Genomic imprinting at the \textit{WT1} gene involves a novel coding transcript (\textit{AWT1}) that shows deregulation in Wilms’ tumours

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The Wilms’ tumour suppressor gene, \textit{WT1}, is mutated in 10–15% of Wilms’ tumours and encodes zinc-finger proteins with diverse cellular functions critical for nephrogenesis, genitourinary development, haematopoiesis and sex determination. Here we report that a novel alternative \textit{WT1} transcript, \textit{AWT1}, is co-expressed with \textit{WT1} in renal and haematopoietic cells. \textit{AWT1} maintains \textit{WT1} exonic structure between exons 2 and 10, but deploys a new 5′-exon located in intron 1 of \textit{WT1}. The \textit{AWT1} gene predicts proteins of approximately 33 kDa, comprising all exon 5 and exon 9 splicing variants previously characterized for \textit{WT1}. Although \textit{WT1} is not genomically imprinted in kidney, we have previously shown monoallelic expression of a \textit{WT1} antisense transcript (\textit{WT1-AS}) that is consistent with genomic imprinting. Here we demonstrate that both \textit{WT1-AS} and the novel \textit{AWT1} transcript are imprinted in normal kidney with expression confined to the paternal allele. Wilms’ tumours display biallelic \textit{AWT1} expression, indicating relaxation of imprinting of \textit{AWT1} in a subset of WTs. Our findings define human chromosome 11p13 as a new imprinted locus, and also suggest a possible molecular basis for the strong bias of paternal allele mutations and variable penetrance observed in syndromes with inherited \textit{WT1} mutations.

\textbf{INTRODUCTION}

The Wilms’ tumour 1 gene (\textit{WT1}) encodes a multifunctional zinc-finger protein with diverse cellular functions critical for normal nephrogenesis, haematopoiesis, and sex-determination. Disruption of the gene in WAGR syndrome patients (Wilms’ tumour, aniridia, genitourinary abnormalities, mental retardation) and in sporadic Wilms’ tumours indicated that \textit{WT1} is the Wilms tumour (WT) suppressor gene, and \textit{WT1} mutations have subsequently also been shown to be involved in leukaemogenesis and developmental abnormalities such as Denys–Drash syndrome (DDS) and Frasier syndrome. During kidney development, \textit{WT1} expression is stringently regulated, displaying low expression in the condensing mesenchyme which increases as cells progress towards an epithelial phenotype (1).

An absolute developmental requirement for \textit{WT1} is emphasized by the demonstration of embryonic lethality in \textit{wt1} null mice (2). In humans, the \textit{WT1} gene is located on chromosome 11p13 and consists of 10 exons. As well as four major protein variants produced via combinatorial splicing at exons 5 and 9 (1), additional \textit{WT1} proteins also arise from alternative translational initiation sites. In addition to the primary translational initiation site which generates proteins of 52–54 kDa, upstream (3) and downstream (4) translational initiation sites have also been identified, generating proteins of ~62 and 36 kDa, respectively. Along with RNA editing, 24 \textit{WT1} isoforms may be generated (1). These possess transcription factor activity with amino-acids 180–294 associated with transcriptional activation and amino-acids 84–179 with repression (5). \textit{WT1} isoforms with three additional exon 9-encoded amino acids lysine, threonine and serine (+KTS) are also involved in RNA metabolism (6).

Although mutations and deletions of \textit{WT1} conform to Knudson’s two-hit model, they are only found in 10–15% of Wilms’ tumours. This has prompted investigation of other loci and the involvement of epigenetic lesions in Wilms’ tumourigenesis. The best characterized epigenetic lesion is imprinting...
changes at chromosome 11p15, especially those associated with the IGF2–H19 locus (7). In the case of WT1, mosaic and polymorphic imprinting has been demonstrated in brain and placenta (8). Importantly, although the selective loss of the maternal allele in WTs undergoing LOH indicated the presence of an imprinted gene at 11p13, WT1 is not imprinted in kidney (9).

In a recent study, we demonstrated monoallelic expression of an antisense RNA, WT1-AS, from the WT1 locus, suggesting, for the first time, imprinting at chromosome 11p13. WT1 is not imprinted in kidney (9).

