The Role of \textit{p16} and \textit{MDM2} Gene Polymorphisms in Prolactinoma: \textit{MDM2} Gene Polymorphisms May Be Associated with Tumor Shrinkage

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Abstract. Aim: Prolactinomas are thought to arise from clonal expansion of a single mutated cell which is subjected to growth stimuli of several permissive factors, although the pathogenetic mechanisms underlying tumorigenesis remain unclear. The present study aimed to investigate the role of \textit{p16} (540C\textrightarrow{}G and 580C\textrightarrow{}T) and mouse double minute 2 (\textit{MDM2}) (SNP309T\textrightarrow{}G) gene polymorphisms in tumorigenesis and characteristics of prolactinoma. Patients and Methods: A total of 74 patients with prolactinoma and 100 age- and gender-matched healthy individuals were enrolled in the study. Serum prolactin levels were measured by enzyme-linked immunosorbent assay (ELISA). \textit{p16} and \textit{MDM2} polymorphisms were determined by polymerase chain reaction-restriction fragment polymorphism and agarose gel electrophoresis. Results: \textit{p16} 540C\textrightarrow{}G genotype distribution was found to be: CC: 66.2\%, CG: 28.4\%, GG: 5.4\%; \textit{p16} 580C\textrightarrow{}T genotype distribution was found to be: CC: 82.4\%, CT: 17.6\%, TT: 0\% and \textit{MDM2} genotype distribution was found to be: TT: 31.1\%, TG: 47.3\%, GG: 21.6\% in patients with prolactinoma. Tumor diameter before treatment was correlated with prolactin levels before treatment and percentage of prolactin decrease with treatment (\textit{r}=0.719, \textit{p}<0.001, \textit{p}=0.034 \textit{r}=0.256, respectively). The number of patients with tumor size decrease of more than 50\% in those with homozygous genotype (TT+GG) of \textit{MDM2} SNP309T\textrightarrow{}G was significantly higher than in heterozygous genotype (TG) carriers (odds ratio(OR)=0.18, 95\% confidence interval(CI)=0.06-0.58; \textit{p}=0.003). Conclusion: This study showed that \textit{p16} and \textit{MDM2} polymorphisms do not play a decisive role in tumorigenesis, but some genotypes of these polymorphisms might be associated with follow-up characteristics of prolactinoma.

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is thought to be caused by uncontrolled cell proliferation and delayed differentiation as a result of genetic alterations, polymorphisms and damage in CDK and CKI families (8).

p16 protein is a member of the CKI family, composed of 156 amino acids and encoded by p16 tumor suppressor gene (also known as CDKN2/INK4A) on chromosome 9p21 of the human genome. p16 gene products lead to blocking of G1/S transition by inhibiting the activity of CDK4, CDK6 and cyclin D1 complex which phosphorylates the retinoblastoma (Rb) protein and precipitates the synthesis of transcription factors required for S phase. Therefore, inactivation of the p16 gene increases cell proliferation and probably contributes to loss of growth control leading to tumorigenesis (9). Two polymorphisms of the p16 gene, 540C→G (rs11515) and 580C→T (rs3088440) located in the 3’ untranslated region (3’UTR) were recently identified (10). Several studies showed that these polymorphisms may play a crucial role in tumor development, prognosis and aggressiveness by affecting the function of p16 protein (11, 12).

Mouse double minute 2 (MDM2) gene, located in the 12q14.3-q15 chromosomal region, produces MDM2 protein. MDM2 binds to p53 tumor-suppressor protein and blocks its roles in cell-cycle arrest at the G1 and G2 checkpoints and apoptosis (13). Various modifications, that may occur in the p53 and MDM2 gene, can disrupt the interaction between these two gene products. It has been shown that the overexpression of MDM2 gene leads to cell-cycle arrest and apoptosis by increasing the degradation rate of p53 protein, and hence contributes to tumorigenesis (14). In addition, MDM2 overexpression is thought to be able to have a direct effect on tumorigenesis independently of the p53 pathway (15). As a single nucleotide polymorphism, the base pair change of a G to T at nucleotide 309 in the MDM2 promoter region (rs2279744; SNP309T→G) alters the level of expression of MDM2 (16). It was reported that MDM2 SNP309T→G leads to loss of p53 tumor-suppressor activity and enhanced tumorigenesis in humans (17, 18).

