Molecular analysis of aggressive renal cell carcinoma with unclassified histology reveals distinct subsets

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Renal cell carcinomas with unclassified histology (uRCC) constitute a significant portion of aggressive non-clear cell renal cell carcinomas that have no standard therapy. The oncogenic drivers in these tumours are unknown. Here we perform a molecular analysis of 62 high-grade primary uRCC, incorporating targeted cancer gene sequencing, RNA sequencing, single-nucleotide polymorphism array, fluorescence in situ hybridization, immunohistochemistry and cell-based assays. We identify recurrent somatic mutations in 29 genes, including NF2 (18%), SETD2 (18%), BAP1 (13%), KMT2C (10%) and MTOR (8%). Integrated analysis reveals a subset of 26% uRCC characterized by NF2 loss, dysregulated Hippo–YAP pathway and worse survival, whereas 21% uRCC with mutations of MTOR, TSC1, TSC2 or PTEN and hyperactive mTORC1 signalling are associated with better clinical outcome. FH deficiency (6%), chromatin/DNA damage regulator mutations (21%) and ALK translocation (2%) distinguish additional cases. Altogether, this study reveals distinct molecular subsets for 76% of our uRCC cohort, which could have diagnostic and therapeutic implications.
Renal cell carcinoma (RCC) encompasses a heterogeneous group of tumours and is mainly categorized based on unique histopathological features. Major subtypes are clear cell RCC (cRCC, ~75%), papillary RCC (pRCC, ~15%) and chromophobe RCC (cchRCC, ~5%)1-3. uRCC accounts for 4–5% of RCC that is not classifiable as one of the major (>5%) or the rare (<1%) subtypes such as medullary, collecting duct, mucinous tubular and spindle cell carcinoma, and MiTF family translocation RCC4-6. uRCC represents a large proportion of metastatic RCC that exhibits non-clear cell histology (nccRCC), has no standard therapy4-6, and presents formidable diagnostic and management challenges7-9. Large collaborative genomic efforts, including The Cancer Genome Atlas projects, have greatly extended our molecular understanding of common RCC subtypes, including cRCC10-13, chRCC14,15 and pRCC15-17. However, as a rare and heterogeneous group of tumours, uRCC currently remains as the largest molecularly uncharacterized RCC category with unknown oncogenic pathways.

To gain knowledge towards this unmet need in the diagnosis and management of aggressive nccRCC, we conducted the first in-depth molecular characterization of uRCC in a cohort of 62 primary tumours with high-grade histologic features, all of which were re-reviewed by experienced genitourinary pathologists to ensure their proper classification based on the current World Health Organization and International Society of Urologic Pathology consensus diagnostic criteria20-22. To study the spectrum of this heterogeneous group of tumours and not to exclude cases with only formalin-fixed, paraffin-embedded (FFPE) archival tissue, we employ an integrated and step-wise approach, combining targeted cancer gene sequencing, RNA sequencing (RNA-seq), single-nucleotide polymorphism (SNP) array, fluorescence in situ hybridization (FISH), immunohistochemistry and cell-based assays to focus on identifying molecular alterations and pathways that are potentially clinically informative. We find recurrent somatic mutations in 29 genes, and identify distinct molecular subsets that are characterized by NF2 loss, hyperactive mTORC1 signalling, FH deficiency, chromatin/DNA damage regulator mutations or ALK translocation and associated with varying clinical outcomes.

**Results**

**Mutation landscape of uRCC by targeted gene sequencing.** The clinicopathologic features and outcomes of this 62-patient uRCC cohort are summarized in Supplementary Table 1. At the time of nephrectomy, 58% of cases were locally advanced (pT3 and above), with 32% showing regional lymph node involvement. Overall, 42% (n = 26) of patients developed metastatic disease and 35% (n = 22) died of RCC, underscoring the aggressive clinical behaviour and poor response to systemic therapies observed in this uRCC cohort.

To investigate the molecular aberrations in uRCC, we first employed the Integrated Mutation Profiling of Actionable Cancer Targets (IMPACT) assay, a customized ultra-deep targeted next-generation sequencing platform designed to capture all exons and selected introns of 230 oncogenes, tumour suppressor genes, and targets of actionable mutations. Of the 16 samples that were sequenced, 13 were uRCC with NF2 loss and dysregulated Hippo–YAP signalling.

