RESEARCH ARTICLE

Frequent LOH at Chromosome 12q22-23 and Apaf-1 Inactivation in Glioblastoma

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Glioblastoma (GB) often has loss of heterozygosity on the chromosomes, 1p, 10p, 10q, 11p, 17p, 19q, 22q, and several others. In the case of chromosome 12q; however, it remains to be seen whether LOH occurs. Apaf-1, the apoptotic protease activating factor-1, located at chromosome 12q22-23, is a major effecter of the p53 mediated apoptosis pathway, and Apaf-1 inactivation due to chromosome 12q22-23 LOH and hypermethylation may be involved in some of the neoplasms in malignancy. However, little is known about the frequency of the 12q22-23 LOH or the state of Apaf-1 in GB. To elucidate their involvement in GB, we analyzed a series of 33 GBs for chromosome 12q22-23 LOH, Apaf-1 mRNA expression, and Apaf-1 protein expression, using microsatellite analysis, reverse transcription (RT) - PCR analysis, and immunohistochemical (IHC) analysis, respectively. We also evaluated if and how the 12q22-23 LOH correlated with the *p53* gene mutation and *EGFR* gene amplification. Chromosome 12q22-23LOH was detected in 14 (42%) of 33 cases. Among the examined cases with LOH at 12q22-23, a low expression of Apaf-1 mRNA was detected in 9 (69%) of 13 cases, and a low expression of Apaf-1 protein was detected in 12 (86%) of 14 cases. The 12q22-23 LOH was significantly correlated with low expression of mRNA and protein ($p<0.05$, $p<0.001$ respectively). The *p53* gene mutation and *EGFR* gene amplification were found in 13 cases (39%) and 8 cases (24%), respectively, and these gene alterations were inversely correlated. However, 12q22-23 LOH had no correlations with the *p53* gene mutation or *EGFR* gene amplification. Six of 9 GBs (67%) with neither *p53* gene mutation *nor EGFR* gene amplification tested positive for 12q22-23 LOH. These GBs are likely to belong to another subset independent from the 2 common genetic subsets in GB (one with *p53* gene mutation and without *EGFR* gene amplification, and the other with *EGFR* gene amplification and without *p53* gene mutation). Twenty-three (70%) out of the 33 GBs with the 12q22-23 LOH also tested positive for Apaf-1 inactivation or *p53* gene mutation. This high frequency of alterations in the apoptosis-associated factors prompts a speculation that abrogation of the Apaf-1 and p53 mediated apoptosis pathway may play an important role in the tumorigenesis of GB.

Introduction

Glioblastoma (GB) is one of the most aggressive brain tumors (WHO grade IV). Despite progress in surgery and adjuvant therapy, patients with GB still have a dismal prognosis (6, 25). Malignant transformation in GB involves a stepwise accumulation of genetic alterations, such as amplification of the *EGFR*, *CDK4*, and *MDM2* and inactivation of *p53*, *Rb*, and *PTEN* (23, 25, 45). Moreover, loss of heterozygosity (LOH) has been reported to frequently occur on chromosomes, 1p, 10p, 10q, 11p, 17p, 19q, 22q, and others, and that on chromosome 10 has been confirmed to be a common and specific gene alteration (10, 43). It was recently suggested that GB can be divided into a few subsets based on these gene alterations (22, 26). Given that the *p53* gene mutation and *EGFR* gene amplification occur in a mutually exclusive manner, they are considered separate subsets independent from each other (24, 44, 45). Clinically, GB with the *p53* gene mutation occurs in younger patients and in a majority of secondary GBs that develop through malignant progression from diffuse astrocytoma WHO grade II or anaplastic astrocytoma. On the other hand, GB with *EGFR* gene amplification occurs in older patients and generally in primary GB (30, 39, 43-45, 47).