In a recent study, we demonstrated monoallelic expression of an antisense RNA, WT1-AS, from the WT1 locus, suggesting, for the first time, imprinting at chromosome 11p13 in human kidney (10). The antisense transcript was also shown to be biallelically expressed in WTs, with altered expression being associated with loss of differential methylation at an antisense regulatory region (WT1 ARR) in the first intron of the WT1 gene. The WT1 ARR comprises the WT1-AS promoter and cis-acting regulatory elements (11) and the imprinted WT1-AS transcript is a putative regulator of WT1 (12). In this study, we prove that WT1-AS is functionally imprinted in normal kidney by proving the parent-of-origin dependence of (a) differential methylation and (b) allele-specific expression. Expression is restricted to the hypomethylated paternal allele. Using methylation-sensitive Southern blot analyses of the WT1 locus, we show that differential allelic methylation and its loss in WTs are largely restricted to the WT1 ARR. Interestingly, we also observed that apart from the WT1 promoter, only one other CPG island (out of five examined) was consistently hypomethylated in normal kidney and WT DNAs. As regions of this CPG island (CPG1) located in intron 1 showed extensive conservation with the murine wt1 locus (68.5% over 815 nt) and northern blotting had previously demonstrated the presence of a shorter WT1 transcript expressed in testis (13), we assessed whether alternative WT1 isoforms may arise from within intron 1. Here we provide evidence that an alternative WT1 transcript, which we refer to as AWT1, is expressed in parallel with WT1 in cell lines, kidney and WTs. Importantly, unlike WT1, AWT1 is subject to genomic imprinting, with AWT1 expression restricted to the paternal allele.

RESULTS

The parental origin of WT1-AS expression and the hypomethylated WT1 ARR allele

The 5’-end of the WT1 gene is illustrated in Figure 1A, together with the position of the WT1-AS transcript analysed. Figure 1B shows the WT1 ARR (GenBank accession no. S79781), together with enzymes employed for methylation-sensitive Southern blots. The differentially methylated Bsh1236I site which produces either 731 or 542 bp bands diagnostic for methylation and hypomethylation, respectively, is asterisked.
Normal tissues show differential methylation of the ARR, with equally intense hypo- (542 bp) and hypermethylated (731 bp) bands (10).

To demonstrate unequivocally the parent-of-origin dependence of WT1 ARR methylation we used a panel of Beckwith–Wiedemann syndrome (BWS) genomic DNAs. Some of these BWS patients have uniparental disomy (UPD) of the short arm of chromosome 11, including the 11p13 region (14,15). As shown in Figure 2A, BWS samples with paternal UPD have an increased intensity of the hypomethylated allele (542 bp band, Fig. 2A lanes 3–5) in comparison to non-UPD samples (Fig. 2A, lanes 1 and 2). The predominance of hypomethylated alleles in UPD indicates that the hypomethylated allele must be paternally derived. The presence of some hypermethylated alleles is expected due to the mosaicism of UPD in the BWS samples as observed for loci at chromosome 11p15 (14,15).

To determine the parental origin of the expressed WT1-AS allele, lymphoblastoid cell lines (LCLs) were prepared from an individual and family heterozygous for an MnlI polymorphism in the WT1-AS transcript. As shown in Figure 2B (DNA lanes), the patient inherited allele A1 (286 bp) from his mother (M) and allele A2 (222 bp) from his father (F). RT–PCR of the patient’s LCL RNA shows that, despite having a genomic copy of each allele, only A2 is expressed, demonstrating the sole expression of the paternal allele. These results are consistent with genomic imprinting of WT1-AS in humans.

### Table 1. Location of WT1 CpG islands and their respective probes

<table>
<thead>
<tr>
<th>Region of WT1 gene</th>
<th>GRAIL CpG island</th>
<th>Probe name and location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upstream WT1</td>
<td>−3948 to −3529</td>
<td>CGPGO (−4424 to −2518)</td>
</tr>
<tr>
<td>WT1 promoter/exon 1</td>
<td>+44 to +1701</td>
<td>CGPGE1 (−513 to +802)</td>
</tr>
<tr>
<td>Intron 1 WT1 ARR</td>
<td>+1 to +833</td>
<td>WT1 ARR (+2043 to +2893)</td>
</tr>
<tr>
<td>Intron 1</td>
<td>+6771 to +7014</td>
<td>CGP25 (−5938 to +6541)</td>
</tr>
<tr>
<td>Intron 1/exon 2</td>
<td>+6911 to +7040</td>
<td></td>
</tr>
<tr>
<td>WT1 exon 3</td>
<td>+7637 to +7575</td>
<td></td>
</tr>
<tr>
<td>WT1 exon 4</td>
<td>+7904 to +8490</td>
<td>CGP23 (−7476 to +8465)</td>
</tr>
</tbody>
</table>

