

MET Is a Potential Target across All Papillary Renal Cell Carcinomas: Result from a Large Molecular Study of pRCC with CGH Array and Matching Gene Expression Array

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Abstract

Purpose: Papillary renal cell carcinomas (pRCC) are the most common nonclear cell RCC subtype. Germline mutations of the *MET* oncogene at 7q31 have been detected in patients with hereditary type I pRCC and in 13% of sporadic type I pRCC. Recent report of *MET* inhibition strengthened the role of c-Met inhibition across pRCC.

Experimental Design: We collected 220 frozen samples of sporadic pRCC through the French RCC Network and quality controlled for percentage of malignant cells >70%. Gene expression was assessed on 98 pRCC using human whole-genome Agilent 8 × 60K arrays. Copy number alterations were analyzed using Agilent Human 2 × 400K and 4 × 180K array for type II pRCC and comparative genomic microarray analysis method for type I pRCC. *MET* gene sequencing was performed on type I pRCC.

Results: *MET* expression level was high across all pRCC. We identified copy number alterations (gain) in 46% of type II pRCC and in 81% of type I pRCC. Correlation between DNA copy number alterations and mRNA expression level was highly significant. Eleven somatic mutations of *MET* gene were identified amongst 51 type I pRCC (21.6%), including 4 new mutations. We validated LRRK2 co-kinase as highly correlated to *MET* expression.

Conclusion: The present report expands the role of *MET* activation as a potential target across all pRCC subtypes. These data support investigating *MET* inhibitors in pRCC in correlation with *MET* activation status. *Clin Cancer Res*; 20(13); 3411–21. ©2014 AACR.

Introduction

Papillary renal cell carcinomas (pRCC) are the second most common histologic subtype of renal carcinoma (1, 2),

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after clear cell renal cell carcinoma (ccRCC). pRCCs account for 10% to 15% of RCC and gather a heterogeneous population with no recommended systemic treatment. Pathologic classification by Delahunt and Eble divides pRCC population in two morphologically different subtypes (3). Type 1 consists of predominantly basophilic cells, whereas type 2 contains mostly eosinophilic cells. Type 1 architecture is described as a single line of cells along the papillary axis, whereas type 2 generally exhibits several cell strata on the axis. Furthermore, type 2 cells demonstrate more aggressive characteristics, such as the presence of nucleoli and increased nuclear size. The papillary cores often contain edema fluid, foamy macrophages, and psammoma bodies. Several clinical reports identified type I tumors as being more frequently multiple, bilateral, indolent, low-grade pRCC, whereas type II were associated with higher grade and poor prognosis tumors related to metastatic spread (4–7).

Because *MET* germline and somatic mutations have been identified in type I pRCC (8–11), there is an increasing interest in the development of *MET* inhibitors in metastatic pRCC (12, 13). However, the discovery and the optimal use of these selective treatments require an adequate knowledge of the target activation across the

Translational Relevance

In the past few years, VEGF-targeted therapies and mTOR inhibitors have become the new standards of care in metastatic clear cell RCC treatment, showing significant improvements in clinical outcome. In contrast, no specific systemic treatment has been developed for the treatment of papillary renal cell carcinomas (pRCC), the second most common form of kidney cancer. MET has been identified as constitutively activated in inherited syndrome of pRCC and in few sporadic pRCC. Recent report of Met inhibition may widen its interest in all pRCC subtypes. Elucidation of the *MET* proto-oncogene status across a large cohort of pRCC is therefore highly relevant to develop the current targeted therapies and to determine which patients with pRCC are most likely to benefit from MET inhibitors.

different subtypes to better determine whether its inhibition has scientific rationale.

The activation of the HGF/MET pathway is commonly reported to promote proliferative and antiapoptotic activities, two common features shared with many growth factor receptors. More specifically, MET activation is involved in tumorigenesis, metastases through cell–cell detachment, migration, and invasiveness (14). MET kinase activation results in trans-autophosphorylation and binding of adaptor proteins such as GRB2 (growth factor receptor–bound protein 2) and GAB2 (GRB2-associated binding protein) that prompt recruitment and activation of several signaling pathways (15). The downstream response to MET activation relies on stereotypical signaling modulators common to many tyrosine kinase receptors, including the Ras/Raf/MEK/ERK pathway, FAK activated through phosphorylation by SRC family kinase, and PI3K/Akt and STAT3-mediated JNK pathways.

Recent clinical report of MET inhibitor assessing foretinib in pRCC population according to *MET* gene status [*MET* germline mutation, *MET* somatic mutation, or *MET* copy number alteration such as amplification (16)] exhibited significant antitumor activity in patients carrying *MET* germline mutation as well as some interesting progression-free survival in patients without mutation. Similarly to what is known from other tumor types such as gastric (17) and lung cancer (18), it could be postulated that copy number alterations are a potential MET activation mechanism, besides *MET* mutation in this population.

Under physiologic condition, MET is activated when its extracellular domain binds to hepatocyte growth factor (HGF) also known as scatter factor (SF), its only known ligand (19). In contrast with MET, primarily expressed by epithelial cells, HGF/SF is produced by mesenchymal cells. To properly bind to the extracellular part of the MET protein, HGF needs to be activated from its inactive form bounded to heparin proteoglycans within the extracellular matrix, by HGF activator (HGFAC), an extracellular serine protease. In addition, it has been previously demonstrated

that MET-triggered pathway implicates several upstream regulators and coreceptors that are physically associated with MET either at the cell surface or intracytoplasmic domain that might consequently induce MET transactivation (15). For instance, leucine-rich repeat kinase 2 (LRRK2) has recently been reported as a required kinase for MET oncogenic signaling in pRCC (20). We therefore investigated the potential impact of both ligand and coactivators required for MET activation in pRCC.

