Loss of Parafibromin Immunoreactivity Is a Distinguishing Feature of Parathyroid Carcinoma

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ABSTRACT

Purpose: A reliable method for diagnosing parathyroid carcinoma has remained elusive over the years, resulting in its under-recognition and suboptimal therapy. Obtaining an accurate diagnosis has become an even more pressing matter with recent evidence that germline HRPT2 gene mutations are found in patients with apparently sporadic parathyroid carcinoma. There is a high prevalence of HRPT2 gene mutations and biallelic inactivation in parathyroid carcinoma. We hypothesize that loss of parafibromin, the protein product of the HRPT2 gene, would distinguish carcinoma from benign tissue.

Experimental Design: We generated a novel antiparafibromin monoclonal antibody and performed immunostaining on 52 definite carcinoma specimens, 6 equivocal carcinoma specimens, 88 benign specimens, and 9 hyperparathyroidism-jaw tumor (HPT-JT) syndrome-related adenomas from patients with primary hyperparathyroidism from nine worldwide centers and one national database.

RESULTS

We report that the loss of parafibromin nuclear immunoreactivity has 96% sensitivity [95% confidence interval (CI), 85−99%] and 99% specificity (95% CI, 92–100%) in diagnosing definite carcinoma. Inter-observer agreement for evaluation of parafibromin loss was excellent, with unweighted kappa of 0.89 (95% CI, 0.79–0.98). Two equivocal carcinomas misclassified as adenomas were highlighted by parafibromin immunostaining. One of these tumors has since recurred, satisfying criteria for a definite carcinoma. Similarly, eight of nine HPT-JT syndrome-related adenomas showed absent nuclear immunoreactivity.

Conclusions: Parafibromin is a promising molecular marker for diagnosing parathyroid carcinoma. The similar loss of parafibromin immunoreactivity in HPT-JT syndrome-related adenomas suggests that this is a pivotal step in parathyroid tumorigenesis.

INTRODUCTION

Primary hyperparathyroidism is a common disorder, diagnosed with increasing frequency (1). The prevalence of parathyroid carcinoma has ranged in various studies from <1 to 5% of cases of primary hyperparathyroidism (1, 2) with especially high rates reported in Japan (3) and Italy (4). The causes of this variation are unclear but may reflect a true geographical difference, referral bias, or differences in the histologic criteria used. Apart from the presence of local invasiveness or metastasis, there is no definitive standard for pathologic diagnosis of parathyroid carcinoma; in the absence of these features, such a diagnosis may have subjective elements (1, 2, 5). As a result of this difficulty in recognition, up to 86% of cases are not initially recognized intra-operatively even in expert institutions and receive inadequate surgical resection (6). The diagnosis of carcinoma is often retrospectively made after relapse (2, 7), when treatment options are limited. In one series, half of all recurrent or metastatic carcinomas were initially diagnosed as benign (8). Indeed, it has been concluded by some authors that systematic diagnosis of parathyroid carcinoma may rest upon ongoing postoperative follow-up of patients who have undergone resection of apparently benign adenomas (9, 10). About 20% of patients with an apparently sporadic carcinoma may be manifesting a forme fruste of hyperparathyroidism-jaw tumor (HPT-JT) syndrome (11), a hereditary multitumor syndrome characterized by HRPT2 gene mutations (12). In this light, an accurate diagnosis is even more critical. Timely diagnosis would allow definitive surgery and targeted genetic screening of individuals and their families (13). From a clinical viewpoint, it is unsatisfactory to rely on tumor extension for diagnosis, as early recognition and treatment by en-bloc resection are the main determinants of prognosis (2, 3, 6, 14).
in 66 to 100% of sporadic parathyroid carcinomas, and biallelic inactivation is present in the majority of these cases (11, 16). We thus hypothesized that loss of parafibromin immunoreactivity would distinguish parathyroid carcinoma from benign pathologies of primary hyperparathyroidism.