Apaf-1, the apoptotic protease activating factor-1, is a major effecter of p53-mediated apoptosis pathway. Apaf-1 is derived from a 27-exon gene located on chromosome 12q22-23, and it is activated by cytochrome c from mitochondria. Apaf-1 activation leads to caspase-9 activation and initiation of a protease cascade. Apaf-1 is broadly expressed in numerous tissue types, including adult spleen, leukocytes, kidney, lung, and brain. In light of its apoptotic activity, Apaf-1 is classed as one of the tumor suppressors (2, 4, 19, 31, 36, 49, 50). A recent investigation found that Apaf-1 inactivation with the 12q22-23 LOH plays an important role in tumorigenesis of certain tumors, and that the abrogation of the apoptosis pathway due to Apaf-1 inactivation leads to severe chemoresistance (18, 37). However, the status

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Gene	Senseprimer sequences	Antisenseprimer sequences	Product size (bp)
SHGC-17541 (D12S393)	5′-ATTAATGCCAGGACATTAAACG-3′	5′-CCTCACACAATGTTGTAAGGG-3′	249
AFMb293ye5 (D12S1657)	5′-TCCTAAAGATGGTGTGCAT-3′	5′-AAGTTCCAATGTTAGTGAACC-3′	150-160
p53 exon 5a	5′-TCAACTCTGTCTCCTTCCTC-3′	5′-CTGTGACTGCTTGTAGATGG-3′	155
p53 exon 5b	5′-GTGGGTTGATTCCACACCCC-3′	5′-AACCAGCCCTGTCGTCTCTC-3′	162
p53 exon 6	5′-AGGCCTCTGATTCCTCACTG-3′	5′-AGAGACCCCAGTTGCAAACC-3′	168
p53 exon 7	5′-GGCCTCATCTTGGGCCTGTG-3′	5′-GAGGCTGGGGCACAGCAGGCCAGTG-3′	191
p53 exon 8	5′-AATGGGACAGGTAGGACCTG-3′	5′-ACCGCTTCTTGTCCTGCTTG-3′	225
EGFR	5′-AGCCATGCCCGCATTAGCTC-3′	5′-AAAGGAATGCAACTTCCCAA-3′	110
IFNG	5′-GCAGAGCCAAATTGTCTCCT-3′	5′-GGTCTCCACACTCTTTTGGA-3′	85
Apaf-1 (for RT-PCR)	5′-GATGGAACAGTGAAGGTATGG-3′	5′-CTCCAGATCTTTGCAGTCTTGTC-3′	149
beta actin	5′-TCATGAGGTAGTCAGTCAGG-3′	5′-AGCCATGTACGTTGCTATCC-3′	182

EGFR, epidermal growth factor receptor; IFNG, interferon gamma; Apaf-1, apoptotic protease activating factor-1

Table 1. Oligonucleotide primers.

of Apaf-1 expression and relationship between Apaf-1 expression and p53 remain unclear in GB. Furthermore, little is known about the frequency of chromosome 12q LOH, the site of the *Apaf-1* gene (32, 43, 48). To elucidate the involvement of those alterations in GB, we analyzed a series of 33 GBs for the chromosome 12q22-23 LOH, Apaf-1 mRNA expression, and Apaf-1 protein expression using microsatellite analysis, reverse transcription (RT)-PCR analysis, and immunohistochemical (IHC) analysis, respectively. We also evaluated if and how the 12q22-23 LOH correlated with the *p53* gene mutation and *EGFR* gene amplification.

Materials and Methods

Tissue samples. Tumor and blood samples were obtained from 33 patients with GB. All of the cases were treated and followed up at the Department of Neurosurgery, Kanazawa University Hospital, Japan, between 1986 and 2002. All of the tumor specimens were examined microscopically and graded according to the guidelines of the World Health Organization (WHO) (21). All tumor specimens were microdissected with the aid of a microscope. Genomic DNA from leukocytes and tumor tissues was extracted by standard methods.

Analysis of LOH at chromosome 12q22-23. LOH at chromosome 12q22-23 was studied by PCR-based microsatellite analysis. Two microsatellite markers located both end of Apaf-1 locus, D12S1657 at centromere side and D12S393 at telomere side (27, 37), were purchased from Sigma Genosis (Hokkaido, Japan). All primers were labeled with Cy5 (Amersham Pharmacia Biotech) at the 5′ end (Table 1). Fluorescent PCR products were separated on a Long Ranger acrylamide gel (Takara, Tokyo, Japan), and analyzed on an automated DNA sequencer (Amersham Pharmacia Biotech Model ALFred). A quantitative analysis of the signal intensity was carried out with the fragment analysis program (Amersham Pharmacia Biotech; AlleleLinks version 1.00). LOH was defined by calculating the allelic ratio (AR) of both normal (N) and tumor (T) DNA according to the following formula: AR = (N2/N1)/(T2/T1), where AR is the ration of the peak height of the longer allele (N2 or T2) to that of the shorter allele (N1 or T1). An LOH was indicated when the ratio was greater than 1.3 or smaller than 0.7, presenting loss of the shorter or longer allele, respectively (35, 41).

