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Telomerase activity in benign and malignant human thyroid nodules

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Abbreviations: TRAP, telomeric repeat amplification protocol; ITAS, internal telomerase amplification standard

Abstract

Telomerase is a ribonucleoprotein polymerase which contains an integral RNA and synthesizes TTAGGG nucleotide repeats at the ends of chromosomes in vertebrates. Accumulated evidence has indicated that telomerase is stringently repressed in normal human somatic tissues but reactivated in immortal and cancer cells, suggesting that activation of telo-merase may play an important role in carcinogenesis. In order to assess the role of telomerase in the development of thyroid cancer, we measured the telomerase activity in 19 frozen samples obtained from patients with benign and malignant thyroid nodules by employing a recently developed sensitive PCR-based telomerase assay (telomeric repeat ampli-fication protocol: TRAP). Telomerase activity was detected in all the thyroid cancer tissues (9 of 9) and 4 out of 8 benign thyroid nodules showed telomerase activity. Three of thyroid cancers with high telomerase activity had an unfavorable prognosis, whereas six cancers with low telomerase activity were associated with a favorable prognosis. Interestingly, Hashimoto's thyroiditis tissues also showed telomerase activity. These results indicate that telomerase may play a key role during thyroid carcinogenesis.

Keywords: telomerase cancer, thyroid nodules, PCR, Hashmoto's thyroiditis, carcinogenesis

Introduction

Human cancers are characterized by malignant transformation and immortalization (Shay *et al*., 1991). However, difficulty in establishing cell lines from cancer tissue samples has led many investigators to question whether cancer cells *in vivo* are immortal or not (Stamps *et al*., 1992). It has been shown that telomere DNA shortens at chromosome ends with both *in vivo* and *in vitro* division of human somatic cells unless the termini are extended specifically by telomerase (Harley *et al*., 1990; Lindsey *et al*., 1991; Allsopp *et al*., 1992).

Telomerase is a ribonucleoprotein, RNA-dependent DNA polymerase and acts as a reverse transcriptase-like enzyme which maintains telomere length by adding telomeric repeat units of TTAGGG to the telomere end (Greider *et al*., 1985; Yu *et al*., 1990). It has been suggested that deregulation of telomerase may participate in cellular immortality and oncogenesis. Cells with indefinite replicative potential such as germline cells and almost all tumor cell lines and cancer tissues express telomerase activity (Counter *et al*., 1994; Kim *et al*., 1994; Nilsson *et al*., 1994), while normal human somatic cells show low or undetectable telomerase activity and progressively lose their telomeric sequences both with senescence *in vitro* and with normal *in vivo* aging (Hastie *et al*., 1990; Vaziri *et al*., 1994). Therefore, telomerase activation can be directly involved in cell immortalization and telomere maintenance.

The recently developed sensitive PCR-based telomerase assay (telomeric repeat amplification protocol, TRAP) makes it possible to detect the enzyme in a variety of human tumors and tumor-derived cell lines (Kim *et al*., 1994). With this method, we measured telomerase activity in benign and malignant human thyroid nodules to determine whether malignant progression of thyroid cancer may correlate with expression of telomerase.

Materials and Methods

Detergent extracts from tissues

Benign and malignant thyroid nodules were obtained from each of 19 patients during surgery (Hanyang University KURI Hospital). Each tissue sample consisting of 100 mg of frozen tissue (-70˚C) was washed in ice-cold wash buffer [10 mM Hepes-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM

KCl, 1 mM dithiothreitol (DTT)], and then homogenized in Kontes tube with matching pestles rotated at 450 rpm with 200 μl of ice-cold lysis buffer [10 mM tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol]. The lysate was incubated for 30 min on ice and then centrifuged for 30 min in a microcentrifuge (16,000 *g*, 4˚C). Human 293 kidney cells (immortalized cell line) and NB4 cells (acute promyelocytic leukemia cell line) which would serve as a positive control were collected by scraping 100-mm dishes, washed twice with ice-cold phosphate buffered saline (PBS), once with ice-cold wash buffer, and centrifuged at 3,000 *g* for 5 min at 4˚C. The pellets were resuspended in 200 μl of ice-cold lysis buffer per 1 × 106 cells, and processed in the same way as the preparation of tissue extracts. The supernatant was obtained, quickly frozen in liquid nitrogen, and stored at -70˚C.

