

Association between p16(CDKN2A) C540G polymorphism and tumor behavior in prolactinoma: A single-center study

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Abstract. Pituitary tumors usually originate as benign sporadic adenomas and develop into invasive and aggressive tumors such as prolactinomas, which are common functioning pituitary adenomas. The aim of the present study was to examine the association between the tumor behavior in prolactinomas and the p16(CDKN2A) gene polymorphism occurring at the 3'-untranslated region of exon 3 (C540G). A total of 104 patients with prolactinoma were included and assigned to two groups based on invasive vs. non-invasive tumor behavior. Ki67 indices were recorded according to histopathology results. Genotypic analysis of the p16(CDKN2A) C540G polymorphism was carried out using a modified polymerase chain reaction-restriction fragment length polymorphism assay. The corresponding frequencies for CC, CG and GG genotypes in non-invasive vs. invasive tumors were 61.5, 30.8, 7.7 and 64.1, 28.2, 7.7%, respectively (not significant). The observed CG genotype frequency was higher compared with previous studies. In addition, the patients with giant adenomas or a high Ki67 index had a higher frequency of the CG genotype as compared with the other subgroups, although the differences were not significant (46.2 and 42.9%, respectively). In conclusion, a higher frequency of the C540G CG genotype of the CDKN2A gene was found among patients with prolactinoma in comparison with previous studies. These frequencies were also higher in the subgroups with elevated Ki67 or giant adenomas. Further studies are required to improve the definition of the role of the CG genotype in the development and progression of tumors in prolactinomas.

Introduction

Pituitary tumors comprise 10-15% of the primary intracranial tumors in adults aged 30-60 years. The majority of the pituitary tumors originate as benign sporadic adenomas, but may also occur in the context of multiple endocrine neoplasia-1, Carney's complex or familial isolated pituitary adenomas (1). Clonal analysis has shown that the origin of pituitary tumors is mainly monoclonal, indicating that the tumors arise from clonal expansion of a single genetically altered cell, in which growth advantage occurs from the activation of protooncogenes or the inactivation of tumor suppressor genes (2). The search for specific genetic alterations primarily leading to the monoclonal expansion of abnormal pituitary cells has been unfruitful (3). However, an exception should be made for a significant subset of growth hormone-secreting tumors that harbor stimulatory G protein mutations (4).

The molecular analysis of human pituitary neoplasia has revealed numerous molecular alterations in the regulation of the cell cycle. The G1/S transition checkpoint, which is a significant step of the cell cycle, frequently undergoes alterations in tumors. This transition is controlled by cyclins and cyclin-dependent kinases (CDKs), which form a complex to induce the transition of cells to the S-phase via phosphorylation of the retinoblastoma protein (pRb) (5). The p16INK4a protein that is coded by the CDKN2A genes on chromosome 9p21 suppresses the catalytic activity of the cyclin D/CDK4 complex responsible for the phosphorylation of pRb, resulting in the release of transcription factors (including E2F) associated with the transition to the G1 checkpoint. Thus, the pRb pathway can be blocked through the loss of p16INK4a, pRb expression or CDK4 amplification. Such changes in the pRb pathway are expected to promote cell proliferation (6,7).

Point mutations, small deletions, large heterozygous or homozygous deletions and silencing of the CpG islands in the promoter region by methylation result in the formation of an aberrant p16 protein, which has been shown to exist in numerous human tumors, including certain intracranial tumors (particularly pituitary adenomas). These findings indicate that these factors are closely associated with tumor development (8-14).

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Studies in human pituitary tumors have shown that point mutations and/or deletions involving the p16INK4a gene are uncommon or absent (14-16). Methylation of the tumor suppressor genes, p16INK4a and RB1 CpG islands, is associated with the significant loss of cognate proteins in non-functional pituitary tumors and somatotrophinomas, respectively (13), with p16-associated methylation being reported in 70% of pituitary tumors. In addition, the absence of p16 immunoreactivity has recently been studied in pituitary adenomas (17).

The development and progression of certain types of cancer have previously been linked to p16 polymorphisms, which are two in number (C540G, rs11515; and C580T, rs3088440) and occur at the 3'-untranslated region of exon 3. Despite the demonstration of altered p16 protein function in conjunction with certain polymorphisms, no such association could be established for the other polymorphisms. Although the association between the altered protein function of p16 and progression of certain malignancies, including ovarian and upper gastrointestinal cancer (18-20), has been demonstrated, a similar correlation has not been established for pituitary adenomas and their progression.

