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5′ CpG Island Methylation of p16 is Associated with Absence of p16 Expression in Glioblastomas

Recent evidence shows that transcriptional silencing as a consequence of hypermethylation of CpG islands is an important mechanism in the inactivation of $p16^{INK4a}$ tumor suppressor gene. This study is designed to clarify the significance of $p16^{INK4a}$ hypermethylation in 23 cases of glioblastomas (GBMs) by methylation-specific polymerase chain reaction (PCR) and p16 immunostaining. Fourteen cases (60.9%) out of 23 GBMs revealed hypermethylation on *p16*. p16 immunostaining revealed that 13 (93%) of these 14 hypermethylation cases showed complete loss of immunoreactivity and only one (7%) case retained immunoreactivity. Among 9 methylation-negative cases, 4 were immunonegative, which might be related to mutations or deletions other than hypermethylation. The most significant finding was that of 17 cases with immunonegativity, 13 cases (76.5%) showed hypermethylation. We reconfirmed that *p16* hypermethylation may be one of the major mechanisms of tumorigenesis of GBMs and the results between the methylation specific-PCR study and p16 immunostaining had a good correlation.

Key Words: *p16; Hypermethylation; Glioblastoma; Immunohistochemistry; Methylation Specific-PCR Study*

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INTRODUCTION

Malignant transformation of human solid tumors is thought to require a series of genetic alterations, including tumor suppressor genes, oncogenes, and chromosomal abnormalities. The p16 protein, located on 9p21, previously identified as an inhibitor of the cyclin-dependent kinase 4 (cdk4), blocks cdk4-dependent phosphorylation of the retinoblastoma (Rb) protein (1) and acts as a negative regulator of cell proliferation. The *p16* gene has been found to be homozygously deleted in a variety of human malignant tumors, suggesting that *p16* gene is a putative tumor suppressor gene (2, 3).

Different mechanisms have been reported for inactivation of the *p16* gene, including homozygous deletion, missense mutation, frame-shift mutation and hypermethylation of the *p16* gene promoter (4-7). The *p16* homozygous deletion and hypermethylation of the *p16* gene are frequently observed in various human malignant tumors, such as squamous cell carcinoma of head and neck cases (4, 8-12). Among gliomas, loss of p16 expression or alteration of *p16* genes are frequently observed in high grade astrocytic tumors but infrequently in non-astrocytic tumors in human (13, 14).

This study was performed to clarify the significance of $p16^{INK4a}$ hypermethylation in 23 cases of glioblastomas (GBMs) by methylation-specific PCR and p16 immunostaining.

MATERIALS AND METHODS

Twenty-three cases of GBMs were retrieved from the files of the Asan Medical Center between 1997 and 1999. All were primary GBMs. The mean age was 45.3 year old (age range: 1 to 74) (Table 1). The male to female ratio was 11 to 12. The majority of lesions were located in the cerebral hemisphere.

P16 immunohistochemistry

Expression of p16 protein was determined by a polyclonal p16 antibody (Santa Cruz Co., CA, U.S.A.). After deparaffinization and rehydration, the sections were subjected to high temperature antigen unmasking in citrate buffer in a microwave for 3 min After blocking in 2% skim milk, the sections were incubated with the p16 antibody at a dilution of 1:300 for 60 min at room temperature. The sections were incubated with mouse anti-rabbit immunoglobulins (dilution 1:25: DAKO) and ex-

Table 1. Summary of 23 glioblastoma cases with results of p16 immunostaining and methylation specific PCR

Patient No.	Sex/Age (year)	Site	p16 immunostaining	Methylation specific PCR
1	F/41	Lt P-O-temporal	+	+
2	F/57	Rt temporal	-	+
3	M/54	Lt temporal	-	+
4	F/32	Rt frontal	-	+
5	M/42	Rt frontal	+	-
6	M/57	Not available	+	-
7	F/61	Rt medial temporal	-	+
8	M/45	Rt temporal	+	-
9	M/43	Lt parietal	-	+
10	M/39	Not available	+	-
11	M/53	Lt P-occipital	-	+
12	F/74	Rt P-occipital	-	+
13	F/40	Rt F-parietal	-	+
14	F/29	Lt frontal	-	+
15	M/62	Rt frontal	-	+
16	F/48	Lt & Rt frontal	-	+
17	M/1	Rt P-occipital	-	+
18	M/23	Rt temporal	-	+
19	M/56	Lt parietal	-	-
20	F/51	Rt frontal	+	-
21	M/24	Lt parietal	-	-
22	M/46	Rt temporal & basal ganglia	-	-
23	M/63	Rt, temporal	-	-

