Diagnosis of parathyroid carcinoma using immunohistochemical staining against hTERT

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Abstract. The differential diagnosis of parathyroid carcinoma from benign adenoma is often difficult when its typical clinicopathological features are absent, even with the aid of various molecular markers. We recently demonstrated that telomerase activation through hTERT expression is a unique characteristic that is limited to parathyroid carcinoma and not seen in benign tumors. In the present study, we investigated hTERT expression in parathyroid tumors using immunohistochemistry in an attempt to determine its clinical utility. There was no evidence of immunoreactivity in the 4 normal parathyroid glands and the 18 typical adenomas. In contrast, one atypical adenoma stained positively and homogeneously, and the disease recurred three times clinically. All of the 6 carcinomas demonstrated a clear positive nuclear staining of hTERT. Every hTERT-positive tumor showed a high Ki-67 index, i.e., greater than 4%. Nucleolin, an hTERT-binding protein, was abundantly and homogeneously expressed in all specimens examined independent of the pathological diagnosis and hTERT or Ki-67 expression. Therefore, it is possible that immunostaining with an anti-hTERT antigen (NCL-L-hTERT) individually may distinguish parathyroid carcinoma from benign tumors.

Introduction

The majority of cases of primary hyperparathyroidism is caused either by benign adenoma or hyperplasia. However, in some rare situations, it accounts for approximately 1-5% of the cases in which parathyroid carcinomas are encountered (1-3). Parathyroid carcinoma often causes local recurrences or distant metastases, and a radical cure is very difficult once the disease recurs (3). The chance for a cure is dependent on whether or not a complete en bloc resection is performed as the initial surgical therapy (4). Therefore, it is particularly important to diagnose parathyroid carcinoma correctly during the initial surgical operation for proper treatment. For cases demonstrating typical clinical features such as a palpable neck mass, an extremely high serum calcium level (hypercalcemic crisis) and painful bone lesions (ostelitis fibrosa cystica), a preoperative diagnosis of parathyroid carcinoma is not difficult (5,6). However, some of these clinical features frequently occur in cases of benign adenoma. Imaging studies may be helpful to obtain an accurate diagnosis in some cases when evidence of metastatic diseases or of invasive growth to the adjacent structures is apparent (7,8). Pathologically, parathyroid carcinoma may be distinguished from benign lesions by examining the histological findings, such as capsular penetration, vascular invasion, or frequent cellular mitosis accompanied by necrosis and macronucleoli (9). The problem is that these typical clinical or pathological features are absent in a considerable number of cases of parathyroid carcinoma. Therefore, a correct diagnosis of parathyroid carcinoma is commonly made only after a recurrence.

In order to overcome the difficulty in making a correct diagnosis of parathyroid carcinoma, the utility of immunochemical examinations by recruiting molecular markers has been widely investigated. However, a consensus has yet to be reached in identifying parathyroid carcinoma from adenoma using these markers. Only Ki-67 demonstrated a universally accepted clinical significance (10-14). However, several false-positive and false-negative results in diagnosis have been reported (10,11,15,16). and there have been conflicting results with respect to the potential role of Ki-67 in predicting clinical outcomes (15,17). Since it is not sufficient to use Ki-67 practically as a single diagnostic marker, the combination with several other markers has been recommended (5,16,19).

Several studies, including ours, have found specific telomerase activation in parathyroid carcinoma with a

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telomeric repeat amplification protocol (TRAP) assay using freshly obtained surgical samples (19-21). Telomerase is an enzyme nucleo-protein that adds telomeric repeats to the terminal regions of the chromosome. Cells can become immortal by acquiring the ability to promote additional telomeres by telomerase activation. Numerous studies have demonstrated that cancer cells possess telomerase activity from the initial stages. hTERT is a catalytic subunit of telomerase and is essential for the activation of enzymatic activity of telomerase (22). We previously demonstrated specific hTERT expression in parathyroid carcinoma by quantitative real-time PCR (19) and *in situ* hybridization methods (23), but we did not observe hTERT expression in benign tumors. These results clearly suggest that telomerase activation through hTERT expression is limited only in carcinoma but not in benign parathyroid tumors with an accuracy of 100%. Although there is significant clinical utility in determining hTERT expression i.e., as a promising marker for the accurate diagnosis of parathyroid carcinoma as has been clearly indicated from these investigations, the former methods require complex techniques and freshly procured samples. Therefore, an easier method has been sought to investigate telomerase or hTERT expression in parathyroid tumors. In this study, we attempted to apply immunohistochemical staining to parathyroid tumors and investigated its utility in identifying parathyroid carcinoma.

Materials and methods

Patients. A total of 26 cases with primary hyperparathyroidism were investigated in this study. The pathological diagnoses of these cases included 20 adenomas and 6 carcinomas. Four of the 6 cases of parathyroid carcinoma were operated on in Kuma Hospital (cases 26-29). Other specimens were collected from surgical samples from the Osaka City University Hospital. Five recurrent sites of 2 cases were also employed in the study. Four normal parathyroid glands obtained during thyroid surgery and a hyperplastic gland obtained from a case of secondary hyperparathyroidism were included as controls. Therefore, a total of 36 specimens were investigated in this study. The details of the cases are described in Table I.

