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Advances in Brief

Identification of Differential Methylation of the WT1 Antisense Regulatory Region and Relaxation of Imprinting in Wilms' Tumor[1]

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Abstract

Wilms' tumor (WT) is associated with loss of heterozygosity at chromosome 11p13, the site of the Wilms' tumor suppressor gene, *WT1*. Although the preferential loss of maternal alleles suggested that differential allelic expression of *WT1* might occur, this has not been evident in normal fetal tissues or WTs. In this study, we show that the *WT1* antisense regulatory region is differentially methylated, with Southern blot analysis of four loss of heterozygosity-negative WTs and their corresponding normal kidneys indicating that allelic methylation is lost in WTs. Reverse transcription-PCR expression analysis correlates methylation with monoallelic expression of the antisense *WT1* transcript (*WT1-AS*) in normal kidney. However, WTs display hypomethylation and biallelic expression of *WT1-AS*. Our findings are consistent with imprinting of *WT1-AS* in normal kidney and the relaxation of imprinting in Wilms' tumorigenesis. This identifies the *WT1* antisense regulatory region in intron 1 as a primary site for epigenetic deregulation at chromosome 11p13 in WTs.

Introduction

Intragenic loss-of-function mutations of tumor suppressor genes are associated with the development of various cancers. One such gene is the WT[3] suppressor gene, *WT1*, which is disrupted by intragenic deletions and mutations in ~20% of WTs, embryonal kidney tumors arising from malignant transformation of renal stem cells. The *WT1* gene encodes up to 16 M_r 52,000–56,000 protein isoforms, generated via alternative mRNA splicing, RNA editing, and non-AUG translational initiation; the proteins act predominantly as transcription factors, regulating the promoters of several genes critical for cell growth, including *IGF2*, *platelet-derived growth factor-A*, *epidermal growth factor receptor*, and *retinoic acid receptor-α*. The WT1(+KTS) isoforms are also thought to be involved in RNA metabolism. The cellular requirement for a diverse and tightly regulated array of WT1 isoforms is reflected in an elaborate multicomponent gene regulatory system, which includes an autoregulatable 5′ promoter, 5′ and 3′ enhancer elements, and an intron 3 silencer (1, 2). Furthermore, our previous work has reported the identification of an antisense *WT1* promoter located in intron 1 that is transactivated by WT1 (3). Antisense *WT1* transcripts (*WT1-AS*) overlapping the 5′ end of the *WT1* gene, with no apparent open reading frames, have been detected in fetal kidney and WTs, suggesting a regulatory role for these RNAs (4, 5). Although WT1 has been shown to be critical for normal renal development (6), the relatively low incidence of *WT1* mutations compared with tumor suppressor genes such as RB, has led to the search for other loci that may be deleted or mutated in WTs. Additionally, other deregulatory mechanisms that may affect candidate loci, such as the loss of genomic imprinting, have been sought. Genomic imprinting is the phenomenon by which maternal or paternal copies of a gene can be selectively expressed, with epigenetic modification of DNA serving as the regulatory signal. The chromosome 11p15 locus serves as a paradigm for genetic and epigenetic alterations associated with WTs. This region harbors a cluster of imprinted genes including *IGF2*, *H19*, and *KvLQT1* and not only shows WT-associated LOH, with the selective loss of maternal alleles but also exhibits loss of genomic imprinting control of *IGF2* and *H19* in WTs (7). The fact that WTs undergoing LOH at 11p13 also preferentially lose the maternal allele and retain the paternal allele (8) suggested that differential allelic expression of *WT1* might also occur. On the contrary, however, equivalent expression of parental alleles was found in normal fetal tissues and WTs (9), with only mosaic and polymorphic imprinting of *WT1* evident in brain and placenta (10). Several lines of evidence have led us and others (11) to postulate that *WT1-AS* may be the candidate imprinted gene on chromosome 11p13: (*a*) part of the *WT1* antisense promoter locus was independently identified as a hypermethylated sequence in human breast cancers (12); (*b*) the discovery of an imprinted antisense RNA for *Igf2r* (13); and (*c*) our demonstration that *WT1-AS* can affect the levels of WT1 protein in an *in vitro* system (14). Our examination of the *WT1* ARR reveals a striking pattern of differential allelic methylation in WTs and their matched normal kidneys. Analysis of allele specificity of expression for *WT1-AS* is consistent with genomic imprinting. Furthermore, whereas normal kidneys show monoallelic *WT1-AS* expression, WTs display biallelic expression, thereby suggesting a relaxation of imprinting in WTs. Our findings identify the *WT1* ARR as a primary site for epigenetic deregulation at chromosome 11p13 in WTs.

