

Single Nucleotide Polymorphism 309 Affects Murin-Double-Minute 2 Protein Expression But Not Glioma Tumorigenesis

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Abstract

Murin-double-minute 2 (MDM2) is an important negative regulator of the p53 tumor suppressor, and affects the p53 protein level and transcriptional activity. The genotype of the single nucleotide polymorphism in the promoter region of MDM2 (single nucleotide polymorphism [SNP] 309) is associated with the MDM2 protein expression level and the onset age of several types of cancer. The SNP309 genotype was investigated in 254 Japanese patients with glioma and 50 healthy subjects. The genotype frequency of SNP309 was T/T homozygous in 62 patients (24%), T/G heterozygous in 126 (50%), and G/G homozygous in 66 (26%) of the glioma patients, and was similar in the healthy subjects. The G/G ratio was higher in our Japanese subjects than in Western populations. Immunohistochemical study of glioma tissues showed that the G/G genotype was associated with higher expression of MDM2 protein compared to the T/T genotype, suggesting that SNP309 attenuates MDM2 protein expression *in vivo*. However, no association was found between the SNP309 genotype and the histological grade of glioma, age at disease onset, or p53 gene mutation rate. In our study population, SNP309 affected MDM2 protein level, but had no significant involvement in glioma tumorigenesis.

Key words: single nucleotide polymorphism, murin-double-minute 2, glioma

Introduction

The tumor suppressor gene p53 is known to affect many cellular functions such as cell-cycle arrest, apoptosis, and transcriptional regulation,¹⁶⁾ and is important in the suppression of glioma tumorigenesis. Approximately half of all examined gliomas manifested p53 mutation, and p53 inactivation is an early event of glioma tumorigenesis,¹⁰⁾ but the mechanisms underlying tumorigenesis in gliomas with wild-type p53 remain to be elucidated.

Murin-double-minute 2 (MDM2) is an important negative regulator of p53,⁸⁾ and MDM2 gene amplification was observed in 10–15% of malignant gliomas.¹¹⁾ MDM2 overexpression leads to inhibition of p53-mediated transactivation and enhancement of tumor progression. In addition, MDM2 promotes rapid degradation of p53 protein via the ubiquitin-proteasome system.⁸⁾ As inactivation of p53 is an

early event in glioma tumorigenesis, the expression level of MDM2 may be an important factor in tumor progression in gliomas with wild-type or mutant p53.

A single nucleotide polymorphism (SNP) in the promoter region of human MDM2 (SNP309) affects the binding activity of transcriptional factor Sp1 and attenuates the MDM2 messenger ribonucleic acid and protein expression level.³⁾ Moreover, the SNP309 genotype was associated with the onset age in several types of cancer. As p53 is important in glioma tumorigenesis and tumor progression, the SNP309 genotype may also be involved.

We examined the SNP309 genotype in Japanese patients with glioma and healthy subjects to determine whether there is a correlation between the expression of SNP309 and that of MDM2 and p53. We also analyzed the association between the SNP309 genotype and the age at onset and the

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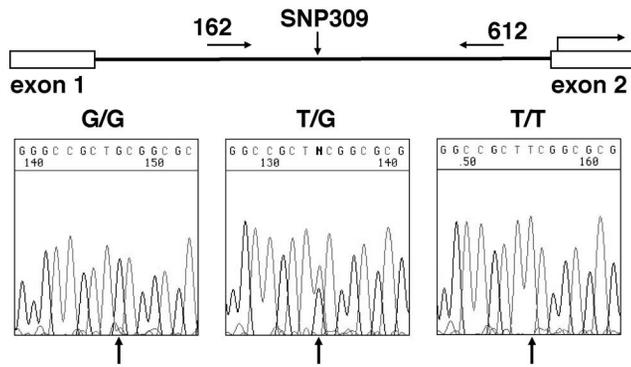


Fig. 1 Sequencing analysis of single nucleotide polymorphism (SNP) 309. Upper: SNP309 is located at the 309th nucleotide in the first intron of the murin-double-minute 2 gene, G is substituted for T. The arrows indicate the position of the primer sets and the amplified 451-bp fragment for direct sequencing of SNP309. Lower: Illustrative results of SNP309 direct sequencing. The arrows show the SNP309 peaks.

clinicopathological features of Japanese patients with glioma.

