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Imbalanced Expression of Functionally Different WT1 Isoforms May Contribute to Sporadic Unilateral Wilms' Tumor

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Functional loss of the product of the Wilm's tumor suppressor gene (wt1) has been identified in subsets of familial Wilms' tumors. Previously, four alternative splice products of WT1 were recognized and each was found to regulate transcription of effector genes differently, suggesting that disruption of the normal ratio of these spliced products will disrupt the normal expression patterns of WT1 effector genes and perhaps lead to Wilms' tumor. In support of these suggestions, we found that four of seven cases of sporadic unilateral Wilms' tumor had striking differences in the ratios of the spliced products of WT1 compared with each other and normal kidney. These data indicate that in addition to structural mutations, alterations in the relative amounts of the mature WT1 isoforms may also be important in the etiology of sporadic Wilms' tumor.

Wilms' tumor, or nephroblastoma, is a blastemal cell malignancy of kidney that affects approximately 1 in 10,000 children worldwide [1]. Genetic analysis of Wilms' tumors led to the identification of the Wilms' tumor susceptibility gene, *wt1* [2,3]; *wt1* contains ten exons spanning 50 kb of DNA and encodes a 3 kb mRNA [4]. The predicted WT1 amino acid sequence has several features that are characteristic of transcription factors, including four contiguous Cys_2–His_2 type zinc-finger motifs clustered near the C-terminus and an amino terminus that is rich in proline and glutamine [2,3] and domain(s) that functionally facilitate dimerization [13].

Two alternative splicing events give rise to four distinct WT1 isoforms [4]. Importantly, the different isoforms of WT1 function differently as transcriptional regulators and recognize different DNA elements [5]. For this reason, the relative contributions of each of the WT1 isoforms is of great importance in determining which and at what levels downstream effectors is expressed. Alternatively spliced form I contains a separate exon (exon 5) that introduces 17 amino acids between the proline-rich domain and the zinc-finger region [4]. This domain alone functions as a transcription repressor and alternate splice form I functions differently in transcription regulation than the other alternative splice forms of WT1 [5]. Alternative splice II introduces three amino acids (lysine, threonine, serine) that disrupt the normal spacing between zinc fingers 3 and 4 and extend the DNA recognition sequence that is required for binding beyond the previously described consensus DNA binding site [5]. The relative ratio of the four *wt1* mRNA species is constant in all human and mouse tissues studied that express WT1 [4]. As a consequence, when the normal ratios of the WT1 isoforms are altered, disruption in normal WT1 transcriptional regulation results and deregulation of the pathways normally regulated by WT1 is likely to alter expression levels of important target genes.

In this report, we demonstrate that the ratios of the WT1 isoforms that contain exon 5 with those that lack exon 5 differ in four of seven sporadic, unilateral Wilms' tumors in comparison to the ratio of a normal control. Based on previous findings that the product of exon 5 contains a potent negative regulatory domain, the results point clearly to the possibility that differences in the expression levels of exon 5 of WT1 may contribute to imbalanced expression of its effector genes and to Wilms' tumor.

MATERIALS AND METHODS

Samples of sporadic, unilateral Wilms' tumor were obtained from the Zin Hua Hospital, the Ren Jin Hospital, and the Children's Hospital at Shanghai Medical University at Shanghai, P.R. China. The samples were snap frozen in liquid nitrogen at the time of

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surgical resection and kept at −70°C. Total cellular RNA was extracted from frozen tumor tissue according to the method described by Chirgwin et al. [9], and first-strand synthesis was performed according to Noonan and Roninson [10]. Initial PCR amplification proceeded for 3 min. at 94°C, followed by 35 cycles of 1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C, finished by a 5 min. final extension at 72°C. PCR products were analyzed on 1.5% agarose gels. The gels were photographed with the gel documentation system 100 (Bio Rad) and the resulting digitized images were analyzed using densitometry software (Molecular Analysis, Bio Rad).

RESULTS AND DISCUSSION

Since alternatively spliced exon 5 encodes a powerful negative regulatory domain [5], increased levels of expression of exon 5 in WT1 isoform I relative to WT1 isoforms that lack exon 5 are likely to significantly reduce the levels of expression of effector genes otherwise expressed at higher levels. In support of this hypothesis, we previously showed that expression constructs that encode WT1 isoforms that lack exon 5 activated a reporter gene but the isoforms act as powerful repressors in the presence of exon 5 [5]. To seek the possible relevance of these findings to Wilms' tumor, we measured the ratios of the *wt1* isoforms in normal fetal kidney (as control) by RT-PCR analysis and in samples from seven patients with unilateral, spontaneous Wilms' tumor. Patients with the related WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities and mental retardation), the Beckwith-Wiedmann Syndrome (enlargement of the tongue and visceral organs, umbilical hernia, and neonatal hypoglycemia), or the Denys-Drash syndrome were excluded.

