Chapter 10

Gene Expression in Wilms Tumor: Disturbance of the Wnt Signaling Pathway and MicroRNA Biogenesis

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Doi: http://dx.doi.org/10.15586/codon.wt.2016.ch10

Abstract

Wilms tumor (WT) originates from the metanephric blastemal cells that are unable to complete the mesenchymal–epithelial transition, resulting in a tumor with triphasic histology, including blastemal, epithelial, and stromal components. WT shows morphological and molecular characteristics that resemble the fetal kidney. Thus, the study of molecular pathways relevant to normal kidney differentiation provides insight into the events that drive

In: *Wilms Tumor*. Marry M. van den Heuvel-Eibrink (Editor)
ISBN: 978-0-9944381-1-9; Doi: http://dx.doi.org/10.15586/codon.wt.2016
Codon Publications, Brisbane, Australia

Wilms tumorigenesis. The Wnt signaling pathway has been shown to be crucial for correct kidney differentiation. This pathway is activated by WNT proteins and consists of two highly connected main branches: the canonical (or β-catenin dependent) and the noncanonical (or β-catenin independent). Both branches are essential for controlling embryonic development and adult cell homeostasis. The activation of the canonical Wnt pathway leads to the nuclear accumulation of β-catenin, which acts as a coactivator for transcription factors. In the absence of WNT ligands, this pathway is inactivated by a destruction complex that phosphorylates β-catenin, leading to ubiquitination, proteasomal degradation, and the prevention of β-catenin accumulation in the nucleus. In this context, the expression and mutation analyses of genes involved in Wnt signaling pathways constitute an important approach for understanding WT etiology. Although the activation of the Wnt pathway is well understood in WT samples relative to normal kidney tissue or differentiated kidney cells, there is a remarkable variation among subgroups of WTs. Recently, five WT subgroups were identified, mainly through the use of gene expression data, and only two of them showed clear evidence of Wnt pathway activation, as measured by the presence of β-catenin in the nucleus. Interestingly, some of these subgroups exhibited recurrent germline or somatic mutations in genes involved in microRNA biogenesis, such as *DROSHA* and *DICER*. Here, we will review relevant findings regarding Wilms tumorigenesis as revealed by gene expression and mutation analyses, mainly in genes belonging to the Wnt signaling and microRNA biogenesis pathways.

Key words: β-catenin; microRNA biogenesis; Nephrogenesis; Wilms tumor; Wnt signaling pathway

Introduction

Wilms tumor (WT) is an embryonic tumor that is initiated from primitive renal cells that are incapable of completing kidney differentiation. The result is a tumor that recapitulates the earliest step of nephrogenesis and is morphologically and molecularly similar to the fetal kidney. As a consequence, WTs are composed of varying proportions of three morphologically distinct cell types: undifferentiated blastemal cells, epithelial cells ordered into primitive structures, and stromal cells (1). Accordingly, the blastemal component displays an expression profile similar to the earlier stages of kidney development (2).

Signal transduction pathways control signaling from the outside to the inside of a cell through interactions between proteins and cell surface receptors, triggering specific cellular processes, mainly via changes in gene expression. The precise control of gene activation or inactivation is crucial for correct kidney differentiation and function. Disturbances in this process through the mutation of genes that directly or indirectly control gene

expression result in the failure of precise kidney development and may in turn lead to renal disease in children, including renal agenesis, dysplasia, hypoplasia, and WT (3). In WT, mutations have been identified in tumor suppressor genes (TSG) and oncogenes (4) (mainly from the WNT signaling pathway) or in genes involved in microRNA (miRNA) biogenesis (5, 6). Thus, as carcinogenesis requires gene mutations, the morphological aspects of WT seem to be dependent on where and when mutations occur during the process of kidney differentiation. In this chapter, we present the current molecular and morphogenetic knowledge about nephrogenesis and WT, focusing on the Wnt signaling and miRNA biogenesis pathways.