Overall, we aimed at verifying the hypothesis that MET activation can be triggered in pRCC through different mechanisms, such as gene copy number gain alteration alone or in combination with known coactivators upregulation. We believe that this work is relevant to better understand the rationale and to optimize the ongoing development of MET inhibitors in this setting.

Materials and Methods

Collection, assessment of tumor samples, and frozen tissue control

The frozen tissue samples from patients with pRCC who underwent tumor radical surgical resection at eight French centers (St Joseph, Tenon, Foch, Necker in Paris, Gustave Roussy Institute in Villejuif, Henri Mondor in Creteil, Bordeaux and Rennes, France) between 1993 and 2011 were collected. Each diagnostic sample was independently reviewed by two specialized pathologists who were blinded to any clinical patient data. All patients had previously provided written informed consent for tumor collection and analysis. Pathologic data were collected and clinical data were retrospectively retrieved from urological and oncology files in each institution.

All frozen tissues had been stored under quality-controlled condition and retrieved. When collected, each frozen tissue has been controlled for tumor necrosis and percentage of tumor cells of at least 70%.

DNA and RNA extraction

DNA was extracted from frozen tumors using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Quality and quantity were assessed both by NanoDrop spectrophotometer (Thermo Scientific) and Qubit (Life Technologies). RNA was extracted from frozen samples using Trizol's protocol (Life Technologies). RNA purity was determined by using the LabOnChip (2100 Bioanalyzer System; Agilent Technologies) before labeling and hybridization.

CGH array profiling

High-resolution oligonucleotide comparative genomic hybridization array (CGH) arrays were performed following standard operating procedures from Agilent Technologies (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis G4410-90010).

DNA was hybridized to Agilent Sure Print G3 Human CGH either to 2 × 400K (G4449A amadiid 21850) or to 4 × 180K whole-genome Agilent arrays (G4448A amadiid 22060) at the Genomic Unit of the Gustave Roussy Institute,

Villejuif, France. About respectively $2 \times 400\text{K}$ and $4 \times 180\text{K}$ arrays, for each sample, 1,500 and 500 ng of DNA were fragmented by a double enzymatic digestion (Alu I+ Rsa I) and checked with LabOnChip (2100 Bioanalyzer System; Agilent Technologies) before labeling and hybridization. Tumor DNA and control DNA from Promega (Human Genomic DNA Female N° 30742202/male N° 30993901) were labeled by random priming with CY5-dCTPs and CY3-dCTP, respectively, and hybridized at 65°C for 48 or 24 hours depending on slides format at 20 rpm. The chips were scanned on an Agilent G2565BA DNA Microarray Scanner and image analysis was done using the Feature-Extraction V9.1.3 software (Agilent Technologies). Feature-Extraction was used for the fluorescence signal acquisition from the scans. Raw data have been submitted to the ArrayExpress database with the accession number E-MTAB-1807.

A \log_2 intensity ratio of test to reference was used to define relative copy number alterations as subsequently: In a normal situation, the \log_2 ratio of normal (copy-neutral) clones is $\log_2(2/2) = 0$; a single copy loss is $\log_2(1/2) = -1$, and a single copy gain is $\log_2(3/2) = 0.58$. We defined amplification as a value of $\log_2(5/2) \geq 1.5$ (meaning 3 additional copies of the gene for a diploid genome).

Gene expression array

Gene expression profiling was done with human whole-genome Agilent 28004, $8 \times 60\text{K}$ v1 (G4851A) following standard operating procedures from Agilent Technologies (One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling G4140-90040). A total of 100 ng of each RNA was used to perform reverse transcription and one-color labeling steps. Amplified and labeled samples were purified using the RNeasy Mini Kit from Qiagen (74106). Purified samples were measured using Nanodrop ND2000 in microarray mode. The labeled RNA was hybridized at 65°C for 17 hours at 10 rpm. Raw data have been submitted to the ArrayExpress database with the accession number E-MTAB-1805.

Data analysis

For gene expression arrays, raw data files from Feature Extraction were imported into R with LIMMA (21), an R package from the Bioconductor project, and processed as follows: gMedianSignal data were imported, controls probes were systematically removed, and flagged probes (gIsSaturated, gIsFeatpoptnOL, and gIsFeatNonUnifOL) were set to Non Available. Interarray normalization was performed by quantile normalization. A single value was obtained for each transcript, taking the mean of each replicated probes summarized data. Missing values were inferred using k-Nearest Neighbors algorithm (KNN) algorithm from the package "impute" from R bioconductor. Normalized data were then analyzed. To assess differentially expressed genes between two groups, we started by fitting a linear model to the data. Then we used an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. The top-ranked genes were selected with the following criteria: an abso-

lute fold change >2 and an adjusted P value [false discovery rate (FDR)] < 0.05 .

Resulting raw signals and \log_2 (ratio) profiles were normalized and centered using an in-house method, according to their dye composition (Cy5/Cy3) and local GC% composition. These profiles were segmented with the CBS (circular binary segmentation) algorithm (22) through its implementation in the DNACopy v1.30 package for R v2.15.1 (default parameters). DNA copy number imbalances were detected considering a minimum of 3 consecutive probes and a minimal absolute amplitude threshold that was specific for each profile, accordingly with its internal background noise. This specific internal noise was computed as one fourth of the median of the absolute \log_2 (ratio) distances across consecutive probes on the genome. All genomics coordinates in this study were mapped against the human genome as defined by the UCSC build hg19.

