ORIGINAL ARTICLE

Sequential *WT1* and *CTNNB1* mutations and alterations of β-catenin localisation in intralobar nephrogenic rests and associated Wilms tumours: two case studies

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J Clin Pathol 2007;**60**:1013–1016. doi: 10.1136/jcp.2006.043083

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Accepted 25 October 2006
Published Online First 15 December 2006

Background: Intralobar nephrogenic rests (ILNRs) are precursor lesions for Wilms tumours and are associated with *WT1* gene mutations. ILNR-associated Wilms tumours have a co-clustering of *WT1* and β-catenin (*CTNNB1*) mutations and unique histological features characterised by a stromal-predominant histology.
Aim: To determine the order in which *WT1* and *CTNNB1* mutations occur to understand the ILNR–Wilms tumour sequence.
Methods: Of nine Wilms tumours with *WT1* and *CTNNB1* mutations, three ILNRs lesions in two Wilms tumours were available for analysis of *WT1* and *CTNNB1* mutations using microdissection. Immunohistochemistry was also performed to investigate how the mutations in β-catenin alter the localisation in Wilms tumour development.
Results: *WT1* mutations were present in the ILNRs, however *CTNNB1* mutations were absent. Immunohistochemistry for WT1 confirmed inactivation of WT1 in both ILNRs and Wilms tumours. Both the ILNRs and the associated Wilms tumours had similar immunostaining patterns for β-catenin in the blastemal and epithelial components. Although rhabdomyoblasts were not included in ILNRs, the associated Wilms tumours showed rhabdomyogenic differentiation with a positive β-catenin nuclear staining.
Conclusions: The results suggest that *CTNNB1* mutation is a later event in Wilms tumourigenesis. *CTNNB1* mutations might be associated with rhabdomyogenesis.

Nephrogenic rests are dysplastic tissues that occur during kidney development.[1] They also have potential for occurrence of Wilms tumours.[1] There are two types of nephrogenic rests according to the location of the renal lobe. Intralobar nephrogenic rests (ILNRs) are situated within the renal lobe, while perilobar nephrogenic rests are located at the periphery of the renal lobe.[1] ILNRs are strongly associated with deletion of chromosome 11p13 or *WT1* mutation since Wilms–aniridia–genitourinary retardation syndrome and Denys–Drash syndrome frequently have ILNRs.[2] ILNR-associated Wilms tumours have unique histological and genetic features characterised by a stromal-predominant histology with rhabdomyogenesis[1 3] and co-clustering of *WT1* and β-catenin (*CTNNB1*) mutations.[4] Therefore, it is also important to analyse *CTNNB1* mutations to understand the sequential events and the role of the Wnt signalling pathway in ILNR-associated tumours. Recently, it has been reported that *CTNNB1* mutations play a critical role in tumour formation after loss of *WT1* expression, based on the results that the expression profiling of WT1-mutant tumours shows over-expression of β-catenin target genes rather than WT1 target genes.[5] Determining whether *CTNNB1* is mutated in nephrogenic rests should provide firm evidence for the temporal sequence of *WT1* and *CTNNB1* mutations, however this has not yet been reported. In this study, we therefore performed sequencing analysis for *WT1* and *CTNNB1* in the kidneys, ILNRs and Wilms tumours.

PATIENTS AND METHODS

Eleven Wilms tumours with *WT1* mutations were selected from a collection of tumours obtained with informed consent from the North Health Ethics Committee, Auckland, New Zealand. Eight tumours have been reported to have the common *CTNNB1* mutations.[6] All *CTNNB1* exons were sequenced for the remaining three tumours. In one of three WT1-mutant tumours without a *CTNNB1* exon 3 mutation, we detected a mutation in *CTNNB1* exon 8 using the previously reported primers.[5] Of a total nine Wilms tumours with *WT1* and *CTNNB1* mutations, two Wilms tumours containing three ILNRs were available for microdissection analysis. Cases 1 and 2 have been previously referred to as cases 25 and 35 respectively.[6] In this study, we found that case 2 had a constitutional heterozygous *WT1* mutation.