Morphogenetic process of kidney development

Kidney development, also known as nephrogenesis, refers to the embryologic origins of this organ. Kidney morphogenesis begins at gastrulation, in the third week of gestation in humans, when the embryo exhibits the three germ layers: ectoderm, mesoderm, and endoderm. The intermediate mesoderm gradually forms the urogenital system, including the pronephros, mesonephros, and metanephros; the first two proceed to develop into transitory kidneys and the third differentiates into the mature and functional kidney (7). The metanephros originates through interactive signals in bidirectional communication between epithelial and mesenchymal cells that ultimately form the nephrons, the functional unit of the kidney. Thus, the entire process of the differentiation of the kidney, with its multifaceted functional structures, involves close interaction between epithelial and mesenchymal cells, in which the signal transduction pathway is imperative. Mesenchymal–epithelial transition (MET) is a crucial process operating during kidney differentiation (7), which comprises the transition from a multipolar or a spindle-shaped mesenchymal cell to a planar assembly of polarized cells known as epithelia. Epithelial cells are stationary and are characterized by apical–basal polarity, tight junctions, and the expression of cell–cell adhesion markers, such as E-cadherin (8), whereas mesenchymal cells do not form cell–cell contacts. Mesenchymal cells can invade through the extracellular matrix and express markers, such as vimentin, fibronectin, N-cadherin, basic helix-loop-helix transcription factor (TWIST), and zinc finger protein SNAI1 (SNAIL) (9).

The exact mechanism that triggers MET in kidney progenitor cells is not entirely known although it has been shown to depend on the silencing of specific genes (e.g., *Osr1* and *Six2*) (7). The morphological result of this process is the formation of a vesicle composed of the metanephric blastema, which further forms the comma-shaped body, followed by the S-shaped body, and then Bowman's capsule, finally culminating in the functional nephron (10). Next, we present some important aspects of WNT signaling, which is a key pathway in MET.

Wnt signaling pathway

The Wnt signaling pathway encompasses a variety of signaling cascades activated by the secreted WNT proteins with major involvement in nephrogenesis. The Wnt signaling pathway has been divided into two main branches: canonical and noncanonical. The canonical Wnt pathway (or the Wnt/β-catenin pathway) operates with the involvement of β-catenin, encoded by *CTNNB1*, whereas the noncanonical (or β-catenin independent) pathway does not involve β-catenin (11, 12). The noncanonical Wnt signaling pathway is mainly divided into the Wnt/calcium (Wnt/Ca^{2+}) and planar cell polarity (PCP) pathways. Despite several differences between the two branches, both are activated by the binding of a Wnt ligand to a Frizzled (FZD) family receptor (13, 14).

The canonical Wnt pathway is characterized by its intracellular mediator β-catenin and plays a crucial role in cell fate. In the canonical Wnt pathway, β-catenin can accumulate in the cytoplasm and either be directed to the membrane as a part of the cell–cell adhesion complex or be translocated into the nucleus, acting as a transcriptional coactivator of TCF/LEF family of transcription factors. Thus, β-catenin plays a dual role, either regulating the coordination of cell–cell adhesion (in the inactivated Wnt signaling pathway) or acting as a transcriptional cofactor when translocated to the nucleus (in the activated Wnt signaling pathway). Thus, the regulation of cytoplasmic levels of β-catenin by the APC/AXIN1 (adenomatous polyposis coli/) destruction complex (DC) represents a fundamental control step of the canonical Wnt pathway.

The DC is composed of AXIN1, PP2A, GSK3, CK1, Dishevelled (DSH), and APC and marks β-catenin for degradation by the proteasome through ubiquitination. The heterodimer formed by a Wnt ligand and an FDZ (frizzled) receptor (WNT–FDZ heterodimer) interacts with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6), another cell surface protein, recruiting cytoplasmic AXIN1 and preventing the formation of APC/AXIN1. The WNT–FDZ heterodimer recruits and interacts with a series of cytoplasmic proteins to prevent the DC from ubiquitinating β-catenin and targeting it to the proteasome (11, 15). Conversely, in the absence of Wnt proteins, the DC phosphorylates β-catenin, which is further ubiquitinated by an E3 ubiquitin ligase (B-TrCP) and degraded in the proteasome (15). More recently, it was observed that the APC membrane recruitment protein 1 (AMER1) interacts with the APC/AXIN1 DC although its role is not yet completely understood (16). Additionally, controversies about β-catenin ubiquitination and degradation in the context of the AXIN1 complex and about the disassembly of the DC are noted in the literature (17).