Materials and Methods

Tissue Samples and Nucleic Acid Preparation. All tumors were typical WTs unless otherwise indicated, ranging from stage II to stage V, and were obtained snap frozen from the Bristol Children's Hospital, along with adjacent histologically normal kidney. The fetal kidney sample was obtained at 22 weeks gestation. DNAs were extracted by standard phenol/chloroform extraction, and total RNAs were purified using TRI reagent (Sigma Chemical Co.) according to the manufacturer's instructions.

LOH Analysis. LOH was examined using PCR-based tetranucleotide polymorphisms as described previously (15), together with additional polymorphisms in the *WT1* gene (16–18).

Southern Hybridization Analysis. DNAs were digested with an excess of *Kpn*I, *Spe*I, and *BstUI/Bsh1236I* (New England Biolabs and MBI Fermentas). *BstUI* and *Bsh1236I* are isoschizomers recognizing the sequence CGCG, with digestion being blocked by methylation. After transfer from 2% analytical agarose gels onto Hybond-N^+ nylon membranes (Amersham), filters were hybridized overnight with probes radiolabeled with [α^{32}-P]dCTP (3000 Ci/mmol) by random primer extension labeling. For Southern blotting of matched normal kidney and WT DNAs, we used an ARR probe spanning four *BstUI* sites (Fig. 1*a*). The 850-bp probe corresponds to the region directly upstream

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[3] The abbreviations used are: WT, Wilms' tumor; IGF, insulin-like growth factor; LOH, loss of heterozygosity; ARR, antisense regulatory region; RT-PCR, reverse transcription-PCR; CCSK, clear cell sarcoma of the kidney; PNET, primitive neuroectodermal tumor; DMR, differentially methylated region.