Table 1 Primers and conditions for PCR

Primer	AT (℃)	CT	PCR product size
WT1 exon1			
F: CTG TGC CCT GCC TGT GAG	54	40	159 bp
R: GGC TCC TGT TTG ATG AAG GA			
WT1 exon 9			
F: TGT CCA TTT AGG TGT GAA ACC A			
R: TGA AGA AAA GTT TAC GCACTT GTT	52	40	123 bp
D11S1392			
F: TTG CAT CCA TAC GGA AAG TC	58	40	200–220 bp
R: ACA TCT GAG ACT TGT AGT AGA AGG			
CTNNB1 exon 3			
F: CGG CTG TTA GTC ACT GG	58	40	156 bp
R: AAA ATC CCT GTT CCC ACT CA			
CTNNB1 exon 8			
F: GAA CTT CAC CTG ACA GAT CCA	58	40	179 bp
R: CTA TTC CCA TGG CAC CAG TT			

AT, annealing temperature; CT, cycle times.

Abbreviations: ILNR, intralobar nephrogenic rest; LOH, loss of heterozygosity

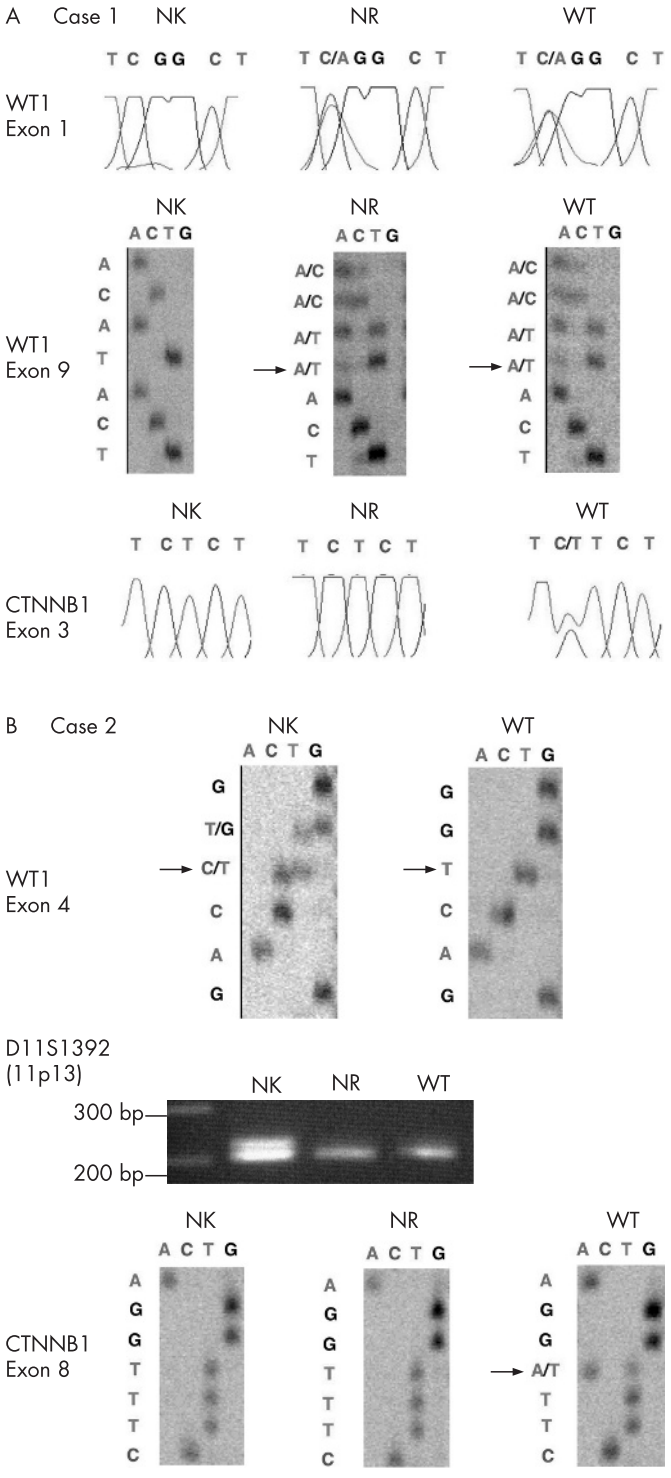

Figure 1 (A) Sequencing results of *WT1* and *CTNNB1* in normal kidney (NK), intralobar nephrogenic rest (NR), and Wilms tumour (WT) in case 1. A heterozygous *WT1* mutation in exon 1 (C to A transition) and in exon 9 mutation (insertion A) in NR and WT. The *CTNNB1* mutation in exon 3 (C to T transition, heterozygous) is confined to WT. (B) Sequencing results of *WT1* and *CTNNB1*, and 11p13 loss of heterozygosity (LOH) analysis in NK, intralobar NR, and WT in case 2. The mutation in *WT1* is heterozygous (deletion C) in NK but is homozygous in WT. NR and WT have LOH at 11p13 (the middle). The *CTNNB1* mutation (exon 8, T to A transition) is limited to WT.

Microdissection and analysis of *WT1* and *CTNNB1* mutations

Sections (7 μm) were prepared from paraffin embedded blocks for microdissection. Microdissection was performed using a Leica AS LMD laser capture system (Leica Microsystems, Wetzlar, Germany) according to a method on the NIH Laser Capture Microdissection website (http://dir.nihd.nid.gov/lcm/lcm.htm) with a modification, ie, the sections were stained with 0.05% toluidine blue.