The enrichment of cases with NF2 mutations (11 of 62) discovered in our uRCC cohort suggests that NF2 loss could potentially define a molecular subset of uRCC. To assess the NF2 status in uRCC beyond mutations, we next assessed the status of chromosome 22q12 where NF2 resides. Based on copy-number plots generated by the IMPACT sequencing, 22q12 loss was evident in 14 cases (23%), among which 9 also carried NF2 mutations. High-resolution genome-wide SNP array analysis was performed for 15 of the 16 uRCC cases carrying NF2 mutations and/or exhibiting 22q12 copy-number loss (referred to as the 'NF2 loss' subset from here onwards; Fig. 2a,b; Supplementary Fig. 1). Thirteen cases were confirmed to exhibit hemizygous loss of 22q and the remaining two tumours (T22 and T64), known to carry NF2 somatic mutations, showed copy-neutral loss of heterozygosity (LOH) of 22q (Supplementary Fig. 1). Of note, frequent, concurrent NF2 somatic mutation and chromosome 22q loss has not been reported in RCC. Furthermore, a three-probe FISH assay was performed, which validated the 22q hemizygous loss cases (n = 14) within the NF2 loss subset of uRCC (Fig. 2c). Consistent with genomic analyses, the NF2 protein level assessed by immunohistochemistry was significantly lower in the NF2 loss subset than in the remaining uRCC (Fig. 2d).

Germline mutation of NF2 is the principal genetic event underlying the human neurofibromatosis type 2 cancer predisposition syndrome24,25. The role of NF2 as a tumour suppressor gene is further demonstrated by mouse models in which genetic loss of NF2 results in various cancers26-28. NF2, a pleotropic factor, plays key roles in cell–cell contact inhibition, growth factor signalling, stem cell and Hippo developmental pathways29. We first focused on the NF2–Hippo tumour suppressor network, which was highlighted by a series of reports showing that NF2 enforces the Hippo tumour suppression signalling pathway by phosphorylating, sequestering, degrading and suppressing YAP/TAZ nuclear translocation, thereby disrupting oncogenic transcription30-32. To evaluate whether YAP/TAZ is active in subsets of our uRCC cohort, we determined YAP/TAZ protein expression, phosphorylation and intracellular localization by immunohistochemistry. When comparing uRCC with NF2 loss (n = 16) to those without (n = 43), there was a statistically significant stronger nuclear YAP/TAZ signal, correlating with negative to very low phospho-YAP signal, in NF2 loss tumours (Fig. 2e). To confirm that nucleus-accumulated YAP/TAZ denotes an aberrant YAP/TAZ transcription program, we performed RNA-seq on seven uRCC (four with NF2 loss and three without). Gene Set Enrichment Analysis (GSEA) demonstrated a significant enrichment of an established YAP/TAZ transcription signature33 in the NF2 loss uRCC (Fig. 2f; Supplementary Data 3). Taken together, these data suggest a novel subset of 16 (26%) uRCC cases with NF2 loss that demonstrates dysregulated Hippo signalling and YAP activation.

The importance of YAP/TAZ signalling in NF2 loss kidney cancer was further assessed using ACHN and LB996 RCC cells, two NF2 loss, nccRCC cell lines. Knockdown of YAP in ACHN or
LB996-RCC cells resulted in a decrease of proliferating cells (S and G2/M phases; Fig. 2g; Supplementary Fig. 2), as well as a reduced colony formation in soft agar (Fig. 2h).

Among this NF2 loss subset of uRCC, chromosome 1p and/or 3p losses were also detected in 450% of cases (Fig. 2a,b). Interestingly, while concurrent 3p loss and VHL inactivation were reported in 90% ccRCC, our uRCC with 3p loss did not carry VHL mutation or display histologic features of ccRCC. Furthermore, the occurrence of SETD2 (3p21) mutation was significantly higher in the NF2 loss than in the remaining uRCC tumours (44% versus 9%, Fisher’s exact test, P = 0.004). SETD2 encodes a histone H3 lysine 36 (H3K36) methyl transferase. A complete functional loss of SETD2 determined by the respective loss of histone H3K36me3 mark was detected in all seven NF2 loss, SETD2-mutated cases (Supplementary Fig. 3). In contrast, 54 of the remaining 55 uRCC tumours retained the H3K36me3 mark. Recurrent mutations of the other chromatin modulating genes including BAP1 did not show significant enrichment in the NF2 loss subset.

The NF2 loss uRCC exhibited a range of architectural patterns with multinodular or infiltrative growth (Supplementary Fig. 4). The morphologic spectrum of our NF2 loss uRCC did not fulfil diagnostic criteria of type 2 pRCC or collecting duct RCC2,3. Nevertheless, as small number of RCC with NF2 mutations have been recently reported in pRCC15,16 and collecting duct RCC34, it remains to be determined whether these tumours were distinct from or overlapped with our NF2 loss uRCC.

