Pergamon

European Journal of Cancer Vol. 31A, Nos 13/14, pp. 2270–2276, 1995
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0959–8049/95 $9.50+0.00

0959-8049(95)00474-2

Original Paper

Splicing of Exon 5 in the *WT1* Gene is Disrupted in Wilms' Tumour

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Using a reverse transcriptase polymerase chain reaction to examine alternate splicing at site I (exon 5) and site II (exon 9) in the Wilms' tumour suppressor gene, *WT1*, we found that in seven of the 10 Wilms' tumours examined, splicing at site I was disrupted. This is predicted to result in isoform imbalance in Wilms' tumours, with an increase in isoforms in which the 17 amino acids encoded by exon 5 are missing. These observations could not be explained by mutations or rearrangements in flanking introns. Disrupted alternate splicing of exon 5 may play a role in the aetiology of Wilms' tumour.

Key words: *WT1* gene, alternate splicing, Wilms' tumour
Eur J Cancer, Vol. 31A, Nos 13/14, pp. 2270–2276, 1995

INTRODUCTION

WILMS' TUMOUR is a paediatric nephroblastoma that arises from the metanephric blastema of the kidney. The *WT1* suppressor gene has been implicated in the aetiology of Wilms' tumour by observations of homozygous deletions and critical point mutations in functionally important regions of the gene in a subset of Wilms' tumours, reviewed in [1]. The gene encodes a protein with four zinc fingers of the Cys_2-His_2 type at the C-terminus, both repressor and activator domains near the N-terminus, and undergoes alternate splicing in exons 5 and 9, generating four distinct isoforms. RNA editing, converting a leucine residue to proline in exon 6 may also confer further complexity to the array of *WT1* isoforms expressed in human tissues with a total of eight isoforms theoretically possible [2]. Exon 9, encoding the third zinc finger, is alternately spliced yielding + and − KTS amino acid insertions and exon 5 is alternately spliced yielding isoforms with or without 17 amino acids (aa). Four transcripts are expressed in human fetal kidney.
Splice D: + 51 base pairs (bp)/ + 9 bp (encoding + 17 aa)/ + KTS),
Splice C: − 51 bp / + 9 bp (encoding − 17 aa + KTS)
Splice B: + 51 bp / − 9 bp (encoding + 17 aa − KTS)
Splice A: − 51 bp / − 9 bp (encoding − 17 aa − KTS).

Alternate splicing in exon 9 alters the configuration of the zinc fingers allowing + KTS isoforms to bind to DNA target sequences distinct from those bound by − KTS zinc fingers [3]. The − KTS isoforms bind to DNA targets with EGR1-like consensus sequences and are hypothesised to regulate the transcription of genes with these elements in their promoters such as insulin-like growth factor (IGF)-2, IGF-1 receptor and platelet-derived growth factor (PDGFA). The physiological targets of the predominant + KTS isoforms are currently unknown. Maintenance of the correct balance between + KTS and − KTS isoforms is apparently critical in sustaining correct *WT1* gene function and normal urogenital development, with point mutations reported in the splice donor site flanking exon 9 in patients with Denys–Drash syndrome [4, 5]. These mutations are predicted to alter the distribution of KTS isoforms, reducing the expression of + KTS relative to − KTS isoforms, and thus to contribute to the developmental abnormalities seen in the genitourinary systems of these individuals.

Despite the evidence that the balance between the KTS isoforms of the *WT1* gene is important in normal genitourinary development, there have been few systematic studies examining splicing patterns of the *WT1* gene in Wilms' tumours. In an early study, Haber and associates [6] examined five Wilms' tumours by RNAase protection and reported no significant differences in *WT1* splicing in this group. Brenner and associates [7] developed a polymerase chain reaction (PCR)-based method to examine alternate transcription in the *WT1* gene, and reportedly observed alterations in exon 5 splicing between individual tumours, although no quantitative analysis was done. However, neither of these previous studies compared normal paired kidney tissue with tumour tissue, so that the variations noted between individual tumours could have been attributed to normal individual sample variation. Haber and associates [8] more recently identified novel transcripts of the *WT1*, WT*del2*, in Wilms'

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Revised 7 Aug. 1995; accepted 8 Aug. 1995.

tumour cell lines, in which both exons 2 and 5 were skipped. The WT*del2* splice variant was postulated to compete with the transcriptional repressor properties of the normal *WT1* splice forms by activating transcription, and to thus abrogate *WT1* gene function *in vivo*.

In this study we have examined the splicing patterns of the *WT1* gene in Wilms' tumour tissue and in the adjacent normal kidney tissue using a PCR-based methodology. We report here that, in a significant number of sporadic Wilms' tumours, splicing in exon 5 is disrupted. This is predicted to result in an imbalance in *WT1* isoform ratios with −17 aa *WT1* isoforms becoming dominant in tumours. No evidence for the presence of WT*del2* transcripts was found in these tumours. Our results suggest that disrupted splicing of exon 5 in the *WT1* gene may, therefore, contribute to the aetiology of Wilms' tumour.

