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Significant Correlation of Telomerase Activity in Thyroid Papillary Carcinomas with Cell Differentiation, Proliferation and Extrathyroidal Extension

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Telomerase activity was examined by telomeric repeat amplification protocol assay in thyroid disease states, including adenomas and carcinomas, and correlated with clinicopathological features. Of a total of 26 papillary carcinomas, 16 cases (61.5%) were positive, with the poorly differentiated subtype being predominant ($P<0.05$). A significantly more shortened terminal restriction fragment length ($P<0.05$), higher incidence of extrathyroidal extension ($P<0.001$), and more elevated Ki-67 labeling indices ($P<0.002$) were also found in telomerase-positive than in telomerase-negative papillary carcinomas. Of four follicular carcinomas, 3 cases (75.0%) were positive. Positive telomerase activity in follicular adenomas (9/23 cases, 39.1%) and lymphocytic thyroiditis (12/22 cases, 54.5%) appeared to be mainly caused by infiltrating lymphocytes. However, three cases of atypical adenoma with relatively increased Ki-67 labeling indices were positive, suggesting a possibility of malignant potential. The good correlations with extrathyroidal invasiveness, Ki-67 labeling indices and poor differentiation of papillary carcinomas, established by multivariate analysis, suggest that this parameter might have potential application in the estimation of tumor progession and prognosis, and in clinical management.

Key words: Telomerase — Papillary carcinoma — Ki-67 — Differentiation — Thyroid carcinoma

The activity of telomerase, an enzyme which is prerequisite for continued proliferation and immortality of cells, has been shown to be increased in carcinomas of various sites, including the stomach,[1)] colorectum,[2)] liver,[3)] kidney,[4)] lung,[5)] brain,[6)] prostate,[7)] breast,[8,9)] and hematopoietic system.[10)] However, data on changes occurring in thyroid diseases are limited. Umbricht *et al.* reported that telomerase activity is a good marker to distinguish follicular thyroid adenoma from carcinoma.[11)] Furthermore, the relations of telomerase activity with papillary carcinoma and clinicopathological features have yet to be well documented. Accordingly, the present study was conducted to ascertain whether telomerase activity is linked to thyroid disease, with particular attention to thyroid adenomas and carcinomas. While many papillary carcinomas in the thyroid are generally associated with a relatively good prognosis, some poorly differentiated lesions can cause rapid mortality.[12–16)] A comparison of clinicopathological features and the level of proliferation, assessed by Ki-67 immunohistochemistry, was therefore included in the present study.

MATERIALS AND METHODS

Patients and histological diagnosis Fifty-eight cases of surgically resected thyroid glands were selected from the records of Kitasato University Hospital. Histological observations were performed on hematoxylin-eosin-stained 4-μm-thick sections of 10% formalin-fixed and paraffin-embedded tissues. The tumors were diagnosed and classified using WHO criteria.[17)] Papillary carcinomas were divided into well and poorly differentiated subtypes on the basis of histological features, including solid structure, trabecular pattern and scirrhous invasion, according to Sakamoto *et al.*[15)] Aggressive and non-aggressive subtypes were distinguished by applying Damiani's criteria.[16)] Lymphocytic thyroiditis was histologically identified and classified after Williams and Doniach[18)] by counting foci of infiltration per standard representative section (2 cm^2) (i.e., grade 0 [none], <1 focus; grade 1 [slight], 2–8 foci; grade 2 [moderate], 9–40 foci; grade 3 [severe], >40 foci; grade 4 [very severe], more than half of the glandular parenchyma replaced by lymphocytes). A 'focus' was defined as an aggregate of 50 or more lymphocytes.

Cellularity and immunohistochemistry As a measure of cellularity, epithelial cells in 4-μm-thick sections stained with hematoxylin/eosin were counted under a light microscope. The average number of epithelial cells per 0.0625 mm^2 was determined for each case. Immunohistochemistry for Ki-67 labeling and p53 accumulation was performed by a combination of the ordinary streptavidin-biotin peroxidase complex method and microwave oven heating. Antibodies used were rabbit anti-human Ki-67

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(×150 diluted, Dako, Copenhagen, Denmark) and Do-7 monoclonal for p53 protein (×500 diluted, Novocastra Laboratories, Newcastle, UK). Ki-67 labeling indices were calculated as percentage values by counting more than 1,000 nuclei of cancer cells in randomly selected areas of sections. Positivity for p53 protein in cancer cells was defined as diffuse (30%<), focal (10–30%), scattered (<10%) and negative (0%).

