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Significant Reduction of *WT1* Gene Expression, Possibly Due to Epigenetic Alteration in Wilms' Tumor

Yuji Satoh[1,2], Tetsuji Nakagawachi[1], Hisaya Nakadate[3], Yasuhiko Kaneko[4], Zenjiro Masaki[2], Tsunehiro Mukai[1] and Hidenobu Soejima[*,1]

[1]Division of Molecular Biology and Genetics, Department of Biomolecular Sciences, Saga Medical School, 5-1-1 Nabeshima, Saga 849-8501; [2]Department of Urology, Saga Medical School, 5-1-1 Nabeshima, Saga 849-8501; [3]Department of Pediatrics, Kitasato University Hospital, 1-15-1 Kitasato, Sagamihara, Kanagawa; and [4]Department of Cancer Chemotherapy, Saitama Cancer Center Hospital, 818 Komuro, Ina, Saitama 362-0806

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***WT1* at 11p13 is a tumor suppressor gene, an aberration of which causes Wilms' tumor (WT). Since *WT1* expression is reduced in a certain proportion of WTs and its mutation is found only in 10–20% of WTs, we examined *WT1* gene silencing due to epigenetic alteration in a total of 22 WTs. *WT1* expression was significantly reduced in half of WTs without any mutation in the *WT1* gene itself, suggesting that the reduction of expression was possibly epigenetic. We found promoter hypermethylation in one WT with loss of heterozygosity (LOH) and showed that promoter methylation reduced reporter gene activity by a reporter assay. These data suggested that methylation was an epigenetic mechanism leading to *WT1* silencing and that the expression-reduced allele by hypermethylation combined with LOH was consistent with the revised two-hit model. In addition, as the β-*catenin* mutation is frequently associated with the *WT1* mutation, the association of *WT1* silencing with the β-*catenin* mutation was also investigated. β-*catenin* mutated in only one WT without *WT1* silencing, suggesting that the β-*catenin* mutation was not associated with the reduction of *WT1* expression.**

Key words: epigenetics, gene expression, promoter methylation, *WT1*, Wilms' tumor.

Abbreviations: WT, Wilms' tumor; LOH, loss of heterozygosity.

Wilms' tumor (WT), also known as nephroblastoma, is one of the most common solid tumors of childhood, accounting for approximately 6% of all childhood malignancies. Since histological features of WT mimic cell types and structures that are seen during differentiation of fetal kidney, WT is thought to arise from renal blastemal cells. The *WT1* gene at chromosome 11p13 is a tumor suppressor gene whose aberration causes Wilms' tumor (*1*, *2*). The gene encodes a zinc finger DNA-binding protein that can function as a transcription factor and plays a pivotal role in early kidney development (*3*). Mice heterozygous for *Wt1* null allele, in which the *Wt1* expression level is 95% of that of wild-type mice, do not generate tumors (*3–5*). However, some $Wt1^{-/-}$ mice carrying the *WT1* transgene, in which the *WT1* expression level is approximately 10% or more of that of wild-type mice, show renal dysplasia (*5*, *6*), suggesting the possibility that the silencing of *WT1* is involved in Wilms' tumorigenesis. It has been reported that more than half of WTs show reduced *WT1* expression (*7*, *8*). The *WT1* mutation, however, is observed in approximately 10–20% of WTs, and homozygous *WT1* mutations are found in only a few WTs (*9*, *10*). It is unclear whether the reduction of *WT1* expression is due to *WT1* mutation or other mechanisms.

DNA hypermethylation of the CpG island in the promoter region has been recognized as one epigenetic mechanism capable of reducing gene expression in many tumors (*11*). Aberrant DNA hypermethylation of the CpG island in the promoter region is an alternative to genetic mutation as a cause of inactivation of tumor suppressor genes (*12*). Thus, the possibility cannot be dismissed that the reduction of *WT1* expression in WTs results from epigenetic alteration. Two hits are required for the full inactivation of a tumor suppressor gene (*13*), and this is usually achieved by a mutation in one allele and loss of heterozygosity (LOH) in another allele. Jones and Laird (*12*) revised this two-hit model, pointing out that methylation of one allele with coordination of mutation or LOH in another allele, or methylation of both alleles, would cause the same effect.