### Methylation changes at the WT1 locus

In order to ascertain whether loss of methylation in WT1 was restricted to the WT1 ARR, we carried out a survey of DNA methylation across the WT1 locus. Computational analysis of 100 kb of genomic sequence spanning the WT1 gene predicted five further GRAIL/CpG islands located across the 5′ end of WT1 (Table 1 and Fig. 3A). Southern analysis of fetal kidney and paired non-loss of 11p13 heterozygosity normal kidney and WT samples was carried out [Fig. 3B(i)–(vi)]. The matched tissues NK84 and T84 represent a WT subset that does not display tumour-specific hypomethylation and concomitant relaxation of imprinting of WT1-AS, whereas matched samples 69 and 72 are from patients exhibiting biallelic WT1-AS expression (data not shown).

Southern analysis reveals that the WT1 ARR produces restriction patterns conformation to allelic differential methylation in normal kidney samples [normal kidneys 84, 69 and 72 in Fig. 3B(iii)]. Regions CPG0, CPG25′ and CPG23′ also show some evidence of methylation changes, but in these cases, the intensity of hypo- compared with hypermethylated bands does not appear to correspond to clear allelic ratios. For example in Figure 3B(v) (CPG25′), the hypomethylated 1080 bp band in NK84 is clearly less intense relative to the lower, hypomethylated band. This data suggests that the WT1 ARR is a key epiregulatory element, although other CPG-rich regions at the WT1 locus may also be involved in imprinting regulation. Interestingly, only probes CGPGE1, which spans the WT1 promoter, and CPG1 gave results consistent with complete hypomethylation [Fig. 3B(i) and (iv)]. The CPG1 region showed high conservation with murine sequences in the same region (~68.5% over 815 nt). As our methylation analysis suggested that CPG1 was probably not involved in epigenetic regulation, we speculated that it may represent an alternative WT1 exon and/or promoter element, especially as shorter transcripts (13) and smaller WT1 proteins (4,16) have been described previously.

### Expression of a novel alternative WT1 transcript, AW1

Database analysis revealed an expressed sequence tag with 5′-homology to CPG1 in a testis cDNA library (GenBank accession no. BC032861). This clone retained exons 2–10 of the WT1 gene, but had an alternative 5′ end in the first intron of WT1 (Fig. 4A). As testis cDNA libraries can contain aberrant
Figure 3. Location and methylation status of CpG islands at the WT1 locus. (A) Five further CpG islands located at the 5’ end of WT1 were analysed using Southern blotting with methylation-sensitive restriction enzymes. The arrows located above and below the horizontal line represent the WT1 and WT1-AS transcriptional start sites respectively, and sense WT1 exons 1–3 are shown joined by the broken line. The CpG-rich regions are shown by filled rectangles and the bars below represent corresponding probes. (B) Southern analyses with methylation-sensitive restriction enzymes. Panels (i)–(vi) show methylation patterns of fetal kidney (FK) and matched normal kidney (NK) and Wilms’ tumour (T) DNAs with CpG island probes. To the right of each panel is a summarising diagram (thick horizontal line) of the methylation at each site of individual CpG loci in NK samples (open square, flanking restriction enzyme; open circle, unmethylated site; grey circle, partial methylation; black/white split circle, differential methylation). The line diagrams below the summary explain the origin of the hybridization patterns observed for each probe; dashed lines represent fragments not detectable in this analysis due to their small size or the probe location.
Figure 4. Location of AWT1 relative to WTI. (A) Schematic of the 5'-end organization of the WTI gene. Numbering commences at the major WTI TSS previously identified (18). A small splice occurring within exon 1a is also indicated. (B) DNA sequence of the AWT1 first exon (exon 1a) and putative promoter. Exonic sequences are bold and upper case, primers used in RT–PCR analyses are shown by arrows, TSSs are underlined and the respective ATG start codons of WTI and AWT1 are indicated by underlining. The AWT1 RPA probe is enclosed by brackets. The sequences limiting the 5'- and 3'-end of the 56F promoter construct are italicized (nucleotides 4105–4110, and 4686–4691). Consensus binding sites highly conserved in human and murine sequences for MZF-1, PRX2 and OCT-1 and PAX-8 transcription factors are indicated by boxes. (C) A comparison of human and mouse sequences at exon 1a.