Materials and Methods

I. Deoxyribonucleic acid (DNA) samples

Genomic DNA from 254 Japanese patients with glioma, 150 males and 104 females aged 1–78 years (mean 47.7 years, median 51.0 years), and 50 healthy subjects, 25 males and 25 females aged 22–47 years (mean 32.2 years, median 31.5 years), was obtained from the Department of Neurosurgery of Kumamoto University Hospital and its affiliated hospitals. All protocols were approved by the Internal Review Board of Kumamoto University and prior informed consent was obtained from all patients and subjects. All histological diagnoses were confirmed by standard histological analysis of surgical specimens as previously described.¹²⁾ Of the 254 patients, 119 had glioblastoma, 51 anaplastic astrocytoma, 13 anaplastic oligodendroglioma, 30 anaplastic oligoastrocytoma, four anaplastic ependymoma, two anaplastic ganglioglioma, 14 pilocytic astrocytoma, 12 low grade astrocytoma, four oligodendroglioma, one oligoastrocytoma, and four ganglioglioma. Genomic DNA was extracted from whole blood using the QIAamp[®] DNA Mini Kit (Qiagen K.K., Tokyo), dissolved in 100 μ l TE (10 mM Tris and 0.5 mM ethylenediaminetetra-acetic acid), and stored at 4°C until use. A pilot study showed complete matching between the genomic DNA extracted from blood

samples and glioma specimens from 15 patients.

II. Identification of SNP309 genotype

To confirm sequence variations in the MDM2 promoter region, a 451 bp fragment that included SNP309 from genomic DNA was amplified and the 309th nucleotide in the first intron of the MDM2 gene was sequenced (Fig. 1). The forward and reverse primers were 5'-TTTTTGTGGACTGGGGCTAG-3' and 5'-AGCAAGTCGGTGCTTACCTG-3', respectively. The polymerase chain reaction (PCR) products were separated by electrophoresis in 2% agarose gels and extracted with a PCR Purification Kit (Qiagen K.K.). Each fragment was sequenced on an ABI PRISM[®] 377 DNA Sequencer (PE Biosystems, Foster City, Calif., U.S.A.) using the ABI PRISM[®] Big Dye Terminator Cycle Sequencing Kit (PE Biosystems).

III. p53 Mutation analysis

The yeast functional assay was used as previously described.¹²⁾ Briefly, using p53 PCR products from the tissues of the patients as templates, the linearized p53-expression vector pSS16 was co-transfected into the yIG397 reporter yeast strain. The transformed yeast cells were plated, incubated at 30°C for 2 days to allow the formation of colonies, and stored overnight at 4°C for color development. At least 200 colonies were examined on each plate. If more than 15% of the colonies were red, we judged the sample positive for p53 functional loss and proceeded to sequence analysis.

IV. Expression of p53 and MDM2 protein

Formalin-fixed, paraffin-embedded tissue sections were cut, deparaffinized in xylene, rehydrated through a graded series of alcohol-to-water, and incubated for 5 minutes at room temperature with 0.3% hydrogen peroxide in distilled water to inhibit endogenous peroxidase. After washing with phosphate-buffered saline, the sections were completely immersed in 0.01 M citrate buffer (pH 6.0 and pH 7.0), processed for 15 minutes in a microwave oven at 500 W to enhance immunoreactivity, saturated with 10% normal serum for 20 minutes, and incubated at 4°C for 18–22 hours with monoclonal anti-human p53 protein (Ab-2, 1:50; Oncogene Research Products, Boston, Mass., U.S.A.) and monoclonal anti-human MDM2 protein (Ab-1, 1:50, SMP-14; Neomarkers Inc., Fremont, Calif., U.S.A.). Biotinylated antibody and Vectastain[®] Elite ABC (Vector Laboratories Inc., Burlingame, Calif., U.S.A.) were successively applied at room temperature for 30 minutes each. The immune reactions were developed with diaminobenzidine as the chro-

mogen. The sections were counterstained with hematoxylin. Sections not exposed to the primary antibodies served as negative controls. The percentages of p53- and MDM2-positive tumor cells were estimated by counting the number of immunoreactive cells in 10 high-power microscopic fields in areas with the highest visually determined immunopositivity. The absence of any positive-signal tumor cells was scored as negative, <5% immunopositive tumor cells as ±, 5–20% as +, 21–50% as ++, and >50% as +++.