Normal parathyroid glands were collected from surgically dissected specimens from 4 cases of papillary thyroid carcinoma operated on during the period from January, 2006 to May, 2006. All cases were female with an average age of 60.8 years (range 40-72). There was no elevation observed in either the serum calcium (S-Ca) or PTH level in these cases.

Twenty parathyroid adenoma cases were operated on between January, 2004 and December, 2004 consisting of 7 males and 13 females with a median age of 55.4 years (range 17-83). A total of 15 cases showed clinical symptoms such as urinary calculus (9 cases) and bone pain (6 cases). One case was associated with papillary thyroid carcinoma. The remaining 4 cases were asymptomatic, and in these cases hyperparathyroidism was diagnosed with an examination of blood biochemistry for other reasons. S-Ca and intact-parathyroid hormone (i-PTH) levels of these cases were 11.2-14.4 mg/dl (normal range 7-10) and 70-1198 pg/ml (normal range 10-65), respectively. The location of the tumors were right upper in 2, right lower in 7, left upper in 6, and left lower in 5 cases, respectively. The sizes of the tumors ranged from 8 to 47 mm, and they weighed 200 mg to 15.0 g. In one case of parathyroid adenoma (case 25), local recurrences were observed twice at one and two years from the initial surgery. In both situations, the recurrent tumors did not display any pathological features of parathyroid carcinoma, such as invasions to adjacent organs, mitosis or a palisade-like arrangement. Therefore, the pathological diagnoses in each situation were adenoma. However, the tumor was large enough to palpate in the patient's neck. The S-Ca and i-PTH levels of the patient were as high as 14.4 mg/dl and 1158 pg/ml at the time of the initial surgery. Furthermore, the tumor showed a fibrous adhesion with an abnormal small vasculature to the thyroid gland, but it was not invasive, suggesting possible malignant clinical features at initial presentation. We thought that this case had potentially malignant characteristics (atypical adenoma).

Six parathyroid carcinoma cases were surgically operated on between 1991 and 2004, consisting of 5 males and 1 female with an average age of 49.8 years (range 28-67). Five cases showed clinical symptoms such as a palpable neck tumor (1 case), urinary calculus (1 cases) or both (3 cases). Another case was asymptomatic. All patients were affected by primary hyperparathyroidism. S-Ca and i-PTH levels in these cases were 12.5-17.2 mg/dl and 181-1253 pg/ml, respectively. These levels were significantly higher than those of the adenoma cases (S-Ca, $p<0.01$; i-PTH, $p<0.05$). One case of parathyroid carcinoma (case 30) was initially present as a large mediasternal mass (24) and was diagnosed as carcinoma by pathological evidence of capsular penetration in a small limited area. Another case (case 31) developed lung metastases three times at 4, 8 and 9 years from the initial surgery. Fifteen lesions of lung metastases were excised, and the pathological features of these lesions were compatible with parathyroid carcinoma. The patient died of hypercalcemia 3 months after the last surgical operation. The other 4 cases were diagnosed as parathyroid carcinoma by pathological evidence of invasive growth to adjacent tissues.

Immunohistochemistry. Immunohistochemical staining was performed as described previously (25). In brief, all the tissue samples were fixed in buffered formalin and embedded in paraffin. The serial sections measuring 4 μm were obtained from a representative block of each case for immunohistochemical studies. Each sample was de-waxed, and the antigen retrieval was performed by autoclaving at 121˚C for 5 min. The sections were incubated for 25 min in 3% hydrogen peroxide to quench endogenous tissue peroxidase. After blocking for nonspecific staining, the slides were incubated with primary antibodies. The primary antibodies used were mouse monoclonal antibody against Ki-67 (MIB-1, Dako Cytomation, Glostrup, Denmark), mouse monoclonal antibody against hTERT (NCL-L-hTERT clone 44F12, Novocastra, Newcastle upon Tyne, UK), and mouse monoclonal antibody against human nucleolin (clone 4E2, MBL, Nagoya, Japan). The sections were then incubated with secondary antibody. Immunohistochemical staining with the Liquid DAB Substrate-Chromogen System (Histofine, Nichirei, Tokyo, Japan) was performed. Nuclear staining

Table I. Details of the examined cases and results of immunohistochemistry.