uRCC with hyperactive mTORC1 signalling. Somatic mutation analysis of our uRCC cohort demonstrated that potentially mTORC1 pathway activating mutations comprising MTOR (5), TSC1 (4), TSC2 (3) and PTEN (4) occurred mutually exclusively in 16 (26%) cases, which might indicate another distinct subset (Fig. 3a). Mutations of these genes have been described in ccRCC (12%)10, pRCC (8%)16 and chRCC (9%)14. Of the MTOR mutations seen in this cohort (Fig. 3b), I1973F has been described and shown to be hyperactive in cell-based assays35,36, whereas L2427R (recurred three times in our uRCC cohort) and V2475M mutations have not yet been reported. To interrogate the functional impact of individual MTOR mutations, we generated MTOR L2427R and V2475M mutants, and assessed the mTORC1 activity by phosphorylation of S6K and 4EBP1, two key mTORC1 downstream substrates37. When the MTOR

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**Figure 1 | Recurrent somatic mutations identified in high-grade uRCC.** (a) Key clinicopathological characteristics of our 62-patient uRCC cohort. Pathological (pT) stage was determined according to the 7th edition of the American Joint Committee staging system for renal cancer. The status of metastasis for individual patients was determined at their last follow-up visits or death. (b) Mutational landscape of recurrent somatic mutations based on IMPACT assays. Mutated genes are listed on the left, and denoted by individual rows. Sixty-two individual patient tumours are presented as columns and labelled at the bottom (T#). Mutation frequency (%) and absolute number of cases with non-silent mutations detected on individual genes are listed on the right. Mutation frequency was calculated as the percentage of individual tumours with mutation(s) in the indicated genes.
**Figure 2 | Molecular characterizations of the NF2 loss uRCC subset.** (a) Genome-wide frequency plot of DNA copy-number gains (blue) and losses (red) across all chromosomes was determined by OncoScan SNP assay in 15 of the 16 uRCC tumours carrying NF2 mutations and/or 22q loss. The y axis denotes frequencies of alteration in individual chromosomal regions. Copy-neutral loss of heterozygosity (CN-LOH) is shown in Supplementary Fig. 1. (b) Summary of NF2 mutations and frequent (>50%) arm-level copy-number alterations (22q, 1p and 3p) detected by sequencing and SNP array analyses of the NF2 loss subset (n = 16). Truncating mutations include nonsense mutations, insertions or deletions that alter the reading frame and splice-site mutations. (c) Representative hemizygous losses of chromosome 22q and the NF2 locus were demonstrated by a custom three-probe FISH assay (red, NF2; orange, 22q11; green, chromosome 10 centromere). Scale bar, 10 μm. (d) Representative immunohistochemical stains of NF2 on NF2 wild-type (WT) and NF2 loss tumours are shown. Scale bars, 50 μm. Semiquantitative IHC scores (0—negative; 1—focal/weak staining; 2—moderate staining; 3—strong and diffuse staining) comparing the NF2 loss subset and the other uRCC tumours are presented as a bar graph. Bars, mean values; error bars, 95% CI. (e) Representative images of NF2 WT and NF2 loss uRCC tumours stained by YAP/TAZ and p-YAP antibodies (left panel) are shown. Scale bars, 100 μm. Immunostaining scores (H-scores) for YAP/TAZ and p-YAP nuclear and cytoplasmic staining were determined and presented as a bar graph on NF2 loss (n = 16) or other uRCC (n = 43) tumours. H-Scores (H = intensity (0–3) × percentage of positive cells (1–100)). Bars, mean values; error bars, 95% CI. (f) GSEA plot of the ranked list of differentially expression genes in uRCC with NF2 loss and those with WT NF2 generated using a previously established YAP/TAZ-regulated gene set. (g) Immunoblots with the indicated antibodies (left) and a bar graph of cell cycle analysis in ACHN cells with YAP1 or control (GFP) knockdown are shown. Bars, mean values; error bars, s.e.m.; replicates n = 3. (h) Representative images (left) and a bar graph of colony formed in the soft agar after plating 10^5 YAP1 or control (GFP) knockdown cells are shown. Scale bars, 200 μm. Bars, mean values; error bars, s.e.m.; replicates n = 3. Statistical significance was determined by Mann–Whitney U-test in (d) and (e), and by Student’s t-test in (g) and (h). Statistical significance is indicated as ***P < 0.001; **P < 0.01; *P < 0.05.
type II pRCC, we performed 2SC (2-succino-cystein) and leiomyomatosis RCC (HLRCC) and a small number of sporadic and somatic mutations of Additional molecular subsets detected in uRCC exclusive (Fig. 4a).

While mTORC1 has been shown to be hyperactive in NF2-deficient mesothelioma and meningioma cell lines38,39, we did not observe hyperactive mTORC1 signalling in NF2 loss uRCC (Fig. 3e). Within our uRCC cohort, the identified NF2 loss (26%) and mTORC1 hyperactive (21%) subsets were mutually exclusive (Fig. 4a).

