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# P16 and p27 tumor suppressor gene methylation status in childhood Wilms tumor cases

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**Aim:** To analyze the methylation status of the promoter regions of p16 and p27 genes in Wilms tumor patients. These tumor suppressor genes are associated with 2 main pathways regulating the G1/S transition of the cell cycle.

**Materials and methods:** Sixteen patients with Wilms tumor were included in the study. The methylation status of CpG islands in the p16 and p27 genes was analyzed by the polymerase chain reaction (PCR) technique in the tumor tissue samples obtained from all patients. Five tissue samples of normal kidney were obtained from pathology department archives.

**Results:** P16 gene promoter methylation was detected in 2 of 16 (12.5%) patients, one of which was heterozygous and the other homozygous. P27 gene promoter methylation was also found in 2 of 16 (12.5%) patients, one of which was heterozygous and the other homozygous. No methylation status was observed in normal kidney tissues.

**Conclusion:** Our results showed that the incidence of CpG island promoter region methylation of the p16 and p27 tumor suppressor genes in Wilms tumor was low. However, larger series are needed to determine the prognostic value of DNA methylation of p16 and p27 in Wilms tumor patients.

**Key words:** Wilms tumor, p16 gene, p27 gene, methylation, epigenetic

## Çocukluk çağı Wilms tümör olgularında p16 ve p27 tümör süpressör gen metilasyon durumu

**Amaç:** Bu çalışma ile çocukluk çağı Wilms tümörü olgularında p16 ve p27 genlerinin promoter bölgesinin metilasyon durumlarının incelenmesi amaçlanmıştır. Bu tümör supresör genler hücrenin G1 fazından S fazına geçişini kontrol eden iki ana yolda rol almaktadır.

**Yöntem ve gereç:** Bu çalışmaya Wilms tümör tanılı 16 hasta alınmıştır. Tüm hastaların alınan patolojik dokularının p16 ve p27 genlerinin CpG adacık bölgelerinin metilasyon durumu polimeraz zincir reaksiyonu (PCR) ile incelendi. Ayrıca 5 adet normal böbrek dokusu çalışılmak üzere patoloji bölümü arşivinden alındı.

**Bulgular:** 16 hastanın 2'sinde (% 12,5) p16 gen promoter bölgesinde biri homozigot diğeri heterozigot olmak üzere metilasyon durumu gözlenmiştir. P27 gen promotor bölgesinde 16 hastanın 2'sinde (% 12,5) biri homozigot diğeri heterozigot olmak üzere metilasyon gözlenmiştir. 5 adet normal böbrek dokusunda metilasyon gözlenmemiştir.

**Sonuç:** Çalışmamızda Wilms tümör olgularında p16 ve p27 gene promotor bölge metilasyonu düşük düzeyde görülmüştür. Düşük olgu sayımız nedeniyle Wilms tümörü olgularında P16 ve P27 gen metilasyonun prognostik önemini belirlemek için daha geniş serili çalışmalara ihtiyaç vardır.

**Anahtar sözcükler:** Wilms tümörü, p16 geni, p27 geni, metilasyon, epigenetik

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## Introduction

Wilms tumor (WT) is a childhood embryonal cancer of the kidney. WT is the original model for studying the etiology of cancer. The functions of the WT1 and WT2 suppressor genes have been shown in WT, but a large part of WT etiology is still unknown. Epigenetics can be defined as a heritable change in gene expression that is not accompanied by changes in DNA sequence. Epigenetic dysregulation may have an important role in cancer initiation and progression. These epigenetic changes may lead to activation of genes that increase tumor invasion and metastasis and inactivation of tumor suppressor genes (1). An important epigenetic regulation mechanism is the methylation of the DNA CpG island. DNA methylation results from the addition of a methyl group to the carbon 5 position of the cytosine ring. The methylation of CpG-rich genes triggers the locking off mechanisms that may turn a gene off or block its activation (2). DNA methylation is also important for other mechanisms, such as mammalian development, embryogenesis, cellular differentiation, chromosome integrity, and control of DNA replication and repair (3). As a general rule, excessive methylation (hypermethylation) and loss of appropriate methylation (hypomethylation) can lead to carcinogenesis. Hypermethylation can be factor via silencing of tumor suppressor genes and hypomethylation by the inappropriate activation of or proto-oncogenes.