Case no.	Age	Gender	Pathological diagnosis	S-Ca (mg/dl)	i-PTH (pg/ml)	Ki-67 index	hTERT (%)	Nucleolin (%)
1	72	F	Normal	9.6	37	1.9	0	45
2	40	F	Normal	9.7	n.d.	0.2	0	55
3	59	F	Normal	9.6	n.d.	0.2	0	55
4	72	F	Normal	10.4	n.d.	0.2	0	50
5	43	M	HP	9.4	702	1.4	0	70
6	68	F	AD	11.2	101	0.6	0	70
7	26	M	AD	11.4	175	2.5	0	75
8	62	F	AD	11.6	95	0.8	0	70
9	76	F	AD	11.2	112	0.1	0	75
10	17	F	AD	13.0	508	0.3	0	70
11	66	F	AD	11.6	160	3.1	0	70
12	60	F	AD	13.6	228	0.7	0	75
13	62	F	AD	11.2	70	3.7	0	70
14	19	F	AD	13.0	96	0.9	0	70
15	46	F	AD	13.2	481	2.4	0	80
			Nodule[a]			17.0	70	80
16	73	F	AD	11.2	107	0.9	0	70
17	41	F	AD	11.2	181	0.7	0	70
18	83	F	AD	12.2	140	1.0	0	70
19	83	F	AD	13.6	750	1.4	0	75
20	49	M	AD	11.6	179	1.1	0	70
21	74	M	AD	14.0	350	0.9	0	75
22	57	M	AD	12.0	121	0.8	0	70
23	62	M	AD	11.6	153	1.0	0	70
24	38	M	AD	11.4	107	0.2	0	70
25	51	M	ATP AD	14.4	1198	30.0	80	80
			1st Rec	11.2	300	22.0	95	80
			2nd Rec	11.4	200	10.0	90	80
26	38	M	PTC	17.2	1253	15.0	80	80
27	67	M	PTC	12.5	181	25.0	90	80
28	28	F	PTC	14.1	430	30.0	90	80
29	49	M	PTC	12.6	946	8.0	75	75
30	61	M	PTC	13.8	294	4.0	50	60
31	56	M	PTC	15.0		5.0	80	75
			1st Rec	12.8	159	5.0	80	75
			2nd Rec	11.4	78	5.0	85	80
			3rd Rec	12.2	96	10.0	50	80

S-Ca, serum calcium level; i-PTH, intact-parathyroid hormone; n.d., not described; HP, hyperplasia; AD, adenoma; ATP AD, atypical adenoma; Rec, recurrence; PTC, parathyroid carcinoma. [a]A nodule in the center of the adenoma of case 15 demonstrating a different staining pattern.

with diamino benzidine was considered positive. The ratio of positively stained tumor cells was determined for hTERT and nucleolin, and the expression was judged positive when >10% of the tumor cells were immunoreactive. The rate of Ki-67-positive nuclei was counted in the representative areas by counting at least a thousand cells and was expressed as a percentage of the positive nuclei (Ki-67 index; KI).

Statistical evaluation. The differences in each value were assessed using the Mann-Whitney's U test, and the difference

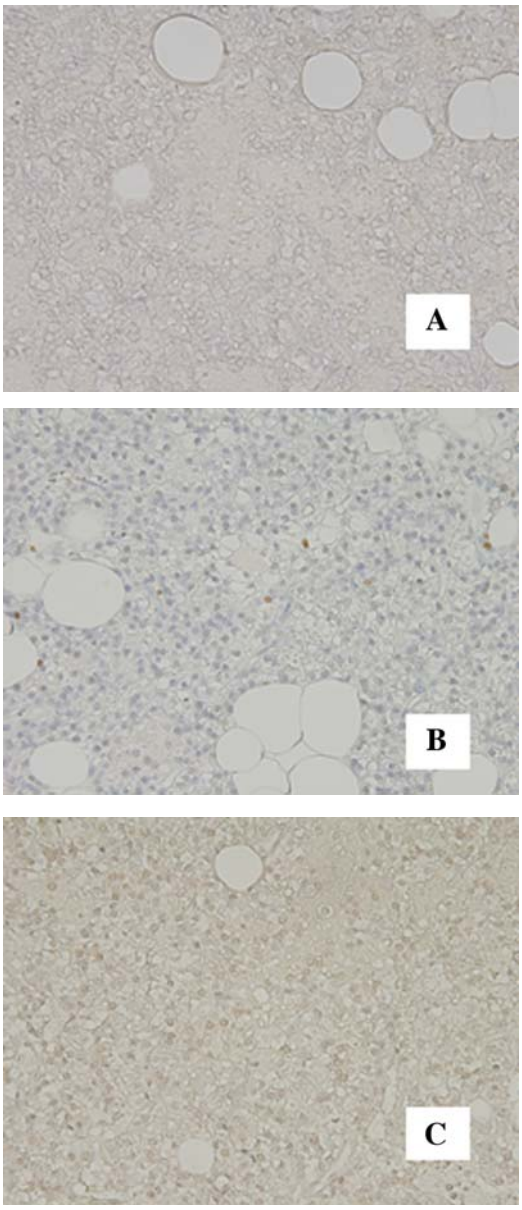

Figure 1. Results of the immunohistochemical staining of the normal parathyroid gland (case 3). MIB-1- positive cells are scarce (A). The normal glands are negative for hTERT staining (B). Nucleolin is positively stained in ~50% of the cells (C). (original magnification x400).

