TFE3 Xp11.2 Translocation Renal Cell Carcinoma Mouse Model Reveals Novel Therapeutic Targets and Identifies GPNMB as a Diagnostic Marker for Human Disease

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Abstract

Renal cell carcinoma (RCC) associated with Xp11.2 translocation (TFE3-RCC) has been recently defined as a distinct subset of RCC classified by characteristic morphology and clinical presentation. The Xp11 translocations involve the TFE3 transcription factor and produce chimeric TFE3 proteins retaining the basic helix-loop-helix leucine zipper structure for dimerization and DNA binding suggesting that chimeric TFE3 proteins function as oncogenic transcription factors. Diagnostic biomarkers and effective forms of therapy for advanced cases of TFE3-RCC are as yet unavailable. To facilitate the development of molecular based diagnostic tools and targeted therapies for this aggressive kidney cancer, we generated a translocation RCC mouse model, in which the PRCC-TFE3 transgene is expressed specifically in kidneys leading to the development of RCC with characteristic histology. Expression of the receptor tyrosine kinase Ret was elevated in the kidneys of the TFE3-RCC mice, and treatment with RET inhibitor, vandetanib, significantly suppressed RCC growth. Moreover, we found that Gpnmb (Glycoprotein nonmetastatic B) expression was notably elevated in the TFE3-RCC mouse kidneys as seen in human TFE3-RCC tumors, and confirmed that GPNMB is the direct transcriptional target of TFE3 fusions. While GPNMB HIC staining was positive in 9/9 cases of TFE3-RCC, Cathepsin K, a conventional marker for TFE3-RCC, was positive in only 67% of cases. These data support RET as a potential target and GPNMB as a diagnostic marker for TFE3-RCC. The TFE3-RCC mouse provides a preclinical in vivo model for the development of new biomarkers and targeted therapeutics for patients affected with this aggressive form of RCC.

Implications: Key findings from studies with this preclinical mouse model of TFE3-RCC underscore the potential for RET as a therapeutic target for treatment of patients with TFE3-RCC, and suggest that GPNMB may serve as diagnostic biomarker for TFE3 fusion RCC.
Introduction

Xp11.2 translocation, t(X;1)(p11.2;q11.2), was first described in a pediatric renal cell carcinoma (RCC) case in 1986, and the fusion gene was confirmed to be PRCC-TFE3 in 1995 (1, 2). TFE3 Xp11.2 translocation RCC (TFE3-RCC) was defined as an independent subtype of RCC by WHO in 2004 and is characterized by distinctive morphologic features and Xp11.2 rearrangements that create TFE3 gene fusions with a variety of partner genes (PRCC, SFPQ, ASPSCR, CLTC, NONO, RBM10, PARP14, LUC7L3, KSHSP etc.; refs. 2–17). TFE3 encodes a transcription factor that has a basic helix–loop–helix leucine zipper (bHLH-Zip) structure through which TFE3 dimerizes and interacts with M-box DNA sequences (TCAYRTGA) in transcriptional target genes. All TFE3 fusion genes encode in-frame chimeric proteins, which retain the bHLH-Zip domain of TFE3 (16, 18). Nuclear accumulation of TFE3 is one of the most significant histopathologic characteristics of TFE3-RCC (19, 20). The evidence is strong for TFE3 fusions to be oncogenes with constitutively active transcriptional activity.

TFE3-RCC is more common than was previously thought, comprising from 2% to 5% of adult cases (21, 22) and from 25% to 40% of pediatric RCC cases (14, 23). TFE3-RCC is known for its aggressive malignant nature with a propensity to metastasize when the primary tumor is small. There is currently no standard or effective form of therapy for patients with advanced disease (4, 16). Reduced awareness of TFE3-RCC and the technical complexity of diagnosis including TFE3 staining and TFE3 gene break-apart FISH have led to a decrease in awareness and early diagnosis of this disease (21, 24–26). It is, therefore, important to develop novel diagnostic methods for TFE3-RCC. Although several diagnostic markers for TFE3-RCC have been reported, such as Cathepsin K, melan A, and HMB45, the sensitivity and specificity of these conventional markers are limited and not robust enough to confirm the diagnosis of TFE3-RCC (20, 26, 27). Transcriptional target genes of TFE3 that are upregulated following TFE3 nuclear localization and activation could potentially be useful markers for the diagnosis of TFE3-RCC.

In this study, we have generated a TFE3-RCC mouse model that expresses PRCC-TFE3, which is the first reported TFE3 fusion partner and frequently observed in human disease, specifically in kidney epithelial cells, and develops a variety of kidney epithelial neoplastic lesions including hyperplastic cysts, adenomas, and solid tumors. This mouse model provides a preclinical system for development of new diagnostic markers and targeted therapeutics. Genes that were upregulated in the kidneys of this mouse model were identified. We determined that GPNMB (glycoprotein nonmetastatic B) is directly transcribed and upregulated by chimeric TFE3 and performed GPNMB IHC staining in human TFE3-RCCs to investigate its potential in the diagnosis of this form of RCC.

Materials and Methods

Generation of TFE3-RCC mouse model

The cDNA of the human PRCC-TFE3 chimeric gene, which is composed of exons 1 of PRCC and exons 4–10 of TFE3, was generated by overlap extension PCR, subcloned into an entry vector of the Gateway Protein Expression System (Invitrogen), and sequence verified. PRCC-TFE3 cDNA was cloned into a targeting vector, pRosA26-DEST (Addgene plasmid # 21189; ref. 28), which has a LoxP-Stop-LoxP (LSL) cassette preceding the gene of interest, using the Gateway Protein Expression System according to the manufacturer’s protocol. The targeting vector (pRosA26-DEST-PRCC-TFE3) was electroporated into mouse embryonic stem (ES) cells and selected for G418 resistance as described previously. ES cells (LSL-PRCC-TFE3), in which the Rosa26 locus was correctly targeted, were identified by Southern blot analysis and injected into blastocysts to produce chimeras. Backcrossing to C57BL/6 mice produced heterozygous F1 offspring with germline transmission of the Rosa26-LSL-PRCC-TFE3 knockin (KI) allele. Cadherin 16 (KSP)-Cre transgenic mice, which express Cre recombinase under the cadherin 16 promoter specifically in renal epithelial cells (29), were crossed with Rosa26-LSL-PRCC-TFE3KI (LSL-PRCC-TFE3) mice to generate PRCC-TFE3;KSP-Cre mice. Mice were housed in Frederick National Laboratory for Cancer Research animal facilities and euthanized by CO2 asphyxiation for analyses according to the NCI-Frederick Animal Care and Use Committee guidelines. Animal care procedures followed the NCI-Frederick Animal Care and Use Committee guidelines.

Drug treatment

All animal studies were conducted in accordance with the institutional guidelines for animal care and experimental neoplasia and according to a protocol approved by the Animal Care and Use Committee of the Frederick National Laboratory for Cancer Research. PRCC-TFE3;KSPCre mice, 6–12 months of age, were randomly assigned to vandetanib-treated group (n = 4) and vehicle-treated group (n = 4). Vandetanib-treated animals received vandetanib at 100 mg/kg by oral gavage 5 days/week for 16 weeks. Control animals received vehicle (PBS) by oral gavage at the same volume and frequency. MRI scans were obtained at 4-week intervals and maximum tumor dimensions were measured from the scans using Image J software and plotted to calculate tumor growth rates.

