Distribution and Effects of *CDKN2* p16 540 C>G and 580 C>T, and *MDM2* SNP309 T>G Polymorphisms in Patients with Primary Brain Tumors

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Abstract. *Background/Aim: Primary brain tumors are unique tumors due to their different pathobiological behavior, while they rarely metastasize outside the central nervous system. Regarding the oncogenesis of primary brain tumors, it was shown that changes in functions of p16 and mouse double minute 2 homolog (MDM2) are related to tumor pathogenesis by enhancing cell proliferation and malign development. The present study aims to evaluate the possible associations between cyclin-dependent kinase 2 (CDKN2) p16 540 C>G and 580 C>T, MDM2 single nucleotide polymorphism 309 (SNP309) T>G polymorphisms and primary brain tumor. Materials and Methods: Using polymerase chain reaction-restriction fragment length polymorphism technique, we determined SNPs in 67 patients with primary brain tumors and 71 healthy volunteers without malignancy. Results: The frequency of CC genotype for CDKN2 p16 540 C>G was significantly two-fold higher (p<0.001) and possessing a C allele conferred a ~7-fold increased risk (p=0.005) of primary brain tumor. We also found that the CC genotype produced a higher ~4-fold risk of glioma (p=0.001) and the G allele had a possibly protective role against meningioma (~4.8-fold reduced risk, p=0.001). We found no significant associations for CDKN2 p16 580 C>T and MDM2 SNP309 T>G variants between cases and controls. CGT haplotype was significantly less frequent in patients with primary brain tumors and glioma cases (p=0.009 and p=0.028, respectively) than controls. CGG haplotype was significantly less frequent in patients with meningioma versus the control group (p=0.023). Conclusion: These findings show that CDKN2 p16 540 C>G, CDKN2 p16 580 C>T and MDM2 SNP309 T>G variants and their haplotypes may be risk factors for the development of primary brain tumors, especially of glioma.*

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Classification of primary brain tumors is mainly based on histopathological characteristics. Due to the peculiarity of the central nervous system (CNS), the location of the tumor is also used in the naming of the CNS tumors. Both features, histopathology and location, determine the main prognostic factors for patients with these tumors. Increased molecular and genetic studies in the past two decades have accumulated vast knowledge on the biological behavior, response to treatment and consequently prognosis of CNS tumors. The most commonly encountered primary brain tumors develop from glial cells and are named gliomas. Glioblastoma, anaplastic astrocytoma, oligoastroctoma, oligodendroglioma, diffuse glioma and brainstem glioma are listed under the name of gliomas, whereas each tumor has a different prognosis. The other most common primary brain tumor is grade I meningioma, which has a benign course compared to gliomas (1, 2).

The risk factors for primary brain tumors that are considered heterogeneous are not yet completely explained. However, epidemiological studies show that diet; smoking; exposure to neurotoxic and carcinogenic chemicals such as formaldehyde, acrylonitryl, phenolic compounds and pesticides; infections; viruses and ionizing radiation are considered as risk factors. Studies have emphasized that 5-10% of brain tumors are related to inheritance, and apart from DNA damage caused by chemical, physical and biological agents, genetic differences in genes encoding proteins involved in de-toxification and

potential oxidative metabolism of neurocarcinogenic agents are thought to be the initiating factor of tumorigenesis in the brain. It was also reported that inherited or acquired changes in cell-cycle control genes such as *p53* and retinoblastoma (*Rb*) may also be risk factors for carcinogenesis, as well as tumor-suppressor genes, proto-oncogenes and signaling pathways that are controlled by these molecules. These play crucial roles in cell differentiation, development and prognosis of cancer. Indeed, a fair number of studies have shown that mutations that cause the activation or inactivation of these genes or pathways are linked to tumor development (3-7).

Among the cell-cycle control mechanisms in humans, cyclin-dependent kinase inhibitor 2A (*CDKN2A*; INK4A/ARF locus) gene of 9p21 locus encodes for two different tumor-suppressor proteins, $p16^{INK4A}$ and $p14^{ARF}$ that block cell-cycle progression (8, 9). Both proteins, which have different targets during cell-cycle blockade, are encoded by different first exons (exon 1α and exon 1β) and common second and third exons (9). $p16^{INK4}$ inhibits cyclin D1-CDK, whereas $p14^{ARF}$ prevents degradation of p53 (8, 9).

p16 protein with 156 amino acids (10) is an important member of the Rb signaling pathway that contributes to cell-cycle progression. p16 competes with cyclin D1 to interact with CDK4 and CDK6, during the G_1 phase, preventing the formation of active cyclin complexes through inhibiting catalytic kinase activity of the enzyme. This leads p16 to act as a negative regulator of the cell cycle by preventing the phosphorylation of Rb protein, thus sustaining the complex formed between hypo-phosphorylated Rb and the transcription factor E2F which is necessary for proceeding to S phase. As a result, the cell cycle is halted at the late G_1 phase and necessary controls are carried-out and in this way, abnormal cell enlargement and cell division are prevented (8, 9, 11). Studies show that when there is a lack of p16 expression, cyclin D1-CDK4 complex phosphorylates Rb releasing E2F, thus leading the cell to move toward to the S phase (12). It was also indicated that *CDKN2* mutations block the formation of a stable complex and thus it can no longer inhibit mitotic divisions efficiently. Its importance in being a regulator in the cell cycle was detected in many tumor types by analysis of *CDKN2* gene mutations (13). In glial oncogenesis, it was shown that changes in functions of p16, pRb and members of CDK4 are related to tumor pathogenesis by enhancing cell proliferation and malign at development (14, 15). Coding of p16 and polymorphisms in the 3'UTR areas of the gene, such as *CDKN2* p16 540C>G (rs11515) and 580 C>T (rs3088440), were shown to be related to cancer development, prognosis and tumor aggressiveness. Indeed, some polymorphisms altered p16 function, while others were observed not to be influential (16, 17).

