ANTICANCER RESEARCH *25*: 509-514 (2005)

Telomerase Activity in Thyroid Neoplasms Evaluated by the Expression of Human Telomerase Reverse Transcriptase (hTERT)

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Abstract. *Background: The recent availability of a monoclonal antibody against human telomerase reverse transcriptase (hTERT) has enabled us to investigate the telomerase activity of tissue specimens more easily than by classical TRAP assay. In this study, we studied teromerase activity in thyroid tumors using this antibody. Materials and Methods: We immunohistochemically investigated hTERT expression in 166 thyroid neoplasms. Results: Normal follicular cells did not express hTERT. In papillary carcinoma, high hTERT expression was observed in 34.5% of cases and it was directly linked to stage and the presence of lymph node metastasis. In follicular carcinoma, 39.2% were classified as the high hTERT expression group, showing a significantly higher incidence than that in follicular adenoma, 9.8%. In anaplastic carcinoma, 73.7% were regarded as the high hTERT expression group, which was larger than the percentages in papillary and follicular carcinomas. We investigated the diagnostic usefulness of hTERT measurement for discriminating follicular carcinoma from adenoma. Its specificity and positive predictive value were high at 90.2% and 83.3%, respectively, while the sensitivity and negative predictive value were low. Conclusion: These results suggest that: i) telomerase activity contributes to anaplastic transformation of differentiated carcinoma and ii) hTERT measurement may contribute to diagnosing follicular carcinoma, but whether it can be applied to preoperative diagnosis by fine-needle aspiration biopsy specimens remains unclear.*

Thyroid carcinoma is one of the most common malignancies originating from the endocrine organs. There are two prominent histological types of thyroid carcinoma originating from follicular cells: papillary carcinoma and follicular carcinoma, the latter being comparably rare (1). Follicular carcinoma is thought to arise from preexisting follicular adenoma, although this hypothesis has not yet been confirmed. It is associated with iodine deficiency, suggesting that this type of tumor can develop from chronically proliferating glands. On the other hand, the precursor lesion of papillary carcinoma has still to be identified, and risk factors for this carcinoma remain unknown except for a history of radiation exposure in childhood (2). Generally, the biological character of such carcinomas is mild but, when tumor cells dedifferentiate and become anaplastic (undifferentiated), they display extremely rapid growth and patients usually have a dire prognosis (3).

For thyroid carcinoma, two important and interesting points remain to be elucidated. One is whether and how follicular carcinoma can be diagnosed by preoperative examinations. Indeed, it is difficult to distinguish follicular carcinoma from benign nodules, including follicular adenoma, on the basis of ultrasonographic findings and fine-needle aspiration biopsy (FNAB). Another is the mechanism triggering anaplastic transformation of papillary and follicular carcinomas. Although some differences between anaplastic and differentiated carcinomas such as p53 gene alterations are known (4), knowledge regarding this point is still lacking.

For the first step of this study, it is important to investigate differences in characteristics between follicular carcinoma and adenoma and between anaplastic and differentiated carcinomas from various aspects. Telomerase activity is one of the important factors in evaluating the biological aggressiveness of human neoplasms, because telomerase is an enzyme required for continued cell proliferation and immortality of cells, which is an important characteristic of tumor cells (5, 6). In some malignancies, its activity is known to be elevated (7-12). In thyroid carcinoma, telomerase activity has been investigated, but

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Key Words: hTERT, follicular tumor, immunohistochemistry.

studies involving a large series have not yet been performed (13-21). This may be because the technique for evaluating telomerase activity, the TRAP assay, is difficult and complicated.

Previous studies demonstrated that human telomerase reverse transcriptase (hTERT) plays a role in the reconstitution of telomerase activity, and the measurement of hTERT expression level reflects telomerase activity (22-24). A few groups investigated hTERT mRNA expression by RT-PCR or *in situ* hybridization in thyroid neoplasms (25-27). Furthermore, the recent availability of anti-hTERT protein monoclonal antibody applicable to immunohistochemistry has enabled us to investigate the telomerase activity of a large number of cases much more easily than by TRAP assay and measuring hTERT mRNA expression level. Thus, we investigated whether telomerase activity is significantly related to the malignancy of follicular tumor and to anaplastic transformation of thyroid carcinoma.

