

# Frequency and Timing of Loss of Imprinting at 11p13 and 11p15 in Wilms' Tumor Development

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## Abstract

**Epigenetic changes occur frequently in Wilms' tumor (WT), especially loss of imprinting (LOI) of *IGF2/H19* at 11p15. Our previous results have identified imprinted transcripts (*WT1-AS* and *AWT1*) from the *WT1* locus at 11p13 and showed LOI of these in some WTs. In this article, we set out to test the relationship between LOI at 11p13 and 11p15 and their timing in WT progression relative to other genetic changes. We found a higher level (83%) of 11p13 LOI in WT than of 11p15 LOI (71%). There was no correlation between methylation levels at the 11p13 and 11p15 differentially methylated regions or between allelic expression of *WT1-AS/AWT1* and *IGF2*. Interestingly, retention of normal imprinting at 11p13 was associated with a small group of relatively late-onset, high-stage WTs. An examination of genetic and epigenetic alterations in nephrogenic rests, which are premalignant WT precursors, showed that LOI at both 11p13 and 11p15 occurred before either 16q loss of heterozygosity (LOH) or 7p LOH. This suggests that these LOH events are very unlikely to be a cause of LOI but that LOH may act by potentiating the effects of overexpression of *IGF2* and/or *WT1-AS/AWT1* that result from LOI. (Mol Cancer Res 2008;6(7):1114–23)**

## Introduction

Wilms' tumor (WT) is a pediatric renal malignancy, affecting ~1 in 10,000 children (1). It is associated with several well-characterized genetic defects, including the mutational inactivation of the *WT1* (2) or *WTX* (3) tumor suppressor genes. *TP53* is also sometimes mutated in a rare aggressive subset of WTs (4). Activating *CTNNB1* proto-oncogene mutations have been found in WT, often in conjunction with *WT1* mutation in the same tumor (5). The involvement of other tumor suppressor genes is predicted in WT on the basis of loss of heterozygosity (LOH) studies that have found LOH at 1p, 7p, 11q, 16q, and

other loci, although the relevant genes remain unidentified as yet (1).

WT has been instrumental in unraveling the role of tumor suppressor genes in cancer because the *WT1* gene was the second tumor suppressor gene to be cloned and shown to conform to Knudson's two-hit model. Moreover, studies of WT have also been pivotal in understanding how epigenetic changes contribute to carcinogenesis, WT being the first malignancy in which loss of imprinting (LOI) was shown. The insulin-like growth factor 2 gene (*IGF2*) at 11p15 is normally imprinted such that only its paternal allele is expressed. However, it was found that in many WTs both copies of *IGF2* were expressed, with overexpression of *IGF2* presumably giving WT cells a selective growth or survival advantage (6, 7). This phenomenon was termed "loss of imprinting" (LOI) or "relaxation of imprinting" (LOI will be used here) and it has subsequently been reported in a wide range of human malignancies, making it one of the commonest epigenetic alterations in human cancer (8). *IGF2* imprinting is controlled by a differentially methylated region (DMR), containing a CTCF-dependent boundary element, close to the oppositely imprinted *H19* gene (9, 10). Hypermethylation of the *H19* DMR leads to LOI in WT, manifested as biallelic expression of *IGF2* and concomitant loss of expression of *H19* (11, 12).

Investigations of nephrogenic rests (NR), which are premalignant precursors of WT, have shown that *IGF2* LOI can be found at this early stage of WT development, suggesting that LOI is a cause, rather than a consequence, of malignant transformation (13–15). Additional proof for a causative role for LOI has come from a mouse model of pancreatic cancer, in which *Igf2* LOI was found in hyperproliferative lesions before the development of frank malignancy (16). Studies of the human overgrowth syndrome Beckwith-Wiedemann syndrome, in which patients are predisposed to pediatric malignancies including WT, have shown that germ-line LOI of *IGF2* is a feature of some cases, which further implicates LOI as a major epigenetic factor in the development of WT (17). Thus, somatic epigenetic defects lead to LOI of *IGF2* in sporadic WT, and similarly, germ-line epigenetic defects can lead to constitutional LOI of *IGF2* in Beckwith-Wiedemann syndrome, giving WT predisposition.

The first piece of evidence implicating imprinted genes in WT development came before the discovery of LOI, when it was noted that LOH of the 11p region in WT invariably involved loss of the maternal allele (1) and that 11p15 duplications and uniparental disomy in Beckwith-Wiedemann syndrome were always of paternal origin (17). This was explained by the presence of imprinted genes on 11p, such that

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**Note:** Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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maternal LOH or paternal uniparental disomy led to the loss of maternally expressed growth-suppressing genes and/or the retention of paternally expressed growth-promoting genes (18). The discovery that the 11p15 cluster of imprinted genes contained a paternally expressed growth factor (*IGF2*) and maternally expressed growth-inhibitory genes (*H19* and *CDKN1C*) supported this hypothesis and implied that maternal LOH was entirely driven by the properties of the imprinted cluster at 11p15 (8).

Interestingly, most cases of 11p LOH in WT involve both the 11p13 and 11p15 regions (19), and although it was originally shown that the *WT1* gene at 11p13 was not imprinted in kidney (20), we have subsequently shown that paternally expressed imprinted transcripts are transcribed from the *WT1* locus. These imprinted transcripts are the noncoding antisense RNA *WT1-AS* (21) and the alternate coding transcript *AWT1*, which gives rise to NH<sub>2</sub>-terminally truncated WT1 proteins (22). We have shown that in a subset of WTs, LOI of these imprinted *WT1* transcripts occurs, leading to their biallelic expression (21, 22). It is therefore clear that imprinting alterations in WT are not just confined to 11p15 but occur at 11p13 as well. In WTs with LOH, the frequent reduplication of the retained paternal allele (19) will result in increased expression of all paternally expressed imprinted genes on 11p, including *IGF2*, *WT1-AS*, and *AWT1* and loss of expression of maternally expressed genes such as *H19* and *CDKN1C*. However, in WTs without 11p LOH, it is not known whether LOI at 11p13 and 11p15 are linked in any way or are completely independent events. LOI at 11p13 and 11p15 could be caused by a common factor affecting imprinting control at both loci, or alternatively, LOI at these loci could be completely unrelated mechanistically. To determine which of these hypotheses is correct, we have characterized a cohort of WTs for LOI at both 11p13 and 11p15 and related these changes to LOH events at other loci associated with WT progression. Our results suggest that LOI at 11p13 and 11p15 are not linked mechanistically. Importantly, however, each may have effects on the phenotype of WTs. Furthermore, we show that LOH at 16q or 7p are unlikely to be controlling events underlying these imprinted gene defects because they occur after LOI in WT development.

