Telomerase activity in benign and malignant human thyroid tissues

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Summary Telomerase is a specialized ribonucleoprotein polymerase that directs the synthesis of telomerase repeats at chromosome ends. Accumulating evidence has indicated that telomerase is stringently repressed in normal human somatic tissues but reactivated in cancers and immortal cells, suggesting that activation of telomerase activity plays a role in carcinogenesis and immortalization. In this work, the status of telomerase activity during the development of human thyroid cancer was determined using telomeric repeat amplification protocol (TRAP) in 14 nodular hyperplasia, 14 adenomas, 23 papillary carcinomas and 11 follicular carcinomas. Positive telomerase activity was detected in 2 of 14 nodular hyperplasias (14%), 4 of 14 adenomas (29%), 12 of 23 papillary carcinomas (52%) and 10 of 11 follicular carcinomas (91%). The cancers that are negative for telomerase activity are mostly in early stage (stage I or II). These results suggest that telomerase reactivation plays a role during the development of thyroid cancer.

Keywords: telomerase; thyroid cancer; follicular; papillary, adenoma

Normal human somatic cells have a limited proliferative capacity (Hayflick, 1965). Circumstantial evidence suggests that acquisition of extended proliferative capacity, and even of immortality, may occur during the development of tumours (Stamps et al, 1992). Telomere length and telomerase activity have recently been implicated in the control of the proliferative capacity of normal and malignant cells (Harley et al, 1994). The telomeres of human chromosomes consist of hundreds to thousands of tandem repeats of the sequence TTAGGG that are specifically extended by telomerase, a specialized ribonucleoprotein polymerase that synthesizes telomeric DNA onto chromosomal ends using a segment of its integral RNA component as a template (Blackburn, 1992). Normal human somatic cells express low or undetectable telomerase activity and progressively lose their telomeric sequences with replicative senescence or with ageing (Harley, 1991; Allosopp et al, 1992; Vaziri et al, 1993). In contrast, most immortal cells contain telomerase activity and show no net loss of telomere sequence with cell division (Counter et al, 1992; 1994). Therefore, the telomerase activity appears to be stringently repressed in normal human somatic cells but reactivated in immortal cells, suggesting that the activation of telomerase expression may participate in cellular immortality.

Although it is not clear how shortened telomeres may contribute to cellular senescence, the association of telomerase activation with immortalization in vitro raised the possibility that the immortal cells in tumours may be derived by telomerase reactivation. Telomerase activity was first demonstrated in metastatic cells from human ovarian carcinomas (Counter et al, 1994) and malignant human haematopoietic cells (Nilsson et al, 1994). The development of a polymerase chain reaction (PCR)-based assay

for detecting telomerase activity, termed TRAP (Kim et al, 1994), increased the sensitivity of detection and allowed investigators to examine telomerase activity in a large number of tumour biopsies. A high percentage of telomerase activity was detected in primary tumour specimens from malignancies of diverse tissue origins, less frequently in premalignant and benign proliferative tissues, and rarely or none in normal somatic tissues (Shay and Bacchetti, 1997). Therefore, activation of telomerase expression appears to play an important role during carcinogenesis.

Thyroid neoplasm is the most common neoplastic disorder encountered in endocrine clinics. The neoplasm usually occurs with painless thyroid nodules. These thyroid nodules are quite common in the general population (Vander et al, 1968; Ezzat et al, 1994), yet clinical thyroid cancer develops only in a small fraction of the population (Mazzaferi, 1992). Thyroid cancer is, however, the most lethal endocrine neoplasm, excluding that of ovary, and accounts for about 1% of all cancer deaths (Robbins et al, 1991). Among the thyroid malignant neoplasms, the most common types are papillary and follicular carcinomas (Hrafnkeisson et al, 1988; Lin et al, 1996). At present, little is known about the malignant progression in thyroid cancers. Mutations in ras and p53 genes were detected during the progression of normal thyroid tissues to follicular carcinoma (Lemoine et al, 1988; Ho et al, 1996). On the other hand, mutations in Ret and other tyrosine kinase oncogenes were detected in the development of papillary carcinoma (Bongarzone et al, 1989; Santoro et al, 1992). These results suggest that oncogenesis in thyroid tissues is multistep and multi-route. As yet, little is known about the status of telomerase activity in the development of thyroid cancers.