A highly significant correlation was reported between the *WT1* mutation and β-*catenin* (*CTNNB1*) mutation in WTs, which suggested that mutations of these genes acted to alter different cellular pathways in Wilms' tumorigenesis (*14*). Among WTs harboring a β-*catenin* mutation, 95% of tumors showed *WT1* mutations, and 50% of WTs carrying a *WT1* mutation also showed the β-*catenin* mutation. β-*catenin* is involved in the regulation of cell adhesion and in signal transduction through the WNT pathway. Abrogation of the WNT pathway by β-*catenin* mutations, resulting in reduced serine/threonine phospholyration, has been recognized as playing an important role in the development of many tumors.

In this study, we investigated epigenetically the *WT1* expression level and the methylation status of the *WT1* promoter, and genetically the *WT1* and β-*catenin* mutations in a total of 22 WTs. *WT1* expression was reduced in approximately 50% of WTs without any mutation in the

[*]To whom correspondence should be addressed. Tel: +81-952-34-2264, Fax: +81-952-34-2067, E-mail: soejimah@post.saga-med.ac.jp

WT1 gene itself. One tumor showed the promoter hypermethylation combined with LOH. The reporter gene assay showed that methylation of the promoter caused gene silencing. The β-*catenin* mutation was found in only one WT without the reduction of *WT1* expression. Our data suggested that *WT1* gene silencing in WTs may have been caused by epigenetic alteration and that the β-*catenin* mutation was not associated with the *WT1* epigenetic alteration. The data also suggested that methylation was an epigenetic mechanism leading to *WT1* gene silencing and that the epigenetic silencing of *WT1* by promoter hypermethylation with LOH was consistent with the revised two-hit model in Wilms' tumorigenesis.

MATERIALS AND METHODS

DNA and RNA Samples and Genetic Analyses—Twenty-two sporadic WT tissues including one associated with WAGR syndrome, and four of mid-gestational fetal kidney, were obtained from Saitama Cancer Center Hospital and the fetal tissue bank at the University of Washington, respectively. Genomic DNAs and total RNAs were extracted with a QIAamp DNA mini kit (Qiagen) and Isogen (Nippon Gene), respectively. Genetic analyses of *WT1* were carried out as previously described (*10*). Briefly, the *WT1* mutation was screened by SSCP analysis for all exons and splice-donor/acceptor-sites. When an aberrant band was seen by SSCP, the band was excised and sequenced. LOH were also analyzed using polymorphic DNA markers on 11p13, such as D11S16, D11S325, *PAX6*, D11S324, *WT1*, and *CAT*, comparing tumor and adjacent normal tissue or peripheral blood. A mutation in exon 3 of the *β-catenin* gene (*CTNNB1*) was investigated by PCR-direct sequencing with the primer pair (sense primer in intron 2, 5′-TGGCTATCATTCTGCTTTTCTTG-3′; antisense primer in intron 3, 5′-CTCTTTTCTTCACCACAACATTTT-3′).

Quantitative Real-Time RT-PCR Analysis—Total RNA (500 ng) was treated with RNase-free DNase I (Roche) and reverse-transcribed with ReverTra Ace reverse transcriptase (Toyobo) and random primers (Takara). RT-PCR of *WT1* was performed using an exon connection primer pair (sense primer in exon 2, 5′-TTCCCCAACCACTCATTCAA-3′; antisense primer in exon 3, 5′-GGCGTCCTCAGCAGCAAAGC-3′). RT-PCR of β-*actin* was also performed using an exon connection primer pair (sense primer in exon 4, 5′-CAAGAGATGGCCACGGCTGCT-3′; antisense primer in exon 5, 5′-TCCTCCTGCATCCTGTCGGCA-3′). Quantitative RT-PCR was performed with the LightCycler™ system (Roche) according to the manufacturer's protocol. The expression of *WT1* was normalized with that of β-*actin*. The average *WT1* expression of four individual fetal kidneys was employed as a standard. All experiments were performed three times independently.

