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Original contribution

Novel concept of Wilms' tumor development: involvement of pluripotential cells of ureteric bud☆

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Summary It is acknowledged that nephron develops after bilateral induction of the metanephric mesenchyma and branching ureteric bud (UB), and that nephrogenic rest and Wilms' tumor (nephroblastoma) arises from impaired differentiation of metanephric blastema. The aim of this study was to obtain more information on the involvement of UB derivatives in nephrogenic rest and Wilms' tumor. We applied immunohistochemistry to analyze nephrogenic rests and Wilms' tumors with mixed histology, including regressive and blastemal types. We used antibodies recognizing UB tip cells (ROBO1, SLIT2, RET), principal cells (AQP2), α- and β-intercalated cells (SLC26A4, SLC4A1, ATP6V1B1, ATP6V0D2), and their precursors (CA2). Tubules surrounded by tumorous blastemal cells resembling UB tip were positive for RET, ROBO1, and SLIT2 in Wilms' tumor. Moreover, CA2-positive tubular structures and ATP6V1B1- and ATP6V0D2-positive immature non-α- and non-β-intercalated cells were detected in nephrogenic rest and Wilms' tumor. We suggest that Wilms' tumor is more than *nephroblastoma* and propose a definition that *Wilms tumor is a malignant embryonal neoplasm derived from pluripotential cells of nephrogenic blastema and of ureteric bud tip*.

☆ Disclosures: None.

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1. Introduction

Nearly 150 years ago, Cohnheim [1] has postulated that a "developmental error and impaired differentiation within the embryonal Anlage may lead to tumor development. The newborn does not bring the tumor itself but merely the superabundant undifferentiated cell material into the world, from them a tumor may develop." Wilms' tumor (WT) is one of the best examples of arrested cellular differentiation and tumor development. After cessation of kidney development at the 36th week of pregnancy, due to failure in bilateral induction between ureteric bud (UB) and metanephric mesenchyma (MM), metanephric blastemal cells may remain over, from which WT may develop [2,3]. WT recapitulates the morphology and molecular biology of developing kidney [4,5]. The arrested differentiation of MM results in WT of triphasic histology with nests of blastemal cells, epithelial tubules, and stromal elements. In other cases, one cell type may predominate, resulting in blastemal, epithelial, or stromal predominant WT [6].

Based on this presumption, WT has been determined by the World Health Organization (WHO) in 2004 as follows: "Nephroblastoma is a malignant embryonal neoplasm derived from *nephrogenic blastemal cells*. It mimics developing kidney and often shows a divergent pattern of differentiation" [7]. This definition was challenged in 2007 by a report presenting UB-like tubular structures in triphasic WT [8]. Despite the new finding, the latest WHO classification in 2016 retained the 2004 definition [9]. Comparative gene expression analysis identified overlapping fingerprint of gene expression in developing human kidney and WT and detected UB-like epithelial structures in tumor tissue by immunohistochemistry [10]. Recently, UB derivatives were found in nephrogenic rest (NR) [11].

The aim of our study was to obtain more information on the involvement of UB in development and histology of WT. Furthermore, we asked whether pluripotent cells of UB-tip (UBT) can differentiate under tumorigenic condition. First, we searched for UB-derived tubular structures by RET, ROBO1, and SLIT2 immunohistochemistry in NR and WT. Subsequently, we have analyzed the expression of CA2 marking progenitors of intercalated (IC) and principal (PC) cells, ATP6V1B1, ATP6V0D2, SLC4A1, and SLC26A4, which are specific for immature and mature α- and β- IC cells and AQP2 specific for PCs [12].

2. Materials and methods

2.1. Tissue samples

Formalin-fixed, paraffin-embedded fetal kidneys from 10 weeks (n = 2), 12 weeks (n = 2), 15 weeks (n = 2), 17 weeks (n = 5), and 21 weeks (n = 5) gestational ages were obtained from the Department of Pathology, Medical School, University of Pecs, Hungary. Tissue samples were fixed in 4% buffered formaldehyde and embedded in paraffin for the histological report. Histological samples of 12 WTs were obtained from the Wilms' Tumour Registry, Department of Pathology, University of Szeged, Hungary. All WT cases were pretreated and evaluated according to the UMBRELLA SIOP-RTSG 2016 protocol [13]. Eight mixed-type WTs, 2 WTs of regressive type, one each of blastemal type and with focal anaplasia were included in this study. Moreover, we have analyzed 6 perilobular nephrogenic rests (PLNRs), including a hyperplastic one of 6 mm in diameter, and 3 intralobular nephrogenic rests (ILNRs).