MRI

MRI was implemented for noninvasive detection of kidney lesions as described previously (30, 31), monitoring their progression and following the therapeutic response. The images were acquired on a 3.0T Clinical Scanner (Philips Intera Achieva) using a 40-mm diameter mouse-dedicated solenoid receiver coil (Philips Research). A T2 weighted (T2w) Turbo Spin Echo (TSE) sequence was acquired with an in-plane resolution of 0.180 × 0.180 mm and 0.5-mm slice thickness. A contrast medium gadolinium chelate, Dotarem, (Guerbet) was administrated intravenously at 0.1 mmol/kg and a postcontrast T1weighted (T1w) image was obtained using a gradient echo scan with the same geometry as the T2w sequence. A fat saturation technique (Spectral Presaturation with Inversion Recovery, SPIR) was implemented for both T1w and T2w sequences to suppress the fat and create a dark background around the kidneys to enhance contrast and distinguish fat from cystic and tumor masses in the kidneys.

DNA microarray analysis

Total RNA was isolated from flash-frozen mouse kidneys using TRIzol reagent (Invitrogen). cDNA preparation and hybridization of the probe arrays were performed according to the manufacturer’s instructions (Affymetrix). Affymetrix GeneChip Mouse
Gene 2.0 ST Arrays were applied. Partek Genomics Suite 6.6 was used for RMA-based data normalization and the subsequent data analysis, including PCA and ANOVA. Data are available at the NCBI GEO database under accession number (GSE130072). Gene set enrichment analysis (GSEA) was performed with GSEA software as described previously (32, 33).

Cell lines and biochemical analysis
HEK293 cell lines that express HA-TFE3 and HA-PRCC-TFE3 in a doxycycline-dependent manner were established using the Flp-In T-Rex System (Invitrogen) as described previously (34) and cultured in DMEM with 10% tetracycline-free FBS (Clontech) and selection antibiotics, 15 μg/mL blasticidin S and 150 μg/mL Hygromycin B (Invitrogen). UOK105, UOK120, UOK124, and UOK146 cell lines were derived from primary tumors of 4 patients with TFE3-RCC treated in the Urologic Oncology Branch (UOB), NCI (Bethesda, MD) and carry the NONO-TFE3 or PRCC-TFE3 gene fusions, as described previously (3, 8, 15). UOK111, UOK115, and UOK140 are cell lines derived from ccRCC tumors with VHL gene mutations that were established in the UOB, NCI (35, 36). All cell lines were maintained in vitro in DMEM media supplemented with 1-glutamine (4 mmol/L), sodium pyruvate (110 mg/L), glucose (4.5 g/L), and 1X essential amino acids, Penicillin–Streptomycin (100 U/mL; Gibco), with 10% FBS (Sigma Aldrich). Cell lines were authenticated using short tandem repeat DNA profiling (Genetica DNA Laboratories) and confirmed to be Mycoplasma free. For gene expression profiling, HEK293 cell lines were cultured with or without 250 ng/mL doxycycline. For qRT-PCR, total RNA was isolated using TRIzol reagent (Invitrogen) and was reverse transcribed to cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo). qRT-PCR was performed with a LightCycler 96 Instrument (Roche) using THUNDERBIRD SYBR qPCR Mix (Toyobo) as described previously (37). All reactions were performed with RPS 18 as an internal control. Primer sequences are as follows: Gpnmb forward, 5'-GCTAICTCGAGCGCCACCATCACAA-3'; Gpnmb reverse, 5'-GGAGATGATCGTACAGGCTTCCA-3'. Gpnmb promoter region was amplified using pGEM-T Easy Vector (Promega). PCR of the GPNMB promoter region containing two M-box motifs are as follows: forward, CGGCCCTTTAGGCTGTCG; reverse, AACTTGATAAGCTTTGAATTCTCACGGACG-3'. Western blotting analysis was performed as described previously (34). 

TCGA data analysis
The Cancer Genome Atlas (TCGA; refs. 12, 38) dataset was used to validate candidate transcriptional target genes of chimeric TFE3, which were identified by RNAseq analysis. The dataset of four TFE3-RCC samples [TCGA-AK-3456-01, TCGA-BP-4756-01, TCGA-BB-3546-01 (all SFPQ-TFE3), TCGA-CJ-5681-01 (KHSRP-TFE3)] was compared with the dataset of 465 ccRCC for gene expression levels of candidate genes using cBioPortal (39) and six TFE3-RCC samples [TCGA-BQ-5882-01, TCGA-BQ-5887-01, TCGA-BQ-7050-01 (all PRCC-TFE3), TCGA-DZ-6131-01 (RM10-TFE3), TCGA-G7-7501-01 (SFQ-TFE3), TCGA-J7-8537-01 (DV12-TFE3)] were compared with the remaining 283 samples with gene expression data within the papillary RCC dataset. The papillary RCC dataset was further subdivided into papillary type I (161 samples) and papillary type II RCC (79 samples).

Luciferase reporter gene assay
A 527 bp fragment of the 5' region of the human GPNMB gene was amplified by PCR using KOD-Plus-Neo from the human BAC clone (RP11-469017; Advanced Geno Techs Co.). The PCR product amplified with primers containing restriction enzyme site (forward, ATGTACAGTCTACGACATGGAAACCTGTCCTCTACT; reverse, AACITGTAAGGTTGATATTCGACCAGCAGG) was digested by Nhel and HindIII, and ligated into a pGL3-Basic Vector (Promega). Two M-box sequences in the GPNMB promoter construct were mutated using PrimeSTAR Mutagenesis Basal Kit ( Takara) following the manufacturer's protocol. Primers for mutagenesis were as follows: M-box Mt1 Forward: TAAACCTCGAGATGGTATTAAGCGGCAC. M-box Mt1 Reverse: CAACTTGGATCCTATGCTCTCTCTC; M-box Mt2 Forward: CAATTCGAGATCTCCGGAGCCCCT; M-box Mt2 Reverse: AGGAATCAGATGCTTTAAACCGGCAC. Reporter plasmids were cotransfected with pHL vector as an internal control into a HEK293-Dx-PRCC-TFE3 cell line, which expresses PRCC-TFE3 in a doxycycline-dependent manner. Luciferase activity was measured using Dual-Luciferase Reporter Assay System according to the manufacturer's protocols (Promega).

Chromatin immunoprecipitation assay
HEK293-Dx-HA-PRCC-TFE3 cell lines were cultured with or without doxycycline for 24 hours, and cross-linked with 1% formaldehyde at room temperature for 5 minutes followed by incubation with 125 mmol/L glycine. Nuclear lysates were sonicated with a Bioruptor USD-200 (Diagenode) for 10 minutes twice. To purify HA-PRCC-TFE3–bound chromatin, 50 μg of chromatin was subjected to immunoprecipitation with Anti-HA Affinity Matrix (Roche) at 4°C overnight. Immunoprecipitates were washed five times, eluted, and reverse–cross–linked. DNA was purified with NucleoSpin Gel and PCR Clean-up ( Takara) following the manufacturer's protocol. DNA enrichment in the chromatin immunoprecipitation (ChIP) samples was determined by qRT-PCR with THUNDERBIRD SYBR qPCR Mix (Toyobo) and a LightCycler 96 Instrument (Roche) following the manufacturer's protocol. Primers for the GPNMB promoter region containing two M-box motifs are as follows: forward, GATGCCAAGAAGCCCGCTGTCG; reverse, ATCCTGCTGTGCCCTCCTCTC. As an internal negative control, a 142 bp region, which is 2 kb upstream from the aforementioned GPNMB promoter region was selected. Primers for this negative control region are as follows: forward, TCAGTCGAGTCCTAGATCACT; reverse, AGGCCATTTTGGGTCTAAAGGA. Data are expressed as percentage of input DNA.

