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Diagnostic utility of Wilms' tumour-1 protein (WT-1) immunostaining in paediatric renal tumours

Surbhi Goyal, Kiran Mishra, Urvee Sarkar, Satendra Sharma & Anita Kumari

Department of Pathology, University College of Medical Sciences, Delhi, India

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***Background & objectives*: Renal tumours constitute about 7 per cent of all neoplasms in children. It is important to differentiate Wilms' tumour (commonest tumour) from non-Wilms' tumours. The aim of this study was to evaluate the immunoexpression and diagnostic role of Wilms' tumour-1 protein (WT1) in paediatric renal tumours.**

***Methods*: A total of 53 cases of renal tumours in children (below 18 yr) who underwent total nephrectomy were included in this retrospective study. WT1 immunostaining was done using mouse monoclonal WT1 antibody (clone: 6F-H2).**

***Results*: Of the 53 cases, 38 (72%) were of Wilms' tumour. Non-Wilms' group (15) included six cases of mesoblastic nephroma (MN), two each of clear cell sarcoma (CCSK), renal cell carcinoma (RCC) and peripheral neuroectodermal tumour (PNET) and one each of angiomyolipoma (AML), rhabdomyosarcoma (RMS) and malignant rhabdoid tumour (MRT). Proportion of WT1 positivity in Wilms' tumour was 100 per cent in contrast to 26.7 per cent in non-Wilms' tumours (P<0.001). Epithelial and blastemal components of Wilms' tumour showed moderate (2+) nuclear and cytoplasmic staining in 80 (24/30) and 75 per cent (24/32) cases, respectively. MN, PNET, CCSK and AML were negative for WT1. RMS, RCC and MRT showed cytoplasmic staining, strongest in RMS. No significant association was seen between WT1 expression and NWTSG (National Wilms' Tumor Study Group) stage.**

***Interpretation & conclusion*s: WT1 helps to differentiate Wilms' tumour from other paediatric renal tumours. It may help in differentiating the two subgroups of Wilms' tumour which have distinct molecular pathogenesis and biological behaviour, however, further prospective studies are required for validation of this hypothesis.**

Key words Immunostaining - non-Wilms' - paediatric renal tumours - Wilms' tumour - WT1

Renal tumours constitute about 7 per cent of all neoplasms in children[1,2]. Most common renal tumour of childhood is Wilms' tumour (WT), affecting children predominantly in age group of less than 5 yr. It is important to differentiate non-Wilms' tumours from Wilms' tumour as these have different presentation and prognosis. Mesoblastic nephroma (MN) occurs usually in the first year of life and generally has a good prognosis. Rhabdoid tumour affects children upto two years of age and is the most malignant renal

tumour. Clear cell sarcoma of kidney (CCSK) has a bad prognosis, but with introduction of adriamycin the survival rate has improved. Primitive neuroectodermal tumour (PNET), renal cell carcinoma (RCC), angiomyolipoma (AML), rhabdomyosarcoma (RMS) and lymphoma are some of the rare tumours found in kidney in paediatric age group[2]. Under normal circumstances, various tumours are easily differentiated morphologically with clinical correlation, but at times particularly in post-chemotherapy cases certain tumour patterns may pose a diagnostic challenge. In these cases, immunohistochemistry is helpful to a great extent.

Approximately 10-15 per cent of sporadic Wilms' tumours harbour mutations in the Wilms' tumour-1 protein (*WT1*) gene. Overexpression of both wild-type and mutant *WT1* has been reported[2,3]. WT-1 protein is encoded by *WT1* gene located on chromosome 11p13. It is a 4 zinc finger DNA binding transcription factor that plays a critical role in kidney development and differentiation[2]. The *WT1* gene was originally recognized as a tumour suppressor gene[3], but later studies have shown the overexpression of *WT1* mRNA in various kinds of solid tumours highlighting its oncogenic potential as well[3-9]. *WT1* mRNA expression appears to be developmentally restricted, being highest during embryogenesis, predominantly in urogenital system (foetal kidney, genital ridge and gonads)[4]. In normal adult tissue, it is expressed in mesothelium, glomerular podocytes, CD34 positive haematopoietic stem cells, Sertoli cells of the testis, stromal cells, surface epithelium and granulosa cells of the ovary, myometrium and endometrial stromal cells of the uterus[4-6]. The WT1 protein has been demonstrated in myeloid leukaemias, solid tumours like desmoplastic small round cell tumour (DSRCT), malignant mesothelioma, glioblastomas, soft tissue sarcomas like malignant peripheral nerve sheath tumour, RMS, osteosarcoma, malignant melanoma, endometrial cancer and ovarian serous adenocarcinoma[6].

