Absence of Canonical WNT Signaling in Adult Renal Cell Tumors of Embryonal Origin

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Abstract. *Background/Aim: The canonical β-catenin pathway is involved in the development of Wilms' tumor, but its role in adult renal cell tumors (RCT) of embryonal origin is not yet known. Materials and Methods: We sequenced the catenin beta 1 (CTNNB1) gene in papillary RCTs, applied the TOPflash/FOPflash reporter plasmid system on cell lines, and examined the β-catenin protein expression by immunohistochemistry. Results: The absence of mutations in CTNNB1 and low TOPflash/FOPflash ratio in tumor cell lines indicated the absence of active Wingless-type MMTV integration site family (WNT) signaling in RCTs. The weakly cytoplasmic tending towards membranous expression of β-catenin in RCT is analogous to cellular differentiation in the embryonal kidney rather than tumorigenic activation of WNT signaling. Conclusion: The localization of β-catenin in papillary RCT, metanephric adenoma and mucinous tubular and spindle-cell carcinoma corresponds to that of emerging tubules of kidney at distinct stage of maturation, indicating their embryonal origin.*

The definition of papillary renal cell tumor (RCT) by the World Health Organization Classification suggests its origin from renal tubular epithelium (1). However, the high number of microscopic lesions accompanying clinically detected papillary RCT and mucinous tubular and spindle-cell carcinoma (MTSCC) suggest an association between precursor lesions and tumor development (2, 3). Metanephric adenoma (MA) consists of small uniform acini of undifferentiated blasteme-like cells, or sometimes papillary structures similar to those occurring in nephrogenic rest and in Wilms' tumor (4). MTSCC is characterized by elongated tubules of 'spindle cells' resembling the emerging loop of Henle in the developing kidney (5). Papillary RCT displays the whole spectrum of cellular differentiation from small blasteme-like 'blue' cells towards large differentiated eosinophilic epithelial cells (6). Therefore, we propose that not only Wilms' tumor, but also papillary RCT, MTSCC and MA develop from embryonal rests, albeit from lesions at distinct stage of cellular differentiation (2, 3, 7).

The link between nephrogenic rests and Wilms' tumor in children is well documented (8-10). The connection of Wilms' tumor to impaired differentiation in embryonal kidney is confirmed by molecular genetic studies (11). Terminal differentiation of meta-nephric mesenchyme (MM) cells into specialized epithelial cells of the nephron, *e.g.* mesenchyme to epithelium transition is a critical step in the developing kidney. WNT9B expressed in ureteric buds, activates the canonical WNT signaling pathway in the surrounding MM, inducing the expression of genes controlling the formation of renal vesicles and thus, the development of nephrons (12-14). β-Catenin encoded by the *CTNNB1* gene is a key downstream component of WNT signaling. Binding the WNT ligand to its transmembrane receptor frizzled and low-density lipoprotein receptor-related protein (LRP) 5 and 6 leads to disruption of the β-catenin destruction complex, which allows accumulation of stabilized β-catenin and subsequent nuclear translocation (15, 16). Dysregulation of genes including those of WNT signaling may lead to 'overgrowth' of cells in the MM niche and to impaired tubular differentiation of blastemal cells, *e.g.* to 'nephrogenic rests' (12).

It has already been shown that a subset of Wilms' tumors displays activated WNT signaling caused by mutation of *CTNNB1* (17, 18). However, no data are available on the activation of WNT signaling in adult tumors of embryonal origin. Taking into account the suggested relationship between development of Wilms' tumor, MA, MTSCC and papillary RCT, we analyzed the possible role of β-catenin in these tumors. We analyzed exons 3, 7 and 8 of the *CTNNB1*

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gene for mutation by sequencing, used a reporter gene assay to analyze β-catenin mediated transcription in cell lines, and applied immunohistochemistry to detect the cellular localization of β-catenin.