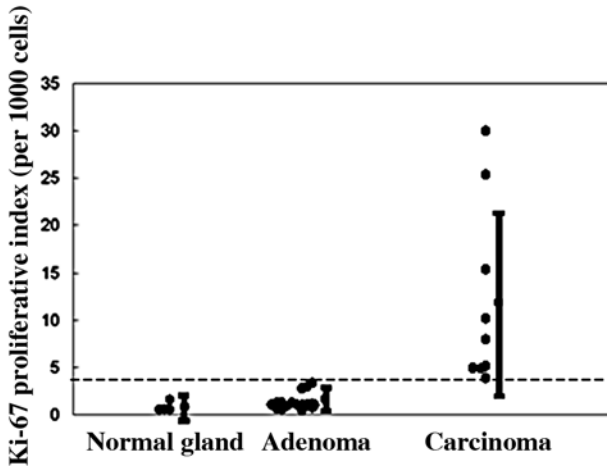

Figure 2. Ki-67 indices in normal parathyroid glands, typical adenomas, and carcinomas. Each circle indicates a case. There are significant differences in KI between the normal glands and carcinomas ($p<0.01$), or adenomas and carcinomas ($p<0.01$). However, no difference in KI was found between the normal glands and adenomas ($p=0.17$).

was considered to be significant when a value of $p<0.01$ was obtained.

Results

Immunoreactivity in the control cases. Fig. 1 shows the representative results found in the normal parathyroid glands. The cells in the normal glands rarely reacted with Ki-67; the KI ranged from 0.2 to 1.9, with a mean of 0.6. The normal parathyroid glands were universally negative for the hTERT antibody. There were no immunoreactive cells observed in the present study. In contrast, ~50% (range 45-55%) of the cells were positive for nucleolin homogeneously throughout the gland. In one case of secondary hyperparathyroidism, the KI was 1.4, and the cells were negative for hTERT. These findings were consistent with those found in the normal parathyroid glands. The immunoreactivity for nucleolin was positive in ~70% of the cells in the hyperplastic gland and was slightly more common than that found in the normal parathyroid gland.

Immunostaining for Ki-67 in parathyroid tumors. The KI ranged from 0.1 to 30 in the parathyroid tumors (Fig. 2). The index score was >4 in every parathyroid carcinoma (4-30; mean 11.9; Fig. 3A) including the recurrent lesions. In the parathyroid carcinomas, a strong nuclear staining was observed in every specimen examined. One case of parathyroid carcinoma (case 30; Fig. 4B) showed a heterogeneous distribution of Ki-67-positive cells. The KI was 4.0 in the area of the extracapsular invasion and 0.7 in the other areas. In the remaining carcinomas, Ki-67-positive cells were homogeneously distributed throughout the tumor. Similar patterns were also observed in the metastatic lung tumor in case 31. In two adenomas, KI scored >4. In one atypical adenoma (case 25; Fig. 5B), KI scored as high as 30. This patient had two recurrences in the local region, and both recurrent lesions had KIs as high as 22 and 10, respectively. This patient was alive and had a third local recurrence 36 months after the last surgical operation. Another case (case 15) demonstrated a heterogeneous distribution of Ki-67-positive cells. As shown in Fig. 6B, MIB-1-positive cells formed a clearly distinguishable nodule in the center of the tumor without a distinct capsule. There was a KI of 17 within the nodule, and 2.4 in the other part of the tumor. There was no morphological difference between tumor cells within or outside of the nodule. There were no cellular features of carcinoma evident within the nodule, such as mitosis or macronucleoli. This patient was also alive without any evidence of recurrent disease 50 months after surgery. Excluding these 2 cases, every adenoma scored a KI <4, ranging from 0.1 to 3.7 with a mean of 1.15. There was no difference in the KI between the normal glands and adenoma. However, a significant difference in KI was found between benign disease and carcinoma ($p<0.01$). Furthermore, no