MATERIALS AND METHODS

Reverse transcriptase (RT) PCR

Wilms' tumour and autologous normal kidney tissue were snap frozen in liquid nitrogen immediately after surgical resection. Total RNA was extracted according to the method of Chomczynski and Sacchi [16] and was reverse transcribed using 200 U of Moloney murine leukaemia virus RT in 1 × RT buffer (Promega) containing 6.5 U RNAsin, 1 mM dNTPs and 1 nmol random hexamers. Reactions were incubated at 37°C for 60 min. PCR amplification across the exon 5, 51 bp *WT1* cDNA insert, was performed with primer pairs P1 and P2 and P1 and P3 and across the 9 bp, exon 9 splice, with primers 920 and 921. Primers W5 and W12 were used to amplify across exon 2. Amplification was performed in a DNA thermal cycler (Perkin Elmer/Cetus) using Promega reaction buffer and Taq polymerase. [^{35}S]dCTP (0.25 mM) replaced cold dCTP in the reaction mix in PCR where densitometry was later performed on the products. All other dNTPS were 0.25 mM. Primer sequences used were as follows:

P1 (exon 2), 5′ ATGAGGATCCCATGGGCCAGCA 3′; P2 (exons 9 and 10), 5′ ACAGCTGAAGGGCTTTTCAC TTGTTTTAC 3′; P3 (exons 9 and 10), 5′ ACAGCTGAA GGGCTTTTCAC CTGTATGAG 3′; P920 (exon 8), 5′ TAC CAGTGTGACTTCAAGGAC 3′; P921 (exon 10), 5′ CATGTTGTGATGGCGGACTAA 3′; W5 (exon 1), 5′ CCGCCTCACTCCTTCATCAAACAGGAGCC 3′; and W12 (exon 8), 5′ CCTTTGGTGTCTTTTGAGCTGGTC TGAACG 3′.

Amplification conditions were as follows: P1 and P2, P1 and P3, 30 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 2 min; P920 and P921, 30 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 2 min; W5 and W12, 30 cycles of 94°C for 1 min, 65°C for 2 min, 72°C for 2 min. Unlabelled products were visualised on either 2% agarose or 12% polyacrylamide gels containing 10% glycerol run in 1× TBE buffer following staining with ethidium bromide. ^{35}S-labelled products were run on polyacrylamide gels as described above and subjected to autoradiography using sensitive film.

Autoradiographs were subsequently scanned using an ultrascan laser densitometer (LKB) for band quantitation. The average of three scans was taken as the measure of band intensity in the calculation of band ratios. The phase of the PCR reaction that best approached exponential was established by the quantitation of ^{35}S-labelled product from a single reaction performed in duplicate, where aliquots were removed after 22, 24, 26, 28, 30, 32, 34 and 36 PCR cycles. Product could not be detected below 24 cycles. This was performed on samples containing 1000 ng of cDNA (the maximum amount of cDNA used in any reaction), such that an upper limit of cycle number could be established beyond which the reaction approached saturation. Thirty cycles were within the maximal amplification phase in the samples examined, and this was, therefore, selected as the optimum number of amplification cycles for alternate transcript ratio calculations.

Mouse cDNAs encoding the four alternate transcripts were amplified after mixing −51 bp and +51 bp cDNAs in the following proportions: +51 bp/−51 bp, 4/1, 2/1, 1/1 and 1/2. Serial dilution of cDNA template was performed to give cDNAs in the range 100–1000 ng for experiments where −51 bp/+51 bp ratio variation with template concentration was examined.

Competitive PCR

Internal standards for competitive PCR were generated as described by Forster and colleagues [17]. Quantitation of −51 bp transcripts was performed according to the following protocol. Primers P4 and P5 were used to amplify a 292 bp product from *WT33* cDNA following which a second, reverse 3′ linker primer, P6, was used to generate a nested, 172 bp product, from a 10^3 dilution of the original 292 bp product. This 172 bp product was then reamplified, following a 10^3 dilution, using the original primer pair P4 and P5. Primer P5 contained a region of 10 bases at the 3′ end homologous to 10 bases at the 5′ end of the linker primer, P6. The 182 bp product generated was used as the internal standard in competitive reactions where sample cDNA of fixed concentration was coamplified with selected dilutions of the internal standard. Normal tissue and tumour PCR reactions from the same individual were compared at fixed cDNA and internal standard concentrations. ^{35}S-labelled products were separated on 12% polyacrylamide gels and quantitated using scanning densitometry as described above. Quantitation of +51-bp transcripts was conducted using primer Pex 5 as the forward primer, P5 as the reverse primer and primer P6 as the linker primer, following essentially the same protocol as described above. Primer sequences were:

P4, 5′ CACCTTAAAGGGCCACAGCAC 3′
Pex, 5 5′ AGCTCCAGCTCAGTGAAATG 3′
P5, 5′ GTCCTTGAAGT**CACACTGGTA** 3′
P6, 5′ **CACACTGGTA**CTCACTGGTCTCAGATC 3′.