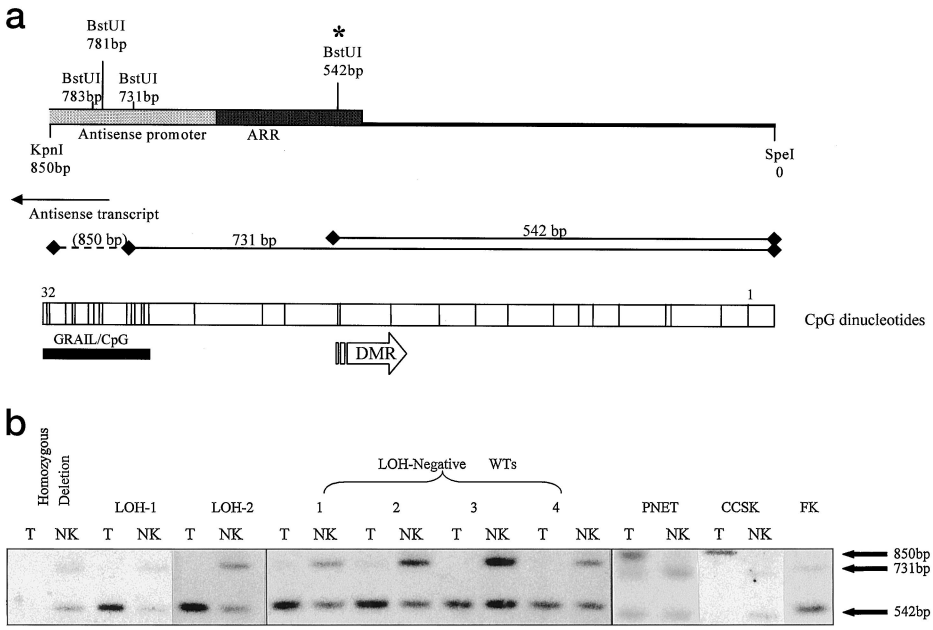

Fig. 1. Differential methylation of the *WT1* ARR. *a*, map of the ARR probe and schematic diagram of major fragments produced when DNA is digested with *Spe*I, *Kpn*I, and *BstUI*. The differentially methylated *BstUI* site is shown with an *asterisk* and marks one edge of the DMR. The distribution of CpG dinucleotides is shown, numbered relative to Table 2. The 3′ end of the CpG island predicted by the GRAIL program is also indicated. *b*, Southern blot analysis using 10 μg of genomic DNAs digested with *Kpn*I, *Spe*I, and *BstUI* and probed with the *WT1* ARR. DNAs are: *T*, WT; *NK*, matched normal kidney; *FK*, fetal kidney. *Arrows*, DNA band sizes according to molecular weight markers.

from the *Kpn*I site located at position −422 in the antisense promoter (Ref. 3; position 1253 in the ARR sequence; GenBank accession number AF233371), extending to the *Spe*I site (position 403 in AF233371). The genomic region between the antisense promoter and the 3′ end of exon 1 is part of a CpG island and was used to assess the possibility of global methylation changes. The insert of plasmid pINE-3 (3) was used for this purpose (nucleotides 1318–2495 in AF233371). Hybridizations were carried out at 65°C in buffer containing 6× SSC, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA (Sigma), and 5× Denhardt's buffer [0.1% each of polyvinylpyrrolidone (PVP-360; Sigma), Ficoll (Pharmacia), and BSA (fraction V; Sigma)]. The filters were washed in 2× SSC, 0.5% SDS at room temperature for 20 min with one change of solution, and then the filters were given three 30-min washes in 0.1× SSC, 0.5% SDS. After washing, the filters were autoradiographed with intensifying screens at −70°C.

Bisulfite Sequence Analysis. Bisulfite analysis used a modification of the protocol of Olek *et al*. (19). Briefly, ~700 ng of genomic DNAs were digested with *Sac*I, boiled for 5 min, quickly chilled, and subsequently incubated in 0.3 M NaOH for 15 min at 50°C. This sample was mixed with 2 volumes of molten 2% NuSieve agarose (SeaKem). Ten-μl aliquots of the agarose/DNA mixture containing ≤100 ng of DNA were pipetted directly into 750 μl of chilled paraffin liquid overlaying 1 ml of a 2.5 M sodium metabisulfite (BDH) solution (pH 5), thereby forming agarose beads. These were incubated for 3.5 h at 50°C in the aqueous phase, after which treatments were stopped by equilibrating the beads against 1 ml of TE (pH 8; 4 × 15 min), followed by desulfonation in 500 μl of 0.2 M NaOH (2 × 15 min). The reactions were neutralized by washing with TE (pH 8; 3 × 10 min). Beads were either used directly for PCR or stored for up to 4 weeks at 4°C. Prior to amplification, beads were washed with H_2O (2 × 15 min). All reactions were subjected to two rounds of amplifications using a seminested primer approach, and the 5′ and 3′ halves of the ARR were separately amplified from bisulfite-modified DNA. Primer sequences, with nucleotide positions referring to GenBank entry AF233371, were as follows. Top-strand primers and nucleotide positions were: TF, 5′-GGGTGGAGAAGAAGGATATATTTAT-3′, 371–395; TFN, 5′-GATATATTTATTTATTAGTTTTGGT-3′, 385–409; TMR, 5′-CACCTTTATTTAACAAACTAATATTAC-3′, 940–914; TR, 5′-AAACCCCTATAATTTACCCTCTTC-3′, 1463–1440; TRN, 5′-CTATTAAAAACCTAAACCAATT-3′, 1415–1394; and TMF, 5′-GGGATATTGAGATTTAGAAATTTTT-3′, 884–908. Bottom-strand primers and nucleotide positions were: BF1, 5′-CATTCACCTACTAATCTAATCCC-3′, 389–413; BFN, 5′-CCTACTAACTACAAAACCC-3′, 487–505; BMR, 5′-TTAGTAGGTTGGTGTTATTTTTGGG-3′, 931–907; BR2, 5′-TTTAGTTAGGATATAAGGAGGGAAT-3′, 1580–1556; BRN, 5′-TTATTGAGAATTTAAGTTAGTT-3′, 1415–1394; and BMF, 5′-CAAAAATAACACCAACCTACTAAACAAA-3′, 908–935.