Materials and Methods

Tissue specimens. Tissue specimens of thyroid neoplasms were obtained from 166 patients who underwent surgery in the Department of Surgery, Kuma Hospital, Japan. This project was approved by the ethical committee of the hospital and informed consent was obtained from the participating patients. These consisted of 19 anaplastic (undifferentiated) carcinomas, 55 papillary carcinomas, 51 follicular carcinomas (14 widely invasive and 37 minimally invasive) and 41 follicular adenomas. For immunohistochemical study, tissues were fixed with 10% formalin and paraffin-embedded.

Antibody. The mouse monoclonal antibody against hTERT was purchased from Novocastra (Newcastle, UK) . It was applied at the dilution of 1:50.

Immunohistochemistry. Tissue sections 4-μm-thick were dewaxed and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15 min. For antigen retrieval, sections were immersed in 0.03 mol/L citrate buffer and incubated at 95˚C for 40 min. After rinsing in phosphate-buffered saline pH 7.2 (PBS), 10% bovine serum (Wako, Osaka, Japan) was applied for 20 min to block nonspecific reactions. Sections were then incubated with a primary antibody at 4˚C overnight. After rinsing in PBS, the sections were treated with peroxidase-labelled anti-mouse and anti-rabbit immunoglobulins (Nichirei, Tokyo, Japan) for 30 min. The peroxidase reaction was visualized by incubating the sections with 0.02% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer with 0.01% hydrogen peroxide (Nichirei). The sections were counterstained with hematoxylin. Sections for the negative control were prepared using mouse immunoglobulins instead of the primary antibody.

Immunohistochemical evaluation. We regarded cells as positive for hTERT when immunoreactivity was clearly observed in their nuclei. We graded positivity by the percentage of positive cell as follows: (+++), more than 50 % positive cells; (++), 25 to 49% positive cells; (+), 5 to 24% positive cells; (-) less than 5% positive cells. Cases graded as (++) or (+++) were assigned to the high group, while those graded as (+) or (-) were assigned to the low group.

Statistical analyses. Fisher's exact probability test was adopted to examine the relationship between variables. A p value less than 0.05 was considered significant.

Results

Follicular cells in normal epithelia adjacent to tumor nests only occasionally expressed hTERT (Figure 1A). In infiltrating lymphocytes, hTERT expression was often observed (not shown).

Then, we investigated hTERT expression in various thyroid neoplasms. Of the 55 cases of papillary carcinoma, 19 (34.5%) were classified as the high hTERT expression group (Figure 1B). Table I shows the relationship between hTERT expression and clinicopathological features of papillary carcinoma. The hTERT expression level was significantly higher in cases with advanced stage (p=0.0410) and those showing lymph node metastasis (p=0.0410), but was not linked to other parameters such as tumor size, extrathyroidal invasion (Table I), or to gender, age and multiple tumor formation (not shown).

The hTERT expression level of other thyroid tumors are summarized in Table II. In follicular carcinoma, hTERT expression was high in 20 out of 51 cases (39.2%), including 6 out of 14 cases (42.8%) showing the widely invasive type (Figure 1C). There was no significant difference observed in the incidence of hTERT expression between widely and minimally invasive follicular carcinomas. However, hTERT was observed in only 4 out of 41 follicular adenomas (9.8%) (Figure 1D), and its incidence was significantly lower than that in follicular carcinoma (p=0.0017).

In carcinoma after anaplastic transformation, hTERT expression was frequently observed, and 14 of the 19 cases (73.7%) were assigned to the high group (Figure 1E) (Table II). The incidence was significantly higher than that in papillary carcinoma (p=0.0064) and follicular carcinoma (p=0.0151).

Lastly, we investigated the diagnostic values of hTERT expression for discriminating follicular carcinoma from adenoma. Its specificity and positive predictive value were high, 90.2% and 83.3%, respectively, whereas its sensitivity and negative predictive value were low. The diagnostic accuracy was 62.0% (Table III).

