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Polymerase Chain Reaction-based Enzyme Immunoassay for Quantitation of Telomerase Activity: Application to Colorectal Cancers†

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Telomerase is a specialized reverse transcriptase that synthesizes telomeric sequences onto human chromosomal ends. It appears to be present in the majority of primary human cancer tissues, and may have potential as a universal tumor marker. In this report, we describe a sensitive, non-radioactive, polymerase chain reaction (PCR)-based enzyme immunoassay (EIA) for the quantitation of telomerase activity in human cells. This PCR-EIA is convenient and can be easily completed within 3 h. The correlation coefficient between the results of PCR-EIA and the conventional telomeric repeat amplification protocol (TRAP) method, as measured on 4 different cell lines, was over 0.98. Evaluation of this method for clinical application was conducted with tissues obtained from patients with colorectal cancers and the results were compared with those of the conventional TRAP method. Our data indicate that telomerase activities measured by conventional TRAP and PCR-EIA are highly correlated, and we suggest that the PCR-EIA method can substitute for conventional TRAP.

Key words: Telomerase activity — Polymerase chain reaction — Enzyme immunoassay — Colorectal cancer

Telomerase is a specialized reverse transcriptase that directs the synthesis of telomeric repeats at chromosome ends.[1)] In most human somatic cells, telomerase activity is undetectable and the telomeric length is progressively shortened during cell proliferation. Cell senescence is thought to occur when the telomeric length is critically shortened.[2, 3)] On the other hand, immortalized human cells exhibit stabilized telomeric lengths and are positive for telomerase activity.[3, 4)] Accumulating evidence indicates that telomerase activity is stringently repressed in normal human somatic cells but reactivated in various types of cancers and immortalized cells.[5)] Levels of telomerase activity have been shown to vary in many diseased human tissues. For example, telomerase activity is detectable in the liver tissues from patients with chronic liver diseases, as well as hepatocellular carcinoma. However, a high level of telomerase activity is detected only in the carcinoma.[6)] Similarly, weak telomerase activity is present in the cortex of normal ovaries from premenopausal women, but a high level of activity is found in malignant ovarian tumor.[7)] Recent studies show that a threshold level of telomerase activity must be acquired to overcome cellular senescence.[8)] All of these reports indicate that quantitative measurement of telomerase activity is important to assess the status of diseased tissues. Development of a sensitive and reliable method to quantitate telomerase activity becomes an important task.

At present, the method commonly used for the detection of telomerase activity employs the "telomeric repeat amplification protocol" (TRAP), in which the telomerase-synthesized products are amplified by a subsequent polymerase chain reaction (PCR).[9)] This method, although highly sensitive, involves the analysis of PCR products by polyacrylamide gel electrophoresis (PAGE). Moreover, quantitation of telomerase activity by TRAP usually requires the use of ^{32}P-labeled primers or incorporation of radioactive deoxyribonucleotides, which poses potential hazards for handlers. Several modifications of the original TRAP have been described to overcome the limitation of conventional TRAP assay. These include the use of different primers to improve the specificity of PCR amplification,[10–12)] the inclusion of an internal standard,[12, 13)] and the use of fluorescent primers or probes.[14–16)] These modifications have improved the quantitation of telomerase, but required time-consuming post-PCR procedures. In this report, we describe a sensitive PCR-enzyme immunoassay (EIA) for the quantitation of telomerase activity. We have compared our PCR-EIA with conventional TRAP and shown that our method is rapid, convenient and accurate, and should be useful in basic research and clinical applications.