Table 1 lists primers and conditions for PCR for detecting *WT1* and *CTNNB1* mutations, and 11p13 loss of heterozygosity (LOH) (D11S1392) in microdissected ILNRs. Sequence was done with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3100. To confirm the sequencing results, the samples were re-sequenced using the Thermosequenase Cycle Sequencing Kit (USB Corporation, Cleveland, OH, USA) and reactions were analysed using a LiCor 400L DNA sequencer (LiCor Inc, Lincoln, NE, USA).

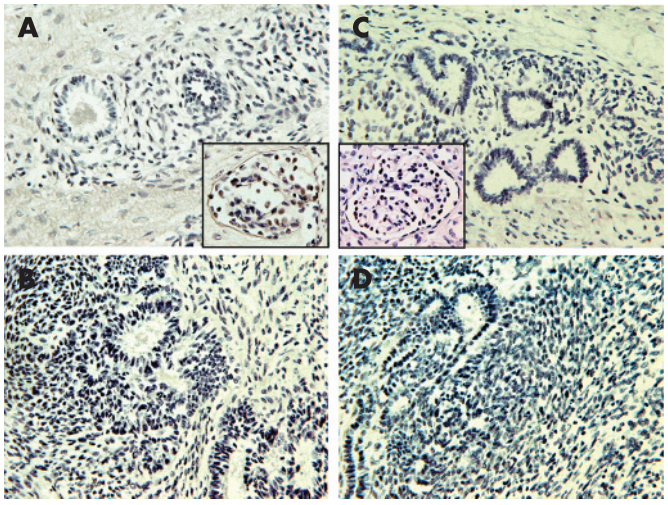

Figure 2 Immunohistochemistry for WT1 in intralobar nephrogenic rests (ILNRs) (case 1 (A), case 2 (C)), and Wilms tumours (case 1 (B), case 2 (D)). There is absence of WT1 expression in ILNRs and Wilms tumours. Insets (A) and (C) show the positive WT1 expression in podocytes in the tumour-bearing kidney tissues as an internal control. Original magnifications, ×400.

Immunohistochemistry

Immunohistochemistry for WT1 was performed to confirm loss of WT1 expression in ILNRs and Wilms tumours. The adjacent tumour-bearing normal kidney (podocytes) was used as an internal positive control. Immunohistochemistry for β-catenin was also carried out to investigate how localisation of this protein was altered in between ILNRs and Wilms tumours. WT1 (6F-H2, DAKO, CA, USA) and β-catenin (E-5, sc-7963, Santa Cruz Biotechnology, CA, USA) were used as primary antibodies, and the DAKO Envision horseradish peroxidase system (K4001, DAKO Cytomation, CA, USA) was used to detect the primary antibodies.