It is well-known that the p53 protein is not only a tumor suppressor that regulates cell proliferation, but also a protein that triggers apoptosis as a response to DNA damage. p53 is actively synthesized in cells that normally divide. However, under physiological conditions, p53 activity should be kept in balance to maintain homeostasis, and one essential cellular agent regulating p53 activity is mouse double minute 2 homolog (MDM2) protein, encoded by *MDM2* gene located at the 12q13-14 chromosomal locus, MDM2 works by binding p53 protein and activating its E3 ubiquitin ligase activity to cause p53 degradation and regulation, helping p53 activity to be kept in balance (18-23).

In many pathological situations, such as exposure to stress conditions including hypoxia, ultraviolet light and radiation, the fragmentation of p53 is halted either by binding of MDM2 with $p14^{ARF}$, which prevents the formation of p53-MDM2 complex, or by leading to a structural change of the p53-binding domain of MDM2 and thus liberating p53 by acetylation or phosphorylation through enzymes activated after DNA injury. p53 then localizes to the nucleus and possesses transcriptional activatory roles that stalls the cell cycle between G_1 and G_2 checkpoints. After these processes, the cell undergoes apoptosis through activation of *BAX* gene and thus prevents accumulation of further mutations (18).

It was shown that in particular with the existence of oncogenic stimulus, MDM2 protein functions independently from p53. It is well-known that the G_1/S transition depends on E2F/DP1 activation and inactivation of $p14^{ARF}$. As inactivated p14ARF cannot bind to MDM2, the migration of MDM2 to cytoplasm is inevitable. This process is followed by the reduction of p53 activity. On the other hand, as MDM2 activates E2F, the cell moves to the S phase from the G_1 phase (18-23).

Recent studies reported a thymidine-guanidine base exchange in the 309th nucleotide of the first intron of the *MDM2* gene, which leads to an increase in mRNA and protein levels, and causes the suppression of the p53 pathway, thus playing a role in carcinogenesis (24). Under normal homeostasis, p53, with a short half-life and rapid turnover, is held at a low level, ensured by MDM2. It was reported that in 20% of sporadic soft-tissue sarcomas, p53 mutations and overexpression of both p53 and MDM2 proteins worsened the prognosis of the disease and led to a decrease in survival (25, 26).

Consequently, studies of gene polymorphisms thought to be amongst risk factors are carried-out for most cancer types for genes related to cell proliferation. Polymorphisms and the variant frequencies that are found in genes for proteins that regulate the cell cycle can differ in different races. In our study, *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP309 T>G gene polymorphisms and the association of these polymorphisms with brain tumor pathogenesis were analyzed in patients in Turkish society with primary brain tumor.

Materials and Methods

Study participants and clinical investigation. In the present study, 67 primary brain tumor cases (43.3% female, 56.7% male) and 71 healthy (54.9% female, 45.1% male) volunteers were enrolled. Primary brain tumors were grouped into two sub-groups as

gliomas (56.72%) and meningiomas (43.28%). The whole patient group was diagnosed and recruited by the Department of Neurosurgery, Istanbul University Cerrahpasa Medical School (Istanbul, Turkey). All patients were newly diagnosed with histopathologically confirmed primary brain tumor, glioma or meningioma, and surgically treated before any radiotherapy and chemotherapy. Pathological grade information for patients was determined according to manual review of the pathology reports and clinical charts. Healthy volunteers without any malignancy were included as controls. Individuals with a negative family history of cancer and lacking any symptoms of brain tumor were selected for the control group. Blood samples were collected after the pathological diagnosis and prior to any chemotherapeutic or radiation therapy.

The study protocol was approved by both the Ethical Committee of the Istanbul Faculty of Medicine and the Research Fund of Istanbul University, Project number: 44725. The protocol was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical research Involving Human Subjects). All participants in the study signed informed consent forms in accordance with ethics guidelines regarding the study.