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MATERIALS AND METHODS

Patients, tissues and cell culture Tissue samples from colorectal carcinoma patients admitted to Chang Gung Memorial Hospital (Tao-Yuan, Taiwan) were collected and immediately frozen in liquid nitrogen or at −80°C. For each patient, one tumor specimen and one piece of normal mucosa were obtained. Informed consent was obtained from all patients who participated in the study. Human cell lines used in this study were nasopharyngeal carcinoma-derived NPC076 cells,[17] lung cancer-derived CaLu-1 cells, melanoma-derived CM73-36 cells,[18] and cervical cancer-derived HeLa cells. NPC076 and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin and 0.25 mg/ml amphotericin B) in a humidified incubator with 5% CO_2 at 37°C. CaLu-1 and CM73-36 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics under the same conditions as described above.

Preparation of tissue and cell extracts For the preparation of cell extracts, cells of each cell line were suspended at 3000 cells per μl of lysis buffer (10 m*M* Tris-HCl, pH 7.5, 1 m*M* $MgCl_2$, 1 m*M* ethyleneglycol bis(2-aminoethylether)tetraacetic acid (EGTA), 0.5% 3-[3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 10% glycerol, 5 m*M* mercaptoethanol, and 0.1 m*M* phenylmethanesulfonyl fluoride) and incubated on ice for 30 min. For preparation of tissue extracts, about 100 mg tissue samples were homogenized in 500 μl of lysis buffer in Kontes tubes with matching pestles rotated at 450 rpm and incubated on ice for 30 min. After centrifugation at 15,000 rpm for 30 min at 4°C, the supernatants were transferred to fresh tubes and used for telomerase activity assay. The protein concentrations of tissue and cell extracts were determined using Coomassie protein assay reagent (Pierce, Rockford, IL).

Primers The sequence and source of TS and CX primers were previously described.[18] When needed for quantitation, TS primer was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Biotinylated TS (TS-B) and digoxigeninated CX (CX-D) oligonucleotides were purchased from Genasia Scientifics Inc., Taipei, Taiwan.

Assay of telomerase activity with conventional TRAP method Assay of telomerase activity by TRAP was slightly modified as follows. First, TS and CX primers were heated at 95°C for 5 min before being added to the reaction mixtures. Then, aliquots of cell extracts containing 1–300 ng protein were added to 30 μl reaction mixtures containing 0.5 μ*M* ^{32}P-labeled TS primer, 0.5 μ*M* CX primer, 2 units of *Taq* DNA polymerase (HT Biotech. Ltd., Taipei, Taiwan), 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, and 0.1 mg/ml bovine serum albumin. RNase digestion was performed as a control to confirm that the activity was that of telomerase. For these reactions, cell extracts were preincubated with 200 μg/ml of RNase A (Boehringer Mannheim, Mannheim, Germany) at room temperature for 20 min before being added to the reaction mixtures. The TRAP reaction mixtures were incubated at 25°C for 15 min, and then amplified by 30 cycles of PCR at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). The PCR products were resolved by electrophoresis on a nondenaturing 10% polyacrylamide gel in a buffer containing 54 m*M* Tris-HCl, pH 8.0, 54 m*M* boric acid, and 1.2 m*M* EDTA. Detection of TRAP assay products on PAGE and quantitation of telomerase activity by using [γ-^{32}P]-labeled primers were as described.[18]

Assay of telomerase activity by PCR-EIA method The PCR-EIA method was performed as follows. First, the TS-B and CX-D primers were heated at 95°C for 5 min before being added to the reaction mixtures. The reaction mixtures and conditions were essentially the same as described above for TRAP assays, except for the use of TS-B and CX-D primers instead of TS and CX. After PCR, 5 μl of the PCR products was dispensed into streptavidin-coated wells (Boehringer Mannheim), and incubated with 100 μl of anti-digoxigenin antibody conjugated with horseradish peroxidase (15 mU/ml, Boehringer Mannheim) at 30°C for 60 min in EIA reaction buffer, which contained 100 m*M* Tris, pH 7.4, 150 m*M* NaCl, 1% bovine serum albumin, 5% fetal bovine serum, 0.1% Tween-20, 0.1% Nonidet P-40, and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin, 0.25 μg/ml amphotericin B). The plates were washed 4 times with 200 μl of washing solution (100 m*M* Tris, pH 7.4, 150 m*M* NaCl, 0.1% Tween-20, 0.1% Nonidet P-40), and enzyme reactions were initiated by the addition of 100 μl of tetramethylbenzidine substrate solution (Sigma Chemical Co., St. Louis, MO) to each well. Ten minutes later, the reactions were stopped by the addition of 100 μl of 2 *N* HCl to each well. Colorimetric signals were determined by measuring the absorbance at 450 nm using an automatic microwell reader (ThermoMax, Molecular Devices Co., Sunnyvale, CA). Mock control was the reaction mixture which contained all of the reaction components except cell extract. The absorbance value of each sample was reported after subtraction of the mock control value.