RESULTS AND DISCUSSION

Case 1 was a sporadic Wilms tumour with *WT1* mutations in exon 1 (137C→A, Ser 46→Term, heterozygous) and 9 (1215 ins A, frame shift-stop (40+89), heterozygous), and a *CTNNB1* mutation in exon 3 (348 TCT→TTT, Ser45Phe, heterozygous) (fig 1A). The appropriate polymorphisms were not available to determine whether two *WT1* mutations occurred on different alleles or the same ones. We confirmed loss of expression of WT1 in the ILNRs and the associated Wilms tumour by immunohistochemistry (fig 2A,B), consistent with WT1 inactivation due to the mutation of both alleles. Neither *WT1* nor *CTNNB1* mutations were detected in the normal adjacent kidney (fig 1A). The tumour had a stromal-predominant

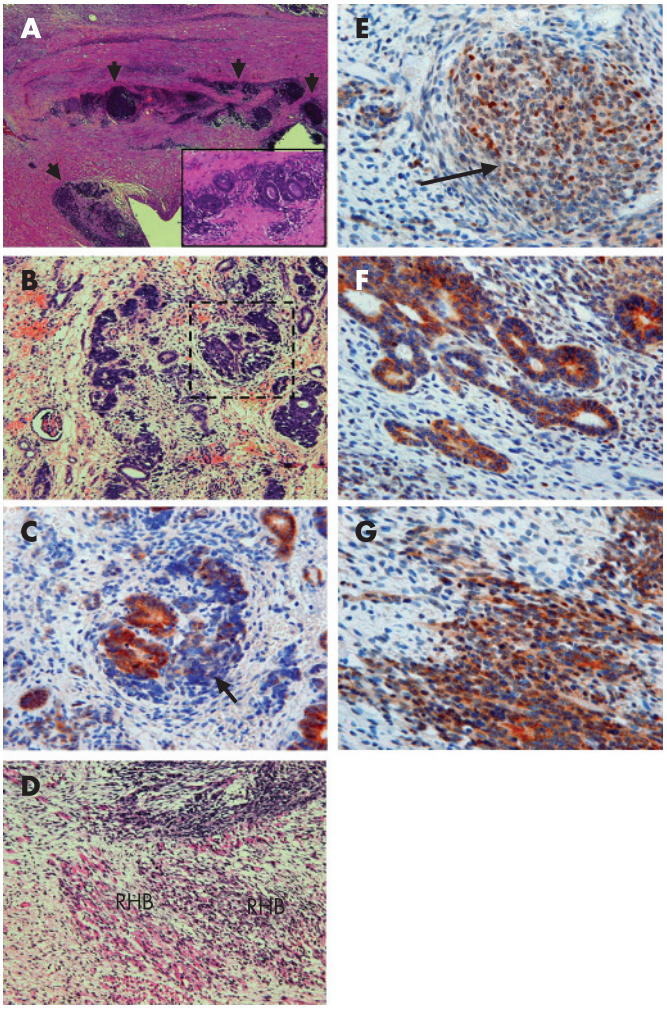

Figure 3 Histopathological and immunohistochemical features of case 1: H&E (A, inset A, B, D), β-catenin (C, E–H). First (indicated by arrowheads) (A) and second intralobar nephrogenic rests (ILNRs) (B). Inset A shows a hyper-power view of the blastemal cells around the epithelial structures in the sclerosing stroma. Immunohistochemistry for β-catenin in the ILNR (a hyper-power view of the dashed rectangle in (B)). The tumour histology shows a stromal predominant histology with rhabdomyogenesis (D). β-Catenin is localised in the cytoplasm of the blastemal cells (indicated by arrows) and in the cytoplasm and the cell membrane of the epithelial structures in the ILNR and the associated tumour (C, F). β-Catenin is occasionally localised in the nuclei of the blastemal cells and the rhabdomyoblasts in the tumour tissue (D, G). Original magnifications, ×40 (A); ×200 (B, inset A); ×400 (C–H).