2.2. Immunohistochemistry

After removing the paraffin and rehydration, the 4 μm-thick sections were subjected to heat-induced epitope retrieval in citrate buffer, pH 6.0, or in EnVision FLEX Target Retrieval Solution, high pH (DAKO, Glostrup, Denmark) in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, The Netherlands). Endogenous peroxidase activity was blocked with EnVision FLEX Peroxidase-Blocking Reagent (DAKO) for 10 min at room temperature. Slides were then incubated for 1 h with antibodies listed in Table 1. EnVision FLEX horse-radish-peroxidase conjugated secondary antibody (DAKO) was applied for 30 min at room temperature, and color was developed using the DAB or AEC substrate (DAKO). Tissue sections were counterstained with Mayer's hematoxylin (Lillie's modification, DAKO), and after 10 s of bluing in ammonium-hydroxide solution, they were mounted by Glycergel

Table 1 Pertinent data of antibodies used in this study.

Antibody	Reference	Species	Clonality	Dilution	Source
RET	Anti-RET-HPA008356	Rabbit	Poly	1:200	Atlas Antibodies
SLIT2	Anti-SLIT2-PA5-31133	Rabbit	Poly	1:500	Thermo Fisher
ROBO1	Anti-ROBO1-PA5-34822	Rabbit	Poly	1:500	Thermo Fisher
AQP2	Anti-AQP2-PA5-38004	Rabbit	Poly	1:500	Thermo Fisher
CA2	Anti-CA2-PA5-28267	Rabbit	Poly	1:1000	Invitrogen
SLC26A4	Anti-SLC26A4-NBP1-60106	Rabbit	Poly	1:200	Novus Biologicals
SLC4A1	Anti-SLC4A1-HPA015584	Rabbit	Poly	1:200	Sigma Aldrich
ATP6V1B1	Anti-ATP6V1B1-EPR16401	Rabbit	Mono	1.2000	Abcam
ATP6V0D2	Anti-ATP6V0D2-NDBP2-31600	Rabbit	Poly	1:100	Novus Biologicals

