Identification of Differential Methylation of the WT1 Antisense Regulatory Region and Relaxation of Imprinting in Wilms’ Tumor

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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 WT1 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 factor receptor, including tors, regulating the promoters of several genes critical for cell growth, tumor; DMR, differentially methylated region. transcription-PCR; CCSK, clear cell sarcoma of the kidney; PNET, primitive neuroectodermal tumor; 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.

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The abbreviations used are: WT, Wilms’ tumor; IGF, insulin-like growth factor; KTS, Kaposi Sarcoma-associated herpesvirus transforming protein 1; IGF2R, insulin-like growth factor 2 receptor; c-myc, c-myc oncogene; 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.

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 KpnI, SpeI, and BstUI/Bsh1236f (New England Biolabs and MBI Fermentas). BstUI and Bsh1236f are isoschizomers recognizing the sequence CCGG, 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 [32P]-P32CTP (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. 1a). The 850-bp probe corresponds to the region directly upstream

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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 *Bst*UI. The differentially methylated *Bst*UI 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 *Bst*UI 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.

PCR was performed using 1 unit of SuperTaq (HT Biotechnology) in the manufacturer’s buffer in a Hybrid 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 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 transcribe 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 38 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 15% Tris-borate-EDTA gel and bands analyzed as described above.

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. As outlined above, there

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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 KpnI and SpeI 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 (CCGG) is unmethylated (Fig. 1a).

As shown in Fig. 1b, 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 hypermethylation 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. 1a). 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. 1b). 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. 1b 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. 2a). As shown in Fig. 2b, 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 KvlQ1 (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).
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. 1b) 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. 2b). 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 tumors fail to either acquire or maintain the methylation imprint prerequisite for normal neoprogenesis, 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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References

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