RT-PCR analysis for Apaf-1. The mRNA was extracted from the frozen tissue samples using the RNeasy Mini kit (QIAGEN, Hilden, Germany). Five of 33 tissue samples were not available for RNA extraction. Random hexamer-primed single-strand cDNA was synthesized using the First strand cDNA synthesis kit (Amersham Pharmacia Biotech, Tokyo, Japan) according to the manufacturer's instructions. Ten μl of cDNA were employed for PCR. The analysis of beta actin was used as measure for ubiquitous gene expression. The Apaf-1 specific primers for a 149 bp fragment encompassing exons 22-24 including boundary regions were purchased from Sigma Genosis (Table 1). The products

were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. A quantitative analysis of the signal intensity was carried out with a public domain soft, NIH Image (NIH, United States, *http://rsb.info.nih.gov/nih-image/*). To determine mRNA expression, the ratio of coamplified Apaf-1 to beta actin PCR product was compared tumor and normal brain. Loss or markedly reduction of amplification of the Apaf-1 is noted when the coamplified Apaf-1/beta actin PCR product ratio of tumor was less than 30% of that of normal brain. (29, 42). The Apaf-1/beta actin PCR product ratios were mean values obtained from 3 independent experiments.

Immunohistochemical (IHC) Apaf-1 staining. The antibody to Apaf-1 (2E12) was purchased from SILENUS (Boronia, Australia). Immunohistochemical analysis was examined using the avidin-biotin (ABC) technique. Tissue sections of colon and kidney were used as positive controls for Apaf-1. Negative controls were made by omitting the primary antibody during the immuno-staining and using phosphate-buffered saline PBS. A tumor was scored as positive if unequivocal cytosol staining was observed, in more than 20% of the tumor cells.

Analysis of p53 gene. *p53* gene was analyzed by fluorescent based-SSCP and direct sequencing (17). The 5 pairs of sense and antisense primers for exons 5 to 8 of *p53* gene were used. All primers were labeled with Cy5 (Amersham Pharmacia Biotech) at the 5′ end (Table 1). The materials with variant SSCP were reamplified and sequenced bidirectionally. Sequence analysis was carried out with a semiautomated sequencer 3100 Genetic Analyzer (Applied Biosystems).

Analysis of EGFR gene. *EGFR* amplification was analyzed with a differential PCR method as described previously with some modifications (46). In brief, a 149-bp fragment of EGFR gene was coamplified with an 85-bp fragment of *interferon gamma* (*IFNG*) gene. One primer of each pair was labeled with indodicarbocyamine (Cy5) fluorescent dye (Amersham Pharmacia Biotech) at the 5′ end (Table 1). Fluorescent PCR products were separated on a Long Ranger acrylamide gel (Takara, Tokyo, Japan), and analyzed on an automated DNA sequencer (Amersham Pharmacia Biotech Model ALFred). A quantitative analysis of the signal intensity was carried out with the fragment analysis program (Amersham Pharmacia Biotech; AlleleLinks version 1.00). To determine the variation in the ratio of *EGFR* to *IFNG* gene PCR products in normal DNA, we studied 56 genomic DNA samples from peripheral lymphocytes. The mean ratio and standard deviation (SD) of the coamplified *EGFR/IFNG* was 1.015 and 0.115. All *EGFR/INFG* ratios greater than twice the mean of the *EGFR/INFG* ratio plus three SDs were taken as evidence of *EGFR* gene amplification. All values in our series of control DNAs were within the range of the mean value ± 3 SD.