Quantitative reverse transcriptase PCR analysis

Gene expression was assessed by quantitative reverse transcriptase (RT)-PCR, using total RNA from frozen tumors. The amplification of the cDNA was performed from 1 μg of total RNA with the Maxima cDNA Synthesis Kit (Thermo Scientific). A total of 20 ng cDNA was amplified in a 25- μL RT reaction using SYBR Green PCR master mix (Applied Biosystems) containing 0.3 $\mu\text{mol/L}$ of specific primers. 18S primer sequences have been previously described by Schmittgen and Zakrajsek (23). To analyze Met transcript expression, we designed primers (exon 15 forward: 5' CATGCCGACAAGTGCAGTAT 3', exon 16 reverse: 5' CACAACCAAAAATGCCCTCTT 3'). The total reaction was performed on the ABI Prism 7000 Detection System TaqMan (Applied Biosystems). Each sample was analyzed in duplicates in every run. Cycling conditions were as follows: 95°C for 5 minutes, and then 45 cycles of 30 seconds at 95°C and 1 minute at 62°C . Dissociation kinetic analyses of the amplification products were performed to ensure specific amplification by heating and cooling the samples at 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. MET expression was normalized to the 18S gene expression.

Sequencing and *in silico* mutation prediction

Mutations in the MET gene were screened by direct sequencing of exons 16 to 19 (primer sequences available upon request). PCR products were first purified using the enzyme ExoSAP-it (GE Healthcare Europe GmbH) for purification and then sequenced with the Big Dye Terminator v.3.1 Kit (Applied Biosystems; Life technologies). Resin Sephadex G50 superfine (GE Healthcare Europe GmbH) was used for purification. Sequencing was performed on an ABI 3730 automatic DNA sequencer (Applied Biosystems) in 96-well plates. Variants and mutations were identified by visual inspection of the sequences using the Sequencher 4.10.1 Demo software (Gene Codes, Corporation).

The NM_001127500 sequence corresponding to the long isoform (1408 amino acids) was used to determine the identified mutations. The NM_000245 sequence corresponding to the short isoform was used for *in silico* tools

namely Polyphen (<http://genetics.bwh.harvard.edu/pph2/index.shtml>) and SIFT (<http://sift.jcvi.org/>). These tools allow to predict the possible impact of an amino acid substitution on the structure and function of a protein, based on chemical and physical characteristics and conservation degree among species.

Results

Overall, 220 frozen pRCCs were collected at eight French centers. Double pathologic review, including pRCC confirmation and control of percentage of tumor cells >70%, excluded 71 samples (32.3%). Reasons for sample exclusion was mostly insufficient number of tumor cells related to tumor necrosis ($n = 29$), infiltration by predominant inflammatory cells ($n = 8$), degradation related to frozen processing ($n = 8$), or nonspecified causes of insufficient tumor cells ($n = 16$), but also inadequate pRCC classification ($n = 3$), inadequate sampling/labeling of nontumor kidney tissue ($n = 7$). Characteristics of the cohort, including clinical features such as age and gender and pathologic stage and grade, are presented in Table 1.

To assess the various potential mechanisms of increased MET activity and its relevance in pRCC, we investigated the MET oncogene at (i) expression level, (ii) gene copy number alteration, (iii) screening for mutations, and (iv) ligand-dependent activation and coactivators in our cohort. Overall samples included in our cohort and different methods used in this report are presented in Fig. 1.

Gene expression across subtypes

Gene expression of the MET oncogene in pRCC was analyzed in 98 pRCC samples, including 47 pRCC type I, 45 pRCC type II, and six unclassified pRCC. For comparison purpose, gene expression in the same analysis profile was performed also for 6 ccRCC and 1 chromophobe tumor. For

10 patients, both tumor and normal kidney tissue were processed. MET expression was significantly higher in all RCC when compared with normal kidney: (fold change = 5.8/3.1/1.8; $P = 1.4 \times 10^{-14}$ / $P = 1.8 \times 10^{-4}$ / $P = 0.1$ respectively for pRCC I/pRCC II/ccRCC when compared with normal kidney expression; Fig. 2A). MET expression was significantly higher in type I versus type II ($P = 1.23 \times 10^{-5}$) and both pRCC expression levels were higher than ccRCC.

To confirm our results from microarray studies, MET expression was verified by an independent gene expression profiling method. Using real-time qRT-PCR, MET expression level was analyzed in 36 pRCC I, 39 pRCC II, and 5 ccRCC (Fig. 2B). Upon analysis, the expression level of the target gene is computed relative to the expression level of the 18S reference gene. The results of real-time qRT-PCR experiments demonstrated that the relative expressions of the MET gene were significantly higher in RCC samples (fold change = 6.4/3.8/3.1; $P = 9.64 \times 10^{-6}$ / $P = 4.05 \times 10^{-4}$ / $P = 9.99 \times 10^{-3}$ respectively for pRCC I/pRCCII/ccRCC) in comparison with normal kidney.

Copy number alterations

To further identify whether MET RNA expression was related to copy number alteration or other mechanisms, we performed a correlation analysis between copy number alteration from the CGH array results available from 37 type II pRCC and matching gene expression results. The correlation between copy number alterations of the MET gene located on 7q31 and gene expression was significant (correlation coefficient = 0.6; $P = 8.2 \times 10^{-4}$; Fig. 3A and Supplementary Fig. S2). Seventeen tumors (46%) presented a gain in MET gene with a median value of \log_2 (ratio) = 0.41 (0.11–0.83). No amplifications, as defined per a stringent threshold of \log_2 (ratio) > 1.5, of MET gene were