Polymerase chain reaction (PCR)-based detection of CDKN2 p16 540 C>G, p16 580C>T and MDM2 SNP309 T>G genotypes. Blood specimens were collected in tubes containing ethylene diaminetetra-acetic acid (EDTA), and genomic DNA samples were extracted from leukocyte nuclei by a previously described method based on a salting-out procedure (27). The genotyping of *CDKN2* p16 540, *CDKN2* p16 580C>T and *MDM2* SNP309 T>G polymorphisms were detected by PCR with locus-specific primers and subsequent analysis of a restriction fragment length polymorphism (RFLP) as previously reported (28, 29). All study protocols were applied under sterile conditions. A concentration of 0.4 μmol/l of each primer was used for the reaction. The primers for PCR amplification of *CDKN2* p16 540 C>G and 580 C>T locus were 5'- GAT GTG CCA CAC ATC TTT GAC CT -3' and 5'- CTA CGA AAG CGG GGT GGG TTG T - 3', and for *MDM2* SNP309 T>G region were 5'- CGC GGG AGT TCA GGG TAA AG-3' and 5'- AGC TGG AGA CAA GTC AGG ACT TAA C -3'. After the amplification the PCR products were digested with the proper restriction endonucleases, *Msp*I, *Hae*III, and *Msp*A1I (MBI Fermentas, Ontario, Canada) for detection of the genotypes of 540 C>G and 580 C>T substitutions of *CDKN2* p16 gene and the promoter substitution of *MDM2* gene, respectively. The digested DNAs were then separated on 2% agarose gel in 1×Tris borate EDTA buffer followed by staining with ethidium bromide solution. The genotypes were typed by visualization under ultraviolet light.

Statistical methods. Statistical analysis was performed by using SPSS software package (revision 21; SPSS Inc., Chicago, IL, USA). A univariate analysis was performed to compare the distribution of age and gender and the frequencies of alleles and genotypes. Clinical laboratory data are expressed as the mean±SD. Mean values were compared between patients and controls by unpaired Student's *t*-test. Differences in the distribution of genotypes and alleles between cases and controls were tested using the Chi-square-statistic and Fisher's exact tests. The Hardy–Weinberg equilibrium was tested for all polymorphisms. Allelic frequencies were estimated by gene counting methods. A multivariate logistic regression model was performed to investigate the associations between the genotypes of the studied polymorphisms, patient characteristics and clinicopathological parameters. Values of $p<0.05$ were considered statistically significant. Linkage disequilibrium and haplotype frequencies among *CDKN2* p16 540, *CDKN2* p16, *CDKN2* p16 580 C>T and *MDM2* SNP309 T>G polymorphisms were evaluated by using the logarithm of the likelihood odds ratio (LOD) and the correlation coefficient between the two loci (r2) values calculated by Haploview 4.2 programme (http://haploview.software.informer.com/4.2/ internet access date: 1st December, 2014).

Results

Clinical investigation. The baseline characteristics of the study groups including age, gender, cigarette smoking, tumor histology, tumor grade, tumor localization, necrosis pathology and vascular endothelial proliferation status are shown in Table I.

The age distribution of the study groups were balanced ($p>0.05$), and cigarette smoking was found to decrease in the order of glioma>meningioma>controls ($p=0.040$). When primary brain tumor and control cases were compared, whether there was any statistically significance detected, it was found that male gender may contribute to the development of primary brain tumor. In glioma cases, the frequency of male gender was higher than that for female gender; it was found that female gender had a ~1.5-fold protective role on the risk of glioma ($p=0.039$).

In the group of patients with primary tumor, 38.8% of patients had tumor with grade I, 17.9% with grade II, 17.9% with grade III and 25.4% with grade IV (Table I). In patients with glioma, the frequency of high-grade tumor (grade III+IV) was 71.1% and in patients with meningioma it was 6.9%. The localization of tumor in glioma cases was 40.5% in the right hemisphere, 54.1% in left hemisphere, and 5.4% on the midline of brain; it was 30.8%, 42.3% and 26.9%, respectively, in patients with meningioma (Table I).

Distribution of CDKN2 p16 540 C>G, CDKN2 p16 580 C>T and MDM2 SNP309 T>G genotypes and alleles. The genotypic and allelic distributions of *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP309 T>G are shown in Table II. The distributions of *CDKN2* p16 540 C>G and 580 C>T, and *MDM2* SNP309 T>G genotypes and alleles were in agreement with the Hardy–Weinberg equilibrium in all cases (primary brain tumor cases or its subgroups: gliomas and meningiomas) and controls ($p>0.05$). In addition, *MDM2* SNP309 T>G variants were not significantly different between controls and patients with primary brain tumors or its sub-groups ($p>0.05$). However, it was found that possessing G allele (TG+GG genotypes) of *MDM2* SNP309 T>G may have a protective role on the pathogenesis of meningioma (OR=1.271; $p=0.082$). Interestingly, it was found that the TT genotype of *CDKN2* p16 580 C>T was not detected in the