Only a few studies have evaluated WT-1 immunostaining in paediatric renal tumours in the past and have shown controversial results owing to the use of different clone of monoclonal WT1 antibody[4,6-8]. Limited studies have explored the diagnostic and prognostic value of WT1 monoclonal antibody (clone: 6F-H2) raised against the N-terminal portion of this protein in paediatric renal tumours[6,8,9].

Therefore, we conducted this study with the aim to assess the immunoexpression of WT1 and to evaluate its diagnostic role in paediatric renal tumours.

Material & Methods

This retrospective study was conducted in the department of Pathology, University College of Medical Sciences, Delhi, India. The study was approved by the Institutional Ethics Committee. All consecutive 53 nephrectomy cases of renal tumours in children (below 18 yr), which were sent to the Pathology department over a period of 25 yr (1988-2012), were included in the study. Nephrectomy specimens were fixed in 10 per cent neutral buffered formalin overnight. Representative tumour sections were taken, routinely processed, paraffin embedded and were stained with hematoxylin and eosin (H&E). Clinico-radiological and morphological findings were noted from the records. Tumour staging was done according to NWTSG (National Wilms' Tumor Study Group)[2].

Immunohistochemistry (IHC) for WT1: Streptavidin-biotinylated immunoperoxidase method[8] was used for immunostaining. Briefly, 4-5µm thick sections, taken on 0.01 per cent poly-L-lysine coated slides were dried, deparaffinized in xylene and rehydrated in graded alcohol. For antigen retrieval, sections were placed in 0.01 M citrate buffer (*p*H 6.0) for 10 min at 98°C in EZ Retriever system (v.2.1, Biogenex, Fremont, California, USA). Sections were cooled to room temperature, and washed with tris buffer (*p*H 7.6). Endogenous peroxidase was blocked by 4 per cent H_2O_2 in methanol for 15 min. Sections were incubated with primary mouse monoclonal antibody in 1:20 dilution (WT1: clone: 6F-H2, Biocare, Concord, California, USA) for one hour. Sections were treated with biotinylated antimouse link antibody (Sigma-Aldrich, USA) for 30 min, followed by preformed streptavidin conjugated horseradish peroxidase complex (Sigma-Aldrich, USA) for 30 min. Diaminobenzidine tetrahydrochloride (DAB) as chromogen substrate solution (0.6 mg/ml in Tris buffer saline, *p*H 7.6 containing 0.04 per cent hydrogen peroxide) was used to develop brown colour. Slides were counterstained with Harris' haematoxylin, dehydrated and mounted.

A negative control (without primary antibody) was taken along with each batch. Glomeruli of adjacent normal kidney acted as positive internal controls. Immunohistochemical analysis was performed in a blinded fashion by two pathologists. The fraction of positively stained tumour cells was scored semi-quantitatively after examining under 10 high power fields (x400) for each case. Nuclear/cytoplasmic staining in >10 per cent of tumour cells was required

to define WT1 positivity. Immunohistochemical results for WT1 were scored as weak (11-25%-1+), moderate (26-50%- 2+) and strong (>50% -3+)[8].

Statistical analysis: Statistical analysis was performed using SPSS software (version 17.0, SPSS, Chicago, Illinois, USA). Proportion of WT1 positivity in Wilms' versus non-Wilms' tumours was compared using Fisher's exact test. Association was assessed between WT1 expression and NWTSG stage using Fisher's exact test.

Results

Age group of the children with renal tumours ranged from 17 days to 17 years. The oldest child was 17 years old with PNET, having bone marrow metastasis, while the youngest was 17 days old girl with mesoblastic nephroma. Age range of patients with Wilms' tumour (n=38) was two months to 15 years, with a mean age of 2.3±1.4. Of these, 27 (71%) were in the age group less than four years, whereas six (15.8%) children were above six years. Mean age in non-Wilms' tumour group was six years. Overall boys were affected more with a sex ratio of 3:2. Boys outnumbered girls in Wilms' tumour as well as non-Wilms' group with sex ratio of 5:4 and 5:2, respectively. Of the 53 patients, 38 (72%) were of Wilms' tumour, six had MN, two had PNET, CCSK and RCC each. Distribution of cases according to histological type along with mean age and sex ratio is shown in Table I.