PCR was performed using 1 unit of SuperTaq (HT Biotechnology) in the manufacturer's buffer in a Hybaid PCR Express thermal cycler. Round 1 cycle parameters were as follows: 94°C for 3 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 90 s, and then 72°C for 5 min. For round 2, between 1 and 10 μl of round 1 product was used as a template, and cycle parameters were as for round 1, except 35 cycles were performed (for the primer pair BRN-BMF, an annealing temperature of 52°C was used). PCR products were isolated from 1.5% NuSieve agarose gels and cloned into pGEM T-Easy (Promega) according to manufacturer's protocol. Automated DNA sequencing was provided by the Department of Biological Sciences DNA sequencing service (Durham University, United Kingdom), and data were compiled from independent clones with ≥99% conversion (assessed on the basis of non-CpG cytosines).

RT-PCR. The reverse primer was annealed to 1 μg of total RNA by heating to 60°C for 5 min and then quenched on ice. Reverse transcription was carried out with Super RT (HT Biotechnologies, Cambridge, United Kingdom) reverse transcriptase at 50°C for 60 min, followed by PCR cycling as follows: 95°C 3 min (1 cycle); 94°C 15 s, 60°C 30 s, 72°C 60 s (2 cycles); 94°C 15 s, 58°C 30 s, 72°C 60 s (2 cycles); 94°C 15 s, 56°C 30 s, 72°C 60 s (10 cycles, 20 for antisense product); 94°C 15 s, 56°C 30 s, 72°C 60 s with 20 s extension per cycle (20 cycles). PCR products were digested by adding the appropriate restriction enzyme directly to the PCR mix and incubating for 60 min at 37°C. Products were separated on 1% agarose/1% NuSieve gels and then alkali blotted onto Hybond N^+ membrane and hybridized with ^{32}P-labeled antisense cDNA probe generated using the primers given below. Primers either side of the antisense *WT1* RNA splice (20) were used for RT-PCR: WT18, CTTAGCACTTTCTTCTTGGC]; and WITKBF2, TTGCTCAGTGATTGACCAGG; primers used for DNA controls were WITKBF2 and WITKBR2, TTGGCTGGAAAGCTTGCAGC. The *Mnl*I polymorphism (16) used is marked by an asterisk in Fig. 2*a*. On digestion of the RT-PCR products, biallelic expression is indicated by bands of 286 bp (*Mnl*I undigested) and 222 bp (*Mnl*I digested). Alternatively major allelic bands of 286 or 222 bp are evident in the case of monoallelic expression.

Results and Discussion

Our previous work has characterized *WT1* regulatory elements including the 5′ promoter and the antisense promoter, with a view to establishing whether quantitative deregulation of *WT1* expression may arise through mutations within these regions. However, we have detected no mutations in either promoter.[4] As outlined above, there

[4] K. Malik and A, Salpekar, unpublished data.