In summary, in the canonical Wnt pathway, the binding of WNT proteins to FZD receptors suppresses β-catenin degradation, resulting in its cytoplasmic accumulation, followed by nuclear translocation, which finally releases the expression of certain genes involved in important cellular processes.

The noncanonical branch of the Wnt pathway resembles the canonical pathway only in its requirement for Wnt ligands [e.g., silberblick (WNT11) and pipetail (WNT5)], FZD receptors, and the cytoplasmic signal transduction molecule DSH. Upon binding of the noncanonical WNT proteins, complexes belonging to the PCP pathway are asymmetrically distributed in the proximal and distal cell membranes [reviewed in reference (18)]. The other downstream interactions remain unclear, but it is well established that the strabismus (STBM) and prickle (PK) proteins are involved, whereas AXIN1, GSK-3, and β-catenin are not. Despite some similarity between the vertebrate noncanonical Wnt pathway and the *Drosophila* PCP pathway, such as the involvement of DSH, no Wnt ligands are known to be involved in *Drosophila* PCP signaling.

Wnt signaling pathway in nephrogenesis

The Wnt/β-catenin pathway is one of the multiple signaling pathways that cooperates in the initiation and progression of MET (19). Several members of the Wnt family have been implicated in the induction of epithelial renal vesicles. WNT4 is required and sufficient for the transition of the metanephric mesenchyme to epithelial cells (20). *Wnt4* is also required for tubulogenesis, and it acts through a noncanonical Wnt pathway (21). WNT9B, which is secreted by cells from the ureteric bud, induces the expression of WNT4, and its loss can be rescued with WNT1, a putative canonical Wnt signaling activator (22). Similarly, WNT6 induces tubulogenesis by activating *WNT4* transcription, which leads to the expression of early markers of kidney tubulogenesis PAX2, PAX8, SFRP2, and E-cadherin genes (23).

Another key protein involved in nephrogenesis is *SIX2* (*Sine oculis homeobox 2 gene*), a transcription factor essential for maintaining the self-renewing and multipotent characteristics of nephron progenitor cells (24). *WNT4* is an upstream regulator of *SIX2*, and decreased expression of *SIX2* results in the commitment of the progenitor cells to undergo differentiation via MET (25).

Wnt/β-catenin signaling also regulates MET through the transcription repressor *SNAIL1*, which is downregulated during embryogenesis, and allows mesenchymal cells to differentiate into epithelia through MET (8, 26, 27).

Wilms tumors and the Wnt signaling pathways

Several studies have confirmed the activation of canonical Wnt signaling pathways in WT. The canonical Wnt signaling pathway, observed by nuclear positivity of β-catenin, is activated in approximately 15–25% of all WTs with a favorable histology (28).

Interestingly, nuclear accumulation of β-catenin in WTs is associated with a mutation in Wilms tumor 1 gene (*WT1*), which in turn is associated with WT cases that show stromal predominance,

where the nuclear positivity of the protein is largely confined to the mesenchymal cells (2, 28). However, the genetic and molecular mechanism that underlies these associations is not understood. The *WT1* is known as an inhibitor of Wnt/β-catenin signaling (29, 30). The antagonistic activities of WT1 and β-catenin probably arises because the two proteins bind to a common transcriptional coactivator, CREB-binding protein. Additionally, the WT1 protein is essential for MET and, hence, for normal embryonic kidney development (31), and genetic deletion and/or inactivating mutations in WT1 cause severe kidney disorders in mice (32).

APC is a tumor suppressor gene whose protein product is a component of the DC for β-catenin, thus its action negatively regulates the canonical Wnt signaling pathway, and this modulation appears to be essential in nephrogenesis. The APC protein exhibits distinct cellular localization during the differentiation process from the fetal kidney to the mature normal kidney. In earlier stages of kidney development, APC expression is nuclear; in later stages, it is cytoplasmic; and in intermediate stages, it is both nuclear and cytoplasmic. Interestingly, in WT samples, the localization of APC recapitulates that in the earliest stages of the fetal kidney, where APC expression is exclusively nuclear—a pattern that resembles the earliest stage in undifferentiated blastemal cells (2). Thus, the nucleocytoplasmic shuttling of APC may be critical in the context of the activation of the canonical Wnt pathway in WT, as well as in kidney development. Given that APC shuttles into and out of the nucleus (33), it is reasonable to speculate that the nuclear localization of APC might interfere with the export of β-catenin from the nucleus in Wnt-stimulated cells. In this context, nuclear APC positivity could be an indirect indication of Wnt signaling activation in WT (2). Although the involvement of the noncanonical Wnt pathway has been demonstrated in nephrogenesis (25) and cancer (34), few studies directly associate disturbances in this pathway and WT.