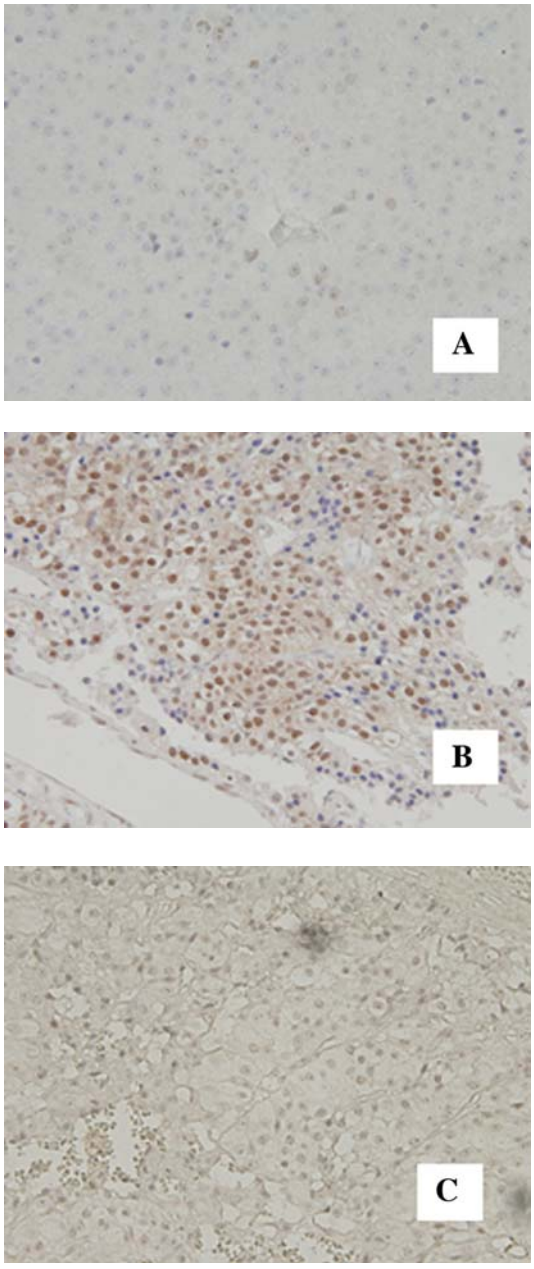

Figure 3. Representative results of immunochemistry in parathyroid carcinoma (case 31). Results from the primary lesion are shown. Immunoreactive cells to the anti-Ki-67 (5%) (A), anti-hTERT (80%) (B) and anti-nucleolin (75%) (C) antibodies are shown, respectively.

evident overlap was observed between them when a cut-off value of 4 was employed (Fig. 2).

Immunostaining against the hTERT antibody in parathyroid tumors. In the parathyroid carcinoma cases, every tumor was positive for hTERT. Approximately 50-95% of the cells were immunoreactive for the hTERT antibody (Fig. 3B). There was no difficulty in distinguishing hTERT-positive tumors from hTERT-negative tumors. In one patient with parathyroid carcinoma (case 30; Fig. 4C), a heterogeneous distribution of hTERT-positive cells was also observed, identical to that found upon Ki-67 staining. Approximately 50% of the cells were positive in the area of invasion, but cells were almost negative (<1%) in the other areas.

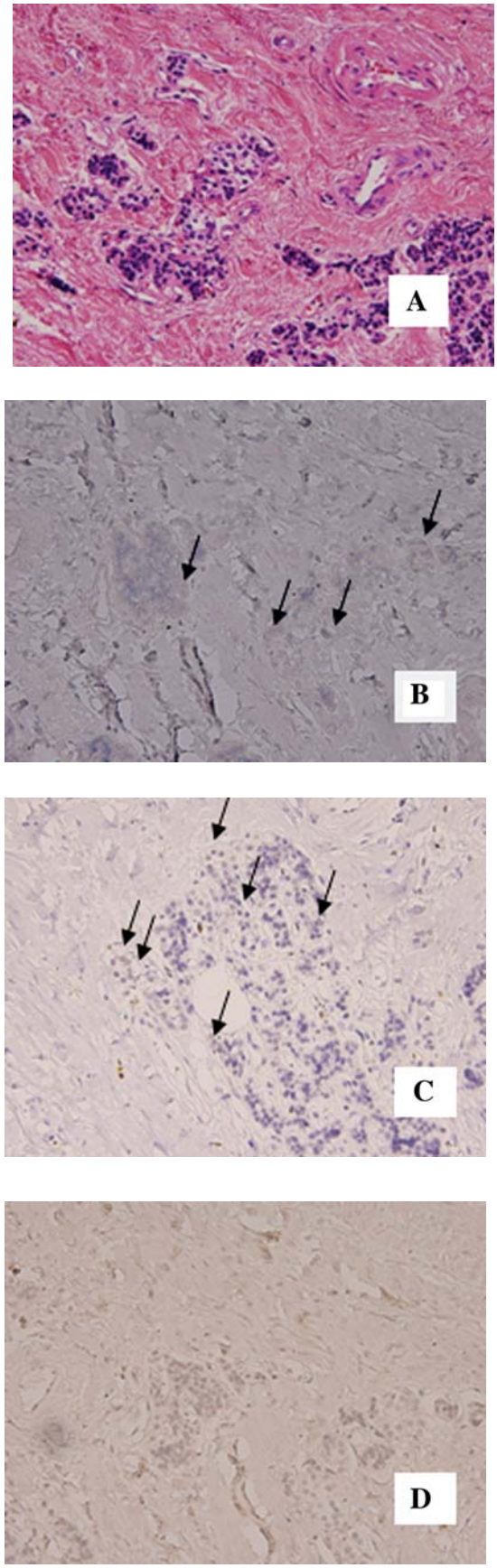

Figure 4. Results of case 30, a large tumor with a limited area of capsular penetration. Hematoxylin and eosin staining indicates histological evidence of cellular infiltration to adjacent fatty tissue (A). Immunoreactive cells to the anti-Ki-67 (4%) (arrows in B), anti-hTERT (50%) (arrows in C) and anti-nucleolin (60%) (D) antibodies are shown, respectively. Ki-67- and hTERT-positive cells are found only in the region of cellular infiltration.