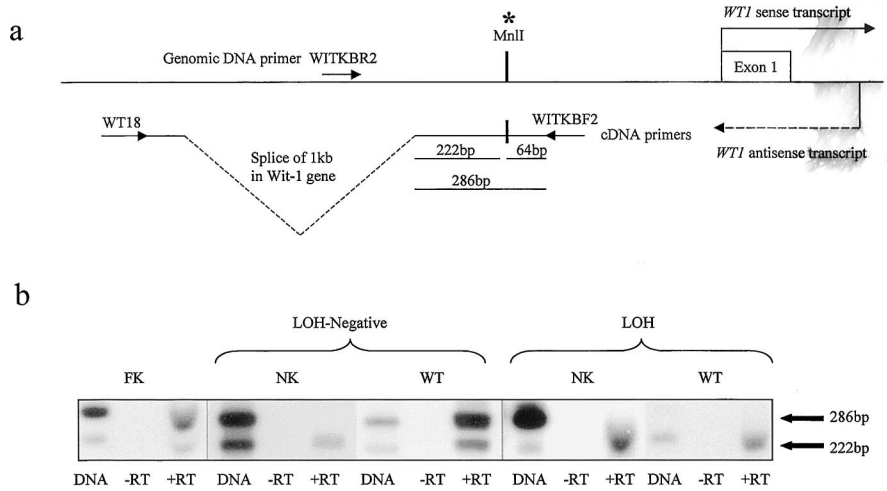

Fig. 2. Allele-specific expression of antisense *WT1* RNA. *a*, schematic diagram showing the primers either side of the antisense *WT1* RNA splice used for RT-PCR. *, *Mnl*I polymorphism (16) used to distinguish between alleles. *b*, RT-PCR of DNA and RNA from fetal kidney (*FK*), WTs, and matched normal kidney (*NK*). *−RT*, minus reverse transcriptase control; *+RT*, plus reverse transcriptase. Biallelic expression is indicated by bands of 286 bp (*Mnl*I undigested) and 222 bp (*Mnl*I digested), whereas only one band can be produced in the case of monoallelic expression

are several reasons to presuppose that epigenetic changes may also take place at the *WT1* locus on chromosome 11p13, and we therefore examined WTs with and without LOH at chromosome 11p13 for altered methylation in the *WT1* ARR (Table 1). Our analysis focused on a region independently demonstrated as hypermethylated in breast cancer (12) and abutting a CpG island (as predicted by the GRAIL/CpG algorithm). This region is delimited by *Kpn*I and *Spe*I sites and contains 32 CpG dinucleotide residues. Digestion of genomic DNAs with these enzymes plus the methylation-sensitive enzyme *BstUI* yields a variety of bands depending on the methylation status of the *BstUI* sites, because the enzyme only cuts when its recognition site (CGCG) is unmethylated (Fig. 1*a*).

As shown in Fig. 1*b*, normal kidney DNA samples display a 542-bp band and a 731-bp band, whereas WT samples show the smaller band only, irrespective of their LOH status. This indicates differential methylation of the *BstUI* site at 542 bp in normal kidney and hypomethylation of this site in tumors without LOH at 11p13. WTs undergoing LOH at 11p13 have been shown to preferentially lose the maternal allele (8). Therefore, the comparison of normal kidney DNA with LOH-positive WT DNAs strongly suggest that the 731-bp band corresponds to the maternal allele (distinguishable from the paternal allele at 542 bp because of methylation of the *BstUI* site, marked with an asterisk in Fig. 1*a*). To date, we have found hypomethylation in 8 of 10 LOH-negative WTs, but in contrast, a CCSK and a PNET did not exhibit hypomethylation of both alleles; instead, they exhibited a degree of hypermethylation (Fig. 1*b*). No differences in banding were seen between any samples when the CpG island probe adjacent to the ARR was used on the same Southern blots (data not shown). These data strongly suggest that the hypomethylation of the ARR is specific to WTs and is not a consequence of global epigenetic modifications.