Table I. *Baseline characteristics of the study groups.*

Characteristic	Controls	Primary brain tumors	Glioma cases	Meningioma cases
Mean age±SD, years	51.76±2.43	46.74±1.84	44.27±2.58	50.00±2.51
Gender, n (%)				
Male	32 (45.1%)	38 (56.7%)	25 (65.8%)	13 (44.8%)
Female	39 (54.9%)	29 (43.3%)	13 (34.2%)	16 (55.2%)
Cigarette smoking, n (%)				
Present	N/A	20 (29.9%)	13 (34.2%)	7 (24.1%)
Absent	N/A	47 (70.1%)	25 (65.8%)	22 (75.9%)
Tumor histology, n (%)				
Astrocytoma+oligoastrocytoma	-	28 (75.7%)	28 (75.7%)	-
Oligodendroglioma	-	9 (24.3%)	9 (24.3%)	-
Tumor grade, n (%)				
I	-	26 (38.8%)	3 (7.9%)	23 (79.3%)
II	-	12 (17.9%)	8 (21.1%)	4 (13.8%)
III	-	12 (17.9%)	10 (26.3%)	2 (6.9%)
IV	-	17 (25.4%)	17 (44.7%)	-
Tumor localization, n (%)				
Right hemisphere	-	23 (36.5%)	15 (40.5%)	8 (30.8%)
Left hemisphere	-	31 (49.2%)	20 (54.1%)	11 (42.3%)
Midline	-	9 (14.3%)	2 (5.4%)	7 (26.9%)
Necrosis pathology, n (%)				
Present	-	16 (42.1%)	16 (40.5%)	N/A
Absent	-	22 (57.9%)	22 (59.5%)	N/A
Vascular endothelial proliferation, n (%)				
Present	-	35 (92.1%)	35 (92.1%)	N/A
Absent	-	3 (7.9%)	3 (7.9%)	N/A

N/A: Not available.

patient group, moreover, only three controls had this rare genotype. Therefore T allele-bearing (TT+CT) genotypes and CC genotypes of *CDKN2* p16 580 were compared for the evaluation of significance. However, no significant difference was detected between cases and controls. On the other hand, significant associations were found in the distributions of *CDKN2* p16 540 C>G genotypes and alleles between cases *versus* controls ($p<0.05$). As seen in Table II, the homozygous CC variant of *CDKN2* p16 540 C>G was more frequent in primary brain tumor cases than controls (70.1% *versus* 35.2%, $p<0.05$), and it was found that possessing CC genotype rather than G allele (~2-fold; $p<0.001$) or possessing C allele (CC and CG genotypes) rather than the GG genotype (~7-fold; $p=0.005$) increases the risk of primary brain tumor (data not shown). In addition, GG genotype of *CDKN2* p16 540 C>G was significantly more frequent in the control group than in those with primary brain tumors ($p<0.001$) or its sub-groups: glioma ($p=0.002$) and meningioma ($p=0.002$). In glioma cases, while possessing a G allele (CG and GG genotypes) and GG genotype were protective [~2-fold ($p=0.001$ and ~7-fold ($p=0.032$), respectively], possessing CC genotype rather than a G allele increased risk of the disease by ~4-fold ($p=0.001$). In meningioma cases, possessing a G allele rather than CC genotype had also a protective effect (~4.8-fold; $p=0.001$).

Association of the CDKN2 p16 540 C>G, CDKN2 p16 580 C>T and MDM2 SNP309 T>G alleles with clinicopathological parameters. The distributions of clinical features according to *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP309 T>G variants in primary brain tumor patients group and its subgroups: gliomas and meningiomas are shown in Table III and IV, respectively. There was no statistical association between *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T or *MDM2* SNP309 T>G variants and tumor histology, tumor grade or presence of necrosis or vascular endothelial proliferation ($p>0.05$). In patients with glial tumors, the distribution of *MDM2* SNP309 T>G homozygous genotypes (TT or GG) according to tumor histology were balanced, therefore astrocytomas and oligoastrocytomas were found in 37.8% patients with TG genotype, and 18.9% of patients with TT or GG genotypes, separately oligodendrogliomas were found in 13.5% with TG genotype, and 5.4% of patients with TT or GG genotypes. On the other hand, the frequency of astrocytoma/oligoastrocytoma or oligodendroglioma were in the order of CC>CG>GG genotypes of *CDKN2* p16 540 C>G.

In Table V, the individual and combined effects of male gender and polymorphisms on the development of primary brain tumor and its sub-groups are presented. The individual

Table II. *The distribution of cyclin-dependent kinase 2 (CDKN2) p16 540 C>G, CDKN2 p16 580 C>T and mouse double minute 2 homolog (MDM2) SNP309 T>G genotypes and alleles in the study groups.*

Genotype and alleles	Control	Primary brain tumors	Meningioma cases	Glioma cases
p540				
CC	25 (35.2%)	47 (70.1%)†	21 (72.4%)	26 (68.4%)
CG	33 (46.5%)	18 (26.9%)	7 (24.1%)	11 (28.9%)
GG	13 (18.3%)†‡§	2 (3.0%)	1 (3.4%)	1 (2.6%)
C Allele	83 (58.45%)	112 (83.58%)	29 (84.48%)	63 (82.89%)
G Allele	59 (41.55%)‡§	22 (16.42%)	9 (15.52%)	13 (17.11%)
p580				
CC	51 (71.8%)	53 (79.1%)	24 (82.8%)	29 (76.3%)
CT	17 (23.9%)	14 (20.9%)	5 (17.2%)	9 (23.7%)
TT	3 (4.2%)	-	-	-
C Allele	119 (83.80%)	120 (89.55%)	53 (91.38%)	67 (88.16%)
T Allele	23 (16.20%)	14 (10.45%)	5 (8.62%)	9 (11.84%)
MDM2				
TT	15 (21.1%)	20 (29.9%)	11 (37.9%)	9 (23.7%)
TG	35 (49.3%)	30 (44.8%)	11 (37.9%)	19 (50.0%)
GG	21 (29.6%)	17 (25.4%)	7 (24.1%)	10 (26.3%)
T Allele	65 (45,8%)	70 (52.2%)	33 (56.9%)	37 (48.7%)
G Allele	77 (54.2%)	64 (47.8%)	25 (43.1%)	39 (51.3%)