V. Statistical analysis

All statistical analyses were conducted using JMP® software (SAS Institute Inc., Cary, N.C., U.S.A.). Statistical differences in variables were tested by the χ^2 test. Genotype frequencies in all groups were checked for Hardy-Weinberg equilibrium using the χ^2 test. Kaplan-Meier incident curves for the three genotypes were used to analyze the age of glioma onset. The log-rank test was applied to compare the homogeneity of the incident curves between genotype groups. A p value < 0.05 was considered as significant.

Results

I. SNP309 genotype in the Japanese population

Investigation of genomic DNA from the 50 subjects found 15 (30%) were homozygous for T/T, 18 (36%) were heterozygous for T/G, and 17 (34%) were homozygous for G/G. The frequencies were consistent with Hardy-Weinberg equilibrium. Investigation of genomic DNA from the 254 glioma patients found that 62 (24%) were T/T homozygous, 126 (50%) were T/G heterozygous, and 66 (26%) were G/G homozygous. These frequencies were also consistent with Hardy-Weinberg equilibrium. There was no statistically significant difference between glioma patients and healthy subjects with respect to the distribution of SNP309 genotype (p = 0.21, χ^2 test).

II. SNP309 genotype and expression of MDM2 and p53

A total of 54 glioma samples, 17 T/T, 22 T/G, and 15 G/G, were randomly selected and the expression of MDM2 and p53 examined (Fig. 2). Table 1 shows that glioma homozygous for G/G expressed significantly more MDM2 protein than glioma homozygous for T/T (p = 0.0009, χ^2 test). In contrast, SNP309 genotype had no effect on p53 protein expression (p = 0.89, χ^2 test). Moreover, Table 2 shows no obvious correlation between the expression of p53 and that of MDM2 (p = 0.57, χ^2 test).

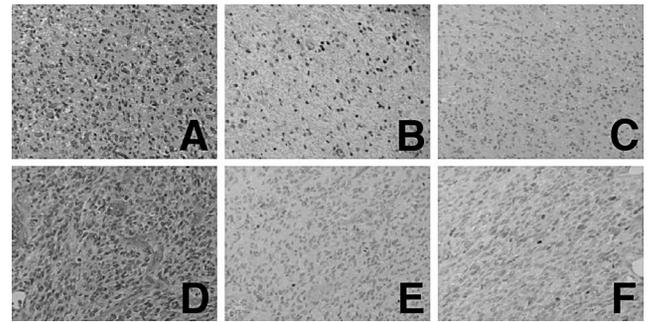


Fig. 2 Photomicrographs showing immunohistochemical examination of illustrative cases of glioblastoma with G/G genotype (A–C) and with T/T genotype (D–F). Hematoxylin and eosin stain (A, D), and immunohistochemical stain for murin-double-minute 2 (B, E) and p53 (C, F), ×200.

Table 1 Single nucleotide polymorphism (SNP) 309 genotype (T/T, T/G, G/G) and expression of murin-double-minute 2 (MDM2) and p53 in 54 glioma samples

	T/T (n = 17)	T/G (n = 22)	G/G (n = 15)
MDM2 expression			
– or ±	14	8	3
+ or ++	3	14	12
p53 expression			
– or ±	9	12	7
+ or ++	8	10	8

The SNP309 genotype affects the protein expression of MDM2 (p = 0.0009) but not that of p53 (p = 0.89, χ^2 test).

Table 2 Relationships between murin-double-minute 2 (MDM2) protein expression and p53 mutation, and p53 protein expression in 54 glioma samples

	p53 expression	
	– or ± (n = 28)	+ or ++ (n = 26)
MDM2 expression		
– or ±	14	11
+ or ++	14	15
p53 mutation		
–	25	5
+	3	21

Expression of p53 protein and p53 mutation were related (p < 0.001), but not MDM2 protein expression (p = 0.57, χ^2 test).