Amplification of telomeric repeat

Telomerase activity was assayed by using the modified TRAP method (Kim *et al*., 1994). In brief, 0.1 μg of lyophilizing CX primer (5'-{CCCTTA}3 CCCTAA-3) was dropped onto the bottom of a tube and we sealed it with 100 μl of wax (Ampliwax, Perkin-Elmer Cetus Corp., Foster City, CA) to prepare the assay tubes. After the wax was allowed to solidify at room temperature, the tubes were stored at 4˚C. Fifty microliter of TRAP reaction mixture above the wax barrier consisted of 20 mM tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 μM deoxynucleoside triphosphates, 0.1 μg of TS primer (5'-AATCCGTCGAGCAGAGTT-3), 2U of *Taq* polymerase (Promega, Madison, WI) and 6 μg of protein from each CHAPS cell extract. To control for the presence of *Taq* polymerase inhibitors (Wright *et al*., 1995), the internal telomerase amplification standard (ITAS) primers were included in each analysis. Given 10 min at 30˚C for telomerase to extend oligonucleotide TS, the assay tubes were transferred to the thermal cycler (Robocycler 40; Stratagene, La Jolla, CA) for 35 rounds at 94˚C for 30 s, 50˚C for 30 s, and 72˚C for 45 s. The resulting solution was analyzed by using electrophoresis in 1 × tris-borate EDTA (TBE) buffer on 15% polyacrylamide nondenaturing gels. Silver staining method was used for visualizing the PCR products after electrophoresis. For an approximate estimation of telomerase activity, positive extracts were re-examined by serial dilution. Extracts that had been diluted both 10 times and 100 times contained 0.6 μg and 0.06 μg of protein, respectively (Hiyama *et al*., 1995).

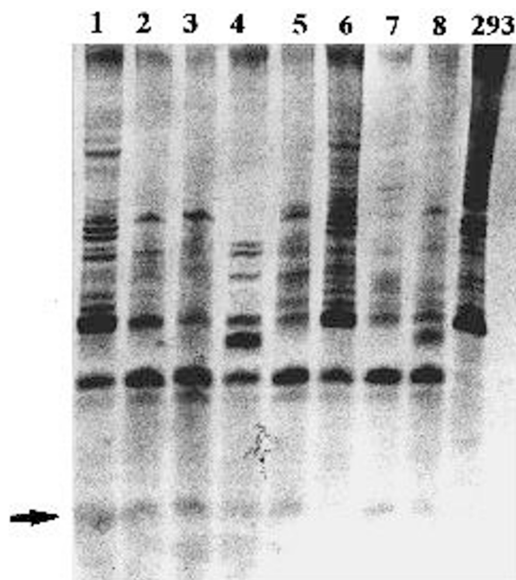

Figure 1. Telomerase activity in thyroid cancer tissues. All the thyroid cancer tissues revealed telomerase activity which appeared as characteristic 6 base pair ladder. Human 293 kidney cells served as a positive control (293). Enzyme assay was performed by using TRAP method (Kim *et al*., 1994). Reaction products were electrophoresed on 15% polyacrylamide nondenaturing gels and visualized with silver staining. Arrow indicates the ITAS primers to control for the presence of *Taq* polymerase inhibitors.