Telomerase assay Fresh tumor and non-tumor tissues were immediately frozen in dry ice-isopentane and stored at −80°C until subjected to the telomeric repeat amplification protocol (TRAP) assay, following the method described by Kim *et al.*[7,19] Briefly, lysates were prepared by powdering tissues frozen in liquid nitrogen, followed by homogenization in 200 μl of ice-cold lysis buffer [10 m*M* Tris-HCl (pH 7.5), 1 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1 m*M* phenylmethylsulfonyl fluoride, 5 m*M* β-mercaptoethanol, 0.5% CHAPS-10% glycerol] and incubation for 30 min on ice. The lysates were then centrifuged at 10,000*g* for 20 min at 4°C and the supernatants and precipitates were rapidly frozen separately and stored at −80°C. The protein concentration of each supernatant was determined by the Bradford assay (Bio-Rad, Hercules, CA).

Assay tubes were prepared by sequestering 0.1 μg of CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) under a wax barrier (Ampliwax; Perkin-Elmer Cetus, Foster City, CA). Supernatant samples equivalent to 6 μg of protein were assayed in 50 μl of reaction mixture (20 m*M* Tris-HCl [pH 8.3], 1.5 m*M* $MgCl_2$, 63 m*M* KCl, 0.005% Tween 20, 1 m*M* EGTA, 50 μ*M* dNTPs, 150 kBq of [α-^{32}P]dCTP, 0.1 μg of TS primer [5′-AATCCG-TCGAGCAGAGTT-3′], 1 μg of T4gene32 protein [Boehringer Mannheim, Germany] and 2 units of Taq DNA polymerase [GIBCO-BRL, Gaithersburg, MD]). After a 30 min incubation at 23°C for telomerase-mediated extension of the TS primer, the reaction mixture was heated at 90°C for 90 s and then subjected to 31 polymerase chain reaction (PCR) cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s. The PCR products were electrophoresed on 10% polyacrylamide gels.

Telomere restriction fragment (TRF) length by Southern blotting[20] Genomic DNA was isolated from the frozen precipitate by incubation with 400 ml of lysis buffer (10 m*M* Tris-HCl [pH 7.4], 50 m*M* EDTA, 50 m*M* NaCl, 2.7% sucrose) containing 0.3 mg of proteinase K (Merck, Darmstadt, Germany) and 0.8% sodium dodecyl sulfate (SDS) at 37°C for one night. Then, the DNA was extracted twice with phenol and chloroform, and precipitated with isopropanol. The DNA precipitate was dissolved in TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0). Fifteen micrograms of the DNA was digested with *Hin*f I, and 7 mg of the digested DNA was electrophoresed on 0.8% agarose gels and transferred to nylon membranes (Hybond-N+) (Amersham, Buckinghamshire, England, UK). The membranes were prehybridized at 65°C in the hybridization solution containing 6× SSPE, 1% SDS, 100 μg/ml salmon sperm DNA, and hybridized overnight at 50°C with a ^{32}P-end-labeled (TTAGGG)4 telomeric probe. The filters were washed once in 2×SSC at room temperature, then twice in 6× SSC-0.1% SDS at 50°C, and autoradiographed. The length of TRF was estimated at the peak of the hybridization signal.

Statistical analysis Differences in incidence data, Ki-67 labeling indices and TRF length between groups positive and negative for telomerase activity were statistically analyzed by the χ^2-test and nonparametric Mann-Whitney U test, respectively. Multivariate analysis[21] of various factors with telomerase activity was performed with the Statistical Analysis System.[22]