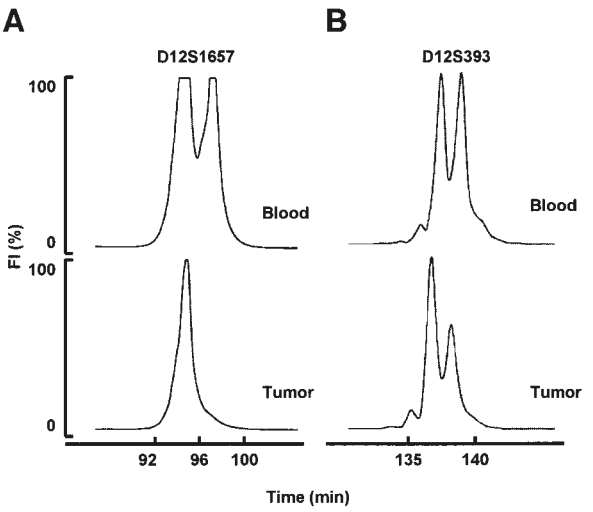

Figure 1. Representative LOH analyses illustrating allelic loss of 12q22-23 at marker D12S1657 in case 27 (**A**) and D12S393 in case29 (**B**). Note the data from blood (upper panel) and tumor (lower panel) were obtained from the same patient. The horizontal axis shows time of electrophoresis (min). The vertical axis shows fluorescent intensity (FI) (%).

Results

Clinical and genetic findings in patients with GB are summarized in Table 2.

Analysis of LOH at chromosome 12q22-23 and Apaf-1 expression. Using 2 microsatellite markers, we examined a total of 66 polymorphic loci at chromosome 12q22-23 and obtained 46 (70%) informative results. LOH at chromosome 12q22-23 was detected in 14 (42%) of 33 cases (Figure 1).We also used RT-PCR to examine 28 cases with available mRNA. Low expression of Apaf-1 mRNA was detected in 12 (43%) of 28 cases (Figure 2). In an IHC analysis of 33 cases, 17 (52%) showed positive reactivity restricted to the cytosol of the tumor cells, and 16 (49%) showed a negative reactivity that means low expression of the Apaf-1 protein (Figure 3). Among 14 cases with the LOH at 12q22-23, low expression of Apaf-1 mRNA was detected in 9 (69%) of

No.	Age	Gender	Site	Region	12q22-23		Apaf-1		EGFR		p53 Mutation			
					S393	S1657	RT-PCR	IHC	amplification		Exon	Codon	Nucleotide	Amino acid
1	34	M	L	F	-	NI	-	+	-	wt				
2	78	M	R	O/T	NI	-	+	+	-	mut	6	218	CTG→GCG	Val→Ala
3	52	M	L	F	-	NI	+	-	-	mut	5	137	CTG→CAG	Leu→Gln
4	51	F	R	O	-	-	+	+	amp	mut	5	137	CTG→CAG	Leu→Gln
5	8	F	L	BG	NI	NI	+	-	-	mut	8	267	CGG→TGG	Arg→Trp
6	25	M	L	O/T	NI	LOH	NE	-	-	wt				
7	57	M	R	F	NI	-	+	+	amp	wt				
8	50	F	L	F	-	-	NE	+	-	wt				
9	34	M	L	P/T	LOH	NI	+	-	-	wt				
10	43	F	L	F	LOH	-	-	-	amp	wt				
11	50	M	R	T/F	-	-	+	+	-	mut	5	151	CCC→TCC	Pro→Ser
12	51	F	R	T	-	-	+	+	amp	wt				
13	66	M	L	T/F	-	-	+	+	-	wt				
14	74	M	L	F	-	-	NE	+	amp	wt				
15	56	F	R	T	-	NI	NE	+	-	wt				
16	44	F	R	T/O	NI	LOH	-	+	amp	wt				
17	76	F	L	P/O	NI	LOH	+	+	-	mut	6	198	GGA→TGA	Gln→stop
18	57	F	L	BG/T	-	LOH	-	-	amp	wt				
19	69	F	R	T	-	-	+	+	-	mut	8	273	CGT→CAT	Arg→His
20	78	M	L	F/P	-	-	+	+	-	wt				
21	59	M	L	T	-	NI	+	+	-	mut	5	146	TGG→TGA	Trp→stop
22	24	F	R	T	LOH	-	-	-	-	mut	8	274	GTT→GCC	Val→Ala
23	43	M	R	F/P	LOH	-	-	-	-	mut	5	149	TCC→ACC	Ser→Thr
24	29	M	L	F/P	-	LOH	-	-	amp	wt				
25	44	F	L	F	LOH	-	+	-	-	wt				
26	34	M	L	F	-	NI	NE	+	-	mut	6	213	CGA→CGG	Arg→Arg
27	46	F	L	T	NI	LOH	-	-	-	wt				
28	33	M	L	T	-	NI	-	-	-	mut	7	249	AGG→AAG	Arg→Lys
29	24	F	L	F	LOH	NI	-	-	-	mut	7	248	CGG→CAG	Arg→Gln
30	30	M	R	F	NI	LOH	+	-	-	wt				
31	32	F	R	F	-	NI	-	-	-	wt				
32	39	M	L	F	-	-	+	+	-	wt				
33	74	F	L	F/P	LOH	-	-	-	-	wt				

