Supplementary Information

Serial ctDNA analysis predicts clinical progression in patients with advanced urothelial carcinoma

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Supplementary Notes

The landscape of ctDNA genomic alterations in advanced UC patients

We analyzed the genomic alterations (GAs) in serial ctDNA samples from each patient. A total of 51/53 (96%) patients had detectable GAs in at least one ctDNA sample, and 44/53 (83%) patients had detectable GAs in their first ctDNA sample. We identified 462 different GAs (450 mutations, 8 copy number alterations, 4 fusions) in 58 genes in 138/182 (75.8%) ctDNA samples (Supplementary Table 3). Of these GAs, 342 (74%) were recurrent in ≥ 2 ctDNA samples. Copy number alterations included *PIK3CA* and *RAF1* amplifications. The frequency of patients in our cohort harboring GAs in each gene detected by the Guardant360 panel was overall similar to the Cancer Genome Atlas-Urothelial Bladder Cancer Study (TCGA-BLCA)(1) (Supplementary Fig. 1). Only *CDKN2A* and *RB1* GAs were significantly enriched in TCGA-BLCA compared to the study cohort (39% vs. 10% and 26% vs. 4%, respectively, $p<0.0001$).

ctDNA and disease burden

To test the relationship between disease burden and ctDNA, we analyzed the association between aggregate ctDNA VAF (aVAF) and the number of identified GAs with patients' clinical characteristics and outcomes. The mean VAF of individual GAs in all samples was 1.73 (SD= 4.29) and was significantly correlated with the number of GAs per sample (r = 0.88, $p<0.0001$). Patients with a visceral disease had a significantly higher mean ctDNA aVAF than patients without a visceral disease (7.64 vs. 2.50, $p=0.05$). Patients who harbored GAs in *FGFR3* or *ARID1A* were found to have higher mean ctDNA aVAF compared to those without these alterations (11.05 vs. 3.46, t-test $p=0.028$, and 12.50 vs. 3.58, t-test $p=0.019$).

Concordance between genomic alterations detected by ctDNA and tissue sequencing

As a result of the extensive UC molecular heterogeneity (2), the concordance between GAs identified in plasma and tissue biopsy is very low, even in patients with synchronous sampling (3). Hence, the molecular profiling of UC based on tissue biopsy is not sufficient to define the intra-patient heterogeneity and clonal evolution throughout the course of the disease. To evaluate whether ctDNA molecular profiling uncovers additional GAs beyond targeted or whole-exome sequencing (WES) of UC tumors, we compared whole-exome or targeted sequencing of tumor tissue to serial plasma ctDNA in a subset of 19 patients (Supplementary Table 7). The mean number of GAs identified in plasma samples was significantly higher than those in tumor samples (5.2 vs. 2.5, paired t-test p= 0.005). Across these GAs, the concordance rate was 46%. Thirteen out of 19 patients were found to harbor therapeutically actionable GAs identified in ctDNA but not in tissue-based testing, including a patient with *FGFR3-TACC3* fusion. In one patient (V033), *FGFR3-TACC3* was detected in ctDNA of two serial samples but was not detected using the CARIS targeted sequencing panel of his tumor tissue. The therapeutically actionable *TP53-NTRK1* fusion was detected in the fifth serial sample of V007, who developed PD on the corresponding scan.

Performance of different ctDNA-derived parameters to predict progression

To assess the optimal ctDNA parameter and cutoff that can be associated with the radiologic response, we performed a receiver operating characteristic curve (ROC) analysis using various parameters, namely ctDNA aVAF per sample, number of GAs, and ctDNA delta VAF. The area under the curve (AUC) for the ctDNA aVAF values that was associated with PD events was 0.73 (95%CI: 0.65-0.81, p<0.0001). The AUC of the

number of GAs was 0.71 (95% CI: 0.62-0.78, p <0.001). The AUC for ctDNA delta VAF >0 (increased) was for the subgroup of patients who have more than two serial samples (AUC = 0.84 (95%CI: 0.65-0.95, p< 00.1) (Supplementary Fig 4a-c).