The noncanonical Wnt signaling pathway directly affects changes in the cytoskeleton, the PCP pathway, and the regulation of calcium release from the endoplasmic reticulum to control intracellular calcium levels via the Wnt/Ca^{2+} pathway.

*PLCG*2, a gene in the Wnt/Ca^{2+} pathway, is involved in the control of external calcium entry and in innate immune responses (35), and its expression is modulated during nephrogenesis (2). The mRNA and the protein levels of PLCG2 are reduced in kidney progenitor cells and increased in the mature kidney, where protein expression has been shown to be strongly positive in some cells of the nephron. Accordingly, the *PLCG2* expression pattern appears to be recapitulated in WT at the mRNA and protein levels, showing decreased or predominately negative expression, respectively, in WTs compared with differentiated kidneys (2).

WNT5A and *WNT5B*, which are members of the noncanonical Wnt signaling pathway, were also recently identified as being altered in WT. *WNT5A* is likely regulated by *PAX2*

and was found to be expressed at lower levels in WT than in the fetal kidney (36). The expression levels of *WNT5B* mRNA decrease during blastemal cell differentiation. *WNT5B* appears to be associated with the formation of cell polarity, as the WNT5B protein expression has only been observed after the renal vesicle formation, when cells are organizing to form kidney structures, such as glomeruli and tubules, where positivity is observed mainly in the apical cell membrane. Although the mRNA levels of *WNT5B* were observed to be elevated in WT (similar to the undifferentiated blastemal cells that give rise to WT) compared with the differentiated kidneys, the protein expression has been only detected in a minority of cases (37). The mechanism underlying WNT5A and WNT5B signaling remains to be elucidated.

These data provide strong evidence for the involvement of Wnt pathway-related genes in WT, whose pattern in the earliest stages of nephrogenesis is recapitulated in the tumor, marking the disruption of the complete differentiation of the kidney progenitor cells.

Wilms tumor and mutation repertoire

Mutations in *WT1*, a TSG, are present in approximately 20% of WTs. Other TSGs with inactivating mutations include *WTX*, which also occur in approximately 30% of cases (38). The WTX protein has been reported to negatively regulate the canonical WNT pathway, as part of the DC (39). Stabilizing mutations in *CTNNB1* (β-catenin), the major regulator of the canonical WNT pathway, are present in 15% of tumors. The well-known *TP53* gene has been found to be mutated in 5% of WT samples (4). Other genes that have been observed to be mutated in lower frequencies are *DIS3L2* (40), *FBXW7*, and *MYCN* (41). Together, mutations in these genes account for approximately 30% of WT samples.

Despite several lines of evidence supporting the overexpression of a number of downstream genes of the canonical WNT pathway in WT (42, 43), only two of the five currently known WT subtypes show clear evidence of canonical WNT pathway activation (44). These five subgroups (S1–S5) were defined by the hierarchical clustering analysis of expression data from genes of the canonical WT pathway. Only the subgroups, S1 and S2, showed evidence of strong WNT activation. Increased expression of LEF1 and FZD2 and decreased expression of CCND1 and JAG1 characterize the S1 subgroup. Interestingly, some samples from S2 subtype, lacking CTNNB1 or WTX mutations, also showed signals of strong Wnt activation. These findings suggest the presence of other mechanisms for canonical Wnt activation.

The repression of miRNA biogenesis through the inhibition of DROSHA and DICER1 expression impairs accurate kidney differentiation (45–47) and promotes tumorigenesis in several cell lines (48). Accordingly, mutations in genes involved in the miRNA biogenesis were recently identified in a higher proportion of WT samples and appear to be associated

with a predominant blastemal histology (5, 6, 49). Mutations in *DROSHA* are found in 12% of WTs, and a recurrent mutation (E1147K) has been shown to affect an RNase IIIb domain of the protein encoded (5, 6). The frequency of the E1147K mutation was further estimated in a validation set of 538 tumors being observed in 11% of the samples (49). If other genes in the miRNA biogenesis pathway are included, such as *DGCR8*, *TARBP2*, *XPO5*, and *DICER*, mutations in this pathway account for approximately 30% of WT samples.

