LETTER TO JMG

Different *CTNNB1* mutations as molecular genetic proof for the independent origin of four Wilms tumours in a patient with a novel germ line *WT1* mutation

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We describe a patient with a novel *WT1* pS50X germ line mutation, who developed bilateral Wilms tumours, both with stromal-type histology. Both tumours showed loss of the wild type *WT1* allele (loss of heterozygosity (LOH)) and a tumour specific mutation in catenin beta1 (*CTNNB1*), S45P in the left and Δ45S in the right tumour. Molecular analysis of microdissected cells from the left tumour revealed the same S45P *CTNNB1* mutation in blastema, tubuli, stroma and muscle, and a different *CTNNB1* mutation (T41A) in stromal cells isolated from another area of the same slide. Microdissection of two areas of muscle cells from the right tumour revealed the same Δ45S mutation and no *CTNNB1* mutation nor LOH of *WT1* in normal kidney cells. One year later, the patient developed a new set of bilateral tumours. Both tumours showed LOH of the wild type *WT1* allele, but different *CTNNB1* mutations as in the first tumours: S45C on the right and S45F on the left side, demonstrating that these developed independently and are not relapses. This case demonstrates the high risk for the development of Wilms tumours in patients with germ line truncation mutations.

Wilms tumour (WT) or nephroblastoma is the most frequent renal tumour of childhood. WT is thought to be derived from a renal stem cell, with impaired differentiation potential. Most tumours have a mixed histology and are composed of three elements: blastema, epithelia and stroma, recapitulating the development of the normal kidney. Tumours may also contain heterotypic cells not normally found in the kidney such as rhabdomyoblasts, fat, cartilage and bone, probably derived from an abnormal mesenchymal differentiation. This type of differentiation is mainly observed in the stromal-type variant of WT. Constitutional or somatic mutations in the *WT1* gene are found in approximately 10–20% of WT, most occurring in stromal-type tumours.[1–4] In addition, approximately 75% of WT carry *CTNNB1* gene mutations, mostly in or close to amino acids that are important for activity and stabilisation of the protein.[5–9]

METHODS

Mutation and allelotyping analysis

DNA was isolated from the tumour and blood by standard methods. *WT1* gene mutation analysis was performed by the DNA-PCR SSCP method including all 10 exons and flanking intron sequences, as described.[2] *CTNNB1* gene mutation analysis of exon 3 was performed as described.[5]

To study the mechanism for LOH, microsatellite analysis was performed with CA-repeat markers from 11q11 (D11S1313), 11p13 (D11S1776) and 11p15.4 (D11S1323). In the PCR one marker was fluorescently labelled with IRD 800 and the products were analysed on an automatic sequencer (Li-Cor Biosciences, Lincoln, Nebraska, USA).

For the molecular analysis of the different cell types the respective cells were identified after HE staining and the corresponding areas were removed from a consecutive unstained slide using a sterile scalpel. After deparaffinisation DNA was extracted with the Pico Pure DNA extraction buffer according to the manufacturer (Arcturus, Mountain View, California, USA). Each sample served as a template for PCR amplification of exon 1 of *WT1* and exon 3 of the *CTNNB1* gene.

CASE PRESENTATION AND RESULTS

A 10-month-old female patient with a 46, XX karyotype presented with large bilateral kidney tumours (fig 1A). She was initially treated in another institution for 8 weeks with empirical chemotherapy including vincristine and actinomycin-D with no clinico-radiological response and then referred to the Hospital Sant Joan de Déu in Barcelona where the tumours were completely excised by kidney sparing surgery. The histology was stromal type (rhabdomyomatous) on both sides and she received no cytotoxic therapy after the surgery and did well for 1 year. At this time she developed bilateral renal tumours again (see MRI in fig 1B). At surgery a very large tumour was found in the right kidney, which led to a complete nephrectomy. In the left kidney there was a small round tumour that was totally resected by tumorectomy (fig 1C). Pathology findings showed in the right kidney a large, classical, mixed WT with no capsular infiltration. The left kidney tumour was round and hard, and pathology confirmed the same rhabdomyomatous nature as in the first set of tumours one year earlier. Nephrogenic rests were not observed in any of the tumours nor in the small amount of adjacent normal kidneys removed during surgery.

Analysis of blood DNA revealed an abnormal SSCP banding pattern of exon 1 of *WT1*. Sequencing identified a heterozygous C>A change at nucleotide position 149, resulting in the creation of a stop codon at Ser50 (S50X) (fig 2A). This mutation has not been described before and represents the most extreme N-terminal germ line truncation mutation in *WT1* to date. Sequencing of the tumour DNA revealed only the mutant allele (fig 2A).