Studies of Diagnostic Markers. The quest for a definitive method for diagnosis started in earnest 30 years ago, when initial histopathologic guidelines for the diagnosis of carcinoma described uniform sheets of cells arranged in a lobulated fashion separated by fibrous trabeculae, capsular or vascular invasion, and the presence of mitotic figures (17). Unfortunately, mitotic features, fibrous bands, and uniform sheets of cells were found not to be pathognomonic for parathyroid carcinoma (5, 14). As a result of the limited applicability of these guidelines, many adjunct investigations for parathyroid carcinoma have been studied, including electron microscopy, immunohistochemistry, DNA flow cytometry, and in situ hybridization (2). Immunohistochemical markers that have been studied include retinoblastoma tumor suppressor gene protein (pRb), calcium-sensing, Ki-67, cytokeratin-14, p27, mdm2, Bcl-2, cyclin D1, p53, and p21 (5, 18–24). However, many markers have not been shown to be useful in this regard (19).

The most extensively studied marker to date is the retinoblastoma tumor suppressor gene RB and the RB protein (pRB). Contrary to initial reports, recent evidence suggests that loss of heterozygosity is not specific to parathyroid carcinoma. Studies of pRB immunostaining have also yielded conflicting results. Some studies have reported that it may be a helpful marker (25, 26), but several other studies have contradicted these findings (22, 23). A recent review of RB gene abnormalities in parathyroid carcinoma concluded that no definitive conclusion could be drawn with regards to pRB staining (26).

Other markers studied include cell cycle-associated antigens. Erickson et al. (21) and Stojadinovic et al. (19) report that although carcinomas, relative to adenomas, have a lower percentage of p27-positive nuclei, there is a considerable overlap in staining percentages. Stojadinovic et al. also report that a multiple-marker phenotype including p27, Bcl-2, Ki-67, and mdm2 was useful in defining a subset of benign tumors but that carcinoma displayed a complex range of multi-marker phenotypes, some of which were not specific. Furthermore, DNA cytometry was shown to be of prognostic but not diagnostic use. It distinguishes a subset of parathyroid carcinomas that are aneuploid and that may behave in a more aggressive fashion (27–29) but is not specific (2, 3, 30, 31) or sensitive (27–30).

In summary, despite many studies over the years, diagnosing parathyroid carcinoma remains a major challenge for the expert pathologist. As there is a high prevalence of HRPT2 gene mutations in parathyroid carcinoma (11, 16), we investigated parafibromin immunoreactivity as a means of differentiating parathyroid cancer from benign tissue, including adenomas, hyperplasias, and multiple endocrine neoplasia types 1 (MEN1)-associated tumors. As the prevalence of parathyroid carcinoma varies geographically for uncertain reasons (2), we selected a geographically diverse, multi-center approach, with a variety of pathologies that may be encountered in the evaluation of primary hyperparathyroidism. As histologic criteria varies because of the lack of standard guidelines, gold-standard criteria of invasion or metastasis was imposed on case selection for standardization. We additionally stained adenomas from patients from HPT-JT syndrome, in view of a common expression profiling signature distinguishing these adenomas and carcinomas from other types of benign tissue that we recently reported (32).

MATERIALS AND METHODS

Patient Samples. We conducted a multi-center, retrospective study involving anonymized formalin-fixed, paraffin-embedded parathyroid specimens from primary hyperparathyroidism cases. The participating centers were Ohio State University, Leiden University Medical Center (Netherlands), Northwestern University, University of Chicago, Seinäjoki Central Hospital (Finland), Singapore General Hospital (Singapore), National University Hospital (Singapore), Shared Pathology Informatics Network (West Michigan), and University of Tasmania (Australia). The study was reviewed and approved by the Van Andel Institute Institutional Review Board.