In the present study, the association between the p16 gene polymorphism C540G (rs11515) at the 3'-untranslated region of exon 3 was investigated, along with the invasiveness or aggressiveness of the prolactinomas, which are the most common functioning pituitary adenomas.

Materials and methods

Study subjects. A total of 104 patients (25 male and 79 female) with a diagnosis of prolactinoma who were followed up at the Department of Endocrinology and Metabolism, Uludag University (Gorukle, Bursa, Turkey) were included in the study. Demographic characteristics, pre-treatment and the most recent plasma prolactin levels and sellar magnetic resonance imaging (MRI) findings were recorded. Tumors were categorized as follows based on the sellar MRI findings: Microadenoma (longest diameter, <1 cm), macroadenoma (longest diameter, 1-4 cm) and giant adenoma (longest diameter >4 cm). In addition, the patients were assigned into groups according to invasive or non-invasive tumor behavior. Extrasellar diffusion of adenomas was assessed using the Hardy classification in conjunction with the cavernous sinus invasion (21), and the presence of the latter was ascertained by detailed coronal sellar MRI. Involvement of $\geq 2/3$ (67%) of the internal carotid artery at the cavernous sinus segment was defined as cavernous sinus invasion (22). Using the Hardy classification, grade 3, grade 4, stage D and/or stage E tumors and those that surrounded or infiltrated $\geq 2/3$ of the cavernous sinuses were considered to be invasive. In patients who had previously undergone surgery, the Ki67 indices were recorded using histopathology reports. The patients with a Ki67 index of $\geq 3/100$ were considered to have a high proliferative index (23).

The study was conducted in accordance with the Declaration of Helsinki and Principles for Good Clinical Practice and was approved by the local Ethics Committee. Prior to the study inclusion, all patients read and signed the informed consent form.

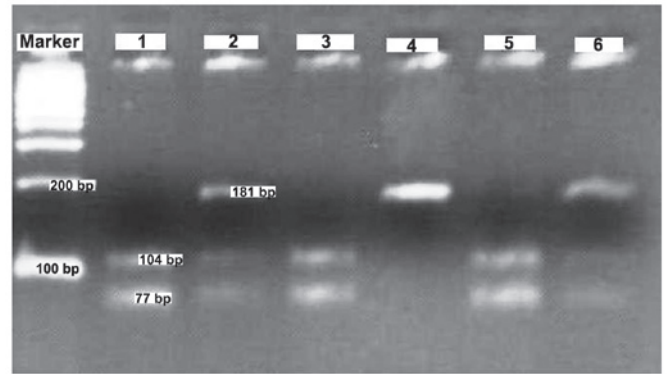


Figure 1. Polymerase chain reaction products of the p16(CDKN2A) gene (C540G) following *MspI* enzyme digestion and on a 4% agarose gel. The lane marker shows the 100-bp DNA ladder; lanes 1, 3 and 5 are the C/C genotype (104 and 77 bp); lanes 2 and 6 are the C/G genotype (181, 104 and 77 bp); and lane 4 is G/G genotype (181 bp). Bp, base pair.

DNA isolation and genotyping of p16(CDKN2A). Blood samples obtained from the patients were collected into EDTA tubes. Genomic DNA was extracted from whole blood using a DZ DNA isolation kit (Dr Zeydanlı Life Science Ltd., Ankara, Turkey) according to the manufacturer's instructions. Genotypic analysis of the p16(CDKN2A) C540G polymorphism was performed using a modified polymerase chain reaction (PCR)-restriction fragment length polymorphism assay, reported previously (20). The primers for amplifying the p16(CDKN2A) C540G polymorphism were forward, 5'-GATGT GCCACACATCTTTGACCT-3'; and reverse, 5'-CTACGAAAG CGGGGTGGGTGT-3'. PCR primers were used to generate a 181-base pair (bp) product containing the polymorphic sites. Following an initial denaturation at 94°C for 10 min, there were 38 cycles of 30 sec at 94°C, 45 sec at 61°C and 45 sec at 72°C; and then a final extension step of 10 min at 72°C. The PCR products were digested with *MspI* (Bioron GmbH, Ludwigshafen am Rhein, Germany) at 37°C overnight and analyzed on a 4% agarose gel. C/C individuals had 104- and 77-bp fragments, C/G individuals had 181-, 104- and 77-bp fragments and G/G individuals had only a 181-bp fragment (Fig. 1).