Case selection
TFE3-immunoreactive RCCs were collected through consultation systems by The Japanese Society of Pathology (http://pathology.or.jp/). Histologic features were evaluated by two pathologists with expertise in renal tumors (N. Kuroda and Y. Nagashima). Clinicopathologic findings including immunostaining were summarized in Table 1. Sporadic clear cell RCCs (n = 56) and papillary RCCs (n = 20), among which 20 ccRCC and 11 papillary RCC were previously reported by us (40), were used for comparison of GPNMB staining. The possibility that these cases represented patients with von Hippel–Lindau disease,
Birt–Hogg–Dubé (BHD) syndrome, tuberous sclerosis complex, hereditary papillary RCC, or hereditary leiomyomatosis RCC was carefully examined and excluded in all patients by thorough medical examination and family history. This study was approved by the Institutional Review Board of the Kumamoto University (Kumamoto, Japan; #1245), Yokohama City University (Yokohama, Japan), Saitama Medical University International Medical Center (Saitama, Japan; #16-226), and Kochi Red Cross Hospital (Kochi, Japan).

**FISH**

To assess the status of TFE3 in the TFE3-RCCs, FISH analysis was performed by using a dual-color FISH break-apart probe (GSP Laboratory), which was labeled with FITC at 5’ side (550 kb) and with Texas Red at 3’ side (570 kb) of TFE3. In each case, at least 60 nonoverlapping nuclei in the tumor area were counted. The signal pattern of a normal cell depends on sex, that is, one fused signal in men, and two fused signals in women. Typical TFE3 rearrangement pattern of a TFE3-RCC cell is one split signal in men, and one split signal and one fused signal in women. The split signal was defined according to the evaluation method described in previous studies (25, 27). When the case exhibited a TFE3 split but did not show a chimeric band of known partners in RT-PCR, a dual-color SFQ-TFE3 fusion FISH Probe (Cyto Test Inc) was used. If a yellow signal was observed in 30% or more of 100 counted cells, the tumor was defined as harboring SFQ-TFE3.

**RT-PCR**

RNAs were extracted from the 9 TFE3-RCCs using QIAGEN RNeasy FFPE Kit (Qiagen) according to the manufacturer’s instructions. Gene fusion products were amplified by RT-PCR using the following primers: ASPSCR1 (exon 7) forward, 5’-CCTTGACTACTGTACACATC-3’; TFE3 reverse (exon 8), 5’-ATGCCTAAGCCTGGGGACGACTA-3’; PRCC (exon 3) forward, 5’-ATGCCCTAAGCCTGGGGACGACTA-3’; TFE3 reverse (exon 4), 5’-TGACAGGTACTGTTTCACCTG-3’; and TFE3 reverse (exon 6), 5’-CTTGTACTCTGTCACATC-3’.

**IHC**

The resected tissues were fixed with 10% formalin and embedded in paraffin. Four-micron-thick paraffin sections were subjected to IHC. Sections were autoclaved at 121°C for 15 minutes. The sections were treated with the diluted antibodies at 4°C overnight. Working dilutions were 1:200 for GPNMB (goat polyclonal antibody AF-2550 from R&D Systems) and Cathepsin K (Abcam), and 1:500 for TFE3 (Sigma). The GPNMB immunostaining was scored as (–), (1+), and (2+) according to the method described previously (40).

**Statistical analysis**

Experimental data are summarized as the mean values with SD. Statistical analyses were performed using a two-tailed unpaired t-test with or without Welch correction using GraphPad Prism 6. When the P value of the F test was less than 0.05, Welch correction was applied. Differences were considered to be statistically significant at a value of P less than 0.05. Survival data were estimated and plotted with the Kaplan–Meier method; differences between survival groups were assessed with the log-rank test. Nonlinear regression analysis for % tumor growth was performed with GraphPad Prism 6.

**Results**

**PRCC-TFE3 expression in mouse kidney epithelial cells produces RCC**

PRCC-TFE3 knockin mice, generated by inserting human PRCC-TFE3 cDNA preceded by a loxP-flanked neomycin cassette into the Rosa26 locus, were crossed with cadherin 16 Cre (KSP-Cre) transgenic mice to express PRCC-TFE3 specifically in mouse kidney epithelial cells (Fig. 1A). PRCC-TFE3;KSP-Cre+ mice and PRCC-TFE3;KSP-Cre− littermate control mice were evaluated for their phenotype. PRCC-TFE3;KSP-Cre+ mice showed significantly larger and heavier kidneys at 4 and 7 months of age (Fig. 1B and C). MRI imaging revealed a disorganized kidney structure, multiple cysts, and renal tumors in PRCC-TFE3;KSP-Cre− mice (Fig. 1D). Histopathologic analysis revealed a variety of proliferating morphologies, dilation of tubules, cystic lesions, neoplastic regions protruding into the cystic lumen, and solid tumors of various sizes (Fig. 1E). Complete necropsy with histopathology revealed no metastasis or primary tumors in organs other than kidney. All pathologic lesions in kidneys showed strong nuclear staining of TFE3 (Fig. 1F). TFE3 nuclear staining was observed without obvious phenotype in renal papillae, pelvis, and ureter, but not in bladder, consistent with the CDH16-Cre (KSP-Cre) expression pattern (Supplementary Fig. S1A–S1F; ref. 29). The cyst lumens were lined by monolayers of cuboidal and/or hyperplastic cells with occasional adenomatous lesions proliferating in multiple layers (Fig. 1E). Solid tumors displayed characteristics of RCC with eosinophilic cytoplasm, clear cytoplasm, and occasional mixture of both populations (Fig. 1G and H). The eosinophilic

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**Table 1. Summary of clinicopathologic information of patients with TFE3-RCC.**

| Case | Sex | Age | Size (cm) | Stage | Fuhrman grade | Histologic finding | FISH | Fusion | TFE3 | GPNMB | Cathepsin K | RET |
|------|-----|-----|----------|-------|---------------|-------------------|------|--------|------|--------|-------------|------|
| 1    | M   | 11  | 2.2      | 3     |               | Clear and papillary| +    | ASPL-TFE3 | 2    | 2      | -           | 1    |
| 2    | F   | 16  | 9.0      | 3     |               | Clear and tubular  | +    | ASPL-TFE3 | 2    | 1      | 1           | 2    |
| 3    | F   | 19  | 2.5      | 3     |               | Clear and papillary| +    | SFQ-TFE3  | 2    | 1      | 1           | 2    |
| 4    | F   | 20  | 6.0      | 3     |               | Eosinophilic, alveolar, tubular| +    | PRCC-TFE3  | 2    | 1      | 1           | 1    |
| 5    | M   | 26  | 3.1      | 3     |               | Eosinophilic, alveolar, tubular| +    | PRCC-TFE3  | 2    | 1      | -           | 1    |
| 6    | F   | 33  | 1.0      | 3     |               | Clear and papillary| +    | SFQ-TFE3  | 2    | 1      | 1           | 1    |
| 7    | M   | 52  | 2.7      | 3     |               | Clear and multicytic, calcification| +    | SFQ-TFE3  | 2    | 2      | 2           | 2    |
| 8    | F   | 66  | 5.5      | 1     |               | Alveolar, papillary, mixed clear and eosinophilic, psammoma body| +    | ASPL-TFE3  | 2    | 2      | 1           | 1    |
| 9    | F   | 73  | 2.5      | 3     |               |                   |      | ASPL-TFE3  | 2    | 2      | 1           | 1    |
cells tended to be arranged in tubules, trabeculae, and lobules, and were supported by a fine fibrovascular stroma. On the other hand, clear cells were arranged in lobules, and frequently exhibited an alveolar and/or multicystic pattern. Psammoma bodies, calcified lesions that are characteristic of TFE3-RCC (41), were occasionally observed (Fig. 1I). An increased cell proliferation rate in the kidneys of PRCC-TFE3;KSP-Cre− mice was indicated by a significantly increased BrdU incorporation (Fig. 1J and K) as well as higher number of Ki67+ cells (Supplementary Fig. S1G and S1H). Increased BrdU incorporation and Ki67+ cells in cyst-lining cells indicated that the cystic regions were produced by uncontroled hyperproliferation of tubular epithelial cells. Intriguingly, multiple stages of RCC development from cystic regions with abnormally proliferating monolayers to solid tumors were observed in a single kidney of PRCC-TFE3;KSP-Cre+ mice. The renal tumor growth rate was calculated from MRI measurements (Fig. 1L and M). Renal neoplastic lesions in this mouse model display heterogeneous histologic features, which are also seen in human TFE3-RCC. As expected, growth rates of individual tumors measured by MRI imaging were variable, probably because of their heterogeneous features. As indicated from the histology and MRI imaging, kidney structures were disorganized because of abnormal epithelial cell proliferation and enlarged tumors in aged mice, resulting in renal failure and early death compared with control PRCC-TFE3;KSP-Cre− mice. The mean survival of PRCC-TFE3;KSP-Cre+ mice was 11 months (Fig. 1N). The blood urea nitrogen (BUN) levels were significantly higher in PRCC-TFE3;KSP-Cre+ mice when moribund over the age of 10 months, compared with PRCC-TFE3;KSP-Cre− control mice (Fig. 1O). This newly established PRCC-TFE3;KSP-Cre+ mouse model will be useful for development of novel therapeutics, identification of potential diagnostic biomarkers, and as a pre-clinical model for testing new therapies for TFE3-RCC.