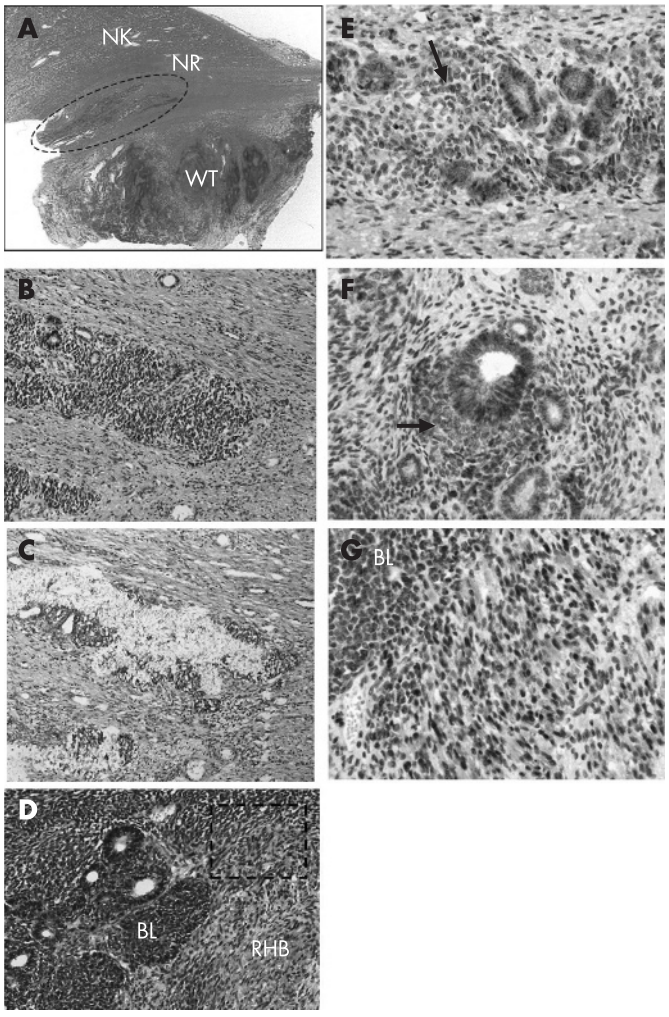

Figure 4 Histopathological and immunohistochemical features of case 2: H&E (A, B, D), toluidine blue (C), β-catenin (E, F, G). An intralobar nephrogenic rest (ILNR) (surrounded by the dashed circle) is irregularly located between the normal kidney and the Wilms tumour (A). A hyper-power view of the ILNR (B) and the microdissected area within the nephrogenic rest (C). The tumour histology includes an area of blastemal and epithelial components with rhabdomyogenesis (D). Expression of β-catenin in the blastemal cells (indicated by arrows) and the epithelial component in the ILNR (E) is similar to that in the tumour (F). Nuclear staining of β-catenin in the rhabdomyoblasts (hyper-magnification of the dashed rectangle in (D)) (G) and weak cytoplasmic expression in the blastemal cells (G). Original magnifications, ×1 (A); ×200 (B, C, D); ×400 (E, F, G).

histology (fig 3D), which contained two ILNRs. The first ILNR was located between the renal calyx and the tumour and was thought to be a regressing rest (fig 3A). The second ILNR was a microscopic lesion (a dormant rest), which was isolated from the tumour tissue (fig 3B). Two ILNRs were microdissected and examined for *WT1* and *CTNNB1* mutations. Both ILNRs had the same heterozygous *WT1* mutations in exons 1 and 9 as well as the tumour; however the *CTNNB1* mutation was not detected (fig 1A).