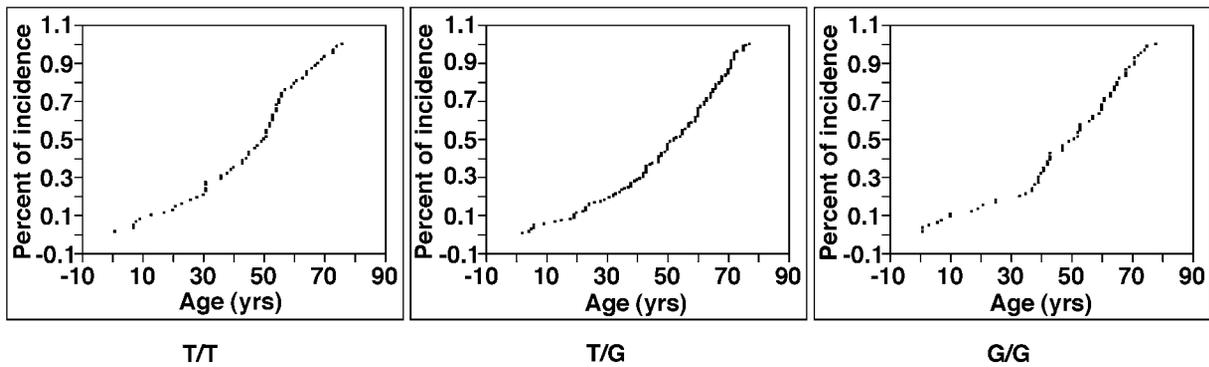


Fig. 3 Cumulative incidence and age at disease onset in 254 glioma patients.

Table 3 Classification of the 254 glioma patients according to the glioma grade and type, and the single nucleotide polymorphism (SNP) 309 genotype (T/T, T/G, G/G)

Histological classification	T/T (n=62)	T/G (n=126)	G/G (n=66)
High grade (WHO grades III and IV)	54 (25%)	112 (51%)	53 (24%)
anaplastic astrocytoma	6	25	20
anaplastic oligodendroglioma	6	5	2
anaplastic oligoastrocytoma	7	20	3
anaplastic ganglioglioma	1	1	0
anaplastic ependymoma	0	3	1
glioblastoma	34	58	27
Low grade (WHO grades I and II)	8 (23%)	14 (40%)	13 (37%)
pilocytic astrocytoma	3	6	5
astrocytoma	3	4	5
oligodendroglioma	0	3	1
oligoastrocytoma	0	1	0
ganglioglioma	2	0	2

There was no significant association between SNP309 genotype and glioma grade and type ($p=0.76$ for low grade glioma, $p=0.14$ for high grade glioma, and $p=0.21$ for all gliomas, χ^2 test). WHO: World Health Organization.

However, 21 of 26 gliomas with higher p53 expression had p53 gene mutations whereas 25 of 28 gliomas with lower p53 expression had wild-type p53, so there was a strong correlation between p53 gene status and protein expression ($p < 0.001$, χ^2 test). These findings suggest that the SNP309 genotype affects expression of MDM2 but not that of p53.

III. SNP309 genotype and clinicopathological features

We postulated that if SNP309 affects the pathway of p53 via MDM2 expression, the genotype may affect the clinicopathological features of the patients. Table 3 shows that there was no significant association between SNP309 genotype and glioma grade ($p = 0.76$ for low grade glioma and $p = 0.14$ for high grade glioma, χ^2 test). Table 4 shows that there was no association between the SNP309 genotype and mean ($p = 0.41$) or median age ($p = 0.71$) at

Table 4 Single nucleotide polymorphism (SNP) 309 genotype (T/T, T/G, G/G) and age at glioma onset in 254 glioma patients

Age	T/T (n=62)	T/G (n=126)	G/G (n=66)
Mean (yrs)	45.2	49.2	47.3
Median (yrs)	50.5	52.5	51.5
Range (yrs)	1-76	2-77	1-78

There was no association between SNP309 genotype and mean ($p=0.41$) or median age ($p=0.71$, χ^2 test).

glioma onset.