A total of 160 specimens were examined. One hundred and twenty-three full sections were studied, including sporadic primary carcinomas (n = 19), sporadic metastatic tissue (n = 1), sporadic equivocal carcinomas (n = 2), sporadic adenomas (n = 50), sporadic primary hyperplasia (n = 25), MEN1-associated tumors (n = 13), HPT-JT primary carcinoma (n = 1), HPT-JT adenomas (n = 7), and normal tissue (n = 5). A tissue array containing an additional 37 specimens obtained from the Dutch National Pathology Database (PALGA) was also studied: sporadic primary carcinomas (n = 23), sporadic metastatic tissue (n = 7), sporadic equivocal carcinomas (n = 3), HPT-JT primary carcinoma (n = 1), HPT-JT adenomas (n = 2), and normal tissue (n = 1). Triplicate tissue cores with a diameter of 0.6 mm, as selected by a pathologist (H. M.), were taken from each specimen (Beecher Instruments, Silver Spring, MD) wherever possible, and a standard procedure was used to array tissue cores on a recipient paraffin block (33). The full sections of all arrayed specimens were examined (H. M.) to ensure concordance with criteria. In all cases (both full sections and arrays), lesions were diagnosed as definite carcinomas only if vascular invasion, invasion of surrounding tissue, or distant metastasis were evident. Equivocal carcinomas were defined as tumors exhibiting histopathologic features of carcinoma without the presence of vascular invasion, invasion of surrounding tissue, or distant metastasis (22, 28). Clinicopathologic data for the definite and equivocal carcinoma cases, following reclassification as described below, are shown in Table 1. In summary, 52 definite carcinoma specimens were obtained from 48 patients; multiple specimens from single patients were obtained at separate clinical events. 49 of 52 definite carcinoma specimens had surrounding tissue or vascular invasion on histopathologic examination. For 25 specimens from 21 patients with available data, 19 specimens were from 15 patients who initially or eventually developed metastases, 5 specimens were from 5 patients who relapsed locally, and only 1 patient, who was followed up for 1 year, did not have local or systemic relapse during follow-up. Twenty-eight arrayed definite carcinoma specimens had been further characterized previously (5): 10 of 28 were cystic; 4 of 28 trabecular; 19 of 28 had fibrotic bands; 8 of 28 had >1 of 10 mitoses/high power field; 14 of 28 showed positive cyclin D1
staining; 20 of 27 showed positive calcium-sensing receptor staining; 14 of 22 had loss of heterozygosity of chromosome 1q (HRPT2 gene loci); 13 of 22 had loss of heterozygosity at chromosome 11q (MEN1 gene loci); Ki-67 index ranged from 0.1 to 27.5. All HPT-JT adenomas had been sequenced previously (15, 33) and confirmed to have HRPT2 gene mutations. All MEN1 tumors had been confirmed previously to have MEN1 gene mutations.

### Table 1: Clinicopathologic data and evaluation of immunostaining for carcinoma specimens

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histo-pathological assessment of carcinoma</th>
<th>Tumor location</th>
<th>Presence of local or vascular invasion at initial surgery</th>
<th>Presence of metastasis at initial surgery</th>
<th>Clinical progression during follow-up</th>
<th>Presence of metastasis or relapse during clinical history</th>
<th>Parafibromin immunoreactivity</th>
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staining; 14 of 22 had loss of heterozygosity of chromosome 1q (HRPT2 gene loci); 13 of 22 had loss of heterozygosity at chromosome 11q (MEN1 gene loci); Ki-67 index ranged from 0.1 to 27.5. All HPT-JT adenomas had been sequenced previously (15, 33) and confirmed to have HRPT2 gene mutations. All MEN1 tumors had been confirmed previously to have MEN1 gene mutations.
Monoclonal Antibody Generation and Validation. We first generated a novel murine monoclonal antibody to parafibromin targeting the peptide RRDPDKDLLGYNLC, corresponding to amino acid positions 87 to 100. BALB/c mice were immunized with intraperitoneally administered keyhole limpet hemocyanin-conjugated synthetic peptides in complete Freund’s adjuvant, followed by two additional injections in incomplete Freund’s adjuvant at an interval of 2 weeks. After a month, injections without adjuvant were administered both intravenously and intraperitoneally. Spleen cells were fused with P3X63AF8/653 myeloma cells 3 days after the final injection. An immunofluorescence assay was used to screen positive hybridomas with COS7 cells transfected with a green fluorescent protein (GFP)-HRPT2 DNA fusion construct, validated by ELISA, subcloned to establish stable monoclonal antibody-secreting hybridomas, and revalidated by ELISA. The antibody was purified by fast performance liquid chromatography in a protein G affinity column.

Indirect immunofluorescence was used to show antibody specificity (Fig. 1). HEK293 cells transfected with a GFP-HRPT2 DNA fusion construct and control cells transfected with GFP-empty vector were fixed by immersion in cold acetone/methanol (1:1) for 1 hour and rehydrated through 70% ethanol, 50% ethanol, and PBS. Anti-parafibromin antibody was applied and washed off, followed by Rhodamine Red-conjugated goat antimouse IgG (Jackson ImmunoResearch Lab, West Grove, PA) at 1:100 dilution for 1.5 hours at 37°C. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. In addition, Western blotting was done on COS7 cells transfected with empty pcDNA3 vector and pcDNA3-HRPT2 (Fig. 2). To further characterize parafibromin immunoreactivity, we did immunohistochemical studies in a variety of formalin-fixed paraffin-embedded human tissues, including lung, kidney, testis, thyroid, adrenal, thymus, and lymph nodes.