paf-1, apoptotic protease activating factor-1; IHC, immunohistochemistry; EGFR, epidermal growth factor receptor; M, male; F, female; L, left; R, right; F, frontal lobe; O, occipital lobe; T, temporal lobe; P, parietal lobe; BG, basal ganglia; NI, not informative; LOH, loss of heterozygosity; NE, not examined; amp, ampification; wt, wild type; mut, mutation

Table 2. Clinical, genetic and immunohistochemical findings in patients with glioblastoma.

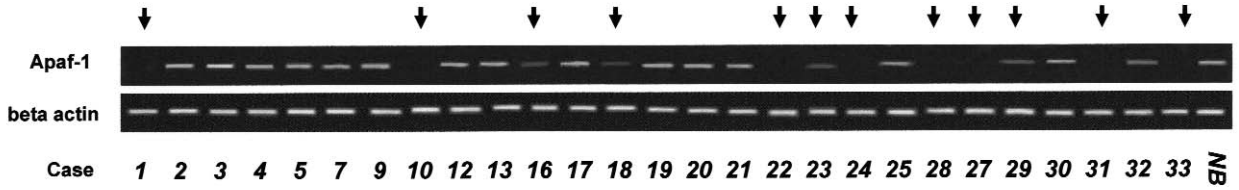

Figure 2. Results of differential RT-PCR analysis. Low expression of Apaf-1 was present in cases, 1, 10, 16, 18, 22, 23, 24, 27, 28, 29, 31, and 33 (arrows). NB = normal brain.

13 examined cases, and low expression of the Apaf-1 protein was detected in 12 (86%) of 14 cases. The 12q22-23 LOH was significantly correlated with low expression of mRNA and low expression of protein ($p<0.05$, $p<0.001$ respectively, Fisher's exact probability test) (Table 3).

12q22-23	Average age*	Apaf-1 mRNA**		Apaf-1 protein***	
		RT-PCR (-)	RT-PCR (+)	IHC (-)	IHC (+)
LOH (+)	42.36 ± 16.97	9	4	12	2
LOH (-)	51.11 ± 18.14	3	12	4	15

* p = 0.169, ** p < 0.05, *** p<0.01
LOH, loss of heterozygosity; Apaf-1, apoptotic protease activating factor -1; IHC, immunohistochemistry.

Table 3. Chromosome 12q22-23 LOH and Apaf-1 expression in glioblastomas.

In an analysis of patient age, the patients with the 12q22-23 LOH in GB were younger than the patients with the GB lacking the 12q22-23 LOH (mean age 42.4 versus 51.1 years), but this age difference was not statistically significant (Table 3).

Correlation with p53 gene mutation or EGFR gene amplification. Of the 33 cases, 13 (39%) had the *p53* gene mutation (Figure 4), 8 (24%) had the *EGFR* gene amplification (Figure 5), and 13 (39%) had neither. The *p53* gene mutation and *EGFR* gene amplification were mutually exclusive ($p<0.05$, Fisher's exact probability test). These findings are similar to those previously reported. However, the 12q22-23 LOH had no correlations with the *p53* gene mutation or *EGFR* gene amplification.

Allelic information of 12q22-23 was obtained from 9 of the 13 cases lacking both the *p53* gene mutation and the *EGFR* gene amplification. Among these 9 cases, 6 (67%) had the 12q22-23 LOH.