Identification of GPNMB and RET as upregulated genes in PRCC-TFE3;KSP-Cre− kidneys

To identify candidate genes for biomarkers or therapeutic target molecules that could be useful for the diagnosis or molecular based therapy of TFE3-RCC, we performed microarray analysis of kidneys from 4-month-old and 7-month-old PRCC-TFE3;KSP-Cre− mice and control PRCC-TFE3;KSP-Cre+ mice (Fig. 2A). Most of the significantly upregulated genes in kidneys from 4-month-old PRCC-TFE3;KSP-Cre− mice were also upregulated in kidneys from 7-month-old PRCC-TFE3;KSP-Cre− mice (Fig. 2A; Supplementary Table S1A; Table S2A). A previous report of a TFE3-driven RCC mouse model demonstrated activation of the Wnt-β-catenin and ErbB signaling pathways (42). However, GSEA analysis did not demonstrate either a Wnt-β-catenin or ErbB activation signature in PRCC-TFE3;KSP-Cre− mouse kidneys, indicating that TFE3 and PRCC-TFE3 have distinct oncogenic functions (Supplementary Fig. S2). One of the most significantly elevated genes in kidneys from both 4 month and 7-month-old PRCC-TFE3;KSP-Cre− mice was Gpnmb that encodes a type I transmembrane glycoprotein (Fig. 2B–D; Supplementary Table S1A; Table S2A). GSEA analysis demonstrated significant enrichment of genes associated with EGFR signaling activation and RET signaling activation among those genes upregulated in kidneys from PRCC-TFE3;KSP-Cre− mice (Fig. 2E and F). In addition, a Kras-activated signature was seen in PRCC-TFE3;KSP-Cre− mouse kidneys (Supplementary Fig. S3). Although Egfr was not significantly upregulated in PRCC-TFE3–expressing kidneys, expression of the Ret proto-oncogene, a receptor tyrosine kinase that shares downstream signaling with EGFR, including the Kras pathway, was significantly higher in PRCC-TFE3;KSP-Cre− kidneys than in control kidneys (Fig. 2B, C, and G). IHC staining revealed robust Gpnmb staining that overlapped with strong TFE3 nuclear staining in kidneys from PRCC-TFE3;KSP-Cre− mice, while control kidneys demonstrated negative staining for both TFE3 and Gpnmb (Fig. 2H–M; Supplementary Fig. S4). In addition, IHC staining demonstrated strong Ret expression in kidney tumors of PRCC-TFE3;KSP-Cre− mice and negative staining in control kidneys (Fig. 2N and O; Supplementary Fig. S4), which motivated us to test therapeutic agents that target Ret in this TFE3-RCC mouse model. Vandetanib is a multiple tyrosine kinase inhibitor that efficiently inhibits EGFR, VEGFR, and RET. We administered vandetanib to PRCC-TFE3;KSP-Cre− mice. Vandetanib treatment (100 mg/kg) demonstrated a therapeutic effect on tumor growth compared with vehicle treatment with no evidence of toxicity (Fig. 2P–U). Nonlinear regression analysis demonstrated statistically significantly reduced tumor growth in the vandetanib-treated group compared with the control-treated group (Fig. 2V). These data underscore the usefulness of this TFE3-RCC mouse model not only for the development of biomarkers and targeted therapeutics but also as a preclinical model to test drugs or imaging methods. Furthermore, we have performed IHC staining of RET on human TFE3-RCC samples and found that 7 of 9 cases (77.8%) were positive for RET staining (Fig. 2W; Table 1), potentially supporting further evaluation of vandetanib treatment in advanced human TFE3-RCC cases.

GPNMB is a direct transcriptional target gene of chimeric TFE3

To investigate the molecular mechanisms of Gpnmb overexpression in PRCC-TFE3;KSP-Cre+ kidneys, we established stable cell lines derived from HEK293 cells, which express wild-type TFE3 or PRCC-TFE3 in a doxycycline-dependent manner (Fig. 3A and B). Induced wild-type TFE3 localized predominantly in the cytoplasm. On the other hand, PRCC-TFE3 localized predominantly in the nucleus (Fig. 3A). These results support the concept that the PRCC-TFE3 chimeric protein works as an oncogene with constitutively active transcriptional activity. Induction of PRCC-TFE3 and, to a lesser extent, wild-type TFE3, resulted in upregulated GPNMB expression at both mRNA and protein levels (Fig. 3C and D). To determine whether GPNMB is the direct transcriptional target of chimeric TFE3, we inserted the promoter region of GPNMB, from −467 to the transcription start site (TSS), into a luciferase reporter plasmid. Two M-box motifs (M-box1 and M-box2), which are putative TFE3-binding sequences, were included in this construct. We transfected reporter plasmids carrying wild-type or mutant M-box motifs into doxycycline-inducible PRCC-TFE3–expressing HEK293 cells (Fig. 3E). As shown in Fig. 3F, the promoter activity of GPNMB was upregulated by the induced expression of PRCC-TFE3. Importantly, this PRCC-TFE3–dependent GPNMB promoter activity was attenuated in cells transfected with mutated M-box1 or M-box2 reporter plasmids. Furthermore, complete absence of PRCC-TFE3–dependent promoter activity was seen in cells transfected with a reporter plasmid in which both M-box1 and M-box2 were mutated (Fig. 3F). These findings indicate that GPNMB is transcriptionally upregulated by PRCC-TFE3 through both M-box motifs in its promoter. In addition, we have confirmed the direct binding of PRCC-TFE3 to the minimal GPNMB promoter region containing these two M-box motifs by ChIP followed by qPCR
Specific expression of GPMB in TFE3-RCC