Results

Telomerase activity was detected in all the thyroid cancer tissues (9 of 9) and representative results are shown in Figure 1. Irrespective of clinical characteristics, all the papillary cancer tissues showed telomerase activity and one follicular cancer and one squamous cell cancer tissue was also positive to telomerase, whereas three adjacent normal tissues did not have any telomerase activity (Figure 2). The expression of telomerase in thyroid cancers was not correlated to age, histologic type, or the presence of lymph node metastasis (Table 1). However, three tumors of thyroid cancer had high telomerase activity (that is, retained a TRAP signal after a 100-fold dilution of the extract) whose representative results are shown in Figure

Table 1. Patient characteristics in thyroid cancer

Patient code	Sex/age	Pathology	Stage (TNM)	AMES risk[a]
C502	M/70	Papillary	T4N1bM0	High risk
C056	F/19	Papillary	T2N1bM0	Low risk
C580	F/39	Papillary	T2N0M0	Low risk
C154	F/45	Follicular	T3N0M0	Low risk
C957	F/58	Squamous	T4N1bM0	High risk
C799	F/54	Papillary	T4N1bM0	High risk
C278	F/29	Papillary	T2N1bM0	Low risk
C121	F/64	Papillary	T1N0M0	Low risk
C871	F/23	Papillary	T2N1bM0	Low risk

[a] AMES risk: age, metastasis, extracapsular tumor and size (Cady *et al*., 1988)

Table 2. Patients characteristics in benign thyroid diseases

Patient code	Sex/Age	Pathology	Telomerase activity
B119	F/33	Adenomatous hyperplasia	Positive
B200	F/58	Adenomatous hyperplasia	Positive
B116	F/28	Adenomatous hyperplasia	Positive
B456	F/67	Adenomatous hyperplasia	Negative
B002	F/51	Adenomatous hyperplasia	Negative
B932	F/35	Adenomatous hyperplasia	Positive
H586	F/36	Hashimotoís thyroiditis	Positive
H205	F/31	Hashimotoís thyroiditis	Positive
B632	M/32	Follicular adenoma	Negative
B640	F/33	Follicular adenoma	Negative

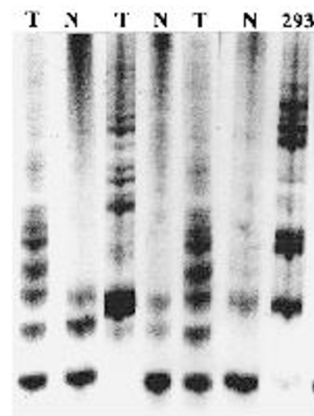

Figure 2. Telomerase activity in thyroid cancer and corresponding normal tissues. Three thyroid cancer tissues showed telomerase activity (T), whereas three adjacent normal thyroid tissues (N) did not have any telomerase activity. Human 293 kidney cells served as a positive control (293).

3 and six tumors had low telomerase activity. Interestingly, three patients with high telomerase activity were high clinical stage and high risk group by AMES risk analysis performed as described previously (Cady *et al*., 1988).

In 4 of 6 adenomatous hyperplasia telomerase activity was detected, whereas all the follicular adenoma were negative for telomerase activity (Table 2, Figure 3). The telomerase activity in benign thyroid nodules was detected in 0.6 μg (10-fold dilution) but not in 0.06 μg (100-fold dilution) of protein (Figure 4).

Hashimoto's thyroiditis tissues also revealed telomerase activity (Figure 3) being characterized by 6 base pair ladder pattern. With serial dilution of protein, the signal was only detected in 10-fold dilution (Figure 4)

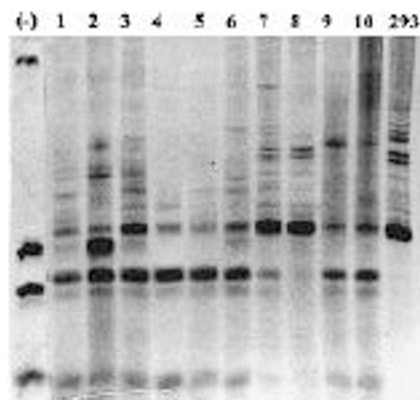

Figure 3. Representative results of telomerase activity in benign thyroid nodules. Four of 8 benign thyroid nodules (lanes 1-3,6) showed telomerase activity, and all the Hashimoto's thyroiditis tissues (lanes 7,8) also revealed telomerase activity.