RESULTS

Telomerase activity was detected in 16 out of 26 papillary carcinomas (61.5%), 3 of 4 follicular carcinomas (75.0%), and 2 of 2 anaplastic carcinomas (100%) (Table I, Fig. 1). TRF length was significantly more shortened ($P<0.05$) in telomerase-positive than in telomerase-negative papillary carcinomas, as follows. TRF length: telomerase-positive ($n=8$), 7.5±2.5 kb (mean± SD); telomerase-negative ($n=8$), 10.0±1.7. Lymphocyte infiltration (grades 2 and 3) was found within tumors in 2 of the telomerase-positive papillary carcinomas. Follicular adenomas also showed telomerase activity in 9 of 23 cases, 6 of these exhibiting lymphoid cell infiltration and the other 3 being atypical. Atypical adenomas were diagnosed on the basis of architectural or cytological atypicalities, according to WHO criteria.[17] Ki-67 labeling indices were significantly higher ($P<0.005$) in telomerase-positive follicular adenomas [0.53± 0.25% (mean±SD)] than in -negative adenomas (0.28 ±0.20%), although histological observation showed no difference between them (hematoxylin/eosin staining). Ki-67 labeling indices of atypical adenomas were 0.77± 0.17%, higher than those for telomerase-positive follicular adenomas and telomerase-negative papillary carcinomas. With regard to non-tumor thyroid parenchyma, telomerase activity was observed in 12 out of 22 cases of lymphocytic thyroiditis, while all of the adenomatous goiter and normal thyroid cases were negative. Moderate to severe lymphoid cell infiltration (grades 2, 3 and 4) was evident in 12 of 14 cases of telomerase-positive thyroiditis (85.7%).

Results of clinicopathological comparison of telomerase-positive and -negative papillary carcinoma groups are summarized in Table II. No significant differences were observed with regard to age and the male/female ratio, although the patients in the telomerase-positive tended to

Table I. Telomerase Activity in Thyroid Diseases

Thyroid disease	Number of cases	Telomerase activity		
		Positive	Negative	%
Papillary carcinoma	26	16	10	61.5
Follicular carcinoma	4	3	1	75.0
Anaplastic carcinoma	2	2	0	100.0
Follicular adenoma	23	9	14	39.1
without lymphocyte infiltration	14	0	14	0.0
with lymphocyte infiltration (grades 3–4)	6	6	0	100.0
atypical adenoma	3	3	0	100.0
Lymphocytic thyroiditis[a)]	22	12	10	54.5
grade 1	8	0	8	0.0
grade 2	3	2	1	66.7
grade 3 or 4	11	10	1	90.9
Adenomatous goiter	3	0	3	0.0
Normal	26	0	26	0.0

a) Grade 1, foci of lymphocyte infiltration 2–8/2 cm^2; 2, foci of lymphocyte infiltration 9–40/2 cm^2; 3, foci of lymphocyte infiltration 41– /2 cm^2; 4, more than half the parenchyma replaced by lymphocyte infiltration.

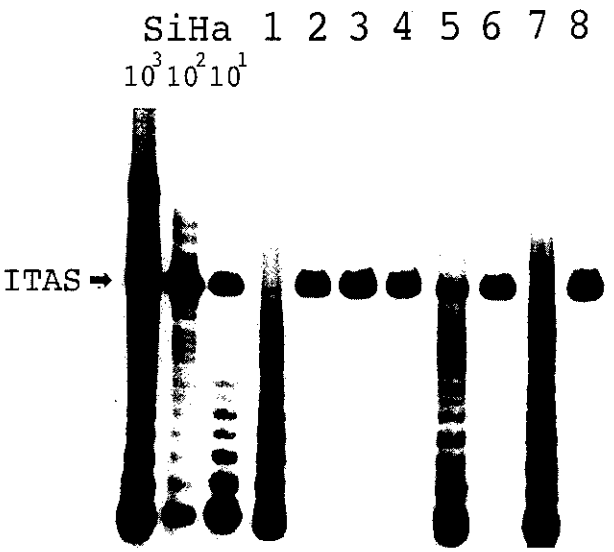

Fig. 1. Telomerase activity in thyroid diseases measured by the TRAP assay. Tissue extracts containing 6 μg of protein and the extracts from 10^3, 10^2 and 10^1 SiHa cells (as controls) were analyzed by means of the TRAP assay with an internal telomerase standard (ITAS)(13). Line 1, papillary carcinoma, poorly differentiated type, with positive telomerase activity; line 2, normal thyroid tissue with negative telomerase activity from the same patient as line 1; line 3, papillary carcinoma, well differentiated type, with negative telomerase activity; line 4, normal thyroid tissue with negative telomerase activity from the same patient as line 3; line 5, follicular adenoma (lymphocyte infiltration within adenoma, grade 3) with positive telomerase activity; line 6, follicular adenoma (no lymphocyte infiltration within adenoma) with negative telomerase activity; line 7, lymphocytic thyroiditis, grade 3, with positive telomerase activity; line 8, lymphocytic thyroiditis, grade 1, with negative telomerase activity.