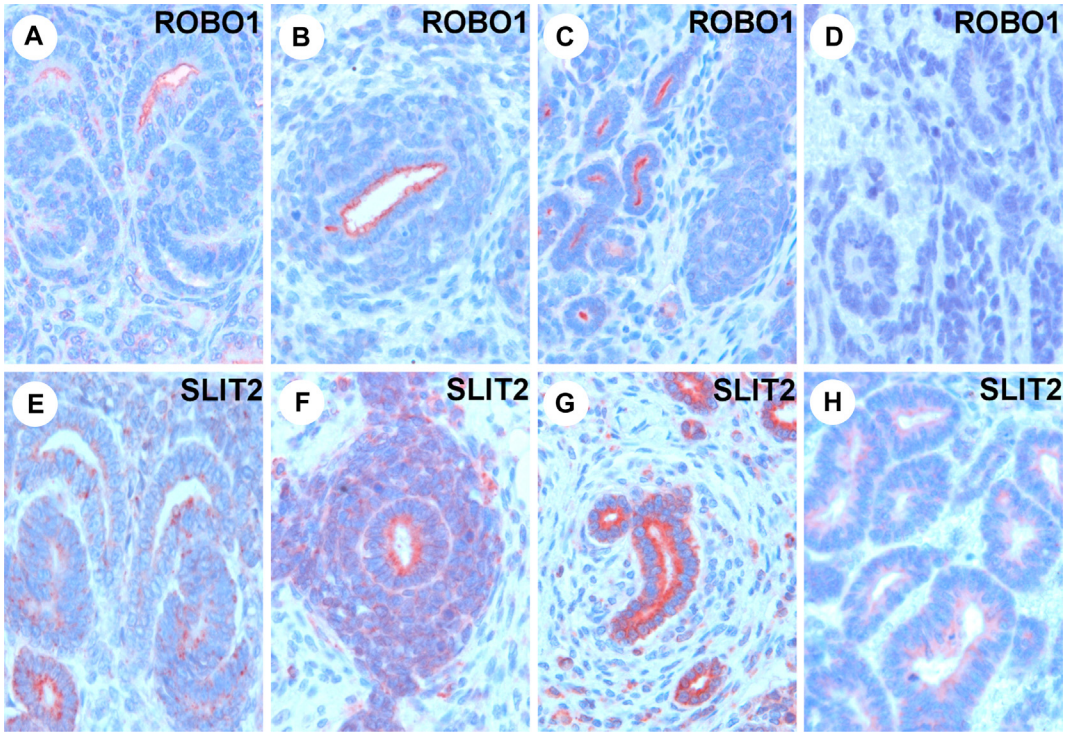

Fig. 1 Expression of ROBO1 and SLIT2 in fetal kidney and WT. A, ROBO1 is expressed on the luminal surface of UBT. B, Strong ROBO1 expression of UB-like tubulus surrounded by blastemal cells in WT. C, Small tubules in fibrotic WT stroma also display ROBO1 staining. D, No immunoreaction was seen in RV-like tubules. E, SLIT2 is expressed in cells of UBT, blastemal cells, and MM-derived SSB in fetal kidney. F, SLIT2 expression in UB-like tubulus and surrounding blastemal cells. G, Tubules embedded in fibrotic WT stroma display SLIT2 expression. H, RV-like tubules in WT show a weak SLIT2 expression in their luminal surface. a-h, ×200 magnification.

(DAKO). Each antibody was validated for specificity by immunohistochemistry of normal adult kidneys [12]. For negative control, the primary antibody was omitted. Photographs were taken by a Leitz DMRBE microscope equipped with a HC PLAN APO 20×0.70 objective and a ProgRes C14 camera.

3. Results

3.1. UB markers in fetal kidney

We have analyzed the cellular localization of proteins encoded by genes listed in Table 1 in fetal kidney. We observed ROBO1 and RET expression in UBT and UB trunk, but no expression has been seen in metanephric mesenchyme (MM)−derived structures such as condensed blastemal cells, renal vesicles (RVs), or S-shaped body (SSB). (Figs. 1A and 2A). SLIT2 showed a weak expression in cells of UBT and UB trunk and in MM-derived blastemal cells and emerging epithelial structures, including RV and SSB (Fig. 1E).

CA2 identifies progenitor cells committed to differentiate into PC and IC cells in adult kidney [12]. The CA2-positive cells were seen in developmentally younger cortical domain of the UB in close vicinity to but not in the UBT (Fig. 2E). Immature IC cells in cortical UB trunk showed positive staining with IC cell markers ATP6V1B1 and ATP6V0D2. Most of the positive tubules displayed a thin line of ATPase positivity on the luminal surface of cells, and only a few showed a cap-like positivity as seen in adult kidneys. Strong AQP2 protein expression was observed in the medullary UB trunk corresponding to the medullary collecting duct (CD), but the cortical UB trunk and UBT were negative. The expression of genes in distinct type of cells and areas in fetal kidneys is summarized in Table 2. None of the genes, with exception of the SLIT2, have been detected in MM-derived structures of fetal kidney.

3.2. UB derivatives in WT

ROBO1, which is expressed in UBT in fetal kidneys (Fig. 1A), showed a strong expression in UB-like tubules embedded in nodular- or serpentine growing blastemal cells or in mesenchymal stroma (Fig. 1B, C). However, no ROBO1 expression was seen in MM-derived tubular structures resembling RVs (Fig. 1D). The expression of SLIT2, corresponding to its expression in normal fetal kidney (Fig. 1E), was detected not only in the UB-like structures but also in the surrounding blastemal cells as