Different *CTNNB1* mutations were found in the tumour DNA from both sides of the initial as well as the second tumour samples. The initial tumours had a S45P on the left side and a ΔS45 on the right side. In the second set of tumours the left side harboured a S45F and the right side a S45C mutation, clearly demonstrating that the second set of tumours occurred independently. Besides the germ line *WT1* mutation, present in

Abbreviations: CTNNB1, catenin beta1; LOH, loss of heterozygosity; WT, Wilms' tumour; *WT1*, Wilms Tumour 1

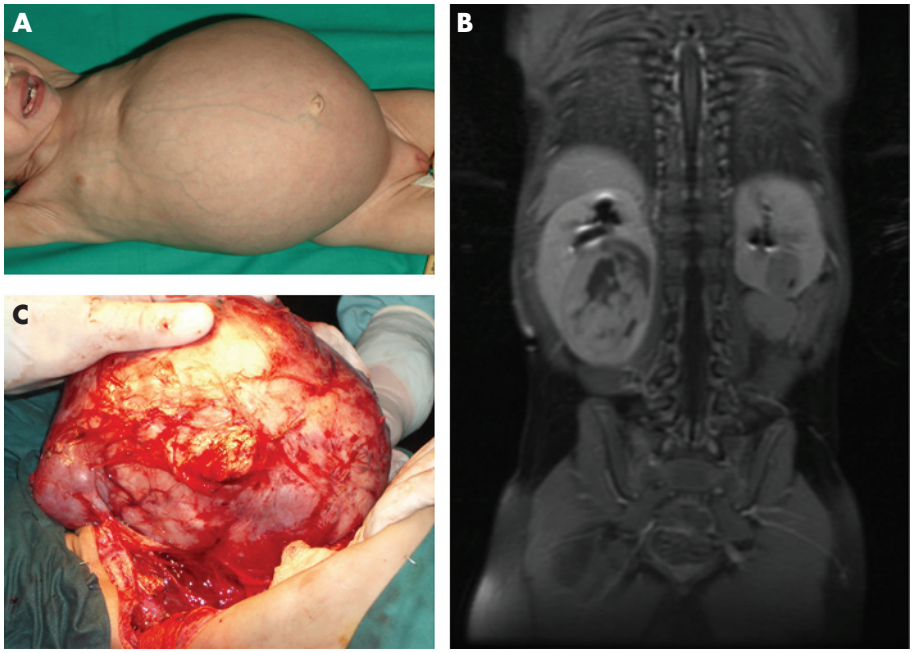

Figure 1 Clinical presentation of the case. (A) Patient at first presentation after chemotherapy (2004). (B) MRI of recurrent tumours. (C) Second tumour, removed in 2005. Parental informed consent was obtained for publication of this figure.

every cell, each tumour had two additional genetic events: loss of the wild type *WT1* allele and a mutation in *CTNNB1*.

To study whether all different cell types of the tumours, ie, blastemal, stromal, muscle and tubules have the same or different genetic events, we performed manual microdissection. Extracted DNAs were first analysed for *CTNNB1* gene mutations. Two different areas from the initial left tumour were used to prepare the A3 and A1 slides. Different cell types from both slides had the same S45P mutation, except one area of stromal cells from the A3 slide, which had a T41A mutation, whereas stromal cells from another part of the same slide had a S45P mutation. From the initial right tumour only one section (C2) was analysed and all tumour cell subtypes contained the same Δ45S mutation.

In normal kidney cells from the right tumour no *CTNNB1* mutation was found, confirming by molecular methods that these were indeed normal kidney cells. The same types of analyses were performed with the recurrent bilateral tumours. All cells from two different slides (1E and F1) from the right side had the same S45C mutation except in two normal kidney sections with epithelial morphology, which were wild type for *CTNNB1*, again confirming that these were normal cells. From the left side two different areas with muscle cells were isolated from one slide and both had a S45F. The *CTNNB1* mutations found on both sides were not seen in any cells of the primary set of tumours, demonstrating that the recurrent tumours on both sides occurred independently and had acquired new *CTNNB1* gene mutations in addition to loss of wild type *WT1*. In addition the presence of identical *CTNNB1* mutations in the different cell types of each tumour clearly shows that these are derived from one parental transformed cell.

