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Advances in Brief

IGF2 but not *H19* Shows Loss of Imprinting in Human Glioma[1]

Shinji Uyeno, Yasuhiro Aoki, Masayuki Nata, Kaoru Sagisaka, Takamasa Kayama, Takashi Yoshimoto, and Tetsuya Ono[2]

Departments of Radiation Research [S. U., T. O.], Neurosurgery [T. K., T. Y.], and Forensic Medicine [Y. A., M. N., K. S.], Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-77, Japan

Abstract

Genomic imprinting is a gamete-specific modification resulting in the allele-specific expression of genes in somatic cells. A loss of imprinting (LOI) has been found in many embryonal and adult tumors, suggesting that it plays a role in tumor development. The incidence of LOI, however, does not seem to be ubiquitous among tumors because neuroblastoma and colorectal cancer revealed no LOI. We examined the involvement of LOI of *IGF2* and *H19* genes in human gliomas. The two genes were imprinted in normal brain subcortex tissues. In glioma, 8 of 14 informative cases (57%) revealed LOI in *IGF2*. The frequency did not depend on the tumor grade. For *H19*, in contrast, all 13 informative cases maintained imprinting. These results suggest that LOI of *IGF2* but not *H19* plays a role in the development of human glioma.

Introduction

In mammals, some genes are differentially expressed between paternal and maternal alleles, and this is called genomic imprinting. It is vital for embryo development, but not much is understood about its role after birth (1–7). Recent studies, however, indicate that a LOI[3] in the *IGF2* and *H19* genes could be a factor involved in tumor development (8–10). *IGF2* has growth-stimulating activity (11, 12), whereas *H19* may have tumor suppressor activity (13). Frequent LOI in these genes was initially found in childhood tumors, but it was later identified in adult tumors. These include Wilms' tumor (1, 2), embryonal rhabdomyosarcoma (1, 14), hepatoblastoma (15, 16), Ewing's sarcoma (17), testicular germ cell tumor (18), choriocarcinoma (19), lung cancer (20, 21), and esophageal cancer (22). On the other hand, a lack of LOI has been identified in neuroblastoma (23) and colorectal cancer (22). This suggests that LOI is involved in some types of tumor. We therefore examined human glioma. It is the most common brain tumor originating from a glial cell, and it appears mostly during adulthood. Here we show that a LOI of *IGF2* but not *H19* is associated with human glioma.

Materials and Methods

Tissues. Normal brain subcortex tissues from the frontal lobe were obtained at forensic autopsy and frozen at −70°C. We did not use the tissues surrounding the tumors because their DNA methylation profiles suggested that they were not normal (24). Tumor samples were collected from surgically resected tissues at the initial diagnosis and stored at −70°C. In sampling normal and tumor brain tissues, we avoided including choroid plexus and leptomeninges because imprinting is reported to be absent in these areas (25, 26). Patients' leukocytes were isolated from the peripheral blood by centrifugation after lysis of RBCs and stored frozen.

The gliomas were classified into five groups histopathologically based on the WHO brain tumor scheme: (*a*) astrocytoma (grade I); (*b*) astrocytoma (grade II); (*c*) oligodendroglioma (grade II); (*d*) anaplastic astrocytoma (grade III); and (*e*) glioblastoma (grade IV; Ref. 27).

Identification of Genomic Polymorphism. Genomic DNA was extracted from frozen tissues as described (28). Heterozygosity in the *IGF2* and *H19* genes was determined by the presence or absence of *Apa*I and *Rsa*I sites, respectively (1, 2). The genotype of the tumor was always compared with that of blood leukocytes because some gliomas show instability in these regions (29). Genomic heterozygosity was studied as follows. The polymorphic region of *IGF2* was amplified by PCR using the primers 5′-TCCTGGAGACGTACTGTGCTA-3′ (P1) or 5′-CTTGGACTTTGAGTCAAATTGG-3′ (P2) for the 5′ end and 5′-GGTCGTGCCAATTACATTTCA-3′ (P3) for the 3′ end. Genomic DNA (200 ng) was mixed with a pair of primers, denatured at 94°C for 4 min, and amplified by 35 cycles of polymerization (94°C for 30 s, 55°C for 1 min, and 72°C for 1 min). The reaction was completed by a final extension at 72°C for 4 min. The polymerase was 2.5 units of *Taq* (Takara, Kyoto, Japan), and the reaction volume was 50 μl [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM each deoxynucleotide triphosphate]. The amplified DNA was extracted with phenol-chloroform, precipitated with ethanol, digested with *Hinf*I + *Apa*I, run through a 2% agarose gel, and stained with ethidium bromide. *H19* polymorphism was similarly analyzed using primers 5′-TACAACCACCTGCACTACCTG-3′ (H1) for the 5′ end and 5′-TGGAATGCTTGAAGGCTGCT-3′ (H2) for the 3′ end (1). The amplification proceeded as follows: 94°C for 4 min for denaturation and 35 cycles of polymerization (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) followed by extension at 72°C for 4 min.