Figure 3 shows the cumulative incidence and age at onset, with no statistical difference between the SNP309 genotype and onset age ($p = 0.2776$, log-rank test). These results suggested that SNP309 genotype had no strong association with the age at

Table 5 Relationship between single nucleotide polymorphism (SNP) 309 genotype (T/T, T/G, G/G) and p53 mutation in 190 glioma patients

p53 mutation	T/T (n = 47)	T/G (n = 93)	G/G (n = 50)
+	14	32	14
-	33	61	36

There was no association between SNP309 genotype and p53 mutation ($p = 0.70$, χ^2 test).

onset.

Although the inactivation of p53 is an early event in glioma tumorigenesis, our findings that the SNP309 genotype had no strong association with patient age at disease onset suggests that p53 mutation may mask the effect of SNP309 genotype on glioma progression. To test this hypothesis we examined the relationship between SNP309 genotype and p53 mutation in 190 of the 254 glioma patients (Table 5). However, we found no association between SNP309 genotype and p53 mutation ($p = 0.70$, χ^2 test). In addition, there was no association between the age at onset and SNP309 genotype in patients without p53 mutation (data not shown). Our results indicate that there was no strong association between the SNP309 genotype and patient age at onset, histological diagnosis, or p53 mutation.

Discussion

The presence of SNP309 homozygous for G/G affects the binding activity of the transcriptional factor Sp1 and results in higher MDM2 protein expression levels and attenuation of the p53 pathway.³⁾ Patients with Li-Fraumeni syndrome and sporadic soft tissue sarcoma who manifested SNP309 homozygous for G/G were younger at disease onset.³⁾ As p53 inactivation is an early event of glioma tumorigenesis, we analyzed the SNP309 genotype in our Japanese patients with glioma. Our immunohistological analysis showed that MDM2 protein expression was attenuated in the glioma tissues. However, we found no strong association between the SNP309 genotype and the histological diagnosis or the age at glioma onset. There is also no significant association between SNP309 genotype and age at disease onset in patients with other cancers.^{1,4,6,7,14)}

The SNP309 genotype distribution for Western populations is 48% T/T, 40% T/G, and 12% G/G.³⁾ Our study found a higher incidence of SNP309 homozygous for G/G in the Japanese population of 34% for healthy subjects and 26% for glioma

patients. The reason is probably ethnicity-related differences in allele frequencies. The incidence of SNP309 homozygous for G/G was 24.8–29.3% in Chinese patients with breast and lung cancer,^{6,7)} similar to our patients and healthy subjects, and 13–24% in Caucasian patients with colorectal, breast, and ovarian cancer.^{3,4,14)} However, the incidence of SNP309 homozygous for G/G was also reported as 16–19% in the European Caucasian population.⁴⁾

MDM2 is an important negative regulator for p53 protein and overexpression of MDM2, with or without gene amplification, is a genetic marker of primary glioblastoma without a p53 mutation,¹¹⁾ but MDM2 overexpression and p53 mutation may not be mutually exclusive events. Our study found that gliomas with SNP309 homozygosity for G/G exhibited relatively higher levels of MDM2 protein, but there was no correlation between p53 protein expression and p53 mutation rate. Our immunohistochemical study showed that overexpression of p53 protein depended on the mutated gene status, not on MDM2 overexpression, suggesting that mutation of the p53 gene strongly affects p53 protein expression and overcomes the protein degradation caused by MDM2. MDM2 regulates p53 by two mechanisms: promotion of the rapid degradation of p53 protein by the ubiquitin-proteasome system, and direct binding to p53 and inactivation of the transactivating capability.⁸⁾ Transcriptional inactivation of p53 occurs via a p53-MDM2 binding complex and p53 is not excessively degraded even in cells with SNP309 homozygous for G/G.²⁾ Therefore, it is not surprising that tumors, especially gliomas, may overexpress both MDM2 and p53 and that overexpression of MDM2 and p53 mutation are not mutually exclusive.