Chi-square test was used to compare genotypes in the study groups. For determining allelic frequencies, gene count method was used. n, Number of individuals. Significantly different at $p<0.05$ between controls and †primary brain tumor cases, ‡meningioma cases, and §glioma cases.

effect of male gender was not different among the study groups. Even possessing T allele or G allele of *MDM2* SNP309 T>G did not lead to differences between patient cases overall *versus* the control group. However, in primary brain tumor cases, male gender and possessing *MDM2* SNP309 G allele led to a ~3.5-fold increase in the risk of the disease ($p<0.05$). While there was no effect detected for meningiomas, the risk was ~6.2 for glioma ($p<0.05$). For *CDKN2* p16 540 C>G , while the individual effects of mutant G allele were significant in all study groups ($p<0.05$), the significance of the C allele was only detected in the primary brain tumor group, and meningioma cases ($p<0.05$). In addition, no combined effect of *CDKN2* p16 540 C>G and gender was found ($p>0.05$). Finally, *CDKN2* p16 580 C>T and male gender had no individual or combined effects on the odds ratio for the development of primary brain tumor, glioma or meningioma ($p>0.05$).

Combined genotype analyses of the association of CDKN2 p16 540 C>G, CDKN2 p16 580 C>T and MDM2 SNP309 T>G polymorphisms with brain tumors. As shown in Table VI, haplotype comparison analysis revealed that the frequency of combined genotype CCT of *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP309 T>G polymorphisms was significantly higher in brain tumor cases than healthy controls [χ^2 of 9.289 ($p=0.002$), 4.411 ($p=0.036$) and 7.827 ($p=0.005$) for primary brain tumor, glioma and meningioma, respectively]. On the other hand,

Table III. *The distribution of clinical features according to cyclin-dependent kinase 2 (CDKN2) p16 540 C>G, CDKN2 p16 580 C>T and mouse double minute 2 homolog (MDM2) SNP309 T>G variants in patients with primary brain tumor.*

Clinical feature	*CDKN2* p16 540 C>G				*CDKN2* p16 580 C>T			*MDM2* SNP309 T>G			
	CC	CG	GG	*p*-Value	CC	CT	*p*-Value	TT	GT	GG	*p*-Value
Tumor histology, n (%)											
A+OA	18 (64.3%)	9 (32.1%)	1 (3.6%)	0.694	22 (78.6%)	6 (21.4%)	0.960	7 (25.0%)	14 (50.0%)	7 (25.0%)	0.960
OD	7 (77.8%)	2 (22.2%)	-		7 (77.8%)	2 (22.2%)		2 (22.2%)	5 (55.6%)	2 (22.2%)	
Tumor grade, n (%)											
I	20 (76.9%)	5 (19.2%)	1 (3.8%)	0.701	21 (80.8%)	5 (19.2%)	0.947	9 (34.6%)	11 (42.3%)	6 (23.1%)	0.947
II	7 (58.3%)	4 (33.3%)	1 (8.3%)		10 (83.3%)	2 (16.7%)		5 (41.7%)	3 (25.0%)	4 (33.3%)	
III	9 (75.0%)	3 (25.0%)	-		9 (75.0%)	3 (25.0%)		2 (16.7%)	7 (58.3%)	3 (25.0%)	
IV	11 (64.7%)	6 (35.3%)	-		13 (76.5%)	4 (23.5%)		4 (23.5%)	9 (52.9%)	4 (23.5%)	
Necrosis pathology, n (%)											
Yes	11 (68.8%)	5 (31.2%)	-	0.682	11 (68.8%)	5 (31.2%)	0.556	6 (37.5%)	6 (37.5%)	4 (25%)	0.556
No	14 (63.6%)	7 (31.8%)	1 (4.5%)		17 (77.3%)	5 (22.7%)		3 (13.6%)	13 (59.1%)	6 (27.3%)	
Vascular endothelial proliferation, n (%)											
Yes	24 (68.6%)	10 (28.6%)	1 (2.9%)	0.391	27 (71.1%)	8 (22.9%)	0.098	9 (25.7%)	18 (51.4%)	8 (22.9%)	0.098
No	1 (33:3%)	2 (66.7%)	-		1 (33.3%)	2 (66.7%)		-	1 (33.3%)	2 (66.7%)	

Chi-square test was used to compare variants in the study groups. n, Number of individuals; p<0.05 denoted statistical significance; A, astrocytoma; OA, oligoastrocytoma; OD: oligodendroglioma.

