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Loss of Heterozygosity on Chromosome 10, 13q(Rb), 17p, and p53 Gene Mutations in Human Brain Gliomas

Seung-Hoon Lee, M.D., Ph.D.,[1] Jong-Hyun Kim, M.D., Ph.D.,[1]
Chang-Hun Rhee, M.D.,[1] Young-Soon Kang, M.S.,[2] Je-Ho Lee, M.D., Ph.D.,[2]
Seok-Il Hong, M.D., Ph.D.,[3] Kil-Soo Choi M.D., Ph.D.[4]

Department of Neurosurgery,[1] Laboratory of Biochemistry,[2]
Laboratory of Cell Biology,[3] Korea Cancer Center Hospital,
Department of Neurosurgery, College of Medicine, Seoul National University,[4] Seoul, Korea

Using the methods of restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) analyses, we have examined 33 cases of human gliomas with various malignant grades to detect the deletions of putative tumor suppressor gene loci, chromosome 10, 13q(retinoblastoma gene, Rb), 17p, and p53 mutation. We observed loss of heterozygosity (LOH) at loci on chromosome 10 (36 %), 13q(Rb) (54 %), and 17p(50 %) in malignant gliomas. There, however was no allelic loss on chromosome 10 and 17p in low-grade gliomas. Rb gene deletions were seen in low-grade gliomas, including oligodendroglioma and ependymoma. This finding suggests that Rb inactivation may be an early genetic event in the development and progression of gliomas. We correlated the results of LOH on chromosome 17p and p53 mutation. Among the 8 cases which showed LOH on chromosome 17p, only three cases (38 %) revealed p53 mutations. Low incidence of p53 mutations in cases with chromosome 17p deletions suggests that some other tumor suppressor genes may be located on chromosome 17p.

Key Words : *Loss of heterozygosity(LOH), Glioma, Tumor suppressor gene, p53 gene, Rb gene*

INTRODUCTION

Gliomas, the most common primary tumors of the human central nervous system, are usually malignant and virtually incurable(Walker et al., 1978 ; Russel and Rubinstein, 1989). They can be classified according to their cellular differentiation ; astrocytoma, oligodendroglioma, and ependymoma(Russel and Rubinstein, 1989). The majority of glial tumors are astrocytomas, which typically progress through three histologically defined stages : one premalignant stage, astrocytoma, and two malignant stages, anaplastic astrocytoma and glioblastoma multiforme(GBM)(Mueller et al., 1977).

LOH studies in malignant astrocytomas have revealed consistent and frequent allelic losses for the loci on chromosome 10, 13q(Rb), 17p and 22q, suggesting the presence of tumor suppressor genes on these chromosomes(James et al., 1988 ; El-Azouzi et al., 1989 ; Reissman et al., 1989 ; Venter et al., 1991 ; Venter and Thomas, 1991 ; Ahmed Rasheed et al., 1992 ; Frankel et al., 1992 ; Fults et al., 1992 ; von

Address for correspondence : *Seung-Hoon Lee, M.D., Ph.D., Department of Neurosurgery, Korea Cancer Center Hospital, 215-4 Gongneung-dong, Nowon-gu, Seoul 139-240, Korea. Tel : 82(02)974-2501, Fax : 82(02)978-2005.*

Deimling et al., 1992). In addition, point mutations at exon 5—8 of the p53 gene have been detected in GBMs(Fults et al., 1990 ; Mashiyama et al., 1991 ; Ohgaki et al., 1991 ; Frankel et al., 1992). Furthermore, several groups reported LOH for the loci on chromosome 17p and p53 mutations in astrocytoma as well as GBMs(Venter and Thomas, 1991 ; Sidransky et al., 1992). Sidransky et al.(1992) identified new additional point mutations of the p53 gene in recurrent astrocytomas and GBMs.

On the basis of these data, p53 mutation and/or LOH for the loci on chromosome 17p may be important genetic changes in the earlier stage, and LOH for the loci on chromosome 10, 13q (Rb), and 22q may be necessary in the progression to the most malignant phenotype, GBM, in the later stage(James et al., 1988 ; Venter and Thomas, 1991 ; Fults et al., 1992 ; Sidransky et al., 1992). However, there are few reports about inactivations of these tumor suppressor genes in oligodendrogliomas and ependymomas. We evaluated 33 gliomas of various degrees of malignancy for LOH on the regions of chromosome 10, 13q (Rb), and 17p. We also looked for mutations in the p53 gene using PCR-SSCP analysis. The goal of this study was to determine if there were differences between low-grade and malignant gliomas in the frequency of LOH at these putative tumor suppressor genes loci. In addition, we wanted to determine the correlationship between LOH on chromosome 17p and p53 mutation. Finally, we tried to determine differences between oligodendrogliomas and astrocytomas, if any.