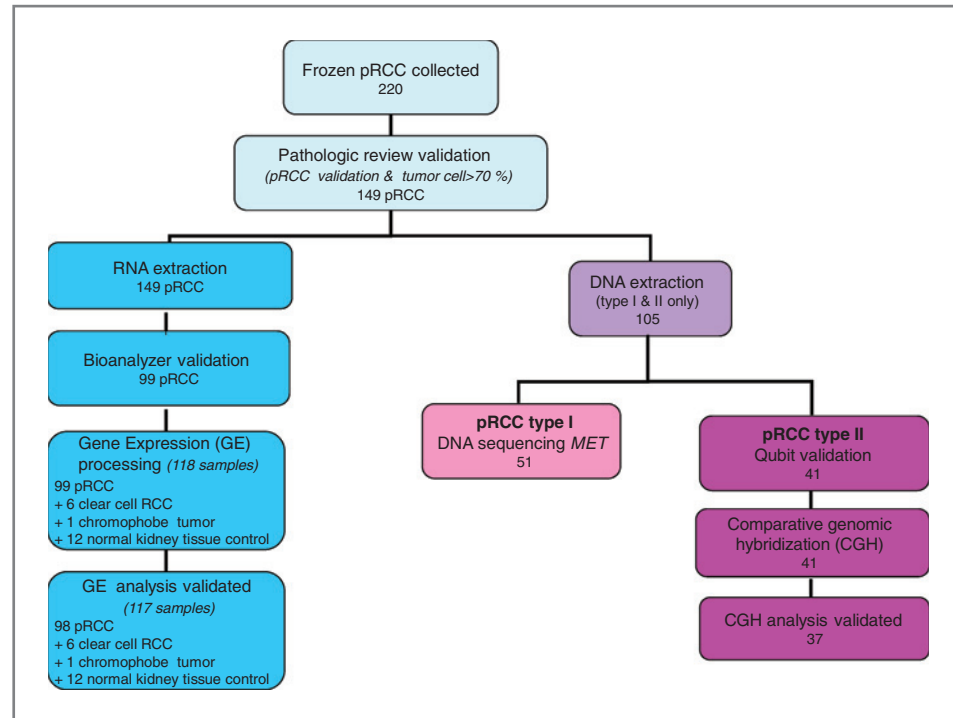
Table 1. Characteristics of the 220 pRCC population

| | Overall population ($n = 220$) | % | Type I ($n = 85$) | % | Type II ($n = 80$) | % | Non-specified ($n = 54$) ^a |
|---|---|----|------------------------|----|-------------------------|----|--|
| Gender | 220 | | 85 | | 80 | | 55 |
| Male | 175 | 79 | 64 | 75 | 69 | 86 | 42 |
| Female | 45 | 21 | 21 | 25 | 11 | 14 | 13 |
| Median age at diagnosis year (min-max) | 61 (26–86) | | 60 (26–85) | | 61 (31–86) | | 59 (33–81) |
| Stage | 189 (30 ukn) | | 75 (10 ukn) | | 70 (10 ukn) | | |
| pT1/pT2 | 129 | 68 | 62 | 83 | 30 | 43 | |
| pT3/pT4 | 60 | 32 | 13 | 17 | 40 | 57 | |
| Lymph nodes invasion (N+) | 25 (out of 108 informative patients) | 23 | 6/44 (41 ukn) | 14 | 18/40 (20 ukn) | 45 | |
| Grade | 207 (12 ukn) | | 81 (4 ukn) | | 77 (3 ukn) | | |
| Grade 1 + grade 2 | (9 + 72) 81 | 39 | (4 + 54) 58 | 72 | (0 + 8) 8 | 10 | |
| Grade 3 + grade 4 | (100 + 26) 126 | 61 | (22 + 1) 23 | 28 | (50 + 19) 69 | 90 | |

Abbreviation: ukn, unknown.

^a+1 pRCC reclassified to chRCC.

Figure 1. Flowchart of tumor collection and tissue processing.



detected. Chromosome 7 alterations observed in the 37 CGH profiles are represented on Fig. 3B. Furthermore, to assess the copy number alteration frequency observed in type I pRCC, we performed a comparative genomic microarray analysis (CGMA; predefined P value 5×10^{-3}) method (24) from the gene expression results of the 47 type I pRCCs and we identified a 81% incidence (40 cases of 47) of 7q copy number alterations (polysomy, gain) in type I pRCC (Fig. 3C).

MET sequencing

To determine whether MET activation relies on mutations, exons 16 to 19—corresponding to the intracellular tyrosine kinase domain of MET protein—were sequenced on 51 DNAs from type 1 pRCCs. Eleven missense mutations were identified out of 51 (21.6%) DNAs and confirmed by further sequencing (Table 2). Among these 11 *MET* mutations, 4 are novel variants: c.3476C>G, p.S1159W and c.3538C>T, p.H1180Y; about the 2 others, they affect the

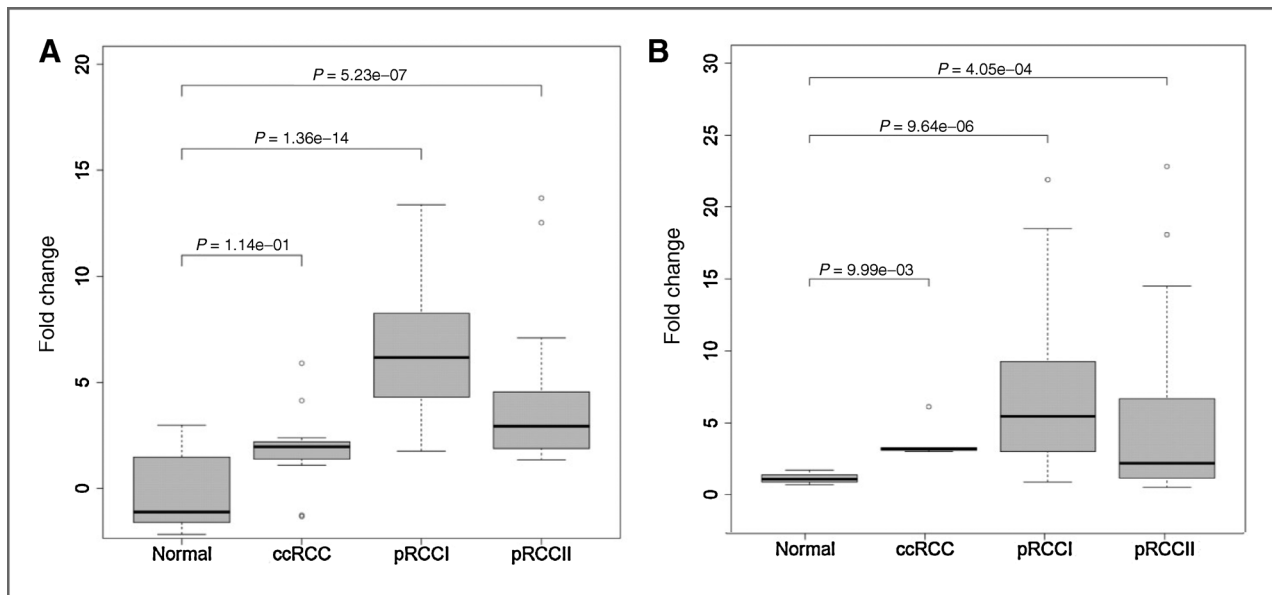


Figure 2. A, box plot of MET gene expression level. B, box plot of MET expression using qRT-PCR validation.