Discussion

Telomerase activity has been detected in a wide variety of human tumors and tumor derived cell lines, whereas it was not detected in both normal cells *in vitro* and normal somatic tissues *in vivo* (Counter *et al*., 1994; Kim *et al*., 1994). Our study presented here is a systematic examination of telomerase activity in human benign and malignant thyroid nodules.

Telomerase activity was positive in 9 of 9 human thyroid cancer tissues (100%), being characterized by the 6 base pair ladder pattern which has been seen consistently in human 293 kidney cell line as a positive control, whereas normal tissues did not show any telomerase activity. Such high positive rates were also demonstrated in our previous reports tested with head and neck cancer (89%) (Ahn *et al*., 1995), gastric cancer (89%) (Ahn *et al*., 1997), and cervical cancer tissues (89%) (Noh *et al*., 1996). These findings suggest that telomerase activation may play a significant role in establishment and progression of cancer, irrespective of the tumor type.

All the six early staged tumors and three advanced staged tumors showed telomerase activity. These results indicate that telomerase activation may be present not only

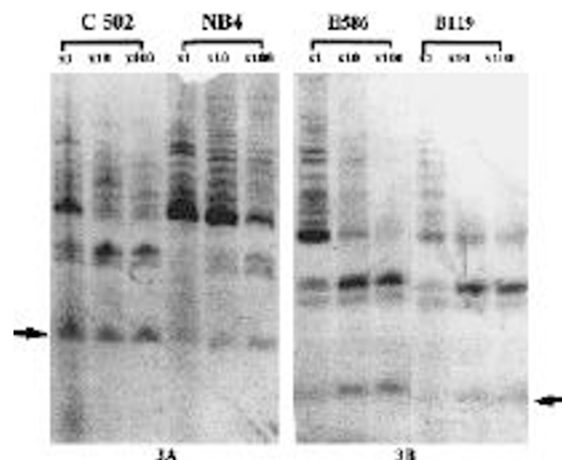

Figure 4. A 10- and 100-fold dilution of each of the extracts designated in Figure 1. Each extract prepared from tumor tissue was subjected to serial dilution and analyzed at 6 μg, at 0.6 μg and at 0.06 μg of protein. Compared to the NB4 leukemia cell line (NB4) and thyroid cancer tissue (C502) which have telomerase activity in 100-fold dilution, telomerase activity was detected only in 10-fold dilution in benign thyroid nodules (B119) and Hashimoto's thyroiditis tissues (H586).

in early but also in late stage of cancers. The incidence of telomerase activity in the thyroid cancer tissues was not correlated to age, tumor size, tumor stage, histologic findings or lymph node metastasis. Thyroid cancers with high telomerase activity by semiquantitative methods were high stage and an unfavorable prognosis, whereas tumors with low telomerase activity were associated with a favorable prognosis, suggesting that there may be different mechanisms regulating telomerase activity in thyroid carcinogenesis.

Some of the benign thyroid nodules (4 of 8) did show telomerase activity in three independent experiments. With semiquantitative methods, the telomerase activity found in benign thyroid nodules was lower compared to that of thyroid cancer tissues. However, further studies to determine the mechanism and the biological significance of telomerase activity in benign thyroid nodules should be warranted.

Hashimoto's thyroiditis tissues also revealed telomerase activity indicating that immune activated lymphocytes infiltrating in Hashimoto's thyroiditis tissues may account for telomerase positivity. Recent reports have shown that normal peripheral blood cells such as granulocytes, T and B lymphocytes, and monocytes have weak telomerase activity and strong telomerase activity was also demonstrated even in normal mature cells of the immune system (Nisslon *et al*., 1994; Broccoli *et al*., 1995; Counter *et al*., 1995; Brousset *et al*., 1997).

In conclusion, the present study demonstrated that telomerase activity is highly positive in human thyroid cancer tissues, irrespective of the tumor size, stage, or histologic findings. These findings indicated that telomerase may be involved in malignant transformation and in progression of thyroid cancers. Further studies are necessary in order to fully understand the biological role of telomerase in thyroid cancinogenesis.

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