The overlapping 10-bp sequence between P5 and the linker primer P6 is indicated by bold type.

Northern blotting

Ten microgrammes of total RNA were subjected to electrophoresis under denaturing conditions in 1% agarose gels containing 3% formaldehyde. RNA was then blotted on to Nylon membranes (Hybond N) which were then fixed in 50 mM NaOH. Filters were prehybridised in 6 × SSPE/2.5 mM NaPPi pH 6.5/5 × Denhardt's /0.5% SDS / 150 μg/ml salmon sperm DNA at 65°C and then hybridised under the same conditions with the 1.8 kb fragment of probe *WT33* radiolabelled using the random primer method of Feinberg and Vogelstein [18]. After overnight hybridisation, blots were washed once with 2 × SSPE/0.1% SDS, once with 1 × SSPE / 0.1% SDS and once with 0.5 × SSPE/0.1% SDS at 65°C.

Southern blotting

Genomic DNA was extracted from snap frozen tissues, digested with EcoR1, BgIII or Sac1, and subjected to electrophoresis and Southern blotting as described previously [19]. Filters

were probed with a 187 bp fragment of *WT1* cDNA prepared by digestion of a PCR product, derived from the amplification of normal kidney cDNA, with Bsml. This probe was designed to span exons 4, 5 and 6.

Primers W7 (forward), 5'ACGCCCTCGCACCATGCGG CGCAGTTCCCC3' and W12 (reverse) 5'CCTTTGGTGTC TTTTGAGCTGGTCTGAACG3' were used for PCR of 1000 ng of normal kidney cDNA under the following conditions: 94°C for 1 min, 65°C for 2 min and 72°C for 1 min. The 689 bp product amplified was then digested with Bsml and the 187 bp fragment gel-purified and labelled using the random priming method. Filters were hybridised as described for northern blotting. Bands were identified using the *WT1* restriction map information in Tadokoro and colleagues [9].

Densitometry was performed and the relative intensities of bands corresponding to exon 5 genomic fragments were compared between normal and tumour tissue relative to the intensities of bands representing exons 4 and 6.

Sequencing

Exon 5 and flanking splice donor and acceptor sites were amplified with intronic primers 5' ex 5 and 3' ex 5 from 200 ng of genomic DNA under the following conditions: 30 cycles of 94°C for 1 min, 61°C for 1 min and 72°C for 1.5 min. Primer sequences used were:

5' ex 5, 5'GCTCCATTCCCCACTCCCCACCTCT3'; and

3' ex 5, 5'TTGCTTTGCCATCTCCGCATTGTCC3'.

PCR products were purified for sequencing by electrophoresis through 0.6% SeaPlaque agarose gels (FMC, U.S.A.), followed by phenol extraction and ethanol precipitation. Primers used for the PCR amplification were used as primers in dideoxy cycle sequencing reactions, which were performed using an ABI (U.S.A.) cycle sequencing kit according to the manufacturer's instructions. Sequences were run on an ABI 373 A automated sequencer according to the manufacturer's instructions. The intron upstream of exon 5 was amplified using primer 5' ex 4: 5'GTGGTTATGTGTTCTAACTCTAGAT3', and reverse primer 3' ex 5 (described above) from 200 ng of genomic DNA in long PCR buffer composed of 0.3 M Tricine pH 8.4/0.02 M $MgCl_2$/0.05 M B-mercaptoethanol/0.1% gelatin/1% Triton X-100. Cycling conditions were 30 cycles of 92°C for 1 min, 54°C for 1 min and 72°C for 5 min. The 1.2 kb products were run on 20 cm 1% agarose gels and visualised by ethidium bromide staining.

RESULTS

PCR analysis of WT1 *alternate splicing in Wilms' tumours*

All tumours examined in this study were sporadic and of varying histology. Tumours WT30, WT86, WT71 and WT73 were either purely or predominantly blastemal, WT78 and WT91 were triphasic and WT81 and WT94 were predominantly epithelial. For tumours WT92 and WT98, there was no pathology information available.