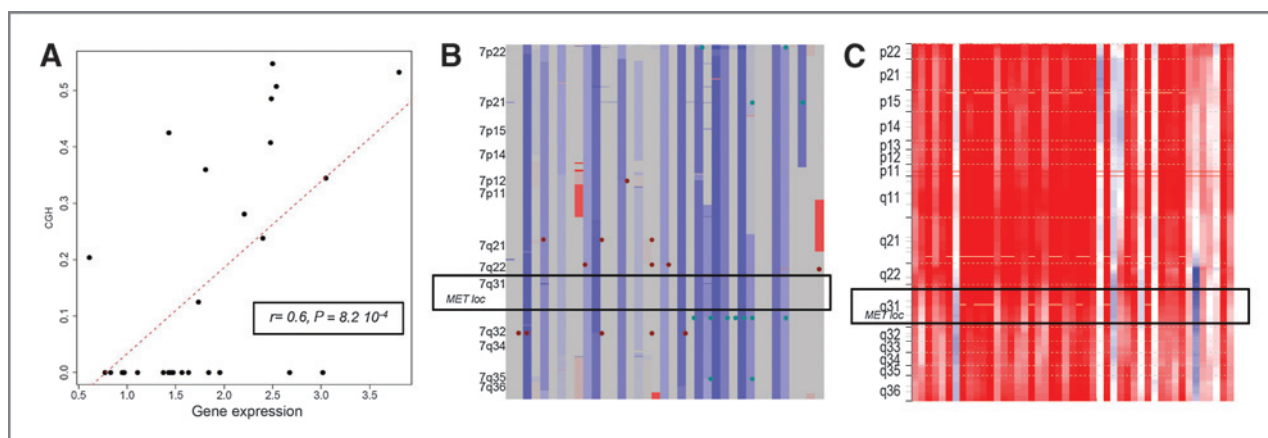


Figure 3. A, scatter plot representation of correlation between copy number alteration and gene expression profiling among type II pRCC. B, CGH array profiles of alteration on chromosome 7 for 37 PRCC type II. Each column represents a tumor sample (gain, blue; loss, red; amplification defined as log₂ (ratio) > 1.5, blue dot; deletion, red dot). C, CGMA approach validates chromosome 7 copy number alteration relevance amongst 47 type I pRCCs (each column: tumor sample; gain, red; loss, blue in the CGMA approach).

same nucleotide and codon: c.3637C>A, p. L1213I and c.3637C>T, p. L1213F. About mutations that were already reported, we detected 4 times the c.3803T>C, p. M1268T. Furthermore, as matched normal DNA was available for 4 tumors, we sequenced them, thus allowing confirmation of the somatic origin of the mutations.

We then performed an *in silico* analysis of the putative functional consequences of the 11 mutations. As shown in Table 2, all these missense mutations are predicted to be damaging by the two tools used.

To further investigate the relevance of the 4 newly identified mutations, we performed a correlation study of each mutated sample in comparison with the other mutated samples using the gene expression available dataset and focusing on the 36 genes involved in the MET pathway currently defined in biocarta_MET_pathway (as of access at http://www.broadinstitute.org/gsea/msigdb/cards/BIOCARTA_MET_PATHWAY on July 28, 2013). Only 10 of 11 mutated samples have been analyzed in Gene Express-

sion data set. We performed a "one-to-one" comparison of each sample level of expression of the 36 genes with each one of the 9 other samples. Figure 4B exhibits both graphic representation and correlation coefficient for each comparison. Correlation coefficients between the 6 previously known mutations ranged from 0.81 to 0.96. Correlation coefficients between each one of the newly identified mutations and the 6 previously described ranged from 0.79 to 0.99. Finally, within the 4 newly diagnosed mutations, the correlation ranged between from 0.89 and 0.94. No sample seemed to be significantly different in terms of MET pathway gene expression level.

The correlation between the samples harboring a MET mutation and the 6 ccRCC samples is presented in Supplementary Fig. S1.

Coactivators

Ligand expression. We analyzed both HGF and HGFAC expressions to identify potential ligand for MET activation.

Table 2. Mutations identified in the MET oncogene in a series of 51 pRCC type I and *in silico* analysis

| Exon | Nucleotide | Amino acid Long isoform NM_001127500 | Amino acid Short isoform NM_000245 | Mutation origin | PolyPhen (score) | SIFT (score) | Previous report |
|------|------------|--------------------------------------|------------------------------------|-----------------|---------------------------|--------------|-----------------|
| 16 | 3328G>A | V1110I | V1092I | ND | Possibly damaging (0.938) | Damaging (0) | (9, 10, 11, 25) |
| 17 | 3476C>G | S1159W | S1141W | Somatic | Probably damaging (1) | Damaging (0) | — |
| 17 | 3538C>T | H1180Y | H1162Y | ND | Probably damaging (0.999) | Damaging (0) | — |
| 18 | 3616G>T | V1206L | V1188L | ND | Probably damaging (0.994) | Damaging (0) | (8, 11) |
| 18 | 3637C>A | L1213I | L1195I | Somatic | Probably damaging (0.996) | Damaging (0) | L1213V (8) |
| 18 | 3637C>T | L1213F | L1195F | ND | Probably damaging (1) | Damaging (0) | L1213V (8) |
| 19 | 3803T>C | M1268T | M1250T | Somatic | Probably damaging (0.999) | Damaging (0) | (8, 10, 11) |
| 19 | 3803T>C | M1268T | M1250T | ND | Probably damaging (0.999) | Damaging (0) | (8, 10, 11, 12) |
| 19 | 3803T>C | M1268T | M1250T | Somatic | Probably damaging (0.999) | Damaging (0) | (8, 10, 11, 12) |
| 19 | 3803T>C | M1268T | M1250T | ND | Probably damaging (0.999) | Damaging (0) | (8, 10, 11, 12) |
| 19 | 3742T>C | Y1248H | Y1230H | ND | Probably damaging (0.998) | Damaging (0) | (8) |