Although previous studies determined the expression of p16 and MDM2 genes in pituitary tumors, as far as we are aware there is no study yet to investigate the association between these two gene polymorphisms and prolactinoma (19-21). The present study aimed to investigate the role of p16 (540C→G and 580C→T) and MDM2 (SNP309T→G) gene polymorphisms in tumorigenesis and characteristics of prolactinoma. This study may contribute to the elucidation of tumorigenesis in order to understand the differences in tumor behavior, development of resistance to medical treatments and tumor recurrence after surgery for prolactinoma.

Materials and Methods

Study participants and prolactin assays. This prospective case-control study was performed at the Department of Endocrinology and Metabolism, Bezmialem Vakif University Hospital, Istanbul, Turkey, between 2013 and 2014. Seventy-four patients with newly diagnosed prolactinoma (59 female and 15 male) and age- and gender-matched 100 healthy individuals (78 female and 22 male) were enrolled in this study. Age under 18 years, hyperprolactinemia as a result of other causes (e.g. chronic disease, medical treatment, other pituitary disease, pregnancy, and lactation) and having a history of operation or radiosurgery were exclusion criteria of the study. Patients were examined with hypophyseal magnetic resonance imaging (MRI) to explore the presence of an adenoma. Tumors were stratified according to the longest dimension as follows: microadenoma: <1 cm, macroadenoma: ≥1 cm. To follow-up disease progression, clinical and laboratory examinations were repeated every 3 months and MRI was performed every 6 months. Patients received the treatment with cabergoline (2×0.25 mg/week) and the dose was adjusted to reach normalization of prolactin levels. Patients were considered to have responded to treatment if more than 50% tumor shrinkage was achieved at the end of the first year.

Serum prolactin levels were measured by enzyme-linked immunosorbent assay (ELISA) with a commercial kit (DKO011-1; Diametra, Pozzuolo, Italy). Reference ranges for prolactin were: female: menstrual cycle 1.2-19.5 ng/ml; menopause 1.5-18.5 ng/ml; male 1.8-17.0 ng/ml.

All participants were volunteers, and their informed consent was obtained. This study was approved by the local Ethics Committee of Bezmialem Vakif University Hospital (Istanbul, Turkey) with 1/3 decision number on 08.01.2014 and all procedures were performed in accordance with guidelines established by the Declaration of Helsinki.

DNA isolation and genotyping. Blood samples (10 ml) were obtained from each participant into EDTA tubes in order to p16 and MDM2 genotyping. DNA was extracted from whole blood using a salting-out technique (22). Genotyping analyzes were performed by polymerase chain reaction and restriction length polymorphism (PCR-RFLP) and defined p16 (540C→G and 580C→T) and MDM2 (SNP309T→G) polymorphisms. Primers used for PCR amplification were: p16 540C→G and p16 580C→T: forward: 5’ GAT GTG CCA CAC ATC TTT GAC CT’3 and reverse: 5’ CTA CGA AAC CGG GGT GGG TTG T’3; MDM2 SNP309 T→G: forward: 5’ CGC GGG AGT TCA GGG TAA AG’3 and reverse: 5’ AGC TGG AGA CAA GTG ACT AGG ACT TAA C’3. After PCR amplification of isolated genomic DNA with specific p16 and MDM2 primer pairs, the SNP309T→G substitution of MDM2 gene

Table I. Baseline characteristics of prolactinoma patients and healthy controls.

|                   | Patients (n=74) | Healthy controls (n=100) |
|-------------------|----------------|--------------------------|
| Age (years)       | 35.4±11        | 32.8±4.1                 |
| Gender (female/male) | 59/15         | 78/22                    |
| BMI (kg/m²)*      | 26.5±5         | 23.1±2                   |
| Prolactin (ng/ml)*| 257.3±366.2    | 13.6±4.9                 |
| Type of adenoma (n, %) | -             | -                       |
| Microadenoma      | 46 (62.2)      | -                        |
| Macroadenoma      | 28 (37.8)      | -                        |
| Galactorrhea (n, %) | 20 (27)        | -                        |
| Visual field defect (n, %) | 2 (2.7) | -                       |

BMI: Body mass index. *p<0.001.

