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Advances in Brief

Somatic Mutations of the *von Hippel-Lindau* Tumor Suppressor Gene and Loss of Heterozygosity on Chromosome 3p in Human Glial Tumors[1]

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Abstract

Molecular genetic analysis of *von Hippel-Lindau* tumor suppressor gene (*VHL* gene) was performed on 38 tissues of human glial tumors (ependymoma, 1; astrocytoma, 6; oligodendroglioma, 1; oligoastrocytoma, 2; anaplastic oligoastrocytoma, 3; anaplastic astrocytoma, 14; glioblastoma multiforme, 11). Somatic DNAs extracted from frozen tumor specimens were examined by single-strand conformational polymorphism analysis and direct sequencing. In addition, loss of heterozygosity (LOH) on chromosome 3p in 15 glial tumor cases, lymphocyte DNAs of which were available, was examined by use of 10 microsatellite probes and two polymorphism markers for the *VHL* gene. Two cases of low-grade gliomas showed somatic sense mutations in exon 3 of the *VHL* gene, and 6 of 15 cases (40.0%) showed LOH of chromosome 3p. The *VHL* gene-mutated cases also showed LOH. The retention of heterozygosity and high pathological grade of glial tumors were correlated significantly. In addition, Kaplan-Meier survival analysis for patients with glial tumors showed that patients with LOH had a significantly longer survival time than those without LOH. These results suggest that somatic mutations on 3p, including the *VHL* gene, may be involved in tumorigenesis of some low-grade glial tumors.

Introduction

The von Hippel-Lindau (*VHL*[3]) disease tumor suppressor gene was isolated by positional cloning at chromosome 3p25-26 (1). The human *VHL* gene encodes a protein of 213 amino acids. The predicted protein contained an acidic pentameric repeat. The *VHL* gene has the characteristics of a classic tumor suppressor gene; *i.e.*, loss of the wild-type allele has been demonstrated in renal cell carcinoma patients with VHL disease, and somatic mutations of the gene have been detected in sporadic renal cell carcinomas and central nervous system hemangioblastomas with a loss of heterozygosity (2–4).

Somatic mutations of other tumor suppressor genes, such as *p53* (5), *Rb-1* (6), and *p16* (7) have been demonstrated in malignant gliomas. Glial tumors do not commonly occur as a manifestation of VHL disease. However, recently, a VHL family that manifested low-grade gliomas was reported (8). Both gliomas and hemangioblastomas are vascular-rich central neuroaxial neoplasms, and glial fibrillary acidic proteins, usually present in gliomas, are sometimes positively stained in hemangioblastomas. Molecular genetic studies in astrocytomas using LOH analysis have shown frequent losses on chromosomes 10 and 17 (9, 10). In addition, LOH on chromosome 3p has also been shown in some gliomas (11). In this report, we document for the first time somatic mutations of the *VHL* gene and also demonstrate LOH on 3p in glial tumors.

Materials and Methods

Tissue Samples. A total of 38 glial tumor tissues was collected at Yokohama City University (Yokohama, Japan). Routine neuropathological evaluation was performed on each specimen by use of the WHO neuroepithelial tumor grading (12). Glial tumors included 1 ependymoma, 6 astrocytomas, 2 oligoastrocytomas, and 1 oligodendroglioma (grade II); 14 anaplastic asytrocytomas and 3 anaplastic oligoastrocytomas (grade III); and 11 glioblastomas (grade IV). From frozen samples, the somatic DNAs were extracted by standard procedures using proteinase K and phenol-chloroform. In addition, lymphocyte DNAs from 15 available blood samples were extracted by the same procedure.

SSCP Analysis and DNA Sequencing. SSCP analysis was performed as described previously (3). PCR products of samples that were positive by SSCP analysis were sequenced directly according to the manufacturer's protocol (United States Biochemical Corp.) using Dynabeads (Dynal, Inc., Oslo, Norway).