Cyclin and cyclin-dependent kinases (CDKs) play an important role in the regulation of cell cycle progression. The activity of Cyclin-CDK complexes is regulated by CDK inhibitors. CDK inhibitors can be divided into 2 groups: the INK4 family, composed of p16, p15, p18, and p19, which specifically inhibits CDK4 and CDK6 (4), and the Kip/Cip family, composed of p27, p21, and p57. A member of the Kip/Cip family is able to inhibit a wide variety of CDKs (5). P16 and p19 tumor suppressor genes increase the tumor suppressor effect of the retinoblastoma (Rb) protein and the p53 tumor suppressor genes, respectively (6). Activated CDK inhibitors, p16 or p27, bind CDKs that result in cell cycle arrest (7). Promoter hypermethylation of p16 inversely correlates with the presence of Rb mutations in multiple tumors. Hypermethylation-mediated inactivation of p16 has been demonstrated in tumors of the brain, breast, colon, head, neck, and non-small cell lung cancers, and in high-grade non-Hodgkin's lymphoma (8). Decreased loss of p27 tumor suppressor function is associated with tumor invasiveness and negative results in cancer cases, especially in hepatocellular carcinoma (9). Although there are some studies on p16 gene methylation in Wilms tumor cases, the loss of imprinting (LOI) of the IGF2 gene (which encodes insulin-like growth factor II) is the most common genetic or epigenetic alteration in these tumors. Our aim in this study was to assess the methylation status of the p16 and p27 tumor suppressor genes in Wilms tumor samples.

## Materials and methods

**Patients and samples**: The study was approved by the institutional ethic committee. The study group consisted of 16 patients (8 female, 8 male; age range 18 to 87 months; mean age 42 months) with sporadic Wilms tumor. All patients underwent surgical resection of the tumor between 1999 and 2007 at Dr. Sami Ulus Children's Hospital. Besides these 16 tissue samples from Wilms tumors, 5 normal kidney tissue samples were obtained as control group from pathology archives. All patients with Wilms tumor were staged and treated according to the Turkish Pediatric Oncology Group Wilms Tumor Study Protocol. The patients with Wilms tumor were followed up until April 2009 and they had all survived to that date. Clinical and histological data are summarized in the Table.

**Molecular analysis**

Genomic DNA was extracted from formalin-fixed paraffin-embedded tissues following the deparaffinization procedure, using QIAamp DNA Mini kit (QIAGEN, Hilder, Germany) according to the manufacturer's instructions. Methylation-specific PCR (MSP) assays were performed to determine the methylation status of CpG islands in the p16 and p27 gene promoters (11). Before methylation-specific PCR (MSP) assays, DNA samples were modified with the sodium bisulfate reaction to convert unmethylated cytosine to uracil according to the manufacturer's instructions (CpG Genome DNA modification kit, Chemicon, Temacula,

USA). Previously reported primer sequences and PCR conditions were used to amplify the p16 and p27 genes (10). For the amplification of the p16 promoter, the DNA was amplified with Taq DNA (1 unit/50 µL, Fermentas, Vilnius, Lithuania) (PCR conditions, 35 cycles at 95 °C for 30 s, at 62 °C for 30 s, and at 72 °C for 30 s) giving a 151 bp product for the unmethylated allele and 150 bp product for the methylated allele, whereas PCR products specific for p27 promoter (PCR conditions, 5 cycles at 95 °C for 30 s, at 56 °C for 30 s, and at 72 °C for 30 s) were 195 bp for unmethylated allele and 201 bp for methylated allele.

CpG genome universal methylated DNA supplied with the kit was used as positive controls whereas PCRs performed with water instead of template DNA was served as PCR-negative controls. DNA from normal kidney specimens was used as normal tissue control for unmethylated alleles for each PCR reaction. PCR products were separated on 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

**Results**

All tumor tissue samples were evaluated to determine the histological type of Wilms tumor. No tumor samples had anaplastic criteria. Of the 16 patient tumor samples, 9 had mixed cell, 1 had the epithelial-mesenchymal, 1 had the epithelial-blastemal, 1 had the teratoid, and 1 had the rhabdomyomatosis subtype of Wilms tumor. It was not possible to subclassify 3 of the 16 Wilms tumor specimens while 2 out of 16 patients had additional urogenital anomalies (horseshoe kidney, hydrocele). One patient has aniridia. Two patients had a family history. No tumor samples showed methylation status (Table).