Sequencing of *WT1* exon 1 from the microdissected cell types revealed only the mutant allele in all cases that were analysed (for example in table 1, sections A3 and C2 and fig 2A). To study the mechanism which led to loss of the wild type *WT1* allele we performed a microsatellite analysis with markers from chromosome 11. For this we first used a tumour cell line that we have established from the left recurrent tumour. The tumour cells had a 46, XX karyotype with two normal appearing copies of chromosome 11. Sequencing of the cell line DNA showed that these were homozygous for the *WT1* mutation, and RNA analysis revealed that they expressed the mutant transcript (fig 2A). In addition they carried a S45F *CTNNB1* mutation, the same as was observed in the microdissected tumour samples. The DNA from the tumour cell line was heterozygous for the 11q11 marker and homozygous for the 11p13 and 11p15 markers (fig 2B), demonstrating that loss of wild type *WT1* was due to a mitotic recombination event between 11q11 and 11p13. Analysis of microdissected blastemal and muscle cells from the second tumour of the right side also showed LOH of 11p13 and 11p15 and no LOH for 11q11, whereas normal appearing epithelial cells did not show LOH of these markers (table 1). Therefore, in both second tumours from the left and right side, a similar mitotic recombination event resulted in loss of wild type *WT1*.

DISCUSSION

The patient presenting here with a novel *WT1* germ line truncation mutation developed four independent WT: bilateral synchronous WT and bilateral synchronous second tumours. The molecular proof for their independent origin was derived from the *CTNNB1* mutation analysis as the four tumours were associated with five different mutations and each showed loss of the *WT1* wild type allele. The *WT1* S50X mutation represents the most extreme N-terminal truncation of the protein described so far. Another child with a F40fsX90 *WT1* germ line mutation who developed a bilateral WT at age 8 months was described as having Denys–Drash syndrome due to a female phenotype but a male karyotype.[10] At the time of surgery this child did not have glomerulosclerosis or proteinuria. The authors discuss the possibility that the short WT1 protein acted in a dominant negative fashion to produce some aspects of the Denys–Drash syndrome phenotype. In our patient the tumours showed loss of the wild type allele excluding the possibility of a dominant negative effect for tumour development and suggesting either gain or loss of function as the mechanism involved in tumour formation. As our patient is female, and so far has had no proteinuria, we cannot exclude the possibility that this truncation mutation would act as dominant negative for sex development or nephropathy.

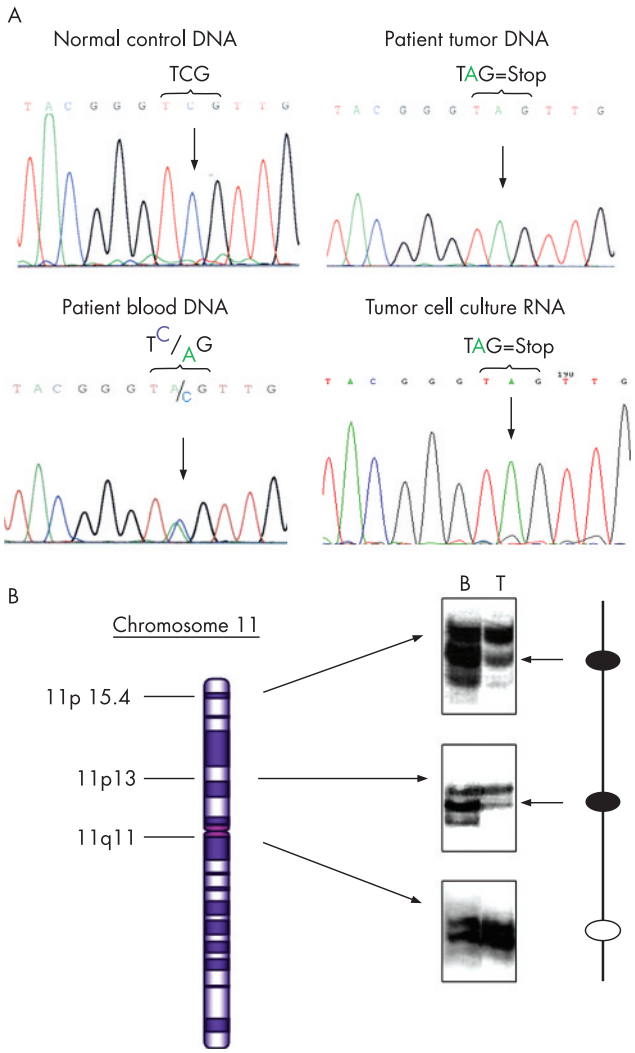

Figure 2 *WT1* sequence of exon 1. (a) normal control DNA, patient's blood DNA, patient's tumour DNA, tumour cell line RNA. (b) LOH analysis with markers from 11q11, 11p13 and 11p15. Open circle no loss, filled circle LOH. B, blood DNA; T, tumour DNA.