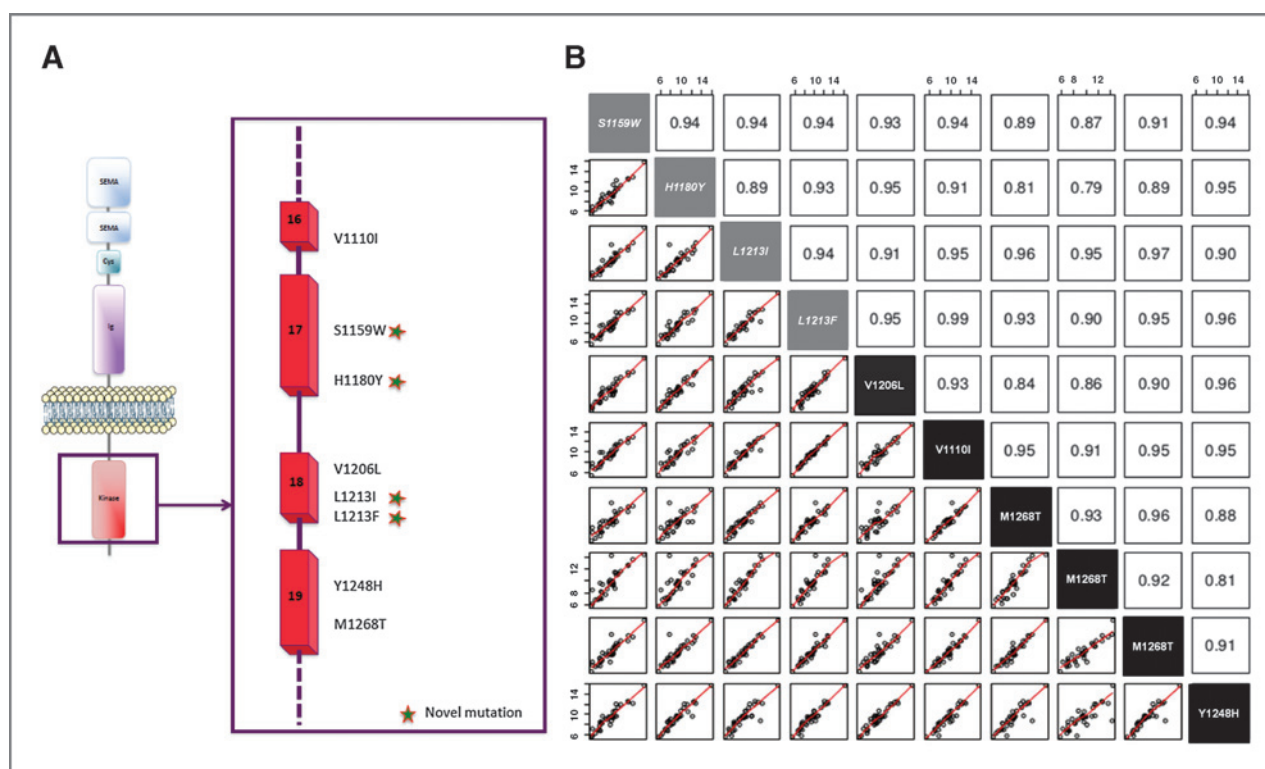


Figure 4. A, schematic representation of the 11 identified mutations in exons 16–19 of the *MET* gene; similar M1268 T mutation in exon 19 was isolated in 4 different cases. B, correlation analysis of the gene expression level of 36 genes—involved in the *MET* pathway—to assess the downstream effect of the 4 newly identified mutations with each one of the 9 other mutations (4 newly identified and 6 previously reported mutations). Cumulative correlation value of the expression of the 36 genes is indicated in each box (one-to-one comparison).

HGF expression level was very low across pRCC with no significant difference in terms of fold change. Unlike HGF, HGFAC was upregulated in pRCC with median fold change of +3.8 ($P = 6.25 \times 10^{-6}$) and +1.9 ($P = 3.03 \times 10^{-02}$) when compared with normal renal tissue, amongst type I and type II pRCC, respectively. HGF and HGFAC expression levels in correlation with *MET* expression level are presented in Fig. 5A and B.

Co-required kinase. We further investigated the relevance of LRRK2 as a co-kinase for *MET*, reported to be required for *MET* oncogenic signaling in pRCC. Gene expression analysis demonstrated higher expression of LRRK2 RNA across all pRCCs: fold change = 7.4/3.6/2.8; $P = 7.38 \times 10^{-11}/P = 2.4 \times 10^{-5}/P = 5.82 \times 10^{-2}$ for pRCC I/pRCCII/ccRCC, respectively. Expression level was higher in type I than in type II tumors (Fig. 5D). These results were subsequently confirmed on the qRT-PCR performed in 73 pRCC samples (Fig. 5E). Its expression was higher in RCC samples, compared with nontumor tissues (fold changes = 6.4/3.3/2.8; $P = 1.70 \times 10^{-9}/P = 4.07 \times 10^{-4}/P = 5.28 \times 10^{-3}$ for pRCC I/pRCCII/ccRCC, respectively).