MATERIALS AND METHODS

Tissue specimens

Surgical tumor specimens were obtained from 33 glioma patients and were stored at —70℃. Constitutional DNA was obtained from peripheral leucocytes of the corresponding patients. The group of 33 gliomas consisted of 6 astrocytomas, 2 anaplastic astrocytomas, 13 GBMs, 6 oligodendrogliomas, 3 anaplastic oligodendrogliomas, and 3 ependymomas. (WHO classification)

Southern blot analysis

The isolation of high molecular weight DNA from tumor and leucocytes, appropriate restriction endonuclease digestion, agarose gel electrophoresis, and transfer on to nylon membrane were performed according to the standard protocols(Southern, 1975). Probes were radiolabeled by using the random primer method(Feinberg and Vogelstein, 1984). The nylon membranes were hybridized, washed, and autoradiographed at —70℃. The following polymorphic DNA probes were used in the RFLP analysis : chromosome 10, pMHZ15(D10S17), pEFD70.2 (D10S26) ; chromosome 13q, H3-8(Rb 1) ; chromosome 17p, pYNZ22.1(D17S5), p144D6(D17S34). The probes used in this analysis were obtained from the Repository of Human DNA Probes and Libraries (American Type Culture Collection, Rockville, MD).

PCR-LOH analysis in Rb gene

Normal and tumor DNAs were amplified with primers flanking an Xbal RFLP within intron 17 as described by McGee et al.(1990). PCR products were digested with Xbal and electrophoresed on 2 % agarose gel.

Primers used in PCR-LOH analysis were as follows : 5′-TTCCAATGAAGAACAAATGG-3′, 3′-TTGAACCTAACACGTTAACG-5′

PCR-SSCP analysis to detect p53 mutations

This analysis was performed to detect p53 mutations in the region between exon 5 and 8 as described by Orita et al.(1989) with a slight modification. Each exon was amplified by PCR from 100ng of genomic DNA using a GeneAmp kit (Perkin-Elmer Cetus) and labeled with [^{32}P]dCTP. Thirty five cycles of amplification were carried out in an automatic DNA Thermal Cycler (Perkin-Elmer Cetus). After heat denaturation, the diluted PCR products were loaded onto 6 % polyacrylamide nondenaturating gels. Gels were electrophoresed at 4℃ for 3—5 hours at 30 W and exposed to X-ray films at —70℃ for 6 to 72 hours.

Primer sets used in PCR-SSCP analysis of the p53 gene : exon5 : 5′-GGATCCTTACTCCCCTGCCCTCAACAA-3′, 3′-GAATTCAACCAGCCCTGTCGTCTCTC-5′, exon 6 : 5′-ACCATGAGCTGCTCAGAT-3′, 3′-AGTTGCAAACCAGACCTCAG-5′, exon 7 : 5′-GTGTTGTCTCCTAGGTTGGC-3′, 3′-CAAGTGGCTCCTGACCTGGA-5′, exon 8 : 5′-CCTATCCTGAGTAGTGGTAA-3′, 3′-CCAAGACTTAGTACCTGAAG-5′

RESULTS

We divided the total 33 cases into two groups, malignant gliomas (18 cases) and premalignant, low-grade gliomas (15 cases). Anaplastic astrocytoma, anaplastic oligodendrogliomas and GBMs belonged to the group of malignant gliomas. The group of low-grade gliomas consisted of astrocytomas, oligodendrogliomas and ependymomas(Table 1).

LOH on chromosome 10

LOH for these loci were seen in 5 out of 14 informative malignant gliomas(36 %), and this figure including LOH in 4 out of 9 informative GBMs (44 %), and 1 out of 2 informative anaplastic astrocytomas(50 %). There was no evidence of LOH on chromosome 10 in 11 informative low-grade gliomas(Table 1).

LOH on chromosome 13q(Rb)

To determine the frequency of deletion of Rb gene, we did a RFLP analysis with Southern blot and a PCR-LOH study. LOH at Rb loci were found in not only 7 out of 12 informative cases of malignant gliomas (54 %), but also 3 out of 12 informative cases of low-grade gliomas(25 %)(Table 1).

