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DETECTION OF HUMAN TELOMERASE ACTIVITY BY TELOMERASE TRAP — ELISA ASSAY*

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Objective: **Telomeric repeat amplification protocol (TRAP) is now a conventional assay for detecting telomerase activity. However, this method presents problems related to tedious quantifying, radioisotopic handing and the limited number of samples to be examined. In order to alleviate these inconveniences, we have developed and evaluated a novel telomerase TRAP-ELISA assay detecting human telomerase activity.** ***Method:*** **Telomerase TRAP-ELISA assay is a system based on the combination of PCR-ELISA with TRAP. Comparing with the conventional TRAP assay, we detected telomerase activity in 293 cell and negative controls (RNase-pretreated or heat-treated).** ***Result:*** **Telomerase activity in 293 cell is positive. The OD value of serial extracts from 10 、 10^2 、 10^3 and 10^4 cells assayed by telomerase PCR-ELISA depended on the number of 293 cells in assay. RNase-pretreated or heat-treated control was negative. Telomerase TRAP-ELISA detection yields results within one day and is handled without radioisotopes.** ***Conclusion:*** **Telomerase TRAP-ELISA assay is non-radioisotopic, fast and quantitative method for detecting human telomerase activity.**

Key word: Telomerase, Detection, ELISA

Telomeres are the end structures of eukaryotic linear chromosomes. Human telomeric DNA is composed of tandem arrays of (TTAGGG) repeat sequences.[1] Telomerase is the ribonucleoprotein enzyme, which synthesized telomeric repeats (TTAGGG)n. Germline cells and most malignant tumor cells express telomerase activity. Recently, telomere and telomerase received much attention in biological research field due to their involvement in cell senescence and tumorigenesis.[2] Detection of the telomerase activity is essential in telomerase study and may have some clinical diagnostic significance. The increasing interest in telomerase studying required the development of sensitive and reliable protocol for detection of telomerase. Telomeric repeat amplification protocol (TRAP) with radioisotopes is often employed in detection of telomerase activity. Radioisotopic TRAP presents problems related to tedious quantifying and radioisotopic handling. In our study, telomerase TRAP-ELIZA assay was developed which based on the combination of PCR-ELISA with TRAP.

MATERIALS AND METHODS

Cell Lines

293 CSH, SMMC-7721, BEL-7402, BEL-7404 and QGY-7701 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Science.

Preparation of Telomerase Extracts

Harvested cells are counted and an aliquot

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containing 100,000 cells is pelleted (3,000×g in a 1.5ml Eppendorf tube for 6 min) in culture medium. The pellet was washed with PBS, and the pellet stored at -80 ℃. In brief, for preparation of telomerase extracts, the cells are removed from the -80 ℃ freezer, then resuspended in 20μl cell lysis buffer [0.5% CHAPS, 10mM Tris-HCl(pH7.5), 1mM $MgCl_2$, 1mM EGTA, 5mM β-mercaptoethanol, 0.1mM AEBSF (4-(2-aminoethy 1)-benzenesulfonyl fluoride) hydro-chlorine and 10% glycerol] by retropipeting at least three times and kept on ice for 30 min. The lysate is centrifuged at 12,000×g for 20min at 4 ℃ and the supernatant is collected for protein assay with Folim-phenol method. The resulting extract is stored at -80 ℃ or detected instantly.

Amplification of Telomeric Repeats

The assay was performed as described[3] with minor modification.[4] In this assay, biotinylated TS primer was utilized for immobilization with ELISA microtiter plate. One microliter S-100 extract (or 6 μg protein) is assayed in 50μl TRAP reaction buffer [20mM Tris-HCl(pH8.3), 1.5mM $MgCl_2$, 68mM KCl, 0.05% Tween 20, 0.1mM EGTA, 50μM dNTP], containing 0.1μg biotinylated TS primer (5'-AATCCGTCGAGCAGAGTT), 0.5 μM T4g32 protein, 2U Taq DNA polymerase at room temperature for 30 min. As control, RNase A was added to parallel reactions before test. The S-100 extract disposed by heating at 65 ℃ for 10min may be also as parallel controls. After extension at room temperature for 30min, 0.1μg CX primer (5'-CCCTTACCCTTA-CCCTTACCCTAA) was added. The reaction mixtures are subjected to denaturalization for 2 min at 97 ℃, and 30sec at 50 ℃, 30sec at 72 ℃, 30sec at 94 ℃ for 30 cycles.

