Supplementary Case Description

All 16 patients had VTT extending at least into the renal vein, and in nine it extended into the inferior vena cava. Nine of the 16 patients exhibited metastases on diagnosis, and two additional patients (PT1 and PT16) recurred with metastasis. The primary tumor regions and VTT samples were all derived from the initial VTT thrombectomy, which was performed shortly after diagnosis (in all but one case). Metastatic regions were sampled both from metastasectomies concomitant to the initial surgery and from recurrences (see Tables S2 and S3). In three of the six cases where recurrences were sampled, one or more non-surgical interventions preceded the sampling (PT1, PT4, PT11; see Figure S1 and Tables S2 and S3).

Supplementary Methods

AVENIO Millisect tissue harvest

AVENIO Millisect automated dissection for tumor enrichment was performed on all cases. Formalin Fixed Paraffin Embedded (FFPE) tissue blocks were serially sectioned with one section at 4µm, followed by 7 sections at 10µm, followed by 3 sections at 4µm, collected onto Superfrost Plus positively charged slides (Thermo Scientific, Runcorn, UK) and allowed to dry at room temperature overnight. Serial sections 1 and 9 (4µm) were baked at 60℃ for 30 minutes and stained with Hematoxylin and Eosin (H&E) on an automated Leica Autostainer XL using a routine protocol. H&E stained slides were scanned on a NanoZoomer 2.0 HT whole slide imager (Hamamatsu, Bridgewater NJ) at 20X magnification. Scanned slide images were annotated by a pathologist for tumor regions of interest, percent tumor area necrosis (% necrosis/total tumor area) was captured and digital masks were created as a dissection reference.

Tissue sections were dissected using the reference mask image from serial section 1 to collect regions of interest using medium or large AVENIO Millisect milling tips (Roche Sequencing Solutions, Pleasanton, CA), collected with Molecular Grade Mineral Oil (Sigma-Aldrich, St. Louis, MO) as dissection fluid and dispensed into nuclease-free 1.5mL Eppendorf tubes. Dissections from slides 2 through 5 and 6 through 8 were centrifuged for 10 minutes at 20,000rpm to pellet tissue. Portions of mineral oil were removed from the tissue pellets. Pellets from slides 2 through 5 were pooled in a 1.5mL Eppendorf tube and held for DNA extraction and pellets from slides 6 through 8 were pooled in a separate 1.5mL Eppendorf tube and held for RNA extraction. Post AVENIO Millisect dissected tissue slides were baked at 60℃ for 30 minutes and stained with Hematoxylin and Eosin (H&E) on an automated Leica Autostainer XL

using routine protocols and scanned on a NanoZoomer 2.0 HT whole slide imager (Hamamatsu, Bridgewater NJ) at 20X magnification. DNA extraction was performed using the Qiagen AllPrep DNA/RNA tissue kit (Qiagen, Germantown, MD) at Q^2 Solutions (Valencia, CA).

Tumor content ranged from 5 to 99% in analyzed tissue regions. Tumor enrichment was performed using AVENIO Millisect for semi-automated dissection, resulting in tumor input of 5.9-1439.81mm^2 (Table S2) that excluded the majority of surrounding normal tissue and necrotic regions from capture and analysis.

Estimating lesion emergence timing

We first evaluated the relationship between mutational signature contribution and mutation burden, in order to establish whether the contribution of MMR deficiency-associated or other signatures was likely to violate the assumption of a constant mutation rate. In order to do so, we regressed mutation burden against age and mutational signature contribution using the lme4 package (39). To do so we fit a linear mixed effect model with age and mutation signature contribution as fixed effects and patient as a random intercept. We assessed the significance of each contribution using the *lmerTest* package (56), and found that after adjusting for multiple comparisons using the method of Benjamini and Hochberg (57), no effects were significant.

In order to estimate absolute emergence times of immediate ancestors for specific tumor lesions, we adapted the linear mixed modeling approach of Mitchell et al (16), using the *lme4* package (34). Patient age was modeled as a function of sample mutation counts as both a fixed and random slope, with intercept set to zero:

Eq (1) $$y_{p,s} = \beta \times x_{p,s} + b_p \times x_{p,s}$$

where $y_{p,s}$ is the age of patient p upon collection of sample s, β a fixed slope term, $x_{p,s}$ is the number of mutations detected in sample s from patient p, and b_p is a random slope term for patient p.

Lesion ancestor emergence timing was estimated by predicting the patient age given the estimated number of mutations present at the branch point immediately upstream of the lesion on the phylogenetic trees. The number of mutations was estimated by calculating the sum of the branch lengths leading to that branch point in each bootstrapped tree, and then taking the mean over all the resulting sums. For example, the number of mutations at VTT emergence in PT4 was estimated by summing over the branch lengths leading to the node immediately upstream

of the VTT (i.e. the branch point leading to both P1 and VTT in the representative tree in Figure 3A) in each of the 100 bootstrapped trees generated for that patient, and then calculating the mean of the resulting sums.

Emergence times were estimated for both the VTT (in all patients) and for the earliest metastasis (i.e. the metastasis with the fewest estimated mutations according to the above approach) in all patients from which a metastatic sample was collected. Confidence intervals were estimated using parametric bootstrapping of the model residuals with the *bootMer()* function from the *lme4* package.

Supplementary References

16. Mitchell TJ, Turajlic S, Rowan A, Nicol D, Farmery JHR, O'Brien T, et al. Timing the Landmark Events in the Evolution of Clear Cell Renal Cell Cancer: TRACERx Renal. Cell. 2018;173:611-623.e17.
34. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. 2015;67:1–48.
37. Alexandrov LB, Kim J, Haradhvala NJ, Huang MN, Tian Ng AW, Wu Y, et al. The repertoire of mutational signatures in human cancer. Nature. Nature Publishing Group; 2020;578:94–101.
39. Pau G, Reeder J. HTSeqGenie: A NGS analysis pipeline. [Internet]. Bioconductor version: Release (3.13); 2021 [cited 2021 Jun 7]. Available from: https://bioconductor.org/packages/HTSeqGenie/
56. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest Package: Tests in Linear Mixed Effects Models. J Stat Softw. 2017;82:1–26.
57. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B Methodol. [Royal Statistical Society, Wiley]; 1995;57:289–300.