Immunohistochemistry. Immunohistochemistry was done with standard procedures: deparaffinized 5-μm sections were steamed in citrate buffer (pH 6) for 30 minutes. Sections were incubated in succession with 0.3% hydrogen peroxide in water for 30 minutes; 5% goat serum for 30 minutes; Avidin D solution for 15 minutes (SP2001, Vector Labs, Burlingame, CA); biotin solution for 15 minutes (SP2001, Vector Labs); primary antibody diluted in diluting buffer (M35, Biomeda Corp., Foster City, CA) at 10 μg/mL for 1 hour; biotinylated goat antimouse antibody (BA-9200, Vector Labs) at 6 μg/mL for 1 hour; streptavidin-biotinylated horseradish peroxidase complex (Vectastain Elite kit, PK-6100, Vector Labs) for 30 minutes; dianimonobenzidine tetrahydrochloride for 5 minutes and counterstained in hematoxylin for 3 seconds. Sections were washed in 2 cycles of PBS (pH 7.4) between each step. Positive controls were transfected COS7 cells expressing parafibromin. Negative controls included experiments omitting primary antibody and experiments with primary antibody preabsorbed with a 20-fold excess of immunizing peptide. A random selection of duplicate slides were stained by a Dako LV-1 automated immunostainer (Dako, Carpinteria, CA) by a separate author (C.M.) in another laboratory.

Pathologic Evaluation. Slide sections were examined independently by two blinded investigators (C. M. and J. R.). The tissue array was examined unblinded by two investigators, who reached a common agreement. The staining pattern of each specimen was classified in three categories: diffuse positive, focal loss, or diffuse loss. Diffuse positive staining was defined as staining of all parathyroid tissue nuclei; heterogeneity of staining without loss was included in this category. Focal loss was defined as the absence of nuclear staining in variably sized regions. Diffuse loss was defined as the absence of nuclear staining in all tumor tissue. Where staining was diffuse, the overall staining intensity was evaluated on a semi-quantitative
RESULTS

Antibody Characterization. The anti-parafibromin monoclonal antibody co-localized with GFP-parafibromin fusion protein in the nuclei of transfected HEK293 cells (Fig. 1). Control cells transfected with GFP-empty vector did not show staining (data not shown). Western blotting done on transfected COS7 cells and empty vector control showed increased intensity of a single crisp band with the expected molecular mass (Fig. 2). We examined a range of human tissues to characterize its range of immunoreactivity and observed that parafibromin immunoreactivity was present in all organs but was cell-type specific (data not shown). Parafibromin was localized to the nucleus in all tissue examined. Controls with antibody pre-absorbed with a 20-fold excess of immunizing peptide did not show any immunoreactivity.

Sequence Analysis. Analysis of the peptide sequence of parafibromin showed the presence of bipartite nuclear localization signal domains at residues 76 to 92 and 393 to 409.

Parathyroid Tissue Immunohistochemistry. We report that parathyroid carcinoma may be distinguished from other benign pathologies by the loss of parafibromin nuclear immunoreactivity (Fig. 3). Table 1 shows individual case evaluation alongside clinicopathologic data for carcinoma cases, and Table 2 shows a summary of the evaluation of all specimens. Eleven benign cases from the adenoma, hyperplasia, and MEN1 tumor groups displayed heterogeneity of staining without absence of parafibromin immunoreactivity, and these were classified as diffusely positive. Loss of parafibromin immunoreactivity for arrayed carcinoma specimens was independent of tissue architecture, frequency of mitoses, Ki-67 index, loss of heterozygosity, cyclin D1 and calcium-sensing receptor immunostaining results. The assay had a calculated sensitivity of 96% [95% confidence interval (CI), 85–99%] and specificity of 99% (95% CI, 92–100%) for differentiating parathyroid carcinoma from sporadic benign proliferations (Table 3). Table 3 also shows the calculated positive predictive values and negative predictive values with confidence intervals for common estimates of prevalence (1% in countries of low reported prevalence, ref. 1; 5% in countries of high reported prevalence, such as Japan and Italy, ref. 3, 4). The data showing inter-observer variation is presented in Table 4. There was exceptional inter-observer agreement with regards to the blinded assessment of any immunoreactivity loss, with an unweighted kappa statistic of 0.89 (95% CI, 0.79–0.98). Agreement with regards to staining pattern was also excellent, with an unweighted kappa statistic of 0.77 (95% CI, 0.65–0.89). The H&E-stained sections of benign specimens that had at least one investigator assessing as having loss (n = 6) were re-evaluated by two independent pathologists.