Table I. Paediatric renal tumours- histological type, age and gender characteristics

Paediatric renal tumour	No. of cases (%)	Mean age	Sex ratio (M:F)
Wilms' tumour			
Favourable histology	38 (72)	2.3 yr	5:4
Non-Wilms' tumour			
Mesoblastic nephroma	6 (10)	5.4 months	2:1
PNET	2 (4)	13 yr	1:1
CCSK	2 (4)	2.25 yr	2:0
Clear cell RCC	2 (4)	11 yr	2:0
RMS	1 (2)	2 yr	1:0
Angiomyolipoma	1 (2)	16 yr	1:0
Malignant rhabdoid tumour	1 (2)	2 yr	0:1

PNET, primitive neuroectodermal tumour; CCSK, clear cell sarcoma kidney; RMS, rhabdomyosarcoma; RCC, renal cell carcinoma

Histology: The most common morphological pattern was triphasic among Wilms' tumour, seen in 25 of 38 cases (65.8%), followed by blastemal (6 cases), epithelial (4 cases), stromal (2 cases) and biphasic pattern (1case) (Table II). Only one tumour showed biphasic morphology which consisted of blastemal and epithelial components. Tubular pattern was the predominant morphology in cases with epithelial predominance. Eight cases of Wilms' tumour showed heterologous epithelial and stromal elements. Of these, all had skeletal muscle/rhabdomyoblastic differentiation, three showed squamous epithelial differentiation, one showed cartilage and columnar epithelium, and one showed smooth muscle.

All the Wilms' tumour cases were of favourable histology. None showed any evidence of anaplasia. Of the non-Wilms' group, one case of clear cell sarcoma showed anaplastic features. Fourteen of 38 (36.8%) cases of Wilms' tumour showed presence of nephrogenic rests (NRs), and all of them were intralobar nephrogenic rests (Fig.1a, Table II).

WT1 Immunostaining

Wilms' tumor - Normal kidney showed a very intense WT1 nuclear staining of glomerular podocytes but faint cytoplasmic staining of the tubules (Fig.

Table II. Morphological patterns in Wilms' tumour with distribution of nephrogenic rests

WT pattern		Total (n=38)	Nephrogenic rests			
			Present	ILNR	PLNR	Absent
Triphasic		25	11	11	0	14
Monophasic	Blastemal	6	1	1	0	5
	Epithelial	4	0	--	--	4
	Stromal	2	1	1	0	1
Biphasic		1	1	1	0	0

ILNR, intralobar nephrogenic rests; PLNR, perilobar nephrogenic rests

1b). Two patterns of WT1 positivity were found. One group (30 cases) showed predominantly diffuse strong to moderate blastemal (75%) and epithelial (80%) positivity which was both nuclear and cytoplasmic (Fig. 2). Stroma showed only focal mild WT1 positivity in 44 per cent of these cases. Other group comprised eight Wilms' tumour cases with prominent heterologous elements (>50%) which showed strong cytoplasmic positivity in skeletal muscle only (Fig. 3, Table III). Blastemal, epithelial and homologous stromal components did not show WT1 staining in these cases (Table III). Cases in which scant heterologous stromal elements were not prominently seen on H&E staining, WT1 immunostaining highlighted those areas (Fig.4). There was variability in the intensity of WT1 staining in the different components of the same patient and among the tumours having the same stage. Nephrogenic rests also showed positive moderate nuclear staining similar to the tumour in their respective cases.

Non-Wilms' tumour: Of the non-Wilms' tumours, cytoplasmic WT1 positivity was seen in RCC, RMS and MRT (Figs 5 and 6). Rest CMN, PNET, CCSK and AML were completely negative on immunostaining (Figs 7 and 8). WT1 immunostaining in Wilms' and non-Wilms' groups is shown in Table IV.

Proportion of WT1 positivity in Wilms' tumour was 100 per cent, while in non-Wilms' tumour was 26.7 per cent, which was found significant (P<0.001). No significant difference of WT-1 expression was found between the three NWTS stages as shown in Table V.