LOH Analysis. Seven microsatellite probes [D3S 1278, D3S 1603, D3S 1228, D3S 1067, D3S 643, D3S 647, D3S 1038 (13), D3S 1317 (14), and D3S 1304] for 3p, two polymorphism markers (A1149G (15) and K54/MA3B) for the *VHL* gene, and three microsatellite probes for 3q (D3S 1603, D3S 1271 and D3S 1278) were used for analysis of LOH (K54/MA3B, upstream GAAATACAGTAACGAGTTGGCCTAGC and downstream GTCGACCTCCGTAGTCTTCG). D3S 1278 is positioned at 3q13, D3S 1603 and D3S 1271 at 3q11; D3S 1228 at 3p14.1–14.3; D3S 1067 at 3p14.3–21.1; D3S 643 at 3p21.3; D3S 647 at 3p23; and D3S 1038, D3S 1317, and D3S 1304 at 3p25. Microsatelite probes except for D3S 1304 are positioned upstream of the *VHL* gene. In contrast, D3S 1304 is positioned downstream of it. Primers of microsatellite probes and VHL polymorphism markers were used for the LOH analysis.

Results

SSCP analysis was carried out completely for 33 out of the 38 glial tumors, but the remaining 5 tumors could not be analyzed for all exons because of unsuccessful PCRs. The available 33 glial tumors included 10 low-grade and 23 high-grade glial tumors. The SSCP analysis revealed alterations at exon 3 of the *VHL* gene in 2 (6.1%) of the available 33 glial tumor DNAs (Fig. 1), but none of them show any alteration at exon 1 or 2. Both tumors showing the alterations were low-grade gliomas in the supratentorial region. Direct sequencing of exon 3 was carried out on PCR products from the tumor DNAs with SSCP alterations. The detected sequence change from CGG to TGG in glial tumor 25 (a 44-year-old male with oligoastrocytoma grade II in the left frontal lobe) was positioned at nucleotide 841 in the *VHL* gene. This change is predicted to cause a missense mutation from arginine to tryptophan (codon 210; Fig. 2; Table 1). In addition, glial tumor case 14 (a 73 year-old-female with astrocytoma grade II in the right temporal lobe) showed a sequence change from GCA to GTA, a change predicted to cause a missense mutation from alanine to valine (codon 207; Table 1). Both of the glial tumors with *VHL* gene mutations were grade II astrocytic tumors.

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[3] The abbreviations used are: VHL, von Hippel Lindau; LOH, loss of heterozygosity; SSCP, single-strand conformation polymorphism.

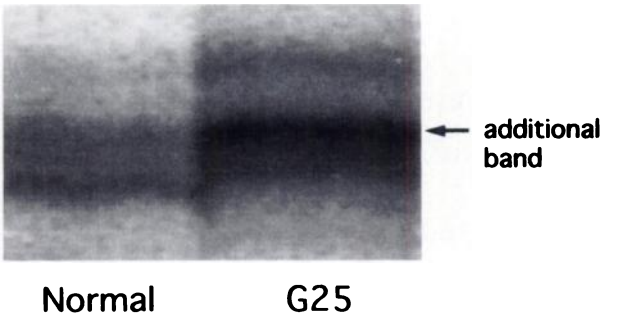

Fig. 1. SSCP analyses of DNAs from tumor samples. A typical result of SSCP analysis is shown. *G25*, glial tumor case 25 (oligoastrocytoma); *Normal*, normal blood DNA. PCR and subsequent gel electrophoresis were performed with a primer set, which covers exon 3. Case 25 shows an abnormal band in SSCP analysis (*additional band*).