Allele-specific Gene Expression. RNA was isolated from frozen tissues using guanidium thiocyanate (28). The RNA extracted from tissues (20 μg) was routinely digested with 20 units of RNase-free DNase I (Promega Biotec, Madison, WI) for 1 h at 37°C in the presence of 20 units of RNase inhibitor (Promega Biotec) to remove contaminating DNA. The DNase I-treated RNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in diethylpyrocarbonate-treated water. RNA (2 μg) was denatured at 70°C for 10 min, cooled on ice, and incubated with 200 units of Moloney murine leukemia virus RT (Life Technologies, Inc., Gaithersburg, MD) for 1 h at 37°C in the presence of 200 ng of random hexamer (Takara), 0.5 mM deoxynucleotide triphosphate, 40 units RNase inhibitor (Promega), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT in a total volume of 20 μl. After cDNA synthesis, the RT was inactivated by heating for 5 min at 98°C. The cDNA was amplified by PCR using primers P1 and P3 for *IGF2* or H1 and H2 for *H19*. The conditions for amplification were the same as those described above, except that 40 PCR cycles were performed. Genomic DNA contamination of the RNA samples was routinely checked by two criteria, the absence of a DNA band after PCR amplification without RT and the absence of a genomic DNA-specific 1400-bp DNA product after PCR amplification of *IGF2* cDNA using primers P1 and P3. Because primer P1 was in exon 8 and primer P3 was in exon 9, the amplified product of genomic DNA should be 1400 bp, whereas the product derived from cDNA should be 1120 bp. After PCR amplification of cDNA for *IGF2*, the DNA was resolved in a 2% agarose gel, excised as a 1120-bp band, digested with *Hinf*I + *Apa*I, separated in an 8% acrylamide gel, blotted to a charged nylon membrane, and hybridized to a labeled DNA probe. The probe was the 256-bp fragment of the *Hinf*I-digested PCR product of genomic DNA amplified with primers P2 and P3 (Fig. 1*A*). The 25-bp fragment of allele *b* in Fig. 1*A* was not detectable under our conditions.

The expression of *H19* was analyzed similarly. The cDNA was amplified by

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[2] To whom requests for reprints should be addressed. Phone: 81-22-717-8132; Fax: 81-22-717-8136.

[3] The abbreviations used are: LOI, loss of imprinting; LOH, loss of heterozygosity; IGF2, insulin-like growth factor II; RT, reverse transcriptase.