Materials and Methods

Tissue samples and cell lines. One part of fresh human kidney and tumor samples obtained during surgery at the Department of Urology, University of Heidelberg, Germany were snap-frozen in liquid nitrogen immediately after nephrectomy and stored at −80˚C. The second part was used to establish tumor cell lines as described earlier (19) and the remaining tissue was fixed in 4% buffered formaldehyde for histological report. Each tumor cell line was karyotyped during the second to third passage and also before being used for this study to confirm their origin. The tumors were diagnosed by a urological pathologist (GK). The cell lines were established and maintained in the Laboratory of Molecular Oncology, Heidelberg. Fetal kidneys were obtained after legal aborts at the Department of Gynaecology, University of Pecs, Hungary. The collection and use of all tissue samples for this study was approved by the Ethics Committee of the University of Heidelberg and University of Pecs, Hungary (no. 5343).

DNA extraction and sequencing. Genomic DNA was isolated from snap-frozen tissue with a standard method. Exons 3, 7, and 8 of the *CTNNB1* gene were independently amplified by PCR. The sequence of primers is available upon request. PCR reactions contained 2 mM $MgCl_2$ and 1 unit Platinum-Taq Polymerase (Invitrogen, Karlsruhe, Germany). PCR fragments were purified using High Pure PCR Purification kit (Roche Diagnostics, Mannheim, Germany) essentially as recommended by the manufacturer. Sequencing reactions were set up following a dye terminator protocol (BigDye Terminator v3.1 Cycle Sequencing Kit, Perkin Elmer, Foster City, CA, USA). The sequencing reactions were run on an ABI Prism 310 DNA Sequencer (Perkin Elmer).

Transfection and luciferase assay. To test the WNT signaling in tumor cells we applied the TOPflash/FOPflash TCF Reporter Plasmid Kit (Upstate Biotechnology, Lake Placid, NY, USA). The reporter plasmid TOPflash contains six copies of the wild-type transcription factor (TCF) binding site upstream of the luciferase open reading frame, while the reporter plasmid FOPflash carries copies of a mutant TCF-binding site and therefore inhibits activation of downstream genes. The latter serves as a negative control to TOPflash. After testing the transfection efficiency, 10 renal tumor cell lines were included in this study (Table I). The human colorectal adenocarcinoma cell line HCT-116 with mutation in the *CTNNB1* gene was used as positive control, whereas kidney cell line HEK293T represented a basal level of WNT signaling. All cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Budapest, Hungary) and 1% glutamine at 37˚C in 5% CO_2. Twenty-four hours before transfection 2.5×10^5 cells/per well were plated into 6-well plates. Each well received 1.0 μg of pTopflash and 1.0 μg pFopflash and as an internal control for transfection efficiency 0.2 μg pRSV-lacZ, with 9.6 μl Enhancer and 25 μl Effectene (Qiagen Inc., Valencia, CA, USA). These specifications were based on the manufacturer's protocol and optimized after preliminary investigation with X-galactosidase staining as the most effective balance between high numbers of transfected cells and a reasonable number of cells killed by cytotoxicity. After 24 h of incubation at 37˚C the transfection medium was removed, cells were washed with phosphate-buffered saline and covered with reporter lysis buffer (Promega Corp., Madison, WI, USA). After 15 min of incubation/freeze and thaw cycles the lysate was taken off, centrifuged and the supernatant used for luciferase and β-galactosidase assay (according to the manufacturer's protocol). Through the β-galactosidase assay, the number of transfected cells was ascertained and the relative luciferase activity was calculated.

Table I. *Origin of renal cell carcinoma cell lines*.

Cell line	Tumor type	Tumor size (mm)	TNM classification
38T	pRCC	105	T3a,N1,M1,G3
394T	pRCC	92	T3b,N1,MX,G3
454T	pRCC	130	T4,N1,M1,G3
2T	cRCC	60	T1b,N1,M1G3
49T	cRCC	32	T3a,N0,M1,G3
136T	cRCC	82	T3a,NX,MX,G2
181T	cRCC	95	T3a,N1,M1,G3
359T	cRCC	45	T1b,NX,MX,G2
390T	cRCC	51	T1b,N0,MX,G1
84T	CDC	22	pT1a,N1,MX,G3
4T	RTK	Unknown	Unknown

pRCC, Papillary RCC; cRCC, conventional RCC; CDC, collecting duct carcinoma; RTK, rhabdoid tumor of the kidney.