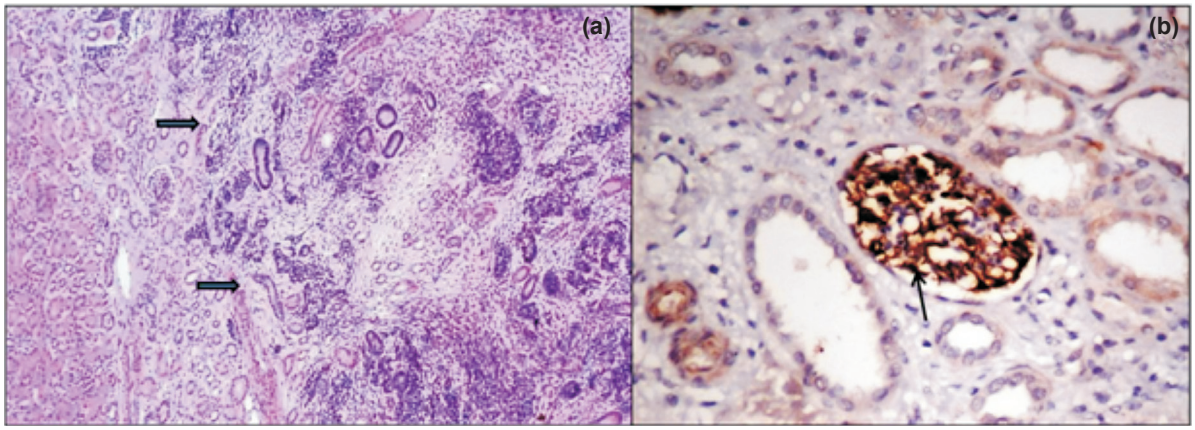

Fig. 1 (a). Microphotograph of intralobar nephrogenic rest (ILNR) comprising blastema, tubules and stromal components (H&E x100). **(b)** WT1 immunostaining in normal kidney - strong positive WT1 immunoexpression in glomerular podocytes (arrow) and faint cytoplasmic staining in the tubules (Immunostain x400).

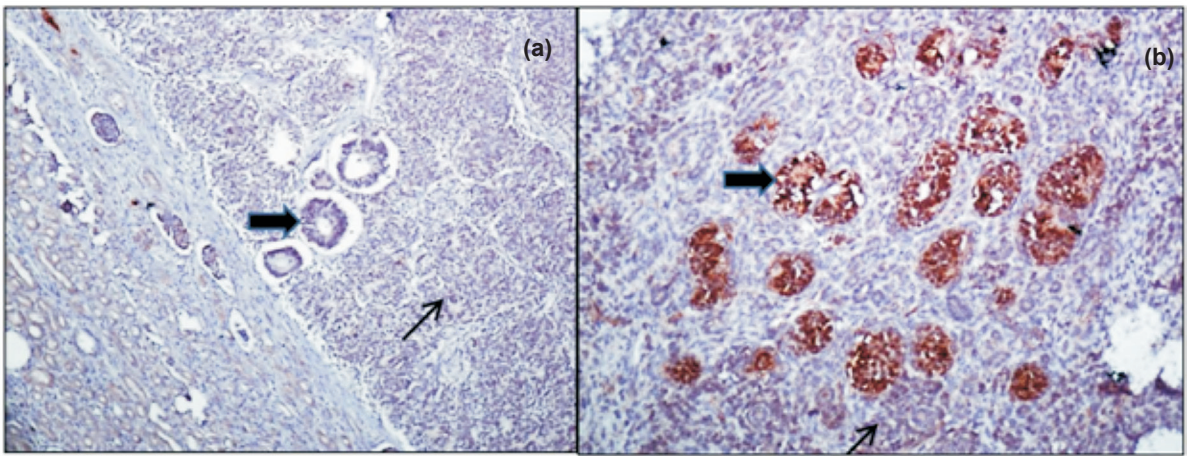

Fig. 2. WT1 expression in first group of Wilms' tumours. **(a)** and **(b)** nuclear-cytoplasmic WT1 immunostaining in epithelial component (tubules) shown by thick arrows. **(a), (b)** Diffuse WT1 immunostaining in blastema, shown by thin arrows (Immunostain x200).