Discussion

Recent molecular genetic studies have uncovered several structural alterations of specific genes and chromosomal instabilities that appear to play a role in the pathogenesis of astrocytic tumors. GB can be divided into several genetic subsets, the most common of which are one subset with the *p53* gene mutation and without EGFR gene amplification, and one subset with EGFR gene amplification and without *p53* gene mutation (24, 44, 45). The former is found in younger patients, while the latter is found in older adults and may act more aggressively (10, 24, 26, 44, 45). Several other genetic alterations, such as homozygous deletion of the *INK4a/ARF* gene and *Rb* gene mutation, correlate with the *p53* gene mutation and *EGFR* gene amplification (1, 11, 14-16, 42). In this way, classification of GB subsets based on genetic alteration reflect the network of biological features associated with each gene alteration, and give us important information on clinical and biological markers (33). In the present study, we focused our attention on Apaf-1, the product of the *Apaf-1* gene located in 12q22-23, and discussed the associations of the 12q22-23 LOH with the *p53* gene mutation and *EGFR* gene amplification, 2 major factors of the different subsets of GB.

The LOH of chromosome 12q has so far been reported to have a frequency of no more than 5 to 6% in GB (43). However, compared with the microsatellite markers used previously, those used in this study were located at different sites at both ends of the Apaf-1 locus, and the frequency of 12q22-23 LOH in our GB series reached as high as 42%. It remains uncertain whether the 12q22-23 LOH occurs specifically in GB, as it has also been observed at a high frequency in metastatic melanoma developing from the ectoderm (37). The 12q22-23 LOH is not a genome-wide event, and its