Methylation Analysis—Genomic DNAs from WTs and fetal kidneys were modified by urea/bisulfite treatment (*15*). The *WT1* promoter region spanning –258 nt to +442 nt, relative to the transcriptional start site (GenBank accession no. X74840), was amplified by hemi-nested PCR. The primers used were: MU-F1 (5′-AGATTTAAGGGTGTAAAGTAAGG-3′) and MU-TR1 (5′-TCCCGAACTCCCTACTACTCTAAC-3′) for primary PCR, and MU-F1 and MU-TR2 (5′-AAAAACCGAATCCTACAACAAA-3′) for secondary PCR. Primary PCR was carried out with an initial denaturation at 96°C for 5 min, followed by 40 cycles of 96°C for 30 s, 58°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 5 min. Secondary PCR was performed under the same conditions as the primary PCR except for 30 cycles of the amplification. The secondary PCR products were cloned into pT7Blue T-vector (Novagen). At least ten clones for each sample were sequenced.

Plasmid Construction and Reporter Gene Assay—Luciferase reporter assay was performed to investigate whether the promoter hypermethylation affected gene expression. First, three reporter vectors were constructed: PBV, PBV^{WT1}, and entMe-PBV^{WT1} (Fig. 2a). The *WT1* promoter region spanning –291 nt to +509 nt was amplified by PCR with the primers containing artificial *Mlu*I and *Xho*I restriction sites (F1, 5′-TACGCGTCTCCCCTACCCGACAGTTC-3′; R1, 5′-TCTCGAGCTGCTCTGGCTGCTGTAGG-3′). The PCR product was subsequently cloned into pT7Blue T-vector (Novagen). After large-scale preparation of the plasmid DNA, the DNA was digested with *Mlu*I and *Xho*I, then fractionated on 1.2% agarose gel followed by purification with Ultrafree®-DA (Millipore). The purified insert DNA was cloned into PicaGene Basic vector (Toyo Inki) at *Mlu*I and *Xho*I sites (PBV^{WT1}). After linearization with *Bam*HI digestion, PBV^{WT1} was entirely methylated with *Sss*I methylase (New England Biolabs) (entMe-PBV^{WT1}). To exclude the effect of ectopic methylation in entMe-PBV^{WT1}, such as the methylation of the luciferase transcriptional unit or of ordinary vector sequences, a further three vectors were constructed: lig-PBV, lig-PBV^{WT1}, and insMe-PBV^{WT1} (Fig. 2c). The insert DNA solely methylated by *Sss*I and unmethylated insert were ligated to PicaGene Basic vector, respectively (insMe-PBV^{WT1} nd lig-PBV^{WT1}). As a negative control, PicaGene Basic vector was digested with MluI followed by the self-ligation (lig-PBV). The ligation mixtures were digested with BamHI, then fractionated on 0.8% agarose gel followed by the purification. The efficiency of methylation was confirmed by resistance to cleavage by methylation-sensitive restriction enzymes *Hpa*II and *Bss*HII. Each linearized reporter vector (500 ng)—PBV, PBV^{WT1}, entMe-PBV^{WT1}, lig-PBV, lig-PBV^{WT1}, and insMe-PBV^{WT1}—was independently transfected into 293T cells (*16*), which were derived from human embryonic kidney and constitutively expressed *WT1* without exogenous stimuli (*17*), using LipofectAMINE PLUS Reagent (Life Technologies). PicaGene Seapansy control vector (Toyo Inki) was co-transfected as an internal control. After transfection followed by incubation for 48 h, luciferase activity was measured with the Dual Luciferase System Assay Kit (Toyo Inki) according to the manufacturer's instructions. The results were confirmed by three independent experiments.