PCR was used to amplify the alternately spliced transcripts of *WT1* in Wilms' tumour samples and their paired normal control kidney tissue, using a strategy similar to that previously described by Brenner and associates [7]. Figure 1 depicts the location of PCR primers with respect to *WT1* exons. Primers were designed to amplify across the alternately spliced exon 5 region and to amplify transcripts with or without the 51 bp insert and including (P1 and P2) or excluding (P1 and P3) the 9 bp exon 9 splice. Primers 920 and 921 were designed to amplify across the alternately spliced exon 9 region. In addition,

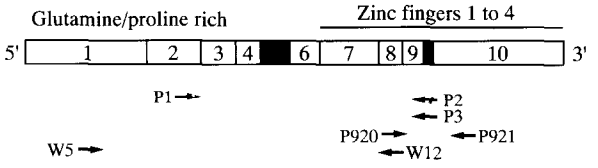


Figure 1. Schematic diagram of the *WT1* gene. The two alternative splice sites are shown in black and the positions of the exonic primers P1, P2, P3, P920, P921, W5 and W12 are shown below. The hatched arrow (P2) represents the KTS insert between zinc fingers 3 and 4.

a forward primer located in exon 1, W5, and a reverse primer located in exon 8, W12, were used to identify transcripts in which exon 2 was alternately spliced, as described by Haber and colleagues [8]. ^{35}S-labelled dCTP was incorporated into the PCR reaction to enable quantitation by densitometry of the relative levels of *WT1* transcripts amplified within each paired sample, following electrophoresis of PCR product on 12% polyacrylamide gels. Relative proportions of *WT1* transcripts within each individual PCR sample were examined after 30 cycles of PCR, which was previously determined to be within the maximal phase of amplification in the reaction under the conditions used (Figure 2). Paired samples were amplified simultaneously using the same reagent mix to reduce reaction variation. The suitability of PCR as a reliable method for the analysis of the true proportions of *WT1* alternate transcripts within individual samples was assessed by subjecting known combinations and amounts of purified mouse *WT1* splice variant cDNAs to PCR. There were two mismatches in forward primer P1, with the mouse cDNA sequence, at positions 2 and 8 relative to the 5' end with a T in the human primer sequence replaced by a C in the mouse sequence. There were no mismatches in reverse primers P2 and P3. Despite the mismatches in primer P1, the mouse *WT1* cDNA amplified efficiently. There were no mismatches between primer 920 and the mouse cDNA sequence, and one mismatch, with an A in the human sequence substituted by a G, in the mouse sequence at position 19 from the 5' end of primer 921. When the purified mouse cDNAs were amplified in these

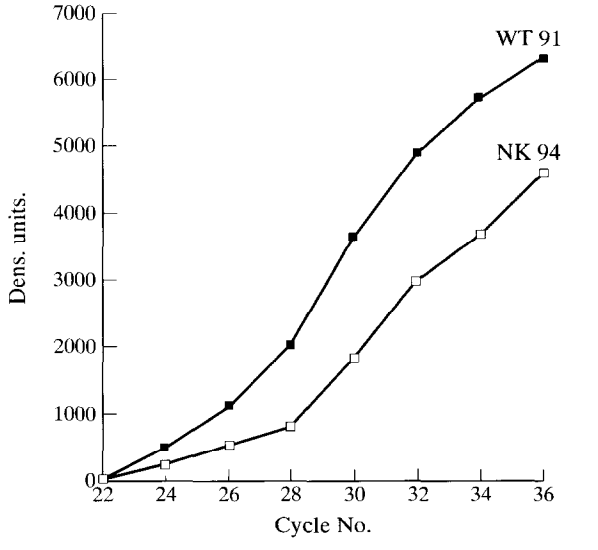


Figure 2. Curve showing amplification of the *WT1* gene from 1000 ng Wilms' tumour (WT91) and normal kidney (NK94) cDNA in the range 22 to 36 PCR cycles. Alternate transcript ratio calculations were made at 30 PCR cycles within the maximum amplification phase.

reactions, the individual alternately spliced transcripts were amplified in the same proportions in which they had been added at the outset of the reaction, suggesting that there was no bias towards the amplification of shorter transcripts. Furthermore, increased concentrations of *WT1* template cDNA in tumour samples did not contribute to aberrant amplification of transcript cDNA as transcript ratios remained essentially constant following serial dilution of cDNA from 1000 to 100 ng. There were no significant differences in the transcript ratio data obtained when amplification cycle number was varied below 30 cycles with 25-cycle PCR data being essentially the same as that at 30 cycles.

Differential alternate splicing in the WT1 *gene in Wilms' tumours*

The relative proportions of *WT1* splice variants with and without the exon 5, 51 bp insert, were calculated in 10 tumours and their paired normal kidney. Of these, seven tumours showed an increase in the proportion of −51 bp to +51 bp amplified transcripts, relative to this ratio calculated in the paired normal kidney. Splicing of the 9 bp insert in exon 9 was unchanged in these tumours, suggesting that the distribution of the 9 bp splice in the −51 and +51 bp transcripts was unchanged relative to that in the normal kidney. No evidence for transcripts in which exon 2 was absent could be found in this tumour panel. Table 1 summarises the average values obtained for *WT1* transcript ratios in our panel of tumours relative to their normal kidney controls. The *P* value was calculated using the Student's paired *t*-test and illustrates that the changes noted in exon 5 splicing in the tumour population were statistically significant. Figure 3 shows graphical representation of experimental data obtained for seven tumours from the sample population and includes a tumour, 81, for which little change in exon 5 splicing could be found. Figure 4 shows representative autoradiographs of exon 5 splicing patterns in two tumours and their paired normal tissue. The relative increase in −51 bp transcript can be clearly seen in the tumour samples.