Serial ctDNA measurements trace the clonal evolutionary trajectories of UC patients

Patient C001 was initially diagnosed with muscle-invasive bladder cancer, received neoadjuvant chemotherapy, and underwent radical cystoprostatectomy. Three months later, the patient developed pelvic nodal recurrence. Targeted sequencing of the primary bladder tumor tissue from this patient showed a *PIK3CA* mutation (M1043I) and an *FGFR3-TACC3* fusion. He was screened and enrolled in a clinical trial investigating an FGFR3 inhibitor (erdafitinib). He initially responded to erdafitinib (Supplementary Fig. 5a). When the disease progressed on erdafitinib, a ctDNA sample identified six GAs at that time. Later, the patient achieved a durable response to pembrolizumab, and the six GAs decreased to an undetectable level in subsequent ctDNA samples. Interestingly, a second activating *PIK3CA* mutation (T727R) was acquired during the initial progression on erdafitinib (Supplementary Fig. 5a). This mutation was only detectable in a serial ctDNA sample but not in primary tumor tissue. This suggests a potential role for composite mutations (e.g., two or more mutations in the same oncogene) in the progression of the disease (4–6). Patient V033 was initially diagnosed with metastatic urothelial carcinoma of the bladder (UCB) with nodal and visceral metastases. He was started on cisplatin-based chemotherapy. ctDNA showed a reduction of the VAFs of three identified GAs on a subsequent sample after starting chemotherapy corresponding to radiologic stable disease (SD) (Supplementary Fig. 5b). Two of the collected three ctDNA samples from this patient identified *FGFR3-TACC3* fusion, which was not identified by targeted

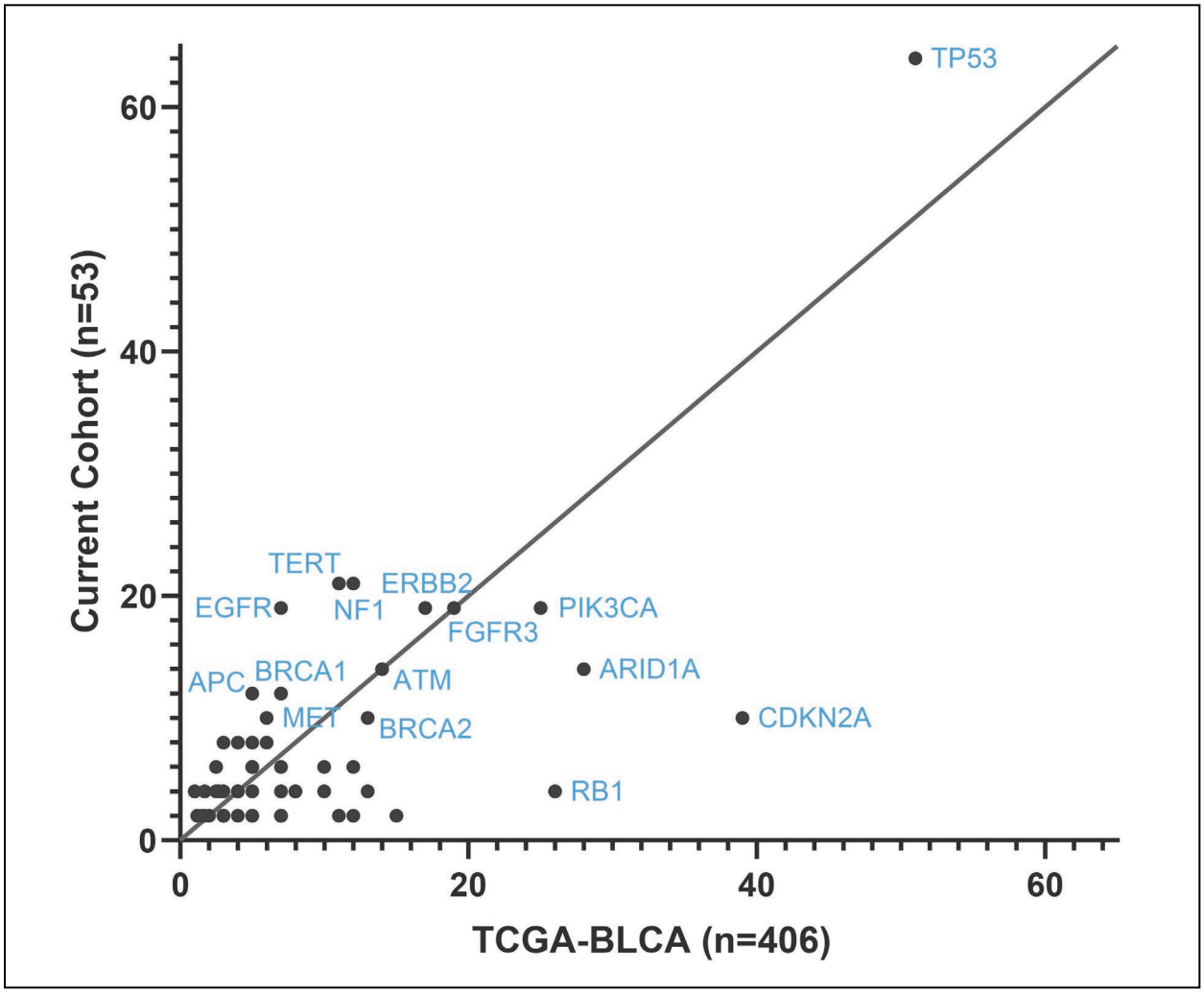
sequencing of his primary bladder tumor tissue. Upon progression on chemotherapy, he enrolled in a clinical trial of ADC. Patient V036 was initially diagnosed with metastatic UCB. The disease progressed on an immune checkpoint blockade (ICB) concurrent with the detection of five new GAs in a ctDNA sample. He was shifted to an ADC. Subsequent ctDNA sampling revealed clonal regression of these five identified GAs that was associated with partial response (PR) on the corresponding scan (Supplementary Fig. 5c). These examples highlight how serial ctDNA tracks the evolution of the disease during progression and response.