LOH on chromosome 17p

Eight out of 16 informative cases of malignant gliomas (50 %) showed LOH for loci on chromosome 17p and this figure included LOH in 4 out of 11 informative GBMs(36 %), 2 out of 2 informative anaplastic astrocytomas (100 %) and 2 out of 3 informative anaplastic oligodendrogliomas (67 %). However, there was no LOH in 12 informative cases of low-grade gliomas(Table 1).

p53 Mutations in Human Gliomas

A total of 33 gliomas was analyzed by SSCP to screen for sequence mutations at exon 5−8 of the p53 gene. The significant mobility shifts that indicate sequence mutations of p53, were observed in 3 out of 13 cases of GBMs(23 %), 1 out of 2 cases of anaplastic astrocytomas (50 %), and 1 out of 3 cases of anaplastic oligodendrogliomas (33 %). Overall frequency of p53 mutations in malignant gliomas was 28 % (5 out of 18 cases). However, there was no p53 mutation in low-grade gliomas. The mutations were located in exon 5 (3 cases) or exon 7 (2 cases).

Comparison of LOH on Chromosome 17p with Frequency of p53 Mutation

Among the 8 cases which showed LOH on chromosome 17p, only three cases(38 %) revealed p53 mutations. All of 5 cases with p53 mutation were informative for polymorphic DNA probes on chromosome 17p, and three of them(60 %) showed LOH on chromosome 17p.

Differences between oligodendrogliomas and astrocytomas

LOH for loci on chromosome 10 was demonstrated in 4 out of 9 GBMs and 1 out of 2 anaplastic astrocytomas, but not in 3 cases of anaplastic oligodendrogliomas. There was no remarkable difference in frequencies of LOH on chromosome 13q and 17p between oligodendrogliomas and astrocytomas(Table 2).

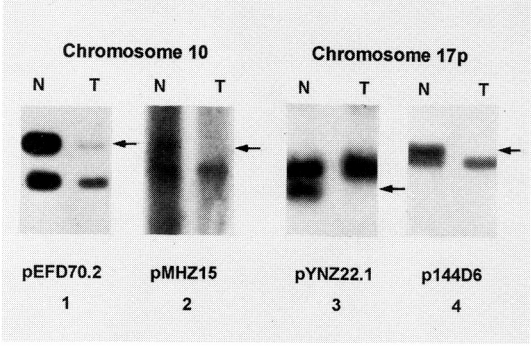

Fig. 1. Loss of heterozygosity on chromosome 10 and 17q. Southern blots on normal (N) and tumor (T) from four cases of glioblastoma multiforme probed with pEFD70.2 (lane 1), pMHZ15 (lane 2), pYNZ22.1 (lane 3), and p144D6 (lane 4). Arrows indicate allelic losses in tumor DNA.

DISCUSSION

We observed frequent LOH at loci on chromosome 10 (36 %), 13q (Rb) (54 %), 17p (50 %) in malignant gliomas. Although our results are in agreement with the findings of other authors, we found several interesting points. Our study demonstrated that Rb gene deletions occurred in low-grade gliomas as well as malignant gliomas. The individuals who have inherited a germline mutation, have high incidences of

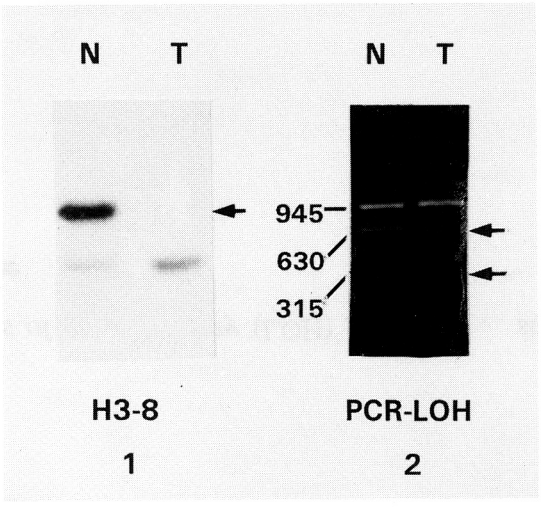

Fig. 2. Loss of heterozygosity at Rb locus. Southern blot on normal (N) and tumor (T) from one case of glioblastoma multiforme probed with H3-8 (lane 1). In lane 2, one case of low-grade oligodendroglioma, DNA was amplified using PCR primer set flanking a XbaI RFLP site in intron 17 of the Rb gene, A 945- base pair amplicon was created. PCR products were digested with XbaI and run on an agarose gel. In normal DNA (N), two cut allele are observed, 630-and 315-base pair bands. As shown, the intensity of the cut bands is decreased in tumor (T). Arrows indicate allelic deletions.