RESULTS

Comparison of PCR-EIA with conventional TRAP assay A typical autoradiogram of conventional TRAP assay using NPC076 cell extract is shown in Fig. 1. The density of telomeric repeats on this autoradiogram was quantitated and compared with the results obtained by the

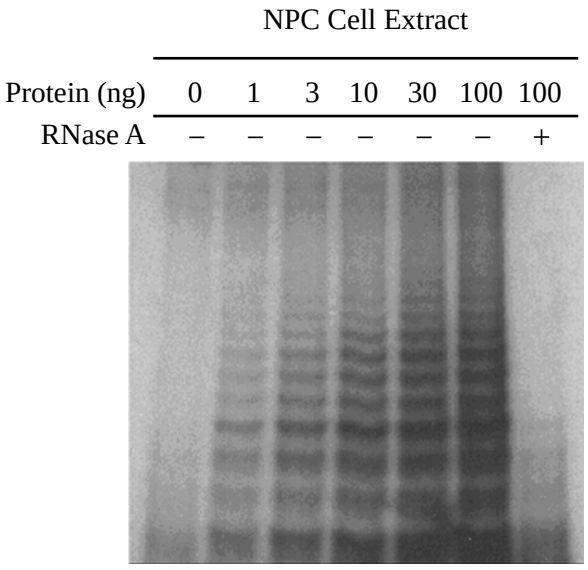

Fig. 1. Telomerase activity in the cell extract of NPC076 cells using the conventional TRAP method. Telomerase activities were determined as described in "Materials and Methods." The amount of protein extract used in each sample is indicated at the top.

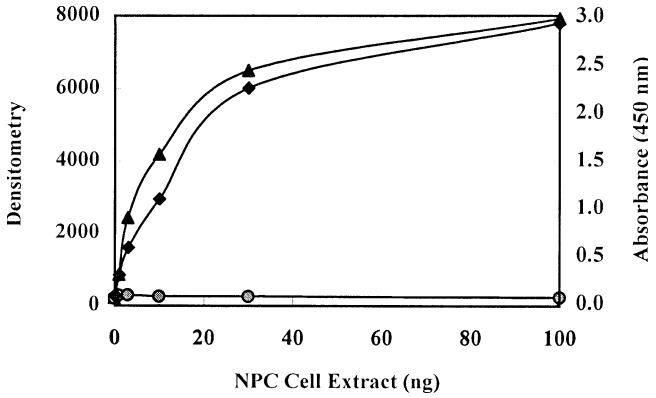

Fig. 2. Comparison of TRAP and PCR-EIA methods for the quantitation of telomerase activity. Telomerase activities in NPC076 cell extracts were quantitated as described in "Materials and Methods." Results from TRAP assays were expressed as relative densitometry unit (◆), while the results from PCR-EIA were expressed as absorbance unit (▲). Results from samples pretreated with RNase A in PCR-EIA were expressed as absorbance unit (●). The correlation coefficient of the results obtained by the two methods was 0.981.