Figure 3 | uRCC subset with hyperactive mTORC1 signalling. (a) Schematic overview of indicated mutational and immunohistochemical analyses with annotation of mTORC1 hyperactivation on a subset of uRCC (n = 16). (b) Depiction of MTOR missense mutations identified in 5 (8%) uRCC tumours. MTOR L2427R mutation recurred in three individual tumours. (c) Functional analyses of MTOR L2427R and V2475M mutants. 293T cells were transfected with the indicated Flag-tagged MTOR expression constructs in conjunction with HA-S6K. Cellular extracts were collected 48 h later and probed with the indicated antibodies. (d) Representative images of p-4EBP1 immunostaining in L2427R and V2475M MTOR mutant tumours. Scale bars, 100 μm. (e) Immunostaining scores (H-scores) of p-4EBP1 and p-S6 were determined and presented as a bar graph on mTORC1 hyperactivation (n = 13). NF2 loss (n = 16) or the other uRCC (n = 31) tumours. H-Scores (H = intensity (0–3) × percentage of positive cells (1–100)). Bars, mean values; error bars, 95% CI. Statistical significance was determined by Mann–Whitney U-test. Statistical significance is indicated as ****P < 0.0001.

mutant was co-expressed with HA-S6K in 293T human embryonic kidney cells, L2427R exhibited higher activity, whereas V2475M showed baseline mTORC1 kinase activity comparable to the wild-type MTOR (Fig. 3c). Consistent with cell-based assays, immunohistochemistry of the uRCC with L2427R mutation displayed strong p-4EBP1 and p-S6 staining, whereas that of V2475M did not (Fig. 3a,d). These findings suggest that the recurrent I1973F and L2427R MTOR mutations are likely pathogenic, whereas V2475M could be a passenger mutation. Notably, all seven tumours with TSC1 or TSC2 mutations had high level of p-4EBP1 (H score = 300), whereas only two of four tumours with PTEN mutations exhibited such staining (Fig. 3a). Altogether, our integrated analysis demonstrated that 13 of the 16 uRCC tumours with MTOR, TSC1, TSC2 or PTEN mutations exhibited hyperactive mTORC1 signals (Fig. 3a,e).

While mTORC1 has been shown to be hyperactive in NF2-deficient mesothelioma and meningioma cell lines38,39, we did not observe hyperactive mTORC1 signalling in NF2 loss uRCC (Fig. 3e). Within our uRCC cohort, the identified NF2 loss (26%) and mTORC1 hyperactive (21%) subsets were mutually exclusive (Fig. 4a).

Additional molecular subsets detected in uRCC. As germline and somatic mutations of FH have been described in hereditary leiomyomatosis RCC (HLRCC) and a small number of sporadic type II pRCC,6,16,40, we performed 2SC (2-succino-cystein) and FH immunohistochemistry to investigate the recurrent FH somatic mutations observed in three of our uRCC cases (Fig. 1a). FH protein loss and 2SC (detects aberrant protein succination) are highly specific markers for FH-deficient RCC41–43. FH and 2SC assays were inversely correlated in our uRCC cohort, and they identified four tumours that were positive for 2SC and negative for FH staining (Supplementary Fig. 5). Genetic testing revealed FH germline mutations in three of these four patients, confirming that they indeed represent HLRCC cases. The remaining FH-negative/2SC-positive tumour (T41) harboured somatic homozygous deletion of the FH gene, revealing a somatic mechanism that can lead to FH functional loss (Supplementary Fig. 5). On the other hand, one tumour (T71) with FH G401V somatic mutation was found to be FH positive/2SC negative, and lacked histologic features of HLRCC or FH-deficient RCC40,42, suggesting that FH G401V might be better categorized as a passenger mutation (Fig. 4a; Supplementary Fig. 5).

We also discovered by IMPACT that one uRCC (T12) carried a TPM3–ALK fusion, which was further confirmed by FISH analysis (Supplementary Fig. 6). The TPM3–ALK fusion has been reported in human cancers44, including in one uRCC case45. Reported rarely in children and adults, ALK translocation-associated RCC is currently considered as an emerging entity, awaiting further characterization.

Collectively, these four distinct molecular subsets (NF2 loss, mTORC1 hyperactive, FH-deficient and ALK translocation)
accounted for 55% of our uRCC cohort (Fig. 4a). Of the remaining 28 (45%) uRCC, 8 cases carried mutations of genes involved in chromatin modulation (SETD2, BAP1, KMT2A/C/D and PBRM1); 5 in DNA damage response (TP53, CHEK2 and BRCA2); and 15 without recurrent molecular features based on our analyses (Fig. 4a). The possibility of these tumours representing other RCC subtypes (for example, TFE3/TFEB translocation or SDHB deficiency) was also excluded by established diagnostic assays.