WT1 expression was increased in all tumours relative to the expression in normal kidney as demonstrated by Northern blotting (results not shown). The observed increase in the −51 bp/+51 bp transcript ratio was, therefore, predicted to occur as a result of an increase in −51 bp transcripts rather than as the result of a relative decrease in +51 bp transcripts. We predicted that the increase in −51 bp transcripts in these tumours would be greater than the increase in +51 bp transcripts

Table 1. *Mean alternate transcript ratios in normal kidney/Wilms' tumour pairs*

Transcript ratio	Normal kidney	Wilms' tumour	*P* value
+/− 51 bp (− KTS)	0.63 ± 0.07	1.12 ± 0.13	< 0.001
+/− 51 bp (+ KTS)	0.64 ± 0.05	1.12 ± 0.13	< 0.001
−/+ KTS	1.57 ± 0.15	1.56 ± 0.24	N.S.

Figures are average values and their 95% confidence interval for the ratio of −51 bp *WT1* transcripts to +51 bp *WT1* transcripts both with (+ KTS) and without (− KTS) the 9 bp exon 9 splice, in normal kidney and paired tumour tissue in 10 samples. Values for the ratio of transcripts with and without the KTS splice in exon 9 are also shown. When the 51 bp exon 5 ratios were compared between the tumour and normal tissue samples, a statistically significant ratio increase was observed in the tumour population when the Students *t*-test was applied to the data. Splicing in exon 9 was, however, essentially unchanged. N.S., non-significant.

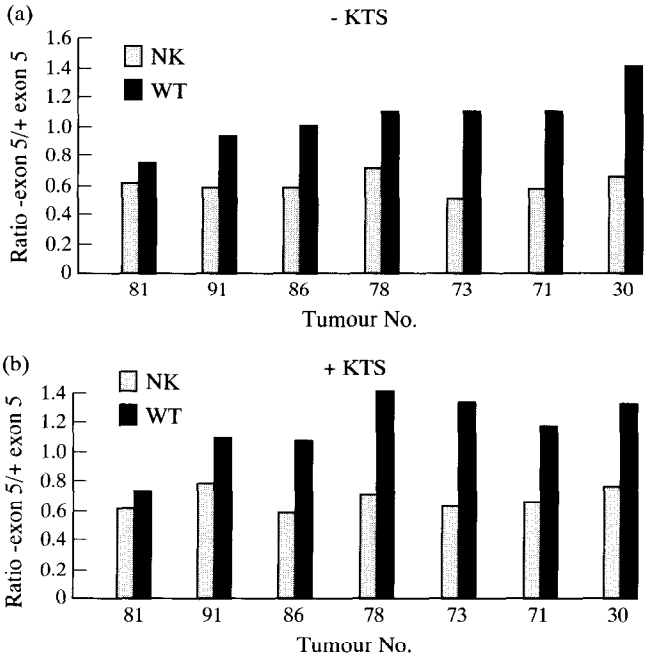


Figure 3. Graphical representation of data for Wilms' tumour and normal kidney pairs showing differences between the ratio of − exon 5 (−51 bp) to + exon 5 (+51 bp) transcripts in tumour and normal tissue. (a) Represents data for transcripts in which the 9-bp KTS splice is absent (−KTS) and (b) represents data for transcripts in which the 9-bp insert is present (+KTS).

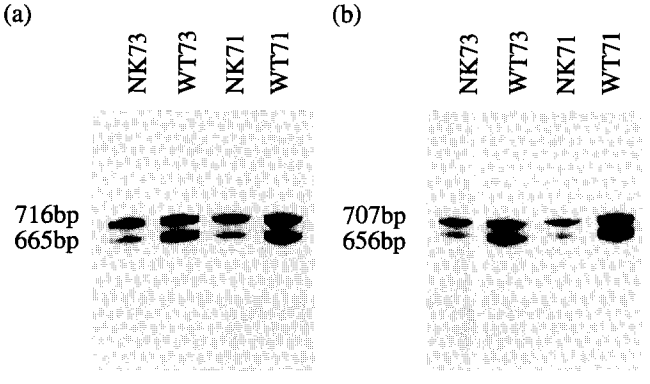


Figure 4. Autoradiographs of ^{35}S-labelled PCR products from two Wilms' tumour (WT) and normal kidney (NK) cDNAs showing a relative increase in −51 bp PCR products in Wilms' tumour samples. (a) PCR primers P1 and P2 amplified splice variants +51 bp/+9 bp and −51 bp/+9 bp (716 bp and 665 bp, respectively). (b) Primers P1 and P3 amplified splice forms +51 bp/−9 bp and −51 bp/−9 bp (707 bp and 656 bp, respectively).