Patient V005 developed nodal and visceral metastases after radical cystectomy. He initially responded to ICB. Upon progression, his ctDNA sample identified an *NTRK1* mutation (H297L), and he was enrolled in a clinical trial of an NTRK inhibitor. Although the *NTRK1* mutation was not identified in the subsequent ctDNA sample, his disease progressed (Supplementary Fig. 5d)**,** suggesting that monitoring a single actionable GA during targeted treatment with the corresponding targeted therapy may not be sufficient to predict progression. The patient's primary bladder tumor tissue tested positive for HER2 with a score of +3 by immunohistochemistry, and he was started on lapatinib (an anti-HER2 inhibitor) without a response. A post-lapatinib ctDNA sample showed an acquired activating *ERBB2* S653C mutation at VAF of 16 (Supplementary Fig. 5d)**,** suggesting a potential resistance mechanism to the anti-HER2 therapy (7). Patient V006 was initially diagnosed with metastatic UCB. Her disease did not respond to platinum-based chemotherapy. She was screened and enrolled in an anti-FGFR3 inhibitor (erdafitinib) trial. Following an initial partial response on Erdafitinib, a progressive disease associated with an increase in the VAF of *FGFR3, CDKN2A*, and *FBXW7* GAs and the

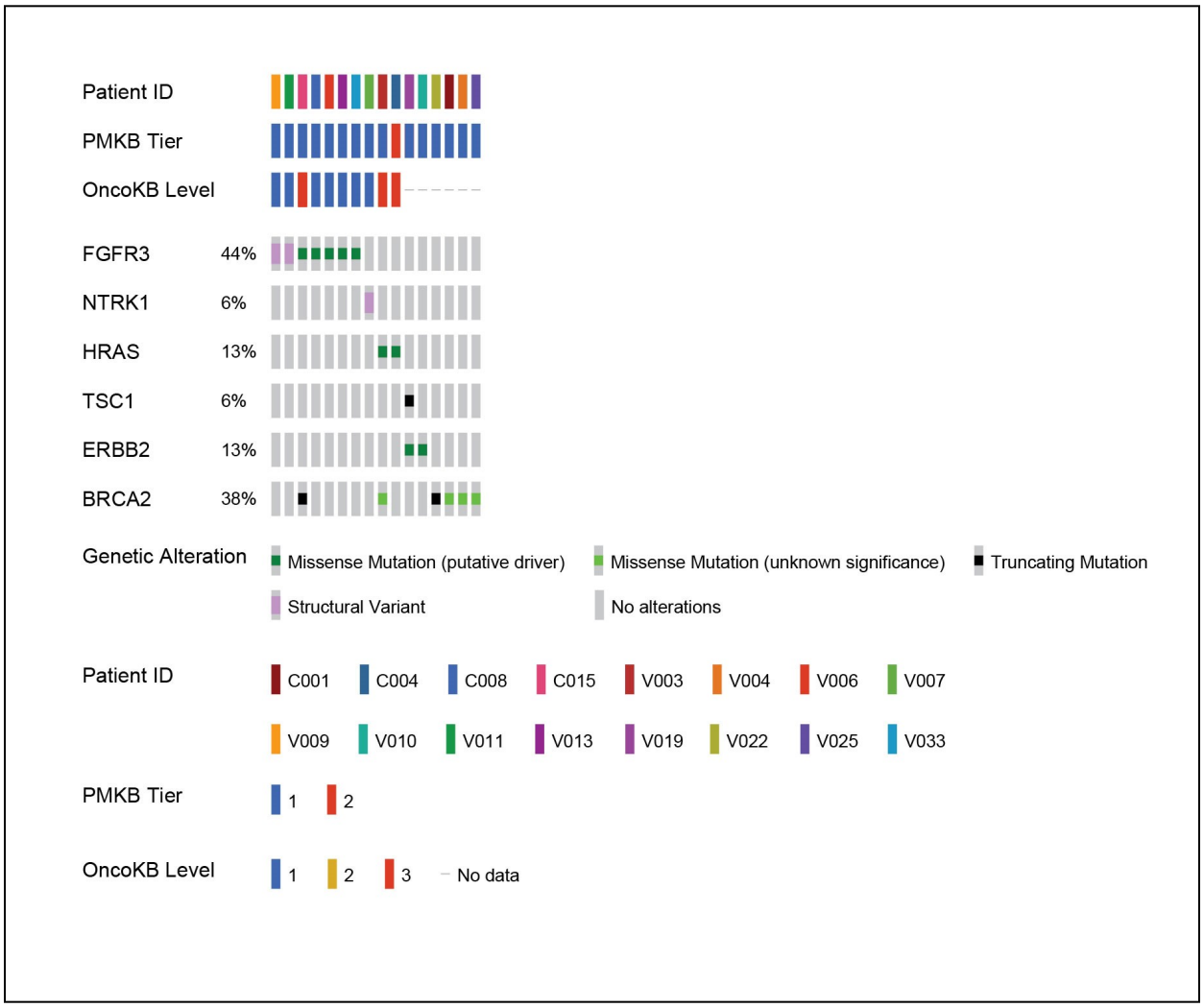
acquisition of three new GAs (Supplementary Fig. 5e). These examples highlight how ctDNA monitoring can capture UC's clonal heterogeneity and provide insights into the molecular characteristics of emerging resistant clones. The real-time insights into clonal evolutionary dynamics can be used for guiding precision medicine approaches for advanced UC patients.