Because GPMB was proven to be a direct transcriptional target of chimeric TFE3, we evaluated the utility of GPMB as a diagnostic marker for TFE3-RCC. To determine whether GPMB expression was specific for TFE3-RCC, we compared GPMB expression in RCC cell lines established from human TFE3-RCCs, and in cell lines from sporadic clear cell RCCs (ccRCC). The TFE3 RCC cell lines UOK109, which we developed from tumor material removed from a 39-year-old female (15), UOK120, developed from tumor material from a 30-year-old male (3), UOK124, developed from a 21-year-old female (3), and UOK146, developed from tumor material from a 45-year-old male (3, 35), expressed significantly higher amounts of GPMB protein, while ccRCC cell lines UOK111, UOK115, and UOK117 (15) expressed no detectable GPMB (Fig. 4A and B). To further confirm the specificity of GPMB expression in TFE3-RCC, we utilized the gene expression database for ccRCC (KIRC) from TCGA project (12, 38) from which there were 4 cases of TFE3-RCC. We compared the GPMB mRNA expression levels in 465 cases of ccRCC with 4 cases of TFE3-RCC. GPMB expression levels were statistically significantly higher ($P < 0.0001$) in TFE3-RCC than ccRCC (Fig. 4C). We also compared the expression levels of Cathepsin K, which has been used as a diagnostic marker for TFE3-RCC, but were unable to see statistically significant Cathepsin K expression differences between ccRCC and TFE3-RCC (Fig. 4G), thereby underscoring the utility of GPMB as a diagnostic marker. Although TFE3-RCC can present with a variety of histologies, the most frequently observed histologic characteristics are papillary, tubular, or alveolar structures composed of clear cells, which may lead to misdiagnosis of TFE3-RCC as ccRCC or papillary RCC. Hence, we compared GPMB mRNA expression in TFE3-RCC and papillary RCC using the TCGA gene expression database for papillary RCC (KIRC; ref. 14) comprised of 6 cases of TFE3-RCC, 161 cases of papillary type I RCC, and 79 cases of papillary type II RCC. As shown in Fig. 4D, while GPMB expression levels were statistically significantly higher ($P < 0.0001$) in TFE3-RCC than in either papillary type I RCC, papillary type II RCC ($P < 0.0001$), or total papillary RCC ($P < 0.0001$), there were no statistically significant differences in Cathepsin K expression between TFE3-RCC and either papillary type I RCC, papillary type II RCC, or total papillary RCC. (Fig. 4D). These data show that GPMB expression is a useful biomarker for distinguishing TFE3-RCC from other types of RCCs.

GPMB immunostaining as potential diagnostic marker for TFE3-RCC

To evaluate the IHC expression pattern of GPMB in formalin-fixed paraffin-embedded (FFPE) TFE3-RCCs, we collected 9 cases with characteristic histologies of TFE3 translocation RCC. They displayed papillary, tubular, or alveolar histology with frequent calcification and were composed of clear cells or mixtures of clear cells and eosinophilic oncocytes (Fig. 5A–C). All cases demonstrated nuclear TFE3 immunoreactivity, and were confirmed to be TFE3-RCC by FISH and RT-PCR analyses (Fig. 5D–F). Clinicopathologic findings are summarized in Table 1. All cases of TFE3-RCC were confirmed to have nuclear TFE3 staining (Table 2). All TFE3-RCCs showed positive immunoreactivity for GPMB (Fig. 5G–I; Table 2), two cases with 1+ staining, and 7 cases with 2+ staining. Cathepsin-K was positively stained in only 6 of 9 TFE3-RCC cases (Fig. 5J–L; Table 2). There was no obvious correlation between Fuhrman grade and immunoreactivity of GPMB or Cathepsin-K (Table 1). To further confirm the specificity of GPMB immunostaining as a potential diagnostic marker for TFE3-RCC, we have stained 56 ccRCCs and 20 papillary RCCs for GPMB ([40] and this report). Comparisons between TFE3-RCC and ccRCC and between TFE3-RCC and papillary RCC were statistically significant ($P < 0.0001$) for GPMB staining (Table 3). Interestingly, the 4 cases from a total of 76 clear cell and papillary RCC that were positive for GPMB were also positive for nuclear TFE3 staining, which suggests that GPMB positivity is the consequence of TFE3 activation by an unknown mechanism other than Xp11.2 translocation. Overall, these data demonstrate that GPMB immunostaining can be a useful diagnostic marker for TFE3-RCC.

Figure 1. Generation and characterization of TFE3-RCC mouse model. A, A schematic diagram showing the generation of kidney-specific PRCC-TFE3-expressing mice. A floxed neomycin resistance cassette with stop codon, followed by a human PRCC-TFE3-DNA, was inserted into the Rosa26 locus (PRCC-TFE3). Kidney-specific PRCC-TFE3 expression was obtained by crossing PRCC-TFE3 mice with Cadherin 16-Cre (KSP-Cre) mice. B, Macroscopic appearance of PRCC-TFE3;KSP-Cre$^-$;Cre(+) and PRCC-TFE3;KSP-Cre$^-$;Cre(-) mouse kidneys at the age of 7 months. C, Relative ratio of kidney to body weight (100 × kidney weight/BW) of 4-month-old mice ($n = 8$ for PRCC-TFE3;KSP-Cre$^-$;Cre(+)), $n = 8$ for PRCC-TFE3;KSP-Cre$^-$;Cre(-)) was calculated. The tumor (red arrow) is well distinguished on both types of images. E, The caudate lobe of the left kidney was selected for immunohistochemical staining with antibodies against Cathepsin-K (Table 1). F, Representative H&E staining of solid tumors in 7-month-old PRCC-TFE3;KSP-Cre$^-$;Cre; PRCC-TFE3;KSP-Cre$^-$;Cre mice. The cysts appear bright on the T2w image and dark on the T1w. The tumor (red arrow) is well distinguished on both types of images. E, A representative histology of hematoxylin and eosin (H&E)-stained kidney from a 7-month-old PRCC-TFE3;KSP-Cre$^-$;Cre mouse. Bottom panels are higher magnified images of the rectangular areas in top panel. F, Representative images of TFE3 IHC of a kidney from a 7-month-old PRCC-TFE3;KSP-Cre$^-$;Cre mouse. Bottom panels are higher magnified images of the rectangular areas in top panel. G, Representative H&E staining of solid tumors in 7-month-old PRCC-TFE3;KSP-Cre$^-$;Cre mice. H, Representative H&E staining of solid tumors in 7-month-old PRCC-TFE3;KSP-Cre$^-$;Cre mice. I, Psmammoma body, a characteristic calcified lesion seen in human TFE3 RCC, is occasionally seen. J, Bromo-2-deoxyuridine (BrDU, 100 μg/g body wt) was injected intraperitoneally into 7-month-old PRCC-TFE3;KSP-Cre$^-$;Cre mice and PRCC-TFE3;KSP-Cre$^-$;Cre mice 2 hours before euthanization. BrDU staining detects few proliferating cells in PRCC-TFE3;KSP-Cre$^-$;Cre kidneys (left) and many proliferating cells in PRCC-TFE3;KSP-Cre$^-$;Cre kidneys (right). K, Greater than 8-fold more BrDU-incorporated cells were detected in PRCC-TFE3;KSP-Cre$^-$;Cre mice kidneys compared with PRCC-TFE3;KSP-Cre$^-$;Cre mouse kidneys ($n = 4$ for each group, mean = 10.0/mm$^2$ vs. 84.4/mm$^2$, unpaired $t$ test; $P < 0.0001$). BrDU-positive cells per field were counted in four randomly selected fields from 4 mice for each group. Data are represented as means and SD. L and M, Representative MRI images of and tumor (red arrow) diameters in PRCC-TFE3;KSP-Cre$^-$;Cre mouse kidneys, which were chronologically examined at 9.5, 10.5, and 11.3 months of age. N, Kaplan–Meier survival analysis shows a statistically significant difference between PRCC-TFE3;KSP-Cre$^-$;Cre and PRCC-TFE3;KSP-Cre$^-$;Cre mice ($n = 31$ for [Cre (+)], $n = 24$ for [Cre (-)], log-rank test; $P < 0.0001$). Median survival time of PRCC-TFE3;KSP-Cre$^-$;Cre mice is 11 months. O, PRCC-TFE3;KSP-Cre$^-$;Cre mice die of renal failure. Blood urea nitrogen (BUN) levels were determined for PRCC-TFE3;KSP-Cre$^-$;Cre mice when moribund at ages ranging from 10 months to 12 months. Statistically significant elevation of BUN levels was observed in PRCC-TFE3;KSP-Cre$^-$;Cre mice compared with PRCC-TFE3;KSP-Cre$^-$;Cre mice ($n = 4$ for each group, mean = 133.8 mg/dL vs. mean = 15.8 mg/dL, unpaired $t$ test; $P < 0.017$). Data are presented as mean ± SD.
Figure 2.
Overexpression of Gpnmb and Ret in TFE3-RCC mouse model. A, Numbers of genes differentially expressed between PRCC-TFE3;KSP-Cre<sup>+</sup> kidneys and PRCC-TFE3;KSP-Cre<sup>-</sup> kidneys. Volcano plot of gene expression changes for 4-month-old (4M; B) and 7-month-old (7M; C) PRCC-TFE3;KSP-Cre<sup>-</sup> kidneys and PRCC-TFE3;KSP-Cre<sup>+</sup> kidneys. The x-axis specifies the fold changes [log₂(Cre<sup>+</sup>)Cre<sup>-</sup>]] and the y-axis specifies the negative log to the base 10 of the t test q-values. Red and blue dots represent genes expressed at significantly higher or lower levels (>2-fold vs. < -2-fold) in PRCC-TFE3;KSP-Cre<sup>-</sup> (Continued on the following page.)
Discussion