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transcripts with scrambled exonic structures, we verified the expression of this alternative WT1 RNA in human kidney. Using forward primers in this region (CPG-S3 and CPG-S4; Fig. 4B) and a reverse primer in exon 10, we cloned cDNAs from human fetal kidney RNA using RT-PCR. Sequencing analysis identified cDNA clones originating within CPG1 extending through to the terminal exon 10, with exon 5 and 9 splicing patterns between AWT1 and WT1 conserved. Alternative splicing of exons 5 and 9 as demonstrated for AWT1, we assessed its conservation in mouse by using RT-PCR to clone cDNAs from mouse kidney. The alternative isoform cDNA (pcDNA-AWT1; Fig. 5). High levels of expression was apparent in renal (293 and 7.92) and haematopoietic cell lines (K562), and high levels of expression were observed in fetal kidney and a subset of WTs. At least three possible transcriptional start sites (TSSs) for AWT1 are seen and are labelled ‘U’. RNAs were also assessed for loading with an 18S rRNA probe (bottom panel). The AWT1 gel is overexposed relative to the WT1 gel to show low level expression. Sizes of protected fragments and the 100 bp DNA marker ladder are shown in bps.

Expression of AWT1 relative to WT1 was assessed by ribonuclease protection assay (RPA), which also provided non-PCR based evidence to confirm the AWT1 transcript. All samples that expressed WT1 also expressed AWT1, suggesting coordinate regulation and function of the proteins (Fig. 6). AWT1 expression was apparent in renal (293 and 7.92) and haematopoietic cell lines (K562), and high levels of expression were observed in fetal kidney and a subset of WTs. At least three possible transcriptional start sites (TSSs) for AWT1 are suggested by protected fragments of 110, 147 and 272 bp. The two former bands correspond closely to the 5′-UTR, the AWT1 mRNA is approximately 2.5 kb, encoding a protein of about 33 kDa. To further confirm the physiological relevance of AWT1, we assessed its conservation in mouse by using RT–PCR to clone awt1 from mouse kidney. The alternative exon 1a is conserved and utilized in mice, and Figure 4C shows the sequence alignment of human and murine 5′-AWT1 sequences.

We evaluated the coding capacity of AWT1 by transfection of 293 cells with an expression construct containing the (+/-) isoform cDNA (pcDNA-AWT1; Fig. 5). High levels of a 33 kDa protein were detected by western blot analysis with an antibody recognizing the carboxyl terminus of WT1. We also transfected 293 cells with pCMV-WT1(+-) to overexpress the full-length WT1(+-) cDNA for comparison. In addition to high expression of protein at ~50 kDa, an additional band of approximately 36 kDa was detected. This band is likely to represent alternative translational initiation at methionine 127 as previously demonstrated (4). Comparison with protein lysates from 7.92 and K562 cells indicate that both types of amino-truncated proteins (i.e. AWT1 proteins and methionine 127-initiated proteins) are expressed. However, unequivocal designation of protein identities is not possible due to the range of similarly sized proteins that might be generated by alternative splicing events. For example, the methionine 127-initiated (-/-) protein isoform would only differ in size from the AWT1 (+/-) isoform by one amino acid.
luciferase plasmids in 293 cells. As shown in Figure 7, orientation specific promoter activity is identified in constructs 400F and 56F. We note the presence of a consensus PAX8 binding site (Fig. 4B) in the putative AWT1 promoter, which has been shown to regulate the WT1 promoter (19), further supporting the possible coordinate regulation of WT1 and AWT1.