Case 2 had a constitutional heterozygous *WT1* mutation in exon 4 (711 del C, frame shift-stop (236+3)] in the tumour-bearing kidney (fig 1B). The tumour had the homozygous *WT1* mutation in exon 4 (fig 1B), and had a stromal-predominant histology with rhabdomyogenesis (fig 4D). A macroscopically visible ILNR (a hyperplastic rest) was found between the tumour and the normal kidney (fig 4A–C). Although the *CTNNB1* mutation was absent in exon 3 in the previous report,[6] we detected a *CTNNB1* mutation in exon 8 (1361TGG→AGG, W383R, heterozygous) in the tumour tissue (fig 1B). The mutation is not a hot spot but has recently been reported in Wilms tumour by another group.[6] Microdissection was repeatedly carried out for the ILNR three times. Although the sequencing of *CTNNB1* was successful, that of *WT1* was not determined. Thus, instead of *WT1* analysis, 11p13 LOH analysis was performed. We detected 11p13 LOH in both the ILNRs and the tumour (fig 1B). Therefore, as the normal kidney contains a heterozygous *WT1* mutation, the ILNR must have the homozygous *WT1* mutation as well as the tumour tissue. The homozygous *WT1* mutation in the ILNRs and the Wilms tumour was confirmed by absence of WT1 expression in both tissues by immunohistochemistry (fig 2C,D). The *CTNNB1* mutation was not identified in the ILNR (fig 1B). The observation that the ILNRs from both cases described here had a mutation status distinct from kidney and tumour

Table 2 Mutation status in *WT1* and *CTNNB1* in kidney, ILNR, and Wilms tumour

	Kidney	ILNR	WT
Case 1		(2)*	
WT1	Negative	Exon 1 137C→A Ser 46→Term Heterozygous Exon 9 1215 ins A Frame shift-stop (40+89) Heterozygous	Exon 1 137C→A Ser 46→Term Heterozygous Exon 9 1215 ins A Frame shift-stop (40+89) Heterozygous
CTNNB1	Negative	Negative	Exon 3 348 TCT→TTT Ser45Phe Heterozygous
Case 2			
WT1	Exon 4 711 del C Frame shift-stop (236+3) Heterozygous	Exon 4 711 del C Frame shift-stop (236+3) Homozygous†	Exon 4 711 del C Frame shift-stop (236+3) Homozygous
11p13 (D11S1392)	ROH	LOH	LOH
CTNNB1	Negative	Negative	Exon 8 1361TGG→AGG W383R Heterozygous

LOH, loss of heterozygosity; ROH, retention of heterozygosity.
*Two ILNR had the same results.
†The result was estimated by 11p13 LOH and *WT1* mutation status in the kidney and tumour.

excluded the possibility that the nephrogenic rests samples were contaminated by normal kidney or tumour tissue (table 2).

The localisation of β-catenin between ILNRs and associated Wilms tumours was compared to investigate changes due to *CTNNB1* mutations in Wilms tumour development. The immunohistochemical localisation of β-catenin in the blastemal and epithelial components in the ILNRs and the associated Wilms tumours (cases 1 and 2) were similar (fig 3C,E,F and fig 4E,F): β-catenin was localised in the cytoplasm of the blastemal cells and in the cell membrane and cytoplasm of the epithelial component in the ILNRs and the Wilms tumour, although nuclear staining of β-catenin was focally positive in the blastemal cells in the tumours (fig 3E). The major difference was that the ILNRs did not contain rhabdomyoblasts but the associated Wilms tumours had occasional differentiation into rhabdomyoblasts with a nuclear accumulation of β-catenin (fig 3G and fig 4G). In general, rhabdomyoblasts are rarely seen in ILNRs,[1] therefore *CTNNB1* mutations might be associated with rhabdomyogenesis. Another interesting result in immunohistochemistry was the mutation in exon 8 resulting in a nuclear accumulation of β-catenin in rhabdomyoblasts in case 2. Mutations of *CTNNB1* have been observed commonly in exon 3, which result in activation of the Wnt signalling pathway by stabilising β-catenin protein, leading to its translocation to the nucleus.[7] However, mutations in exon 8 might also stabilise β-catenin protein by disrupting the APC/axin binding region of this protein.[5]