Table 1 Mutations found in microdissected tumour samples

Slides	Tissue cell types	*CTNNB1* mutation	Nucleotide change	*WT1* mutation*	LOH analysis
Initial left tumour					
A3	Tubuli	S45P	TCT>CCT	m/m	
A3	Stroma	T41A	ACC>GCC	m/m	
A3	Blastema	S45P	TCT>CCT	m/m	
A3	Stroma II	S45P	TCT>CCT	nd	
A3	Muscle I	S45P	TCT>CCT	m/m	
A3	Muscle II	S45P	TCT>CCT	nd	
A1	Muscle	S45P	TCT>CCT	nd	
A1	Stroma	S45P	TCT>CCT	nd	
A1	Muscle/fat	S45P	TCT>CCT	nd	
Initial right tumour					
C2	Tumour pool	ΔS45	ΔTCT	nd	
C2	Muscle I	ΔS45	ΔTCT	m/m	
C2	Muscle II	ΔS45	ΔTCT	nd	
C2	*Normal kidney I*	*wild type*		*m/w*	
C2	*Normal kidney II*	*wild type*		*nd*	
Recurrence left					
3B	Muscle I	S45F	TCT>TTT	nd	
3B	Muscle II	S45F	TCT>TTT	nd	
Cell line		S45F	TCT>TTT	m/m	LOH 11p13 + p15 no LOH 11q11
Recurrence right					
1E	Blastema I	S45C	TCT>TGT	nd	LOH 11p13 no LOH 11q11
1E	Blastema II	S45C	TCT>TGT	nd	
1E	Muscle	S45C	TCT>TGT	nd	
1E	Stroma	S45C	TCT>TGT	nd	
F1	Blastema I	S45C	TCT>TGT	nd	LOH 11p15
F1	Blastema II	S45C	TCT>TGT	nd	LOH 11p15
F1	*Epithelia I*	*wild type*		*nd*	*no LOH 11p15*
F1	*Epithelia II*	*wild type*		*nd*	*no LOH 11p15*
F1	Muscle	S45C	TCT>TGT	nd	LOH 11p15

Microdissected areas labelled in italics correspond to normal kidney cells by molecular analysis, one *WT1* mutant allele as in all cells of the patient and no *CTNNB1* mutation.
**WT1* mutation analysis was performed by sequencing, m/m indicates mutant/mutant allele, and m/w one mutant one wild type allele, nd: not done

Key points

- Germ line mutations in the *WT1* gene result in a high risk for Wilms tumour development, often as a synchronous bilateral tumour.
- Three genetic events occur in these tumours, (1) a mutation in *WT1*, (2) loss of wild type *WT1* and (3) a mutation in *CTNNB1*.
- The timing of these events is at present not known as all analysed tumour sections harboured all three genetic events.
- Patients with germ line *WT1* mutations and tumour sparing surgery should be surveyed for the development of independent second tumours.
- The different independent tumours harboured the same mitotic recombination event between 11q11 and 11p13 as a mechanism for LOH.

The novel findings of the molecular genetic tumour analysis are the high frequency of mitotic recombination events between 11q11 and 11p13 leading to loss of wild type *WT1*, and the strong selection pressure for *CTNNB1* mutations in cells with a complete loss of a functional WT1 protein. These results suggest that WT1 negative kidney precursor cells may not survive without a *CTNNB1* mutation. As a matter of fact in the *Wt1* knockout mouse *Wt1*-negative mesenchymal cells die by apoptosis during kidney development.[11]

It will be interesting to study whether the type of the *WT1* mutation influences the molecular mechanisms, and therefore the risk for tumour development, as has been observed for the age of tumour onset, with the earliest onset in patients with truncation mutations.[4] Also we have observed a higher percentage of bilateral tumours in cases with truncation mutations in the 5′ half of the gene before the nuclear localisation signal.[4] In agreement with our earlier observation, this patient has a termination signal at codon 50. The molecular proof that this patient developed independent second sets of bilateral tumours shows the high risk for WT in patients with germ line *WT1* mutations and the need for a close clinical tumour surveillance of such patients.

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Parental informed consent was obtained for the publication of figure 1.

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