## Results

To investigate the relationship between LOI at 11p13 and LOI at 11p15 in WT, we initially characterized a cohort of 51 WTs for LOH status using RFLP and/or microsatellite polymorphisms on 11p (23, 24). We found 28 WTs that retained heterozygosity at 11p13 and 11p15 (and one that was heterozygous at 11p13 but had LOH at 11p15) and were therefore suitable for LOI analysis. Allelic expression of imprinted genes at 11p13 and 11p15 was then assessed in these heterozygous tumors using transcribed polymorphisms and DNA methylation was assayed by combined bisulfite restriction analysis (COBRA), as illustrated in Figs. 1 and 2. Full results for all individual tumors, including clinical data, are given in Supplementary Table S1.

### *LOI at 11p13*

We have previously shown that the *WT1* locus at 11p13 expresses two imprinted transcripts: the noncoding antisense

RNA *WT1-AS* (21) and the alternate coding transcript *AWT1* (22). Hypomethylation of the DMR in the *WT1* ARR is associated with LOI of the *WT1-AS* and *AWT1* transcripts (21, 22, 25). We therefore designed a COBRA assay to assess methylation of the ARR DMR (Fig. 1A) and used polymorphisms in the *WT1-AS* and *AWT1* transcripts (21, 22, 25) to investigate imprinted expression.

As expected from our earlier work, normal kidney (NK) samples showed ~50% methylation at the ARR (Fig. 1B and C) and monoallelic expression of *WT1-AS*, as expected for an imprinted gene (e.g., NK62; Fig. 1B). Of the 29 WTs that retained heterozygosity at 11p13, 24 (83%) had <40% methylation of the ARR, similar to the level of methylation found in fetal kidney (FK; Fig. 1B and C). Of these 24 samples, 12 were informative for transcribed polymorphisms in *WT1-AS* and/or *AWT1* and all 12 showed biallelic expression of these transcripts, showing LOI (e.g., WT69 and WT45; Fig. 1B). Seven tumors were informative for both *WT1-AS* and *AWT1* polymorphisms and in every case showed concordance for LOI, in agreement with our *in vitro* data suggesting coregulation of *WT1-AS* and *AWT1* imprinting (25). The remaining 5 of the 29 heterozygous WTs (17%) had ARR methylation levels in excess of 40% (Fig. 1B and C). One of these tumors was informative for a *WT1-AS* transcribed polymorphism and one for an *AWT1* polymorphism, and both had monoallelic expression, showing retention of normal imprinting (e.g., WT04; Fig. 1B). For comparison, we also assessed ARR methylation in two representative 11p13 LOH WTs and both had very low levels (Fig. 1C). This is as predicted because the ARR is methylated on the maternal allele (22) and 11p13 LOH WTs invariably lose the maternal allele (1). Two normal kidney samples had *WT1* ARR methylation <40% (Fig. 1C); this may represent an early somatic epigenetic change in a proportion of apparently normal kidney cells, as previously reported for *H19* (12).

By comparing the allelic expression data and ARR methylation analysis, we could divide the 29 heterozygous WTs into two distinct groups: (a) an LOI group consisting of 24 WTs with <40% ARR methylation and biallelic expression of *WT1-AS* and/or *AWT1* in informative tumors and (b) a smaller group of 5 normally imprinted WTs with >40% ARR methylation and monoallelic expression of *WT1-AS* or *AWT1* in informative tumors (Fig. 1C). We then compared the clinical features and LOH status at 16q and 7p between the 11p13 LOH, LOI, and normally imprinted groups (Table 1). There were no significant differences between these three groups for clinical outcome (relapse or death), LOH at 7p, or LOH at 16q (Table 1). However, the small group ( $n = 5$ ) of normally imprinted WTs had a significantly ( $P = 0.012$ ) later age of diagnosis than the LOH WTs and significantly ( $P = 0.014$ ) higher stage distribution than both the LOH and LOI tumors (Table 1). This suggests that retention of normal imprinting at *WT1-AS*/*AWT1* may be associated with a specific phenotype in WT, consisting of high stage with a relatively late age of diagnosis.

### *LOI at 11p15*

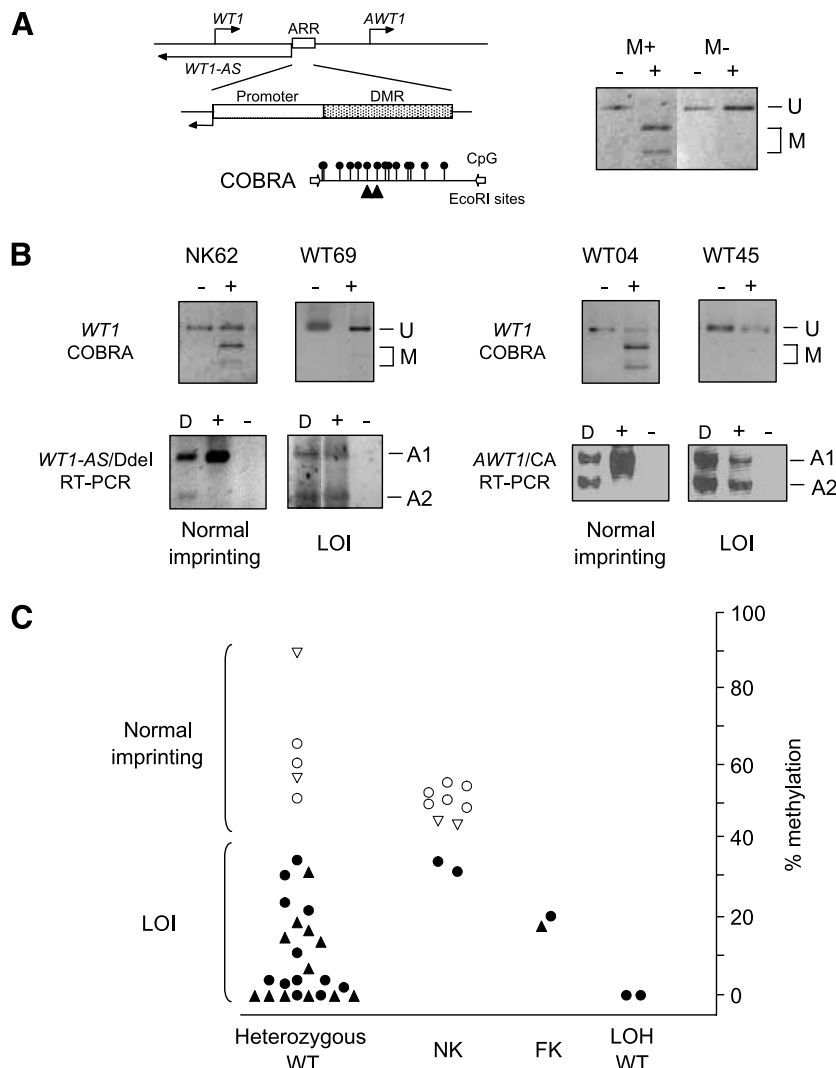
LOI at the 11p15 locus is well established in WT (8), and we therefore assessed the same set of WTs for 11p15 LOI to determine whether LOI was a generalized epigenetic defect or

specific to 11p13 or 11p15. We designed a COBRA assay for the *H19* DMR in an area that has been shown to be consistently hypermethylated in 11p15 LOI WTs (Fig. 2A; ref. 26). Transcribed polymorphisms in *IGF2* were used as previously described in our studies of Beckwith-Wiedemann syndrome (27) to determine the allelic expression of *IGF2* in informative WTs.