The G allele of SNP309 is associated with lower age of onset in patients with sporadic soft tissue sarcoma,³⁾ but the present study found no strong association between the SNP309 genotype and the patient age at glioma onset. Patients with soft tissue sarcoma tend to be younger than patients with glioma at disease onset. In fact, most soft tissue sarcomas develop during childhood whereas gliomas tend to occur in individuals older than 50 years. There is a strong correlation between p53 inactivation and the development of soft tissue sarcoma.^{5,9)} However, although p53 inactivation and development of glioma are related, other biological molecular events, e.g. PTEN mutation, p16 inactivation, and epidermal growth factor receptor amplification, are also involved.^{10,15)} Indeed, p53 mutations are significantly less frequent in primary glioblastoma than in secondary glioblastoma.¹⁷⁾ Moreover, the

coexistence of several genetic mutation in glioblastoma¹³⁾ suggests that whereas a single mutation may suffice for the development of soft tissue sarcoma, this is not true for gliomas.

The present study showed that the SNP309 genotype is involved in the attenuation of MDM2 protein expression in glioma tissues but has no strong effect on the age at onset or histological classification. Overexpression of MDM2 induced by the SNP309 genotype does not appear to be critical in glioma tumorigenesis, although MDM2 attenuation via the p53 signal pathway may be involved in glioma progression.

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Commentary

The authors have reported that single nucleotide polymorphism in the promoter region of MDM2 (SNP309) is involved in the attenuation of MDM2 protein level

but has no significant effect on glioma tumorigenesis. MDM2 is a negative regulator of p53 that is mutated in half of gliomas. P53 inactivation is an early event of glioma tumorigenesis. SNP309 is associated with MDM2 protein expression level in the onset age of several types of cancer. In this study, the SNP309 genotype in 254 patients with glioma and 50 healthy subjects was examined. It was shown that SNP309 affected MDM2 protein level, but no correlation was found between the expression of SNP309 and that of MDM2 and p53, and also between the SNP309 genotype and age at disease onset or the histological grade of glioma. Recently SNPs related to onset or progression have been studied in numerous diseases, but not in glioma. This study showed that SNP309 had an effect on MDM2 protein level, but did not show that SNP309 was associated with glioma tumorigenesis and p53. This finding suggests that glioma tumorigenesis is not affected by SNP309, but whether another SNP affects glioma tumorigenesis remains unknown. SNP study for glioma will be useful, because it can contribute information about the probability of developing glioma in the future. Although no association between SNP and glioma tumorigenesis was established, it is valuable to pioneer a new field in glioma research.

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MDM2 has been shown to be a key controller of p53 which is involved in the genesis of a significant number of gliomas. Moreover, a number of prior studies have demonstrated the importance of the SNP309 genotype in tumorigenesis both in sporadic tumors and in inherited disorders, such as Li-Fraumeni syndrome. Thus, a study of the role of SNP309 in primary glioma tissue is important. However, in this study by Tsuiki et al. in a population of Japanese patients with gliomas, no strong association of SNP309 genotype was noted either in the histological

grade of the tumor nor in the age of onset of the diagnosis. This is an important piece of information in our continual increase of understanding of gliomagenesis. It will be important to confirm this finding in other populations as well.

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The authors have studied the role of the SNP309 genotype in a series of 254 Japanese patients harboring gliomas, and 50 healthy subjects. They show that patients homozygous for G/G have a higher expression of MDM2 protein by immunohistochemistry. However, there was no association between the G/G genotype and histological grade of tumor, disease onset or p53 gene mutation status. It is interesting that the rates of the SNP309 genotype in Japanese subjects differed from that reported in a series of Western subjects.

SNP309 is found in the promoter region of the human MDM2 gene. Its presence alters the binding of transcription factors and ultimately affects the expression of MDM2. The authors' data are convincing that MDM2 protein is upregulated by immunohistochemistry. It would have been nice to see confirmatory evidence of the same using Western blot analysis. Precisely why the upregulated MDM2 did not affect tumorigenesis is an interesting but unanswered question at this stage. Perhaps it is a matter of dosing of MDM2 which could be higher in the context of MDM2 gene amplification than it is in the presence of the SNP309 genotype that makes the difference. I congratulate the authors on their fine genetic analysis.

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