We assessed the regional methylation pattern directly by sequencing bisulfite-modified DNA. This analysis distinguishes between methylated and unmethylated CpG residues by selectively converting cytosine to uracil in unmethylated CpGs, with methylated cytosines remaining unconverted. By subsequent PCR and sequencing of modified DNAs, we have established the pattern of methylation in the normal kidney and WT of LOH-negative sample 3 (Fig. 1*b* and Table 2). In normal kidney, a pattern indicative of one methylated allele and one unmethylated allele is observed, with ~50% methylation apparent at CpG islands 1–13. Tumor DNA, however, displays generalized hypomethylation of CpG dinucleotides (Table 2). The data presented also show that the distinguishing *BstUI* site used for Southern blot analysis demarcates the start of a DMR and lies within a region that we have suggested may contain negative regulatory elements (3). Although the role of such negative regulatory elements remains to be determined, the absence of methylation changes in the core promoter region (3) underlines their possible importance.

The allele-specific methylation pattern observed in normal kidney strongly suggests genomic imprinting of the *WT1* ARR and tumor-specific relaxation of imprinting in WTs. We therefore assessed allele specificity of expression using RT-PCR (Fig. 2*a*). As shown in Fig. 2*b*, normal kidney samples that matched both LOH-negative and LOH-positive WTs displayed monoallelic expression. However, the LOH-negative tumor sample displayed biallelic antisense *WT1* RNA expression, whereas the LOH-positive WT expressed only the paternal allele, as expected after maternal allele loss. Together with the identification of the *WT1* ARR DMR, these results are consistent with imprinting of *WT1-AS*, although formal evidence of genomic imprinting requires assessment of germ-line cells in an animal model. Furthermore, our data suggest that relaxation of *WT1-AS* imprinting is a frequent event in Wilms' tumorigenesis.

As reported previously, normal kidney, WTs, and fetal kidney all showed biallelic expression of the *WT1* sense transcript (Ref. 9 and data not shown). Although very little is known about the cellular functions of the recently reported imprinted antisense transcripts for *Igf2r* (13) and *KvLQT1* (21, 22), they have been postulated to act as allelic silencers, because sense and antisense coexpression from the same allele is not observed. Our observations suggest that *WT1-AS* transcription does not preclude sense mRNA expression in normal kidney or WTs, indicating an alternative role for *WT1-AS* in *WT1* regulation.

Table 1 *LOH data for tumor samples*

Tumor samples were assessed for LOH by PCR using previously reported polymorphic markers (15–18).

	11p13				11p15	
	WT1 5′ *MnlI*[a]	WT1 Exon 1 *Acc*II[a]	WT1 Exon 10 CA[b]	D11S1392 TET[b]	D11S1999 TET[b]	TH TET[b]
Homozygous deletion	D[c]	D	D	+	−	−
LOH 1	NI	NI	NI	+	+	+
LOH 2	NI	NI	NI	+		+
Non-LOH 1	−	−	−	NI		−
Non-LOH 2	NI		NI	−		−
Non-LOH 3	−	−	−	−	NI	NI
Non-LOH 4	NI	NI	NI	−		−
PNET		NI	−	−	−	−
CCSK		NI		−		−

[a] *Mnl*I and *Acc*II, polymorphisms detectable by restriction digests.
[b] CA, CA repeat; TET, tetranucleotide repeat.
[c] D, deleted; NI, noninformative; +, LOH, −, no LOH.

Table 2 *Demarcation of the DMR by bisulfite sequencing analysis: LOH-negative sample 3*

The methylation patterns across the antisense promoter and regulatory region were assessed in normal kidney and WT DNAs corresponding to LOH-negative sample 3 (see Fig. 1*b*). Independent clones A–J are shown on the vertical axis, and CpG dinucleotides assessed are numbered on the horizontal axis. The CpGs are oriented relative to Fig. 1*a* by the *Kpn*I and *Spe*I sites. Methylated CpGs are indicated by + and unmethylated by −.

	CpG probe							(*Kpn*I) ARR probe																														
	39	38	37	36	35	34	33	32	31	30	29	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14[a]	13[a]	12	11	10	9	8	7	6	5	4	3	2	1
Normal kidney																																							
A	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	/[b]
E	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	/[b]
F	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	+	−	−	+	+	+	+	+
G	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	+	+	+	+	+	+	+	+
H	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+	+
I	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+	+
J	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
WT																																							
A	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	/[b]
E	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	/[b]
F	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	/[b]
G	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	/[b]
H	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	/[b]
I	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	/[b]
J	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+

[a] Differentially methylated *BstUI* site (shown with an asterisk in Fig. 1*a*).
[b] The CpG was unassessable because of the bisulfite analysis primer used.