Table IV. *The distribution of clinical features according to cyclin-dependent kinase 2 (CDKN2 p16 540 C>G, CDKN2 p16 580 C>T and mouse double minute 2 homolog (MDM2) SNP309 T>G variants in patients with glioma and meningioma.*

	Clinical feature	*CDKN2* p16 540 C>G				*CDKN2* p16 580 C>T			*MDM2* SNP309 T>G			
		CC	CG	GG	*p*-Value	CC	CT	*p*-Value	TT	TG	GG	*p*-Value
Gliomas	Tumor histology, n (%)											
	A+OA	18 (64.3%)	9 (32.1%)	1 (3.6%)	0.694	22 (78.6%)	6 (21.4%)	0.960	7 (25.0%)	14 (50.0%)	7 (25.0%)	0.959
	OD	7 (77.8%)	2 (22.2%)	-		7 (77.8%)	2 (22.2%)		2 (22.2%)	5 (55.6%)	2 (22.2%)	
	Tumor grade, n (%)											
	I	3 (100.0%)	-	-	0.380	2 (66.7%)	1 (33.3%)	0.818	1 (33.3%)	1 (33.3%)	1 (33.3%)	0.962
	II	4 (50.0%)	3 (37.5%)	1 (12.5%)		7 (87.5%)	1 (12.5%)		2 (25.0%)	3 (37.5%)	3 (37.5%)	
	III	8 (80.0%)	2 (20.0%)	-		7 (70.0%)	3 (30.0%)		2 (20.0%)	6 (60.0%)	2 (20.0%)	
	IV	11 (64.7%)	6 (35.3%)	-		13 (76.5%)	4 (23.5%)		4 (23.5%)	9 (52.9%)	4 (23.5%)	
	Necrosis pathology, n (%)											
	Yes	11 (73.3%)	4 (26.7%)	-	0.642	11 (73.3%)	4 (26.7%)	0.784	6 (40.0%)	6 (40.0%)	3 (20.0%)	0.185
	No	14 (63.6%)	7 (31.8%)	1 (4.5%)		17 (77.3%)	5 (22.7%)		3 (13.6%)	13 (59.1%)	6 (27.3%)	
	Vascular endothelial proliferation , n (%)											
	Yes	24 (68.6%)	10 (28.6%)	1 (2.9%)	0.800	27 (77.1%)	8 (22.9%)	0.384	9 (25.7%)	18 (51.4%)	8 (22.9%)	0.580
	No	1 (50.0%)	1 (50.0%)	-		1 (50.0%)	1 (50.0%)		-	1 (50.0%)	1 (50.0%)	
Meningiomas	Tumor grade, n (%)											
	I	17 (73.9%)	5 (21.7%)	1 (4.3%)	0.907	19 (82.6%)	4 (17.4%)	0.746	8 (34.8%)	10 (43.5%)	5 (21.7%)	0.324
	II	3 (75.0%)	1 (25.0%)	-		3 (75.0%)	1 (25.0%)		3 (75.0%)	-	1 (25.0%)	
	III	1 (50.0%)	1 (50.0%)	-		2 (100.0%)	-		-	1 (50.0%)	1 (50.0%)	

Chi-square test was used to compare variants in the study groups. n, Number of individuals; $p<0.05$ denoted statistical significance; A, astrocytoma; OA, oligoastrocytoma; OD: oligodendroglioma.

compared to the control group, the CGT combined genotype was significantly more frequent in primary brain tumor and glioma cases [χ^2 of 6.82 ($p=0.009$) and 4.794 ($p=0.028$), respectively]. In addition, the frequency of the CGG haplotype was lower in patients with meningioma *versus* the control group ($\chi^2=5.155$, $p=0.023$).

Discussion

Brain tumors comprise 1.4% of all cancer types and account for 2.4% of cancer related-deaths (1). Neuroepithelial tumors in males and meningeal tumors in females are the most common worldwide (3, 6). It was found that males are 40% more at risk of developing glioma than females, and females are 80% more at risk of developing meningioma (4). On the other hand, it is possible that the differences observed in tumor types may also be due to social, environmental or ethnical differences. For instance, fatal brain tumor incidence in Northern Europe is twice as high as the cases observed in Japan. In addition, while the risk for developing glioma for the Whites in the US is greater than the risk for Blacks, the risk for developing meningioma is likely equal for both races (3, 6). These regional differences are generally the basis of most SNP studies.

Epidemiological studies classified risk factors of brain tumors as diet, smoking, exposure to neurotoxic and carcinogenic chemicals, as well as accumulation of genetic and epigenetic defects mostly seen in de-toxification and oxidation enzymes of carcinogenic agents, cell-cycle control genes, tumor-suppressor genes, proto-oncogenes and genes which play crucial roles in cell differentiation and cancer prognosis. Indeed, a number of studies have shown that mutations or polymorphisms that cause the activation or inactivation of these genes andpathways are linked to tumor development and progression. Furthermore, they are involved in the toxicity of anticancer drugs, thus in the efficacy of cancer treatment and prediction of survival (1-7, 30).

Our study investigated *CDKN2* and *MDM2* polymorphisms in relation to primary brain tumors, whose incidence is on the increase worldwide and in our region.