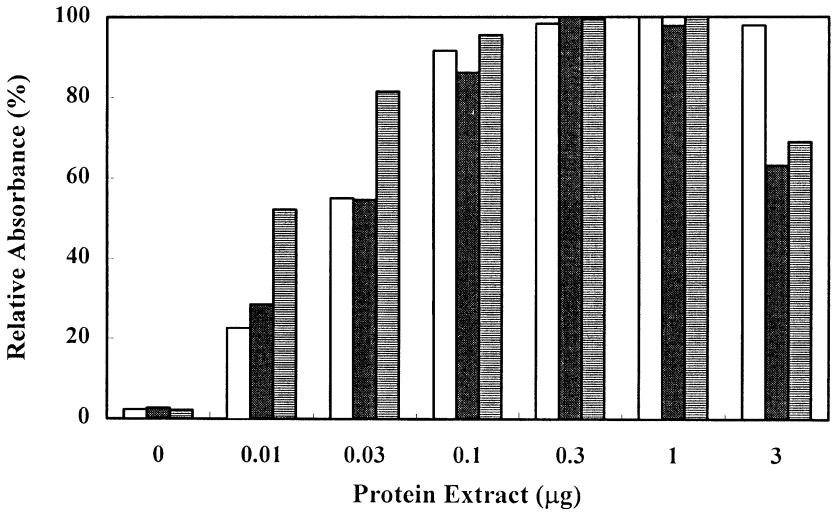

Fig. 3. Titration of telomerase activity in the cell extract of NPC076, CM73-36, and HeLa cells for PCR-EIA. Telomerase activities in these cells were assayed with various concentrations of cellular protein extract up to 3 μg as described in "Materials and Methods." In each cell line, relative absorbance was expressed by taking the highest absorbance value as 100%. □ CM73-36, ■ HeLa, ▤ NPC076.

PCR-EIA method. As shown in Fig. 2, both the absorbance values and densitometric levels increased proportionally with the amounts of protein extracts. The assay sensitivity of PCR-EIA was comparable with that of the conventional TRAP (Fig. 2). In PCR-EIA, pretreatment the cell extract with RNase A dramatically reduced the absorbance values, indicating that the detected activity is authentic telomerase activity. The Pearson's correlation

coefficient of conventional TRAP and PCR-EIA was 0.981. Similar studies were also performed in three other types of cancer cell lines: lung cancer-derived CaLu-1 cells, melanoma-derived CM73-36 cells, and cervical cancer-derived HeLa cells. The correlation coefficients of TRAP and PCR-EIA results in these cells were 0.993, 0.991, and 0.984, respectively (data not shown). All of the above results indicate that telomerase activity measured by the PCR-EIA method is comparable to that obtained from conventional TRAP assay.

Titration of cell extract for PCR-EIA Since it has been reported that cell extract may contain unknown factors which interfere with TRAP assay,[19] titration of each cell extract to obtain an optimal protein concentration for PCR-EIA was indispensable. Aliquots of cell extracts obtained from NPC076, CM73-36, and HeLa cells were assayed for telomerase activity by PCR-EIA. As shown in Fig. 3, when the amount of proteins was less than 0.3 μg, the relative absorbance increased proportionally with the amount of proteins used in the assay. In the case of CM73-36 cells, the absorbance stayed at the same level when more than 0.3 μg of extract proteins was used. However, in NPC076 and HeLa cells, the absorbance level decreased when the amount of extract protein was greater than 1 μg. These results are consistent with previous findings that high concentrations of cell extract may interfere with the assay for telomerase activity.[18, 19] To minimize any possible negative interference by high concentrations of cell extract, we employed 0.3 μg of extract protein in the subsequent analysis of clinical samples by PCR-EIA.