RESULTS

Expression and Genetic Alteration of WT1 in Wilms' Tumors—The expression level and genetic alteration of *WT1* were investigated in a total of 22 WTs (Table 1). Using available RNAs from 19 of 22 WTs, the quantity of *WT1* expressed was determined and normalized with *β-actin* expression (Table 1). Four normal mid-gestational

Table 1. **Expression and genetic alterations of *WT1* and β-*catenine* mutations in Wilms' tumors.**

Tumor Sample	*WT1* expression[a] (%)	*WT1* mutation	11p13LOH	*CTNNB1* mutation	Complication
1	0.0[b]	homo.del.	homo.del.	–	–
2	0.0[b]	–	–	–	–
3	0.0[b]	–	n.d.	–	–
4	0.6	–	n.d.	–	–
5	0.8	–	–	–	–
6	1.2	–	+	–	–
7	14.3	–	+	–	–
8	28.6	–	–	–	–
9	28.6	–	–	–	–
10	28.6	–	+	–	–
11	57.1	–	–	–	–
12	85.7	–	–	–	–
13	85.7	–	+	–	–
14	85.7	–	–	–	–
15	142.9	–	–	–	–
16	142.9	–	–	–	–
17	157.1	–	–	ACC to GCC (T41A)	–
18	300.0	–	+	–	–
19	414.3	–	+	–	–
20	n.d.	–	+	n.a.	WAGR syndrome
21	n.d.	–	+	n.a.	–
22	n.d.	n.d.	+	n.a.	–

[a]Relative expression level of *WT1* to that of fetal kidney. [b]Below the detectable range. n.d., not done; homo.del., homozygous deletion; LOH, loss of heterozygosity; n.a., not amplified.

fetal kidneys were used as controls for *WT1* expression, these being the appropriate stage-matched controls for WT employed in several previous reports (*7*, *18*, *19*). In 10 out of 19 (53%) tumors, *WT1* expression decreased to a level 50% below the level in fetal kidney, and 6 of them showed a reduction to less than 10%. Among them were 1 WT with homozygous deletion, 3 with LOH, and 4 with retention of heterozygosity. However, we could not find any point mutation in any exon of *WT1*.

As we used frozen tumor tissues in this study, the possibility that normal cells were present in samples could not be ruled out. The presence of normal cells might influence the expression of *WT1* and lead to underestimation of the frequency of the reduced expression, because normal counterparts of tumor tissue also expressed *WT1* (*20*). Thus, our data indicated that reduced expression occurred in at least half of WTs and the frequency might increase if the normal cells were completely removed from samples.

Methylation Status of WT1 Promoter and the Reporter Gene Assay—The promoter of *WT1*, spanning from –742 to +443 nt relative to the transcription initiation site, consisted of two regions: distal and proximal promoters. Since the proximal promoter, –258 to +443 nt, had significantly stronger activity than the distal promoter (*17*), and a part of it satisfied the definition of a CpG island (0.68 of G+C content and 0.8 of an observed/expected presence of CpG), we examined the methylation status of 44 CpG sites in it by the bisulfite sequencing method (Fig. 1a). Four fetal kidneys and all WTs preserving *WT1* expression showed hypomethylation of the region. In WTs with reduced *WT1*, only one tumor (sample no. 6) showed all CpG sites to be hypermethylated (Fig. 1b). The expression level in this sample was 1.2% of fetal kidney and it showed LOH, indicating promoter hypermethylation on the remaining allele. The possible presence of normal cells would affect methylation status in the same way as the expression mentioned above.

To assess whether promoter hypermethylation caused transcriptional silencing of *WT1*, we performed a functional analysis of the promoter activity by a dual luciferase assay. This assay was employed to evaluate the influence of promoter methylation on transcription in previous reports (*21–26*), and the methylation by *Sss*I was able to mimic the methylation status of sample no. 6. First, the three reporter vectors, PBV, PBV^{WT1}, and entMe-PBV^{WT1} were transfected (Fig. 2a, see "MATERIALS AND METHODS"). Considerable luciferase activity was detected in PBV^{WT1} transfected cells compared with negative control cells transfected with mock vector (PBV) (Fig. 2b). However, the activity of entMe-PBV^{WT1} was significantly reduced, to the same extent as the mock (unpaired *t*-test, $p < 0.0001$), indicating suppression of promoter activity by the entire methylation. Secondly, three other vectors, lig-PBV, lig-PBV^{WT1}, and insMe-PBV^{WT1}, were used to exclude the effect of ectopic methylation (Fig. 2c, see materials and methods). Considerable activity was also detected in lig-PBV^{WT1} transfected cells, but the activity of insMe-PBV^{WT1} was significantly reduced (unpaired *t*-test, $p = 0.0004$) (Fig. 2d). More than 96 and 85% reduction of luciferase activity were seen in the entMe-PBV^{WT1} and insMe-PBV^{WT1} compared with PBV^{WT1} and lig-PBV^{WT1}, respectively. This result indicated that the methylation of the promoter itself was sufficient for the suppression of gene transcription.