Table II. Clinicopathological Comparison between Telomerase-positive and -negative Papillary Carcinoma

	Telomerase activity	
	Positive ($n=16$)	Negative ($n=10$)
Patients		
Age (years)	55.3±17.8	48.1±10.4
Range (years)	9–80	32–70
Male : Female	2 : 14	2 : 8
Papillary carcinoma		
Well : Poorly differentiated subtype	6 : 10[a)]	9 : 1
Aggressive : Non-aggressive	3 : 13	0 : 10
Size (cm)	2.8±1.3	2.2±0.8
Cellularity (/0.0625 mm^2)	273.1±57.7	270.5±64.4
Extrathyroidal extension (%)	13 (81.3)[b)]	2 (20.0)
Lymph node metastasis (%)	13/15 (86.7)[c)]	9/10 (90.0)
p53 protein accumulation[d)]		
Negative	3	0
Sporadic	10	9
Focal	2	1
Diffuse	1	0

a) $P<0.05$ (χ^2 test).
b) $P<0.001$ (χ^2 test).
c) Data not available for one case.
d) Negative: no positive cells. Sporadic: sporadic distribution of positive cells (<10%). Focal: focal distribution of positive cells (10–30%). Diffuse: diffuse distribution of positive cells (>30%).

be older. Tumor size, cellularity, presence of regional lymph node metastasis and p53 accumulation also did not show significant differences between the two groups. However, poorly differentiated tumors significantly pre-

dominated ($P<0.05$) in the telomerase-positive group, which also exhibited a significantly higher incidence of extrathyroidal extension ($P<0.001$) and greater Ki-67 labeling indices ($P<0.002$) ($1.47\pm84\%/0.41\pm0.26\%$) (Fig. 2). Multivariate analysis of papillary carcinoma revealed close associations of extrathyroidal extension (invasiveness) ($P=0.0009$), Ki-67 labeling indices ($P=0.0011$) and poor differentiation ($P=0.0124$) with telomerase activity, and of telomerase activity ($P=0.0009$), poor differentiation ($P=0.0124$) and Ki-67 labeling indices ($P=0.0145$) with extrathyroidal extension.

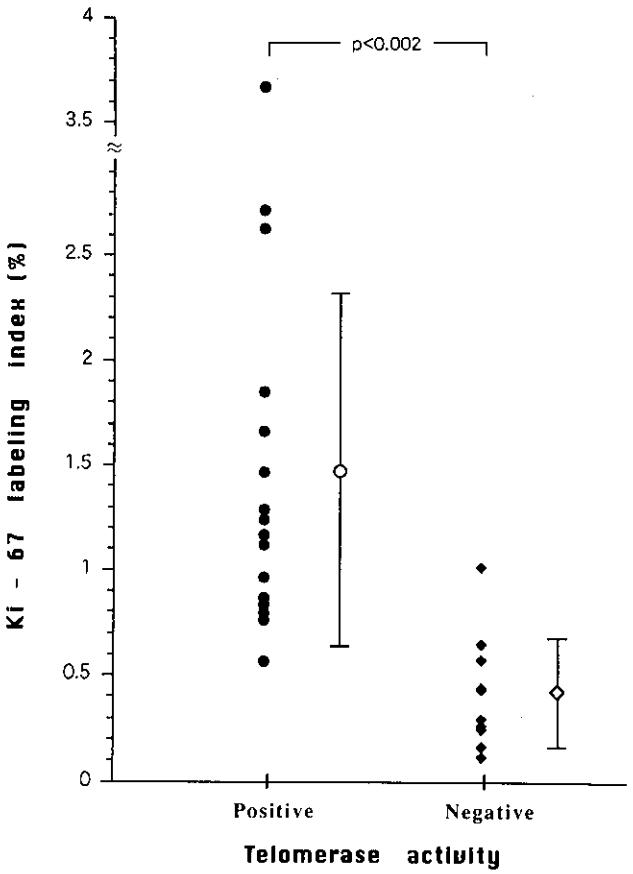

Fig. 2. Comparison of Ki-67 labeling indices between telomerase-positive and -negative papillary carcinomas. Ki-67-positive nuclei were calculated as percentage values after counting in a histologic section immunohistochemically stained with anti-Ki-67 mononuclear antibody. Note high Ki-67 labeling index in telomerase-positive papillary carcinoma, showing increased proliferation activity.