This study investigated the temporal sequence of *WT1* and *CTNNB1* mutations in the development of Wilms tumour. We have shown that mutation in the *CTNNB1* occurs as a later event of Wilms tumourigenesis. It has been reported that mutation of *WT1*, 11p13 LOH, and 11p15 LOH are earlier events, while *p53* mutation and 16q LOH are later events in Wilms tumour development.[8–10] Our results support a sequence involving inactivation of WT1 as a very early event followed by *CTNNB1* mutation and activation of the Wnt signalling pathway.[5] Rhabdomyoblasts were absent in the ILNRs where WT1 was inactivated but the Wnt signalling pathway was not activated, while rhabdomyogenesis was present in the ILNR-associated Wilms tumours where the Wnt signalling pathway is activated. Therefore, the Wnt signalling pathway might be related to myogenesis rather than loss of WT1 function.

Take-home messages

- Intralobar nephrogenic rests (ILNRs) are precursor lesions for Wilms tumours and are associated with *WT1* mutations.
- *WT1* and β-catenin (*CTNNB1*) mutations are frequently co-clustered in Wilms tumour.
- Mutations of *WT1* occur as a first event in ILNRs followed secondarily by *CTNNB1* mutations in Wilms tumour.
- *CTNNB1* mutations might be associated with rhabdomyogenesis in Wilms tumour.

ACKNOWLEDGEMENTS

We thank Dr Benjamin Tycko for the sequence information of the *CTNNB1* primers.

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Funding: This work was supported by the Health Research Council of New Zealand, the Cancer Society of New Zealand, and the NZ Lottery Health Grants Board.

Competing interests: None.

Ethics approval for this study was obtained from North Health Ethics Committee, Auckland, New Zealand.

REFERENCES

1 **Beckwith JB**, Kiviat NB, Bonadio JF. Nephrogenic rests, nephroblastomatosis, and the pathogenesis of Wilms tumor. *Pediatr Pathol* 1990;**10**:1–36.
2 **Beckwith JB**. Nephrogenic rests and the pathogenesis of Wilms tumor: developmental and clinical considerations. *Am J Med Genet* 1998;**79**:268–73.
3 **Beckwith JB**. Precursor lesions of Wilms tumor: clinical and biological implications. *Med Pediatr Oncol* 1993;**21**:158–68.
4 **Maiti S**, Alam R, Amos CI, *et al*. Frequent association of beta-catenin and WT1 mutations in Wilms tumors. *Cancer Res* 2000;**60**:6288–92.
5 **Li CM**, Kim CE, Margolin AA, *et al*. CTNNB1 mutations and overexpression of Wnt/beta-catenin target genes in WT1-mutant Wilms tumors. *Am J Pathol* 2004;**165**:1943–53.
6 **Fukuzawa R**, Heathcott RW, Sano M, *et al*. Myogenesis in Wilms tumors is associated with mutations of the WT1 gene and activation of Bcl-2 and the Wnt signaling pathway. *Pediatr Dev Pathol* 2004;**7**:125–37.
7 **Eberhart CG**, Argani P. Wnt signaling in human development: beta-catenin nuclear translocation in fetal lung, kidney, placenta, capillaries, adrenal, and cartilage. *Pediatr Dev Pathol* 2001;**4**:351–7.
8 **Park S**, Bernard A, Bove KE, *et al*. Inactivation of WT1 in nephrogenic rests, genetic precursors to Wilms tumour. *Nat Genet* 1993;**5**:363–7.
9 **Charles AK**, Brown KW, Berry PJ. Microdissecting the genetic events in nephrogenic rests and Wilms tumor development. *Am J Pathol* 1998;**153**:991–1000.
10 **Maitra A**, Salahuddin S, Timmons CF, *et al*. Allelotyping nephrogenic rests: putative precursor lesions of Wilms tumors. *Pediatr Dev Pathol* 1999;**2**:488–9.