During the course of preparing this manuscript, two articles dealing with the telomerase activity in thyroid tumours have appeared (Haugen et al, 1997; Umbricht et al, 1997). Although telomerase activity was reported to be absent in follicular carcinomas

Table 1 Telomerase activity in human thyroid tissues

Pathology	Total	Telomerase activity		
		Positive	Negative	Positive (%)
Nodular hyperplasia	14	2	12	14
Follicular adenoma	14	4	10	29
Papillary carcinoma	23	12	11	52
Follicular carcinoma	11	10	1	91

Table 2 Clinical data and telomerase activity in thyroid papillary and follicular carcinomas

Classification	Fraction of telomerase-positive tissues	
	Follicular carcinoma	Papillary carcinoma
Stage		
I, II	7/8	8/18
III, IV	3/3	4/5
Tumour size		
< 3 cm	2/2	7/13
3–5 cm	7/7	4/8
5 cm	1/2	1/2
Thyroglobulin		
< 10 ng ml^{-1}	7/8	3/8
10–100 ng ml^{-1}	0/0	3/5
> 100 ng ml^{-1}	1/1	1/2
ND[a]	2/2	5/8
Patient Age		
< 35 years old	3/3	4/11
36–50 years old	3/3	4/5
> 51 years old	4/5	4/7

[a]ND, not determined

and adenomas by Haugen et al (1997), Umbricht et al (1997) reported the detection of telomerase activity in 11 of 11 follicular carcinomas and in 8 of 33 follicular adenomas. The discrepancy between these two reports is currently unresolved. In this work, we report our evaluation of telomerase activity in 14 hyperplasia, 14 follicular adenoma, 23 papillary carcinoma and 11 follicular carcinoma tissues.

MATERIALS AND METHODS

Patients, tissues and cells

Tissue samples were obtained by surgical resection of thyroid tumours from patients admitted at Chang Gung Memorial Hospital (Taiwan). Twenty-eight samples of benign thyroid tissues, which included nodular hyperplasia and follicular adenoma tissues, and thirty-four samples of malignant thyroid tissues, which included papillary and follicular carcinomas were collected and examined in this study. The tissues were washed three times with phosphate-buffered saline (PBS) and stored in liquid nitrogen until use. The disease status of all tissues was verified by histopathological examination. Pathological review was performed for all the thyroid tissues according to the World Health Organization (WHO) classification (Hedinger et al, 1989). In this study, tumour staging was classified as previously described (DeGroot, 1995). Stage I indicates a tumour with a single or multiple intrathyroidal

foci. Stage II indicates a tumour with regional lymph node metastases. Stage III indicates a thyroid tumour with local cervical invasion or fixed cervical metastases. Stage IV indicates lesions metastatic outside the neck. HeLa cells, kindly provided by Dr C Chao, were grown at 37°C, 5% carbon dioxide in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B).

Thyroglobulin determination

Serum thyroglobulin (Tg) levels were detected using a Tg kit (CIS Biointernational, France) according to the manufacturer's instruction. The detection limit of the Tg kit was 0.5 ng ml^{-1}. Interassay coefficient of variation was 8% at Tg level of 4.9 ng ml^{-1}, 6.9% at 232.2 ng ml^{-1} and 5.1% at 312.9 ng ml^{-1}. Data were presented as means ± s.d.