F, frontal; P, parietal; O, occipital

pression was detected by the peroxidase labeled streptavidin biotin complex technique according to the manufacturer's recommendations. Hematoxylin counter staining was performed. More than 10% positive nuclei per high-power field were interpreted as cases with positive staining (15). Variable cytoplasmic staining was considered to be nonspecific. Entrapped lymphocytes were used as positive control and for negative control, primary antibodies were omitted.

DNA extraction

Genomic DNA were extracted from formalin-fixed and paraffin-embedded tissue blocks. 4 μm thick sections were obtained from the paraffin blocks with a disposable scalpel blade. Briefly, the sections were dewaxed and incubated in 50 mM of Tris, pH 8.3, containing 200 ng/μL proteinase K, at 55℃ for about 16 hr. After phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation, pellets were washed with 70% ethanol, dried, and resuspended in sterile water. Extracteded DNA was modified by sodium bisulfite to determine the methylation status by methylation-specific PCR as previously described (8).

Methylation-specific PCR

All samples were evaluated for the presence of 5' CpG island methylation using the recently described method of methylation-specific PCR (8). Primer pairs are shown in Table 2. The PCR mixture contained 1XPCR buffer (10 mM Tris/pH 8.3/50 mM KCl/1.5 mM $MgCl_2$), dNTPs (each at 0.2 mM), primers (10 pmole each), and bisulfite-modified DNA (30 to 50 ng) or unmodified DNA (30-50 ng) in a final volume of 25 μL. Reactions were hot-started at 95℃ for 5 min before the addition of 0.75 units of *Taq* polymerase (TaKaRa Shuzo Company). Amplification was performed in a thermal cycler for 35 cycles (1 min at 94℃, 1 min at 65℃ and 1 min at 72℃), followed by a final 4-min extension at 72℃.

Table 2. PCR primers used for methylation specific-PCR

Primer	Sense, 5'→3'	Antisense, 5'→3'	Size, (bp)	Genomic position
P16-M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	151	+167
P16-U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	150	+167

P16-M, p16 methylated; p16-U, p16 unmethylated

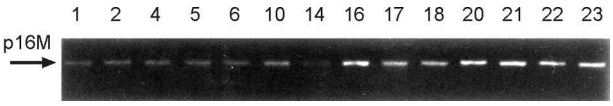

Fig. 1. Result with methylation specific PCR. Fourteen cases reveal hypermethylation bands on *p16*.

Controls without DNA were performed for each set of PCRs. The PCR product (10 μL) was run with 2.5% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

RESULT

Fourteen cases (60.9%) and 17 (73.9%) cases out of 23 GBMs revealed hypermethylation on *p16* (Fig. 1) and p16 immunonegativity, respectively. p16 immunostaining revealed that of 14 hypermethylation cases 13 cases (93%) showed complete loss of immunoreactivity and only one (7%) case showed p16 immunoreactivity (Fig. 2). Among 9 methylation-negative cases, 4 were immunonegative. The most important result in this study was that of 17 cases with negative p16 immunostaining, 13 cases (76.5%) showed hypermethylation (p=0.018) (Table 3).

DISCUSSION

Malignant astrocytic neoplasm arises either from the progression of low grade precursor lesions or de novo origin, and it contains distinct molecular events responsible for tumor formation and progression. According to Louis (16), the growth of low grade astrocytomas gains stimulus from the platelet derived growth factor system coupled with inactivation of the *p53* gene, which may lead to decreased cell death with slow net growth. Such cells would also get defective responses to DNA damage and impaired DNA repair, setting the stage for future malignant change. Anaplastic astrocytomas further display release of a critical cell cycle brake that involves the *CDKN2/p16*, *Rb* and *CDK 4* genes. This results in mitoses and rapid growth of the tumors. Finally, GBMs