Genomic imprinting of AWT1 in human kidney

Patient 84 showed no methylation changes of the WT1 ARR [Fig. 3(iii)] and very low levels of AWT1 and WT1 (Fig. 6). The possible correlation of gene expression with epigenetic status, and the identification of a putative AWT1 promoter proximal to the WT1 ARR in intron 1 prompted us to investigate whether the AWT1 transcript may, like WT1-AS, also be subject to epigenetic regulation. We conducted RT–PCR analysis on WTs with and without LOH at 11p13 (Fig. 8A). Tumours with LOH continued to express AWT1. However, examination of matched normal and non-LOH tumour tissue informative for an AflIII polymorphism in exon 7 revealed that expression of AWT1 in normal kidney was monoallelic, whereas matched tumours expressed AWT1 from both alleles. As LOH in WTs has been demonstrated to involve the maternal allele (20), these results are consistent with genomic imprinting of AWT1 in normal kidney, with only the paternal allele expressed. Importantly, imprinting is relaxed in WTs, with high level expression being apparent from both alleles. Fetal kidney also expresses AWT1 biallelically. WT1 (exons 1–10) was expressed biallelically in all samples examined (data not shown).

In order to determine whether monoallelic expression reflects true genomic imprinting, we assessed the parental origin of the expressed allele (Fig. 8B). Allelic expression of WT1 and AWT1 was analysed using the polymorphic GT repeat in exon 10 in the normal kidney RNA of patient 21 and compared to the paternal allelotype. Three major alleles were detected in the DNA of the patient and father. Patient 21 is heterozygous (A1, A3) with A1 being paternal. In NK RNA both alleles A1 (paternal) and A3 (maternal) of WT1 are expressed, but only the paternal allele is expressed in the normal kidney of patient 21. In addition to the three normal kidneys with monoallelic AWT1 expression shown in Figure 7, two further patients also showed monoallelic expression in kidney (data not shown), strongly suggesting that AWT1 imprinting is not polymorphic. Thus, AWT1 is a novel, paternally expressed imprinted gene, and imprinting is relaxed in Wilms’ tumourigenesis.

DISCUSSION

Although the WT1 gene was identified over a decade ago, many aspects of its function and roles in development and tumourigenesis remain obscure. One reason for this is that the WT1 gene encodes numerous protein isoforms which are closely related in terms of structure and function (1). One major finding
of this study is another sub-family of WT1 variant proteins encoded by a partially overlapping transcriptional unit which we refer to as AWT1. This alternate transcript has the capacity to encode proteins of approximately 33 kDa, which retain the DNA-binding zinc-finger motif and transactivational domain, but lack the domain associated with repression (5). WT1 proteins of similar mass have been detected previously (4,13), and have been attributed to internal translational initiation from methionine 127 (4). We note that the methionine codon of AWT1 (Fig. 4B) has, in contrast to methionine 127, a strong Kozak consensus sequence (21), and our analysis of proteins in transfected cells confirms high levels of translated 33 kDa AWT1 protein, in contrast to lower levels of 36 kDa WT1 protein apparent with cells transfected with a methionine 127-initiating cDNA [Fig. 3 in Scharnhorst et al. (4)]. However, the finite levels of cellular proteins are very likely to be influenced by other factors such as mRNA transcript stability and processing.

In view of the parallel expression pattern of WT1 and AWT1 revealed by RPA analysis, it is interesting to note that complexing of WT1 with a protein of 36 kDa was suggested by purification studies (22), and, although AWT1 does not retain the self-association domain present in the first 182 amino-acids, WT1 self-interaction via the zinc fingers has also been observed (23).

A second major finding of our work is that chromosome 11p13 is a site for developmental regulation of gene expression by genomic imprinting. Both WT1-AS and AWT1 are expressed from the paternal allele. In view of the tumour suppressor activity of WT1, it is perhaps surprising that AWT1 is expressed from the paternal allele, as maternal allele LOH would suggest a maternally expressed tumour suppressor gene. Conversely, retention of the expressed paternal allele might indicate that AWT1 may promote cell-survival. The expression of WT1 in human malignancies such as leukaemia (1) and breast cancer (24) and its anti-apoptotic activities (1,16) has alluded to a possible oncogenic capacity for WT1. In WT, high levels of WT1 expression are considered to reflect blastemal cell persistence. However, our identification of a developmental regulation defect of a probable key intermediary in nephrogenesis (i.e. imprinting failure of AWT1) raises the possibility that deregulated AWT1 expression may also promote cell survival.