Tissue microarray and immunohistochemistry. Original paraffin blocks of fetal and adult kidneys and tissue microarrays (TMA) containing of distinct types of renal cell tumors were used in this study. TMA was constructed using paraffin-embedded material by a Manual Tissue Arrayer (MTA1: Beecher Instruments, Inc., Sun Prairie, WI, USA.) and 0.6 mm core biopsies. After deparaffinization and rehydration of the 4 μm sections, antigen demasking was performed by boiling the slides in 10 mM sodium citrate buffer (pH 6.0) in a 2100-Retriever instrument (Pick-Cell Laboratories, Amsterdam, the Netherlands). Endogenous peroxidase activity and nonspecific binding sites were blocked with 0.3% hydrogen peroxide containing 1% normal horse serum for 15 min at room temperature. Slides were then incubated overnight at 4˚C in a moist chamber with antibody to β-catenin (BD Transduction Laboratories, Lexington, KY, USA) at a dilution of 1:250. Horseradish peroxidase polymer (HRP) -conjugated anti-rabbit secondary antibody (MACH4 Universal HRP-Polymer: BioCare Medical, Concord, CA, USA) was applied for 30 min and color was developed using the 3-amino-9-ethylcarbazole chromogen (DAKO, Glostrup, Denmark). Tissue sections were counterstained with Mayer's hematoxylin. β-Catenin staining was evaluated twice by one of the authors (GK).

Results

Absence of constitutive activation of WNT signaling in RCT. Sequencing failed to detect mutation in exons 3, 7 and 8 of the *CTNNB1* in 8 papillary RCTs. Active WNT signaling is

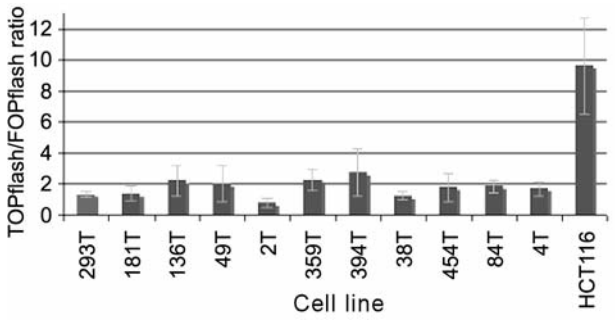

Figure 1. *TOPflash/FOPflash ratio in renal cell carcinoma and control cell lines. The ratio of TOPflash/FOPflash is similarly low to that of negative control 293T cells. In contrast, in HCT-116 colonic carcinoma cells, which harbor an activating mutation in catenin beta 1 (CTNNB1) gene, the transactivation of the TOPflash promoter was about nine-fold higher than that of FOPflash. Designation of tumors corresponds to those shown in Table I.*

characterized by transcriptional activation of WNT-specific target genes which is mediated by nuclear β-catenin-TCF complexes. TOPflash reporter plasmid allows the expression of luciferase under the specific control of an element that binds β-catenin-TCF. In contrast, FOPflash carrying a mutation within the β-catenin-TCF recognition sequence prevents binding as well as transcriptional activation. Cells with active WNT signaling are able to drive the reporter gene expression from TOPflash, whereas FOPflash remains silent. The ratio between the reporter plasmids indicates WNT signaling activity. In our experiment, the ratio of TOPflash/FOPflash expression was low in the negative control of HEK293T cells as well as in the tumor cell lines (Figure 1). In contrast, HCT-116 colonic carcinoma cells harboring an activating mutation in *CTNNB1* displayed nine-fold higher transactivation of the TOPflash promoter than that of FOPflash. The *in vitro* experiment indicates the absence of constitutive activation of the WNT signaling in renal cell carcinomas.