Table 3. Summary of the results of methylation specific PCR & p16 immunostaining

Hypermethylation	p16 protein		
	Immunopositive	Immunonegative	Total (%)
Positive	1	13	14 (60.9%)
Negative	5	4	9 (39.1%)
Total (%)	6 (26.1%)	17 (73.9%)	23 (100%)

p=0.018

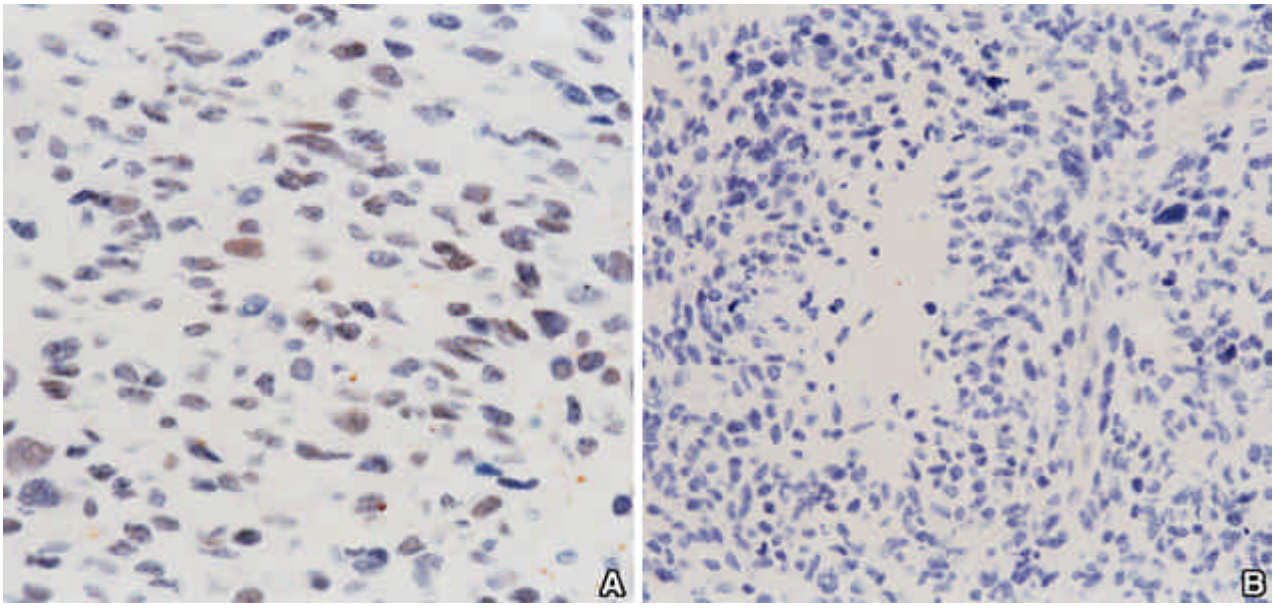

Fig. 2. Glioblastoma with p16 immunoreactivity on A and glioblastoma with p16 immunonegative case on B. Positive reaction reveals brown nuclear staining (p16 immunostaining A: ×400, B: ×200).

may emerge from the microenvironmental outgrowth of more malignant clones in a complex vicious cycle that involves necrosis, hypoxia, growth factor release, angiogenesis and clonal selection; growth signals mediated by activation of epidermal growth factor receptors may precipitate GBMs (16).

In GBMs, deletion of *p16* tumor suppressor gene is common molecular event and found in 40-60% of cases (17-19).

Loss of p16 expression seems to play a role in glioma progression, since loss of expression is infrequent in low-grade astrocytomas (0 to 16%) but common in anaplastic astrocytomas and GBMs (>50%) (15, 20). In pediatric GBMs, loss of p16 protein expression was observed in 63% of cases. Deletions of the gene are a common mechanism of inactivation of p16 gene in GBMs; however, there are cases with loss of p16 protein expression, with wild type alleles (with no deletion of *p16* gene) in about 40% (4, 20, 21). These cases could be explained by other mechanisms and hypermethylation is one possible mechanism for it (4, 20). Interestingly, p16 nuclear expression was not detectable immunohistochemically in GBMs harboring a mutant *p53* gene because 40% of GBMs carrying *p53* mutations revealed loss of p16 expression (6, 15).

In our study, the results between the methylation status and p16 immunostaining pattern showed a good correlation. Loss of p16 protein expression was seen in 73.9% (17/23) of GBMs and among them 76.5% (13/17) showed hypermethylation, suggesting that hypermethylation might be an important mechanism of *p16* gene inactivation in GBMs ($p=0.018$).

Among 9 methylation-negative cases, 4 were immunonegative, which might be related to mutations or deletions other than hypermethylation.

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