Figure 8. Allelic expression of AWT1, in kidney and WTs. (A) RT–PCR (+ and −RT) of AWT1 exons 1a–2 shows expression in a normal kidney (NK36) and two maternal 11p LOH Wilms' tumours (WT36 and WT44). RT–PCR (+ and −RT) of AWT1 exons 1a–10 across an exon 7 AflIII polymorphism in two informative normal kidneys (NK81 and NK62), their matched non-11p LOH tumours (WT81 and WT62), and a 22 week fetal kidney sample (FK). Digestion with AflIII (A) identifies monoallelic expression of AWT1 (allele A2) in both NK81 and NK62. Relaxation of imprinting is observed in both WT81 and WT62, indicated by the presence of both allele A1 and A2 following AflIII digestion. (B) Allelic expression of WT1 and AWT1 in normal kidney RNA (patient 21) was analysed using the polymorphic GT repeat in exon 10. Three major alleles were detected. Patient 21 is heterozygous (A1, A3) with A1 being paternal. In NK RNA both alleles of WT1 are expressed but only the paternal allele of AWT1 is expressed. Stutter bands are labelled with small black diamonds. These minor bands are caused by polymerase slippage, as shown previously for this locus (8).
as has been suggested for WTI. Although the variant biological functions of AWT1 will need to be carefully dissected, we note that the AWT1 transcript is conserved in mice. As the wt1 knockout mouse would not necessarily abrogate awt1 expression, the deletion employed being upstream of exon 1a, awt1 alone may not be able to compensate for the loss of wt1. However, the authors reported that immunohistochemical analysis for WT1 proteins in knockout mice did not yield interpretable results (2), which might be explained by retention of awt1 expression.

Our data add to the complex repertoire of WT1 proteins, with the imprinted status of AWT1 necessitating definition of the parental origin of mutated alleles and the dosages of their respective proteins in developmental abnormalities and carcinogenesis. The cogency of such studies is emphasised by the finding of heterozygous mutations of paternal origin in patients with WT1-related disorders (25–31). Table 2 summarizes patient data where the parental origin of mutations in the WTI/WT1 coding regions has been ascertained. DDS consists of constitutional heterozygous WT1 effects (23), and/or dominant-negative mechanisms. Asymptomatic fathers presumably carry the mutations on the silent (with respect to AWT1) allele, with dominant negative WT1 interactions being insufficient to advance to DDS. Loss of the maternal allele in WTs may then further disrupt WT1/WT1 interactions and functions to provoke tumourigenesis. Although preferential de novo mutations in the male germline have been previously demonstrated for genes such as RB1 (32), consideration of the mutation data together with our imprinting analysis provides a possible mechanistic explanation for the variable penetrance observed with DDS.

Only one WT pedigree has been described showing maternal transmission of AWT1/WT1 mutations (cases 8a and b). In this unusual family, a normal mother with a heterozygous truncating point mutation in exon 7 transmitted this mutation to a son and daughter, who both developed WTs with deletion/defects of the other allele. The son also suffered from genitourinary abnormalities. This case is in stark contrast to those cited above, and it is unclear why the son, having acquired a silent AWT1 mutant allele from the mother, suffered from genitourinary abnormalities. The paucity of such cases suggests the possible involvement of rare genetic or epigenetic events, such as a de novo or paternally inherited mutation of exon 1a in the paternal allele or polymorphic imprinting, as previously described for WTI in some tissues (8).

In conclusion, the identification of a maternally imprinted (paternally expressed) gene within the WTI locus suggests that an alternative non-mutationale lesion that may be significant in carcinogenesis. The biallelic expression of AWT1 in WTs without LOH would be quantitatively mirrored by duplication of the remaining active paternal allele in tumours with LOH. These dosage changes underline the requirement for further analysis of AWT1/WT1 functions and interactions, especially those involved in dominant negative effects.

