ORIGINAL ARTICLE

Presence of Telomerase Activity with Undetectable p16 Gene Mutation in Malaysian Patients with Brain Tumor

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Summary

Recent study has shown that activation of the telomerase and p16 gene mutation are both necessary for tumorigenesis. Our objectives were to detect telomerase activity and investigate the possibility of p16 gene mutations in various types of brain tumor. We analyzed 23 tumor tissues collected in 2000 to 2002. Telomerase activity was detected by a TRAP assay using a TRAPEZE Telomerase Detection Kit (Intergen, Co). PCR-SSCP (Single Strand Conformation Polymorphism) analysis was performed to screen for p16 gene mutation at exon 1 and 2. The activity was detected in 26.1% of the brain tumor samples and mostly present in high-grade tumors. There was a significant association between telomerase activity status and tumor grade but not with patient criteria. Telomerase activity was detected in the analyzed tumors, supporting the fact that activation of telomerase is an important feature for tumorigenesis. There was no mobility shift of p16 gene using SSCP and suggested no mutation at exon 1 and 2 occurred in all samples. These results suggest that another mechanism of p16 gene alterations could be involved and associated with detectable telomerase activity in the progression of tumors.

Key Words: Telomerase activity, p16 gene, Mutations, Brain tumors, Tumorigenesis

Introduction

The telomere and telomerase hypothesis of aging and cancer is based on the findings that most human tumors have telomerase activity while almost all normal human somatic cells do not. This hypothesis has become a new model for some aspects of cancer progression[1]. It also has been reported that telomerase is active in the germ cells, as well as some stem cells, but is inactive in most somatic cells[2]. Telomeres are special structures that provide protection and maintain chromosome stability[3]. It consists of thousands of repetitive sequences of TTAGGG ranging from 5 to 20 kb[4]. Both telomerase and telomeres have been identified as targets for anticancer therapy since there is evidence of a strong correlation between telomerase reactivation, cellular immortalization and cancer[5]. Currently, numerous studies are underway to discover and understand the importance of telomerase in future therapies of human cancer.

Over the last decade, a lot of ideas have emerged about the genetic alterations that occur in human cancers and how they contribute to tumorigenesis[6]. Human brain tumors are among the most rapidly fatal of all cancers and new molecular strategies should be explored to cure these tumors in the future. Gliomas are the most common primary tumors arising in the human brain, and a large majority of them are astrocytic tumors[7]. The progression of malignant gliomas may result from

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multi-step accumulation of genetic alterations, including activation of oncogenes and inactivation of tumor suppressor genes[7]. Tumor suppressor genes are one of the cancer-related genes involved in malignant transformation[8]. Genetic evidence has accumulated that p16 gene (MTS1/CDKN2A/INK4A) which is located at chromosome 9p21 is involved in tumorigenesis and plays a major role in regulating the cell cycle at the G_1-S check point.

The p16 gene was firstly demonstrated in 1994 by Kamb, et al [9]. This gene is composed of three exons, which encode a 156 amino acid, 15.8 kD protein that blocks progression of the cell cycle, have indirectly preventing cells from entering into the S phase through the G_1 phase[10,11]. Loss of this protein function may lead to cancer progression by allowing unregulated cellular proliferation[10]. High frequency mutations and deletions of this gene in human cancer cell lines has been suggested as an important role in the occurrence of many types of cancer. Alterations of p16 gene are known to occur in many primary tumors via different mechanisms including homozygous deletion, point mutation and hypermethylation of p16 gene promoter.

Although scientific research has been carried out on p16 gene and telomerase enzyme separately, few researchers have studied the relationship between them[12,13,14]. According to a previous study, there is a correlation between telomerase activity and homozygous deletions of the p16 gene in liver metastases of colorectal carcinoma, suggesting its crucial role in liver metastases[13]. It has been reported that alteration of cell cycle progression such as the p16/pRb pathway may cooperate with telomerase activation during cellular immortalization and tumor progression[14,15].

In this study, we investigated the detection of telomerase activity and the possibility of p16 gene mutations in human brain tumors from Malaysian patients.

Materials and Methods

Tissue Samples
Tissue samples of brain tumors were obtained from 23 patients undergoing surgery at the Hospital of Universiti Sains Malaysia in 2000 to 2002. These samples were banked at the Brain Tumor Tissue Bank of the Neuroscience Unit in the same institution. The tumors were classified according to the World Health Organization (WHO) brain tumor scheme.

DNA and telomerase extraction
DNA was extracted from tumor tissues using commercial extraction kits, according to standard protocol as suggested by the manufacturer (Boehringer Manneheim, Germany). Telomerase extract preparation was carried out using a CHAPS detergent-based extraction from frozen tissues. Tissues were homogenized using a manual disposable pestles until a uniform consistency was achieved. Tissue extracts were collected and the protein concentration was measured using the Bradford protein assay method.