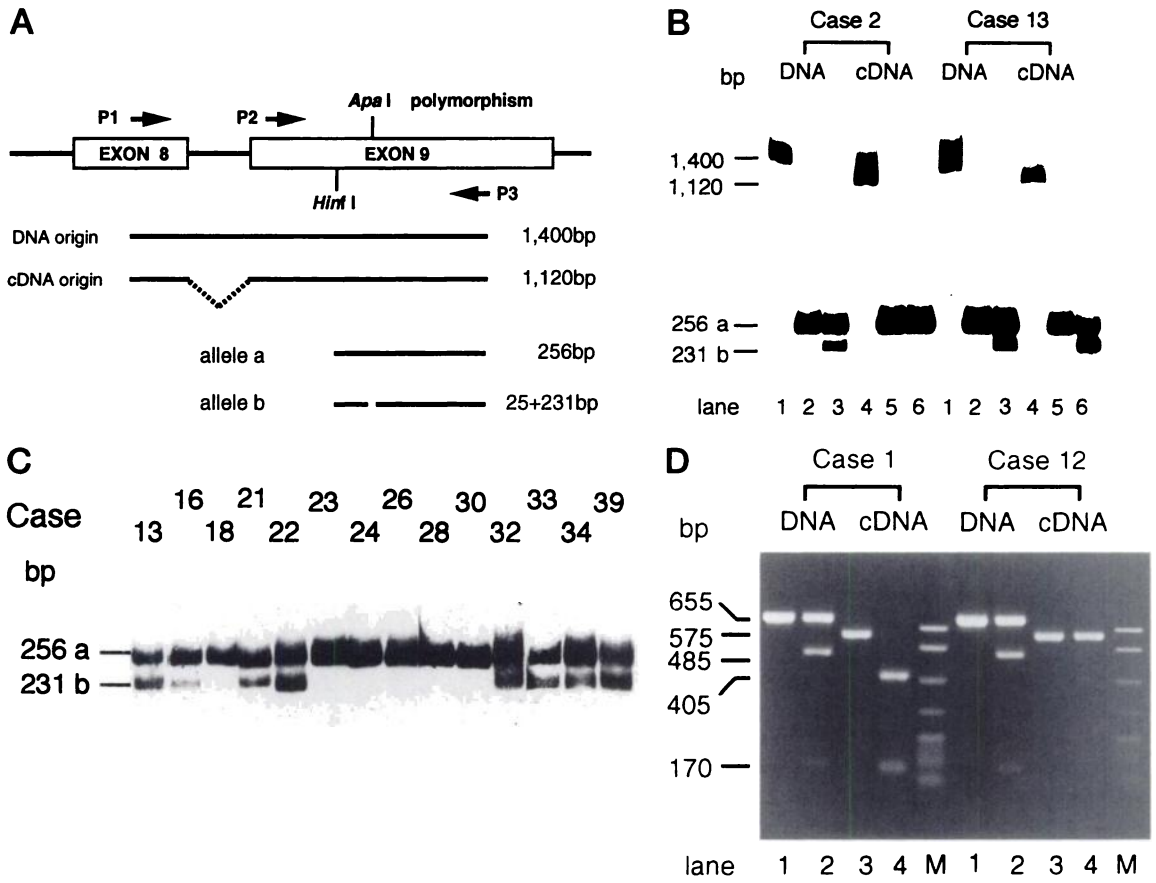

Fig. 1. Allele-specific expression of *IGF2* in human gliomas. *A*, a schematic map of the exon 8–9 region of the human *IGF2* gene. The scale is not accurately proportional. The sites recognized by *Hin*fI and *Apa*I and the PCR primers (P1, P2, and P3) are shown on the map. The DNA amplified by PCR using primers P1 and P3 from genomic DNA and cDNA are shown *under* the map. The sizes of their fragments are indicated on the *right*. Alleles *a* and *b*, observed in the analysis of cDNA for allele specificity, are also shown at the *bottom* with their size. *B*, an autoradiograph showing monoallelic expression observed in a normal brain tissue (*Case 2*) and biallelic expression in a tumor tissue (*Case 13*). *Lanes 1, 2,* and *3*, analyses of genomic DNA. *Lane 1*, the PCR product using primers P1 and P3; *Lane 2*, the *Hin*fI digestion product of *Lane 1*; *Lane 3*, the *Hin*fI + *Apa*I digestion product. The hybridization probe was a *Hin*fI-digested 256-bp fragment of the PCR product made using the P2 and P3 primers. *Lane 3* shows that the genomic DNA is heterozygous. *Lanes 4, 5,* and *6*, the results of similar analysis on cDNA derived from cases 2 and 13. *Lane 4*, the PCR products of cDNA using the primers P1 and P3. *Lane 5*, the *Hin*fI digest of the PCR product. *Lane 6*, the *Hin*fI + *Apa*I digest of the PCR product. *Case 2*, expression is monoallelic; *Case 13*, expression is biallelic. *C*, an autoradiograph analyzing allele specificity of the *IGF2* gene transcript in 14 informative glioma tissues. cDNA was amplified by PCR and digested with *Hin*fI + *Apa*I, run through polyacrylamide gel, transferred to a nylon filter, and hybridized to the 256-bp *Hin*fI fragment shown in *A*. Eight cases (cases 13, 16, 21, 22, 32, 33, 34, and 39) showed biallelic expression, whereas the rest showed monoallelic expression. *D*, maintenance of *H19* gene imprinting in normal brain (*Case 1*) and glioma (*Case 12*). *Lanes 1* and *2*, analyses of genomic DNA. *Lane 1*, PCR product of the *H19* gene using primers H1 and H2; *Lane 2*, *Rsa*I digestion product of *Lane 1*. *Lanes 3* and *4*, analysis of cDNA. *Lane 3*, reverse transcription-PCR product of *H19* mRNA using primers H1 and H2; *Lane 4*, *Rsa*I digestion product of *Lane 3*. Lanes marked *M* show size markers, *Msp*I digest of pBR322 DNA. The bands of 655 bp (DNA) and 575 bp (cDNA) represent the *a* allele, whereas bands of 485 and 170 bp from DNA and 405 and 170 bp from cDNA indicate the *b* allele. In both cases, DNA shows heterozygosity, but cDNA reveals monoallelic expression.