Commonly mutated in VHL-deficient ccRCC, chromatin modulators PBRM1, SETD2 and BAP1 were recurrently mutated in uRCC that lacked VHL mutations. Our finding that these mutations also recur in nccRCC is in line with the recently reported mutations of SWI/SNF and chromatin modifier pathways in type 1 and type 2 pRCC. Given the presence of mutations of chromatin modulation or DNA damage response genes in a wide variety of cancers and their known implications in tumorigenesis, we tentatively grouped together the uRCC cases with mutations in these pathways and lacking other apparent driver alterations.

Among the 15 cases lacking recurrent features (‘other’ group), T62 and T69 had non-recurrent MET (H1094Y) or BRAF (Y472C) pathogenic mutations, respectively (Supplementary Data 2). Together, there were seven cases in which no mutation or other significant molecular alteration was detected by our panel of analyses, but the clinicopathologic features of these cases (for example, high-grade nuclear features, necrosis and so on) excluded the possibility of them being reclassified as renal oncocytomas. In addition, three uRCC tumours with somatic SMARCB1 mutations (T23, T38 and T41) retained the INI1 protein expression (encoded by SMARCB1), and were histologically distinct from renal medullary carcinoma that exhibits characteristic INI1 loss and occurs in individuals with sickle cell trait or other hemoglobinopathies (Supplementary Fig. 7). These three tumours were assigned into different molecular subsets (that is, mTORC1, NF2 loss or FH) based on their other aberrations.
Differential clinical outcomes observed in molecular subsets. Despite the relatively small patient sample size of our high-grade uRCC cohort, differential cancer-specific outcomes were observed among the above-defined molecular subgroups (Fig. 4b). NF2 loss and FH-deficient uRCC appeared to have worse clinical outcome than mTORC1 hyperactive and thus far unspecified uRCC, whereas uRCC with mutations mainly in chromatin modulation or DNA damage response genes fared intermediately (Fig. 4b). SETD2 or BAP1 mutation alone did not discern tumour subsets with significantly different clinical outcomes in this uRCC cohort (Supplementary Fig. 8).

Discussion

This study presents the first in-depth molecular characterization of high-grade uRCC, a rare and heterogenous group of aggressive tumours that poses one of the most important diagnostic and therapeutic challenges among rare kidney cancers. Our integrated, step-wise, molecular approach yields molecularly distinct subsets accounting for ~76% of the uRCC cohort, and we are able to show differential clinical outcomes associated with these molecular subsets. We identified 29 recurrently mutated genes (Fig. 1) including NF2 (18%), SETD2 (18%) and BAP1 (13%) as the most frequently mutated genes. Although some of these mutations are present in certain established subtypes of RCC, the overall mutation profiles, the frequencies of mutations in specific genes and a lack of characteristic molecular features of established RCC subtypes support the notion that these uRCC tumours are largely distinct from the established RCC subtypes and harbour their unique oncogenic pathways.

This study identifies a subset of uRCC that is characterized by NF2 loss, dysregulated Hippo–YAP signalling and aggressive clinical behaviour (Figs 2 and 4). The majority (69%) of this subset demonstrates biallelic inactivation of NF2 with concurrent NF2 mutation and LOH, a molecular feature that has not been reported in RCC. In the remaining cases with only LOH of NF2, low NF2 protein levels were observed. While NF2 has been shown to be a haploinsufficient tumour suppressor in mice24, it is possible that other mechanisms further inactivate its function in these cases. As the regulation of Hippo signalling could differ based on organ or cellular contexts53, the YAP activation we observed predominantly in the NF2 loss subset of uRCC suggests that NF2 inactivation is an essential mechanism dysregulating Hippo signalling in RCC. The high prevalence of NF2 loss in a distinct subset of uRCC suggests it acting as an early driver event in the tumorigenesis, although this remains to be further investigated. Other molecular features found in this subset of tumours include the enrichment of SETD2 mutations, frequent 1p and 3p losses and aberrant histone methylation (absence of H3K36me3) in cases with concurrent 3p loss and SETD2 mutation. The identification of this NF2 loss subset of uRCC provides an opportunity to improve our diagnosis of this particularly aggressive subset of tumours, and test new therapeutic strategies such as those aimed at interfering with YAP activity54, or the synthetic lethal interaction of WEE1 inhibition in H3K36me3-deficient cancer55.

The mTORC1 hyperactive uRCC displayed much higher levels of mTORC1 signalling than the other uRCC, and was associated with a better clinical outcome. This subset harbours molecular alterations similar to those identified in a small cohort of cRCC patients who benefit long term from mTOR inhibitor therapy56, and suggests a readily available targeted therapy venue for patients with advanced uRCC that belong to this subset.