PCR-SSCP Analysis
The p16 gene mutations at exon 1 and 2 were screened by Single-Strand Conformational Polymorphism (SSCP) analysis of PCR products. Four sets of oligonucleotide primers purchased from Operon Inc. (USA) were used to amplify both exons of the p16 gene as previously described[9]. SSCP analysis for exon 1 was performed according to a previous original method with a slight modification[16]. PCR products of exon 2 were digested with 10 U of restriction enzyme Sma I (Boehringer Mannheim, Germany) for 8 hours at 25°C as previously reported[17]. Eight µl of each PCR products was added to 4 µl of sequencing stop solution (95% formamide, 0.5 M EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 94°C for 4 minutes. The samples were chilled on ice before loading onto the gel. PCR products of exon 1 were electrophoresed using 10% polyacrylamide gel at room temperature (22-24°C) using 2 watts constant for 16 hours. While PCR products of exon 2 were loaded onto the 0.5X MDE gel containing 5% glycerol and 1X TBE buffer at 12 watts constant for 6 hours and 30 minutes. Electrophoresis of exon 1 was carried out in the cold room and the temperature was set at 17°C. SSCP analysis was performed using Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories, USA). After the electrophoresles was completed, the gels were stained with silver staining method.

Telomerase activity assay
Telomerase activity was determined based on the PCR-Telomeric Repeat Amplification Protocol (TRAP) assay using the TRAPeze Telomerase Detection Kit (Intergen Co., USA) which is derived from an improved version of the original method described by Kim, et al18. 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 silver staining method.

Results

All telomerase extracts from tissue 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 at 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. When the 36 bp internal control was not visible in the sample lane, it indicated the presence of PCR inhibitors. The intensity of the internal control band was very weak when the telomerase activity in the sample extracts was too high. It has been recommended the sample extracts be diluted with CHAPS buffer and reanalysed to observe the internal control band together with the TRAP products. Conversely, if the extract was telomerase negative, only the 36 bp internal control was observed.

Results of representative TRAP assays and the presence of TRAP products are shown in Figure 1. Telomerase activity in various type of brain tumors is summarized in Table I. Overall, telomerase activity was detected in six cases of these tumors (26.1%) and mostly present in high-grade tumors. Results of Fisher's Exact Test showed that there was a significant association between telomerase activity status with tumor grades of samples (p=0.008) (Table II). There was no significant association between this activity with sex and age of the patients.

In mutation screening analysis of p16 gene at exon 1 and 2, there was no mobility shift observed in all tumors analysed, suggesting that no mutation occurred (Figures 2 and 3).

Table I: Telomerase activity in brain tumors

Tumor type (Grade)	Total	Telomerase activity	
		Positive	Negative
		(%)	(%)
Astrocytoma (I)	2	0 (0)	2 (100)
Pilocytic Astrocytoma (I)	1	0 (0)	1 (100)
Oligodendroglioma (II)	1	1 (100)	0 (0)
Ependymoma (II)	1	0 (0)	1 (100)
Anaplastic astrocytoma (III)	1	0 (0)	1 (100)
Glioblastoma multiforme (IV)	2	2 (100)	0 (0)
Meningioma (I)	8	0 (0)	8 (100)
Schwannoma (I)	4	0 (0)	4 (100)
Paraganglioma (I)	1	1 (100)	0 (0)
Meduloblastoma (IV)	2	2 (100)	0 (0)
Total	23	6	17

Table II: Association between telomerase activity status with criteria of patients and tumors

Criteria		Total	Telomerase activity		p value*
			Positive (%)	Negative (%)	
N		23	6	17	
Age (years)					
	≤ 30	8	3 (50)	5 (29.4)	p = 0.334
	> 30	15	3 (50)	12 (70.6)	
Sex					
	Male	11	4 (66.7)	7 (41.2)	p = 0.275
	Female	12	2 (33.3)	10 (58.8)	
Tumour grade					
	Low (Grade I-II)	18	2 (33.3)	16 (94.1)	p = 0.008
	High (Grade III-IV)	5	4 (66.7)	1 (5.9)	

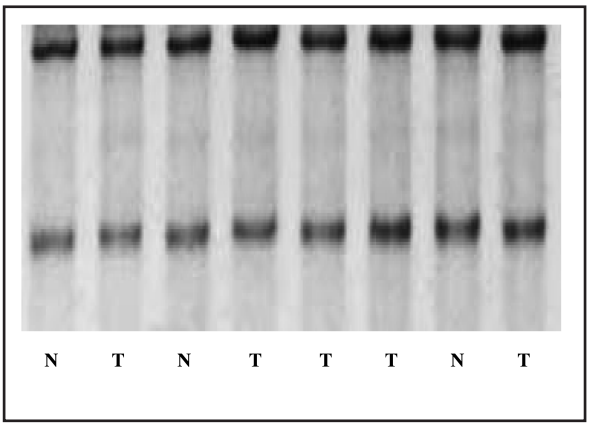

Fig. 1: Results of the TRAP assay show the telomerase activity in controls and brain tumor samples. Lane M DNA ladder; Lane 1 PCR contamination control; Lane 2 GBM; Lane 3 positive control (control cell pellet 10^6); Lane 4 Paraganglioma; Lane 5 and 7 Medulloblastoma (telomerase activity in the tumor extracts is too high, Lane 5 Extract A & Lane 7 Extract B); Lane 6 negative control (heat-treated sample extract) for GBM.