PCR in the same way as genomic DNA. Because the primers spanned the last intron of *H19*, it was simple to determine whether or not the PCR product was derived from genomic DNA (655 bp) or cDNA (575 bp; Ref. 1). Under our conditions, a genomic DNA-derived band was not evident. The amplified DNA was digested with *Rsa*I, resolved by electrophoresis in a 2% agarose gel, and stained with ethidium bromide.

Results

We surveyed *IGF2* and *H19* gene heterogeneity in genomic DNA from normal brain subcortex and glioma tissues. The results are summarized in Table 1. Alleles *a* and *b* indicate restriction enzyme-resistant and -sensitive allele, respectively (Fig. 1*A*).

Because some gliomas show LOH in the *IGF2* region (29), we compared genomic polymorphism of *IGF2* between tumor DNA and matched leukocyte DNA. In four patients (cases 19, 27, 29, and 37 in Table 1), the allelotype was discrepant, indicating a LOH in the tumors. The frequency of the LOH (22%, 4 of 18 informative cases) was comparable to that reported (29). In all other tumors, the genotype was the same as that of the matched leukocyte. Similarly, genotypes of *H19* in gliomas and their corresponding blood leukocytes were examined. Among 13 informative cases, there were no discrepancies between gliomas and leukocyte DNAs (Table 1).

RNA was extracted from the samples that showed heterozygosity in *IGF2* or *H19*. We determined the allele from which the mRNA was transcribed by means of reverse transcription-PCR and sensitivity to restriction enzymes. In normal brains, all of the eight informative cases for *IGF2* and seven cases for *H19* revealed monoallelic expression, indicating the maintenance of imprinting. Examples of the results are shown in Fig. 1*B*, case 2 and Fig. 1*D*, case 1. On the other hand, similar analysis of *IGF2* on gliomas revealed frequent expression of the two alleles, indicating LOI. In case 13, for example, both *a* and *b* alleles were found in the cDNA made from mRNA (Fig. 1*B*, case 13). The results of cDNA analysis of all 14 informative cases are shown in Fig. 1*C*. The frequency of the LOI of *IGF2* in glioma was 57% (8 of 14 cases).

We analyzed *H19* gene expression in the same cDNA preparation. All 13 informative cases revealed the expression of 1 allele (representative data are shown in Fig. 1*D*, case 12). Six of them showed a LOI in the *IGF2* gene but not in the *H19* gene (cases 13, 16, 21, 22,

