

Technical report

A non-isotopic method for the detection of telomerase activity in tumour tissues: TRAP-silver staining assay

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Abstract

Telomerase activity has been observed in a high proportion of specimens from a wide variety of human malignant tumours, but not in non-neoplastic tissues. Measurement of telomerase activity is a possible diagnostic maker for malignant tumours. A non-isotopic telomeric repeat amplification protocol (TRAP) silver staining method for the detection of telomerase activity in human bone tumour tissues is described. The TRAP-silver staining method was quick, safe, and effective, and can be used as a routine diagnostic method for the detection of telomerase activity in bone tumours.

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Keywords: telomerase; TRAP; bone tumours; silver staining

The role of telomeres in tumorigenesis has led to the suggestion that telomerase activity might be of value in the diagnosis of malignant tumours. Using the telomeric repeat amplification protocol (TRAP) developed by Kim *et al*,¹ telomerase activity has been seen in a high proportion of specimens from a wide variety of human malignant tumours, but not in non-

neoplastic tissues. Most studies showed that telomerase activity is found in 70-100% of all malignant tumours including bladder² and prostate carcinomas,³ head and neck squamous cell carcinoma,⁴ breast carcinoma,⁵ hepatocellular carcinomas,⁶ non-Hodgkin's lymphoma,⁷ neuroblastoma,⁸ ovarian carcinoma,⁹ colorectal carcinoma,¹⁰ and lung cancer.¹¹ Measurement of telomerase activity has been considered as a possible diagnostic maker for malignant tumours.^{2-3 10 11} Isotopic labelling for the TRAP assay is not a common practice for the routine laboratory; we describe optimisation of conditions for the detection of telomerase activity and the development of a non-isotopic silver staining method.

Methods

SAMPLES

Fifty bone tumour specimens were obtained from patients who had surgical resection of tumours. HeLa cells harvested from culture were used as positive controls.

TRAP ASSAY

The bone tumour tissues and cultured cells were assayed for telomerase activity using the protocol developed by Kim and colleagues³ with modification. Tumour tissue (100 mg) or about 10⁷ cultured cells were washed once in ice cold wash buffer (10 mM HEPES-KOH pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol), and homogenised in 200 µl ice cold lysis buffer (table 1). The homogenate was

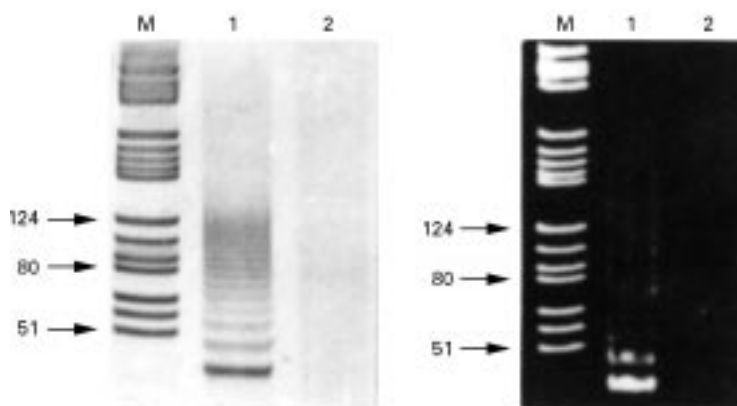


Figure 1 TRAP-silver staining (left) and ethidium bromide staining (right) for telomerase activity in HeLa cells. A clear ladder of telomeric repeats is seen with silver staining. M, PBR322 DNA/Hae III molecular standard; Lane 1, HeLa cell extract; Lane 2, control assay with lysis buffer.