relative to their respective levels in normal kidney. To demonstrate this, a competitive PCR methodology was employed. This involved the construction and use of internal standards which were coamplified in reactions using primers designed to detect transcripts in which only the 51-bp splice insert was present, or those in which the 51-bp insert was absent. Reactions containing fixed concentrations of the internal standard showed a greater increase in the *WT1* transcript/internal standard ratio for −51 bp transcripts in tumour cDNA relative to the transcript/internal standard ratio in paired normal kidney, when this data was compared with the increase in +51 b transcripts in the same

samples. Figure 5 depicts representative data from these analyses in two sample pairs. Tumour WT78 exhibited a 1.5-fold increase in − 51 bp transcripts relative to the increase in + 51 bp transcript in the same sample and tumour WT 73 exhibited a 1.8-fold increase in −51 bp transcript relative to the increase in + 51 bp transcript. Therefore, as predicted, there was a greater relative increase in −51 bp *WT1* transcript in Wilms' tumours compared to the increase in + 51 bp *WT1* transcript.

Mechanisms affecting WT1 *exon* 5 *alternate splicing*

The observed increase in *WT1* transcripts without the 51 bp insert in Wilms' tumours could not be explained by the presence of heterozygous point mutations in the splice donor and acceptor sites flanking exon 5 in genomic DNA. Direct sequencing of the splice donor and acceptor sites flanking exon 5 in all tumours showed normal sequence to be present. A larger region of the *WT1* gene encompassing the region from exon 4 to exon 6 was examined by Southern blotting to identify intronic rearrangements or deletions, or evidence of heterozygous exon 5 deletion in tumours. Restriction enzyme digests with BglII and SacI were designed to produce separate fragments containing exons 4, 5 and 6 and their flanking intron sequence region, and to generate small fragments incorporating exon 5. Filters were probed with a cDNA incorporating sequence from exons 4, 5 and 6 only. *WT1* restriction map information for BglII and SacI digestion was obtained from Tadokoro and associates [9]. There was, however, no evidence of rearrangement or deletion in this region in the tumours examined, as assessed by consistent changes in relative band intensities or mobility shifts when EcoR1, SacI and BglII digests were each compared in paired samples. Representative digests are shown in Figure 6. The intron upstream of exon 5 was then examined in greater detail for evidence of small deletions. This intron, being small, was amenable to PCR amplification. Amplification was performed using an intronic forward primer upstream of exon 4 and a reverse intronic primer downstream of exon 5. Products of 1.2 kb were run out on long 1% agarose gels. No evidence for size differences in tumour samples was obtained. We were unable to examine the entire intron downstream of exon 5 by this method due to its large size (> 16 kb) and the lack of internal intronic sequence information available.

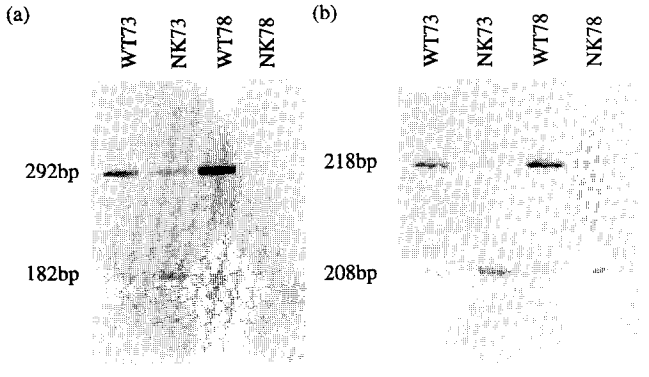


Figure 5. Quantitative RT-PCR detection of −51 bp and +51 bp *WT1* transcripts in two Wilms' tumour and normal kidney cDNA samples. In each sample, constant quantities of standard and cDNA were co-amplified. (a) Upper band: PCR product of −51 bp splice variants (292 bp), lower band: standard (182 bp). (b) Upper band: PCR product of +51 bp splice variants (218 bp), lower band: standard (208 bp). In (a) the *WT1* transcript/internal standard ratio was increased 12.7-fold in tumour WT78 relative to that in the paired normal kidney and was increased 5.5-fold in tumour WT73. In (b), the *WT1* transcript/internal standard ratio was increased 8.4-fold in tumour WT78 and 3.1-fold in tumour WT73. Therefore, there was a 1.5-fold increase in −51 bp transcripts in WT78 relative to the increase in +51 bp transcripts and an increase of 1.8-fold in −51 bp transcripts in WT73 relative to the increase in +51 bp transcripts.