Discussion

To date, several studies have been performed for telomerase activity in thyroid tumors using the most widely available technique, the TRAP assay (13-21). However, as described above, its technical difficulty has prevented

Table I. *Relationships between hTERT expression and clinicopathological features of papillary carcinoma.*

	High	Low	Total
UICC stage			
IV	0	1	1
III	8	12	20
II	8	7	15
I	3	16	19
		$p=0.0410$ (I *vs* II, III, IV)	
Tumor size			
≥ 4.0cm	6	7	13
< 4.0cm, > 1.0cm	10	17	27
≤ 1.0cm	3	12	15
		Not significant	
Lymph node metastasis			
Positive	16	20	36
Negative	3	16	19
		$p=0.0410$	
Extrathyroidal invasion			
Positive	5	8	13
Negative	14	28	42
		Not significant	
Total	19	36	55

Table II. *hTERT expression in thyroid neoplasms.*

	High	Low	Total
Anaplastic carcinoma	14	5	19
Papillary carcinoma	19	36	55
Follicular carcinoma	20	31	51
(widely invasive	6	8	14)
(minimally invasive	14	23	37)
Follicular adenoma	4	37	41

$p=0.0017$ (follicular carcinoma *vs* follicular adenoma)
$p=0.0064$ (anaplastic carcinoma *vs* papillary carcinoma)
$p=0.0151$ (anaplastic carcinoma *vs* follicular carcinoma)

Table III. *Discrimination between follicular adenoma and follicular carcinoma by immunodetection of hTERT.*

Variables	High hTERT expression
Sensitivity (%)	39.2
Specificity (%)	90.2
Positive predictive value (%)	83.3
Negative predictive value (%)	54.4
Diagnostic accuracy (%)	62.0

researchers from investigating large numbers of cases. Furthermore, the assay uses whole tissue samples, indicating that contamination by infiltrating lymphoid cells and aggregated lymphoid tissues with telomerase activity may cause misinterpretation of the data (28, 29). *In situ* hybridization for hTERT mRNA may solve the problem as Chou *et al.* indicated (27), but the technique remains complicated and hardly suitable for the analysis of a large number of samples.

This is the first study using hTERT immunostaining to investigate telomerase activity in a large number of thyroid neoplasms arising from follicular cells. We demonstrated that: i) hTERT expression was more frequently observed in anaplastic carcinoma than in papillary and follicular carcinomas, ii) in papillary carcinoma, the hTERT expression level was directly linked to UICC stage and lymph node metastasis, and iii) follicular carcinoma showed a higher incidence of hTERT expression than follicular adenoma.

Our results for papillary carcinoma showed a tendency similar to those of previous studies with smaller numbers of cases, that is, cases showing biologically aggressive characteristics have significantly higher telomerase activity. It is suggested that telomerase activity plays an important role in the development of papillary carcinoma predominantly in the later phase, possibly by contributing to cell immortality. However, a direct relationship between telomerase activity and progression of follicular carcinoma could not be confirmed because there was no significant difference in hTERT expression levels between widely and minimally invasive types. Furthermore, we demonstrated that cases after anaplastic transformation much more frequently showed high hTERT expression than differentiated papillary and follicular carcinomas. It is, therefore, suggested that telomerase activity is one of the factors reflecting the high biological aggressiveness of anaplastic carcinoma.

Previous studies speculated that telomerase activity measurement has a diagnostic value for thyroid malignancies. Saji *et al.* demonstrated that all 6 cases of follicular carcinoma expressed the hTERT gene, although this phenomenon could be observed only in 2 out of 7 follicular adenomas (25). Similar results have been reported by Umbricht *et al.* by TRAP assay and they indicated that telomerase activity can be a marker to distinguish follicular adenoma from carcinoma (15). However, the number of cases they examined was still too small to derive any absolute conclusion. We examined hTERT protein expression in a larger number of cases and