There was no CPG island methylation in 12 of the 16 samples. Tumor suppressor methylation was detected in only 4 samples. One sample (No: 313-02) showed homozygous p27 gene methylation and another sample (No: 290-01) showed heterozygous p27 gene methylation. In the p16 gene promoter region methylation evaluation, 1 sample (No:1099-02) showed heterozygous and another sample (No:

Table. Summary of the clinical and molecular features of Wilms tumor (nonanaplastic) cases.

| Sample no | Sex | Age at diagnosis (months) | Additional anomaly | Stage Histology | Outcome | p16 | p27 Tumor | Familiy History |
|---|---|---|---|---|---|---|---|---|
| 112-07 | M | 24 | -- | II Mixed cell Wilms tumor | Alive | UM | UM | -- |
| 390-07 | F | 60 | -- | II Mixed cell Wilms tumor | Alive | UM | UM | -- |
| 1099-02 | M | 18 | -- | II Mixed cell Wilms tumor | Alive | HtM | UM | -- |
| 313-02 | M | 24 | -- | III Wilms tumor | Alive | UM | HM | -- |
| 546-02 | M | 30 | Left hidrocele | IV Wilms tumor | Alive | UM | UM | -- |
| 1288-05 | F | 66 | Horse shoe kidney | IV Wilms tumor | Local relapse | HtM | UM | -- |
| 441-06 | F | 42 | -- | III Rhabdomyomatous | Alive | UM | UM | -- |
| 269-05 | F | 54 | -- | III Teratoid Wilms tumor | Alive | UM | UM | -- |
| 579-99 | F | 42 | -- | III Mixed cell Wilms tumor | Alive | UM | UM | -- |
| 216-03 | M | 60 | -- | IV Epithelial-blastemal | Alive | UM | UM | -- |
| 464-01 | M | 36 | -- | III Mixed cell Wilms tumor | Alive | UM | UM | -- |
| 615-03 | F | 24 | -- | II Mixed cell Wilms tumor | Alive | UM | UM | -- |
| 144-97 | F | 36 | -- | III Mixed cell Wilms tumor | Alive | UM | UM | -- |
| 290-01 | M | 20 | -- | III Mixed cell Wilms tumor | Alive | UM | HtM | -- |
| 167-05 | M | 18 | Aniridia | III Epithelial-mesenchymal | Alive | UM | UM | + |
| 742-07 | F | 87 | -- | IV Mixed cell Wilms tumor | Alive | UM | UM | + |

Abbrevations: M:Male, F:Female, UM:Unmethylated, HtM:Heterozygous methylated, HM:Homozygous methylated

1288-05) showed homozygous p16 gene promoter region methylation (Figure). The patient with horseshoe kidney (No: 1288-05) and relapsed Wilms tumor had a homozygous p16 gene methylation.

## Discussion

Epigenetic alterations may increase the vulnerability of the genome to genetic changes and are environmental carcinogenesis. Decreased function of the tumor suppressor genes may also result from epigenetic changes in the DNA structure, such as mutations, rearrangements, and gene amplifications (11).

It was previously thought that an alteration of DNA methylation occurred only as a part of carcinogenesis. However, it is now thought that it is not a secondary result of cancer as alteration of DNA methylation has been shown in the precancerous event. To the contrary, it can facilitate the development of cancer (12).

DNA hypomethylation and hypermethylation are important in the activation of tumor suppressor genes. DNA hypomethylation can cause genetic dysregulation via genetic instability (13). The tumor suppressor gene methylation status affects tumor progression. The hypermethylation status of tumor suppressor genes generally leads to tumor progression. However, some studies have shown that hypomethylation of tumor suppressor genes also affects tumor progression. Hypomethylation of the promoter region of DNA CpG islands has also been shown in rhabdomyosarcoma, Hodgkin lymphoma, and anaplastic large cell lymphoma (14,15). This methylation was thought to lead to tumor progression. Nevertheless, Nishiyama et al. (16) demonstrated that cytosine residues of the NBL2 gene subregion in ovarian cancer cells showed both hypermethylation and hypomethylation status. Erlich et al. (17) observed general hypomethylation at CpG islands but also found hypermethylation at local CpG islands. These interesting changes show some variance in the first tumor cell population and may cause tumor cell heterogeneity and more aggressive tumor cell clones (1).