Expression of β-catenin in fetal kidney, MA, MTSCC and papillary RCT. Immunohistochemical analysis of fetal kidneys revealed the constitutional activation of β-catenin during the mesenchyme to epithelium transition (Figure 2A). The ureteric bud cells displayed strong cytoplasmic β-catenin staining whereas the cap MM cells exhibited a strong cytoplasmic and occasionally nuclear positivity. The renal vesicle, comma-shaped body and distal part of the S-shaped body exhibited cytoplasmic and weak membranous staining. The emerging small tubules displayed cytoplasmic staining, whereas those with more differentiated epithelial cells presented membranous positivity with β-catenin antibody. Stromal mesenchyme cells 'dropping down' along the proximal-distal axis of nephrons

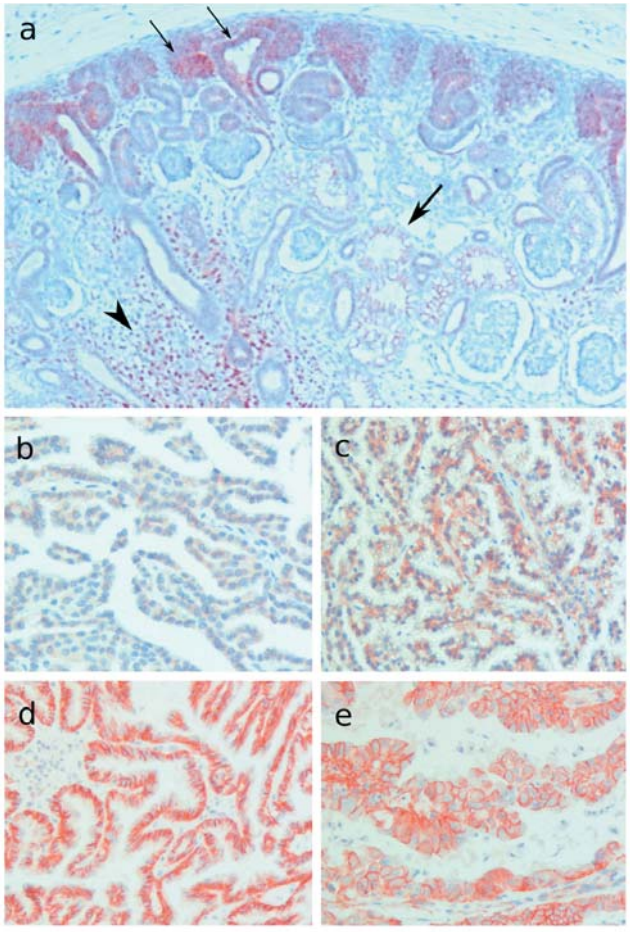

Figure 2. *Expression of β-catenin in fetal kidney (×50) and papillary renal cell tumors (RCT) (×400). β-Catenin is strongly expressed in the tip of ureteric buds and condensed meta-nephric mesenchyme (thin arrow), and stromal cells (arrowhead). There is a weak membranous staining in differentiated tubules (thick arrow). Papillary RCT display weakly cytoplasmic towards strong membranous staining with the increasing cell size and polarity (b-e).*

exhibited weak to strong nuclear staining. In normal adult kidney distal convoluted tubules showed accumulation of β-catenin on their membranes, sometimes in cytoplasm, but never in the nucleus. Faint membranous staining was also seen in cells of collecting ducts.

Small, undifferentiated cells of MA were negative for β-catenin staining whereas cells of larger tubular structures displayed weak cytoplasmic staining. The 'spindle cell' and tubulo-papillary structures of MTSCC exhibited weak and stronger cytoplasmic staining, respectively. Papillary RCT with small 'blue' cells also displayed weak cytoplasmic staining, whereas with cellular differentiation towards larger epithelial cells, membranous accumulation of β-catenin was seen (Figure 2B-E). Results of immunohistochemistry are summarized in Table II.