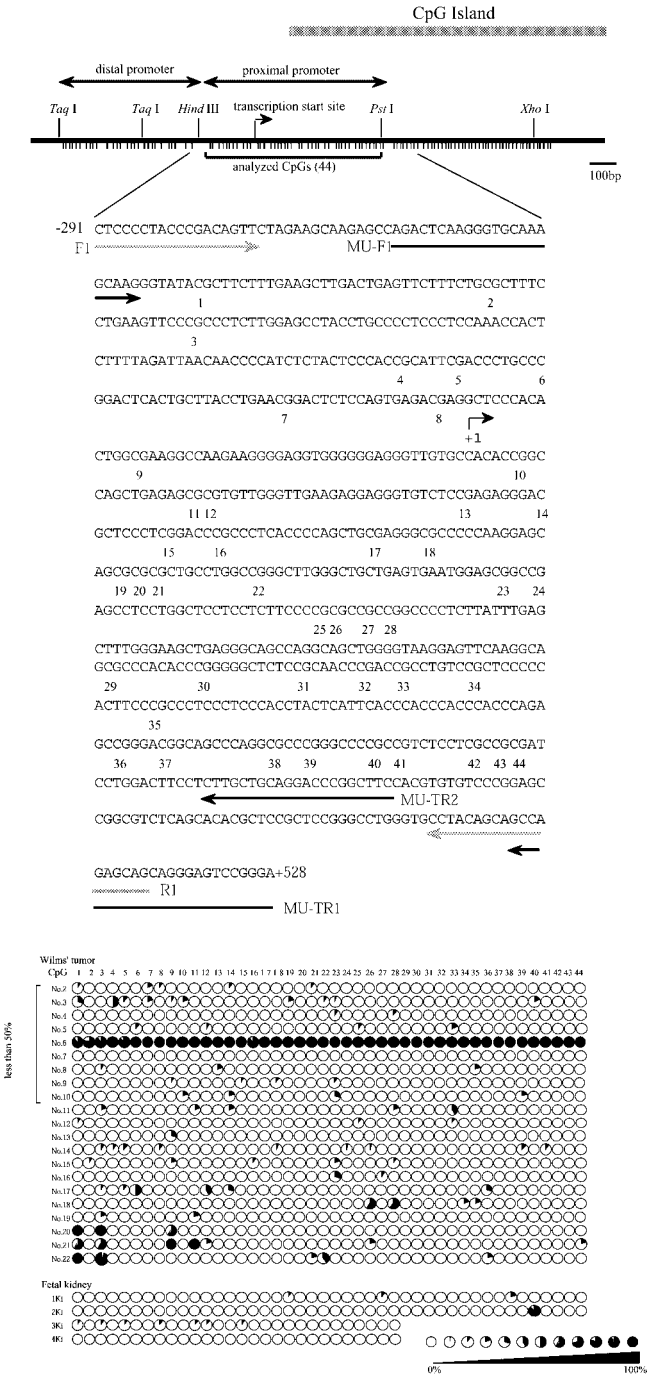

Fig. 1. **DNA methylation analysis of the *WT1* promoter region.** (a) Structure of the *WT1* promoter region and the sequence of the proximal promoter (*17*). Small vertical bars indicate CpG sites. There are 44 CpG sites in the region analyzed, and each CpG is these are numbered in order beneath the nucleotide sequence. The major transcription start site is also marked as +1. Arrows indicate the position of primers used in this study. (b) The result of urea/bisulfite sequencing. *WT1* expression of the tumors no. 2–10 decreased to a level 50% below the level in fetal kidney. The circles represent CpG sites. Each circle graph represents the percentage of methylated clones (number of methylated clones/10 analyzed clones ×100).

