A Murine Model of K-RAS and β-Catenin Induced Renal Tumors Expresses High Levels of E2F1 and Resembles Human Wilms Tumor

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Abbreviations and Acronyms

CTNNB1 = stabilizing/activating mutations of β-catenin
EXALT = Expression AnaLysis Tool
GEO = Gene Expression Omnibus
IHC = immunohistochemistry
IPA = Ingenuity® Pathway Analysis
NCBI = National Center for Biotechnology Information
PCR = polymerase chain reaction
RT = reverse transcriptase
TMA = tissue microarray
WT = Wilms tumor

Accepted for publication April 13, 2015.
Study received institutional animal care and use committee approval.
Supported by Awards K08 CA113452 (PEC) and P30 DK079341 (PEC pilot fund).
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Purpose: Wilms tumor is the most common renal neoplasm of childhood. We previously found that restricted activation of the WNT/β-catenin pathway in renal epithelium late in kidney development is sufficient to induce small primitive neoplasms with features of epithelial Wilms tumor. Metastatic disease progression required simultaneous addition of an activating mutation of the oncogene K-RAS. We sought to define the molecular pathways activated in this process and their relationship to human renal malignancies.

Materials and Methods: Affymetrix® expression microarray data from murine kidneys with activation of K-*ras* and/or *Ctnnb1* (β-catenin) restricted to renal epithelium were analyzed and compared to publicly available expression data on normal and neoplastic human renal tissue. Target genes were verified by immunoblot and immunohistochemistry.

Results: Mouse kidney tumors with activation of K-*ras* and *Ctnnb1*, and human renal malignancies had similar mRNA expression signatures and were associated with activation of networks centered on β-catenin and TP53. Up-regulation of WNT/β-catenin targets (MYC, Survivin, FOXA2, Axin2 and Cyclin D1) was confirmed by immunoblot. K-RAS/β-catenin murine kidney tumors were more similar to human Wilms tumor than to other renal malignancies and demonstrated activation of a TP53 dependent network of genes, including the transcription factor E2F1. Up-regulation of E2F1 was confirmed in murine and human Wilms tumor samples.

Conclusions: Simultaneous activation of K-RAS and β-catenin in embryonic renal epithelium leads to neoplasms similar to human Wilms tumor and associated with activation of TP53 and up-regulation of E2F1. Further studies are warranted to evaluate the role of TP53 and E2F1 in human Wilms tumor.

Key Words: kidney; Wilms tumor; oncogenes; beta catenin; models, animal

Wilms tumor is the most common renal neoplasm of childhood.[1] While current multimodal treatment cures the majority of children,[2] this survival comes at the cost of significant long-term treatment related morbidity.[3] In addition, a substantial number of patients (15%) relapse and half of these tumors resist intensive salvage therapies and progress.[4] Therefore, the main challenge in WT research is to identify novel therapies to address these deficiencies. Such novel approaches critically rely on in-depth

0022-5347/15/1946-1762/0
THE JOURNAL OF UROLOGY®

http://dx.doi.org/10.1016/j.juro.2015.04.090
Vol. 194, 1762-1770, December 2015
Printed in U.S.A.

understanding of the activated pathways underlying WT disease progression.

WTs are triphasic embryonic-like tumors that are generally thought to arise from primitive nephrogenic rests derived from the metanephric mesenchyma, a multipotent progenitor pool in the developing kidney. Some genetic aberrations underlying this process include inactivating mutations of *WT1*, *WTX* (WT gene found on chromosome X) and *CTNNB1*.[5] The mechanisms by which these differing alterations lead to WT have not been fully elucidated but all share an association with canonical WNT/β-catenin signaling.[6,7]

In addition to WNT/β-catenin, aberrations of the TP53 pathway also have an important role in numerous malignancies, including WT and adult renal cell carcinoma.[5,8] However, the specifics by which TP53 and its regulatory network induce malignant transformation and impact disease progression vary across tumor types. In WT while the accumulation of TP53 protein and several alterations in its gene are associated with anaplasia and treatment resistance, the precise mechanisms underlying its influence on disease progression remain unknown.[5] One mechanism could be via the E2F family of transcription factors, which has an important role in regulating the cell cycle through interaction with the Rb family of proteins. As with TP53 the E2F-Rb pathway is also disrupted in a wide range of malignancies.[9] Classically cell cycle regulation is thought to occur through increased levels of free E2F proteins upon release by phosphorylated Rb, leading to transcription of key target genes involved in cell proliferation. This Rb-E2F pathway interacts with the MDM2-TP53 pathway to regulate the cell cycle, particularly in response to cellular stresses such as DNA damage or oncogene activation.[10] Whether these pathways are critical to WT biology has not been fully elucidated.