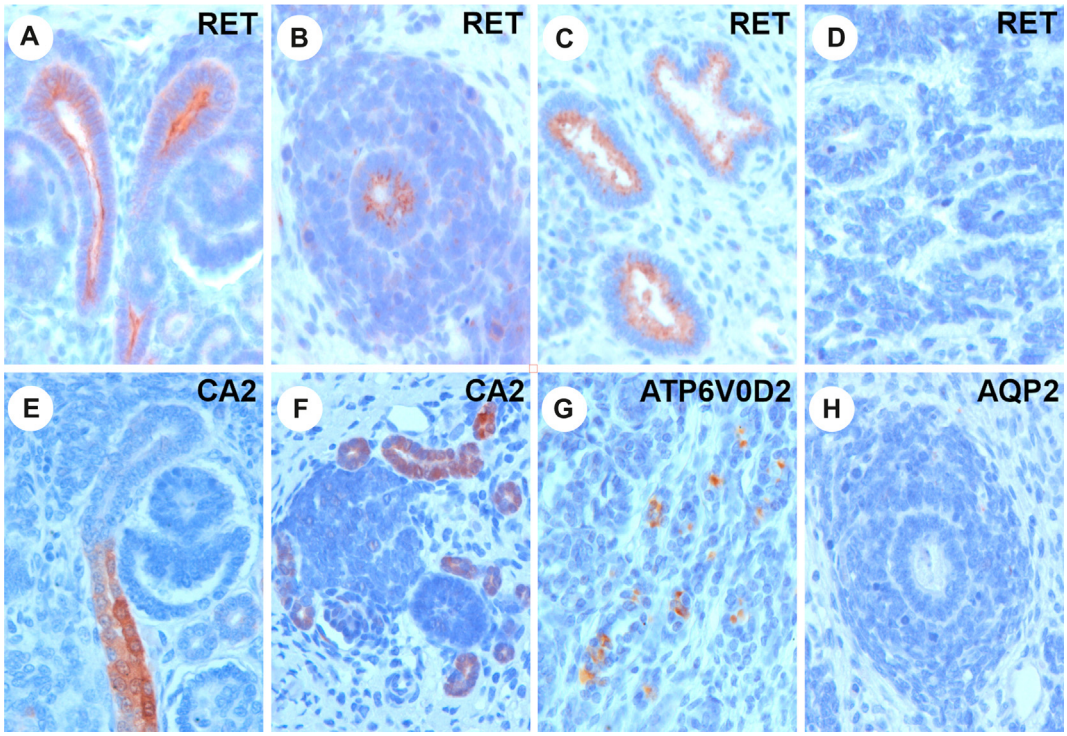

Fig. 2 Expression of RET and CA2 in fetal kidney and WT. A, RET is expressed in UBT in fetal kidney. B, UBT-like tubulus embedded in blastemal cells in WT display RET expression. C, Tubular cells in fibrotic WT stroma express RET protein. D, No RET expression was noticed in RV-like tubules. E, In fetal kidney, CA2 is expressed in cortical UB trunk but not in UBT. F, Small tubules in WT display strong CA2 expression. G, ATP6V0D2-positive immunostaining indicates the occurrence of immature IC cells in WT. H, No positive staining was seen with AQ2 in tubular or blastemal cells in WT. a-h, ×200 magnification.

Table 2 Expression of ureteric bud and cell specific gene products in human fetal kidney.

	RET	ROBO1	SLIT2	CA2	ATPase	AQP2
UBT	+	+	+	−	−	−
C-TRUNK	+	+	+	+	+	−
M-TRUNK	+	−	−	−	−	+
MM-DER	−	−	+	−	−	−

Abbreviations: UBT, ureteric bud tip; C-TRUNK, cortical UB-trunk; M-TRUNK, medullary UB-trunk; MM-DER, metanephric-mesenchyme derivative; −, negative; +, positive.

well (Fig. 1F). SLIT2 immunoreaction was seen in tubular structures embedded in circularly growing proliferative stroma (Fig. 1G) and in MM-derived RV-like tubules (Fig. 1H).

RET is expressed exclusively in UBT cells of fetal kidney (Fig. 2A), and RET immunoreaction in UB-like tubules surrounded by blastemal cells and UB-like tubules in fibrotic stroma in WT (Fig. 2B-C). None of the RV-like tubular structures displayed RET-positive staining (Fig. 2D). Thus, ROBO1, SLIT2, and RET, each of which marks UBT cells in normal fetal kidney, showed a positive immunoreaction in UBT-like tubules in WT. In fetal kidney, UBT cells were negative, whereas cells of cortical UB trunk were positive for CA2 staining (Fig. 2E). In WT, elongated tubular structures resembling cortical UB trunk showed a strong immunoreaction with the CA2 antibody (Fig. 2F). Some tubular structures in WT displayed ATP6V1B1 and ATP6V0D2 immunoreactions, indicating immature IC cells (Fig. 2G). None of the WT showed positive staining with SLC4A1 and SLC26A4, which are markers of mature α- and β-IC cells. No structures or cells in WT displayed positive staining with AQP2 (Fig. 2H).