Precision of PCR-EIA for the detection of telomerase activity To evaluate the precision of PCR-EIA, intra-run and inter-run variations were determined by multiple analyses of 5 colorectal tumor or normal mucosa samples. In these assays, 0.3 μg of tissue extract protein was used. Intra-run precision was assessed by evaluating the coefficient of variation (CV) of each tissue sample using 10 assays within a run. Inter-run precision was assessed by performing 8 different runs of assays over a period of 10 days. Results of the precision study are summarized in Table I. The CVs of intra-run precision in 3 colorectal tissue samples were between 1.5% and 6.5% (Table IA). The inter-run CVs from 2 tissue samples were less than 10%, which are in the acceptable range (Table IB). For

Table I. Precision of PCR-EIA for Determination of Telomerase Activity

A. Intra-run assay

	Sample A	Sample B	Sample C
N	10	10	10
Mean absorbance	2.294	1.095	0.381
Standard deviation	0.035	0.071	0.022
Coefficient of variation (%)	1.547	6.520	5.782

B. Inter-run assay

	Sample D	Sample E	Mock control
N	8	8	8
Mean absorbance	2.507	0.218	0.068
Standard deviation	0.215	0.020	0.004
Coefficient of variation (%)	8.356	9.093	5.957

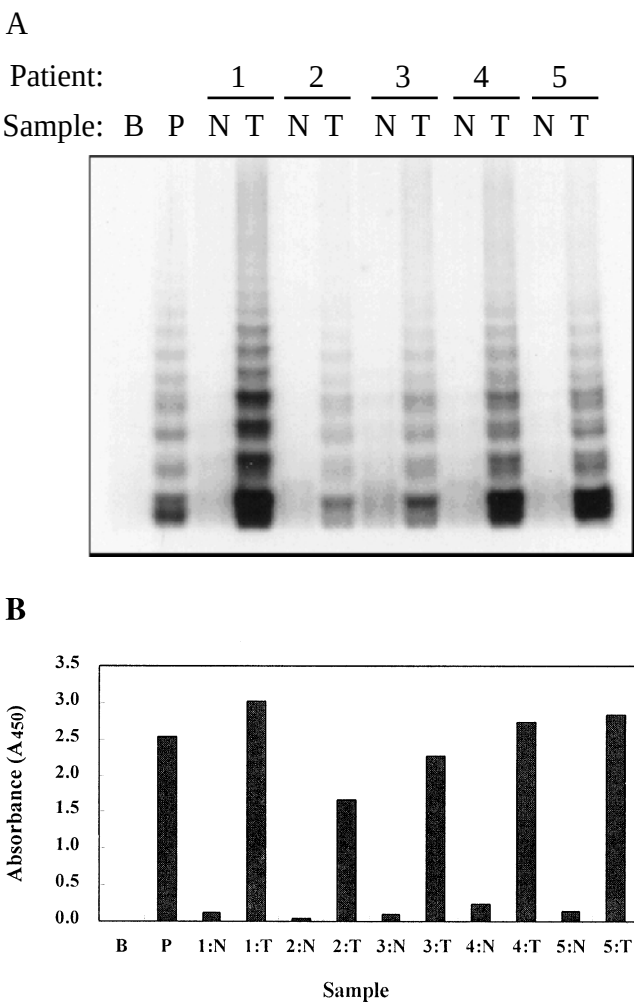

Fig. 4. Determination of telomerase activity in colorectal tissues by conventional TRAP and by PCR-EIA. Samples from 5 pairs of colorectal normal mucosa (indicated as "N" in the figure) and tumor tissues (indicated as "T" in the figure) were assayed for telomerase activity by conventional TRAP (A) and PCR-EIA (B) methods as described in "Materials and Methods." In Fig. B, each sample indicated as "patient:tissue" below each bar represents the specific patient and tissue. Sample B is a buffer control containing all of the reaction mixture without cell extract protein. Sample P is a positive control containing 30 ng of NPC076 cell extract.