β-catenin Mutation in Wilms' Tumors—Among 19 WTs, we found 1 tumor (sample no.17) carrying a missense mutation (ACC to GCC) of the β-*catenin* gene, which replaced threonine with alanine at codon 41. This sample was heterozygous for the mutation and did not carry any *WT1* mutation. The *WT1* expression of the tumor was 157% of fetal kidney.

DISCUSSION

Inactivation of WT1 May Be Due to Epigenetic Alteration in Wilms' Tumor—Genetic and epigenetic analyses were performed on a total of 22 WTs. In 10 out of 19 (53%) tumors, *WT1* expression decreased to a level 50% below the level in fetal kidney, and in 6 of them it was less than 10%. It was noteworthy that one third of WTs showed more than 90% reduction of *WT1* and that the frequency of *WT1* reduction was higher than that of the *WT1* mutation. Although we found some interstitial chromosomal aberrations, such as homozygous deletion and LOH of 11p13 in some tumors, we could not find any point mutation in any exon of the gene. It has been reported that no mutation in the promoter region was found in a total of 39 WTs (*27*). *WT1* has a 5′-enhancer upstream of the promoter, a silencer in the third intron, and a 3′-enhancer downstream of the last exon. Although we did not investigate these elements, none of them would be involved in the silencing of *WT1* in WTs, because none of these elements was effective in cells derived from kidney (*17*, *28*). The evidence, taken as a whole, suggested that the reduction of *WT1* expression was the result of epigenetic alterations, though the regulation of *WT1* transcription was complex (*6*, *29*, *30*).

We found one tumor that showed hypermethylation of *WT1* promoter with significantly reduced expression. This tumor simultaneously possessed LOH. The evidence was consistent with the revised two-hit model described by Jones and Laird (*12*).

Silencing of WT1 Expression by Promoter Methylation—In this study, our luciferase reporter gene assay revealed that the methylation of the promoter itself suppressed *WT1* transcription. Although methylation by *Sss*I methylase may not reflect the situation *in vivo*, this method is the best available to evaluate the influence of promoter methylation on transcription. It has previously been reported that methylation of the luciferase transcription unit or of the luciferase transcription unit and vector sequences others that the promoter reduced the activity significantly, suggesting that methylation of the transcription unit and vector sequences affected the suppression of the gene (*21*, *26*). It was also reported that promoter methylation alone did not significantly suppress transcription using the Rous sarcoma virus long terminal repeat (RSV LTR) promoter. We investigated the effect of methylation of the luciferase transcription unit and vector sequences using a plasmid with *WT1* promoter as the only methylation-free region. We obtained a similar result to that reported earlier, that the luciferase activity was reduced to same level as complete methylation (data not shown). Our study showed, however, that methylation-free *WT1* promoter had considerable transcriptional activity and the expression was significantly reduced with the methylated promoter. This result indi-