TFE3 Xp11.2 translocation RCC (TFE3-RCC) was initially described in 1996 (3, 43) and established as an independent subtype of RCC in 2004 by the World Health Organization (44). Because of the relatively low incidence of TFE3-RCC (14), the biological characteristics of TFE3-RCC have not been fully clarified and effective forms of therapy for patients with advanced disease have yet to be established (16). Although many cases of TFE3-RCC display the characteristic histology, other cases of TFE3-RCC are misdiagnosed as ccRCC, papillary RCC, or unclassified RCC (12, 25, 26). Given the aggressive nature of this disease, it is of great importance to develop a concise, sensitive, and specific method for diagnosis and treatment of TFE3-RCC. Here, we have generated the first TFE3-RCC mouse model, which we have utilized to further characterize the chimeric PRCC-TFE3 protein and identify a robust and reliable diagnostic marker for TFE3-RCC and as a model for the development of targeted therapeutic approaches for this disease.

Results from the evaluation of our PRCC-TFE3-expressing mouse model have confirmed that chimeric PRCC-TFE3 is an oncogene, which is responsible for RCC development in vivo. Because all the chimeric genes reported to date in TFE3-RCC encode the carboxy-terminal half of TFE3, which retains the basic helix–loop–helix leucine zipper structures through which TFE3 dimerizes and binds to DNA (16, 18), it is predicted that these chimeric genes function as oncogenic transcription factors (45, 46). Indeed, overexpressed PRCC-TFE3 and SFPQ-TFE3 (data not shown) demonstrated predominant nuclear localization, while overexpressed wild-type TFE3 localized in the cytoplasm of HEK 293 cells. This finding suggests that chimeric TFE3 proteins acquire the ability to localize in the nucleus and function as constitutively active transcription factors. aberrant upregulation of PRCC-TFE3 transcriptional target genes, followed by perturbation of the transcriptional network most is likely responsible for TFE3-RCC development. Further analysis of the transcriptional network alterations caused by PRCC-TFE3 expression may provide clues to understanding the molecular mechanisms of TFE3-RCC development. Indeed, our TFE3-RCC mouse model demonstrated Ret overexpression and therapeutic responses to vanetanib treatment. We utilized publicly available databases and searched for putative transcription factors, which may regulate Ret gene expression (data not shown). There was no clear evidence for TFE3 mediated Ret regulation. However, among the many putative transcription factors, which may regulate Ret transcription, Nrl4a1 expression was dramatically elevated in PRCC-TFE3–expressing kidneys (Supplementary Fig. S5). Nrl4a1 expression was significantly higher in TFE3-RCC than ccRCC and papillary RCC (Supplementary Fig. S5). It will be of great importance to clarify the details of the transcriptional network, including Nrl4a1, perturbed by chimeric TFE3.

Another type of translocation RCC has been described involving chromosome 6p21 translocation, in which a second MIT family transcription factor, TFE3, is fused to and transcribed by a strong promoter of the MALAT1 gene (47, 48). In chromosome 6p21 translocation RCC, overexpression of wild-type TFE3 drives RCC development. Indeed, kidney-specific overexpression of wild-type TFE3 in a transgenic mouse model produced clear cell and papillary RCCs (42). This mouse model displays aberrant activation of the Wnt signaling and EphB signaling pathways, which was not observed in our PRCC-RCC mouse model by GSEA analysis (Supplementary Fig. S2). The difference between these two models might reflect differences between wild-type TFE3 and chimeric PRCC-TFE3. As we have shown, PRCC-TFE3 localizes in the nucleus in most of the cells, whereas wild-type TFE3 and TFE3 localize predominantly in the cytoplasm under normal conditions (37, 49–51). In addition, PRCC that displaces the N-terminal half of TFE3 may contribute to altered transcriptional activity affecting different transcriptional targets compared with wild-type TFE3. Indeed, when we compared significantly upregulated genes in PRCC-TFE3–expressing kidneys and TFE3-expressing kidneys (42), less than 10% of PRCC-TFE3 upregulated genes were commonly upregulated in TFE3-expressing kidneys (Supplementary Table S3). Our unique PRCC-TFE3 mouse model will provide a powerful tool for further clarification of molecular mechanisms responsible for TFE3-RCC development.

In this TFE3-RCC mouse model, PRCC-TFE3 expression was induced by cadherin 16 promoter–driven Cre recombinase with broad expression in the distal nephron and some regions of the proximal tubules (29, 30). Notably, PRCC-TFE3–expressing kidneys demonstrated different histologic features, dilated monolayer tubules with hyperproliferation, adenomatous epithelial cells growing as multiple layers into the lumen of diluted tubules, and solid tumors with a variety of sizes. The diversity of histologic features resulting from PRCC-TFE3 expression in mouse kidney may represent different stages of RCC development, which have acquired further genetic and/or epigenetic
changes in addition to PRCC-TFE3 expression. Of note, recent advances in next-generation sequencing technology have revealed the heterogeneity of cancer. Clonal heterogeneity with different combinations of driver gene mutations has been well characterized in clear cell RCC (52, 53). We hypothesize that TFE3-RCC may also display clonal heterogeneity with a variety of driver gene mutations. In fact, our TFE3-RCC mice developed variable histologies, tumor doubling times, and responses to targeted therapeutics. This diverse phenotype may result from a variety of driver gene mutations. Identification and characterization of additional TFE3-RCC driver gene mutations will contribute to a better understanding of the causes of TFE3-

Figure 3. 