Hybridization and ELISA Procedure

The assay was performed as described[5] with minor modification. Microtiter plate is coated with streptavidin at 4 ℃ for 12hr, and washed 4 times with 2 × SSC. forty microliter hybridization solution (5 × SSC, 200μg of herring sperm DNA) was dispensed into microtiter wells coated with streptavidin. Ten microtiter of TRAP product was mixed into the hybridization solution in microtiter wells and incubated for 30 min at 37 ℃. The hybridization solution was removed from the well, which was rinsed with 200μl of 2 × SSC. Two hundred microliter denaturalization reagent (0.5 mol/L NaOH) was added into the well and incubated for 5 min at room temperature. The denaturalization reagent was removed from the well, which was rinsed 2 times with 200μl of 2 × SSC. Two pmol of telomeric repeat probe (5'CCCTAACCCTAACCCTAA) dissolved to total of 100μl of hybridization solution was added into the well and incubated at 50 ℃ for 30min, which was rinsed 2 times with 200μl of 2 × SSC. One hundred microliter anti-Fluorescein-POD solution, diluted 1/1000 with incubation solution (0.1mol/L Tris-Cl (pH7.5), 0.3mol/L NaCl, 0.2mol/L $MgCl_2$, 1% bovine serum albumin and 0.05% Tween-20), was added to the well and incubated for 60min at room temperature. After incubation, the well was rinsed 3 times with 200μl of incubation solution, then 100μl of 0-phenylenediamin (OPD) made up in substrate buffer and activated with H_2O_2 just prior to use. Reaction proceeded at 37 ℃ for 20min. The enzyme reaction was stopped with 15μl of 2M H_2SO_4 and the OD of each well was read at 492 nm using a microtiter plate reader.

RESULTS

The Determination of PCR Cycling Number

PCR cycling number for amplification of telomeric repeated sequence needs to be as few as possible to ensure that the detection is performed in the logarithmic phase. From Figure 1, we can tell that from 25 to 35, OD value has a linear correlation with cycling number and reaches plateau range from 35 to 45. No specific response has been found in negative control pretreated with RNase. Taken together, 30 cycles were chosen for PCR procedure.

Comparison of TRAP-ELISA with Conventional TRAP Assays

Telomerase was detected in the same 293 cell samples with conventional TRAP[4] and TRAP-ELISA protocols respectively. PCR-ELISA protocol was repeated for over 3 times. OD value for the sample from 1000 293 cells pretreated either with RNase at 37 ℃ or with heat at 65 ℃ for 10 min was less than 0.2, whereas, OD values for the crude samples from 10, 100, or 1000 293 cells were more than 0.2. In

extension reaction, OD value was below 0.2 with dGTP deletion, in contrast, OD value was over 0.2 with dCTP deletion (Figure 2).

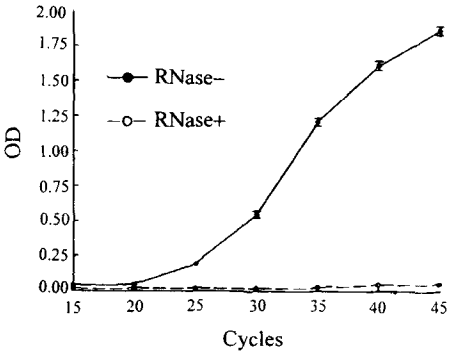

Fig 1. The determination of PCR cycling number with telomerase TRAP-ELISA protocol.

Telomerase Determination in Liver Cancer Cells

Four lines of human liver cancer have been verified to be telomerase positive with conventional TRAP method.[4] All of the OD values for these four human liver vancer cell lines are over 0.2 with TRAP-ELISA method for telomerase. The ratios of OD value without heat pretreatment to that with heat treatment are over 2, showing positive results (Figure 3).