Table 1 Reaction solutions for TRAP assay

Lysis buffer

10 mM Tris-HCl (pH 7.5)
1 mM MgCl₂
1 mM EGTA
0.1 mM AEBSF
[4-(2-aminoethyl)-benzenesulphonyl-fluoride-hydrochlorine]
5 mM β mercaptoethanol
0.5% CHAPS
(3-cholamidopropyl-dimethylammonio-1-propane-sulphonate)
10% glycerol

TRAP reaction solution

20 mM Tris-HCl (pH 8.3)
1.5 mM MgCl₂
63 mM KCl
0.005% Tween-20
1 mM EGTA
0.1 mg/ml bovine serum albumin

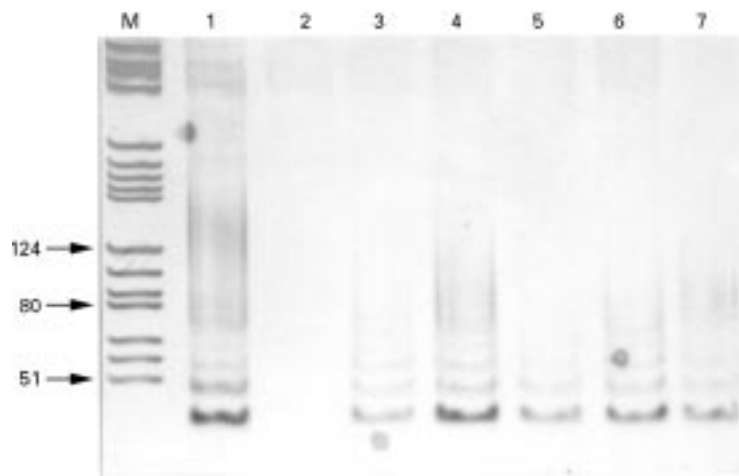


Figure 2 Telomerase activity in aggressive bone tumours demonstrated by TRAP-silver staining. M, PBR322 DNA/Hae III molecular standard; Lane 1, HeLa cells (positive control); Lane 2, lysis buffer (negative control); Lane 3, osteosarcoma; Lane 4, chondrosarcoma; Lane 5, malignant fibrous histiocytoma of bone; Lane 6, rhabdomyosarcoma of bone; Lane 7, giant cell tumour of bone.

incubated on ice for 30 minutes and then centrifuged at $10\,000 \times g$ for 30 minutes at 4°C . The supernatant was collected and the protein content determined by Coomassie brilliant blue assay.

PCR reaction mixture (total $48\ \mu\text{l}$) consisted of $36.6\ \mu\text{l}$ DEPC treated water, $2\ \mu\text{l}$ ($6\ \mu\text{g}$ protein) extract, $5\ \mu\text{l}$ $10\times$ TRAP reaction solution (table 1), $2\ \mu\text{l}$ ($50\ \mu\text{M}$) each dNTP, $0.4\ \mu\text{l}$ ($2\ \text{U}$) Taq DNA polymerase, and $2\ \mu\text{l}$ ($0.1\ \mu\text{g}$) of TS primer sequence ($5'$ -AATCCGTCGAGCAGAGTT- $3'$).

The PCR reaction mixture was incubated at 25°C in a thermal cycler for 30 minutes for extension of TS primer. CX primer sequence ($5'$ -CCCTTACCCTTACCCTTACCCTAA- $3'$) ($2\ \mu\text{l}$ ($0.1\ \mu\text{g}$)) was added at 72°C within 10 minutes. The reaction mixture (total $50\ \mu\text{l}$) was

