TELOMERASE ACTIVITY IN MALAYSIAN PATIENTS WITH CENTRAL NERVOUS SYSTEM TUMORS

Mohd Nizam Isa[1], Sarina Sulong[1], Mohamad Ros Sidek[1], P Jain George[2] and Jafri Malin Abdullah[2]

[1]Human Genome Center, Health Campus, Universiti Sains Malaysia; [2]Neuroscience Unit, Health Campus, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Abstract. Telomerase, the enzyme that stabilizes telomere length is reactivated with almost all cancer types, and may be a useful diagnostic marker for malignancy. Telomerase activity has been detected in germ line cells and most cancer cells, whereas most normal somatic cells have no clearly detectable telomerase activity. In our study, we aim to detect telomerase activity in 20 human central nervous system tumors from Malaysian patients. Telomerase activity was detected based on a highly sensitive procedure consisting of a CHAPS detergent-based extraction from frozen tissues and a PCR-based telomeric repeat amplification protocol (TRAP) using a TRAP$_{EZE}$ Telomerase Detection Kit (Intergen, Co). Telomerase activity was considered positive when a ladder of products was observed starting at 50bp, with 6bp increments. The activity was detected in 30% of the samples analysed, included glioblastoma multiforme, meduloblastoma, paraganglioma and oligodendroglioma. The result of Fisher's exact test indicated that there was a significant association between telomerase activity status with tumor grade (p=0.003). These results suggest that telomerase activity may be an important marker for tumor malignancy.

INTRODUCTION

Telomerase is an RNA-dependent DNA polymerase where it's activity has been detected in cancer cells, germ line cells and immortal cells but not in most normal somatic tissues. This correlation has led to the telomere-telomerase hypothesis of aging and cancer that involve the reactivation of telomerase that is necessary for sustained cell proliferation in many tumors. This unique ribonucleoprotein enzyme is responsible for adding the telomeric repeats onto 3′ ends of chromosomes and contains two major components, which are RNA and human telomerase reverse transcriptase (hTERT) catalytic subunit (White *et al*, 2001). Differences have been observed in telomerase activity in normal versus tumor derived cells resulted in the hypothesis that telomerase may represent a suitable target for specific anti-cancer therapies (Gryaznov *et al*, 2001).

Correspondence: Dr Jafri Malin Abdullah, Neuroscience Unit, Health Campus, School of Medical Sciences, Universiti Sains Malaysia, 16150, Kubang Kerian, Kelantan, Malaysia.
Tel: +609-7664240, 7663000 ext 4240; Fax: +609-765 3370
E-mail: unitneurosainsppspusm@yahoo.com

Telomeres are specialized structures that cap the ends of chromosomes and consist of thousands of repetitive sequences TTAGGG (Herbert *et al*, 1999; White *et al*, 2001; Wright and Shay 2001). With each cell division, telomeres shorten by 50-200 bp because the lagging strand of DNA synthesis is unable to replicate the extreme 3′ end of the chromosome (end replication problem) (White *et al*, 2001). It has been stated that when telomeres become sufficiently short, cells enter an irreversible growth arrest called cellular senescence (Shay *et al*, 2001). Telomere shortening has been proposed as a molecular clock mechanism that counts the number of times a cell has divided, and when telomeres are short, cellular senescence occurs (Shay, 1999). Telomeres have a variety of purposes, including preventing chromosome ends from being recognized as double-strand DNA breaks (Blackburn, 1991).

Human central nervous system tumors are among the most rapidly fatal of all cancers and new molecular strategies should be developed to cure these tumors in the future. Glial tumors, such as astrocytoma, glioblastoma multiforme and oligodendroglioma, are the most common primary tumors in the central nervous system (Leon *et al*, 1994). In a study of 90 glial tumors, telomerase activity was detected in 75% of glioblastomas and

in all oligodendrogliomas, but only 10% of anaplastic astrocytomas were telomerase positive (Langford *et al*, 1995). A previous study has shown that telomerase activity was detected in 65% of the glioma samples (Morri *et al*, 1997).