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**Table 2. Allelic origin of heterozygous AWT1 and WTI mutations**

<table>
<thead>
<tr>
<th>Patient description (karyotype)</th>
<th>Mutations</th>
<th>Origin of mutated allele</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: D1, DDS (46, XY)</td>
<td>377His→Arg; WT: LOH</td>
<td>Paternal de novo</td>
<td>26</td>
</tr>
<tr>
<td>2: D5, DDS (46, XX)</td>
<td>396Asp→Asn; WT: LOH</td>
<td>Paternal de novo</td>
<td>26</td>
</tr>
<tr>
<td>3: D5, DDS (46, XY)</td>
<td>394Arg→Trp; kidneys removed at 18 months</td>
<td>Paternally inherited (father normal)</td>
<td>27</td>
</tr>
<tr>
<td>4: P21, WT + GU (46, XX)</td>
<td>223Ser→Asn</td>
<td>Paternally inherited (father normal)</td>
<td>28</td>
</tr>
<tr>
<td>5: WT + GU (46, XY)</td>
<td>Intron 7 + 2bp, T→G (term. ex9); WT: LOH</td>
<td>Paternal de novo</td>
<td>29</td>
</tr>
<tr>
<td>6: TS, WT + GU (46, XY)</td>
<td>1bp del exon 6 (term ex7); WT: LOH</td>
<td>Paternally inherited (father with WT)</td>
<td>25</td>
</tr>
<tr>
<td>7: Z-2368, WT (46, XX)</td>
<td>362Arg→STOP codon (term.); WT: LOH</td>
<td>Paternally inherited (father normal)</td>
<td>30</td>
</tr>
<tr>
<td>8a: Brother 302 d WT + GU (46, XY)</td>
<td>7bp del exon 7 (term); WT: LOH</td>
<td>Maternally inherited (mother normal)</td>
<td>31</td>
</tr>
<tr>
<td>8b: Sister 301d, WT (46, XX)</td>
<td>7bp del ex7 (term.); WT: 26bp insert ex7 (term.)</td>
<td>Paternal de novo</td>
<td>26</td>
</tr>
</tbody>
</table>

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*aConstitutional mutation information; WT genotype where applicable.
bWT homozygous for mutation.
cThree daughters in family HMK had WT, out of five siblings.
dTwo siblings from family Wilms 5.

term., truncating mutation. LOH, 11p loss of heterozygosity.
MATERIALS AND METHODS

Clinical material

All tissues were obtained with appropriate ethical approval, and processed as described previously (10). All WTs used in this study were sporadic tumours with no associated predisposing syndromes. Eight were unilateral and two bilateral (three stage I, two stage II, one stage III, one stage IV, two stage V and one stage unknown). Five tumours had favourable histology, and another five were of unknown histology. The WTs were from six girls and four boys, diagnosed between the ages of 10 and 120 months. Three patients relapsed, two of whom died. Fetal kidney was obtained at 22 weeks of age.

Southern blot analysis

A 100 kb sample of human genomic sequence spanning the WT1 locus (sequence data from GenBank NT_009237) was analysed using the GRAIL/CPG software located at the Human Genome Mapping Project web site (www.hgmp.mrc.ac.uk). This identified five further CPG islands all located within the 5’ region of WT1. These were designated as CPG0, located upstream of WT1, CPGEX1, spanning WT1 exon 1, CPG1 and CPG25 situated in intron 1, and CPG23, which spans exons 3 and intron 3. The locations of the CPG islands and the probes used for methylation analysis are given in Table 1 and shown in Figure 3A. Numbering is relative to the transcriptional start site of WT1 (designated ‘0’). The locations of WT1 exons 1–4 are included for orientation.

Probes for Southern hybridization were generated by subcloning of PCR products spanning the 5’-end of WT1. Methyltransferase-sensitive Southern analysis was carried out as previously described (10). The use of a particular restriction endonuclease combination for each analysis was determined from the genomic sequence and the specific probe used. Digests used were: CPG0, BamHI, EcoRI, Smal; CPGEX1, KpnI, SpeI, Bsh1236I; CPG1, KpnI, XbaI, Smal; CPG25 and CPG23, BamHI, Bsh1236I. Bsh1236I is a BstUI isoschizomer (Helena Bioscience).