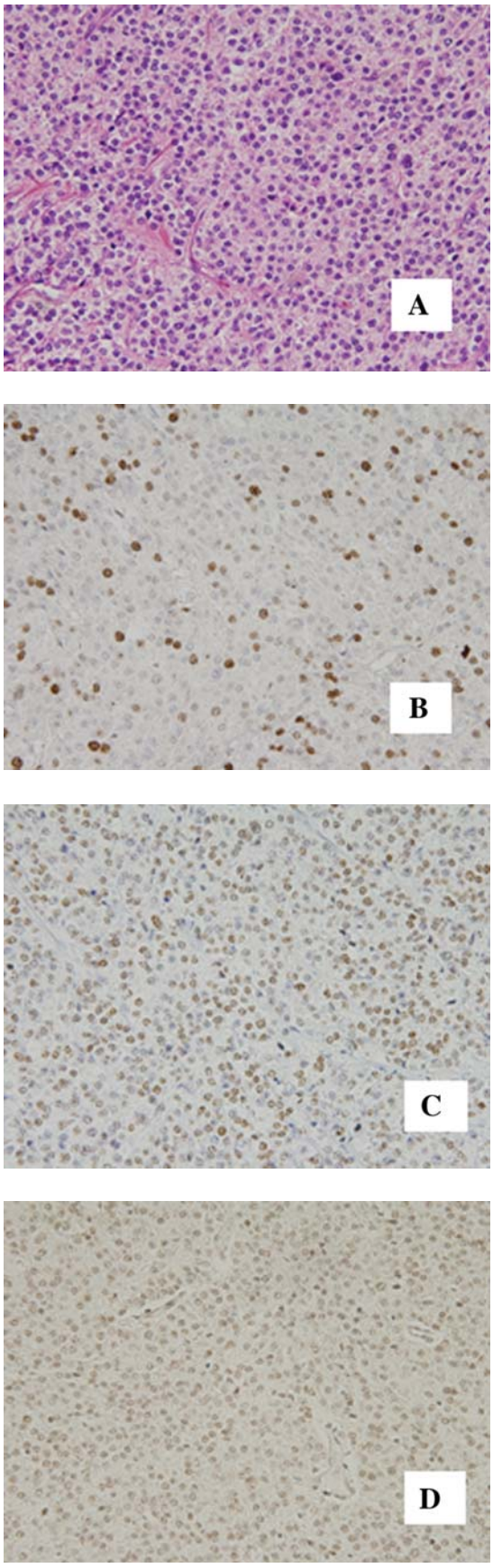

Figure 5. Results of case 25, an atypical adenoma. Results from the initial lesion are shown. Hematoxylin and eosin staining indicates no abnormal mitosis and cellular or histological features of carcinoma (A). Immunoreactive cells to the anti-Ki-67 (30%) (B), anti-hTERT (80%) (C) and anti-nucleolin (80%) (D) antibodies are shown, respectively. These staining patterns are similar to those of the cancer lesions.

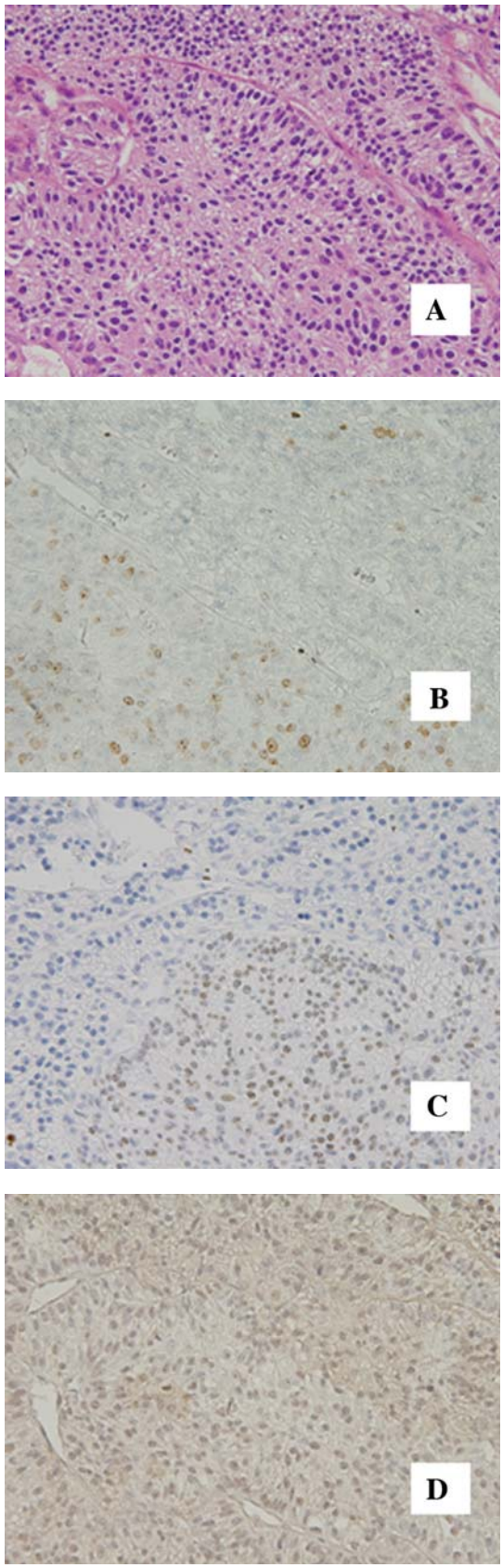

Figure 6. Results of an adenoma (case 15). Several nodules without a clear capsule are noted within the adenoma (A: H&E stain, original magnification x400). In a nodule located in the center of the adenoma, Ki-67 is highly positive (17%). However, the Ki-67-positive cells are found in a scattered manner in the other part of the surrounding adenoma (KI 2.4) (B). Strong nuclear staining against the hTERT antibody is limited within a nodule that shows a high Ki-67 index, but is not in the other part of the surrounding tumor (C).