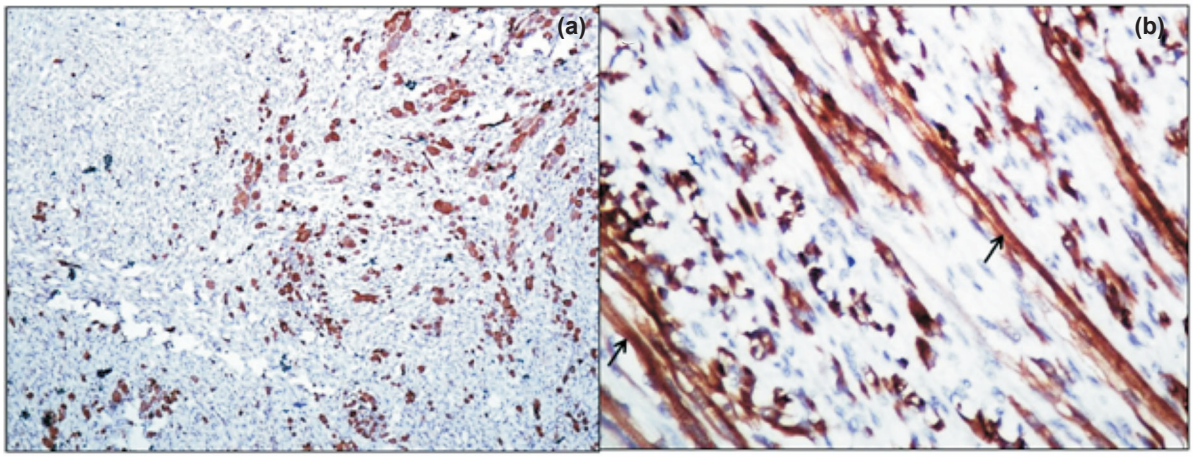

Fig. 3. WT1 expression in second group of Wilms' tumours showing heterologous stromal elements. **(a)** Strong cytoplasmic WT1 expression limited to rhabdomyoblastic stroma (Immunostain x100). **(b)** Higher magnification showed WT1 immunostaining in mature skeletal muscle fibres with cross-striations (arrows) (Immunostain x400).

Discussion

Heterologous components such as striated and smooth muscle, cartilage, bone, or adipose tissues are seen in 10 per cent of Wilms' tumour. Tumours with extensive rhabdomyogenesis have been termed "foetal rhabdomyomatous type", occur in younger children and are frequently bilateral[10]. Rhabdomyoblastic differentiation correlates with poor response to chemotherapy[10].

Perilobar nephrogenic rests have been associated with blastema predominant WTs lacking heterologous elements, bilaterality, mutation in *WT2* gene and are often found in patients with hemihypertrophy and Beckwith-Wiedemann syndrome[11]. Intralobar nephrogenic rests are associated with stromal WTs containing heterologous elements, are mostly unilateral, harbour *WT1* mutation and are found in WT associated with genitourinary anomalies, aniridia, or Denys-Drash syndrome[12]. We found nephrogenic rests in 36.8 per cent of our cases and all of them were ILNRs without anaplasia. These findings were in concordance with a study from Japan[13], suggesting a distinct genetic and molecular biology in Asian population in contrast to Western world. Mishra *et al*[14] have reported ILNR in 45.3 per cent of WT in a multi-institutional study from India.

Traditionally, only nuclear staining for WT1 was considered specific because WT1 is principally a DNA binding transcription factor. Cytoplasmic staining of WT1 has not been counted as positive and, therefore, not evaluated much until now[6]. However, several studies have shown evidence that WT1 is also involved

Table III. Detailed histology, proportion of different components and WT1 staining in second group of Wilms' tumours (n=8)

Heterologous stroma, skeletal muscle (%)	Epithelial (%)	Homologous stroma (%)	Blastema (%)	WT1 staining, intensity
>80	5-10	0	5-10	*Sk. Muscle, 3+, C
>80	5-10	0	5-10	*Sk. Muscle, 3+, C
60	20	0	20	*Sk. Muscle, 3+, C
60	15	0	25	*Sk. Muscle, 2-3+, C
55	20	0	25	*Sk. Muscle, 3+, C
50	20	10	20	*Sk. Muscle, 2-3+, C
45	25	10	20	*Sk. Muscle, 3+, C
35	30	20	15	*Sk. Muscle, 2-3+, C

*WT1 positivity was seen only in skeletal muscle in these cases. All other components were negative for WT1. C- cytoplasmic staining

Table IV. WT1 immunostaining in Wilms' and non-Wilms' tumours

Paediatric renal tumour	No. of cases	Cases positive for WT-1	% positivity	Intensity of immunostaining
Wilms' tumours (38 cases)				
Stromal component	27	12	44.4	1+ (Nuclear, cytoplasmic)
Epithelial component	30	24	80.0	2-3+, (Nuclear, cytoplasmic)
Blastemal component	32	24	75	2-3+, (Nuclear, cytoplasmic)
Heterologous stroma (skeletal muscle)	8	8	100	3+, Cytoplasmic
Non-Wilms' tumours (15 cases)				
RMS	1	1	100	3+ , Cytoplasmic
MRT	1	1	100	2+, Cytoplasmic
RCC	2	2	100	1-2+, Cytoplasmic
Mesoblastic nephroma	6	0	0	--
PNET	2	0	0	--
CCSK	2	0	0	---
Angiomyolipoma	1	0	0	---

RMS, rhabdomyosarcoma; MRT, malignant rhabdoid tumour; RCC, renal cell carcinoma; PNET, primitive neuroectodermal tumour; CCSK, clear cell sarcoma kidney