We reported that constitutive restricted activation of the canonical Wnt/β-catenin pathway in murine renal tubular epithelial cells late in renal development is sufficient to induce small tumors with features of human WT.[11] Metastatic disease progression required the simultaneous addition of an activating mutation of the G-coupled protein/oncogene K-*ras*. This was accompanied by increased activation of oncogenic pathways such as PI3K/AKT and activation of canonical Wnt/β-catenin signalling.

We sought to define the molecular pathways activated in this process and their relationship to human renal malignancies. Using an expression microarray we found that kidneys from mice with simultaneous activation of K-*ras* and *Ctnnb1* restricted to the renal epithelium induce an expression signature similar to that of human renal malignancies and WT in particular. This is associated with a network of genes regulated by TP53, including up-regulation of the transcription factor E2F1, suggesting that further study of the role of E2F proteins in WT biology is warranted.

METHODS AND MATERIALS

Mice

We used mice harboring γGT-Cre recombinase,[12] mice with a conditional activating mutation of *Ctnnb1* in which exon 3 is flanked by lox sites ($Catnb^{lox(ex3)}$)[13] and mice with a conditional activating mutation of *Kras* ($LSL\text{-}Kras^{G12D}$).[14] All mice were bred and housed under an institutional animal care and use committee approved protocol. Mice were crossed to obtain mice with the genotypes γGT-Cre/$Catnb^{+/lox(ex3)}$ (referred to as $Catnb^{\Delta ex3}$), γGT-Cre/$Kras^{+/G12D}$ (referred to as $Kras^{G12D}$), γGT-Cre/$Kras^{+/G12D}$/$Catnb^{+/lox(ex3)}$ (referred to as $Kras^{G12D}$/$Catnb^{\Delta ex3}$) and litter mate controls as previously described.[11]

For the microarray experiment mice were sacrificed at ages 15 to 20 weeks and the kidneys were flash frozen in liquid nitrogen. RNA was extracted from the kidney of 3 mice per group except 2 from the $Kras^{G12D}$/$Catnb^{\Delta ex3}$ group and cDNA was created. For immunoblotting and IHC we bred an independent cohort with the same genotypes, harvested the kidneys at ages 15 to 20 weeks and extracted protein from whole kidneys or fixed and embedded kidneys as described.

WT Tissue Microarray

We used formalin fixed, paraffin embedded renal tumor and adjacent kidney specimens collected prospectively and archived in our institutional review board approved laboratory embryonal tumor repository. Briefly, we created 2 TMAs comprising a total of 148 punches, each approximately 1 mm in diameter, which were derived from 32 consecutive childhood WTs.[15] Serial 5 μm sections of these 2 TMAs were included for IHC analysis, which concentrated on the 32 WT specimens.

Antibodies

The antibodies used for IHC and/or immunoblotting were c-Myc (Epitomics®), Actin (Sigma-Aldrich®), Axin2 (Abcam®), E2F1 (Atlas Antibodies, Stockholm, Sweden), survivin and Cyclin D1 (Cell Signaling Technology®).

Histology and Immunohistochemistry

Murine kidneys were harvested, fixed in 10% buffered formalin, processed and paraffin embedded. Sections were stained with hematoxylin and eosin or underwent IHC. For IHC the slides were incubated with primary antibodies and exposed to biotinylated secondary antibody. They were incubated with avidin-biotin complex/horseradish peroxidase (Vector Laboratories, Burlingame, California) and then with liquid DAB (3,3′-diaminobenzidine tetrahydrochloride, Liquid DAB+ Substrate Chromogen System, No. 2012-02, Dako, Glostrup, Denmark). Stained sections were photographed and processed using an AX10 Imager.M1 microscope and AxioVision, release 4.6 software (Carl Zeiss, Jena, Germany).