Western blot analysis

Cells were grown to 70% confluence, trypsinized and 1 × 10⁶ cells were lysed in 80 µl of sample buffer (60 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.01% bromophenol blue). For WT1 and AWT1 transfected cells, the equivalent of 2 × 10⁶ cells were loaded per well, whereas 1 × 10⁶ cells per well were used for the other samples. The samples were electrophoresed on a 12.5% SDS–polyacrylamide gel. After electrophoresis the proteins were transferred to Immobilon-P (Millipore) with a semidyf transfer apparatus. The Immobilon-P was then transferred to 5% non-fat dry milk (Tesco) in PBS (milk block) and blocked for a minimum of 2 h. Primary antibodies were incubated in milk block for 1 h at RT, followed by the secondary antibody at RT for 1 h. Protein bands were visualized with the ECL Plus reagents (Amersham Pharmacia Biotech) and placed next to MXB autoradiography film (Kodak) for up to 2 h. Antibodies used were WT1 C-19 (Santa Cruz biotechnology) rabbit anti-human WT1 used at a 1/1000 (v/v) dilution. Peroxidase-conjugated goat anti-rabbit IgG (DAKO) was used as secondary antibody at 1/1000.

Ribonuclease protection assays

RPA probes were generated by PCR from a phage artificial chromosome spanning the WT1 gene (312–16). This PAC was isolated from a human gridded genomic library (HGMP, UK). PCR fragments were cloned directly into pGEM-T easy (Promega), and transcribed using Maxiscript T3, T7 and SP6 polymerases (Ambion). Ribonuclease protection assays were carried out with the RPAIII kit (Ambion) according to manufacturers instructions, with hybridizations performed at 60°C.

Cell culture and promoter assays

Cell maintenance and transient transfections of expression constructs were carried out by electroporation as previously described (11). Plasmids pCMV–WT1 and pcDNA–AWT1 were made using expression plasmids pCMV-neo and pcDNA3.1 and contained (+/–) isoforms of WT1 and AWT1 respectively. AWT1 promoter constructs were made in pGL2-E (Promega) and transient transfections of 293 cells were performed with Transfast (Promega) and repeated in triplicate at least three times, and harvested after 48 h and assayed for luciferase activity.

Reverse-transcriptase PCR analysis

Reverse-transcriptase-PCR was performed essentially as previously described (10). For the MnII polymorphism used to trace the parental origin of the expressed allele of WT1-AS, nested primers were used for RT–PCR as follows: round 1, primers R1 (CATGTGGATCCGTTGGGTC) and F2 (TT GCTCAGTGATGTGACGCC); round 2, primer 18 (CTTAGC ACTTTCTTTGGGGC) and F2A (TCAGTGATGACCGAGGGCGGA). Both rounds of amplification were for 30 cycles (94°C, 15 s, 55°C, 30 s and 72°C, 1 min). The template for round 2 was 1 µl of the 25 µl round 1 reaction.

AWT1 expression analysis of LOH tumours used primers CPG-S3 and WT11 (Fig. 4B). Imprinting analysis of AWT1 employed primers CPG-S4 (GTGCAGTGCCCTGGGTCCCT) in exon 1a and primer WT4 (ACTGAAAGCGACTTCA) in exon 10. Amplification conditions for both were 94°C for 3 min followed by 35 cycles of 94°C, 15 s, 55°C, 30 s and 72°C, 1 min, with a 5 min, 72°C final extension. PCR products were digested with an excess of AflIII at 37°C overnight, electrophoresed on a 1.5% agarose gel and alkali-blotted onto Hybond-N+ (Amersham Biosciences, UK). Filters were fixed, and hybridized with a WT1 cDNA probe.

Parental origin of WT1/AWT1 allelic expression was analysed using the polymorphic GT repeat in exon 10 (33), using nested PCR. Round 1 used forward primer 1 in exon 1 for WT1 (AGCAGTGGCCCTGGGACTT) or CPG-S4 in exon 1a for AWT1 (GTGCAGTGCCCTGGGTC) with reverse primer 7B in exon 10 (GTCAAGAGCAAAATCATTAGCAG) for both, amplified at 94°C for 3 min followed by 30 cycles of 94°C 15 s, 55°C, 30 s, 68°C, 2 min, with a 5 min, 68°C final extension. One-fifth of the round 1 product was then amplified with primers flanking the GT repeat in exon 10...
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