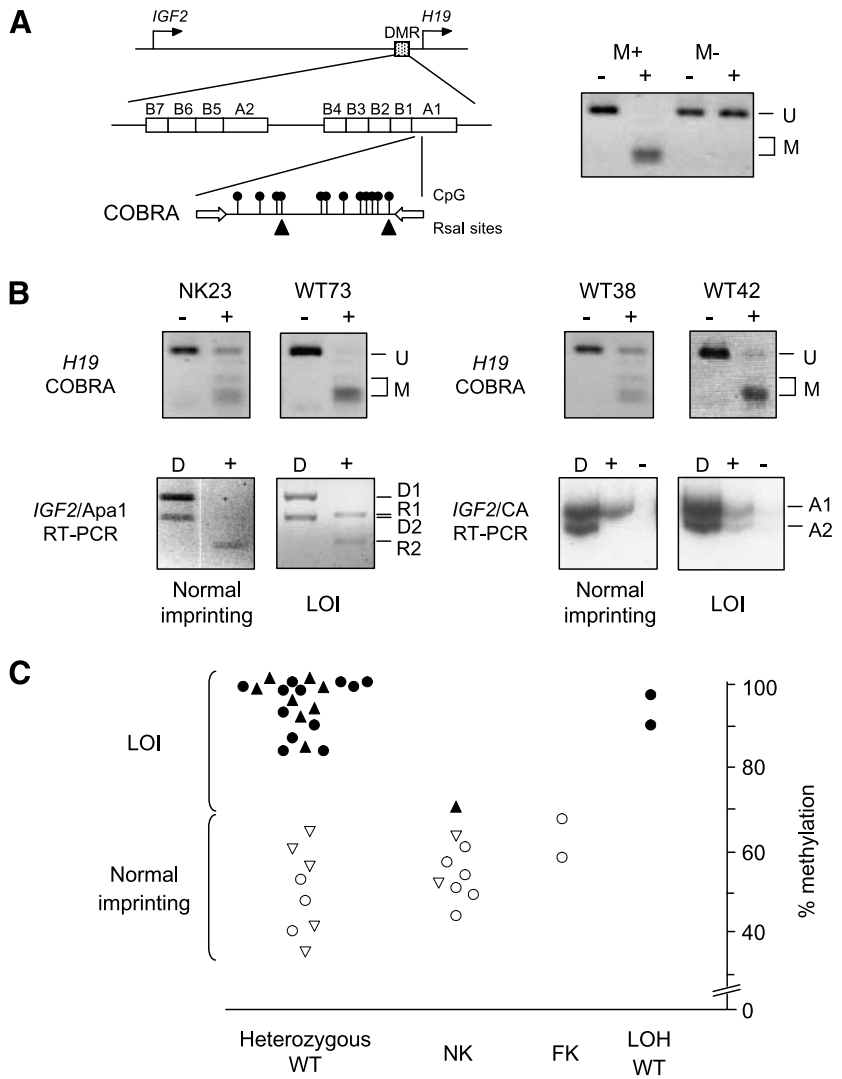
Normal tissues (NK and FK) showed ~50% methylation at the *H19* DMR (Fig. 2B and C) and a representative informative normal kidney sample (NK23) showed monoallelic expression of *IGF2* (Fig. 2B). These results are consistent with imprinted monoallelic expression of *IGF2* and differential methylation at the *H19* DMR, as expected for normally imprinted tissues. Of the 28 WTs that retained heterozygosity at 11p15, 20 (71%) had hypermethylation (>70%) of the *H19* DMR (Fig. 2C), and of these 20, all 8 of the tumors that were informative for transcribed *IGF2* polymorphisms showed biallelic expression, confirming LOI (Fig. 2B and C). Representative LOI tumors (WT42 and WT73) are shown in Fig. 2B. The other 8 (29%) heterozygous tumors had <70% methylation at the *H19* DMR (Fig. 2B and C) and monoallelic expression of *IGF2* in all 5

informative WTs, showing that they had retained normal imprinting at 11p15 (e.g., WT38; Fig. 2B). Two representative 11p15 LOH WTs were assayed for methylation at this locus and both showed hypermethylation (89-96%) of the *H19* DMR, as expected given the invariable loss of the unmethylated maternal allele in WT (Fig. 2C; ref. 1). Interestingly, one NK sample showed *H19* methylation >70% (Fig. 2C), similar to some previous reports (12), and in this case we found biallelic expression of *IGF2*, as further discussed below (Fig. 4).

The 11p15 imprinted cluster is divided into two domains, one controlled by the *H19* DMR in IC1 and another by the *KCNQ1OT1* DMR (*Kv* DMR) in IC2 (17). We therefore investigated methylation at the *Kv* DMR to determine whether LOI was associated with altered methylation outside of the *H19* DMR. We found that the distribution of *Kv* DMR methylation levels in WTs was essentially identical to that in normal tissues (Fig. 3C), although two WT samples had zero methylation at the *Kv* DMR (e.g., WT39; Fig. 3B). These two WTs did not show 11p13 or 11p15 LOH with our markers but we cannot exclude localized 11p15 LOH in these tumors because both were noninformative for polymorphisms within *IGF2* and *H19*.