Statistical analysis. Demographic characteristics, serum prolactin levels and tumor size were summarized using descriptive statistics, including mean \pm standard deviation (the values are presented in parenthesis) for the continuous variables, and the frequency and percentage for the categorical variables. Tumor size was defined on the basis of the longest diameter. The χ^2 test was used for comparison of continuous variables and Kruskal-Wallis, Mann-Whitney U and the Student's t-tests were used to assess the differences among the study groups. In the statistical analyses, the results were considered to indicate a statistically significant difference if the two-tailed P-value was <0.05. Statistical analysis of data was performed using the IBM Statistical Package for Social Sciences software, version 20.0 (IBM, Somers, NY, USA).

Results

Basal characteristics of patients for invasive and non-invasive groups. The mean age of participants was 39.9 ± 9 years

Table I. Basal characteristics of patients with non-invasive and invasive groups.

Characteristics	Total, n=104	Non-invasive, n=65	Invasive, n=39	P-value
Male, % (n)	24.0 (25)	7.7 (5)	51.3 (20)	<0.01
Female, % (n)	76.0 (79)	92.3 (60)	48.7 (19)	
Age, years	39.9±9	38.8±8	41.8±10	NS
Male	44.5±10	44.8±10	44.4±10	NS
Female	38.4±8	38.3±8	39.0±8	NS
Age at diagnosis, years	34.1±9	32.6±7	36.5±11	0.06
Male	39.6±11	40.4±9	39.4±12	NS
Female	32.3±8	31.9±7	33.5±9	NS
Prolactin, ng/dl	1,659.4	126.4	4,171.8	<0.01
Male	5,932.6	138.6	7,636.8	<0.01
Female	371.6	125.3	1,071.5	<0.01
Adenoma, mm	16.9±17	6.8±3	33.5±18	<0.01
Male	37.5±21	9.2±9	44.6±17	<0.01
Female	10.3±8	6.6±3	21.9±10	<0.01

NS, not specified.

and the mean age at the time of diagnosis was 34.1±9 years. There were 25 male (24.0%) and 79 female (76.0%) patients. The mean age at the time of diagnosis was 32.6 years in the non-invasive group (40.4 and 31.9 years in males and females, respectively) and 36.5 years (39.4 and 33.5 years in males and females, respectively) in the invasive group. Mean prolactin levels at the time of diagnosis in the non-invasive and invasive groups were 126.4 (138 and 128 ng/dl in males and females, respectively) and 4,171.8 ng/dl (7,636 and 1,071 ng/dl in males and females, respectively), respectively. The tumor size at the time of diagnosis was 16.9 mm in the overall group (37.5 and 10.3 mm in males and females, respectively), while the corresponding figure for the non-invasive and invasive groups was 6.8 (9.2 and 6.2 mm in males and females, respectively) and 33.5 mm (44.6 and 21.9 mm in males and females, respectively), respectively. The two groups were comparable in terms of gender distribution and age at the time of diagnosis. As expected, prolactin levels and tumor size at the time of diagnosis were significantly higher among patients with invasive tumors compared with patients with non-invasive tumors in both genders (Table I).

Basal characteristics of patients for the genotype groups. In terms of the CDKN2A C540G polymorphism, 65 patients (62.5%) had the CC genotype, 31 (29.8%) had CG and eight (7.7%) had GG. Overall, the G and A alleles were present in 77.4 and 22.6% in female and male patients, respectively. No significant differences in terms of gender distribution, mean age, mean age at the time of diagnosis, prolactin levels and tumor size at the time of diagnosis were detected between the groups with the different genotypes (Table II).