DISCUSSION

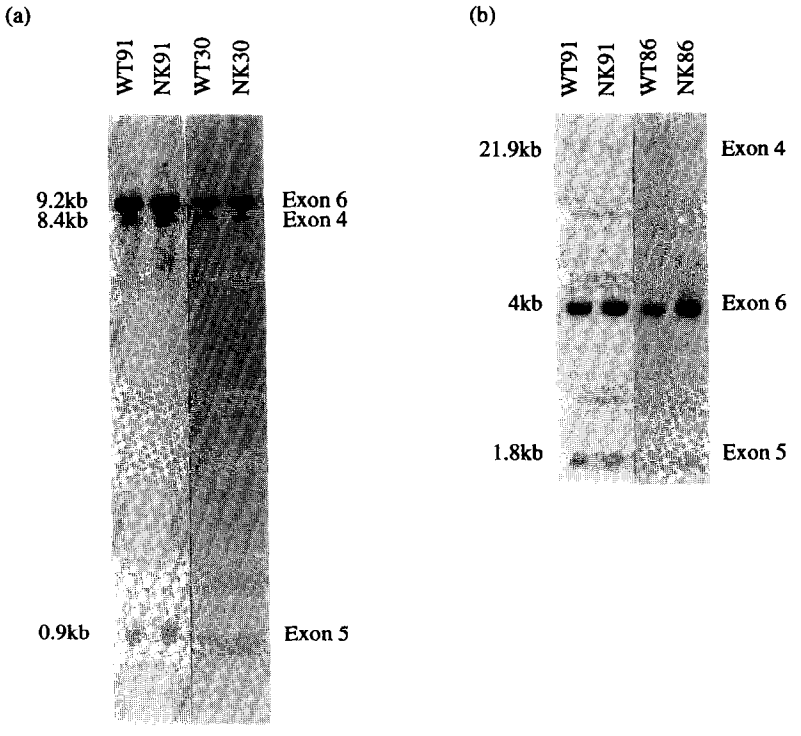
In this study we have demonstrated that a significant proportion of sporadic Wilms' tumours exhibit changes in exon 5 splicing relative to that occurring in normal kidney tissue. Exon 5 splicing in tumours is altered, resulting in an increase in *WT1* transcripts in which exon 5 is skipped, with a consequent increase in the proportion of −51 bp to + 51 bp transcripts in Wilms' tumour tissue. This observed increase in exon 5 skipping in tumour tissue could not be explained by point mutations in the cis-acting splicing elements flanking the exon or by large rearrangements in the adjacent introns.

The relationship between the degree of exon 5 skipping and tumour histology was not clear from this study. However, tumours that were predominantly blastemal appeared to have the greatest increase in −51 bp transcripts. Tumours that exhibited triphasic histology also showed this increase. Interestingly, however, two tumours WT81 (Figure 3) and WT 94, that exhibited predominantly epithelial histology, with little or no blastema present, did not exhibit significant changes in exon 5 splicing.

The relative proportions of exon 5 transcripts determined in this study in normal juvenile kidney tissue were in general agreement with values reported in an earlier study, examining both human fetal and mouse kidney transcripts using RNAase protection [6]. However, the values obtained for the relative proportions of + 9 bp (+ KTS) to − 9 bp (− KTS) transcripts in this study are lower than those reported by Haber and associates [6], although in the latter study there appeared to be considerable variation in this ratio in both Wilms' tumour tissue and in mouse tissues. Our values, however, approximate to those found in mouse ovary in the Haber study.

The present study is the only one to date in which splicing in paired normal kidney tissue has been compared with splicing in Wilms' tumour tissue. Although Brenner and associates [7] noted differences in exon 5 splicing between different Wilms' tumour samples, no comparisons with splicing in adjacent kidney tissue were made. The altered splicing that we observe clearly seems to be a tumour-specific phenomenon, affecting exon 5 splicing, but not splicing in exon 9. On average, approximately equal amounts of − 51 bp and + 51 bp transcripts were seen in our tumours, similar to observations recently made by Walker and coworkers [10], who reported equivalent expression of these two transcripts in human mesothelioma cell lines. No evidence could be found for the presence of WT1*del2* transcripts, in which both exons 2 and 5 are skipped, in our tumour panel. However, the fact that such transcripts have been reported previously, although in a small number of tumours and in tumour cell lines, is in agreement with our observation that normal splicing of the gene is disrupted to some extent in Wilms' tumour.