The detection of telomerase activity is a useful test for the future of cancer diagnosis, prognosis and therapy since it is repressed in almost all normal cells and reactivated in many tumor tissues. Most studies showed that telomerase activity is found in 70-100% of all malignant tumors, including breast cancer, neuroblastoma and lung cancer (Wen *et al*, 1998). Both telomerase and telomere have been identified as targets for anticancer therapy, since there is evidence of a strong correlation between telomerase reactivation, cellular immortalization and cancer (Urquidi *et al*, 2000).

Using a sensitive and efficient PCR-based telomerase activity detection method, TRAP (Telomeric Repeat Amplification Protocol), larger scale surveys of telomerase activity in human cells and tissues can be made. The TRAP assay is considered to be the gold standard (Dalla Torre *et al*, 2002). The TRAP-silver staining method is quick, safe and effective, and can be used as a routine diagnostic method for the detection of telomerase activity in bone tumors (Wen *et al*, 1998).

The aim of this study is to detect the telomerase activity in various types of central nervous system tumor tissues obtained from Malaysian patients using a non-isotopic method, TRAP-silver staining assay.

MATERIALS AND METHODS

Tissue samples

Twenty tumor tissues collected in the years 2001 and 2002 were obtained for this study. These tumor samples were selected consecutively out of 30 tumors stored at the Brain Tumor Tissue Bank of the Neuroscience Unit of the same institution. Both low and high-grade tumors were studied. This study was approved by the Research Development and Human Ethics Committee of the School of Medical Sciences, Universiti Sains Malaysia 2000. The tumors were classified histologically according to World Health Organization (WHO) criteria.

Of 20 tumors tissues, eight were meningiomas (Grade I), three were schwannomas (Grade I), two were meduloblastomas (Grade IV), one

was a paraganglioma (Grade I), two were glioblastoma multiforme (Grade IV)) and four were low-grade gliomas (2 were Astrocytoma Grade I, 1 was a Pilocytic astrocytoma Grade I and 1 was an oligodendroglioma Grade II). The samples were rapidly frozen and stored at -85ºC to -75ºC after washing once with PBS (Mg^{2+} and Ca^{2+} free) until the time for the analysis of telomerase activity.

Extract preparation

Forty to a hundred ng of frozen tissues were cut into slices and were transferred to a sterile 1.5 ml RNase-free micro centrifuge tubes containing 200 µl of 1XCHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM mercaptoethanol, 0.5% CHAPS, 10% glycerol) for telomerase extraction. These mixtures were dispersed by homogenization with manual disposable pestles until a uniform consistency was achieved.

RNase inhibitor (Invitrogen, USA) was added to the 1XCHAPS lysis buffer prior to the extraction for a final concentration of 150 units/ml. Homogenized tissues were then left on ice for 30 minutes and centrifuged at 12,000g for 20 minutes at 4ºC. The supernatant was collected and the protein concentration was measured using the Bradford protein assay method. This protein determination was carried out using BioPhotometer (Eppendorf, Germany). A control cell pellet (10^6 cells) was provided in the kit and used as a positive telomerase extract control.

Telomerase activity assay

Telomerase activity was determined using the TRAP assay with the $TRAP_{EZE}$ Telomerase Detection Kit (Intergen Co, USA) based on an improved version of the original method described by Kim *et al* (1994). Certain procedures in the kit were minimally modified to enhance the detection sensitivity. When performing the TRAP assay, less than 1 µg of the protein extract was used, as recommended by the kit.

The PCR amplification was perform in a final volume of 50 µl reaction volume using less than 1 µg telomerase extracts, 1XTRAP reaction buffer, 1X dNTP mix, TRAP primer mix (RP, K1 primer and TSK1 template), TS primer, 2 units of Taq DNA Polymerase (Fermentas, USA) and PCR-Grade Water. When telomerase was present in the sample extracts, it added a number of

telomeric repeats to the 3′ end of a substrate oligonucleotide telomerase primer (TS primer) (5′-AATCCGTCGAGCAGAGTT-3′). Every sample extract to be evaluated was also tested for heat sensitivity as a negative control by incubating at 85ºC for 10 minutes prior to the TRAP assay. All PCR reactions (TRAP assay) were also performed with other controls, such as a telomerase-positive control, a primer-dimer/PCR contamination control and a quantitation control.

