. The nomogram based on the 5 IHC biomarker expression patterns enabled us to easily calculate the probability of diagnosis of PC versus other parathyroid neoplasms. In this study, we reaffirmed that parafibromin has a pattern of loss of expression that makes it one of the best individual markers of PC, that its combination in a panel with other biomarkers increases the diagnostic sensitivity in PC, and for the first time the diagnostic potential of E-cadherin in an IHC panel for the recognition of PC against other parathyroid neoplasms is included and evaluated. Also, overexpression of galectin-3 was quite common in PCs but rare in other parathyroid neoplasms, and closely associated with PC disease recurrence, either locally or systemically. Therefore, use of these IHC biomarkers by pathologists during the pathologic evaluation may aid in diagnostic classification, especially for difficult cases.

In our study, none of the patients in the atypical or adenoma groups experienced disease persistence or recurrence. Previous studies of atypical neoplasms have also documented low recurrence rates, ranging from 0 to 4.3% [7, 51, 52]. Therefore, the biologic course of atypical neoplasm, while low, remains undetermined warranting continuing close clinical follow-up of patients for at least 5 years after surgical intervention specially in those of large size, complete loss of parafibromin expression, or loading with CDC73 germline mutations [27, 53, 54]. Additionally, in our study, the adenoma with loss of parafibromin did not meet WHO 2017 criteria for parathyroid carcinoma; however it showed increased galectin 3 (60%) and Ki67 of 4% with a total nomogram of 120 points leading to a probability of carcinoma of 80%. Thus, while histologically, in adenoma, the nomogram highlights an increased risk for this patient. Utilizing parafibromin alone, Gill et al. (2019) recently showed that out of 26 parathyroid cases with parafibromin deficiency that did not meet the WHO criteria for parathyroid carcinoma, none behaved

aggressively. The estimated risk of metastasis/recurrence in that cohort did not exceed 4% [55]. Further risk stratification of parafibromin deficient tumors may be facilitated by the nomogram; however, further validation would be required.

Of the biomarkers examined in our study, parafibromin is one of the most investigated in the literature, and its strong molecular link with both germline CDC73 alterations and sporadic mutations in PC supports the correlation with parafibromin protein loss. Therefore, its inclusion in standard biomarker panels for PC is supported. A recent meta-analysis by Hu and colleagues demonstrated that the specificity of parafibromin expression loss is satisfactory for PC diagnosis as a single biomarker, but its sensitivity as an individual biomarker for PC is limited [56]. Our study supports this conclusion, with a specificity rate of 87% but a sensitivity rate of only 64%. These results thus both reaffirm the individual discriminatory utility of parafibromin IHC and also promote investigation of its combination with other biomarkers. In the present study, we identified that the loss of parafibromin staining combined with alterations in other IHC biomarkers, specifically overexpression of Ki67, and/or galectin-3, or alternatively decreased expression of RB and/or E-cadherin, characterizes the spectrum of PC allowing its distinction from other parathyroid neoplasms.

In our study, only eight of the 21 (38%) PC samples, one adenoma, and none of the atypical neoplasms highly expressed galectin-3. This contrasts with previous reports, in which more than 70% of PCs were classified as galectin-3–positive. This discrepancy may be explained by researchers in previous studies using lower threshold for galectin-3–positive staining than the 30% utilized in this study [13, 22, 50]. Prior studies also noted frequent positivity in adenomas and atypical parathyroid neoplasms (10–47%) using the same 30% cutoff; whereas using a higher threshold, we identified only 1 of 20 adenomas (5%) and no atypical (0%) parathyroid expressing this marker [21, 48]. Our ROC analysis was crucial in evaluating the level of galectin-3 associated with PC, facilitating differentiation of PC from other parathyroid neoplasms. In our study, five of the eight PC patients who presented with galectin-3 positivity developed local or systemic recurrence, although there are preliminary results; this finding suggests that galectin-3 positivity may correlate with predisposition for PC recurrence. This is consistent with studies of other cancers identifying association of galectin-3 expression with disease progression and metastasis [57, 58].