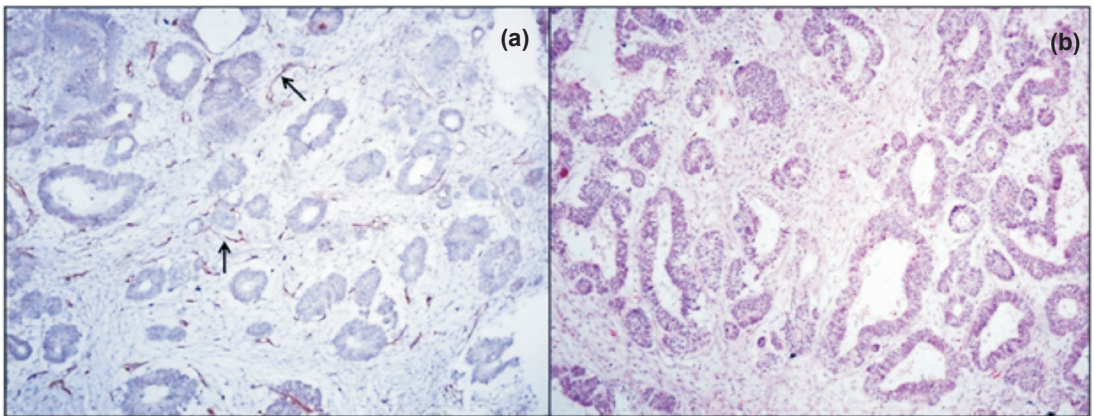

Fig. 4 (a). WT1 immunostaining can highlight scant heterologous stromal components (arrows) (Immunostain x100), **(b)** which otherwise may not be apparent on routine Hematoxylin & Eosin stain (H&Ex100).

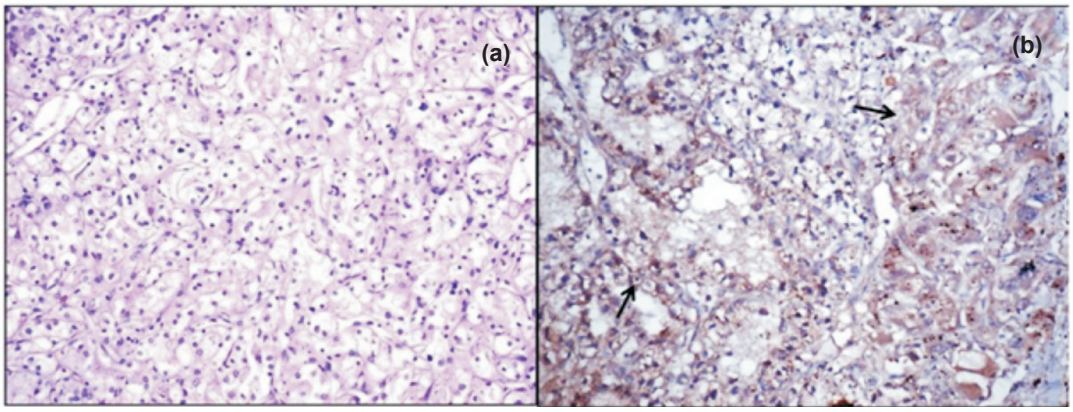

Fig. 5 (a). A case of clear cell renal cell carcinoma in a 12 year old child (H&Ex200). **(b)** Immuostaining shows cytoplasmic WT1 expression in the tumour tissue (arrows) (Immunostain x200).

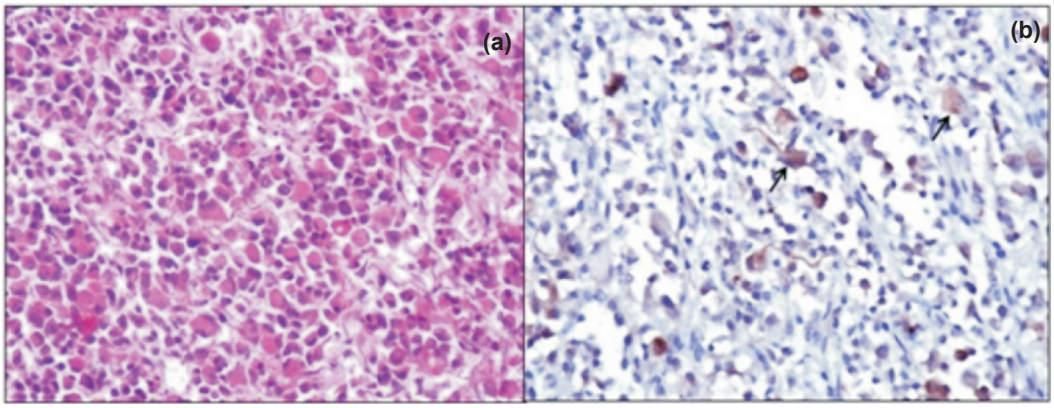

Fig. 6 (a). A case of malignant rhabdoid tumour in a 3 month old boy (H&Ex200). **(b)** WT1 immunostaining shows cytoplasmic WT1 expression in rhabdoid cells (arrows) (Immunostain x200).