The majority (90%, 18/20) of parathyroid adenomas gave negative results against the hTERT antibody. There were no immunoreactive cells found in the tumors of these 18 typical adenomas. Two exceptional hTERT-positive adenomas were the same as those that demonstrated a high KI, as described above. In an atypical adenoma (case 25), the positive cells were distributed homogeneously throughout the tumor (Fig. 5C). A similar staining pattern was also found in the recurrent tumor. The proportion of hTERT-positive cells was 80% in the initial tumor, and was 95 and 90% in the two recurrent tumors, respectively. The positive cell proportion was consistent with those of parathyroid carcinoma. Another case (case15) demonstrated an hTERT-positive-stained nodule within the tumor, and overlapped with the area of the Ki-67-positive nodule (Fig. 6C). The hTERT-positive cell population was 70% within the nodule and 0% in the other part of the tumor.

Immunostaining for nucleolin in parathyroid tumors. We detected abundant nuclear staining of nucleolin in the cells homogeneously in every specimen examined, including the normal parathyroid glands. A total of 45-55% (51.3±4.8) of the cells were positive for nucleolin in the normal parathyroid glands. Seventy to 80% (71.8±3.0) of the tumor cells were positive for nucleolin in the parathyroid adenomas. In the carcinomas, 60-80% (76.1±7.8) of the tumor cells were nucleolin-positive. There was no heterogeneity of nucleolin-positive cell distribution in the tumors, including a carcinoma (case 30; Fig. 4D), with heterogeneous Ki-67- and hTERT-positive cell distributions. An atypical adenoma (case 25; Fig. 5D) also demonstrated a homogeneous distribution of nucleolin-positive cells, with positive cell rates as high as 80%. In addition, there was no heterogeneity in the staining pattern in case 15, which displayed a hTERT-positive nodule within the tumor. A similar staining pattern was displayed within the Ki67- and hTERT-positive nodule and surrounding tumor cells with a positive cell rate of 80% (Fig. 6D).

Discussion

In order to obtain an accurate final diagnosis, previous studies have identified many important immunohistochemical characteristics of parathyroid carcinoma with reference to benign lesions. The apoptotic activity was elevated in carcinomas and was indicated by a negative expression of Bcl-2 (10-12). Several studies have indicated that parathyroid carcinoma is commonly p53-positive. The allelic loss and abnormal protein expression of p53 may imply that p53 plays a role in the pathogenesis of a subset of tumors (26). However, the abnormality of p53 gene expression was found to vary (0-100%) in carcinomas (10-12,16,27). In addition to the p53 abnormality, the loss of Rb (11,14) and p21 (16) expression, and the accumulation of cyclin D1 (16) and p27 (28) expression suggest a disintegration of the cell cycle control, although this was not always present (up to 55%). The accelerated cell proliferation in carcinoma was successfully evaluated by displaying the increase in PCNA- (15) or the Ki-67-positive cell population. Ki-67 is the most widely used molecular marker to estimate the activity of cellular proliferation, and typically more than 5% (4-6%) of cells stain positively in parathyroid carcinoma (5,17,29). Ki-67 is a human nuclear proliferation-associated antigen, expressed in all cells that are not in the G0-phase. Therefore, Ki-67 staining reflects the amount of proliferating cells more sensitively than that evaluated by the mitotic cell counts with a conventional histological examination. Stojadinovic *et al* conducted a study with multiple markers in addition to Ki-67. They found that the molecular phenotype, p27(+)Bcl-2(+) Ki-67(-)mdm2(+), was unique to nonmalignant tumors. However, at the same time, they concluded that multiple marker phenotypes were more complex in carcinomas (16). The most recent study of Fernandez-Ranvier *et al* also attempted to demonstrate the significance of investigating multiple markers, including the HRPT2 gene product, parafibromin, to distinguish parathyroid carcinoma (5). Although these intensive studies with multiple markers using a large panel of parathyroid carcinomas were conducted, no independent diagnostic markers have been identified to distinguish parathyroid carcinoma from benign conditions. In contrast, two recent studies have described candidates for specific markers of parathyroid carcinoma, although the number of cases studied was small. Thomopoulou *et al* showed that expression of Fhit, a protein involved in pro-apoptotic function and cell cycle control, was selectively lost in 2 carcinomas but not in benign tumors (30). Bergero *et al* demonstrated that Galectin-3 was expressed in all carcinomas that developed metastasis (18).

Consistent with these two markers, we found that the immunoreactivity against the hTERT antibody was unique to parathyroid carcinomas. In the previous study, we confirmed that expression of hTERT induces telomerase activation selectively in parathyroid carcinoma, but not in benign tumors (19,21). The case in which we showed telomerase activation and hTERT mRNA expression in our previous report was also included in this study (case 31). The immunoreactivity against hTERT antibody was strongly and homogeneously identified in this tumor. This is the first case of hTERT mRNA, hTERT protein and telomerase expression that has been demonstrated sequentially in the same parathyroid carcinoma specimen. Moreover, in the present study, we demonstrated immunoreactivity against the hTERT antibody in all of the pathologically confirmed parathyroid carcinomas, but not in the normal parathyroid gland, hyperplasia or typical adenoma cases. The hTERT-positive tumors also displayed high KI levels, thus suggesting the highly proliferative nature of the cells. These results suggest that immunostaining with this antibody (NCL-hTERT) may clearly and selectively distinguish parathyroid carcinoma from nonmalignant tumors.