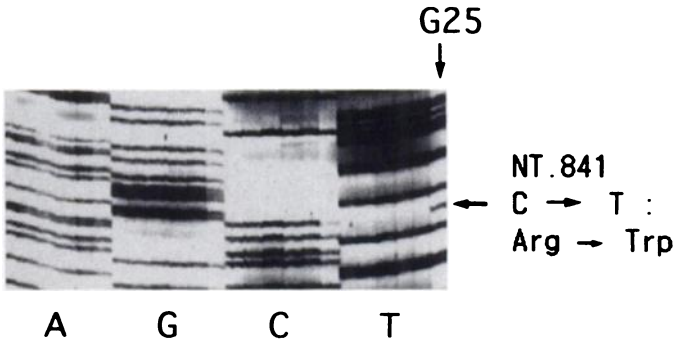

Fig. 2. Direct sequencing analysis reveals a point mutation (transversion from cytosine to thymine) at nucleotide number 841, indicating a missense mutation (from arginine to tryptophan). *NT*, nucleotide; *G25*, glioma case 25.

The LOH analysis was examined by the comparison of DNAs from the leukocytes and tumor tissues by use of 12 probes (7 microsatellite probes for 3p, 3 microsatellite for 3q, and 2 VHL polymorphism markers). The analyses were performed on 15 astrocytic tumor cases (low-grade glial tumor, 5; high-grade glial tumor, 10) for which blood samples were available. The analysis using those probes revealed LOH on chromosome 3p, including the *VHL* gene locus in six cases (40.0%) and retention of heterozygosity in eight cases (60.0%). LOH shown in six cases was positioned in 3q11–3p26. The analysis using D3S 1278 for 3q13 did not show LOH in any of the cases (Fig. 3). All LOH patterns showed a homogeneous defect in chromosome 3p toward the 3p tip. Two cases (cases 14 and 30) showed LOH from 3q11 (D3S 1271) toward the 3p tip, which implicated a whole-arm defect of 3p (Fig. 4). In addition, two cases (cases 14 and 25) out of the six LOH-detecting cases also showed *VHL* gene mutations, as mentioned earlier. The pathohistological investigation revealed dominancy of low-grade gliomas in LOH-detected cases (grade II, four; grade III, one; and grade IV, one) and that of high-grade gliomas in cases of retained heterozygosity (grade II, two; grade III, three; and grade IV, five). As a result, retention of heterozygosity and high-grade tumors were correlated significantly (Student's t test, $P < 0.05$). In addition, Kaplan-Meier analysis for patients analyzed for 3p LOH revealed a significant difference between cases with LOH and those without LOH (Cox-Mantel test; $P < 0.05$). The result showed longer survival time of patients with 3p LOH than of those without LOH (Fig. 5).

Discussion

Mutations in several tumor suppressor genes are predicted to cause the genesis of glial tumors. Frequent somatic mutation of p53 has been found in malignant gliomas (5). In addition, the mutations of Rb-1 (6), and p16 (7) have been demonstrated in glial tumors. Fults *et al.* (11) revealed LOH greater than 10% on 3p, 5q, 7p, 10p, 10q, 11p, 13q, 14q, 15q, 16p, 16q, 17p, 17q, and 18q in malignant glial tumors. The locus of p53 has been located on 17p. However, the relationship between frequency of LOH on the other chromosomes and mutations of other suppressor genes has not been reported in glial tumors. Using one marker probe of EFD145, an 11% frequency of LOH on 3p was found by Fults *et al.* (11). His report suggested that a suppressor gene on 3p might be related to the genesis of glial tumors.

Whaley *et al.* (16) identified *VHL* gene somatic mutations in 33% of sporadic renal cell carcinomas but did not find any in other carcinomas that were usually not associated with the VHL disease. They proposed that the *VHL* gene plays an important role in the etiology of sporadic renal cell carcinomas and suggested that functional domains in the 3′ end of the reading frame of the *VHL* gene were critical to the growth-suppressive function of the VHL protein. The predicted protein of the *VHL* gene contains an acidic pentametric repeat that has homology to the acidic repeat domain in the procyclic surface membrane glycoprotein of *Trypanaosoma brucei* (1).