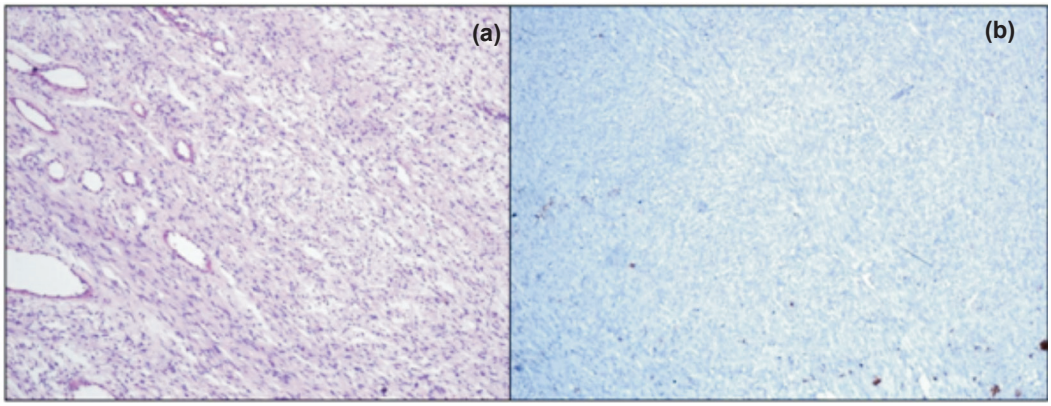

Fig. 7 (a). A case of congenital mesoblastic nephroma in a 3 month old boy (H&Ex100). **(b)** WT1 immunostaining shows negative results (Immunostain x100).

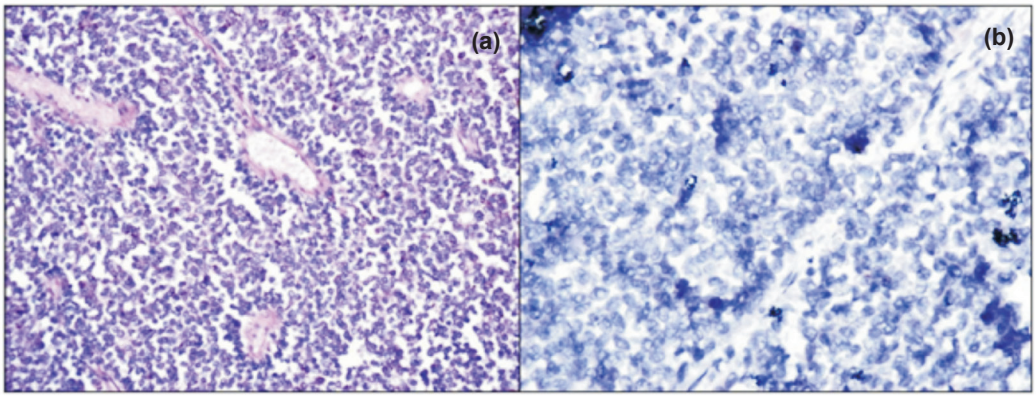

Fig. 8 (a). A case of peripheral neuroectodermal tumour (PNET) of kidney in a 15 year old boy (H&Ex100). **(b)** WT1 immunostaining shows negative results (Immunostain x100).

in RNA metabolism and translational regulation in the cytoplasm[6,8,15,16].

A few studies have correlated the morphology and WT1 expression with molecular subtypes of Wilms' tumour[8,16-18]. Schumacher *et al*[8] demonstrated that *WT1* mutations occurred in a high percentage (63%) stromal predominant Wilms' and these tumours showed aberrant differentiation into heterologous elements instead of epithelial differentiation. Miyagawa *et al*[17] suggested that histology of Wilms' tumour with *WT1* mutation

Table V. WT1 immunoexpression in different National Wilms' Tumour Study Group (NWTSG) stages

WT1 immuno- expression (%)	T1	T2	T3
< 10 (negative)	1	2	1
10-25	2	2	0
26-50	6	10	4
>50	5	3	2
Total	14	17	7

was stromal predominant with rhabdomyogenesis[17]. Fukuzawa *et al*[18] hypothesized that abundant rhabdomyogenesis in *WT1* mutated tumours was attributed to extensive apoptosis of blastema due to reduced Bcl-2 expression resulting from loss of WT1 function. According to Miwa *et al*[19] WT1 protein is expressed at high levels in Wilms' tumours having wild type genotype and blastema/epithelial predominant histology. WT1 expression is limited to stromal component due to developmental arrest mediated by mutated *WT1* gene. It appears that WT1 controls genes which mediate mesenchymal to epithelial transition during embryonic kidney development. Sangkhathat *et al*[16] analyzed the WT1 expression with regard to *WT1* mutation status and compared with other paediatric renal tumours. They showed that WT1 was positive in all of the nephroblastoma components in the Wilms' tumours with wild-type WT1, whereas WT1 protein was confined to only stromal component in the WT1 mutated tumours, suggesting different roles of WT1 in the two nephroblastoma subclasses.