Although various aspects remain controversial (31), Wu *et al* reported, while this manuscript was in preparation, that this widely used antibody (NCL-hTERT) recognized nucleolin, rather than telomerase (32). Nucleolin is a major multi-functional nucleolar protein that was shown to be involved in ribosome assembly, rRNA maturation, nucleocytoplasmic transport and cell proliferation (33,34). Therefore, in the present study we also attempted to identify nucleolin localization in the normal parathyroid glands and tumors by using another monoclonal antibody, and we found abundant expression of nucleolin in every sample examined including

the normal parathyroid gland. There were no significant differences in the population of nucleolin expressing cells between the normal gland, adenoma and carcinoma cases. Furthermore, the staining patterns of nucleolin detected by the anti-nucleolin antibody were completely different from that demonstrated using the NCL-hTERT antibody. Many studies have demonstrated that nucleolin plays critical roles in telomerase activation by binding to and shuttling hTERT (35-37). In other words, telomerase activation is regulated by the status of the nucleolin-hTERT complex. Many reports have described the significance of detecting hTERT expression by using the NCL-hTERT antibody (38-42). Moreover, Yan *et al* described that the protein expression detected by NCL-hTERT correlated significantly with telomerase activation and hTERT mRNA expression (39). Therefore, we continued to explore this avenue of research.

In the present study, a case of atypical adenoma (case 25) demonstrated homogeneous positive staining against the hTERT antibody in a primary and in two recurrent lesions. In this case, the sites of recurrences were limited to the neck, and no lymph node or distant metastasis was found. Parathyromatosis may be considered in this situation. Although parathyromatosis occurs predominantly in cases with familial or secondary hyperparathyroidism after initial surgery, it may be rare in cases with sporadic primary hyperparathyroidism, as in our case (5). This case showed some clinical features that led us to suspect parathyroid cancer at the initial presentation, as described. Furthermore, the KI was as high as 30 and was in the range of parathyroid carcinoma. Moreover, Fernandez-Ranvier *et al* reported that most cases of parathyromatosis are negative for Ki-67 (5). We concluded that this atypical case was not a parathyromatosis, but was a parathyroid carcinoma both clinically and biologically.

Heterogeneity was detected in immunostaining against the hTERT antibody in 2 cases. One case was pathologically diagnosed as carcinoma because of the evidence of invasion to adjacent fatty tissue. It was observed that Ki-67- and hTERT-positive cells were limited in the area of invasive growth, but not in the other parts of the tumor. Heterogenic hTERT immunoreactivity was reported to be found frequently in human cancerous tissues (39). The authors reported that hTERT-negative cells were apoptotic. Yet, it appeared that the cells in our large tumor were not apoptotic. Instead, our findings indicated that only cells with telomerase activity acquired a potential for invasive growth. Another case was an adenoma with a Ki-67- and hTERT-positive nodule in the center of the tumor. This lesion consisted of several nodules, but we did not find any high KI or hTERT-positive cells in the other nodules. These findings suggest that highly proliferating immortal cells may have existed in parts of the benign tumor. The carcinogenic pathway of the parathyroid carcinoma remains to be elucidated. However, our findings indicate that potentially malignant cells may exist within or in part of the benign tumor. In contrast, in case 19, although a marked elevation of S-Ca (13.6 mg/dl) and i-PTH (750 pg/ml) was noted in a large parathyroid adenoma (5800 mg, 28 mm), no positive staining was recognized, and the patient had a full recovery in a short period after surgery. These results indicate that a high KI and high hTERT-positive cell ratio are not markers for the severity of disease, but are indicators of the cellular potential for malignancy. Although hTERT expression is essential for telomerase activation, the mechanism that promotes hTERT expression remains to be elucidated. Several upstream factors which incorporate the hTERT promoter have been identified, including cellular transcriptional activators as well as repressors, many of which comprise tumor suppressor gene products such as p53, WT1 and Menin (43). In addition, epigenetic regulation and several cofactors that influence hTERT expression have been investigated, such as nucleolin. Notably, some of these abnormalities have also been found in parathyroid carcinomas (26,44,45). We propose that further exploration of the upstream regulator of hTERT may be a possible target to elucidate the carcinogenic pathway of parathyroid carcinoma.

In conclusion, we herein demonstrated that immunostaining with an anti-hTERT antigen individually may distinguish parathyroid carcinoma from benign tumors more selectively than Ki-67. hTERT-positive cells displayed a highly proliferative nature and may have malignant potential such as invasive growth or recurrence. This marker therefore appears to be highly useful for identifying parathyroid carcinoma. Further studies should be conducted with respect to hTERT expression and its regulation to clarify and confirm these findings.

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