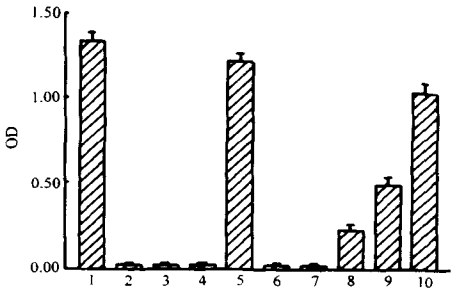

Fig 2. Specificity and sensitivity of telomerase TRAP-ELISA assay in telomerase detection

1. 293 cell 2. $RNase^+$ 3. pretreatment with heat 4. extension without dGTP 5. Extension without dCTP 6. primer control 7. Lysis reagent control 8. 9. 10. 10^1, 10^2 and 10^3 293 cells respectively.

DISCUSSION

Telomeres are special structures at the ends of eukaryotic chromosomes. In human, telomeres are

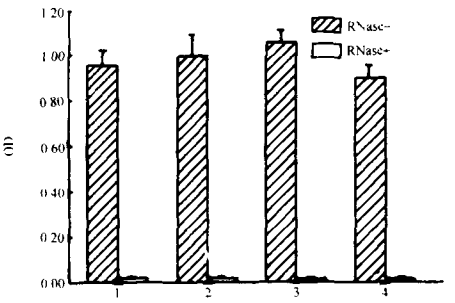

Fig 3. Telomerase activity in liver cancer cell lines with TRAP-ELISA method.

1. QYG-7701 2. BEL-7402 3. BEL-7404 4. SMMC-7721

composed of two parts: tandem arrays of 6 base repeat TTAGGG and telomeric binding proteins. Telomeres exert great biological functions, such as chromosome stabilization, prevention of chromosomal terminus fusion, protection of the structural genes located in chromosomes and modulation of the growth of normal cells. As the proliferation proceeds, the telomeres of normal cells shorten progressively due to the 5'-termini loss of linear DNA during replication. The cells step dividing and keep inactive when telomeres reach a certain length. So the telomeres are regarded as mitotic clock of normal somatic cells.[6] Telomerase, a kind of ribonucleoprotein enzyme, Composed of RNA and protein components, functions as a reverse transcriptional DNA polymerase with 3'-termini as primers in telomere elongation, using its RNA component as a template and its protein component for catalysis to synthesize the telomeric repeats. About 85 — 90% malignant tissues (e.g. lung cancer, gastric cancer, colon cancer, liver cancer and breast cancer) are positive with telomerase.[7] It shows that telomere and telomerase are tightly connected with growth control in normal cells and oncogenesis. Telomerase detection assay is a must for further study and may have significance of diagnostic and prognostic importance. Kim, et al. developed TRAP method in 1994. This method, which unites the techniques of telomeric extension by telomerase and PCR, is employed frequently at present. Polyacrylamide gel electro-phoresis and autoradiography are employed to analyze the amplified 6bp ladder bands of PCR products. This method needs isotopes and long time for determination and is difficult to quantify.

Ericha, et al. connected TRAP method with scintillation proximity assay (SPA) method.[8] A 5'-end

biotinylated oligonucleotide is used as primer for amplification of the telomerase reaction product in the presence of tritiated thymidine. Upon addition of streptavidin coated fluoromicrospheres, the biotinylated ^{3}H-labeled product will bind to the beads which contain a scintillant stimulated by the close proximity of β-emitters. The incorporated tritiated nucleotides are thus able to stimulate the scintillant and produce a signal, whereas the unincorporated free nucleotides, whose energy is absorbed in the assay buffer, cannot. Finally, the results are displayed with liquid scintillation counter directly. This method is fast, sensitive, specific and quantifiable. It is performed in 96-well culture plates and fit for detection of large amount of samples. But it also needs isotopes.

In our study, we combined Kim's TRAP method with conventional ELISA method and developed the TRAP-ELISA assay for telomerase. Compared with conventional TRAP method, it is specific, sensitive and fast in detection of telomerase in 293 cell. In this method, the goal of quantification can be attained and isotopes are not needed, avoiding dangers to man and environment. Telomerase is regarded as a promising target for the development of cancer therapy.[9,10] It will be of great importance to explore the drugs inhibiting the telomerase activity. The method we developed provides a useful tool for telomerase inhibition experiment.

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