An incubation step for telomerase extension was carried out at 30ºC for 30 minutes. The extended products were then amplified by PCR using a telomerase primer and a reverse primer (RP). In addition, each reaction mixture contained a primer (K1) and a template (TSK1) for amplification of a 36-bp internal standard. Thirty-three amplification cycles, consisting of denaturising at 94ºC for 30 seconds, annealing at 59ºC for 30 seconds and extension at 72ºC for 30 seconds and ending with a holding period at 4ºC were used in a Mastercycler® Gradient (Eppendorf, Germany).

The TRAP reaction product was analyzed by electrophoresis in 0.5X Tris-borate EDTA buffer on 12.5% polyacrylamide non-denaturing gels (Mini-PROTEAN®II, Bio-Rad Laboratories) using a loading dye solution which contained 0.25% bromophenol blue, 0.25% xylene cyanol, 0.5 M EDTA, pH 8.0 and 50% glycerol. The electrophoresis was carried out at 80 volts for 2 hours and 30 minutes at room temperature. Then the gel was stained using a silver staining method.

RESULTS

All telomerase extracts from the samples had a protein concentration within the range, 10-500 ng/µl as suggested in the kit. Telomerase activity was considered positive when a ladder of products was observed starting of 50 bp, with 6 bp increments (TRAP products). A 36 bp internal positive control band was detected in every lane and was used to identify the non-informative specimen inhibitors of Taq DNA polymerase. Conversely, if the extract was telomerase negative, only the 36 bp internal control was observed.

The results of representative TRAP assays and the presence of TRAP products are shown in Fig 1. Telomerase activity in central nervous system tumors is summarized in Table 1. Overall,
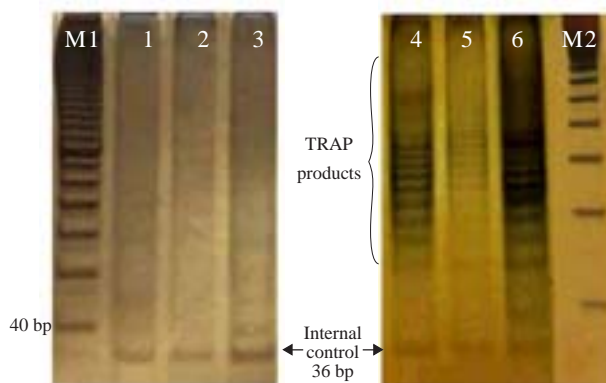

Fig 1–Results of the TRAP assay show the telomerase activity in controls and human central nervous system tumor samples. Lane M1 and M2 DNA ladder; lanes 1 and 2 negative controls for glioblastoma multiforme and meduloblastoma samples respectively; lane 3 contamination control; lane 4 meduloblastoma; lane 5 positive control (control cell pellet 106); lane 6 glioblastoma multiforme.

telomerase activity was detected in six cases of these tumors (30%). All samples of glioblastoma multiforme and meduloblastoma showed telomerase activity, whereas no telomerase activity was detected in any of the meningioma and schwannoma samples. None of the low-grade of gliomas except for the oligodendroglioma sample, demonstrated telomerase activity.

The different detection rates of telomerase activity between the age group 30 years old and below or more than 30 years old were not significant (p>0.05). Results also showed that there was no significant difference in the telomerase activity status between the sex groups of the patients (p>0.05) (Table 2). There was a significant association between telomerase activity status and the tumor grades of the samples (p<0.05).

DISCUSSION

In immortal cancer cells, telomerase activity was found to be reactivated in response to the onset of tumorigenesis (Wen *et al*, 1998). Recent studies demonstrated that an increase in telomerase activity may be associated with tumor progression and that it's level may be of prognostic value. In our study, telomerase activity was detected in 30% of central nervous system tumors, supporting the fact that activation of telomerase is an important feature for tumorigenesis.