**FIGURE 1.** LOI at the *WT1* locus at 11p13 in WT. **A.** *WT1* ARR COBRA. Left, a schematic of the *WT1* ARR. Bent arrows, positions of the *WT1*, *WT1-AS*, and *AWT1* promoters (underneath is an enlarged view of the ARR, showing the DMR and the position of the amplicon used in COBRA analyses). White arrows, primers; filled circles, CpG residues; black arrowheads, *EcoRI* restriction endonuclease sites. Right, an agarose gel of COBRA analysis of fully methylated (M+) and unmethylated (M-) human genomic DNA controls. -, untreated; +, *EcoRI*-treated PCR product. U, unmethylated band (undigested); M, methylated band (digested). **B.** Top, COBRA analysis of *WT1* ARR methylation; gel labeling as in **A**. Bottom, allelic expression of *WT1-AS* using the *DdeI* polymorphism (two left images) and *AWT1* using the CA repeat polymorphism (two right images). D, Genomic DNA product; +, product from RT+ cDNA; -, product from RT-cDNA. A1, allele 1; A2, allele 2. NK62 and WT04 are normally imprinted and WT69 and WT45 show LOI. **C.** Dot plot of *WT1* ARR COBRA results showing percent methylation for all samples. Each dot represents a single tissue sample. Circles, samples that were noninformative for allelic expression analysis; triangles, samples with *WT1-AS* and/or *AWT1* LOI; inverted triangles, samples with normal imprinting. Filled symbols, samples with <40% methylation; unfilled symbols, >40% methylation. Gels are cropped and shown in negative for clarity. Uncropped gels are presented in Supplementary Fig. S1.



**FIGURE 2.** LOI at the *IGF2/H19* locus at 11p15 in WT. **A.** *H19* DMR COBRA. Left, a schematic of the *H19* DMR. Bent arrows, positions of the *H19* and *IGF2* promoters (underneath is an enlarged view of the DMR, showing repeat blocks and the position of the amplicon used in COBRA analyses). White arrows, primers; filled circles, CpG residues; black arrowheads, *RsaI* restriction endonuclease sites. Right, an agarose gel of COBRA analysis of fully methylated (M+) and unmethylated (M-) human genomic DNA controls. -, untreated; +, *RsaI*-treated PCR product. **B.** Top, COBRA analysis of *H19* DMR methylation; gel labeling as in **A.** Bottom, allelic expression of *IGF2* using the *ApaI* polymorphism (two left images) and the CA repeat polymorphism (two right images). D, Genomic DNA product; +, product from RT+ cDNA; -, product from RT-cDNA. For the *ApaI* polymorphism, the PCR is across a small intron, so genomic DNA gives larger products: D1, genomic DNA allele 1; D2, genomic DNA allele 2; R1, cDNA allele 1; R2, cDNA allele 2. For the CA repeat polymorphism, the PCR is within one exon, therefore genomic and cDNA products are of the same size: A1, allele 1; A2, allele 2. NK23 and WT38 are normally imprinted and WT73 and WT42 show LOI. **C.** Dot plot of *H19* DMR COBRA results showing percent methylation for all samples. Each dot represents a single tissue sample. Circles, samples that were noninformative for allelic expression analysis; triangles, samples with *IGF2* LOI; inverted triangles, samples with normal imprinting. Filled symbols, samples with >70% methylation; unfilled symbols, <70%. Gels are cropped and shown in negative for clarity. Uncropped gels are presented in Supplementary Fig. S2.

However, 27 of 29 (93%) WT samples had similar *Kv* DMR methylation to normal tissues and, therefore, it seems to be very unlikely that altered methylation of the *Kv* DMR plays a role in 11p15 LOI in this cohort of WTs.

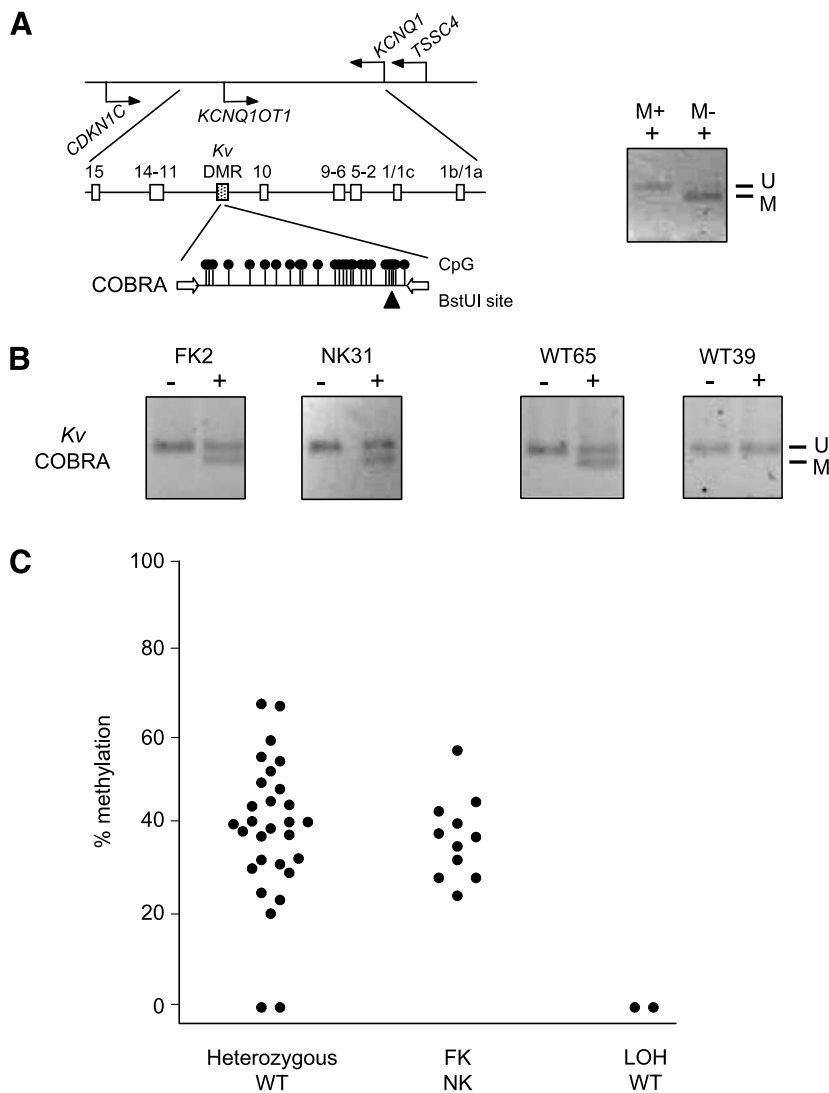
We divided the 11p15 heterozygous WTs into two distinct groups (LOI and normally imprinted) on the basis of *H19* DMR methylation (>70% or <70%) and *IGF2* allelic expression.