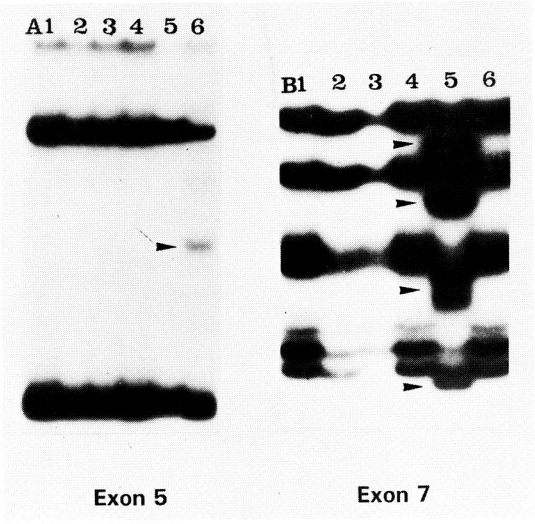

Fig. 3. Results of PCR-SSCP analyses on exon 5 (A) and 7 (B) of p53 gene. Mobility shifts are observed in lane A6 (one case of glioblastoma multiforme) and B5 (one case of anaplastic oligodendroglioma). Arrowheads indicate abnormal shifted bands.

Table 1. LOH and p53 Mutations in Malignant and Low-grade Gliomas.

Tumor type	Loss of heterozygosity(LOH)			p53 mutation
	Chrom. 10	Chrom. 13q(RB)	Chrom. 17p	
Malignant gliomas (18 cases)				
Glioblastoma multiforme	4/9	6/10	4/11	3/13
Anaplastic astrocytoma	1/2	1/2	2/2	1/2
Anaplastic oligodendroglioma	0/3	0/1	2/3	1/3
Total LOH (%)	5/14(36%)	7/13(54%)	8/16(50%)	5/18(28%)
Low-grade gliomas (15 cases)				
Astrocytoma	0/5	1/5	0/6	0/6
Oligodendroglioma	0/4	1/5	0/4	0/6
Ependymoma	0/2	1/2	0/2	0/3
Total LOH(%)	0/11	3/12(25%)	0/12	0/15

Table 2. LOH and p53 Mutations in Different Pathological Diagnoses.

Pathological Diagnosis	Loss of heterozygosity(LOH)			p53 mutation
	Chrom. 10	Chrom. 13q(RB)	Chrom. 17p	
Glioblastoma multiforme & anaplastic astrocytoma(15 cases)	5/11(45%)	7/12(58%)	6/13(46%)	4/15(27%)
Astrocytoma(6 cases)	0/5	1/5(20%)	0/6	0/6
Anaplastic oligodendroglioma(3 cases)	0/3	0/1	2/3(67%)	1/3(33%)
Oligodendroglioma(6 cases)	0/4	1/5(20%)	0/4	0/6
Ependymoma(3 cases)	0/2	1/2(50%)	0/2	0/3

Table 3. Summary of Loss of Heterozygosity and p53 Mutation in Human Gliomas.

	Chromosome 10		Chromosome 13q		Chromosome 17p		
	D10S26 pEDF70.2 PvuII	D10S17 pMHZ15 MspI	RB1 H3-8 EcoRI	RB intron 17 RB PCR-LOH XbaI	D17S5 pYNZ22.1 BamHI	D17S34 p144D6 TaqI	p53 mutation
GBM1	-	A/B	—/B	—/B	A/B	A/B	-
GBM2	—/B	—/B	A/B	-	-	-	-
GBM3	A/—	A/—	—/B	-	-	A/B	+Exon 5
GBM4	-	-	A/B	-	A/—	A/—	+Exon 5
GBM5	-	A/B	NC	NC	A/B	A/B	-
GBM6	-	-	A/B	-	A/—	-	-
GBM7	-	A/B	—/B	A/B	—/B	-	-
GBM8	A/B	-	-	-	-	-	-
GBM9	-	—/B	A/B	A/B	A/B	-	-
GBM10	-	-	—/B	—/B	A/B	A/B	-
GBM11	-	-	—/B	A/—	—/B	-	+Exon 5
GBM12	-	A/—	A/B	A/B	A/B	-	-
GBM13	A/B	-	NC	NC	-	A/B	-
AA1	A/B	-	A/B	A/B	A/—	A/B	-
AA2	—/B	A/B	A/B	A/—	—/B	—/B	+Exon 7
AO1	NC	A/B	-	-	A/—	A/—	-
AO2	-	A/B	-	-	A/—	—/B	-
AO3	A/B	-	-	A/B	A/B	-	+Exon 7
A1	-	A/B	-	A/B	A/B	-	-
A2	-	-	-	-	A/B	-	-
A3	A/B	-	A/B	-	A/B	A/B	-
A4	-	A/B	-	A/B	-	A/B	-
A5	A/B	NC	—/B	A/—	-	A/B	-
A6	A/B	A/B	-	A/B	-	A/B	-
O1	A/B	-	-	-	A/B	-	-
O2	-	A/B	-	A/B	-	A/B	-
O3	A/B	NC	A/B	-	A/B	-	-
O4	-	-	A/B	-	-	-	-
O5	-	-	-	A/—	-	-	-
O6	-	NC	-	A/B	-	A/B	-
E1	-	A/B	A/B	A/B	-	-	-
E2	A/B	-	-	-	-	A/B	-
E3	-	-	—/B	-	A/B	A/B	-