In our study, telomerase activity was detected

Table 1
Telomerase activity in central nervous system tumors.

Tumor type	No. of tumors	Telomerase activity	
		Positive (%)	Negative (%)
Astrocytoma (I)	2	0 (0)	2 (100)
Pilocytic actrocytoma (I)	1	0 (0)	1 (100)
Oligodendroglioma (II)	1	1 (100)	0 (0)
Glioblastoma multiforme (IV)	2	2 (100)	0 (0)
Meningioma (I)	8	0 (0)	8 (100)
Schwannoma (I)	3	0 (0)	3 (100)
Paraganglioma (I)	1	1 (100)	0 (0)
Meduloblastoma (IV)	2	2 (100)	0 (0)
Total	20	6	14

Table 2
Association between telomerase activity status and criteria of patients and tumors.

Criteria		Telomerase activity			
		Positive (%)	Negative (%)	Total (%)	p value[a]
N		6	14	20	
Age (years)					
	≤ 30	3 (50)	4 (28.6)	7 (35)	p = 0.336
	> 30	3 (50)	10 (71.4)	13 (65)	
Sex					
	Male	4 (66.7)	4 (28.6)	8 (40)	p = 0.137
	Female	2 (33.3)	10 (71.4)	12 (60)	
Tumor grade					
	Low (Grade I-II)	2 (33.3)	14 (100)	16 (80)	p = 0.003
	High (Grade III-IV)	4 (66.7)	0 (0)	4 (20)	

[a]Fisher's exact test

in both glioblastoma multiforme (GBM) and oligodendroglioma samples, but undetectable in three (75%) of four low-grade glioma samples. Previously, telomerase activity was found in 83.3% (25/30) of GBM samples and 50% (1/2) of oligodendroglioma samples reported by Falchetti *et al* (1999). GBM are the most frequent glial tumors and represent the highest grade (Grade IV) of malignancy of diffuse astrocytic tumors (He *et al*, 2001). These findings suggest that telomerase is associated with the malignant progression of glial tumors.

In contrast, such activity was not detected in any schwannoma samples. Similarly, it was reported by Hiraga *et al* (1998) that telomerase activity was not detected in five samples of schwannoma. Telomerase activity was undetectable in our meningioma samples. Falchetti *et al* (1999) also reported that all meningioma samples analyzed showed the absence of telomerase activity, but this activity can be detected in anaplastic meningioma samples. These findings suggest that telomerase activity is undetectable in such benign tumor populations. Therefore, telomerase detection may help in differentiating benign meningiomas from meningiomas with aggressive biological behavior, where conventional histology may not be conclusive (Falchetti *et al*, 1999).

In this study, telomerase activity was detected in both (100%) of meduloblastoma samples and showed the same results as reported by Sano *et al* (1998). Their findings indicated that posi-

tive telomerase activity was strongly associated with malignant tumor types and was rare in benign, non-glial tumors. According to Holt *et al* (1996), there are some types of cancer that would be expected to be mortal and be negative for telomerase activity. This is because the cancer is diverse, some tumors may need only a few mutations in order to become malignant and may not exhaust the normal limits of proliferation before they cause disease.

It is important to investigate the correlation between telomerase activity and clinical-pathological features, such as tumor size, histopathological grade, stage, clinical outcome and genetic alterations. Sharma *et al* (1998) found an association between telomerase activity and the histological grading of the tumors analyzed where the more anaplastic the lesions, the greater the telomerase activity. Our results showed that telomerase activity was detected in most of the high-grade tumors (66.7%). Our study revealed there was no significant correlation between telomerase activity status and the age and sex of the patients. Further investigations or a larger number of patients will be required in order to confirm this association.

In conclusion, our study shows that telomerase activity can be detected in various types of central nervous system tumors using this non-isotopic method. This activity may play an important role in the carcinogenesis of these tumors. Telomerase activity status also may have clinical utility in cancer screening, diagnosis and monitoring treatment efficacy.

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