Comparison of 11p15 LOH, LOI, and normally imprinted tumors revealed a highly significant ( $P = 0.001$  and  $P = 0.007$ ) later age of onset in the LOI WTs compared with the other two groups (Table 2). The LOI WTs also showed a higher proportion of late-stage tumors and, compared with the normally imprinted group, had a much higher frequency of 16q LOH, although these differences did not reach statistical significance

**Table 1. Comparison of Clinical and Other LOH Data for 11p13 LOH, LOI, and Normally Imprinted WTs**

|                    | Mean Age at Diagnosis ( $\pm$ SD), mo | <i>P</i>                  | Stage       |               | <i>P</i>                     | Relapsed or Died, % (n) | <i>P</i> | 7p LOH, % (n) | <i>P</i> | 16q LOH, % (n) | <i>P</i> |
|--------------------|---------------------------------------|---------------------------|-------------|---------------|------------------------------|-------------------------|----------|---------------|----------|----------------|----------|
|                    |                                       |                           | I-II, % (n) | III-IV, % (n) |                              |                         |          |               |          |                |          |
| LOH                | 29.4 $\pm$ 17.2 (n = 22)              | —                         | 64 (14/22)  | 23 (5/22)     | —                            | 18 (4/22)               | —        | 14 (3/21)     | —        | 19 (4/21)      | —        |
| LOI                | 40.8 $\pm$ 23.1 (n = 24)              | ns                        | 61 (14/23)  | 17 (4/23)     | ns                           | 39 (9/23)               | ns       | 17 (4/24)     | ns       | 33 (8/24)      | ns       |
| Normally imprinted | 54.8 $\pm$ 26.0 (n = 5)               | 0.012 vs LOH<br>ns vs LOI | 0 (0/4)     | 100 (4/4)     | 0.014 vs LOH<br>0.014 vs LOI | 25 (1/4)                | ns       | 20 (1/5)      | ns       | 20 (1/5)       | ns       |

NOTE: Full data for each tumor are given in Supplementary Table S1. Stage and survival data were unavailable for one LOI WT and one normally imprinted WT. Stage V tumor numbers not included in this table. Statistical analysis was by Student's *t* test for age at diagnosis and Fisher's exact test for other variables. Comparison is to the LOH group unless indicated otherwise. ns, nonsignificant ( $P > 0.017$ , Bonferroni's correction for multiple testing).



**FIGURE 3.** Methylation of the *Kv* DMR at 11p15 in WT. **A.** *Kv* DMR COBRA. Left, a schematic of the *Kv* DMR. Bent arrows, positions of *CDKN1C*, *KCNQ1OT1*, *KCNQ1*, and *TSSC4* promoters (underneath is an enlarged view of the *KCNQ1* gene, showing the position of the exons and the DMR and the position of the amplicon used in COBRA analyses). White arrows, primers; filled circles, CpG residues; black arrowheads, *Bst*UI restriction endonuclease sites. Right, an agarose gel of COBRA analysis of fully methylated (M+) and unmethylated (M-) human genomic DNA controls treated (+) with *Bst*UI. **B.** COBRA analysis of *Kv* DMR methylation in four tissue samples: FK2, NK31, WT65, and WT39. -, untreated; +, *Bst*UI-treated PCR product. **C.** Dot plot of *Kv* DMR COBRA results showing percent methylation for all samples. Each dot represents a single tissue sample. FK NK, combined results for all fetal and normal kidney tissues. Gels are cropped and shown in negative for clarity. Uncropped gels are presented in Supplementary Fig. S3.

when corrected for multiple testing (Table 2). It therefore seems that 11p15 LOI is associated with a group of late-onset WTs that may usually be of higher stage and have 16q LOH, suggesting an association with poor prognosis.

#### Comparison of LOI at 11p13 and 11p15

Methylation at the *WT1* ARR and the *H19* DMR showed no correlation in the 28 WTs that were heterozygous at both 11p13 and 11p15 ( $r = -0.1256$ , Spearman rank order correlation coefficient;  $P = 0.52$ ), and the proportions of WTs with and without LOI at 11p13 and/or 11p15 were almost exactly as expected for two independent variables. Six WTs were informative for transcribed polymorphisms in both *IGF2* and *WT1-AS* or *AWT1*. All six had LOI at 11p13 (*WT1-AS/AWT1*) but only five had *IGF2* LOI. These data suggest that there is unlikely to be a mechanistic link between LOI at 11p13 and 11p15.

Given that a later age of onset was associated with both normal imprinting at 11p13 and LOI at 11p15 (Tables 1 and 2), we divided the 11p15 LOI WTs into two groups on the basis

of their 11p13 LOI status and calculated their mean ages of diagnosis. The 11p13 normally imprinted group had a later age of onset compared with the 11p13 LOI group (65.0 mo compared with 48.1 mo), but this difference was not statistically significant ( $P = 0.19$ , Student's *t* test). It is therefore likely that 11p15 imprinting status is the major factor affecting age of onset of WT in this cohort of patients.

#### Imprinting Changes during WT Progression

NRs are thought to be premalignant precursors that can be found associated with many WTs (28). We therefore examined 11p13 and 11p15 imprinting in two examples of hyperplastic perilobar NRs dissected from adjacent to tumors WT62 and WT65 to determine the timing of LOI at 11p13 and 11p15 and their relationship to LOH at 16q and 7p, two loci associated with WT progression (24, 29). These two patients had multiple perilobar NRs that were macroscopically visible and well separated from tumor tissue (see Supplementary Fig. S6), thus the perilobar NRs could easily be dissected free of contaminating WT. Our molecular results, discussed below, showed a pattern of genetic and

**Table 2. Comparison of Clinical and Other LOH Data for 11p15 LOH, LOI, and Normally Imprinted WT**

|                    | Mean Age at Diagnosis ( $\pm$ SD), mo | <i>P</i>                                     | Stage                |                        | <i>P</i> | Relapsed or Died, % ( <i>n</i> ) | <i>P</i> | 7p LOH, % ( <i>n</i> ) | <i>P</i> | 16q LOH, % ( <i>n</i> ) | <i>P</i> |
|--------------------|---------------------------------------|--|----------------------|------------------------|----------|----------------------------------|----------|------------------------|----------|-------------------------|----------|
|                    |                                       |  | I-II, % ( <i>n</i> ) | III-IV, % ( <i>n</i> ) |          |                                  |          |                        |          |                         |          |
| LOH                | 29.0 $\pm$ 16.9 ( <i>n</i> = 23)      | —  | 65 (15/23)           | 22 (5/23)              | —        | 17 (4/23)                        | —        | 14 (3/22)              | —        | 18 (4/22)               | —        |
| LOI                | 51.5 $\pm$ 23.0 ( <i>n</i> = 20)      | 0.001 vs LOH;<br>0.007 vs normally imprinted | 47 (9/19)            | 32 (6/19)              | ns       | 47 (9/19)                        | ns       | 20 (4/20)              | ns       | 45 (9/20)               | ns       |
| Normally imprinted | 25.6 $\pm$ 14.1 ( <i>n</i> = 8)       | ns   | 71 (5/7)             | 29 (2/7)               | ns       | 14 (1/7)                         | ns       | 13 (1/8)               | ns       | 0 (0/8)                 | ns       |