In the present study a variable pattern of expression of WT1 was observed in Wilms' tumour. Though our study was limited in molecular analysis, we divided our Wilms' tumour cases into two subgroups. First group (30 cases) showed moderate nuclear-cytoplasmic WT1 positivity in blastema and epithelial components. This localization pattern of WT1 was comparable with that reported in foetal kidneys[4,7,18]. The second group (8 cases) of Wilms' tumours had prominent heterologous stromal elements (skeletal muscle), all of whom showed strong cytoplasmic WT1 positivity. Wilms' tumour cases showing strong cytoplasmic positivity in heterologous stromal elements only possibly belonged to the subgroup harbouring *WT1* mutation as described by Schumacher *et al*[8]. Expression of cytoplasmic WT1 expression in this subgroup can be explained by aberrant localization of mutant transcript of WT1 in the cytoplasm of heterologous stromal cells which can be detected by IHC using N terminal antibody. *WT1* acts as tumour suppressor gene in this subclass in contrast to oncogene in other subgroup of tumours which do not have *WT1* mutations. Near uniform and diffuse nuclear expression of WT1 protein in 75-80 per cent of the first subgroup cases can be explained by overexpression of wild type *WT1* gene as hypothesized by previous authors[16,19].

Ghanem *et al*[20] have reported higher WT1 expression in Wilms' tumours with predominant blastemal and epithelial differentiation than stromal predominant tumours. The negative stromal elements in their study included mostly adipose tissue and smooth muscle rather than skeletal muscle. This may be due to the use of C terminal antibody previously.

During embryonic development of the kidney, WT1 is first expressed in both pronephric and mesonephric structures[4]. The metanephric blastema expresses relatively lower amounts of WT1 but during subsequent differentiation, WT1 expression increases in the glomerular podocytes and is lost in the differentiating proximal and distal tubules[4,21]. RCC is derived from proximal tubules which represent a more differentiated product of the metanephric blastema. The two cases of clear cell RCC in our study showed a diffuse cytoplasmic WT1 positivity. Aberrant immunoexpression of WT1 in RCC may be linked to the dedifferentiation to an embryonic phenotype and it may act as a transcriptional regulator in RCC like Wilms' tumour as suggested by Campbell *et al*[21]. Further studies may be of value to clarify this hypothesis.

Apart from Wilms' tumour, strong cytoplasmic positivity was found only in cases of RMS and MRT. Carpentieri *et al*[9] have suggested that functional WT1 nuclear factor is required for inhibition of rhabdomyogenesis and cytoplasmic positivity reflects aberrant localization of mutated *WT1* gene. Other non-Wilms' tumours like PNET, CCSK, AML and MN were completely negative. These findings are in concordance with previous studies[4,7,9]. Positive WT1 expression can be helpful in morphological distinction of blastema predominant Wilms' from PNET (a rare, aggressive tumour of adolescence).

Significant positive correlation of blastemal nuclear expression of WT1 with clinical stage and poor prognosis has been reportd[18,22]. However, in our study no association was seen with NWTS stage (possibly due to small sample size).

WT1 was positive in epithelial and blastema components in majority of the Wilms' tumour cases which had probably wild-type *WT1* oncogene. In contrast, in the other subgroup of cases, WT1 protein expression was limited to heterologous stromal components, mainly skeletal muscle which may reflect aberrant mutated WT1 cytoplasmic protein. This hypothesis needs to be tested in large prospective studies.

In conclusion, WT1 immunostaining may differentiate Wilms' tumours from other paediatric renal tumours. Highlighting of residual stromal component in post-chemotherapy cases by WT1 immunostaining can pick up scant heterologous elements, which may be missed on routine H and E morphology. This may help in confirmation of the diagnosis in post-chemotherapy Wilms' tumour cases. Extensive rhabdomyomatous differentiation and the presence of strong cytoplasmic positivity of WT1 may be used as a surrogate marker for *WT1* mutation, which may identify a tumour subtype that seems to respond poorly to chemotherapy.

***Conflicts of Interest*:** None.

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Reprint requests: Dr Kiran Mishra, Department of Pathology, University College of Medical Sciences, University of Delhi, Dilshad Garden, Delhi 110 095, India
e-mail: drkiranmishra@gmail.com