There are many studies about the facilitating role of CpG island hypermethylation in the initiation

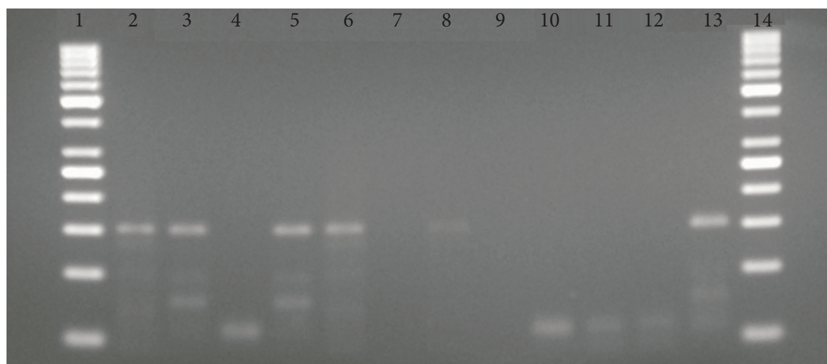


Figure. Methylation-specific PCR gel of p16 tumor suppressor genes.

1- 50 base pair marker.

2- Unmethylated band of the p16 gene that belongs to sample 1099.

3- Methylated band of the p16 gene that belongs to sample 1099.

6- Unmethylated band of the p16 gene that belongs to sample 141.

8- Unmethylated band of p16 gene that belongs to normal kidney sample.

10- No DNA p16 unmethylated band.

11- No DNA p16 unmethylated band.

13- Methylated band of p16 gene that belongs to positive control.

14- 50 base pair marker.

of tumor formation and tumor progression. The inactivation of multiple tumor suppressor genes leads to dysregulation of the cell cycle control and subsequently tumor development. The tissue proliferation index was found to be high during the evaluation of the methylation status of the p16 gene, one of the tumor suppressor genes with an important role in intracellular signal transduction in hepatocellular carcinoma (9) and lymphoma (18). Chim et al. (19) found that p16 gene methylation was associated with a higher lymphocyte count in chronic lymphocytic leukemia patients. Higher levels of p16 gene methylation appear to increase the risk of hepatocellular and colorectal cancer progression (9,20). The p27 tumor suppressor gene plays an important role in the intracellular signal conduction system. It is thought that low levels of the p27 gene are not a cause but a result of carcinogenesis and that this low level affects the progression of the disease (7). Kawauchi et al. (21) found a higher tumor proliferation index in germ cell tumors with high p27 tumor gene methylation status. Qian et al. (22) showed methylation of the p27 tumor suppressor gene in some pituitary cell tumors leading to silencing of the p27 tumor suppressor gene.

WT1 and WT2 gene-related pathology is the first known gene pathology of Wilms tumor. However, this gene pathology is found in only 15% of Wilms tumor cases. Investigators have therefore searched for the presence of any other pathology other than those related to the WT1 and WT2 genes. P53 is a well-known tumor suppressor gene and is also responsible for some familial cancers. Mutations in the p53 tumor suppressor gene were found in 75% of patients with anaplastic Wilms tumors in a study (23). Methylation of the p16 tumor suppressor gene was detected in 22.9% of patients, none of whom had the protein product. There was also no statistical relationship between the methylation status of the p16 tumor suppressor gene and the tumor progression and stage (24). Arcellana-Panlilio et al. (25) reported that methylation of the p16 tumor suppressor gene was present in 9 of 38 Wilms tumor tissues and half of these patients had advanced disease stages. They thought that methylation of the p16 tumor suppressor gene might be an important prognostic sign in Wilms tumor. We did not detect methylation of the p16 and p27 tumor suppressor genes in 87.5% of the tumor tissues in our study. However, statistical analysis was not performed due to the relatively small sample size. Interestingly, one patient with a horseshoe kidney and relapsed tumor showed homozygous p16 methylation. This finding reminded us of the study by Arcellana-Panlilio et al. (25) indicating that the p16 methylation status of the tumor may be related to poor prognosis. However, our p16 methylation status results are consistent with those of the Faussillon et al. (26) and Morris et al. (27) studies on Wilms tumor cases. In terms of the evaluation of methylation status of the p27 tumor suppressor gene, it seems that p27 methylation does not play an important role in Wilms tumor cases. Our findings regarding p27 methylation are consistent with those of Kawauchi et al. (22) in malignant germ cell tumor, of Brakensiek et al. (28) in myelodysplastic syndrome and acute myeloid leukemia, and of Chim et al. (29) in chronic lymphocytic leukemia. This low level may be attributed to an activated post-transcriptional degradation mechanism, such as the ubiqutin-proteasome pathway (30). Our results suggest that the methylation of the p16 and p27 tumor suppressor gene promoter region is at a low level and there is no correlation with the clinical stage. However, larger patient series are needed to determine the prognostic value of the p16 and p27 gene methylation status in Wilms tumors.

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