GBM ; glioblastoma multiforme, AA ; anaplastic astrocytoma, A ; astrocytoma, AO ; anaplastic oligodendroglioma, O ; oligodendroglioma, E ; ependymoma, A/B ; maintain constitutional heterozygosity, A/— and —/B ; loss of heterozygosity, - ; constitutionally homozygous, NC ; not checked

development of non-ocular malignancies, like sarcoma and gliomas as well as retinoblastoma (Meadows et al., 1985 ; Draper et al., 1986). In addition, Rb gene alterations have been found in a variety of other human cancers(Lee et al., 1988 ; Harbour et al., 1988 ; Reissmann et al., 1989). RFLP analyses have shown LOH on chromosome 13 in 15 % of malignant gliomas(James et al., 1988 ; Fults et al., 1990). Venter et al.(1991) have reported LOH at Rb locus in 4 out of 9 cases of GBM (44 %), whereas Frankel et al.(1992) could not find any structural alterations of 13q(Rb) locus in 40 cases of malignant gliomas using cDNA markers. While Venter et al.(1991) observed LOH at RB1 locus only in GBM, we observed Rb gene deletions in not only 7 of 12 malignant glioma(54 %), but also 3 of 12 cases with low-grade gliomas (25 %). Even though we must do further investigations of Rb expression, this finding suggests that the deletion of Rb gene may occur in a relatively early premalignant stage in gliomas.

To date, it is a well-known molecular genetic finding in p53 inactivation that RFLP analysis shows

LOH of one copy on chromosome 17p and sequence analysis reveals a mutation on the remaining copy of p53(Nigro et al., 1989). However, a significant proportion of malignant gliomas which showed LOH on chromosome 17p, does not have a p53 mutation (Frankel et al., 1992; Fults et al., 1992). Similar findings have been reported in medulloblastomas. In spite of a highly frequent LOH on chromosome 17p in medulloblastomas, the p53 gene mutation was very rare(Saylors et al., 1991). In our analysis, we obtained similar results that only 3 out of 8 malignant gliomas (38 %) with LOH for loci on chromosome 17p showed p53 mutations. Of course, there are at least two possibilities. One is that p53 mutations might remain in undiscovered status by this SSCP analysis, and another is that some other tumor suppressor genes on chromosome 17p are the targets for loss in the development of gliomas like medulloblastomas.

There are few reports that have assessed the LOH and p53 mutation in a large number of oligodendrogliomas. Ohgaki et al.(1991) reported p53 mutations in 2 of 17 (12 %) oligodendrogliomas in exon 5 and 7. We observed a Rb gene deletion in one case of oligodendroglioma, LOH on chromosome 17p in 2 out of 3 anaplastic oligodendrogliomas (67 %), and p53 gene mutation in one case out of 3 anaplastic oligodendrogliomas (33 %). This result suggests the possible role of two well-known tumor suppressor genes, Rb and p53 gene, in oligodendrogliomas. The basic genetic pathway in oligodendrogliomas is probably same as the way in astrocytomas. However, the present study demonstrated that the remarkable difference between oligodendrogliomas and astrocytomas was the frequency of LOH on chromosome 10. LOH on chromosome 10 was not observed in oligodendrogliomas. Because of the small number of cases and probes in this study, it will be necessary to do further extended studies to say whether oligodendrogliomas might have different molecular genetic pathways from astrocytomas.

Our data, though based on a relatively small sample size and limited number of additional chromosome markers, demonstrated that a relatively high incidence of LOH on chromosome 10, 13q(Rb), 17p(p53) are characteristics of gliomas and those must be important in the tumorigenesis and progression of gliomas. Rb gene deletions were observed in low-grade gliomas, including a oligodendroglioma and an ependymoma. This finding suggests that Rb inactivation may be an early genetic event in the development and progression of gliomas. The low incidence of p53 mutations in cases with chromosome 17p deletions suggests that some other tumor suppressor genes may be located on chromosome 17p. The inactivations of Rb and p53 gene may play important roles in the tumorigenesis of oligodendrogliomas.

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