Distribution of CDKN2A C540G genotype groups and allele frequencies in the subgroups of prolactinomas. The CC, CG and GG genotype frequencies were similar in the invasive

and non-invasive groups; 61.5, 30.8 and 7.7, and 64.1, 28.2 and 7.7%, respectively. In total, seven of the 13 patients with giant adenomas (53.8%) had the CC genotype and six (46.2%) had the CG genotype. Of the 49 patients with microadenomas, the distribution of the CC, CG and GG genotypes was 65.3, 26.5 and 8.2%, respectively. The corresponding frequencies for patients with macroadenomas were 61.9, 28.6 and 9.5%, respectively. While the CC, CG and GG genotypes were detected in 64.0, 28.0 and 8.0% of the 25 patients with cavernous sinus invasion, the frequencies were 62.0, 30.4 and 7.6% in the 79 subjects with no invasion, respectively. In the 16 patients with a low Ki67 index, the CC, CG and GG genotypes occurred at a frequency of 56.3, 37.5 and 6.2%, respectively, and among the 14 patients with a high Ki67 index the CC and CG genotypes were found in 57.1 and 42.9%, respectively. Except for the absence of the GG genotype and the higher incidence of CG (46.2 vs. 42.9%) in the patients exhibiting a giant adenoma in conjunction with a high Ki67 index, the genotype frequencies were similar in all within-group comparisons. The increased frequency of the CG genotype in the patients with giant adenoma and a high Ki67 index did not represent a significant difference, possibly due to the small sample size. The genotype frequencies and allele distributions in all groups are presented in Table II together with the number of participants in each study group. Overall, the G and A allele frequencies (77.4 and 22.6%) were also similar across the study groups (Table III).

Discussion

Intracranial tumors commonly involving the downregulation of p16 include anaplastic astrocytomas and glioblastomas (24,25), haemangiopericytomas (11), brain lymphomas (12) and pituitary adenomas (13,26-28). In the latter group of tumors, the most common mechanism of CDKN2A inactivation involves methylation, while mutations or deletions are rare (13-16,26-28).

Table II. Basal characteristics of patients with genotype groups.

Characteristics	Total, n=104	CC, n=65	CG, n=31	GG, n=8	P-value
Male, % (n)	24.0 (25)	24.6 (16)	22.6 (7)	25.0 (2)	NS
Female, % (n)	76.0 (79)	75.4 (49)	77.4 (24)	75.0 (6)	
Age, years	39.9±9	41.0±9	38.6±9	35.8±10	NS
Male	44.5±10	44.9±10	44.8±12	40.0±14	NS
Female	38.4±8	39.7±8	36.8±7	34.5±10	NS
Age at diagnosis, years	34.1±9	34.8±8	33.2±10	31.6±10	NS
Male	39.6±11	39.0±10	41.1±14	38.5±14	NS
Female	32.3±8.0	33.4±7	30.9±7	29.3±9	NS
Prolactin, ng/dl	1,659.4	1,832.0	1,570.2	731.7	NS
Male	5,932.6	7,538.3	4,028.4	2,160.5	NS
Female	371.6	183.5	788.0	255.5	NS
Adenoma, mm	16.9±17	16.9±18	18.1±16	13.0±11	NS
Male	37.5±21	37.5±26	41.5±10	23.0±9	NS
Female	10.3±8	10.0±7	11.2±10	9.6±10	NS

NS, not specified.

Table III. Distribution of CDKN2A C540G genotype groups and allele frequencies in the subgroups of prolactinomas.

Subgroups	No.	C/C (%)	C/G (%)	G/G (%)	G (%)	A (%)	P-value
Total	104	62.5	29.8	7.7	77.4	22.6	NS
Non-invasive	65	61.5	30.8	7.7	76.9	23.1	NS
Invasive	39	64.1	28.2	7.7	78.2	21.8	NS
Low Ki67	16	56.3	37.5	6.2	75.0	25.0	NS
High Ki67	14	57.1	42.9	0.0	78.5	21.5	NS
Giant	13	53.8	46.2	0.0	76.9	23.1	NS
Micro	49	65.3	26.5	8.2	78.5	21.5	NS
Macro	42	61.9	28.6	9.5	76.1	23.9	NS
Non-CSI	79	62.0	30.4	7.6	77.0	23.0	NS
CSI	25	64.0	28.0	8.0	78.0	22.0	NS

NS, not specified; CSI, cavernous sinus invasion.