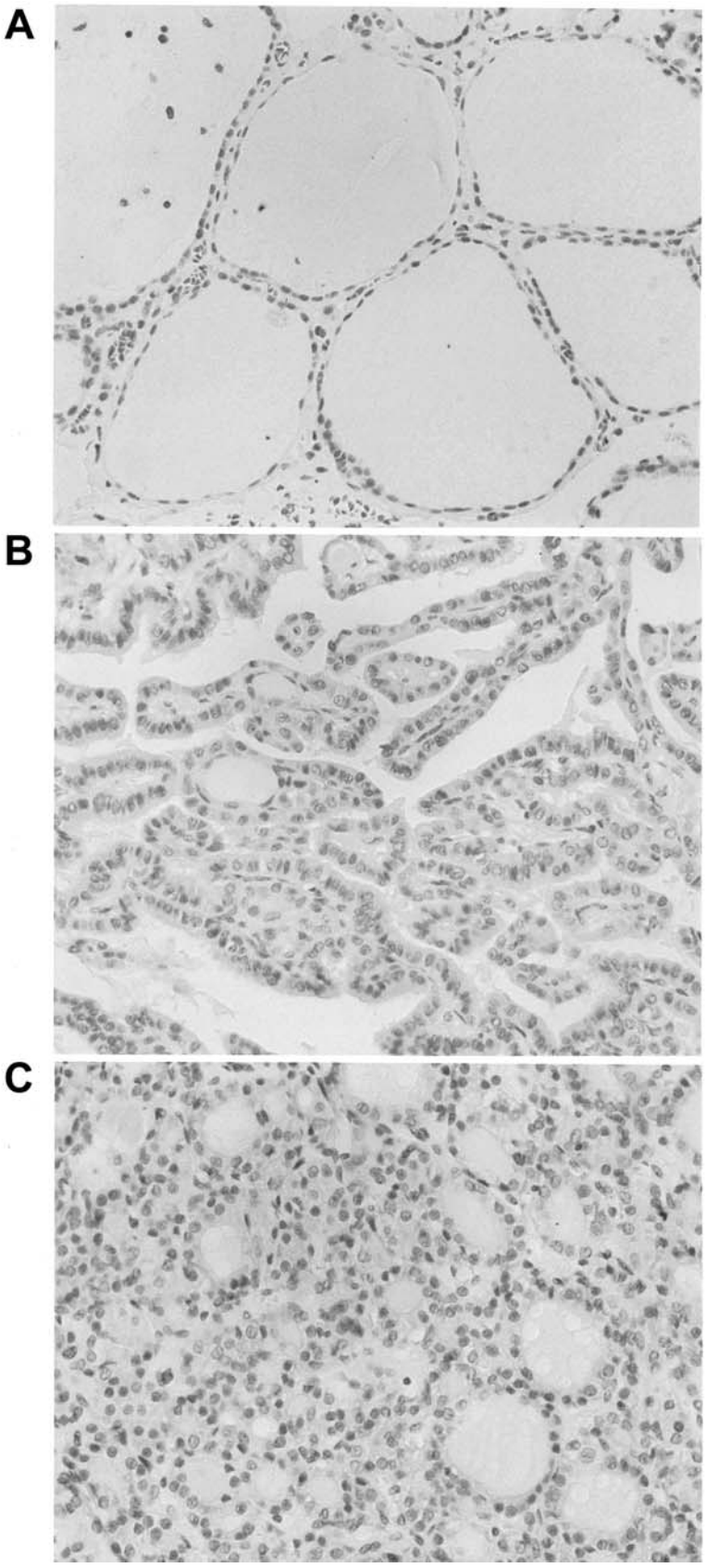

Figure 1 →

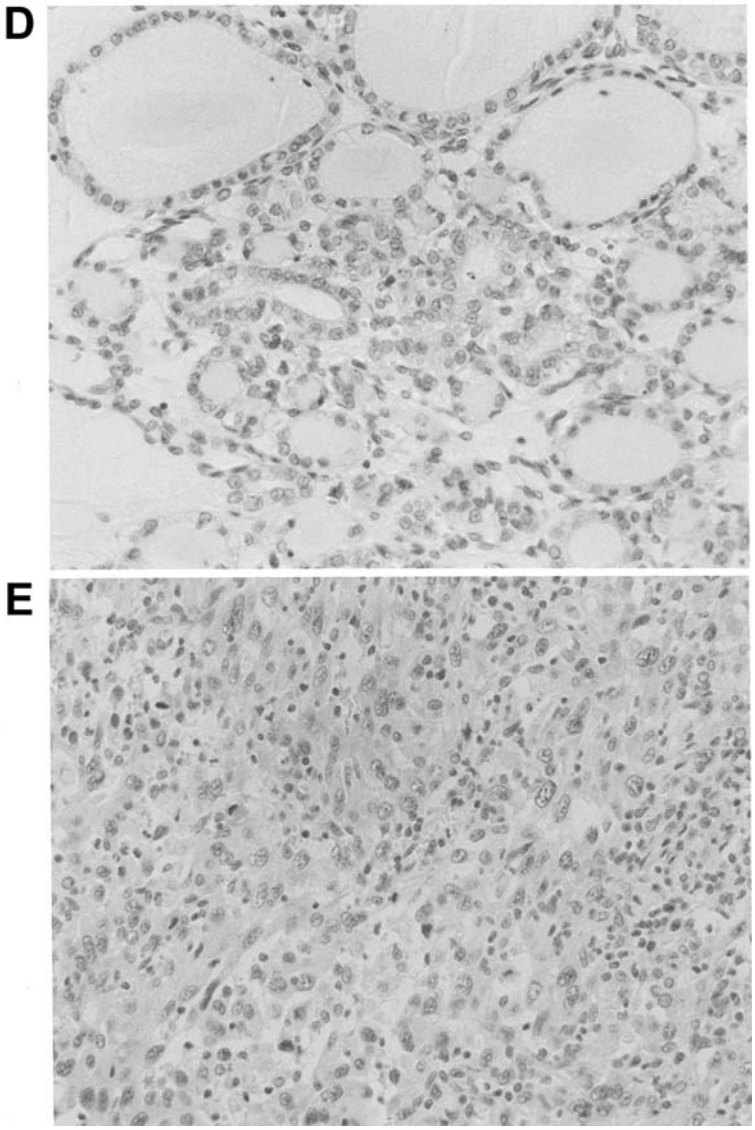

Figure 1. *Immunostaining of hTERT in thyroid tissues. A. hTERT was negative in normal thyroid lesion. B. hTERT expression in papillary carcinoma. C. hTERT expression in follicular carcinoma (minimally invasive type). D. Lack of hTERT expression in follicular adenoma. E. Diffuse hTERT expression in anaplastic carcinoma. Original magnifications: x750*

showed that follicular carcinoma expressed hTERT much more frequently than follicular adenoma. This finding indicates that high telomerase activity is one of the characteristics of follicular carcinoma and, if the hypothesis that follicular carcinoma arises from preexisting adenoma is correct, it concerns, at least in part, malignant transformation of the follicular tumor. Regarding the diagnostic value of hTERT expression in discriminating follicular carcinoma from adenoma, we showed its high positive predictive value, 83.3% using surgical specimens. If this method is applied to FNAB specimen, it could facilitate determination of the surgical indications for follicular tumor. However, in our study, similar numbers of follicular carcinoma and adenoma specimens were examined, 51 and 41 cases, respectively. However, the proportion of follicular carcinoma among thyroid tumors should be much lower than that of benign nodules including follicular adenoma. Therefore, it is suggested that the positive predictive value of hTERT expression for diagnosing follicular carcinoma from FNAB specimens would be lower than that estimated in this study using surgical specimens. Thus, it is still debatable whether hTERT is useful to preoperatively discriminate follicular carcinoma.

In summary, we demonstrated that telomerase activity plays a role in the progression of papillary carcinoma

predominantly in the later phase. Further careful study of large numbers of both FNAB and surgical specimens is needed to determine whether hTERT expression is available for discrimination of follicular carcinoma and useful to determine the surgical indications for thyroid nodules.

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Received July 16, 2004
Accepted November 15, 2004