We detected three HLRCC cases with proven germline FH mutations in our uRCC cohort, emphasizing the wide histological spectrum observed in HLRCC-associated renal tumours40,42. We also found one case harbouring somatic alterations (homozygous deletion) that fully inactivate FH function. Together, these four FH-deficient tumours showed the worst clinical outcome in this cohort. Nonetheless, given the rare incidence of FH somatic alterations reported to this date in RCC16; it remains unclear whether the clinical behaviour of a FH-deficient RCC due to somatic alterations resembles that of HLRCC. The current histological criteria and immunohistochemical markers for FH deficiency (FH and 2SC) appear to be insufficient to reliably distinguish the latter tumour from true HLRCC cases, and it remains critical to recommend genetic counselling when pathologic assessment raises a suspicion of HLRCC.

We also identified a tumour with TPM3–ALK fusion, a second RCC case with this specific fusion reported in adults. Identifying these specific driver events supports dissecting them out from the uRCC category to facilitate future characterization of these emerging RCC subtypes.

Similar to what have been described in ccRCC and pRCC10,11,20,15–17, mutations in chromatin modulation genes are relatively frequent in uRCC, although none of which (for example, SETD2 and BAP1) was found to be significantly associated with clinical outcomes in this cohort. We did not observe specific patterns of distribution for these mutations, except for the enrichment of SETD2 mutations in the NF2 loss subset. Further validation studies are needed to clarify the roles of these mutations in the oncogenesis of various types of RCC.

MET mutations have been predominantly, but not exclusively detected in pRCC based on the recent genomic studies of RCC10,15–17. While the discovery of MET H1094Y mutation in one uRCC may suggest it represents a pRCC with atypical histologic features, more importantly it provides a potential therapeutic option for this patient.

Based on this molecular study of uRCC, it is tempting to speculate that NF2 loss, mTORC1 hyperactivity, FH deficiency and chromatin modulation/DNA damage defects could play key roles in the tumorigenesis and help explain the highly heterogeneous nature of uRCC. In conclusion, this study not only demonstrates shared molecular features between uRCC and other RCC subtypes, but also uncovers unique oncogenic pathways to uRCC, which could have future diagnostic, pathogenic and therapeutic implications for rare kidney cancer patients.

Methods

Human tumour samples. Frozen or FFPE tissue samples were collected from primary nephrectomy specimens performed at Memorial Sloan Kettering Cancer Center (MSKCC) and processed according to MSKCC institutional review board approved tissue collection protocols with informed consent from all patients. The study was approved by our institutional review board. All cases have been reviewed and confirmed to fulfil the diagnostic criteria of renal cell carcinoma, unclassified (uRCC) by three genitourinary pathologists (Y.B.C., V.E.R. and S.K.T.) based on the current World Health Organization classification and consensus diagnostic criteria of International Society of Urological Pathology2,3. Ancillary studies such as TFE3/TFEB immunohistochemistry and FISH were performed to exclude tumours harbouring MiTF family translocations. Tissues were macro-dissected to ensure >70% tumour content. DNA was extracted from tumour or matched normal tissue using DNeasy Blood and Tissue kit or QIAGen DNA FFPE Tissue kit (Qiagen) for frozen or FFPE tissue, respectively, according to the manufacturer’s instructions. Total RNA was purified from tumour and normal tissues using mirVana miRNA Isolation kit (Ambion) according to the manufacturer’s instruction for total RNA isolation. DNA from each sample was quantified using Qubit fluorometer (ThermoFisher). RNA from each sample was analysed using Bioanalyzer assay (Agilent Technologies).

Targeted sequencing and analysis. The IMPACT assay is a next-generation sequencing platform that uses hybridization-based exon capture and massively parallel DNA sequencing to capture all protein-coding exons and selected introns of oncogenes, tumour suppressor genes and members of pathways deemed actionable by targeted therapies18,19. It is suitable for DNA extracted from either
fresh-frozen or FFPE samples. The assay used for this study included 230 key ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13131

average, 80 million paired-end (2

D57.2.2E, Cell Signaling Technology), phospho-4EBP1 (Thr37/46; 1:400, 236B4, YAP/TAZ (1:50, D24E4, Cell Signaling Technology), phospho-YAP (Ser127) were sequenced in parallel with the corresponding tumour DNAs. For the detection by filtering out alterations also present in matched normal samples.

Immunohistochemistry

We performed immunohistochemical staining of FFPE tissue for each tumour sample. The samples were processed according to the manufacturer’s guidelines. In brief, genomic DNA was annealed to MIP probes, followed by gap filling, ligation, digestion, amplification and hybridization to the microarrays using the Affymetrix GeneChip 3000 System. The data were analysed using the Oncocart Console (Affymetrix) and Nexus Express (BioDiscovery) softwares using Affymetrix TuScan algorithm. All array data were also manually reviewed for subtle alterations not automatically called by the software.