Inactivation of the p16INK4a gene by methylation of the CpG islands in the promoter region has been reported to play a role in pituitary tumorigenesis, with a consequent decrease or abolishment of the p16 protein (7,27). Certain studies have shown an absence of the expression of p16INK4a at the level of mRNA and protein in all tumors examined. Additionally, an equal frequency of methylation has been found in non-functioning and functioning adenomas (26,28). However, in their study Seemann *et al* (14) identified a difference in the p16INK4a gene alteration frequency between somatotroph and corticotroph adenomas. The majority of the gonadotroph, lactotroph, plurihormonal and null cell adenomas involved the inactivation of CDKN2A methylations, possibly accounting for the p16 loss. The most important mechanisms responsible for the down-regulation of p16 in somatotroph and corticotroph adenomas

did not appear to include the methylation, homozygous deletion or mutation of the CDKN2A gene. The study concluded that CDKN2A/p16 inactivation is associated with the type and size of the tumor and that p16 downregulation is obtained following the progression of adenoma rather than representing a starting point. The study by Simpson *et al* (13) showed that hypermethylation of the CpG island within exon 1, but not exon 2, of the CDKN2A gene is frequently associated with the loss of protein expression in non-functional pituitary tumors, but not in somatotrophinomas, indicating various tumorigenic pathways. The study by Machiavelli *et al* (29) found that the p16INK4a tumor suppressor gene is not expressed in a number of pituitary adenomas and that the absence of p16INK4a occurs significantly more frequently in clinically non-functioning macroadenomas in comparison with functioning tumors.

Table IV. Distribution of CDKN2A C540G genotype groups and allele frequencies in the previous and current studies.

Study	Patients				Controls				(Refs.)
	CC, %	CG, %	GG, %	C allele, %	CC, %	CG, %	GG, %	C allele, %	
Chansaenroj <i>et al</i>	92.9	7.1	0.0	NA	87.5	12.5	0.0	NA	(35)
Chen <i>et al</i>	72.3	27.7 ^a	27.7 ^a	NA	NA	NA	NA	NA	(33)
Yan <i>et al</i>	94.0	5.4	0.5	96.8	91.0	8.2	0.7	95.1	(20)
Tuna <i>et al</i>	66.7	31.0	2.3	82.2	34.7	48	17.3	58.7	(36)
Present study	62.5	29.8	7.7	77.4					

^aCG+GG. NA, not available.

Therefore, the deregulation of the RB pathway may play a significant role in the development of clinically non-functioning macroadenomas. In a previous study by Abd El-Moneimand and Abd El-Rehim (17), the loss of the p16 protein expression occurred in 19/34 (55.9%) of the pituitary tumor samples and p16INK4a methylation was detected in 14 (41.2%). The methylation of p16INK4a and absence of p16 immunoreactivity were significantly associated with a larger tumor size and increased grade. The age of the patients was significantly associated with the presence of p16 and methylation status of the tumors with older patients exhibiting p16-negative or methylated tumors in comparison with p16-positive or unmethylated tumors. Furthermore, a significant positive correlation between the loss of p16 protein expression and p16INK4a gene methylation was observed. The study concluded that p16 downregulation is a common event in pituitary adenomas and that its alteration may play a significant role in genesis, growth progression and biological behavior of pituitary adenomas.

Genetic polymorphisms in a variety of genes have been examined to establish a correlation between specific allele variants and cancer progression. The p16 gene has two polymorphisms (C540G, rs11515; and C580T, rs3088440) at the 3'-untranslated region of exon 3 and these genetic variations can contribute to cancer development in a number of ways (18). In a previous study, the two polymorphisms were found to be associated with increased disease progression and familial melanoma risk (18). By contrast, a study by Geddert *et al* (30) indicated that these polymorphisms had no association with upper gastrointestinal and oesophageal adenocarcinomas. Previous studies have shown that these polymorphisms are associated with the progression of ovarian and upper gastrointestinal cancers, despite uncertainty regarding the progression of cervical cancer (31,32). A study by Chen *et al* (33) reported that in patients with the polymorphic genotype for C540G there was no significant increase in the hazard ratio (HR) for the age-related pancreatic cancer risk, as compared with those with wild-type genotype [HR, 0.80; 95% confidence interval (CI): 0.56-1.17] and there was a only a marginally significant increase in the HR for C580T (HR, 1.52; 95% CI: 1.00-2.30). In another study examining the p16 gene alterations, a significant association between the p73 and p16 polymorphic genotypes and a shorter time to progression in pancreatic cancer has been observed (log-rank test, $P=0.021$ and 0.039 , respectively). A gene-dosage effect was detected for the time to tumor progres-