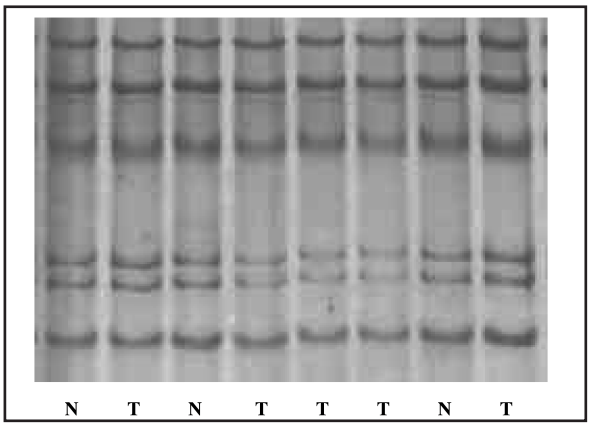

Fig. 2: SSCP analysis of the PCR products of exon 1 p16 gene. No mobility shift was observed. N: Normal sample. 1: Tumor sample

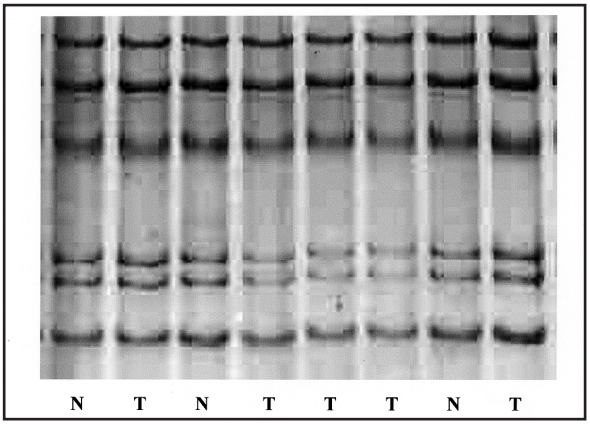

Fig. 3: SSCP analysis of the PCR products of exon 2 p16 gene. No mobility shift eas observed. N: Normal sample. 1: Tumour sample

Discussion

Detection of telomerase activity in analyzed tumors (26.1%) supports the fact that activation of telomerase is an important feature for tumorigenesis. In our study, telomerase activity was detected in both GBM and oligodendroglioma samples. Previously, telomerase activity was found in 83.3% (25/30) of GBM samples and 50% (1/2) of oligodendroglioma samples[19]. A recent study suggested that telomerase activity may be used as a tumor marker and its activation may be associated with tumor progression of astrocytic tumors[7]. In contrast, such activity was not detected in all schwannoma samples. Similarly, it was reported by Hiraga, et al that telomerase activity was not detected in five samples of schwannoma [20].

Telomerase activity was also undetectable in our meningioma samples. It has been reported that in benign and premalignant tumors, including breast fibrocystic disease and fibroadenomas, anaplastic astrocytoma and benign meningiomas, in general no telomerase activity was detected. However, it was found in malignant tumor stages[21,22]. It has been reported that there is an association between telomerase activity and histological grading of the tumors analysed where the more anaplastic the lesions, the greater the telomerase activity[23]. Our results showed that telomerase activity was detected in most of the high-grade tumors (66.7%) and there was a significant association between telomerase activity with high-grade tumors.

In this study, we found no mutation of p16 gene at exon 1 and 2 in our samples, suggesting that this gene could not be involved in the genesis of brain tumors in Malaysian patients. Previous studies also showed that this gene was often mutated in glioblastoma multiforme (GBM) cases and the frequency of p16 gene mutations were still low [24,25]. According to Ueki, et al, out of 24 GBM and 30 anaplastic astrocytoma cases, only one mutation of exon 2 p16 gene was detected[26]. Another inactivation mechanism of p16 gene may involved in the development of these tumors and suggests that p16 gene mutations do not play a major role in development of brain tumors in this study. In conclusion, our study showed the presence of telomerase activity with undetectable p16 gene mutations in a series of Malaysian brain tumor patients. Further studies should be carried out using more brain tumor samples to determined whether another tumor suppressor gene might be involved in brain tumor progression and its association with detectable telomerase activity.

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