Table II. *Staining and cellular localisation of* β*-catenin in adult tumors of embryonal origin.*

Tumor	Number of tumors	Negative	Results of immunohistochemistry		
			Membranous	Cytoplasmic	Nuclear
pRCT	86	15	49	22	0
MTSCC	6	1	1	4	0
MA	7	2	3	2	0

pRCT, Papillary renal cell tumor; MTSCC, mucinous tubular and spindle-cell carcinoma; MA, metanephric adenoma.

Discussion

Activation of WNT signaling through mutation, cytoplasmic accumulation and nuclear transition of β-catenin and subsequent transcription of the *TCF/LEF* target genes has been described in several childhood and adult tumors (17, 18, 20). Previous studies identified exon 3 encoding the consensus glycogen synthase kinase 3 beta (GSK3β) phosphorylation sites as the major mutational target region within *CTNNB1*. It was also suggested that mutation in exon 7 and exon 8 might substitute for exon 3 mutations in a subset of Wilms' tumors (17). By sequencing exons 3, 7, and 8 of *CTNNB1* we have excluded their mutation as a driver of WNT signaling in papillary RCT. Although the TOPflash/FOPflash ratio in distinct RCC cell lines was slightly higher than in the HEK293T normal kidney cell line, constitutive activation of the WNT signaling in renal cell tumors can be excluded.

The constitutive activation of the canonical WNT signaling pathway is instrumental during development of the kidney (12-14). In the absence of WNT, cytoplasmic free β-catenin is recognized by the axin, casein kinase 1, GSK3 and adenomatous polyposis coli destruction complex and rapidly targeted for degradation (16). In the presence of WNT ligand, β-catenin accumulates in the cytosol, enters the nucleus and leads to transcriptional activation TCF/LEF target genes (15). Conditional deletion of *Ctnnb1* in mouse MM cells results in down-regulation of the target genes and renal hypoplasia (12). These data indicate that canonical WNT signaling in MM is necessary for the formation of pre-tubular aggregates and subsequent nephron development. It was also proposed that β-catenin-TCF-LEF1 signaling is not only instrumental in the morphogenesis of kidney epithelial lineage but has an inhibitory effect on terminal differentiation (21).

Immunohistochemical analysis of fetal kidney revealed the constitutional activation of β-catenin signaling in MM cells around the ureteric bud. Weak to strong nuclear expression of β-catenin in cells of the stromal mesenchyme indicates active WNT signaling in the formation of mature renal structures. In addition to its key role in the canonical WNT signaling, β-catenin is involved in the regulation of cell adhesion as a part of protein complex of adherens junctions, which play a role in the maintenance of epithelial cell layers by regulating cell growth and adhesion between cells (15). During transition from the mesenchyme fate towards epithelial characteristics, β-catenin is immobilized by binding to E-cadherin and thus accumulates at the cell membrane of emerging tubules in fetal kidney. In adult kidney, in addition to membranous staining, weak cytoplasmic positivity for β-catenin protein was seen in distal and connecting tubules and membranous staining in collecting duct.

Of interest, MA, MTSCC and papillary RCT recapitulate not only the cellular differentiation of kidney development but also the corresponding localization of β-catenin, with exception of its nuclear staining. Small, undifferentiated cells or slightly elongated cells of the MA and MTSCC, respectively, inhibited weak cytoplasmic staining indicating that at least a fraction of β-catenin is stabilized. The small tubular-papillary growing cells of papillary RCT displayed weak staining in the cytoplasm whereas with increasing size and polarization of tumor cells, the staining intensity increased at the cell membrane. Thus the weak cytoplasmic towards membrane bound expression of β-catenin in MA, MTSCC and papillary RCT analogous to cellular differentiation in embryonal kidney rather than tumorigenic activation of WNT signaling.

In summary, the lack of mutation, signaling activity *in vitro* and also the lack of cytoplasmic or nuclear accumulation of β-catenin exclude the role of canonical WNT signaling in the development of adult renal cell tumors of embryonal origin, such as MA, MTSCC and papillary RCT.