The effect of the E1147K mutation in DROSHA is associated with a predominant reduction in the expression level of mature miRNAs (5, 6). miRNAs are critical regulators of gene expression, and consequently, the defective miRNA biogenesis observed in WT surely makes a crucial contribution to WT development. However, the effects of the deregulation of miRNAs on their target genes, particularly those assumed to be involved in the differentiation of the kidneys, have yet to be established.

Members of the cyclin gene family were recently described as being upregulated in blastemal-type WT samples, especially in samples with recurrent mutations in the SIX1/2 gene (50). This pattern may be an important underlying cause of the continued proliferation of the metanephric mesenchyme.

Interestingly, tumor samples with ectopic mesenchymal elements show upregulation of WNT-related genes, whereas tumors with epithelial elements do not. Moreover, samples from this "WNT-independent" subgroup often show perilobar nephrogenic rests (PLNRs), instead of intralobar nephrogenic rests, which may reflect the likely origin of these tumors in errors that occur later during kidney development (51). More recently, Walz et al. (49) showed a significant statistical association between the presence of PLNR and the mutations in miRNA processing genes. Further analysis may be necessary to clarify the consequences of this probable association.

Conclusion

The origins of WTs are closely related to the processes of kidney development. This is supported by the fact that genes involved in nephrogenesis are altered in WT (Figure 1). The involvement of WNT signaling pathway in kidney embryogenesis was demonstrated by alterations in several genes directly or indirectly. These alterations include mutations (*WT1*, *WTX*, *CTNNB1*, *TP53*, *DIS3L2*, *FBXW7*, and *MYCN*) or altered expression (*WNT5*, *APC*, and *PLCG2*) in WT. Additionally, mutations in genes from the miRNA biogenesis pathway (*DROSHA*, *DGCR8*, *TARBP2*, *XPO5*, and *DICER*) are found in a relatively high frequency in WT cases. Recently, it was demonstrated that one of these mutations affects the function of *DROSHA*, impairing the process of miRNA biogenesis. Currently, there is an urgent need to understand the possible interplay between the miRNA downregulation and the Wnt

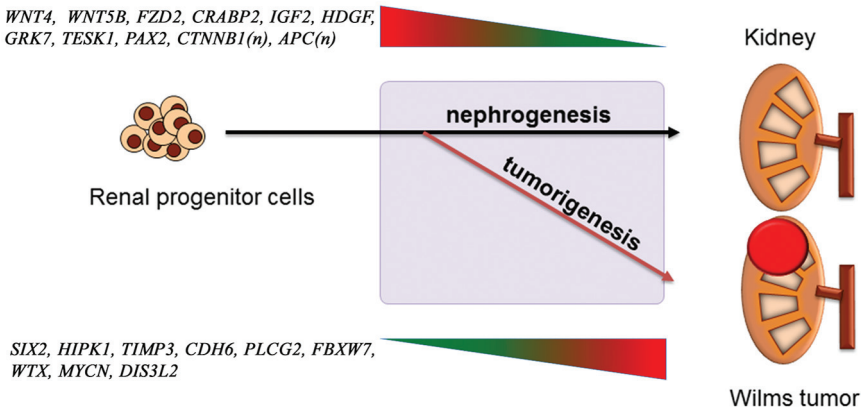

Figure 1. Disruption of differentiation of the precursor cells of the kidney may result in Wilms tumor formation. Several molecules are associated with nephrogenesis and tumorigenesis. The available evidence suggests that the delicate balance in the expression pattern of these molecules during embryogenesis is the determinant of nephrogenesis and that disturbance in the expression leads to tumorigenesis. The differential expression of selected molecules is depicted beside each triangle (red: higher, green: lower). n: nuclear positivity of protein.

signaling pathway. This knowledge may lead to new perspectives in the design of more effective anticancer therapies for WT.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

Acknowledgment

The authors thank FAPESP for financial support (2013/23277-8). MM is funded by FAPESP 2015/06281-7.

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