Recently, the VHL protein was shown to bind tightly and specific to Elongins B and C, which activate trancription elongation by RNA polymerase II, and to inhibit Elongin (S III) transcriptional activity (17), suggesting that the VHL protein may play an important role in the transcriptional regulatory network that controls tumorigenesis. Recent results showed that wild-type VHL protein regulated expression of the hypoxia-induced genes such as vascular endothelial growth

Table 1 *Data of glial tumors with* VHL *gene mutations*

Case no.	14	25
Age/Sex	73/F	44/M
Pathological feature	Astrocytoma	Oligoastrocytoma
Tumor grade	II	II
Region of tumor	Right temporal lobe	Left frontal lobe
Site of SSCP change	Exon 3	Exon 3
VHL gene mutation	GCA → GTA	CGG → TGG
Site of mutation	Nucleotide 833	Nucleotide 841
Consequence	Alanine → Valine	Arginine → Tryptophan
Site of amino acid	Coden 207	Coden 210
LOH	+	+

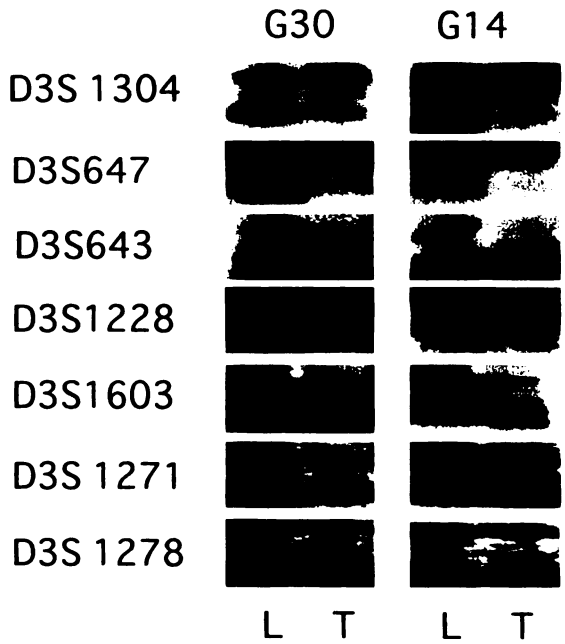

Fig. 3. Cases 30 and 14 showed LOH with microsatellite probes D3S 1304 to D3S 1278. Both cases showed retention of heterozygosity with D3S 1271. *G30*, glioma case 30; *G14*, glioma case 14; *L*, lymphocyte, *T*, tumor.