Using RNAase protection assays, Haber and associates [6] reportedly observed no significant differences between the relative levels of splice variants in a panel of five Wilms' tumours. However, in calculating these ratios, Haber and associates measured band intensities relative to the least abundant −51 bp/−9 bp transcript. Any increase in −51 bp transcripts would, therefore, be reflected in reduced relative amounts of transcripts



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Sample	Exon 4	Exon 5	Exon 6
WT 91	9.8	18.2	72.0
NK 91	12.4	15.3	72.3
WT 30	12.8	8.4	78.8
NK 30	10.6	11.6	77.8

Sac1

Sample	Exon 4	Exon 5	Exon 6
WT 91	6.8	12.1	81.1
NK 91	5.5	13.9	80.6
WT 86	4.8	7.4	87.8
NK 86	2.7	7.6	89.7

Figure 6.

with +51 bp present. One tumour in the Haber study exhibited reduced relative amounts of *WT1* alternate transcripts which might reflect an increase in −51 bp transcripts. As the present study does not exclude the possibility that tumour histology may have a bearing on *WT1* splicing patterns in Wilms' tumour, the lack of histological data in the Haber study makes direct comparisons between this study, and the earlier Haber study, difficult.

Recent evidence suggests that alternate splicing of certain genes may be regulated by the relative antagonistic activity between the serine arginine (SR) family of splicing factors and heterogenous nuclear ribonucleoprotein particle A1 (hnRNPA1), and that tissue-specific differences in these relative activities may determine the patterns of alternative splicing of specific genes in a particular cell type [11]. It would, therefore, be of interest to examine the activity of these factors in malignant tissue and their role in the splicing of cancer-related genes. In this context, it is of interest that Nakai and coworkers [12] have observed that *P53* undergoes aberrant splicing in chronic myelogenous leukaemia without genomic mutations around exon–intron junctions. Whether these factors directly influence alternate splicing in the *WT1* gene remains to be established, but the SR protein SF2/ASF has been shown to influence positively the selection of alternately spliced exons by binding to purine-rich sequences within exons, and the 3′ region of exon 5 in the *WT1* gene is highly purine rich. Consequently, a reduction in SF2/ASF activity or an increase in hnRNPA1 in Wilms' tumour tissue could hypothetically result in reduced selection of exon 5, resulting in a higher number of transcripts in which exon 5 is skipped. The binding of U1snRNP to splice donor sites is also thought to be important in exon selection in alternate splicing, where U1snRNP complexes assist the recognition of exons by the splicesome assembly [13]. The extent of contiguous complimentarity between the U1snRNA and the splice donor site sequences appears to be important in this process [14]. Examination of the complimentarity between the 5′ splice donor sites in the *WT1* gene and U1snRNA sequence suggests that the splice donor sites, flanking exons 5 and the 9 bp insert in exon 9, have relatively poor contiguous complimentarity with the U1snRNA sequence. At the exon 5 splice donor site there are seven complimentary residues out of a total of 11, but only three residues match contiguously. Perhaps not surprisingly, this splice donor site and that flanking the alternately spliced nine bases of exon 9 exhibit the poorest match of contiguous residues for all the *WT1* splice donor sites examined. This observation suggests that the reduced affinity for U1snRNA at these sites may form part of a mechanism regulating alternate splicing of the *WT1* gene. Other tissue-specific splicing factors, possibly

members of the SR family, presumably also play a role in exon selection in the *WT1* gene. As changes in exon 5 splicing patterns occurred independently of splicing in exon 9, we predict that such factors may be specifically associated with recognition of exon 5, and that they may be differentially expressed in malignant tissue. The identity of these factors is currently under investigation.

The question remains as to how altered splicing of exon 5 and the consequent imbalance in *WT1* isoforms could contribute to malignancy in Wilms' tumour. Current knowledge concerning the role of individual *WT1* isoforms is limited. In the main, functional studies on the gene have been conducted using −17 aa / − KTS isoforms and both repressor and activator functions have been reported [1]. In one recent study functional differences between + KTS isoforms attributable to splicing in exon 5 were noted [15]. In this study by Rupprecht and associates, the + KTS isoform, in which the 17 aa splice was absent, was found to have nearly 50-fold weaker suppressor activity when acting on promoter sequences associated with autoregulation of the *WT1* gene *in vitro*. If similar behaviour is translated to the regulation of other *WT1* gene targets then alterations in exon 5 splicing, and isoform imbalance, may be very significant in modifying overall gene function. This evidence and the observation that −51 bp transcripts of the *WT1* gene are not expressed in rat testis, but are expressed in kidney and spleen [10], suggests that *WT1* transcripts in which exon 5 is skipped, encode functionally different isoforms. The physiological function of these isoforms and the role that they play in modifying overall *WT1* gene activity in Wilms' tumour deserves further investigation.

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Acknowledgements—We are indebted to the medical and pathology staff of the Royal Children's Hospital and the Mater Children's Hospital, Brisbane, Australia. This work was supported by the Queensland Cancer Fund, The Royal Children's Hospital Foundation and the National Health and Medical Research Council of Australia.