sion for polymorphisms involving p73, p16 and MDM2 genes. The HRs (95% CIs) for one, two or three adverse genotypes were 2.13 (1.04-4.36), 3.18 (1.37-7.39) and 10.09 (3.17-32.05), respectively, and these findings indicate that polymorphisms in cell cycle genes may represent promising markers for patients with pancreatic cancer (34). Yan *et al* (20) observed no association between the p16 C540G polymorphism and early ovarian cancer and progression risk. Furthermore, no association between haplotypes of two different single-nucleotide polymorphisms and tumor progression has been found. Chansaenroj *et al* (35) have indicated that the frequencies of adjacent polymorphisms are not significantly increased in abnormal cervical lesions.

The literature search in the present study only yielded a single study examining CDKN2A C540G polymorphism in Turkish patients. The study by Tuna *et al* (36) assessed the CDKN2A C540G and C580G polymorphisms in a total of 87 patients with colorectal cancer and 75 healthy controls, and the CC, CG and GG genotypes were detected in 34.7, 48.0 and 17.3; and 66.7, 31.0 and 23% of the samples in the respective groups. The study concluded that the CDKN2A C540G G allele and GG genotype occurred at a lower frequency in colorectal cancer patients as compared with controls. Results of that study differ significantly from the present study in terms of the mean age of patients and controls (58.3 and 54.4, respectively, compared with 39.9 years in the present study) and the genetic testing methods used.

As mentioned earlier, the changes in the CDKN2A gene play a significant role in the growth and progression of tumors. Despite the demonstration of the downregulation of the p16 pathway in association with methylation, homozygous deletion or mutation of the CDKN2A gene in several tumor types, including pituitary adenomas, studies examining the polymorphisms resulting in a change in the p16 pathway are scarce. To the best of our knowledge, no literature data are available on CDKN2A gene polymorphisms in the pituitary adenomas.

In the present study, the frequency of the CC (wild genotype), CG and GG genotypes were 62.5, 29.8 and 7.7% in the overall patient group. The G and A alleles were detected in 77.4 and 22.6% of the samples. Although one of the major limitations of the study is the absence of a healthy control group, previous studies examining genotype distribution and allele frequencies in patients and controls may serve as a model for comparison

(Table IV). Accordingly, a significantly higher frequency of the CG genotype and G allele was detected in the samples in comparison with certain previous reports (20,33,35,36). Subgroup comparisons for the tumor behavior of prolactinoma have also shown that those with a high Ki67 index and with giant prolactinomas had a higher frequency of CG genotype as compared with the other groups (although not statistically significant) in the study and when compared with previous studies.

The absence of a healthy control group is a major limitation of the study. Only patients with prolactinomas were included in the study, examining for the first time the role of the CDKN2A gene C540G (rs11515) polymorphisms in the growth and progression of these lesions. However, these polymorphisms may also play a role in other pituitary tumors. Furthermore, other changes involving the CDKN2A gene (including methylation or deletion) or other polymorphisms (C580T, rs3088440) have not been examined. Therefore, there is a clear requirement for further studies with larger sample sizes involving more than one type of hypophysis tumor with healthy controls. The study is an initial step for a more comprehensive study, in which the aim is to assess these associations in other hypophyseal adenomas, although we believe that the preliminary results deserve consideration. The preliminary data from a study that is the first of its type may be viewed in this context and it may give certain insight for further studies.

In conclusion, the present study is, to the best of our knowledge, the first study to examine the role of the CDKN2A C540G (rs11515) gene polymorphisms on tumor size and behavior in hypophyseal tumors and prolactinomas. The p16 pathway and CDKN2A gene have tumor-suppressing effects that play a major role in cell proliferation processes. Changes involving this gene are important for tumoral growth and progression. In the study, the CDKN2A gene C540G CG genotype has been observed to occur at a higher frequency in patients with prolactinoma compared with previous reports. In addition, in the tumors with a high Ki67 index (a sign of high proliferative capacity) and in giant adenomas (>4 cm), a higher frequency of the CG genotype was found. Future studies are required to confirm the data showing that the CDKN2A gene C540G polymorphism and CG genotype may play a role in the tumor development and growth.

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