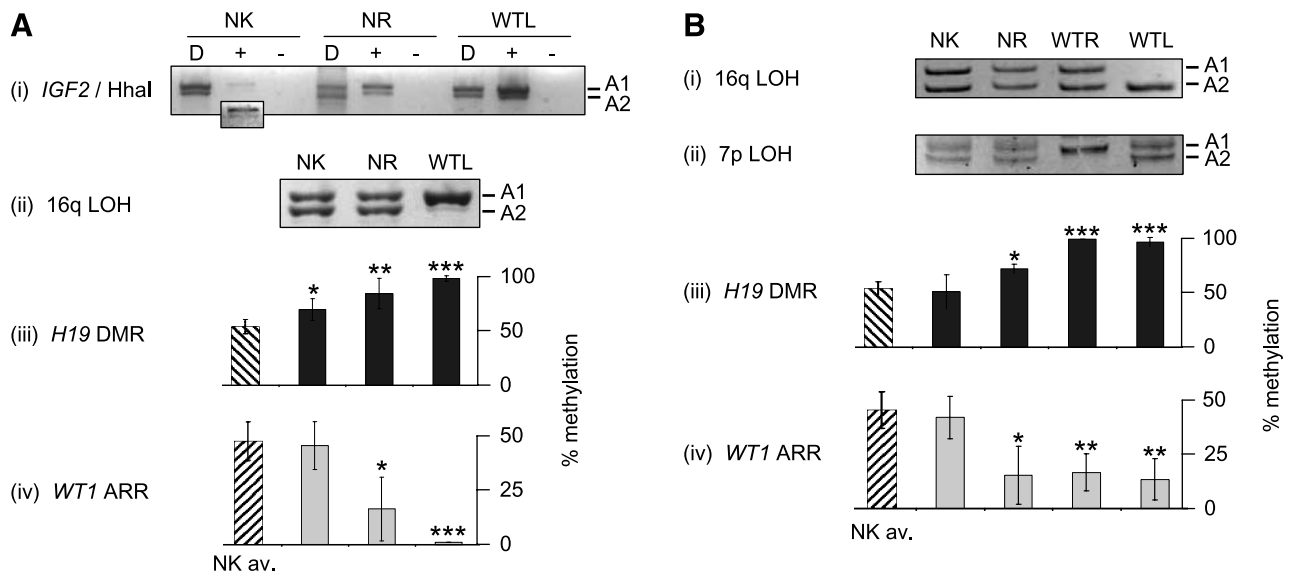
NOTE: Full data for each tumor are given in Supplementary Table S1. Stage and survival data were unavailable for one LOI WT and one normally imprinted WT. Stage V tumor numbers not included in this table. Statistical analysis was by Student's *t* test for age at diagnosis and Fisher's exact test for other variables. Comparison is to the LOH group unless indicated otherwise. ns, nonsignificant ( $P > 0.017$ , Bonferroni's correction for multiple testing).

epigenetic alterations that clearly distinguished these NRs from their corresponding NK and WT, indicating that we dissected these discrete lesions in pure form, uncontaminated by NK or WT tissue.

Patient 62 had bilateral tumors but full analysis is only shown for one WT; the other showed similar changes. This patient was informative for a *HhaI* transcribed polymorphism in *IGF2* and clearly showed LOI in both the NR and WT as shown by biallelic expression of *IGF2* (Fig. 4A-i). This was accompanied by significantly increased *H19* DMR methylation in the NR and WT compared with the average methylation in a group of normal kidney samples (Fig. 4A-iii). Interestingly, apparently histologically normal kidney taken from adjacent to WT62 also showed significantly increased *H19* methylation

(Fig. 4A-iii) and evidence of faint biallelic expression of *IGF2* (Fig. 4A-i), suggesting that there might be an early imprinting defect in patient 62. COBRA analysis of methylation at the *WT1* ARR confirmed our recently published Southern blotting and bisulfite sequencing results (25), showing reduced methylation in the NR compared with the NK, with the WT being almost completely hypomethylated (Fig. 4A-iv). We have shown biallelic expression of both *WT1-AS* and *AWT1* in NR62 and WT62 (25), which is in agreement with the hypomethylation of the *WT1* ARR shown here. WT62 also had LOH of 16q as previously described (29), but this was only found in the WT and not in the adjacent NR (Fig. 4A-ii).

For patient 65, tissues were available from each of two bilateral WTs, and accompanying NR and NK. Like patient 62,



**FIGURE 4.** LOI and LOH in WT progression. **A.** Patient 62. i, LOI of *IGF2* analyzed using the *HhaI* polymorphism. Agarose gel shows genomic DNA product (D), product from RT+ cDNA (+), and product from RT-cDNA (-). Inset under the NK R<sup>+</sup> lane is a higher contrast view. ii, 16q LOH using the D16S539 CA repeat. iii, *H19* DMR methylation as measured by COBRA. Columns, mean percent methylation of three samples for each of NK62, NR62, and WT62; bars, SD. NK av., mean of the results from seven NK samples (excluding NK62 and NK65); bars, SD. iv, *WT1* ARR methylation as measured by COBRA. Columns, mean percent methylation of three samples for each of NK62, NR62, and WT62; bars, SD. NK av., mean of the results from eight NK samples (excluding NK62 and NK65); bars, SD. **B.** Patient 65. i, 16q LOH using the D16S539 CA repeat. ii, 7p LOH using the D7S507 CA repeat. iii, *H19* DMR methylation as measured by COBRA. Columns, mean percent methylation of three samples for each of NK65, NR65, WT65 right (R), and WT65 left (L); bars, SD. NK av., mean of the results from seven NK samples (excluding NK62 and NK65); bars, SD. iv, *WT1* ARR methylation as measured by COBRA. Columns, mean percent methylation of three samples for each of NK65, NR65, WT65 right, and WT65 left; bars, SD. NK av., mean of the results from eight NK samples (excluding NK62 and NK65); bars, SD. In **A**-iii, **A**-iv, **B**-iii, and **B**-iv, asterisks indicate a significant difference compared with the NK av. result. Statistical analysis was by Student's *t* test using Bonferroni's correction for multiple testing. In **A**, \*,  $P < 0.017$ ; \*\*,  $P < 0.0017$ ; \*\*\*,  $P < 0.00017$ . In **B**, \*,  $P < 0.013$ ; \*\*,  $P < 0.0013$ ; \*\*\*,  $P < 0.00013$ . Gels are cropped and shown in negative for clarity. Uncropped gels are presented in Supplementary Fig. S4.

*H19* DMR methylation was significantly increased in the NR and in both WTs, but unlike NK62, methylation in NK65 was within the reference range for other NKs (Fig. 4B-iii). *WT1* ARR methylation was decreased in the NR and both WTs (Fig. 4B-iv), similar to our recently published findings, where we also showed *WT1-AS* LOI in NR65 and in one of the WTs (25). Interestingly, only one of the bilateral WTs showed LOH for 16q (WTL; Fig. 4B-i, and also previously described in ref. 29), and in addition, we found that the other WT had LOH at 7p (WTR; Fig. 4B-ii). The NR had neither 7p LOH nor 16q LOH (Fig. 4B-i and ii).