Telomerase assay

Telomerase activity of tissue samples was assayed blindly with a code number and the results were decoded later. Tissue samples (100 mg) were homogenized in 500 µl of lysis buffer [10 mM Tris-HCl, pH 7.5, 1 mM magnesium chloride, 1 mM EGTA, 0.5% CHAPS (Pharmacia), 10% glycerol, 5 mM mercaptoethanol and 0.1 mM phenylmethylsulphonyl fluoride (PMSF)] in Kontes tubes with matching pestles rotated at 450 r.p.m. For HeLa cell extracts, cells were resuspended at 5×10^3 per µl of lysis buffer. After 30 min at 4°C, the lysate was centrifuged at 16 000 g for 30 min at 4°C. The supernatants of tissue and HeLa cell extracts were transferred to fresh tubes and used for telomerase activity assay. The protein concentrations were determined using Coomassie protein assay reagent (Pierce). Telomerase activity was assayed using PCR-based TRAP (telomeric repeat amplification protocol) (Kim et al, 1994). In brief, 0.05–5 µg of extract protein was added to a 50-µl reaction mixture containing 0.1 µg TS primer (5′-AATC-CGTCGA-GCAGAGTT-3′), 2 units of Taq DNA polymerase (HT Biotech), 20 mM Tris-HCl (pH 8.3), 1.5 mM magnesium chloride, 63 mM potassium chloride, 0.005% Tween-20, 1 mM EGTA, 50 µM dNTPs, and 0.1 mg ml^{-1} bovine serum albumin (BSA). The reaction mixtures were incubated at 24°C for 10 min, and 0.1 µg of CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) was then added to initiate PCR amplification. The condition for PCR amplification was 30 rounds of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min in a DNA Thermal Cycler (Perkin Elmer Cetus). RNAase digestion was performed as a control by the addition of 0.5 µg of RNAase A (Sigma) to the reaction mixture. The PCR products were resolved by electrophoresis on a non-denaturing 12% polyacrylamide gel (PAGE) in a buffer containing 54 mM Tris-HCl, pH 8.0, 54 mM boric acid, 1.2 mM EDTA. The gel was stained with SyBr green DNA stain (Molecular Probes), visualized and photographed by illuminating with 254 nm UV.

RESULTS

The presence of telomerase activity in hyperplastic, benign and malignant thyroid tissues was determined by the standard TRAP method using 0.5 µg of extract protein in the reaction mixture. Typical results are shown in Figure 1. Positive telomerase activity in an extract is determined by the presence of a six-nucleotide ladder of TRAP products in PAGE that are sensitive to RNAase A
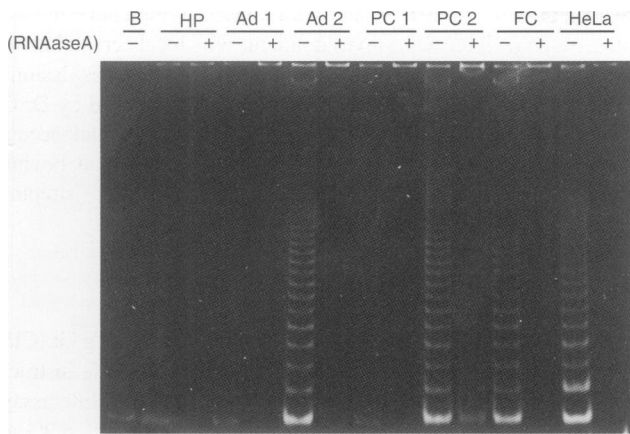
Figure 1 Detection of telomerase activity in thyroid tissues. For the TRAP assay shown in this figure, same amount of protein extracts (0.5 µg) from the tissues of nodular hyperplasia (HP), adenoma (Ad), papillary carcinoma (PC), and follicular carcinoma (FC) was used. Cell extract from HeLa cells was served as positive control, and omission of cell extract (B) was served as negative control