Table 1. Baseline characteristics of prolactinoma patients and healthy controls.
and the 540C→G and 580C→T substitutions of the p16 gene were identified by cutting the PCR products with appropriate restriction enzymes. MspAI, MspI and HaeIII (MBI Fermentas, Ontario, Canada), respectively. The digested DNA fragments were run on 2% agarose gel followed by staining with ethidium bromide solution. The genotypes were determined under ultraviolet light.

Statistical analysis. Clinical parameters and demographic characteristics are expressed as mean±SD, frequency, and the percentage. Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) software (version 20; IBM, SPSS Inc. NY, USA). Differences in the frequencies of alleles and polymorphisms between prolactinoma versus those of the control group were tested using the Chi-square statistic. Mann–Whitney U and Kruskal–Wallis tests were used for comparing continuous variables depending on the number of the group. Student’s t-test and ANOVA were applied if required. Spearman’s correlation was used to assess the relationship between the two variables. The Hardy–Weinberg equilibrium was tested for all polymorphisms. The estimated odds ratios (OR) with 95% confidence interval (CI) were calculated for potential risk. Values were considered as statistically significant if the two-tailed p-value was less than 0.05.

Results

Baseline characteristics of the patients with prolactinoma and healthy controls are shown in Table I. No significant difference was found in gender or age between patients and controls (p>0.05). There were 59 female (79.7%) and 15 male (20.3%) patients with prolactinoma; 78 female (78%) and 22 male (22%) healthy controls in the study. The mean basal prolactin level was significantly higher in males (451.8±462.9 ng/ml) than in females (215.8±337.8 ng/ml) in the patient group (p=0.036). There was no significant difference in prolactin levels according to gender in the healthy controls. Clinical characteristics of patients with prolactinoma according to micro- and macroadennomas are given in Table II. Macroadennoma frequency was significantly higher in male patients than in females (OR=4.55, 95% CI=1.36-15.25; p=0.016).

Percentage of reduction in prolactin level was moderately correlated with pre-treatment prolactin level (r=0.279, p=0.019), negatively well-correlated with post-treatment prolactin level (r=-0.732, p<0.001). Pre-treatment tumor diameter was correlated with pre-treatment prolactin and percentage of reduction in prolactin level (r=0.719, p<0.001;
and r=0.256, p=0.034; respectively). Post-treatment tumor diameter was well-correlated with pre-treatment prolactin level and pre-treatment tumor diameter (r=0.369, p<0.001; and r=0.638, p<0.001; respectively). The mean cabergoline dose was also moderately correlated with the post-treatment prolactin level and pre-treatment tumor diameter (r=0.443, p<0.001; and r=0.284, p=0.017; respectively).

The distribution of p16 (540C→G and 580C→T) and MDM2 (SNP309T→G) genotypes and alleles in prolactinoma patients and healthy subjects is shown in Table III. Genotype distributions of p16 (540C→G and 580C→T) and MDM2 (SNP309T→G) polymorphisms were in agreement with the Hardy–Weinberg equilibrium both in control and patient groups (p>0.05). CC genotype (homozygous wild-type) frequency was higher, without statistical significance, for both p16 540C→G and p16 580C→T polymorphisms among patients with prolactinoma. The comparisons of characteristics among the p16 and MDM2 genotypes in patients with prolactinoma are summarized in Table IV. The number of patients with tumor diameter decreased more than 50% in homozygous genotype (TT+GG) carriers of MDM2 SNP309T→G is significantly higher than in heterozygous genotype (TG) carriers (OR=0.18, 95% CI=0.06-0.58; p=0.003). However, other than that, no significant association was found between characteristics of prolactinoma and p16, MDM2 gene polymorphisms and alleles.

**Discussion**

Although prolactinoma is the most frequent hypophyseal tumor, our knowledge regarding the molecular events that occur during lactotroph cell proliferation is limited. Molecular studies have been performed on more atypical prolactinomas due to surgical removal of prolactinoma is rarely performed compared to other types of pituitary tumors (23). As a consequence, factors related to tumor development and clinical parameters are not clearly understood even if previous studies have investigated cellular mechanisms involving tumor pathogenesis. In our study, we evaluated polymorphisms in the p16 and MDM2 gene and their role in tumorigenesis and clinical characteristics of prolactinoma.