Sequence Analysis. The ScanProSite program (36) was used to search the PROSITE Release 18.26 database.13

astatic carcinoma, showed diffuse nuclear immunoreactivity and was shown by parathyroid hormone immunostaining (37) to be of non-parathyroid origin. All but one HPT-JT adenomas exhibited loss of parafibromin immunoreactivity; the remaining one was evaluated as diffusely weak staining by one investigator and by the other as diffuse loss. It is important to note that reclassification has been incorporated into all tables and statistical calculations.

**DISCUSSION**

This is the first study on parafibromin, the protein product of the *HRPT2* tumor suppressor gene. Our results localize hu-
man paraﬁbromin to the nucleus, which is consistent with peptide sequence analysis, cellular fractionation studies, and immunohistochemistry in a variety of tissues. Paraﬁbromin shares 32% identity with yeast protein cdc73p (15), which is also a nuclear protein and part of the Paﬁ complex mediating cell cycle regulation and transcription (38). Its function in humans is currently unknown.

Pathologic Assessment. This study shows that paraﬁbromin immunoreactivity is a promising adjunct for differentiating carcinoma from benign tissue. Paraﬁbroid carcinoma is often not recognized, even after histologic examination (2, 7). With the approximately 20% (3 of 15) possibility that apparently sporadic paraﬁbroid carcinoma may be a manifestation of hereditary HPT-JT syndrome (11), making an accurate diagnosis is now paramount. To our knowledge, no other immunohistochemical marker for paraﬁbroid carcinoma has been previously assessed in a study of similar size or geographical diversity. The loss of paraﬁbromin immunoreactivity was true regardless of architecture, presence of mitotic ﬁgures, loss of heterozygosity, or immunostaining for Ki67, CASR, and cyclin D1. In addition, this study successfully detected the misclassiﬁcation of two equivocal carcinomas among 50 adenomas, one of which subsequently recurred locally, demonstrating that paraﬁbroid malignancy is often under-recognized.

In the assessment of the value of a diagnostic assay, the positive predictive values and negative predictive value are the most relevant clinically (39). These depend on the prevalence of a disease within a certain population, as well as the sensitivity and specificity. With an estimated prevalence of 1% of primary hyperparathyroidism cases, paraﬁbromin immunostaining has a positive predictive value of 49% (95% CI, 10–100%) and an negative predictive value of 100% (95% CI, 100–100%, with rounding). In countries with a prevalence of 5%, such as Japan and Italy (3, 4), the positive predictive value and negative predictive value would be 83% (95% CI, 36–100%) and 99% (95% CI, 99–100%), respectively. Should these values be validated in additional studies, paraﬁbromin immunostaining is likely to be a helpful diagnostic adjunct for the pathologist. Whereas deﬁnite carcinomas may be recognized on the deﬁnitive criteria of invasion or metastasis, tumors that have histopathologic features of malignancy but lack tumor extension represent challenged clinical and pathologic problems. Levin (40) distinguished between “typical” and “atypical adenomas.” Others have chosen to label this group as “equivocal carcinomas” (22, 28). We prefer the terminology “equivocal carcinoma” in the research and clinical setting, as gold-standard pathologic criteria does not accommodate a localized parathyroid carcinoma (2, 27, 40). Considering such patients as having “equivocal carcinomas” is also logical clinically, because they are followed up in a similar fashion as patients with deﬁnite parathyroid carcinoma (27, 41). Thus, this terminology is more appropriate in view of the potential malignant behavior of these group of tumors, the under-recognition of carcinoma, and the fact that current gold-standard diagnostic criteria of malignancy of invasion or metastasis is limited to advanced disease. Our results support the view that this entity termed “equivocal carcinoma” is heterogeneous (40). Of the five cases initially diagnosed as equivocal carcinomas, three displayed loss of paraﬁbromin immunoreactivity. No cases showing paraﬁbromin immunoreactivity relapsed. In addition, two cases initially diagnosed as adenomas were subsequently reclassiﬁed pathologically as equivocal carcinomas after paraﬁbromin immunostaining and re-evaluation. One case relapsed locally on follow-up and was reclassiﬁed clinically as a deﬁnite carcinoma. The