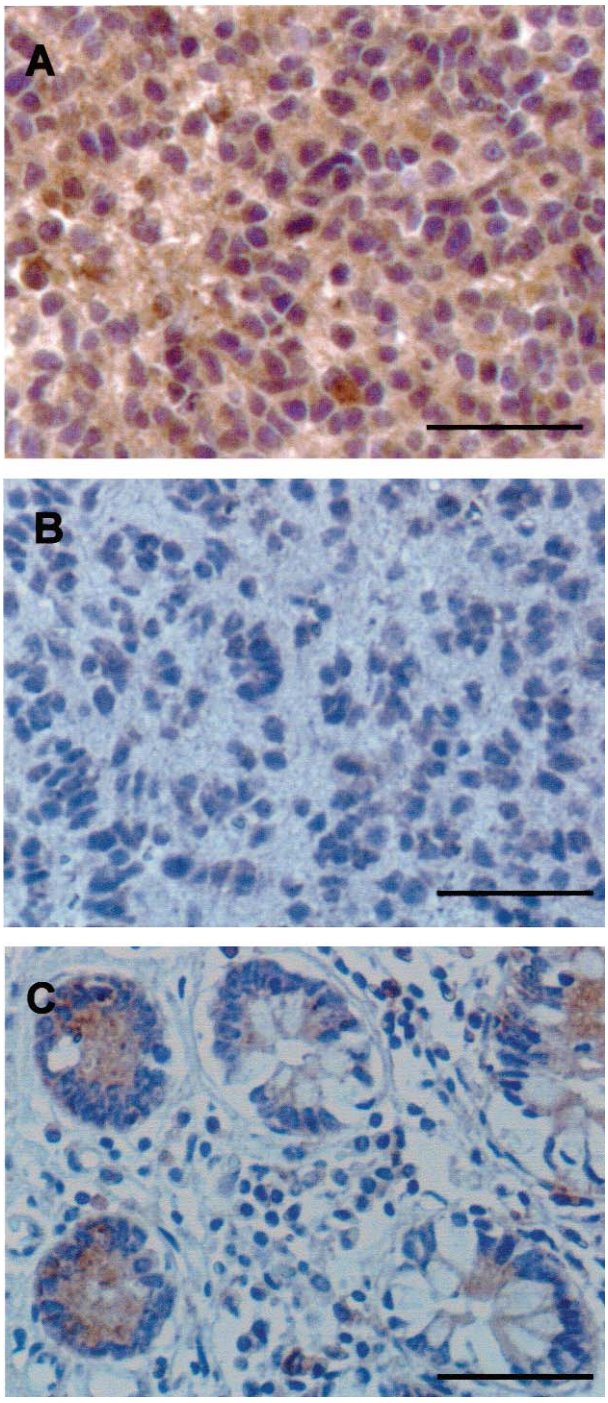

Figure 3. Immunostaining with anti Apaf-1 antibody. Positive Immunoreactivity is found and restricted to the cytosol of the tumor cells of case 13 (**A**). Immunoreactivity is not seen in tumor cells of case 6 (**B**). Immunoreactivity is found in colon tissue (positive control, **C**). **A**, **B**, and **C** are at the same magnification, bar = 20 μm.

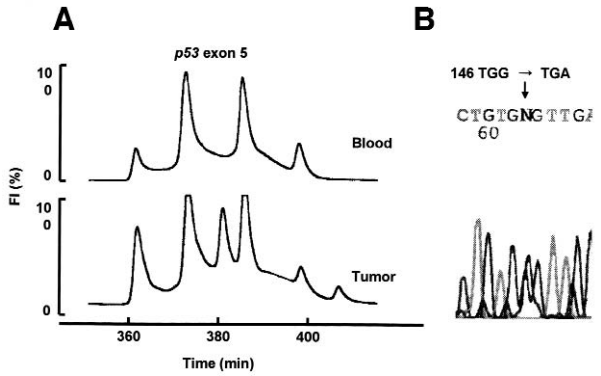

Figure 4. Representative case carrying *p53* mutation (case 21). Fluorescence-based single strand conformation polymorphism both from the same patient shows a different mobility shift in exon 5a in the tumor as compared with blood (**A**). The horizontal axis shows time of electrophoresis (min). The vertical axis shows fluorescent intensity (FI) (%).The sequencing analysis of the tumor DNA reveals a one-base substitution from G to A (**B**).

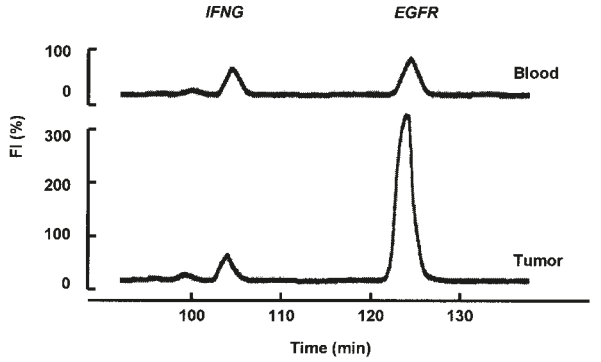

Figure 5. Differential PCR-analysis for *EGFR/IFNG* genes. Representative case from a glioblastoma carrying *EGFR* amplification (case 7, lower panel). The horizontal axis shows time of electrophoresis (min). The vertical axis shows fluorescent intensity (FI) (%). IFNG, interferon gamma; EGFR, epidermal growth factor receptor.

extremely confined localization to 12q22-23 on chromosome 12q explains the discrepancy in frequency between our study and the former one. This discrepancy might also be due to the higher sensitivity of our analysis method using fluorescent based-microsatellite PCR assay detected by automated DNA sequencer. In a metastatic melanoma carrying the 12q22-23 LOH, *Apaf-1* was repressed by methylation of the other allelic gene (37). The present study also indicated significant relationships between the 12q22-23 LOH and repression of Apaf-1 mRNA as well as protein. Although a recent study of leukemia revealed that epigenetic mechanism of

Apaf-1 inactivation was not only due to promoter methylation (9), future studies will seek to clarify whether the other allelic gene is methylated in our GB series. Of the 19 GBs without 12q22-23 LOH, 3 showed low expression of Apaf-1 mRNA, and 4 showed low expression of Apaf-1 protein. In these 7 cases, the *Apaf-1* gene might be silenced by an epigenetic mechanism such as methylation of both allelic genes. On the other hand, in one of the 14 GBs carrying the 12q22-23 LOH, we were unable to determine whether that both the Apaf-1 mRNA and protein expression levels were low. The Apaf-1 gene in this case might have escaped from the methylation, or our method for evaluating the expression level of mRNA in the present study might have been nothing more than comparative.