The p16 gene is an important tumor-suppressor gene that functions in the negative regulation of G1/S transition of the cell cycle (24). Several studies have shown that deletion, promoter hypermethylation, or loss of heterozygosity and homozygosity at the 9p21 chromosome area (coding p16INK4a/p14ARF/p15 tumor-suppressor loci) result in loss of p16 expression (25, 26). The demonstration of increased p16 expression in differentiated adult human brain tissue suggests a role for p16 in the development of human brain and possibly in tumor pathogenesis (27). It has been shown that the presence of mutational changes of p16 gene in several intracranial tumors such as anaplastic astrocytoma, glioblastoma, and pituitary adenoma (28-30). Seaman et al. detected p16INK4a gene alterations in hypophyseal adenomas, and they reported that p16 down-regulation is one of the most important mechanisms involving somatotropic and corticotropic adenomas. The same study revealed that CDKN2A/p16 inactivation was associated with tumor type and size and that p16 down-regulation was observed during the progression of adenoma rather than the onset of tumor development (31). A similar study found a significant association between p16INK4a...
methylated and tumor size. The authors suggested that \( p16 \) inactivation may be important in development, progression, and behavior of hypophyseal tumors (32). The 540C\( \rightarrow \)G (rs=11515) and 580C\( \rightarrow \)T (rs=3088440) polymorphisms that are present in the 3'UTR region of the \( p16 \) gene can lead to tumor development by altering several cellular pathways. One study from Turkey found that C allelic frequency in \( p16 \) 540C\( \rightarrow \)G polymorphism was 77.4% and the G allelic frequency was 22.6% (21). Similar to allelic frequencies in \( p16 \) 540C\( \rightarrow \)G polymorphism that has been shown in a European population (http://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=9:21967700-21968700; v=rs11515; vdb=variation; vf=22717), we found the C allelic frequency of 76% and G allelic frequency of 24% in our study. In addition, the C allelic frequency in \( p16 \) 580C\( \rightarrow \)T polymorphism was 91.5%, and the T allelic frequency was 8.5%. These rates are also consistent with the rates shown in a European population (http://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=9:21967660-21968660; v=rs3088440; vdb=variation; vf=2689541). Tuna et al. investigated the role of \( p16 \) (540C\( \rightarrow \)G and 580C\( \rightarrow \)T) and MDM2 (SNP309T\( \rightarrow \)G) polymorphisms in development and progression of colorectal cancer. They found a significant difference between patient and control groups regarding the distribution of \( p16 \) 540C\( \rightarrow \)G polymorphism (12). Another study by Cander et al. involving patients with prolactinoma, did not find an association between \( p16 \) 540C\( \rightarrow \)G polymorphism and tumor behavior. However, they recorded a higher frequency of CG genotype among patients with giant prolactinoma and patients with increased Ki67 index, which is an indicator of high proliferative capacity, although this did not reach a level of statistical significance (21). Consistent with their results, \( p16 \) 540C\( \rightarrow \)G polymorphism had a similar distribution across patient and control groups in our study. In contrast to their findings, the patient group with macroadenoma had a higher frequency of CC genotype, which is the homozygous wild-type for both \( p16 \) 540C\( \rightarrow \)G and 580C\( \rightarrow \)T polymorphisms in our study. However, the difference did not reach statistical significance. Further larger scale studies are needed to explain the results regarding the significance of this difference.