### Table 2 Summary of specimen evaluation

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Total (N = 159)</th>
<th>Diffuse loss (%)</th>
<th>Focal loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definite carcinoma (sections)</td>
<td>22</td>
<td>9 (41)</td>
<td>11 (50)</td>
</tr>
<tr>
<td>Definite carcinoma (array)</td>
<td>30</td>
<td>17 (57)</td>
<td>13 (43)</td>
</tr>
<tr>
<td>Equivocal carcinoma (sections)</td>
<td>3</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Equivocal carcinoma (array)</td>
<td>7</td>
<td>1 (33)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>HPT-JT adenomas (sections)</td>
<td>7</td>
<td>4 (57)</td>
<td>2 (29)</td>
</tr>
<tr>
<td>HPT-JT adenomas (array)</td>
<td>2</td>
<td>2 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sporadic adenomas</td>
<td>48</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sporadic primary hyperplasias</td>
<td>25</td>
<td>0 (0)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>MEN1-related tumors</td>
<td>13</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>6</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
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</table>

### Table 3 Calculated diagnostic value indices

<table>
<thead>
<tr>
<th>Diagnostic indices</th>
<th>Value/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (95% CI)</td>
<td>96 (86–99)</td>
</tr>
<tr>
<td>Specificity (95% CI)</td>
<td>99 (92–100)</td>
</tr>
<tr>
<td>Positive predictive value at 1% prevalence (95% CI)</td>
<td>49 (10–100)</td>
</tr>
<tr>
<td>Negative predictive value at 1% prevalence (95% CI)</td>
<td>100 (100–100)</td>
</tr>
<tr>
<td>Positive predictive value at 5% prevalence (95% CI)</td>
<td>83 (36–100)</td>
</tr>
<tr>
<td>Negative predictive value at 5% prevalence (95% CI)</td>
<td>100 (99–100)</td>
</tr>
</tbody>
</table>

### Table 4 Results of blinded individual observer evaluation for all sections (N = 123)

<table>
<thead>
<tr>
<th>Pathologist 1</th>
<th>Diffusely positive</th>
<th>Focal loss</th>
<th>Diffuse loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>92</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pathologist 2</td>
<td></td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>12</td>
</tr>
</tbody>
</table>
classification of this case by parafibromin immunoreactivity shows
a utility exceeding that of current gold-standard criteria, which is
restricted to advanced disease. From a clinical point of view, such
a utility is highly desirable, as current gold-standard criteria only
define advanced disease, but surgical intervention is most ap-
propriate with localized disease (2, 3, 6, 14). In addition, this result
suggests that parafibromin loss occurs at an early stage.

Adenomas with HRPT2 mutations also shows diffuse loss of
parafibromin immunoreactivity in our study. These occur at a very
low frequency in sporadic parathyroid adenomas (15, 16). How-
ever, the loss of parafibromin immunoreactivity is useful in detect-
ing these tumors. For patients with HPT-JT syndrome who are at
high risk of carcinoma, deliberate total prophylactic parathyroid-
ecctomy has been considered (42). Whether radical surgery will
benefit the rare patient who has had a somatic HRPT2 mutation
detected in a parathyroid adenoma remains uncertain.