3.3. UB-derivatives in NR

It is generally accepted that WT is associated with and develops from NR. Therefore, we have analyzed 9 NRs, including a hyperplastic one of 6 mm in diameter, by applying antibodies used for analysis of fetal kidney and WT. We found a positive reaction with RET and ROBO1 in

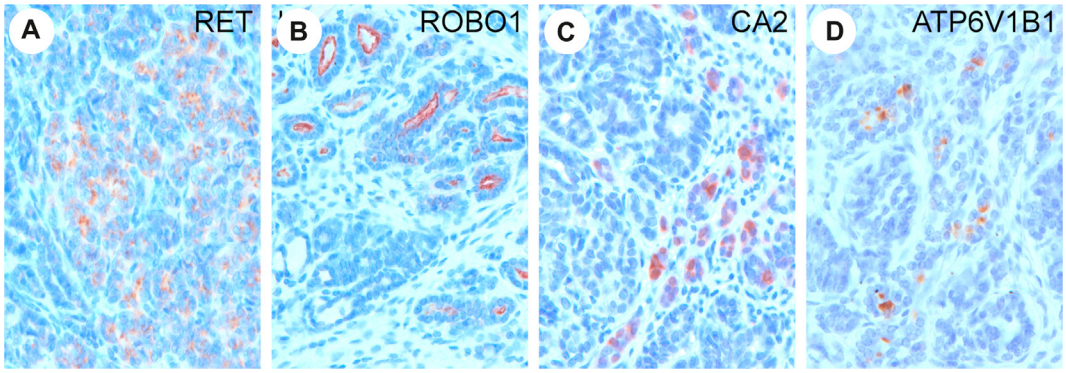

Fig. 3 UBT derivative cells in nephrogenic rest. A, Proliferating epithelial cells in a hyperplastic nephrogenic rest express RET protein. B, ROBO1-positive tubules in intralobular nephrogenic rest. C, CA2-positive epithelial cells in intralobular nephrogenic rest. D, Scattered immature IC cells showing ATP6V1B1 positivity in intralobular nephrogenic rest. a-d, ×200 magnification.

3 of 6 PLNRs, including the hyperplastic ones (Fig. 3A). Two of the 3 ILNRs also showed positive immunoreactions with both RET and ROBO1 antibodies (Fig. 3B). Positive immunoreaction was also seen with CA2 antibody in solid-growing epithelial cells of the hyperplastic rest and tubular structures of 2 ILNRs (Fig. 3C). Moreover, 2 ILNRs contained tubular cells displaying positive staining with ATP6V0A4 and ATP6V1B1 antibodies (Fig. 3D).

4. Discussion

We found the expression of UBT-specific markers RET and ROBO1 as well as SLIT2 in epithelial cells of NR and triphasic WT, indicating the involvement of UBT in the development of WT. We have documented the occurrance of CA2-positive PC and IC precursor cells and immature IC cells in both NR and WT. Our finding strongly suggests that pluripotential cells of UBT are involved in the oncogenesis of WT and that these cells have limited capacity to differentiate under tumorigenic conditions.

The RET, ROBO1, and SLIT2 play pivotal roles in the development of UB. The binding of transmembrane receptor tyrosine kinase RET to its ligands, GDNF, is a requirement for the outgrowth of UB from nephric duct (ND) [14]. The GDNF-mediated RET signaling promotes cell movements within the ND that leads to initial UB formation [15]. GDNF/RET signaling induces not only the budding of UB but also controls the regular UB branching during kidney development. Lack of RET function due to mutation is associated with renal agenesis [16]. In addition to the GDNF/RET signaling, several other genes secreted by the MM regulate the growth and branching of UB [17].

At the initial stage of UB formation, the SLIT/ROBO signaling provides axon guidance clues and represses ectopic UB outgrowth from ND by preventing GDNF expression in MM cells [18]. Mouse mutants lacking either SLIT2 or ROBO2 develop supernumerary UBs that remain inappropriately connected to the ND. The intercellular SLIT2/ROBO1 signaling plays an important role in growing and branching of UB and development of CD system [18,19]. ROBO1 expression in UBT cells mediates a chemorepellent signal to bring the SLIT2 ligand expressing SSB in right position to be connected to UBT, which is the prerequisite for the development of normal nephron. The SLIT2/ROBO1 signaling is involved in the branching morphogenesis in other organs, such as the mammary gland [20].

The nephron develops after bilateral induction of the cap mesenchyme and branching UBT [21]. During the branching phase, UBT cells display rapid cell proliferation, whereas cells of the UB trunk show significantly decreased mitotic activity [22]. The UBT cells express a set of highly specific genes and have a self-renewing capacity to produce enough cells for branching until the kidney development is completed [23]. In fetal kidney, cells of UB trunk gradually lose the genes expressed in UBT and convert into CA2-positive epithelial cells, which later differentiate into AQP2-positive PC.