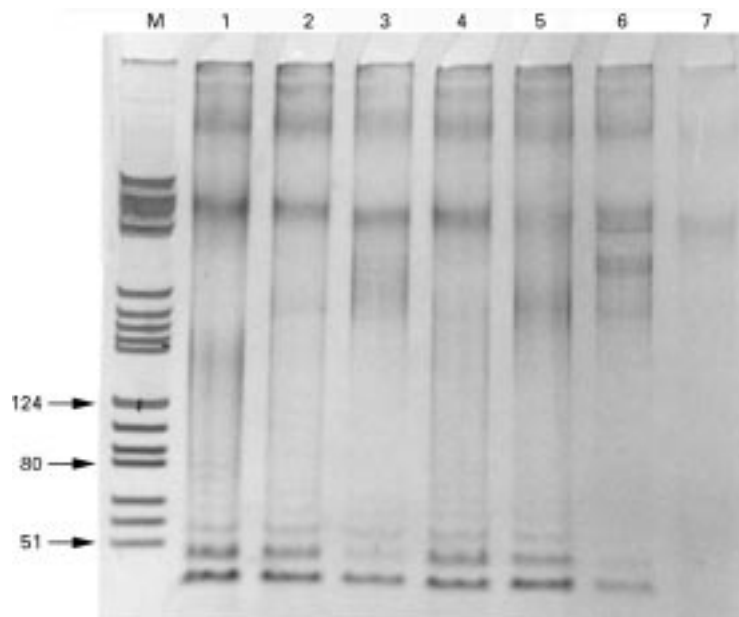


Figure 3 Sensitivity of TRAP-silver staining. Protein extract ($6\ \mu\text{g}$) of an osteosarcoma sample and 10^5 HeLa cells were diluted 10-fold and 100-fold. M, PBR322 DNA/Hae II molecular standard. HeLa cells at densities of 10^5 (Lane 1); 10^4 (Lane 2); and 10^3 (Lane 3). Protein extract from an osteosarcoma samples: $6\ \mu\text{g}$ (Lane 4); $0.6\ \mu\text{g}$ (Lane 5); and $0.06\ \mu\text{g}$ (Lane 6). Lane 8, lysis buffer (negative control).

subjected to PCR cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds (then 10 minutes for the final step). TRAP reaction product ($20\ \mu\text{l}$) was analysed by electrophoresis in 0.5 Tris-borate EDTA buffer on 12% polyacrylamide non-denaturing gels.

SILVER STAINING

As described previously,¹² gels were soaked in 10% ethanol for five minutes, $0.012\ \text{M}$ silver nitrate solution for 20 minutes under subdued lighting, and developed in sodium carbonate solution ($0.28\ \text{M}$) and formaldehyde (0.019%). Development was halted by soaking the gel in 10% acetic acid for five minutes. After photography, the gels were dried for storage.

ETHIDIUM BROMIDE STAINING

Gels were soaked in $0.5\ \text{g/ml}$ ethidium bromide in $1\times$ TAE (Tris acetate EDTA) buffer for 30 minutes, viewed and photographed.

Results

Kim and co-workers¹ have developed an extremely sensitive assay for the detection of telomerase activity, however, it requires radioisotopes for the reaction. In this study, telomerase activity was detected by a non-isotopic TRAP assay combined with silver staining. The characteristic ladder with six base increments starting at 40 nucleotides could be seen more clearly in silver stained gels than ethidium bromide stained gels (fig 1). There was no TRAP product when HeLa cell extract was replaced by lysis buffer (negative control). Figure 2 shows the telomerase activity detected in various aggressive bone tumours. To determine the sensitivity of the silver staining, $6\ \mu\text{g}$ of protein extract of tumour samples and 10^5 HeLa cells were diluted 10-fold and 100-fold. The results show that the telomerase activity, determined by the number of ladders, is concentration dependent. Telomerase activity can be detected at a protein concentration as low as $0.06\ \mu\text{g}$ or 1000 cells (fig 3). It is noteworthy that telomerase activity was maintained in protein extract of tumour tissues for several months after storage at -70°C .

Discussion

Telomeres are specialised protein-DNA structures at chromosomal ends of all eukaryotic cells. They contain multiple tandem repeats of the highly conserved G rich sequence TTAGGG. It is accepted that telomeres protect the end of the chromosome from fusion and recombination events by interaction with DNA binding proteins and those of the nuclear matrix. Human telomeric DNA consists of 5–20 kb pairs of homogeneous repeats. Somatic cell telomeres are progressively shortened by 40–200 bp with each cell division. A reduction of telomere length beyond a critical point leads to subsequent exit from the cell cycle and senescence. The restoration of telomeric repeats to the ends of the chromosome may overcome this limitation and lead to immortalisation of cells. Telomerase is a key enzyme for synthesis of telomeric repeats and

can thus repair truncated, damaged chromosomal ends. In immortal cancer cell, telomerase was found to be reactivated in response to the onset of tumorigenesis. To date, measurement of telomerase activity has been considered as a diagnostic maker for malignant tumours.^{2 3 10 11}

This modified TRAP method effectively detected telomerase activity in bone tumour tissues. The minimal equipment requirements should make this assay available to most routine histology laboratories.

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