The *CDKN2* gene encoding tumor-suppressor p16 protein is located on chromosome 9p21 and is composed of three exons encoding a 156-amino-acid protein (8-15). It was shown that the growth suppression effect of p16 could be inactivated by mutations or polymorphisms of *CDKN2* which lead to the loss of function of this important CDK inhibitor (12, 16, 17, 31). However, there also exist

Table V. *The individual and common effects of male gender and CDKN2 p16 540 C>G, CDKN2 p16 580 C>T and MDM2 SNP309 T>G alleles on the odds ratio (OR) of the possible risks for the development of primary brain tumor and its sub-groups: glioma and meningioma.*

	OR (95% CI)		
	Primary brain tumor × controls	Gliomas × controls	Meningiomas × controls
Male gender	1.258 (0.904-1.752)	0.685 (0.468-0.967)	1.005 (0.623-1.622)
	CDKN2 p16 540		
C Allele (CC+CG genotype)	1.188 (1.056-1.336)*	0.839 (0.743-0.948)	0.846 (0.743-0.963)*
Male gender and C allele	0.947 (0.879-1.021)	0.960 (0.886-1.040)	0.923 (0.789-1.080)
G allele (GG+CG genotype)	0.461 (0.307-0.691)*	2.052 (1.246-3.377)*	2.349 (1.271-4.340)*
Male gender and G allele	1.212 (0.418-3.509)	1.875 (0.407-8.633)	0.660 (0.124-3.499)
	CDKN2 p16 580		
C Allele (CC+CT genotype)	1.044 (0.994-1.096)	0.958 (0.912-1.006)	0.958 (0.912-1.006)
Male gender and C allele	N/A	N/A	N/A
T Allele (TT+CT genotype)	0.742 (0.409-1.346)	1.189 (0.602-2.350)	1.634 (0.678-3.938)
Male gender and T allele	1.490 (0.440-5.044)	2.139 (0.375-12.202)	0.788 (0.111-5.600)
	MDM2		
T Allele (TT+TG genotype)	1.060 (0.863-1.301	0.956 (0.750-1.218)	0.928 (0.720-1.198)
Male gender and T allele	0.891 (0.292-2.719)	0.771 (0.162-3.663)	1.111 (0.200-6.181)
G Allele (GG+TG genotype)	0.889 (0.730-2.524)	1.034 (0.834-1.280)	1.271 (0.933-1.731)
Male gender and G allele	3.598 (1.199-10.801)*	6.286 (1.236-31.956)*	1.750 (0.376-8.140)

CI, Confidence interval; N/A, Not available; *significantly different at $p<0.05$.

controversial results regarding CDKN2 p16 gene and cancer development (32, 33).

Korshunov *et al*. investigated the prognostic significance of $p16^{INK4A}$ locus in meningioma cases and found no association (34). However, Gibson *et al*. reported that while mice lacking p16 had larger and redder colonic tumors with more necrosis, less apoptosis, higher red blood cell density and increased vascular endothelial growth factor production, the progression of tumor was constrained in a human colonic cancer cell line by exogenous p16 expression *via* inhibition of angiogenic signaling (35). Kalamarides *et al*. reported an increased frequency of meningioma and meningothelial proliferation in mice with nullizygosity for $p16^{Ink4a}$ locus (36). Likewise, Barton *et al*. reported that the survival rates were prolonged in INK4A-ARF-deficient mouse model of brainstem glioma after treatment with a compound which inhibits CDK4/6 (37). Song *et al*. reported a hypermethylation of the *CDKN2* p16 gene in gastric cancer and concluded that p16 hypermethylation could be a predictive biomarker for detection of gastric cancer (38). Alves *et al*. investigated p16 hypermethylation in astrocytomas and found that p16 inactivation by promotor methylation is a frequent event in astrocytomas and is related to the age and sex of patients (39). Yan *et al*. investigated the polymorphisms of *CDKN2* p16 gene in Chinese patients with ovarian cancer and did not find any association between p16 540C>G polymorphism and the development and progression of tumor. However, they did report that individual susceptibility to specific subtypes of epithelial ovarian cancer could be affected by p16 580C>T polymorphism (40). Recently, Sibin *et al*. reported less frequency of *CDKN2* p16 gene mutation in Indian patients with glioma (41). In our previous study on Turkish patients with colorectal cancer, we found significant associations between *CDKN2* p16 540 C>G and *MDM2* SNP309 T>G polymorphisms and colorectal cancer (42).

In the present study, homozygous mutant *CDKN2* p16 580 C>T genotype was not detected in any patient and there were only three controls possessing this genotype, and no association was detected between this polymorphism and brain tumor. On the other hand, as in our previous study, significant associations were found between *CDKN2* p16 540 C>G polymorphism and primary brain tumor development. This finding shows that possessing the C allele may be a risk factor for the development of primary brain tumors (gliomas and meningiomas), especially gliomas.

Sauroja *et al*. reported significant associations between *CDKN2* p12 540 C>G or *CDKN2* p12 580 C>T polymorphisms and melanoma aggressiveness (43). In the

present report, we did not observe any association between *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP309 T>G variants and clinical features or gender in primary brain tumor or its subgroups of glioma and meningioma.