HRPT2 Mutation and Parafibromin Loss. Fifty of fif-
ty-two definite parathyroid carcinomas displayed loss of para-
fibromin immunoreactivity. This is consistent with the high rate
of somatic HRPT2 gene mutations with biallelic inactivation
reported in sporadic parathyroid carcinoma (11, 16). HPT-JT
syndrome is characterized by HRPT2 gene mutations, and pa-
ients with HPT-JT syndrome have a high risk of parathyroid
carcinoma (15, 16). In conjunction with microarray studies
showing a common gene signature for parathyroid carcinomas
and HPT-JT-related adenomas, the loss of parafibromin immu-
noreactivity in both groups suggests that parafibromin down-
regulation is an early and pivotal step in parathyroid tumorigen-
esis. It was interesting that focal expression of parafibromin was
retained in a small subset of parathyroid adenomas with docu-
mented HRPT2 gene mutations. It was observed that in tumors
with focal loss of parafibromin immunoreactivity, parafibromin
expression was markedly higher near blood vessels and margins
(both internal and external) such as fibrous septa and capsular
tissue (data not shown). Whether the antibody was binding to
wild-type or mutant parafibromin remains uncertain. A small
subset of carcinomas display normal parafibromin expression.
This may be because of alternative tumorigenic mechanisms,
and there is evidence of that at least one additional tumor suppres-
sor gene may exist on chromosome 13q (25, 43–46). A recent
study of two such candidate genes, RB and BRCA2, did not
identify any mutations in seven specimens of parathyroid car-
icoma (47). Parathyroid carcinoma is a rare manifestation in
MEN1 (48, 49), but our study showed normal parafibromin ex-
pression in MEN1-related benign tumors.

Study Limitations. The evaluation of immunohistochemi-
cally stained slides is inherently subjective, with considerable ob-
erver dependence. To address this, two observers independently
evaluated the slides. The calculated inter-observer agreement was
excellent for assessment of parafibromin loss, with a kappa statistic
of 0.89. However, one limitation of our study was the risk of
subconscious bias. Although the diagnoses were blinded, vascular
or local tissue invasion may have been visible on inspection.
However, we conclude that any biases are unlikely to be substantial
as the majority of benign cases stain with either moderate or strong
intensity, rather than with weak staining (Table 2). Additionally,
adrenomas with HPT-JT mutations were uniformly diagnosed with
loss of parafibromin immunoreactivity, whereas adenomas without
these mutations were not.

CONCLUSION

Parafibromin immunostaining is a promising adjunct for the
diagnosis of parathyroid carcinoma, an often unrecognized
entity that may be hereditary (11). This recognition of carci-
noma is critical for genetic screening (13), and our results
provide direct evidence that carcinoma may not be recognized
during initial histopathologic evaluation. Because en-bloc resec-
tion constitutes definitive therapy for parathyroid carcinoma and
86% of carcinomas may not be detected intraoperatively (6),
studying the intra-operative assessment of parafibromin immu-
noreactivity through ultrarapid immunostaining (50) would be
logical. Finally, the shared loss of parafibromin between para-
thyroid carcinoma and HPT-JT-related adenomas, alongside
evidence of a common gene expression signature (32), suggests
novel tumorigenesis pathways mediated by the HRPT2 gene.

ACKNOWLEDGMENTS

The authors dedicate this report to the late Dr. J. J. Shepherd of
the University of Tasmania.

REFERENCES

2. Shane E. Clinical review 122: parathyroid carcinoma. J Clin Endo-
3. Obara T, Okamoto T, Kane M, Ishara M. Functioning parathyroid
carcinoma: clinicopathologic features and rational treatment. Semin
4. Favia G, Lumachi F, Polistina F, D’Amico DF. Parathyroid carci-
noma: sixteen new cases and suggestions for correct management.
5. Sandelin K, Tullgren O, Fannebo LO. Clinical course of metastatic
6. Hendahl SA, Fleming ID, Frengem AM, Menck HR. Two hundred
eighty-six cases of parathyroid carcinoma treated in the U.S. between
1985–1995: a National Cancer Data Base Report. The American Col-
lege of Surgeons Commission on Cancer and the American Cancer
Society, Cancer (Philad) 1999;86:538–44.
HD. Parathyroid carcinoma: problems in diagnosis and the need for radical
8. Sandelin K, Tullgren O, Fannebo LO. Clinical course of metastatic
10. Wells SA Jr, Debenedetti MK, Doherty GM. Recurrent or persistent
mutations of the HRPT2 gene in sporadic parathyroid carcinoma.
roidism and multiple ossifying jaw fibromas: a clinically and genetically
13. Weinstein LS, Simonds WF, HRPT2, a marker of parathyroid
Parathyroid carcinoma: evaluation and interdisciplinary management.
Cancer (Philad) 2004;100:900–5.
15. Carpten JD, Robbins CM, Villablanca A, et al. HRPT2, encoding
parafibromin, is mutated in hyperparathyroidism-jaw tumor syndrome.


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Min-Han Tan, Carl Morrison, Pengfei Wang, et al.


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