INK4a and *ARF*, common tumor suppressor genes in GB, were also reported to be silenced by both of methylation and a genetic alteration such as a homozygous deletion (5, 28). While it remains unclear why the *Apaf-1* and these specific genes are likely to undergo such conditions of structural alterations, their genetic instability might be closely associated with a certain region of the cancer-linked gene in tumor cells. In the case of the *Apaf-1* gene, the 12q22-23 LOH may be induced by genetic instability due to the epigenetic effects including a methylation during course of tumor progression (3, 7, 8, 12, 13).

In our 33 GB series, the 12q22-23 LOH associated with Apaf-1 inactivation had no correlations with the *p53* gene mutation or *EGFR* gene amplification. However, among the 9 cases lacking both the *p53* gene mutation and *EGFR* gene amplification, 6 (67%) had the 12q22-23 LOH. These 6 cases appeared to be classifiable within another subset that possesses the 12q22-23 LOH with Apaf-1 inactivation, while lacking both the *p53* gene mutation and *EGFR* gene amplification. Additionally, the mean age of the cases with the 12q22-23 LOH, 42.4 years, was substantially lower than that of the cases without the 12q22-23 LOH, 51.1 years. This difference in age, together with the tendency of the GBs carrying the *p53* gene mutation to be younger than the GBs carrying the *EGFR* gene amplification (43, 45, 47), suggests that the younger patients with GB are divided into at least 2 subsets, one with the *p53* gene mutation, and another with the 12q22-23 LOH. However, the accumulation of a larger series of patients with GB will be required to prove whether these speculated differences are significant. Our GB series included 6 cases with secondary GB assessed over the clinical course, and all of them lacked the *EGFR* gene amplification while showing a high rate of the *p53* gene mutation, as previously described. Interestingly, the 12q22-23 LOH was identified in all 6 of these cases. This deviation might have been due to the occurrence of the 12q22-23 LOH younger patients, in whom secondary GBs were likely to arise (21, 26). This group of secondary GBs may belong to a third subset of younger patients carrying both the *p53* gene mutation and 12q22-23 LOH.

Apoptosis regulators play one of the most critical roles of tumorigenesis, and its abrogation causes various tumors, including GB (49, 20, 40, 38). In the "$p14^{ARF}$-p53-Apaf-1" mediated apoptosis pathway, it was reported that an inactivation of Apaf-1 is involved in tumorigenesis, along with inactivation of $p14^{ARF}$ and p53 (4, 49, 37, 18). While co-transduction of Apaf-1 and caspase-9 genes has been reported to strongly augment p53-mediated apoptosis in glioma cell lines (34), there have been no reports on expression of Apaf-1 in actual GB series. The present study is the first to suggest that an inactivation of Apaf-1 may be involved in GB tumorigenesis.

The *p53* gene mutation is not the only common genetic alteration in GB. *INK4a/ARF* gene deletions are also common in GB (11, 21, 25), and they have been demonstrated to be mutually exclusive genetic events in GB (11, 14). Homozygous deletion of *INK4a/ARF* gene can only be involved in the development of astrocytic tumors after a coexistent EGFR activation (1). In addition, GBs carrying the *EGFR* gene amplification tend to have homozygous deletion of the *INK4a/ARF* gene (14, 16). Noting the above relation between *EGFR* gene amplification and *INK4a/ARF* gene deletion, as well as high frequencies of the *p53* gene mutation and the 12q22-23 LOH with Apaf-1 inactivation, it appears that structural genetic alterations involved in the "p14ARF-p53-Apaf-1" apoptosis pathway are likely to be present in almost all GBs. In this study, there were 23 (70%) cases carrying either the 12q22-23 LOH or the *p53* gene mutation. When the cases with *EGFR* gene amplification were included, the total reached 29 (88%).

In summary, the LOH at chromosome 12q22-23 is strongly associated with Apaf-1 expression, and not with the genetic subsets previously known in GB. Inactivation of Apaf-1 could be one of important events associated with tumorigenesis of GB via abrogation of apoptosis pathway.

Acknowledgments

This project was supported in part by a Grant-in-Aid for Scientific Research C (Yutaka Hayashi) and B (Junkoh Yamashita) from Ministry of Education, Science, Sports and Culture of Japan.

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