MDM2, the major regulator of tumor-suppressor p53, negatively regulates the stability and activity of p53 by binding its transactivation domain. It was reported that mutations that were generated in *MDM2* gene could have a possible contributory role to the development of several types of malignant neoplasm (44, 45). Indeed, in the promoter region of *MDM2*, a thymidine (T) to guanine (G) transition at position 309 was found to be associated with several cancer types, including large B-cell lymphoma, connective tissue sarcomas, invasive ductal breast carcinoma, colorectal cancer, lung cancer pancreatic adenocarcinoma, glioma and meningioma tumorigenesis (45-51).

Bond *et al*. found an increase in the affinity of the transcriptional activator SP1 with *MDM2* SNP309 polymorphism that results in higher levels of *MDM2* RNA and protein and consequently the attenuation of the p53 pathway, thus they concluded that this polymorphism has a rate-limiting role in carcinogenesis (45). Grochola *et al*. reported a male-specific association between *MDM2* SNP309 locus and pancreatic adenocarcinoma (48). Wang *et al*. reported an overexpression of *MDM2* protein in glioma progression (50). Das *et al*. reported a strong association between MDM2 overexpression and progesterone receptor expression in patients with meningioma and suggested a novel approach in developing progesterone receptor-tergeted therapy for meningiomas (51). However, Kraus *et al*. reported no association between MDM2 overexpression and childhood primitive neuroectodermal tumors or glioblastomas (52). In the present study, no association was found between *MDM2* SNP309 T>G variants and primary brain tumors or its sub-groups. However, co-existence of *MDM2* SNP309 G allele and male gender were found to be a risk factor in the development of primary brain tumors. This risk was much more significant in patients with glioma compared to those with meningioma. This fact may be explained by the different mechanisms of tumorigenesis of gliomas and meningiomas, whereas grade I meningiomas are benign tumors and confer better survival than gliomas. On the other hand, no association between *MDM2* SNP309 T>G and clinical parameters was found.

The present study was preliminary in evaluating the association of *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP 309 T>G combined genotypes with the development of primary brain tumor or its sub-groups. The presence of combined genotype CCT of *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP309 T>G polymorphisms was found to be more common in those with primary gliomas and meningiomas.

Table VI. *Association of combined-genotype analyses of cyclin-dependent kinase 2 (CDKN2) p16 540 C>G, CDKN2 p16 580 C>T and MDM2 SNP309 T>G single nucleotide polymorphisms (SNPs) in patients with brain tumors. Based on comparison of frequency distributions of all haplotypes for the combination of SNPs. Haplotypes were sequenced as alleles of CDKN2 p16 580 C>T, CDKN2 p16 540 C>G and MDM2 SNP309 T>G, respectively.*

Haplotype	Frequency	Case, Control Ratio Counts	Chi-square‡	*p*-Value
Primary brain tumor				
CCT	0.322	0.411, 0.239	9.289	0.002*
CCG	0.297	0.342, 0.255	2.503	0.113
CGG	0.139	0.086, 0.190	6.216	0.013*
CGT	0.107	0.057, 0.154	6.82	0.009*
TCT	0.044	0.045, 0.044	0.001	0.977
TCG	0.039	0.038, 0.039	0.002	0.966
TGG	0.035	0.011, 0.058	4.408	0.036*
TGT	0.015	0.010, 0.020	0.493	0.483
Glioma				
CCT	0.291	0.380, 0.244	4.411	0.036*
CCG	0.286	0.345, 0.254	2.035	0.154
CGG	0.162	0.107, 0.191	2.609	0.106
CGT	0.115	0.050, 0.149	4.794	0.028*
TCT	0.050	0.054, 0.047	0.045	0.832
TCG	0.043	0.050, 0.040	0.136	0.712
TGG	0.042	0.011, 0.058	2.721	0.099
TGT	0.012	0.003, 0.017	0.79	0.374
Meningioma				
CCT	0.291	0.432, 0.234	7.827	0.005*
CCG	0.287	0.340, 0.266	1.111	0.292
CGG	0.145	0.056, 0.181	5.155	0.023*
CGT	0.137	0.086, 0.158	1.811	0.178
TCT	0.052	0.052, 0.052	0.0	0.997
TCG	0.048	0.013, 0.062	2.158	0.142
TGG	0.030	0.021, 0.034	0.21	0.647
TGT	0.011	0.000, 0.015	0.874	0.350

‡Between cases and controls; *significantly different at $p<0.05$.

The strength of the present study is in highlighting the relationship between *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP309 T>G polymorphisms with the risk of primary glioma and meningioma development and progression in a Turkish population. However, the main limitation of the study is the number of participants, which is relatively small. Therefore the adverse effects of *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP 309 T>G polymorphisms may not be significant with respect to the control group but still may give us clues regarding prognosis of the disease. Additional studies with larger sample sizes are needed to define the influence of *CDKN2* p16 540 C>G, *CDKN2* p16 580 C>T and *MDM2* SNP 309 T>G genotyping

on clinical outcomes. We believe that the results of the present study could be more conclusive with further studies determining the interaction of *CDKN2* p16 and *MDM2* gene. As a conclusion, we believe that our study will shed light on studies that concentrate on the importance of *CDKN2* p16 and *MDM2* genes in etiopathogenesis of primary brain tumors.

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Conflicts of Interests

The Authors declare that no competing interests exist.

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