Gpnmb is a direct transcriptional target of PRCC-TFE3. A, ICC using anti-HA antibody on HEK293-derived stable cell lines, which express HA-tagged wild-type TFE3 and PRCC-TFE3 in a doxycycline-dependent manner. B, Western blotting with anti-TFE3, anti-HA, and anti-β-actin on HEK293-derived doxycycline-inducible cell lines cultured without and with doxycycline. C, GPNMB expression was quantified by qRT-PCR on HEK293-derived doxycycline-inducible cell lines, which were cultured without (open bar) and with doxycycline (solid bar). Data represent means ± SD (triplicate, unpaired t test: WT-TFE3, *P = 0.0084, PRCC-TFE3; **P = 0.0092). Representative data from at least three independent experiments are shown. D, Western blotting with anti-GPNMB and anti-histone H3 on HEK293-derived doxycycline-inducible cell lines cultured without and with doxycycline. E, Scheme of Luciferase reporter constructs with human GPNMB promoter. Putative TFE3 consensus sequences are listed as M-box1 (CACATGA) and M-box2 (TCACATGA). Wt, wild-type GPNMB promoter construct; Mt1: M-box1 is mutated to CTCGAGA; Mt2: M-box2 is mutated to TCTCGAGA; M-box1/2: both M-box1 and M-box2 are mutated to CTCGAGA and TCTCGAGA, respectively. F, Each Luciferase reporter construct and pGL4 as a negative control were transfected into PRCC-TFE3 doxycycline-inducible HEK 293 cell line with phRL internal control. Twelve hours after transfection, medium was changed to new medium with or without doxycycline, followed by additional 24-hour incubation and harvest. The x-axis displays relative Luciferase activity. GPNMB promoter activity is upregulated by PRCC-TFE3 induction in an M-Box-dependent manner. Data represent means ± SD (triplicate, unpaired t test: n.s., not significant; **P < 0.01; ***P < 0.001). Representative data from at least three independent experiments are shown. G and H, ChIP was performed using anti-HA antibody on PRCC-TFE3 doxycycline-inducible HEK 293 cells cultured with or without doxycycline, followed by qPCR on ChIP samples. G, Scheme indicating the primer sets used for qPCR. H, ChIP-qPCR results demonstrate PRCC-TFE3 specifically binds to M-Box containing sequence in GPNMB promoter. Y-axis indicates % of input. Data represent means ± SD (triplicate, unpaired t test; n.s., not significant; ***, P < 0.001).
RCC heterogeneity and facilitate the development of effective targeted therapeutics. This PRCC-TFE3 mouse model can be utilized in future studies to evaluate potential driver gene mutations by crossing with genetically engineered mice for selective gene deletion.

Because the chimeric TFE3 proteins responsible for TFE3-RCC development act as oncogenic transcription factors, transcriptionally upregulated direct targets of chimeric TFE3 could be promising candidates for TFE3-RCC diagnostic markers. *GPNMB* was one of the most significantly upregulated genes in PRCC-TFE3-expressing kidneys, and, with luciferase reporter assays and ChIP-qPCR, we have shown that *GPNMB* is a direct transcriptional target of PRCC-TFE3. *GPNMB* expression was significantly higher in human TFE3-RCCs than in clear cell or papillary RCCs, thereby demonstrating high sensitivity and specificity as a biomarker for TFE3-RCC.

In pathologic diagnosis of TFE3-RCCs using FFPE tissues, strong TFE3 nuclear immunostaining is considered the diagnostic gold standard (19), but TFE3 IHC can be problematic because the antigenicity of TFE3 can be easily altered by improper fixation and sample storage conditions. Therefore, pathologists often face difficulties to evaluate TFE3 staining (24, 26). The break-apart FISH assay is recommended to augment the histopathologic diagnosis of TFE3-RCC (25, 27). However, the FISH result may be judged as negative in cases in which the partner localizes to the vicinity of TFE3 on the short arm of chromosome X (54). Hence, a surrogate marker that can be used for routine pathologic diagnosis in FFPE tissues is desired.

We found that *GPNMB* immunostaining using FFPE tissues was positive in all 9 human TFE3-RCCs in this study. Furthermore, our study showed that 55 of 56 (98.2%) sporadic ccRCCs and 17 of 20 (85.0%) sporadic papillary RCCs were negatively stained for GPNMB (Table 3). Although sporadic chromophobe RCCs and hybrid oncocytic tumors associated with BHD syndrome also stain for GPNMB (40), these tumors are histologically distinctive and rarely need to be distinguished from TFE3-RCCs. Pathologists may find GPNMB immunostaining useful in diagnosing TFE3-RCC when tumors have papillary growth patterns.
and/or clear cell morphologies with indefinite TFE3 staining. This histopathologic study was based on a limited number of human TFE3-RCC cases. A massing additional clinicopathologic data will be necessary for a better understanding of the clinicopathologic signature of TFE3-RCC, which will contribute to the design of potential therapeutic agents for treating advanced cases of TFE3-RCC.

Our study provides a valuable model for the development of targeted therapies for advanced TFE3-RCC. PRCC-TFE3 mouse model kidneys and human TFE3-RCC samples demonstrate significant elevation of RET expression. Moreover, the RET inhibitor vandetanib significantly suppressed TFE3-RCC growth in mice. The receptor tyrosine kinase inhibitor vandetanib is FDA approved for the treatment of advanced cases of medullary thyroid carcinoma (53). Because there is no established treatment for advanced TFE3-RCC with poor prognosis, vandetanib or other multiple tyrosine kinase inhibitors that target RET could be promising candidates as effective therapeutic agents for advanced TFE3-RCC. In addition, GPNMB itself could be a therapeutic target for advanced TFE3-RCC. An antibody–drug conjugate (ADC) targeting GPNMB, glembatumumab vedotin, comprised of an anti-GPNMB antibody conjugated to a cellular toxin, monomethylauristatin E (MMAE), causes death of GPNMB-positive cells. Indeed, glembatumumab vedotin is being evaluated in clinical trials for several cancers, including advanced melanoma and breast cancer (56). It would be of interest to test this ADC in a preclinical study in the PRCC-TFE3 mouse model. GPNMB is also known to promote tumor growth and invasion through integrin signaling, VEGFR activation, or EGFR activation (45, 57). Targeting GPNMB signaling may also be a promising strategy for treatment of advanced TFE3-RCC. Our current work has provided the basis for the development of effective therapies against advanced TFE3-RCC that eventually could lead to clinical trials that may benefit patients with this very aggressive form of RCC.

**Disclosure of Potential Conflicts of Interest**

M. Baba reports receiving commercial research grant from Ono Pharmaceutical Co. Ltd and Bristol-Myers Squibb K.K. No potential conflicts of interest were disclosed by the other authors.

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19. Anguil P, Lal P, Hutchison B, Lui MY, Reuter VE, Ladanyi M. Aberrant nuclear immunoreactivity for TFE3 in neoplasms with TFE3 gene fusions: a sensitive and specific immunohistochemical assay. Am J Surg Pathol 2003;27:750–61.

20. Kuroda N, Mikami S, Pan CC, Cohen RJ, Hes O, Michal M, et al. Review of renal carcinoma associated TFE3 gene fusions: a consideration of FISH translocations/TFE3 gene fusions with focus on pathological aspect. Histol Histopathol 2012;27:133–40.

21. Koma Y, Fujimaru M, Fujii Y, Mokai H, Yonezawa, K, Kawai S, et al. Adult Xp11 translocation renal cell carcinoma diagnosed by cytogenetics and immunohistochemistry. Clin Cancer Res 2009;15:1170–6.

22. Zheng A, De Angelo P, Osborne L, Pariisi Mondolfi AE, Geller M, Yang Y, et al. Translocation renal cell carcinomas in adults: a single-institution experience. Am J Surg Pathol 2012;36:654–62.

23. Cajaiba MM, Dyer IAL, Geller MJ, Jennings LJ, George D, Kirschmann D, et al. The classification of pediatric and young adult renal cell carcinomas registered on the children’s oncology group (COG) protocol ARS03B2 after focused genetic testing. Cancer 2018;124:3381–9.