Finally, we assessed correlation between LRRK2 and *MET* level (Fig. 5C): correlation coefficient = 0.63; $P = 1.31e-13$.

Discussion

In the metastatic setting, pRCC prognosis is worse than ccRCC prognosis, especially in type II subpopulation

(6, 26, 27). Currently, there is no standard of care for systemic treatment of metastatic pRCC. Progression-free survival observed in first line treatment with current available VEGF receptor tyrosine kinase inhibitor agents and mTOR inhibitors ranges from 2 to 7 months (28–30) with an overall survival being around 1 year. Therefore, there is a need to develop specific targeted agents in pRCC. Knowledge from hereditary syndrome biology and first reports on *MET* inhibitors in this tumor subtype may represent the first breakthrough in terms of therapeutic compound available for pRCC. Choueiri and colleagues reported a phase II study investigating foretinib (16; GSK 1363089 and XL880), a dual *MET* and VEGFR2 inhibitor in patients with pRCC with either *MET* mutation or *MET* gene amplification or no *MET* alteration. Very interestingly, besides substantial benefit in *MET* germline-mutated tumors, median progression-free survival was also promising in both mutated and nonmutated pRCC patients, and furthermore irrespectively of subtypes I and II, which were not assessed in this study.

In our report, we explored several variables about the *MET* gene status to provide a preclinical rationale for targeting *MET* in all subtypes of pRCCs. As any proto-oncogene, malignant transformation occurs when *MET* activity is increased inappropriately and/or constitutively activated. The aim of the present work was to assess the differential *MET* gene status and expression across different pRCC tumors that may lead to *MET* activation using

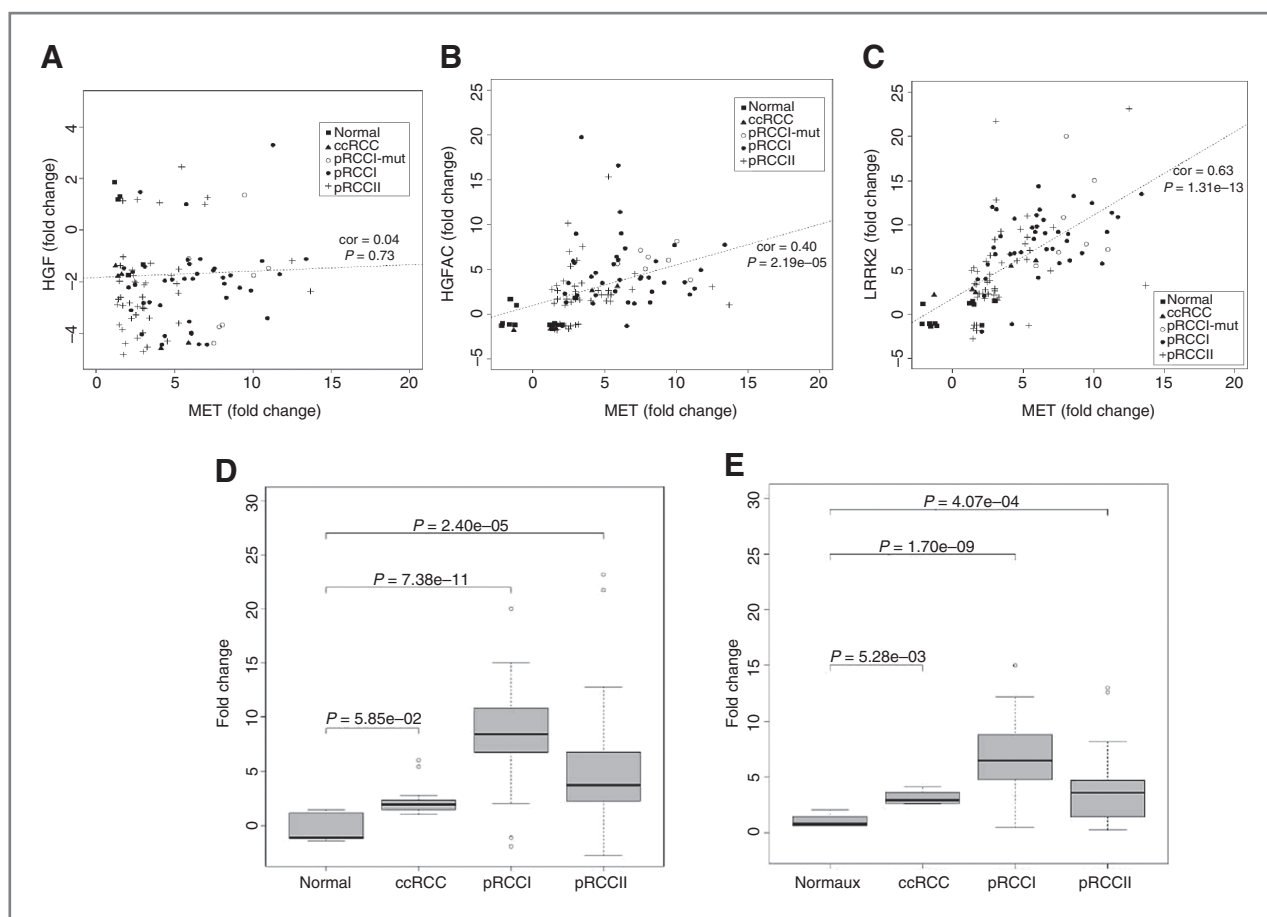


Figure 5. A to C, HGF, HGFAC, and LRRK2 expression in all samples and correlation with MET expression. D, box plot of LRRK2 gene expression. E, box plot of LRRK2 qRT-PCR.

combined (i) gene expression, (ii) matching copy number alterations, (iii) gene sequencing, (iv) and finally assessment of potential ligand-dependent or cofactors activation of MET protein.