RNA-seq and GSEA. RNA-seq libraries were prepared using the TruSeq RNA Sample Preparation kit (Illumina) according to the manufacturer’s protocol. Libraries were sequenced on the Illumina HiSeq2500 platform to obtain on average, 80 million paired-end (2 x 75 bp) reads per sample. Sequence data were processed and mapped to the human reference genome (hg19) using STAR (v2.3)39. Gene expression levels were quantified with HTSeq-count40 and normalized using DESeq41. We used GSEA42 to statistically evaluate pathway or gene set activity that may associate with NF2 status. We tested YAP/TAZ targeted gene sets (differentially expressed genes between si-YAP/TAZ and non-targeting control) derived from the previous study43 in the GSEA analysis (Supplementary Data 3). We first removed genes expressed at low levels in all tumours (read count <20 in all samples) from the analysis. The expressed genes (=16,658) were tested for differential expression between the NF2 mutated (n = 4) and wild-type (n = 3) samples with RNA-seq data available. We used two-tailed t-test method for differential expression43, which applies an empirical Bayes approach suitable for small sample sizes, to compute a moderate t-statistic for the null hypothesis that there is no difference in expression between the two groups. Genes were sorted by this t-statistic, and GSEA was used to evaluate the null hypothesis that genes in selected gene sets were not differentially expressed in mutated versus wild-type samples (using 1,000 permutations).

Fluorescence in situ hybridization. NF2/22q FISH analysis was performed on paraffin section (5 µm) using a three-color probe mix as described in Supplementary Table 2. Clone DNA was labelled by nick translation using fluorochrome-conjugated dUTPs from Enzo Life Sciences Inc., supplied by Abbott Molecular Inc. Hybridization, post-hybridization washing and fluorescent signal detection were performed according to standard procedures. Slides were scanned using a Zeiss Axioplan 2i epifluorescence microscope equipped with a megapixel charge-coupled device camera (CV-M4 Charge, JAI) controlled by Isis 5.2 imaging software (Metasystems Group Inc, Waltham, MA). The entire section was scanned under a x3 objective with a pixel copy-number detection. Representative regions were imaged through the depth of the tissue (compressed/merged stack of 12 z-section images taken at 0.5 µm intervals under the red, green and orange filter, respectively). A minimum of two to three tumour image fields (>100 cells) were selected and the total number of signals scored for each locus. Non-specific or normal tissue signals expected in infiltrating lymphocytes were also analysed and served as the internal control to assess quality of hybridization. A minimum of 100 non-tumour cells were also scored. Interphase FISH analysis on FFPE tumour tissue was perform to evaluate ALK gene rearrangements, using dual-colour break-apart probes targeting ALK gene (Abbott Molecular). The orange fluorochrome direct probe hybridizes distal (3’) to ALK gene; the green fluorochrome direct labelled probe hybridizes proximal (5’) to ALK. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue), and interphase FISH signal scoring was performed. In a normal nucleus, two orange green fusion signals are expected. Signals for ALK gene rearrangement are either ‘broken apart’ signal or ‘single orange’ signal (deleted green signal for 5’ ALK). One hundred interphase cells from the area with high tumour content were analysed.

Plasmiids. Flag-tagged mTOR (pcDNA3-Flag-mTOR wt) was a gift from Jie Chen (Addgene plasmid no. 26063). HA-GST-tagged S6K1 (pRKS-HA-GST-S6K1) was a gift from Dr David Sabatini. The mTOR single mutations were generated by introducing corresponding nucleotide changes into pcDNA3-Flag-mTOR using QuikChange II XL site-directed mutagenesis kit (Agilent). All the constructs were confirmed by DNA sequencing. The primers for site-directed mutagenesis are as follows: mTOR: l2427R, 5’-CATACGACCTCCAGTCCGAGGGG TTCTTATTGAGG-3’; mTOR: V2475M, 5’-AATAGATCTGACCTGGTGTGGC CTTTCCTATGGG-3’. 

short hairpin RNA-mediated knockdown. pK01-shYAP1_1 was a gift from Kunliang Guan (Addgene plasmid no. 27369). pK01-shYAP1_2 was obtained from Sigma-Aldrich (TRCN0000107266). Lentiviral vectors carrying the indicated short hairpin RNA were co-transfected with pCMV.R8.2 and pHCMV.VSVG into 293T cells to generate lentivirus. ACHN and LB996-RCC cells infected with lentivirus were under puromycin selection at 2 and 1 µg/ml, respectively.