24. Srigley JR, Delahunt B, Eble JN, Egevad L, Epstein JI, Grignon DJ, et al. The Cancer Genome Atlas (CGA) project: an update and perspective. Mod Mol Pathol 2010;43:86–90.

25. Green WM, Yonescu R, Morsberger L, Morris K, Netto GJ, Epstein JI, et al. Modelling TFE renal cell carcinoma in mice reveals a critical role of WNT signalling. Elife 2016;5:e17047.

26. Kuroda N, Katto K, Tanaka Y, Yamaguchi T, Inoue K, Ohara M, et al. TFE3 break-apart FISH has a higher sensitivity for Xp11.2 translocation compared to immunohistochemistry. Am J Surg Pathol 2013;37:1150–63.

27. Baba M, Furihata M, Hong SB, Tessarollo L, Haines DC, Southon E, et al. Modelling alveolar soft part sarcoma unveils novel mechanisms of metastasis. Cancer Res 2017;77:897–907.

28. Hohenstein P, Slight J, Ozdemir DD, Burn SF, Berry R, Hastie ND. High-sensitivity TFE3 break-apart FISH for the management of Xp11.2 translocations: (X;p11.2) translocations. Cancer Genet 2013;215:401–4.

29. Baba M, Furihata M, Hong SB, Tessarollo L, Haines DC, Southon E, et al. Adult Xp11 translocation renal cell carcinoma diagnosed by cytogenetics and immunohistochemistry. Clin Cancer Res 2009;15:1170–6.

30. Baba M, Furihata M, Hong SB, Tessarollo L, Haines DC, Southon E, et al. Adult Xp11 translocation renal cell carcinoma diagnosed by cytogenetics and immunohistochemistry. Clin Cancer Res 2009;15:1170–6.

31. Baba M, Furihata M, Hong SB, Tessarollo L, Haines DC, Southon E, et al. Adult Xp11 translocation renal cell carcinoma diagnosed by cytogenetics and immunohistochemistry. Clin Cancer Res 2009;15:1170–6.

32. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression pro

33. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The Cancer genome atlas: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401–4.

34. Sunaya M, Hong SB, Tanaka R, Kuroda N, Nagashima Y, Nagahama K, et al. Distinctive expression patterns of glycoprotein non-metastatic B and TFE3 in renal tumors in patients with Birt-Hogg-Dube syndrome. Cancer Sci 2015;106:315–23.

35. Anglard P, Trahan E, Liu S, Latif F, Merino MJ, Lerman M, et al. The cancer genome atlas: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401–4.

36. Sourbier C, Srivastava G, Ghosh MC, Ghosh S, Yang Y, Gupta G, et al. Targeting HIF(1)alpha translation with Tempol in VHL-deficient clear cell renal cell carcinoma. OncoTarget 2012;3:11.

37. Baba M, Endoh M, Ma W, Toyama H, Hirayama A, Nishikawa K, et al. Folliculin regulates osteoclastogenesis through metabolic regulation. J Bone Miner Res 2018;33:1785–98.

38. Rickerts CJ, De Cubas AA, Fan H, Smith CC, Lang M, Reznik E, et al. The Cancer Genome Atlas comprehensive molecular characterization of renal cell carcinoma. Cell Rep 2018;23:313–26.

39. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The Cancer genome atlas: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401–4.

40. Furuya M, Hong SB, Tanaka R, Kuroda N, Nagashima Y, Nagahama K, et al. Distinctive expression patterns of glycoprotein non-metastatic B and folliculin in renal tumors with Birt-Hogg-Dube syndrome. Cancer Sci 2015;106:315–23.

41. Kuroda N, Katto K, Tanaka Y, Yamaguchi T, Inoue K, Ohara M, et al. Diagnostic pitfall on the histological spectrum of adult-onset renal carcinoma associated with Xp11.2 translocations/TFE3 gene fusions. Med Mol Morphol 2010;43:86–90.

42. Calcagni A, Korn I, Verschuren F, De Cegli R, Zampelli N, Nusco E, et al. Modelling TFE renal cell carcinoma in mice reveals a critical role of WNT signalling. Elife 2016;5:e17047.

43. Weterman MA, Wilbrink M, Janssen I, Janssen HA, van den Berg E, Fisher SE, et al. Molecular cloning of the papillary renal cell carcinoma-associated translocation (X;p11.2) breakpoint. Cytogenet Cell Genet 1996;75:6–10.

44. Lopez-Beltran A, Scarpelli M, Montironi R, Kirklia Z. 2004 WHO classification of the renal tumors of the adults. Eur Urol 2006;49:798–805.

45. Tanaka M, Homme M, Yamazaki Y, Shimizu R, Takazawa Y, Nakamura T. Modeling alveolar soft part sarcoma unveils novel mechanisms of metastasis. Cancer Res 2017;77:897–907.

46. Kobos R, Nagai M, Tsuda M, Merl MY, Saito T, Loe M, et al. Combining integrated genomics and functional genomics to dissect the biology of a cancer-associated, aberrant transcription factor, the AQPSCRI-TFE3 fusion oncoprotein. J Pathol 2013;229:734–43.

47. Davis II, Hsi BL, Arroyo JD, Vargas SO, Yeh VA, Motycka G, et al. Cloning of an Alpha-TFEB fusion in renal tumors harboring the t(6;11)(p21;q13) chromosome translocation. Proc Natl Acad Sci U S A 2003;100:6053–6.

48. Kuiper RP, Schepens M, Thijsen J, van Asseldonk M, van den Berg E, Bridge AJ, et al. Upregulation of the transcription factor TFE in (6;11)(p21; q13) positive renal cell carcinomas due to promoter substitution. Hum Mol Genet 2003;12:1661–9.

49. Hong SB, Oh H, Valera VA, Baba M, Schmidt LS, Linehan WM. Inactivation of the FLCN tumor suppressor gene induces TFE3 transcriptional activity by increasing its nuclear localization. PLoS One 2010;5:e15793.

50. Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, et al. A gene network regulating lysosomal biogenesis and function. Science 2009;325:473–7.

51. BoscaLet–Frengueto A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, et al. The transcription factor TFBX links mTORC1 signaling to transcriptional control of lysosome homeostasis. Sci Signal 2012;5:ra42.

52. Gerlinger M, Horswell S, Larkin J, Rowan AJ, Salm MP, Varela I, et al. Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. Nat Genet 2013;45:223–31.

53. Takjes G, Xu H, Litchfield K, Rowan A, Horswell S, Chambers T, et al. Deterministic evolutionary trajectories influence primary tumor growth. TRECRA renal cell. Cell 2018;173:595–610.

54. Argani P, Zhang L, Reuter VE, Tickoo SK, Antonescu CR. RBM10-TFE3 renal cell carcinoma: a potential diagnostic pitfall due to cryptic intrachromosomal Xp11.2 inversion resulting in false-negative TFE3 FISH. Am J Surg Pathol 2017;41:655–62.

55. Valerio L, Pieruzzi L, Giani C, Agate L, Bottici V, Lorusso L, et al. Targeting GPNMB with the fusion partner I скачал emule на компьютере. Scand J Urol Nephrol 2014;48:355–61.

56. Rose AAN, Biondini M, Curiel R, Siegel PM. Targeting GPNMB with the fusion partner I скачал emule на компьютере. Scand J Urol Nephrol 2014;48:355–61.

57. Taya M, Hammes SR. Glycoprotein non-metastatic melanoma protein B (GPNMB) and cancer: a novel potential therapeutic target. Steroids 2018;133:102–7.
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