Table 1 *IGF2 and H19 allelic expression in 39 normal and glioma tissues*

Case no.	Age (yr)/sex	Histology and grade[a]	*IGF2/ApaI*			*H19/RsaI*		
			DNA[b]	cDNA	LOI/LOH	DNA	cDNA	LOI/LOH
Normal brain tissues								
1	12/M	N	a/b	a		a/b	b	
2	21/M	N	a/b	a		b		
3	30 /F	N	a/b	a		b		
4	40/M	N	a/b	a		a/b	b	
5	41 /F	N	a/b	a		a/b	b	
6	48/M	N	b			a/b	b	
7	54/M	N	a/b	b		b		
8	58 /F	N	a/b	a		a		
9	59/M	N	b			a/b	b	
10	60/M	N	b			a/b	b	
11	68/M	N	a/b	a		a/b	b	
Tumor tissues								
12	28 /F	A I	a			a/b	a	
13	35 /F	A II	a/b	a/b	LOI	a/b	b	
14	37/M	A II	b			a/b	a	
15	40/M	A II	b			a		
16	43/M	A II	a/b	a/b	LOI	a/b	b	
17	54 /F	A II	b			a		
18	65 /F	OG II	a/b	a		a		
19	28/M	AA III	a/−	a	LOH	b		
20	36/M	AA III	b			b		
21	40/M	AA III	a/b	a/b	LOI	a/b	b	
22	51 /F	AA III	a/b	a/b	LOI	a/b	a	
23	72 /F	AA III	a/b	a		a/b	b	
24	54/M	AA III	a/b	a		b		
25	60/M	AA III	b			b		
26	23 /F	GB IV	a/b	a		a/b	b	
27	24/M	GB IV	a/−	a	LOH	a/b	a	
28	37 /F	GB IV	a/b	a		b		
29	38 /F	GB IV	a/−	a	LOH	b		
30	49/M	GB IV	a/b	a		a/b	b	
31	54/M	GB IV	b			a/b	a	
32	54/M	GB IV	a/b	a/b	LOI	a/b	b	
33	58 /F	GB IV	a/b	a/b	LOI	b		
34	61/M	GB IV	a/b	a/b	LOI	b		
35	63 /F	GB IV	b			a		
36	64/M	GB IV	b			b		
37	66 /F	GB IV	−/b	b	LOH	b		
38	67/M	GB IV	a			b		
39	72/M	GB IV	a/b	a/b	LOI	a/b	b	

[a] Histology and grade: N, normal brain tissue. Tumors were classified according to WHO guidelines: A I, astrocytoma grade I; A II, astrocytoma grade II; OG II, oligodendroglioma grade II; AA III, anaplastic astrocytoma grade III; GB IV, glioblastoma grade IV.

[b] a/− or −/b, the genotype of matched leukocyte DNA is a/b and that of tumor is a or b, respectively. a, allele a; b, allele b.

32, and 39 in Table 1). The results suggested the maintenance of *H19* imprinting in glioma.

Discussion

The data presented here suggest a role for LOI in the *IGF2* gene, but not the *H19* gene, in human glioma development. The two genes were imprinted in all informative normal brains examined, whereas frequent biallelic expression was observed in the *IGF2* gene in gliomas. Assuming that the *IGF2* gene was imprinted in the normal brains of the glioma patients, the data suggest a frequent association of LOI of the *IGF2* gene in glioma. The frequency of LOI in *IGF2* (8 of 14 cases, 57%) was comparable to those in lung carcinoma (14 of 30 cases, 47%; Ref. 20) and choriocarcinoma (1 of 2 cases, 50%; Ref. 19) and slightly lower than that observed in testicular germ cell tumor (10 of 11 cases, 91%; Ref. 18). The frequency in the most malignant glioblastoma (4 of 7 cases, 57%) was not largely different from that of either moderately malignant anaplastic (2 of 4 cases, 50%) or low-grade astrocytoma (2 of 3 cases, 67%). It suggests that the LOI of *IGF2* is not involved in the later stages of glioma development but rather occurs early in some tumor fractions. A similar phenomenon has been observed in lung carcinoma (20). A unique characteristic of LOI in glioma in comparison with other tumors is its absence in *H19*. All the tumors thus far examined revealed LOI in both *IGF2* and *H19*, although not necessarily coincidentally in the same tumor. In the present study, no LOI was found in *H19* among 13 informative glioma cases. This supports the notion that a tumor-associated LOI can appear only in one of *IGF2* and *H19* (15). This is in contrast to normal embryogenesis, in which the imprinting of *IGF2* and *H19* is closely linked (30–33). Tilghman and her colleagues (34) have proposed an enhancer competition model for a close relationship between *IGF2* and *H19* imprinting in normal tissues. If the model could apply to the human gliomas showing LOI in *IGF2* but not *H19*, a possible explanation for the phenomenon would be that the enhancer activity in maternal chromosome changes in glioma and both *IGF2* and *H19* genes become activated. The other possible explanation for the phenomenon would be that the glioma consists of two kinds of cells; one maintains imprinting in both *IGF2* and *H19*, and the other has lost imprinting in *IGF2* and *H19* becomes inactive like the cases reported in Wilms' tumor (35, 36). Then *IGF2* mRNA from both alleles could be found, whereas only maternal *H19* mRNA is observed. Of course, these possibilities need experimental evidence to be proved.

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