These results indicate that epigenetic changes at 11p13 and 11p15 occur early in WT development and before either 7p LOH or 16q LOH.

## Discussion

In this article, we have shown that LOI at 11p13 in WT is more common than LOI at 11p15 and that each event is associated with changes in tumor phenotype. The two LOI events both occur at a relatively high frequency (71-83%), which means that most heterozygous WTs will have LOI at 11p13 as well as at 11p15. However, we found that several tumors were discordant for LOI status at 11p13 and 11p15, suggesting that the two events are unlikely to be mechanistically linked. LOI at 11p13 and 11p15 can be found in NRs, before LOH at 7p or 16q, which has important implications for our understanding of how epigenetic and genetic events interact during WT pathogenesis.

### *LOI at 11p13*

LOI at 11p in WT has conventionally been thought of as involving just the 11p15 region because the *WT1* gene at 11p13 was originally shown not to be imprinted in kidney (20) whereas many imprinted genes were found within the 11p15 cluster (8). However, our group has discovered imprinted transcripts at the *WT1* locus (*WT1-AS* and *AWT1*; refs. 21, 22) and characterized a methylation-dependent silencer in the *WT1* ARR that can become deregulated by hypomethylation, leading to LOI of *WT1-AS* and *AWT1* in WT (25). In this article, we have investigated a much larger series of non-LOH WTs than previously examined and showed LOI at 11p13 in >80% (24 of 29) of tumors. We also found that LOI of *WT1-AS* and *AWT1* always occurred together, giving *in vivo* evidence to support our previous *in vitro* experiments, which showed that the silencer in the *WT1* ARR could regulate both the *WT1-AS* promoter and the *AWT1* promoter (25). WTs that retain normal imprinting at 11p13 form a small group of tumors, which seem to have a distinct phenotype of high stage and relatively late age of onset (Table 1), although, clearly, this result needs replicating in a larger series. Overall therefore, these results show that LOI at the *WT1* locus, associated with hypomethylation of the ARR DMR, is a frequent event in WT, in contrast to the very infrequent hypermethylation of the *WT1* sense promoter reported in WT (30, 31). Our results, together with previous reports of methylation changes at or close to the *WT1* ARR in human breast cancer (32), acute myeloid leukemia (33), ovarian clear cell adenocarcinoma (34), and rat mesothelioma and renal cell carcinoma (35), pinpoint the *WT1* ARR as a target for

epigenetic deregulation in a range of cancers. Interestingly, Cooper et al. (36) have found that Beckwith-Wiedemann syndrome patients who have paternal uniparental disomy extending to the *WT1* locus at 11p13 have a higher frequency of WT and nephroblastomatosis, reinforcing the association between deregulated imprinted *WT1* transcripts and carcinogenesis.

The functions of the imprinted *WT1* transcripts are still being investigated, but *WT1-AS* seems to stabilize *WT1* coding transcripts via RNA-RNA interactions (37) and *AWT1* retains transcriptional regulatory activity but with different effects on some target genes and has paradoxical growth-promoting activity compared with the canonical *WT1* proteins (38). Thus, overexpression of these novel *WT1* transcripts could contribute to the development of WT and may be caused by somatic genetic defects (11p13 LOH), somatic epigenetic alterations (*WT1* ARR hypomethylation), or germ-line genetic defects (paternal uniparental disomy in Beckwith-Wiedemann syndrome).

### *LOI at 11p15*

In agreement with previous findings (8), we observed 11p15 LOI in ~70% of WTs. Interestingly, we found a very significantly average older age of diagnosis in WT patients with LOI, compared with both normally imprinted and LOH patients (Table 2), similar to previous reports (15, 39). When we analyzed 11p15 LOI tumors with respect to their 11p13 LOI status, we found no significant difference in age of onset between WTs with and without 11p13 LOI. It therefore seems likely that it is the expression of 11p15 genes, rather than 11p13 genes, that is the major factor affecting age of diagnosis in most WTs.

11p LOH in WT most often involves mitotic recombination events, leading to reduplication of the retained paternal allele (19), and thus both LOI and LOH tumors should be expressing two *IGF2* alleles and lack *H19* expression. Although it was suggested that LOH and LOI are not related to *IGF2* expression levels in WT (40), a more recent study has shown a reproducible and specific 2-fold elevation of *IGF2* RNA levels in LOI WTs compared with normally imprinted tumors (15). This implies that the active gene dosage of *IGF2* does determine its expression level in WT and, therefore, phenotypic differences between 11p15 LOH and LOI WTs are unlikely to be associated with *IGF2* because both will be expressing two *IGF2* alleles.

However, one obvious difference between 11p15 LOH and LOI tumors is that LOI specifically affects the IC1 region of the 11p15 imprinting cluster, which controls *IGF2* and *H19* expression, whereas LOH affects both IC1 and IC2 (containing the *Kv* DMR). This is well illustrated by the difference between *H19* DMR and *Kv* DMR methylation in our series of WTs. LOI WTs showed hypermethylation of the *H19* DMR, with levels of methylation of the *Kv* DMR similar to those seen in normal kidney, whereas LOH WTs showed *H19* hypermethylation and complete loss of *Kv* DMR methylation (Supplementary Fig. S5). Others have also shown that the *Kv* DMR is not often hypomethylated in non-LOH WTs but is hypomethylated in heterozygous tumors (30, 41). Thus, a possible explanation for the late-onset phenotype in 11p15 LOI WTs might be that they lack the alerted expression of imprinted genes in the IC2 domain such as *CDKN1C* that should occur in 11p15 LOH

WTs. Support for this comes from the phenotypic differences that are observed in different groups of Beckwith-Wiedemann syndrome patients, depending on whether they have IC1 and/or IC2 defects (36). However, imprinting of human *CDKN1C* is incomplete in some tissues and the normally less active paternal allele can be expressed in 11p15 LOH WTs, so that often there is no significant difference in *CDKN1C* expression between LOH and heterozygous WTs (42-44). This suggests that if the expression of 11p15 IC2-regulated imprinted genes is responsible for the phenotypic differences between LOH and LOI tumors, then other genes apart from *CDKN1C* are more likely to be responsible; some have already been shown to have altered expression in WT (8).