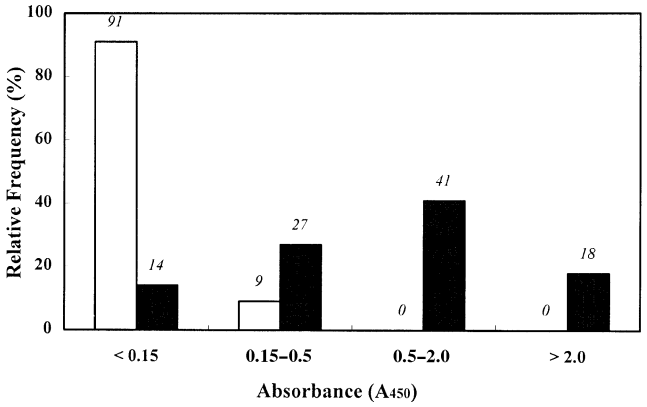

Fig. 5. Relative levels of telomerase activity in 49 colorectal cancers and 53 normal mucosa samples as determined by the PCR-EIA method, as described in "Materials and Methods." The levels of telomerase activity were arbitrarily divided into four groups: less than 0.15, between 0.15 to 0.5, between 0.5 to 2.0, and greater than 2.0. The relative frequency of normal mucosa and cancer in each group is expressed as a percentage, and the actual number is shown on the top of each bar. □ normal tissues, ■ tumor samples.

mock controls, the mean absorbance value was 0.068 with an inter-run CV of 5.96% (Table IB). The low background absorbance allows the present method to detect telomerase activity sensitively.

PCR-EIA for clinical measurement of telomerase activity Determinations of telomerase activity in 5 pairs of colorectal normal mucosa and tumor tissues were performed using both conventional TRAP and PCR-EIA assays. As shown in Fig. 4A and 4B for TRAP assay and PCR-EIA, respectively, normal mucosa tissues had very low or undetectable levels of telomerase activity, while the tumors had high levels of telomerase activity. The relative levels of telomerase activity in the tumor samples as measured by conventional TRAP and PCR-EIA were highly correlated.

Application of PCR-EIA for measurement of telomerase activity in clinical samples was carried out on 49 histologically confirmed colorectal cancers and 53 normal mucosa samples. As shown in Fig. 5, 91% of normal mucosa samples had absorbance values of less than 0.15, and were considered as negative for telomerase activity. Approximately 9% of normal mucosa samples had absorbance values in the range of 0.15 to 0.5, and were considered as weakly positive for telomerase activity. In contrast, only 14% of colorectal cancer samples had an absorbance value below 0.15. The great majority of cancer samples (86%) had absorbance values greater than 0.15. Among these samples, 27% had low levels of telomerase activity (absorbance values 0.15–0.5); 41% had moderate levels of telomerase activity (absorbance value 0.5–2.0); and 18% had high levels of telomerase activity (absorbance greater than 2.0). These results are in agreement with previous reports on the expression of telomerase activity in colorectal cancers.[5, 20, 21]

DISCUSSION

In the development of the PCR-EIA method, it is essential to minimize the background absorbance which might be produced by possible primer-dimer formation. In our experience, the most effective way of reducing background absorbance is to preheat the primers before adding them to reaction mixtures. Application of a modified primer,[11] which had been reported to eliminate primer-dimer formation, made no difference in the PCR-EIA (data not shown).

The PCR-EIA method offers a rapid, quantitative, non-isotopic assay for the determination of telomerase activity. Compared to conventional TRAP, the PCR-EIA method does not require time-consuming post-PCR procedures to quantitate telomerase activity. We have shown that the sensitivity of PCR-EIA is comparable to or greater than that of TRAP, and the quantitative results of PCR-EIA and TRAP are highly correlated. Applications of PCR-EIA for determinations of telomerase activity in clinical specimens produced results (Fig. 5) comparable to those in earlier reports which employed TRAP for analysis.[20, 21] Therefore, the PCR-EIA method described in this work should be a good substitute for conventional TRAP to quantitate telomerase activity.

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