DISCUSSION

Recent studies on telomerase activity in germline cells and cancers using the sensitive TRAP assay have demonstrated that this enzyme, thought to be involved in maintaining telomere length and stability, is associated with immortalization and continuous growth of cells.[2,23] In addition, increased telomerase activity has been found in most, if not all, types of cancers.[1-10] It has been associated with acquisition of malignancy, since it is detectable in colorectal carcinomas, but not in adenomas,[2] and advanced, but not early stage gastric cancers.[1] This is supported by the extremely high levels in acute myelogenous leukemia, in comparison with chronic myelogenous leukemia.[10,24]

Thyroid tumors, including papillary and follicular carcinomas, tend to demonstrate slow proliferation, except for anaplastic forms.[25] However, clinically, some papillary and even follicular carcinomas show a poor prognosis. The present finding of telomerase activity in 61.5% of papillary and 75.0% of follicular malignancies, with a clear link to dedifferentiation (poorly differentiated subtype),[15] and cellular proliferation activity indicated by Ki-67 labeling[26] and extrathyroidal extension is therefore of interest. A positive association between telomerase activity and cell proliferation in thyroid papillary carcinoma was shown in the present study, as had been noted in breast cancer.[9] Further, normal human lymphocytes and hematopoietic progenitor cells strongly express telomerase after mitogenic stimulation.[27] According to a recent study,[28] the endometrium in the proliferative phase expresses telomerase activity, suggesting in turn that telomerase is a regulated enzyme linked to cellular proliferation. Our findings might support that idea.

Poorly differentiated papillary carcinomas have a poorer prognosis than their well-differentiated counterparts.[15] In our study, it would have been difficult to see any difference in prognosis between telomerase-positive and -negative papillary carcinoma groups, since our materials were collected during 1990–1996. Only one patient with papillary carcinoma in this study died (of another disease) in this short period. The multivariate analysis revealed that the association of extrathyroidal invasiveness was the closest with telomerase activity. This might suggest that telomerase activity has an intimate relation with tumor progression. The lack of telomerase activity in some such lesions and the absence of any significant correlation with tumor size, cellularity, regional lymph node metastasis, cytological appearance[16] and p53 protein accumulation suggest that telomerase activation is not a critical factor for cancer development. In addition to the above-described data, telomerase-negative papillary carcinomas showed significantly longer TRF length than telomerase-positive papillary carcinomas in the present study. Another type of genetic alteration is presumably therefore responsible for allowing continued proliferation, particularly in cancers of endocrine organs whose growth is very slow.[25] The finding that telomerase

activity regresses during differentiation of maturation-sensitive human embryonal carcinoma (NTERA-2) cell lines,[29] clearly points to an inverse relationship with the degree of differentiation, as well as potential activity reversibility.

Although the stepwise participation of *p53* gene mutation and overexpression during dedifferentiation of thyroid carcinomas had been suggested,[30, 31] there was no significant difference of p53 accumulation between telomerase-positive and -negative papillary carcinomas, which had a good correlation with cancer cell differentiation, in the present work. This result might suggest that telomerase activation has no intimate relationship with p53 abnormality. However, this point remains to be clarified by means of *p53* gene mutation analysis.

The facts that telomerase was present in adenomas with lymphocytic cell infiltration and in moderate and high grade lymphocytic thyroiditis, in which activated lymphocytes infiltrate as an autoimmune phenomenon,[32] are in line with the literature on hematopoietic cells.[23, 24, 27] Activated human T and B cells show increased activity of telomerase, although it is detectable at low levels in normal hematopoietic cells.[27] In this regard the association between chronic lymphocytic thyroiditis and carcinoma development may be important,[32] although this point also needs clarification. In addition to lymphocytic cell infiltration, telomerase-positive follicular adenomas showed increased Ki-67 labeling indices. Further, atypical adenomas without lymphocytic cell infiltration also showed a tendency for increased Ki-67 labeling indices and telomerase positivity. Accordingly, atypical adenomas and some follicular adenomas may have a malignant potential, although the WHO's histological criteria fail to identify malignancy, which depends on vascular invasion or capsular penetration by tumor cells. Accordingly, telomerase activity may be a good marker for differentiation of follicular carcinoma from other follicular neoplasms, although lymphocytic infiltration may cause false-positive results.

To conclude, the present data point to a special relationship between telomerase activity and cancer cell differentiation, proliferation and local invasion in thyroid tumors.

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