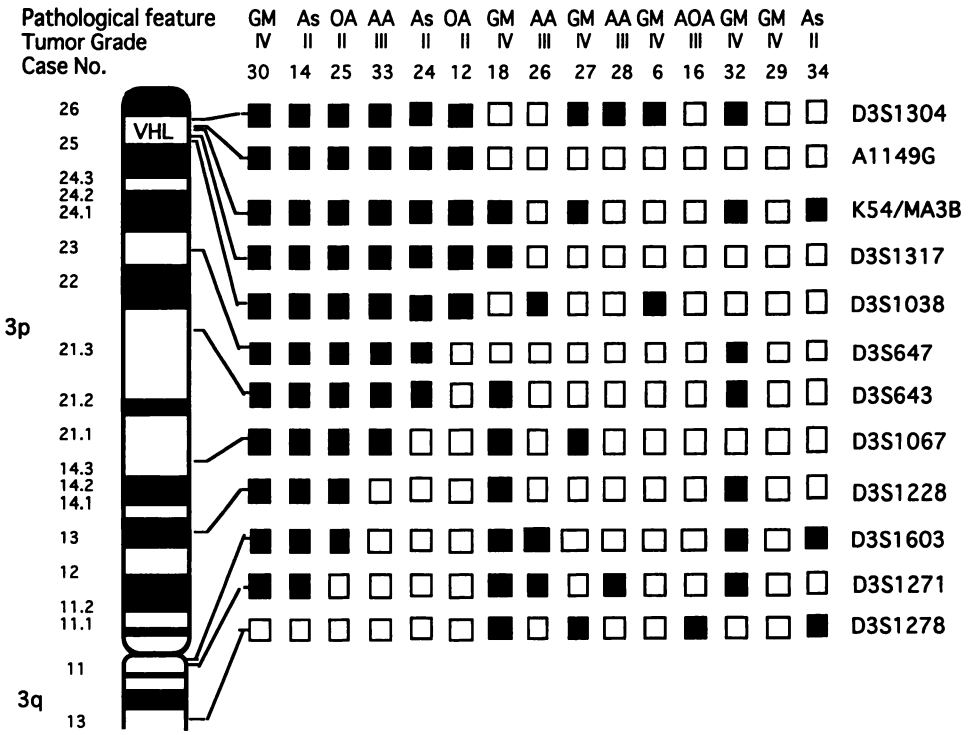

Fig. 4. LOH analysis revealed that LOH was detected in 6 out of 15 glial tumors. ■, LOH; □, retention of heterozygosity; ▨, noninformative. *GM*, glioblastoma multiforme; *As*, astrocytoma; *OA*, oligoastrocytoma; *AA*, anaplastic astrocytoma; *AOA*, anaplastic oligoastrocytoma.

factor. The VHL protein inhibits the cellular expression of vascular endothelial growth factor, platelet-derived growth factor B chain, glucose transporter GLUT1 in hypoxic condition, but not in normoxic condition (18). It is supposed that the VHL protein regulates the mRNA stability of these genes at the posttranscriptional level by interacting with Elongins B and C (19). We detected somatic sense mutations in 2 of 33 glial tumors. Both mutations were positioned at the terminal end of the downstream portion of the *VHL* tumor suppressor gene. In addition, these two cases of glial tumors also showed LOH in 3p. The function of the *VHL* gene has not been elucidated fully. However, these present mutation in glial tumors may cause the loss of function of the VHL protein, a protein that is related to the transcriptional regulatory network. Thus, this process may lead to the genesis of glial tumors. In addition, the high incidence of LOH on 3p in glial tumors may also suggest the involvement of the VHL tumor suppressor gene. Interestingly, many of LOH-detected cases displayed benign low-grade tumors, whereas retained heterozygosity was found to be dominant in the malignant high-grade tumors. This result suggests that some suppressor genes in 3p, including the *VHL* gene, may cause tumorgenesis of benign glial tumors. Recently, a VHL family, some members of which had cerebellar benign astrocytomas, was reported (8). This suggests that *VHL* gene mutations may be related to the development of some astrocytomas.

In addition, *VHL* gene expression in the central nervous system of adult and fetal human tissues was shown by *in situ* mRNA hybridization in nerve cells of the cerebral cortex, midbrain, cerebellum, and spinal cord (20). The widespread expression of the *VHL* gene in the central nervous system suggests that the occurrence of hemangioblastomas and benign glial tumors can be related to a mutation of the *VHL* gene.

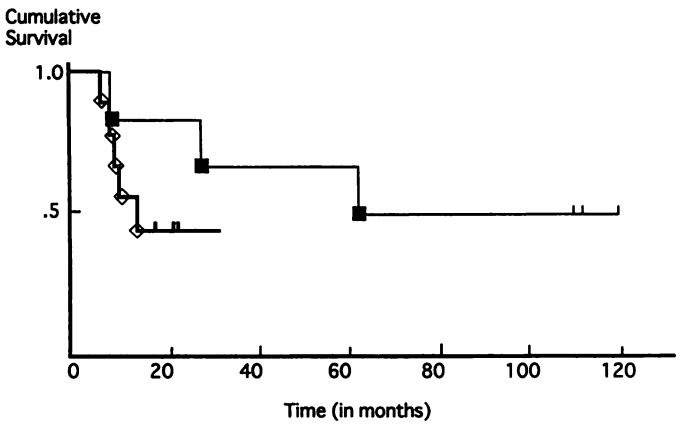

Fig. 5. Kaplan-Meier analysis of patients with glial tumors. Patient with LOH (*thin line*, ■) and without LOH (*thick line*, ◇) on chromosome 3p, including the *VHL* gene locus, were compared. *Bars* on the survival curves indicate patients alive at the indicated time. The Cox-Mantel test showed a significant difference ($P = 0.0192$).

Acknowledgments

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