The potential of MDM2 gene in tumorigenesis has been determined by demonstration of causes of overexpression in human tumors, such as gene amplification, increased transcription, and translation (33). It was thought that factors that can induce cellular stress, such as cytokines and growth factors, which are known to play a role in the pathogenesis of hypophyseal tumors, may contribute to tumor development via acting on \( p53 \) and MDM2 pathways. Based on this hypothesis, MDM2 protein expression in hypophyseal tumors was first demonstrated in a study by Suliman et al. (19). Although \( p53 \) is one of the most frequently inactivated genes in human cancer, \( p53 \) mutation was very rarely detected in hypophyseal adenomas (34, 35). As a possible explanation for this, it was suggested that in the pathogenesis of hypophyseal tumors, the effect of cellular stress on \( p53 \) pathway could be more important than mutations in \( p53 \) and that \( p53 \) may not initiate tumor development, but could play a role in prognosis (36). The T\( \rightarrow \)G base exchange occurring at 309th nucleotide of the first intron of MDM2 gene is expressed as MDM2 SNP309T\( \rightarrow \)G (rs=rs2279744), and this mutation causes an increased level of MDM2 protein, leading to suppression of \( p53 \) apoptosis pathway (18). One study from Turkey found the T allelic frequency of MDM2 polymorphism to be 44.7%, and the G allelic frequency 55.3% (12). Similarly, in our study, we found a T allelic frequency of 50% and G allelic frequency of 50%. In a European population, the T allelic frequency was found to be 64% and that of the G allele was found to be 36%. Conversely, the T allelic frequency was found to be 43% and G allelic frequency 57% in an Asian population (http://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=12:68808300-68809300; v=rs2279744; vdb=variation; vf=1937624). There are several studies that investigated the effect of the MDM2 polymorphism on cancer development risk and prognosis. A large-scale study by Schmidt et al. did not show any association between MDM2 SNP309T\( \rightarrow \)G and \( p53 \) polymorphisms and breast cancer, whereas another study found a strong relationship between MDM2 SNP309T\( \rightarrow \)G polymorphism and survival in patients with breast cancer (37, 38). A study by Hirata et al. involving patients with renal cell carcinoma showed that MDM2 polymorphism was associated with increased renal cell carcinoma risk. The same study reported that MDM2 GG genotype was significantly more frequent compared to TT or TG genotypes in renal cancer tissues (39). In their study, Hori et al. did not find a significant difference between the patient group with pancreatic ductal adenocarcinoma and chronic pancreatitis and healthy volunteers regarding MDM2 polymorphism. However, they found patients with MDM2 GG genotype had shorter total life expectancy compared to those with other (TG, TT) genotypes (40). Another study by Chua et al. reported MDM2 TT genotype was associated with lung cancer in non-smoking women in a Chinese population (41). A similar study in the same population showed the G allele was associated with increased risk of cancer; however, early age of onset was found to be associated with the TT genotype (42). Several points draw attention as the cause of these differences in genotype distribution in terms of disease development risk and prognosis. Regarding MDM2 polymorphism, the basal T allelic frequency was found to be higher in a European population, while the basal G allelic frequency was found to be high in an Asian population. This suggests possible ethnic differences in cancer risk determined by MDM2 gene functions and alleles. These ethnic differences are possibly among the causes of the differences in activity of individual polymorphic variants of the genes present in the
p53 pathway. In our study, we did not find a significant difference between the prolactinoma and control groups regarding MDM2 polymorphism. When we examined the relationship of genotypes with clinical parameters, we found that the number of patients with greater than 50% reduction in tumor size was significantly higher among those carrying homozygous genotypes (TT+GG) in comparison to those with heterozygous (TG) genotype (OR=0.18, 95% CI=0.06-0.58, p=0.003). This result indicates a better response to treatment in patients with a homozygous genotype in comparison to those with a heterozygous genotype. Similarly to what was mentioned about previous population studies, ethnic differences may be responsible for this result. Another important point was the possible confounding effect in polymorphism studies caused by susceptibility of p53 control over cell proliferation to environmental factors. Recent evidence indicates cellular stress factors that could lead to genotoxic injuries such as cytokines, growth factors, hypoxia, and metabolic alterations. These may be effective at the initial phase of p53 response and may cause differences in functionality of polymorphisms (43).

Current single nucleotide polymorphism studies give enlightening results that would aid in the determination of tumor prevalence in the population, the age of tumor incidence, and response of these tumors to treatment. According to our results, while there was no association of p16 and MDM2 gene polymorphisms with prolactinoma development, it was shown that carrying homozygous genotype (TT+GG) of MDM2 SNP309T→G might be associated with tumor shrinkage in patients with prolactinoma. We hope that our results contribute to shedding light on future studies for a better understanding of tumor etiopathogenesis, which is essential to elucidate the distinguishing differences in tumor behavior, to identify new prognostic parameters, and to develop effective treatment options.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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