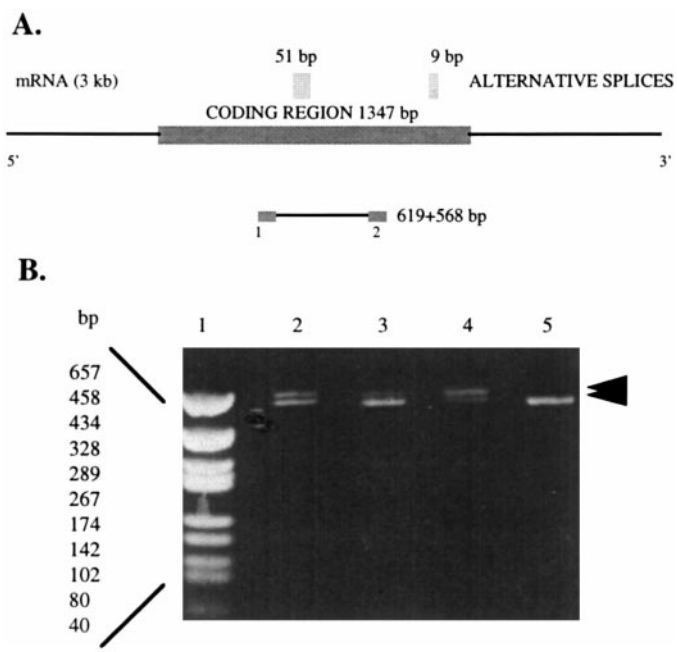

FIG. 1. (A) Schematic diagram of PCR primers used to amplify the *wtl* RNA. The DNA insertions introduced by alternative splicing are indicated. The positions of the primers 1 and 2 in the transcript are shown. Primer sequences are the same as designed by Brown et al. [12]. The size of the fragments obtained with these primers is shown below. (B) Agarose gel electrophoresis of RT-PCR products of WT 1. Lane 1: pGEM72f(+) III Hae molecular weight marker. Lane 2: PCR product from Case 4. Lane3: PCR product from Case 222. Lane 4: PCR product from normal fetal kidney. Lane 5: PCR product from a WTl plasmid containing *wtl* cDNA without exon 5 as positive control.

TABLE 1

Summary of the Ratios of wt1 mRNA With or Without Exon5 in Human Fetal Kidney and Four Cases of Sporadic Unilateral Wilms' Tumor

	Fetal kidney	Wilms tumors(cases)			
		4	37	98	222
WT1 +17aa : WT1 -17aa	2:1	1:1	1:5	1:5	1:5

Figure 1A demonstrates the primers and the anticipated size of the unique PCR products used in this analysis. Amplification of the 3′ end of exon 1 and exons 2–5 results in a band of 619 bp whereas amplification of the identical 3′ end of exon I and exons 2–4 but not exon 5 generates a smaller band of 568 bp. Total RNA from seven Wilms' tumors was examined. The data from two tumor samples and normal fetal kidney tissue are illustrated in Fig. 1 and demonstrate clear differences in the relative ratios of exon 5 containing vs. exon 5 lacking transcripts. In each instance, the results were repeated and tested at two different concentrations of mRNA. In four of seven samples analyzed, the ratio of WT1 containing wt1 isoforms that contain exon 5 relative to WT1 lacking exon 5 differed strikingly from the ratios of these isoforms in normal fetal kidney tissues (Table 1). Figure 1 also illustrates examples of these differences. In Lane 3 (Case 222), the relative level of the WT1 isoform without exon 5 is far greater than the level of the WT1 isoform with exon 5. In Lane 2 (Case 4), the relative levels of these two isoforms are essentially the same. In contrast, the relative ratios of the two isoforms in control normal fetal kidney (Lane 4) are 2:1, the precise ratios previously observed in normal tissues [4]. Furthermore, in Table 1, the ratios of the *wt1* isoforms with and without exon 5 in two other cases are similar to Case 222 illustrated in Figure 1. The results suggest the possibility that this unique alteration in expression levels of the WT1 isoforms is relatively common in sporadic Wilms' tumors.

In these experiments, data is presented to support the possibility that differences in the relative levels of expression of WT1 isoforms containing exon 5 to the WT1 isoforms that lack exon 5 may be commonly found in patients with sporadic Wilms' tumors. The results suggest that these differences in relative levels of expression of WT1 isoforms containing exon 5 are significant and may be characteristic of subsets of sporadic unilateral Wilms' tumor. Alterations in the normal ratios of the alternatively spliced isoforms of WT1 have

also been found in patients with Denys-Drash syndrome [7] and disruption of the normal splicing of exon 5 of wt1 has been found in Wilms' tumor [8]. In previous experiments, we demonstrated that the 17 amino acid residues encoded by exon 5 are capable of acting independently as atranscriptional repressor. When exon 5 is present in the naturally found isoforms of WT1, the repressor activity is dominant to the other regulatory domains of WT1 [5,11]. Thus, the four different WT1 isoforms have different regulatory properties. We have speculated that these isoforms may act as a regulatory switch to differentially regulate expression of different target genes at different times during development. We also speculate that if the relative levels of the *wt1* alternative splice products are changed, the normal levels of expression of the downstream genes are disrupted and lead to tissue specific development arrest and/or neoplasia. Our results suggest that strict maintenance of the precise ratios of different WT1 isoforms may be critical during kidney and gonadal development and that dysregulation of the balance of *wt1* splicing isoforms may contribute to the genesis of Wilms' tumor.

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