Previous studies have demonstrated that Ki67 by itself is not a sensitive enough marker to distinguish PC from other parathyroid neoplasms secondary to the overall low proliferative Ki67 levels in most PCs [15, 22, 44], but its combination with these four biomarkers was associated with a higher diagnostic probability of PC in this study. However, dysregulation of the cell cycle is a critical component in a subset of PC as highlighted by the loss of RB expression [1, 59]. Despite the regular performance of RB alone in our ROC analysis (AUC, 69%), when it was combined with the other biomarkers, a progressive decrease in RB expression was associated with a greater likelihood of diagnosis of PC. Our results were compatible with the findings described in previous studies that used at least 3 biomarkers included in this IHC panel and evaluated biomarkers expression not only in PC but also in atypical adenomas. Fernandez-Ranvier et al. showed that the combination of the loss of expression of parafibromin, RB loss, and galectin-3 overexpression was able to identify 16 PC versus 2 atypical adenomas and 18 adenomas benign. [1]. Also, Hosny Mohammed et al. found that the combination loss of parafibromin and high Ki67 is useful for recognizing 21 PCs versus 3 atypical adenomas and 73 benign adenomas [19]. Finally, Kumari et al found that the combined loss of parafibromin, galectin-3, and PGP9.5 overexpression had a sensitivity of 50% and specificity of 98% for 14 PC versus 19 atypical adenomas and 194 benign adenoma [21].

In the present study, abnormal E-cadherin expression was heterogeneous in the three patient groups. In the ROC analysis, the diagnostic performance of E-cadherin was regular (AUC, 65%), but combining its increasing loss of expression in cell membranes with the other biomarkers in our linear predictor adequately distinguished the probability of diagnosis of PC from the other parathyroid neoplasms. Recently, authors have reported significant differences in E-cadherin expression patterns in adenomas versus PC which showed loss of its expression with associated activation of tumor cell migration and mobility [26].

Overall, our nomogram of the 5 IHC biomarker expression patterns may help predict the risk of malignancy of parathyroid neoplasms. A parathyroid neoplasm may behave malignantly in the presence of high proliferative Ki67 levels, complete loss of membranous E-cadherin expression, and/or galectin-3 overexpression, and even more so when associated with loss of RB and parafibromin expression. Notably, if the sum of each score per biomarker in our nomogram is greater than 140 points, it corresponds to a probability of PC greater than 90%. Moreover, patients with PC expressing galectin-3 may have a high risk of recurrence, emphasizing the potential value of close follow-up for early detection. Further evaluation of galectin-3 as a potential biomarker of clinical aggressiveness, including prospectively, is warranted.

Regarding expression of PGP9.5, authors have reported variable incidence of PC positivity with rates ranging from 33 to 64% [15, 21]. In our study, we observed fewer PGP9.5-positive stains of PC samples (6/23 [26%]). This lower incidence of PGP9.5-positive staining may be explained by differences in tumor biology, methodology, and/ or interpretation of the staining in our cohort. In addition, Howell and colleagues found that atypical neoplasms have at least three IHC PGP9.5 phenotypes related to high risk of malignancy or cancer [20], which may explain the non-consistent

performance of PGP9.5 when it was combined with the other biomarkers in our study. Further research is needed to determine how PGP9.5 evaluation may compliment PC evaluation.

We are aware of the limitations of our study and biases of its retrospective nature, including those shared by other investigations of PC and atypical parathyroid neoplasms, which are each rare tumor. In addition, we used only primary neoplasm samples for the 3 groups to minimize confounding factors that could influence the initial tumor microenvironment. As our focus centered on parathyroid carcinomas and atypical adenomas, the number of adenomas in this study is a limitation; however, this group is widely analyzed in previous literature. We are grateful to our collaborating colleagues and encourage multi-institutional studies to further advance understanding of these tumors. We are looking forward to external validation of our findings. Finally, another significant limitation is that due to the considerable heterogeneity of the scoring criteria used for the different biomarkers which could influence the sensitivity, we decided to use a similar cutoff previously used in other studies with multiple biomarkers for PC, encouraging the standardization of IHC protocols and scoring systems used for PC recognition in future research.

In summary, the panel of biomarkers evaluated here may be a clinically useful tool to improve the characterization and diagnosis of PC versus other parathyroid neoplasms. Our results suggest that the combination of parafibromin, RB, Ki67, E-cadherin, and galectin-3 IHC expression patterns can confer an adequate degree of confidence in supporting a diagnosis of PC versus other parathyroid neoplasms, both atypical and adenomas. We further identified galectin-3 as a potential biomarker of PC aggressiveness. Combining histopathologic features with a biomarker panel may provide a tool for surgical pathologists to group more similar parathyroid neoplasms together, ultimately for improved predication of risk of malignancy.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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