Cell culture and in vitro assays. 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, l-glutamine, sodium pyruvate and antibiotics (Invitrogen). ACHN cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS, non-essential amino acids, l-glutamine, sodium pyruvate and antibiotics (Invitrogen). LB996-RCC cells were cultured in IMDM (Invitrogen) supplemented with 10% FBS, GlutaMAX, G-4 supplements and antibiotics (Invitrogen). The ACHN cells were provided by the National Cancer Institute Developmental Therapeutics Program (Bethesda, MD), and the LB996-RCC cells were provided by Dr Van den Eynde (Ludwig Cancer Research Center, Brussels), whose laboratory established this cell line46. To assay cell proliferation, 1 x 104 cells were seeded onto a 35 mm dish and counted 4 days later. To assay cell cycle, cells were trypsinized, washed with PBS, treated with 20 µg/ml - RNase A, and stained with 25 µg/ml - propidium iodide (PI) for 1 h before being subjected to cell cycle analyses. Flow-cytometric analyses were performed using a FACSCalibur flow cytometer (Becton-Dickinson) to measure DNA contents. And, data were analysed with FlowJo software (Tree Star). To perform soft agar assay, 1 x 105 cells were seeded onto a 6 cm dish containing a top layer of 0.3% noble agar and a bottom layer of 0.6% noble agar base. Cells were fed with media every 3 days. After 3 weeks, colonies with diameter >200 µm were scored. Three independent triplicate experiments were performed. For mTORC1 signalling experiments, 293T cells were seeded at 1 x 104 cells per well (10% media per well) 24 h before transfection by 1.5 µg of vectors expressing wild type or mutant mTOR and 50 ng of vector expressing S6K using Lipofectamine 2000 (Invitrogen). The cells were collected 48 h post transfection.

Protein blot analysis. Cells were collected in ice-cold PBS buffer, pelleted and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 1% Na deoxycholate, 0.1 M Sodium phosphate (pH 7.2), 0.1% SDS, 2 mM EDTA and 50 mM NaF) with complete protease inhibitor (Roche) and phosphatase inhibitors (EMD/Millipore). Protein concentration was determined by the BCA kit (Pierce). Protein samples (20–40 µg) were resolved by 4–12% NuPAGE (Life Technologies), transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore) and detected by the enhanced chemiluminescence method (Western Lightning, PerkinElmer) and LAS-4000 Imaging system (Fujifilm). The blot images were analysed by ImageGage software (FujiFilm).

Antibodies used for immunoblot analysis are as follows: anti-NF2 (ab88957, Abcam), anti-YAP1 (no. 12395, Cell Signalling Technology), anti-pS6R127 YAP1 (no. 13008, Cell Signalling Technology), actin (A1978, Sigma), anti-pThr-389 S6K (no. 9601, Cell Signalling Technology), anti-HA (12CA5), anti-pSer-63/474-EKB1 (no. 9451, Cell Signalling Technology), anti-EKB1 (no. 9452, Cell Signalling Technology), anti-pS389/403 (no. 2900, Cell Signalling Technology), anti-Flag (F1804, Sigma), anti-RAPTOR (no. 2260, Cell Signalling Technology). The dilution for all of the primary antibodies for immunoblot analysis was 1/1,000. The dilution for all of the secondary antibodies for immunoblot analysis was 1/5,000. The full blots from these analyses are shown in Supplementary Fig. 9.
Statistical analysis. Significant co-occurrence or mutual exclusivity was determined using Fisher’s exact test. Statistical significance of quantified or semi-quantified immunohistochemical staining between tumour groups was determined by Mann–Whitney U-test. Statistical significance of cell line experiments was determined by Student's t-test. Significance of survival curves was analysed using the log-rank test.

Data availability. The RNA-seq and Oncoscan SNP array data have been deposited in the database of Gene Expression Omnibus under accession GSE85971. The IMPACT targeted DNA sequencing and clinical data of the cohort are available for public access at cbioPortal (http://www.cbioportal.org/study?studyId=uroc_msckc_2016#summary). Any other data are contained within the article and its Supplementary Information files or available from the authors on request.

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**Author contributions**

Y.B.C., V.E.R., M.F.B. and J.J.H. designed and oversaw the study. Y.B.C., S.K.T. and V.E.R. performed histological assessment. A.I.S., A.R.B., H.H.W., P.I.W., W.L., N.S. and M.F.B. analysed DNA and RNA-seq data. L.W. analysed SNP array data. A.A.I. performed H3K36me3 immunohistochemical study. Y.B.C. analysed the immunohistochemistry data. G.J.N. and L.W. performed FISH studies. J.X., Y.D. and V.O. performed the cell line experiments. F.G.G., E.H.C., Y.B.C. and J.J.H. designed the cell-based assays. W.L. and F.G.G. provided NF2-related gene expression signatures. A.A.H., M.H.V., R.J.M. and P.R. recruited patients and provided clinical database. Y.B.C. and J.I. H. drafted the manuscript. All authors contributed to critical review of the paper.

**Additional information**

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