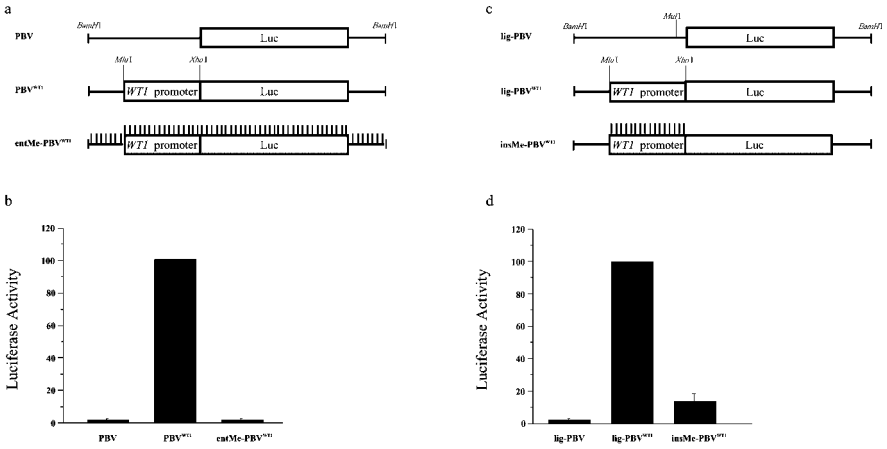

Fig. 2. **Luciferase reporter assay.** (a) Schema of the vector constructs. PBV^{WT1} is a reporter vector containing the *WT1* proximal promoter and is not methylated at all. entMe-PBV^{WT1} is methylated entirely. PBV is a negative control without promoter. The vertical bars show methylated CpG sites but do not indicate the exact CpG position. (b) Relative luciferase activity of the constructs shown in (a). Luciferase activity of PBV^{WT1} was regarded as 100%. entMe-PBV^{WT1} significantly suppressed luciferase activity to the same level as the negative control ($p < 0.0001$). (c) Schema of the constructs for the promoter-specific methylation reporter assay. lig-PBV^{WT1} and insMe-PBV^{WT1} were generated by ligation of the unmethylated and methylated *WT1* promoter with the vector, followed by linearization and gel purification. lig-PBV is a negative control without promoter generated by digestion with *Mlu*I followed by self-ligation. (d) Relative luciferase activity of the constructs shown in (c). Luciferase activity of lig-PBV^{WT1} was regarded as 100%. insMe-PBV^{WT1} significantly suppressed luciferase activity ($p = 0.0004$). All experiments were repeated three times independently.

cates that methylation of *WT1* promoter alone was sufficient to suppress transcription.

Treatment with 5-aza-deoxyC of breast cancer cell line MDA-MB-231 in which the promoter was methylated and *WT1* was silenced, led to the reinitiation of *WT1* expression (*31*), indicating the possibility that promoter methylation was related to the expression of *WT1*. On the other hand, it was reported that methylation was not found in WTs (*32*) and not effective in gene silencing in colorectal or breast tumors (*31*, *33*, *34*). Our data suggested that methylation was an epigenetic mechanism leading to *WT1* gene silencing in kidney-derived cells. Since only one WT with the promoter hypermethylation was found, the deacetylation or methylation of nucleosome histone or some other, unknown epigenetic mechanism might be involved in *WT1* gene silencing in other WTs.

Other WTs showing neither reduced *WT1* expression nor *WT1* genetic aberration should be the result of aberration of other responsible loci, *e.g.*, the WT2 locus at 11p15.5, where the biallelic expression of *IGF2* due to loss of imprinting is frequently seen in WTs; WT3 at 16q; WT4 at 17q12-q21; WT5 at 7p15-p11.2; and others (*35*).

Non-Association of the bCatenin Mutation and Reduction of WT1 Expression—It was reported that the β-*catenin* and *WT1* mutations are frequently associated in WTs (*14*). We found a missense mutation of β-*catenin* in one WT. The mutation gave rise to amino acid substitution, Thr41Ala, which resulted in loss of the functionally important phosphorylation site. This sample was heterozygous for the mutation and did not carry any *WT1* mutation. *WT1* expression was 157% of fetal kidney. In contrast to the *WT1* mutation, the evidence suggested that the β-*catenin* mutation was not associated with *WT1* epigenetic alteration in WTs.

In summary, our data suggest that *WT1* gene silencing in Wilms' tumors may be due to epigenetic alteration and that it occurred more frequently than the *WT1* mutation. The β-*catenin* mutation, however, was not associated with the epigenetic alteration of *WT1*. Although tumor-specific promoter hypermethylation was found in a minority of WTs, methylation was an epigenetic mechanism leading to *WT1* gene silencing, and *WT1* promoter hypermethylation combined with LOH was consistent with the revised two-hit model. Elucidation of the precise genetic and epigenetic regulation of *WT1* expression should help us further in understanding Wilms' tumorigenesis.

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