#### *Relationship between LOI at 11p13 and LOI at 11p15*

In this series of WTs, we did not find any evidence linking LOI at 11p13 with LOI at 11p15. There was no correlation between methylation levels at the *WT1* ARR at 11p13 and the *H19* DMR at 11p15 ( $r = -0.1256$ , Spearman rank order correlation coefficient;  $P = 0.52$ ). This is exemplified by the similar levels of *WT1* ARR methylation found in 11p15 LOI and 11p15 normally imprinted WTs (Supplementary Fig. S5). Additionally, our allelic expression analysis revealed that tumors with *WT1-AS* and/or *AWT1* LOI could have *IGF2* LOI or not. The lack of a relationship between 11p13 and 11p15 LOI is further suggested by our finding that LOI at 11p13 and 11p15 are associated with opposing trends in age of diagnosis in WT (Tables 1 and 2). Thus, it seems that LOI at the *WT1* locus and at the *H19/IGF2* locus are probably mechanistically unlinked and involve the generation of separate epigenetic defects at 11p13 and 11p15.

Other reports also support the view that LOI is not caused by a generalized genome-wide failure of imprinting in WT. These include reports that have excluded imprinting defects in the 14q *DLK1/GTL2* imprinting cluster in WT (45, 46) and a study of allele-specific expression and methylation of imprinted genes in 11p15 LOI WTs, which showed that the only major alterations were confined to the *IGF2/H19* region (47). However, the study we report here represents the first to directly compare two different loci that each show LOI in WT.

#### *LOI in WT Progression*

One way to attempt to identify the possible causes of LOI in WT is to correlate imprinting alterations with other genetic defects and to relate these changes to the WT progression pathway by examining normal tissue, NR, and tumor from the same patient. Previously, we have used this approach to show that 11p LOH and *WT1* mutation are relatively early events in WT development, being found in NRs, whereas 16q and 7p LOH appear as later events, being confined to tumor tissue (24, 29).

In this article, we have found that LOI at both 11p13 and 11p15 can be found in NRs, whereas 7p and 16 LOH are not observed in the NRs, only in the corresponding WTs (Fig. 4). This implies that LOI at 11p13 and 11p15 occur earlier in WT development than either 16q LOH or 7p LOH. Yuan et al. (19) also concluded that 16q and 7p LOH occurred later than 11p15 LOI on the basis of mosaicism for 16q LOH in cultured WT cells that had complete LOI and the finding of LOI in the NK of

a patient who had 7p LOH in their tumor. However, our results are the first to examine histologically distinct premalignant lesions (NR) for both LOI and 16q and 7p LOH and to examine LOI at 11p13 as well as at 11p15.

Mummert et al. (48) had previously hypothesized that 16q LOH might cause LOI in some cases of WT via CTCF haploinsufficiency, based on a very strong association that they detected between 16q LOH and 11p15 LOI. Other studies have not consistently found an association between 11p15 LOI and 16q LOH (19, 39, 49), and in our results we saw an increased incidence of 16q LOH in 11p15 LOI WTs but this did not reach statistical significance (Table 2). Clearly, our comparison of the timing of LOH and LOI in WT progression (Fig. 4) precludes a causative role for either 16q or 7p LOH in the establishment of epigenetic defects leading to LOI. However, it is interesting to speculate why 16q and 7p LOH occur after genetic and epigenetic alterations at 11p. The phenotypic effects of 7p LOH are not yet apparent but 16q LOH is clearly associated with a poor prognosis in WT (1). Given that 16q LOH occurs after 11p LOH or LOI and therefore in the context of up-regulated *IGF2* and/or *WT1-AS/AWT1* expression, it is possible that the gene(s) inactivated by 16q LOH act synergistically with increased *IGF2* and/or *WT1-AS/AWT1* to produce a poor-prognosis phenotype in WT.

## Materials and Methods

### *Tissues*

Human fetal kidney (15-18 wk gestation), kidney taken adjacent to WT, and WT were obtained as frozen tissues from local hospitals with appropriate consent and ethical approval and stored as prescribed under the U.K. Human Tissue Act.

Tumors WT62 and WT65 were from patients with bilateral tumors that had macroscopically visible multiple perilobar NRs adjacent to them. The perilobar NRs were well separated from tumor tissue (see Supplementary Fig. S6) and perilobar NR nodules ~0.5 cm in diameter were directly excised from frozen tissue and used for DNA and RNA extraction.

### *DNA and RNA Extraction*

Genomic DNA was extracted from frozen tissue by conventional proteinase K/phenol-chloroform methods. Total RNA was made using TRI Reagent (Sigma) according to the manufacturer's instructions.

### *LOH Analysis*

LOH was assessed using RFLP and/or microsatellite polymorphisms on 7p, 11p, and 16q as previously described (23, 24).

### *Methylation Analysis*

COBRA was carried out essentially as described by Xiong and Laird (50). Genomic DNA was bisulfite modified using an EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions. Approximately 50 ng of bisulfite-modified DNA were denatured at 95°C for 3 min and then amplified for 40 cycles [94°C 15 s, 53°C ( $K_v$ )-55°C (*H19*, *WT1*) 30 s, 72°C 60 s] by PCR in a volume of 25  $\mu$ L, using either HotStarTaq Plus (Qiagen) or Jumpstart (Sigma) enzymes according to the manufacturer's instructions. Primer sequences



were as follows (all 5'-3'): *H19* DMR, TAGGATTTTTGTGT-TGTTGGAGATA (forward) and ACACCTATAAACAATT-CACCTCTC (reverse); *WT1* ARR, TTGAGGTTTGTGTTT-TTGATT (forward) and ACCCTCTCCATTAACACTTTT (reverse); and *Kv* DMR, GTTATTTTATATTTAGT-TAGTGTTTTATG (forward) and TCTTACTAAAAAATC-CCTAAAAATC (reverse). PCR product (5-10  $\mu$ L) was then digested with 5 units of the appropriate restriction enzyme at 37°C for 2 h. Ethidium bromide-stained agarose gels of digested COBRA PCR products were scanned on a video densitometer (UV products) and band intensities quantified using Scion Image for Windows software.<sup>3</sup> For zero methylation controls, we used human genomic DNA *in vitro* amplified isothermally using  $\phi$ 29 DNA polymerase (Genomiphi V2 kit, GE Healthcare). For 100% methylation control, Universal Methylated DNA was used (Chemicon). Linear response of the COBRA assays was validated by mixing known ratios of 0% and 100% methylated DNA and correlating the percentage of input fully methylated DNA versus the COBRA-measured percentage methylation ( $r^2 = 0.94-0.97$ ).

#### Allelic Expression Analysis

Reverse transcription-PCR of transcribed polymorphisms was used as previously described to investigate imprinted expression of *IGF2* (27), *WT1-AS*, and *AWT1* (21, 22, 25).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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<sup>3</sup> <http://www.scioncorp.com>

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## Frequency and Timing of Loss of Imprinting at 11p13 and 11p15 in Wilms' Tumor Development

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