Conflicts of Interest

None.

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References

1 Moch H, Humphrey PA, Ulbright TM and Reuter VE (eds): WHO Classification of Tumours of the Urinary System and Male Genital Organs. IARC: Lyon, p. 23, 2016.

2 Kovacs G and Kovacs A: Parenchymal abnormalities associated with papillary renal cell tumors: a morphologic study. J Urol Pathol *1*: 301-312, 1993.

3 Szponar A and Kovacs G: Expression of KRT7 and WT1 differentiates precursor lesions of Wilms' tumours, papillary renal cell tumours and mucinous tubular and spindle cell carcinomas. Virchows Arch *460*: 423-427, 2012.

4 Szponar A, Yusenko MV and Kovacs G: High-resolution array CGH of metanephric adenomas: lack of DNA copy number changes. Histopathology *56*: 212-216, 2010.

5 Rakozy C, Schmahl GE, Bogner S and Storkel S: Low-grade tubular-mucinous renal neoplasms: morphologic, immunohistochemical, and genetic features. Mod Pathol *15*: 1162-1171, 2002.

6 Balint I, Szponar A, Jauch A and Kovacs G: Trisomy 7 and 17 mark papillary renal cell tumours irrespectively of variation of the phenotype. J Clin Pathol *62*: 892-895, 2009.

7 Kovacs G: Molecular cytogenetics of renal cell tumors. Adv Cancer Res *62*: 89-124, 1993.

8 Bove K and McAdams AJ: The nephroblastomatosis complex and its relationship to Wilms tumor: A clinicopathologic treatise. Perspect Pediatr Pathol *3*: 185-223, 1976.

9 Beckwith JB: Nephrogenic rests and the pathogenesis of Wilms tumor: Developmental and clinical considerations. Am J Med Genet *79*: 268-273, 1998.

10 Beckwith JB, Kiviat NB and Bonadio JF: Nephrogenic rests, nephroblastomatosis, and the pathogenesis of Wilms tumor. Pediatr Pathol *10*: 1-36, 1990.

11 Rivera MN and Haber DA: Wilms' tumour: connecting tumorigenesis and organ development in the kidney. Nature Rev Cancer *5*: 699-712, 2005.

12 ParkJS, Valerius MT and McMahon AP: WNT/beta-catenin signaling regulates nephron induction during mouse kidney development. Development *134*: 2533-2539, 2007.

13 Carroll TJ, Park JS, Hayashi S, Majumdar A and McMahon AP: WNT9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. Dev Cell *2*: 283-292, 2005.

14 Kispert A, Vainio S and McMahon AP: WNT4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. Development *125*: 4225-4234, 1998.

15 Valenta T, Hausman G and Basler K: The many faces and functions of β-catenin. EMBO J *31*: 2714-2736, 2012.

16 Kimelman D and Xu W: Beta-catenin destruction complex: insights and questions from a structural perspective. Oncogene *25*: 7482-7491, 2006.

17 Koesters R and Doeberitz MV: The WNT signaling pathway in solid childhood tumors. Cancer Letters *198*: 123-138, 2003.

18 Li CM, Kim CE, Margolin AA, Guo M, Zhu J, Mason JM, Hensle TW, Murty VV, Grundy PE, Fearon ER, D'Agati V, Licht JD and Tycko B: *CTNNB1* mutations and overexpression of WNT/beta-catenin target genes in *WT1*-mutant Wilms' tumors. Am J Pathol *165*: 1943-1953, 2004.

19 Kovacs G and Frisch S: Clonal chromosome abnormalities in tumour cells from patients with sporadic renal cell carcinomas. Cancer Res *49*: 651-659, 1989.

20 Polakis P: WNT signaling and cancer. Genes and Dev *14*: 1837-1851, 2000.

21 El-Dahr SS, Aboudehen K and Saifudeen Z: Transcriptional control of terminal nephron differentiation. Am J Physiol Renal Physiol *294*: F1273-F1278, 2008.

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