The self-renewal capacity of cells within the MM and UBT determines the number of nephrons, which is estimated between 200.000 and 2 million per organ [24]. The molecular mechanism underlying the regulation of differentiation is not completely known [25]. Considering the large number of nephrons, an error in the regulation of complex patterning may easily occur. Some of the differentiation arrested cells or cell groups may develop NR and later WT, both containing MM- and UBT-derived cells [8,10,11]. The UBT is a cell reservoir, and the bipotential UBT cells can give rise to trunk cells as well as more tip cells for continuing the branching process [14,21]. The differentiation of UB trunk from UBT cells is evident from their spatial pattern of gene expression [26]. Many tip-specific genes are involved in UBT growth and branching, whereas many trunk-specific genes have specific functions in the mature CD as ion channels.

CA2 marks the progenitors of PC and α- and β-IC cells [12]. We have detected the expression of CA2, ATP6V1B1, and ATP6V0D2 in NR and triphasic WT. Our findings

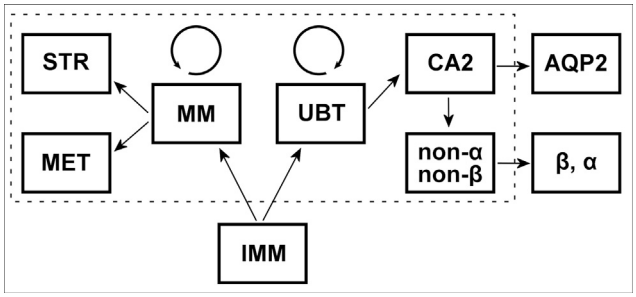

Fig. 4 The suggested pathway of WT development. Both metanephric mesenchyma (MM) and ureteric bud (UB) derive from the intermediary mesenchyme (IMM) and retain self-renewal capacity to generate appropriate number of nephrons. MM differentiates into the proximal nephron through the mesenchymal-to-epithelial transition (MET) and makes up the kidney stroma (STR). The UBT differentiate into CA2-positive precursor cells, which convert into AQP2-positive principal cells (PC) in normal kidney. CA2-positive cells give rise to immature non-α- and non-β-intercalated (IC) cells, which differentiate into mature, functional β- and α-IC cells in normal kidney. Differentiation phases of embryonal kidney which may occur in WT are bracketed. Fully differentiated PC and IC cells cannot be seen in WT.

indicate that UBT cells can differentiate into CA2-expressing cortical UB trunk and ATP6V1B1- and ATP6V0D2-positive immature IC cells in tumorigenic condition. However, UBT derivatives cannot be converted into functional β- and α-IC cells in NR and WT as they differentiate during the development of normal kidney [27,28].

Data from the literature as well as from the present study demonstrate the occurrance of UBT derivatives in NR and WT [8,10,11]. These findings indicate that not only MM-derived cells but also bipotential cells of UBT are involved in the development of NR and WT. Based on these results, we propose a new concept of WT histogenesis (Fig. 4). We suggest that WT is more than *nephroblastoma*. Instead of the WHO definition, we prefer a definition of WT as follows: *Wilms tumor is a malignant embryonal neoplasm derived from pluripotential cells of nephrogenic blastema and UB tip*.

5. Concluding remarks

Recently, the Children's Oncology Group (COG) and International Society of Pediatric Oncology (SIOP) Group included the histologic response to therapy as one of the prognostic factors [29]. The SIOP Renal Tumor Study Group classified WT as low, intermediate, and high-risk tumor based on analysis of viable cellular components that remained over after chemotherapy [13]. We showed here that not only MM- but also UBT-derived cells are involved in the development of NR and WT. How the consideration of viable UBT-derived epithelial cells would modify the post-therapeutic classification of WT is not yet known. Screening many WTs included in the UMBRELLA SIOP-RTSG 2016 protocol with ROBO1 and RET antibodies would give information on the response of UBT derivatives to chemotherapy.

Acknowledgments

Statement of ethics: The collection and use of fetal kidney specimens were approved by the Ethics Committee of the University of Pecs, Hungary (No. 8466-PTE 2020). The use of WT samples for this study was approved by the Ethics Commission of the Hungarian Medical Research Council (IV/8956-3/2020/EKU). All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from the parents of all patients involved in this study.

Author contribution statement: B.S. contributed to conceptualization, investigation, methodology, project administration, and writing the original draft. L.K. contributed to material selection and tumor collections. G.K. contributed to conceptualization, methodology, and writing − review of manuscript. All authors read and approved the final version of the manuscript submitted for publication.

Data availability statement: The data sets are available from the corresponding authors on reasonable request.

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