One of the vital questions arising from our study is how the epigenetic modifications of the ARR may be involved in directing a cell toward cancerous growth. Examination of differential allelic methylation in fetal kidney DNA (Fig. 1*b*) reveals a prevalence of hypomethylation (increased intensity of the 542-bp band) and a lesser degree of hypermethylation (731-bp band). This was also evident in three other fetal kidney samples, with densitometric analysis indicating a 731-bp band:542-bp band ratio of 0.19 (± 0.04; $n = 4$; data not shown). As predicted by this epigenotype, biallelic expression of the *WT1-AS* was detectable in fetal kidney (Fig. 2*b*). Normal kidneys display a 731-bp band:542-bp band ratio of 0.96 (± 0.29; $n = 9$). Comparison of differential methylation and allele-specific expression in fetal kidney with that in normal kidney and WTs suggests that tumor cells fail to either acquire or maintain the methylation imprint prerequisite for normal nephrogenesis, depending on which epigenotypic subset of fetal kidney cells progress along the tumorigenic pathway. We therefore prefer the term relaxation of imprinting, rather than loss of imprinting. It will clearly be of great interest to examine the methylation and imprinting status of premalignant lesions such as nephrogenic rests.

Low WT1 protein levels are observed in normal kidney, contrasting with high levels in fetal kidney and WTs (1, 2), indicating that imprinting of the ARR may represent a genetic switch controlling *WT1* expression such that hypermethylation correlates with low protein and hypomethylation with high protein. In support of this, we have observed previously that altering *WT1-AS* levels can, surprisingly, lead to increased WT1 protein *in vitro* (14). Although the mechanism by which this can occur remains to be elucidated, it is possible to envisage a situation *in vivo* whereby expression of *WT1-AS* may stabilize the sense transcript. Such a mechanism may facilitate the rapid increase and attenuation of WT1 levels that parallel the progression of metanephric mesenchymal cells toward an immature epithelial cell phenotype, and the subsequent maturation of epithelial cells (1, 2).

Although the elevated levels of WT1 in WTs are thought to reflect the arrested differentiation status of tumor cells (1, 2), our work suggests an intriguing functional equivalence between relaxation of imprinting and LOH at the *WT1* locus. Because LOH usually arises from mitotic recombination and is accompanied by duplication of the paternal copy (1), the net effect of both genetic and epigenetic defects would be increased antisense RNA from the paternal allele, a situation analogous to the deregulation of *IGF2* in Beckwith-Wiedemann syndrome and WTs (1, 7). Thus, if relaxation of imprinting can result in increased *WT1* gene expression via altered levels of *WT1-AS*, the LOH "second hit" required for Wilms' tumorigenesis may, paradoxically, also lead to overexpression of *WT1*. Given the potential interaction of WT1 with downstream target genes, it can be envisaged that the loss or alteration of mechanisms controlling *WT1* gene expression could have pleiotropic deleterious effects on regulated cellular growth and differentiation. Quantitative perturbations may, therefore, be alternative or additive to qualitative defects such as deletions and mutations when assessing the role of *WT1* dysfunction in tumorigenesis, and *WT1*/*WT1-AS* may be considered as potentially oncogenic when inappropriately expressed. In this regard, we note with interest that ectopic expression of *WT1* can increase the tumorigenic potential of adenovirus-transformed baby rat kidney cells (23), and that elevated *WT1* expression has also been observed in other neoplasias, including acute leukemias and malignant mesotheliomas (1, 2). Furthermore, *WT1* expression has been shown to be altered in breast cancers (24). Our characterization of ARR imprinting identifies a switch that, when deregulated, may contribute to the development of WTs and other cancers.

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