Our first set of results is about the magnitude of *MET* RNA upregulation (through gene expression profiling and qRT-PCR validation) across pRCC when compared with ccRCC or normal kidney tissue. This information is consistent with the MET staining that has previously been reported by Gibney and colleagues (31). Indeed, they used automated quantitative analysis to characterize the expression of MET protein levels in 317 RCC tumors including 45 pRCC. Protein expression level was not only higher in RCC tissue than in adjacent paired normal tissue but also significantly higher in papillary than in clear cell subtypes ($P < 0.0001$). MET protein expression has been reported in up to 90% of pRCC, including both type I and type II in a pathology report from Choi and colleagues (32).

Copy number alterations were detected in 81% of type I and 46% of type II, where CGH array identified 17 gains and no amplification of *MET*. Copy number alterations highly correlate with upregulation in RNA level. Interestingly, the first report from Glokhova and colleagues (33) demonstrat-

ed that gene copy number increase was associated with upregulation of MET expression. This observation is not limited to kidney cancer: similar correlations between *MET* gene copy number and protein expression level measured by immunohistochemistry have also been reported in non-small cell lung cancer (34). Previous cytologic reports identified chromosome 7 trisomy as a common feature of pRCC (11). Further reports, using both CGH arrays and SNP arrays, did confirm that either chromosome 7 trisomy or partial gain is more likely to be seen in pRCC (35, 36). Noteworthy, some authors considered the chromosome 7 alterations more likely to be seen in type I pRCC (37), as 17q gain and 9p loss are specific features of type I and II, respectively (38). On the contrary, other reports considered chromosome 7 trisomy as shared by all pRCC subtypes irrespectively of grade, size, and hereditary versus sporadic presentation (39–42). More interestingly, patients with *MET* mutations, either inherited or somatic, were reported to present allelic imbalanced duplication on 7q of the mutated allele (43, 44), drawing the hypothesis that both events are required for oncogenic transformation in hereditary and somatic *MET* mutant pRCC. Our results represent the largest CGH array cohort of type II pRCC with copy

number alteration assessment and matching gene expression levels while underlining in both pRCC subtypes the relevance of copy number alteration in relation with gene expression. Interestingly, no amplification was identified in type II pRCC. These results are consistent with penile squamous cell carcinoma where *MET* expression is detected in 87% of tumors and correlates with polysomy-7 and without gene amplification (45), but are in contrast with various tumor types, such as gastroesophageal, gastric, ovarian, and non-small cell lung cancers (17, 46) where amplifications of the *MET* gene have been reported.

Genetic evidence that *MET* plays a crucial role in pRCC came from the identification of families carrying autosomal dominant missense mutations in the tyrosine kinase encoding sequence of *MET* gene (8–12, 25). Some mutations were subsequently identified in somatic tumors with a 13% incidence (9, 10). We obtained a mutation rate of 21.5% for the *MET* gene in type 1 pRCC (11/51), which is higher than in previous studies. As our cohort only includes sporadic cases, we may postulate that these mutations are somatic, as shown for 4 cases. We identified 4 new variants in this study and, interestingly, two are linked to a same position, which is the leucine in 1213, already known to be mutated in a Valine (8). In our cases, the mutations lead to an Isoleucine, which is similar to leucine, and Phenylalanine, which is an aromatic residue probably leading to a different conformation of the tyrosine kinase domain of the HGF receptor. *In silico* tools were used to evaluate the potential effect of these mutations and because mutated residues belong to conserved domains, we can expect a damaging consequence. We also detected 4 already described mutations including M1268T, which is the most frequently reported mutation (8), suggesting a hot-spot or a position which is highly critical for the protein function and may be selected specifically through tumor progression. We chose to sequence only type 1 pRCCs, whereas frequently the two types of pRCCs are considered together. For example in COSMIC website, a mutation rate of 4% is reported for the *MET* gene in 40 pRCCs. However, as we chose to sequence the 4 exons of the tyrosine kinase domain only, our mutation rate might probably be underestimated, even if it is difficult to evaluate the functional consequence of mutations detected in other domains than the tyrosine kinase one.

Our ligand gene expression analysis did not identify HGF upregulation as a potential mechanism for *MET* activation in pRCC, but it is of interest to point out that *MET* upregulation was correlated to *HGFAC* upregulation, therefore suggesting that the serine protease is more relevant than the ligand in this setting of *MET* activation and signaling.

Looyenga and colleagues (20) identified LRRK2 as a mechanism of signaling cross-talk that promotes *MET* activation, tumor cell growth, and survival in papillary renal and thyroid carcinomas. We confirmed that LRRK2 is amplified and upregulated across pRCC and highly correlates with *MET* expression.

There are obviously some limitations in our results. The first main limits of the present report rely on the challenge of

investigating the expression and activation of downstream molecules of *MET* pathways, mainly due to the fact that effectors are highly shared with other growth factor receptors and none may be specifically used as a surrogate of *MET* activity (AKT, ERK, etc.). The second major concern is the use of primary tumor samples of heterogeneous population (localized, locally advanced, and metastatic tumors). Nevertheless, we believe that this report provides new insights on *MET* gene status across pRCC.

Conclusion

This report, to our knowledge, is the largest study on both gene expression and cytogenetic analysis of sporadic pRCC; it emphasizes the upregulation of *MET* gene across pRCC.

We confirmed the occurrence of *MET* mutation in type I pRCC and describe 4 newly identified somatic mutations in *MET* gene. Copy number analysis identified *MET* gain as another potential mechanism of increased *MET* activity relevant in pRCC. Finally, the upregulation of cokinase required for *MET* signaling has also been identified in pRCC and may therefore not only be a potential predictive marker of *MET* inhibitors activity but also a therapeutic target in itself.

Disclosure of Potential Conflicts of Interest

L. Albiges is a consultant/advisory board member for Amgen, Novartis, Pfizer, and Sanofi. No potential conflicts of interest were disclosed by the other authors.

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