treatment. Tissue samples that did not display telomerase activity were further confirmed by repeating the TRAP assay using 0.05 µg and 5 µg of extract protein in the reaction mixtures. In addition, the presence of inhibitors in the extract was ruled out as mixing the telomerase-negative extract with that of HeLa cell extract did not inhibit the telomerase activity (data not shown). A tally of these results from 14 nodular hyperplasias, 14 follicular adenomas, 23 papillary carcinomas and 11 follicular carcinomas is shown in Table 1. Telomerase activity was detectable in 2 of 14 nodular hyperplasias (14%), 4 of 14 adenomas (29%), 12 of 23 papillary carcinomas (52%) and 10 of 11 follicular carcinomas (91%). The frequency of telomerase-positive tissues in papillary carcinoma was considerably less than that of follicular carcinoma. To understand the basis for the absence of telomerase activity in the carcinoma tissues, we examined whether the absence of telomerase activity may be correlated with tumour stage, tumour size, level of thyroglobulin or patient age. As shown in Table 2, no significant correlation was found between the presence of telomerase activity with tumour size, patient age or thyroglobulin level. The only follicular carcinoma tissue that was negative for telomerase activity had a thyroglobulin level of < 10 ng ml^{-1} and was at stage I. In the case of papillary carcinomas, four out of five carcinomas at stage III or IV were found to be telomerase positive, and 8 out of 18 carcinomas at stage I or II were telomerase positive (Table 2).

DISCUSSION

In this work, we have analysed telomerase activity in thyroid tissues derived from nodular hyperplasia, follicular adenoma, papillary carcinoma and follicular carcinoma. Positive telomerase activity was detected in 2 of 14 nodular hyperplasias (14%), 4 of 14 follicular adenomas (29%) and 10 of 11 follicular carcinomas (91%). These results, which are in general agreement with that reported by Umbricht et al (1997), suggest that telomerase reactivation plays a role in the follicular carcinogenesis. In the case of papillary carcinoma, positive telomerase activity was detected in 12 of 23 carcinomas (52%). Evaluation of clinical data revealed that most of the telomerase-negative tissues of papillary carcinoma

were at stage I or II (10/18), whereas only one out of five carcinomas at stage III or IV was found to be telomerase negative (Table 2), suggesting that telomerase reactivation also plays a role in the papillary carcinogenesis. Haugen et al (1997) reported the detection of telomerase activity in 10 of 14 papillary carcinomas (71%). In their analysis for the correlation between tumour invasiveness and telomerase activity, they observed that six out of seven invasive papillary carcinomas had telomerase activity, whereas only three out of seven non-invasive papillary carcinomas had telomerase activity. Therefore, it appears that a significant fraction of papillary carcinomas at earlier stages is negative for telomerase activity. The smaller percentage of telomerase-positive papillary carcinomas observed in this study may be accounted for by the many carcinoma samples at earlier stages, i.e. 18 out of 23 samples.

In the malignant thyroid cancers examined in this study, we observed that the frequency of telomerase-positive tissues in follicular carcinoma (91%) is higher than that of papillary carcinoma (52%). The prognosis of patients with papillary carcinoma is generally more favourable than patients with follicular carcinoma. It is not known whether the prognosis of malignant thyroid disorders may be correlated with the presence of telomerase activity. Prospective observations are being continued in the patients with papillary carcinoma to evaluate whether telomerase activity is correlated with cancer prognosis. In addition, prospective studies are also being conducted in the patients with benign and hyperplasia disorders to investigate whether the presence of telomerase activity in these non-cancer patients may be correlated with further cancer development.

In summary, our results reveal that telomerase activity is detected in the majority of thyroid cancer at advanced stages (stage III or IV), frequently in papillary carcinoma at early stages (stage I or II) and less frequently in benign and hyperplastic tissues. These results suggest that telomerase reactivation plays a role during thyroid cancer development. Although the biological significance of telomerase activity in non-cancerous thyroid tissue is unclear at present, it is possible that this enzyme may serve as an early indication of thyroid cancer development.

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