Supplementary Figures

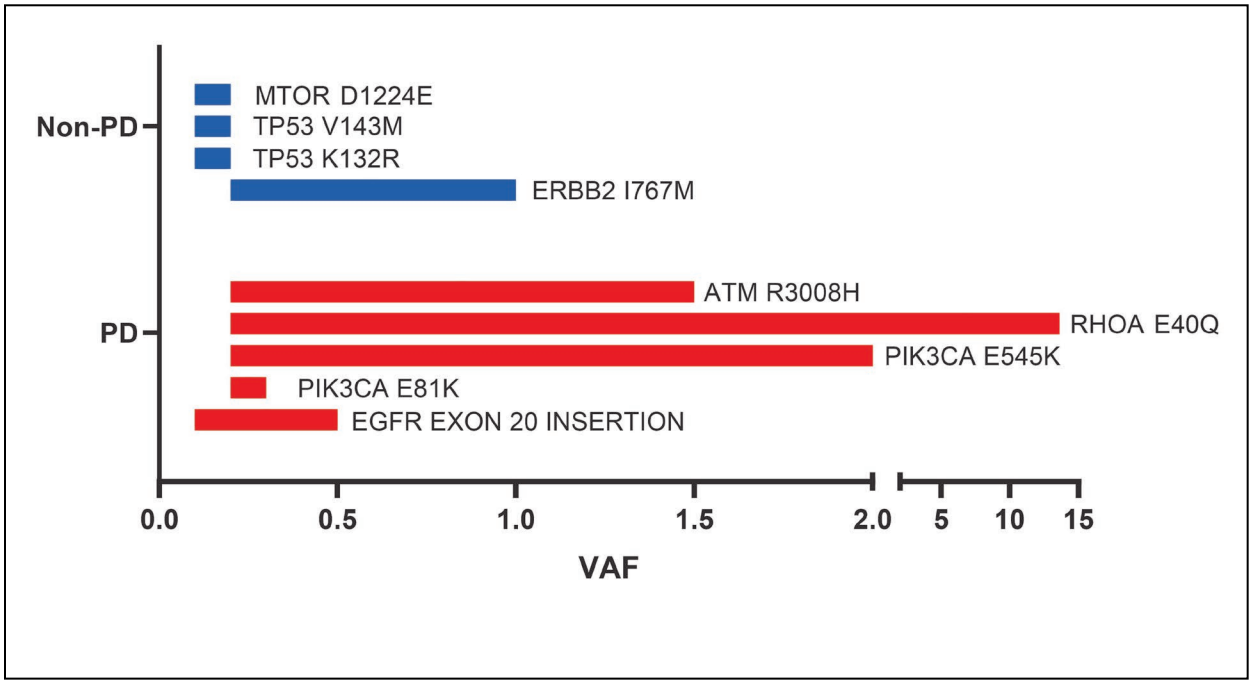
Supplementary Figure 1: Frequency of the altered genes identified in ctDNA samples compared to TCGA-BLCA.



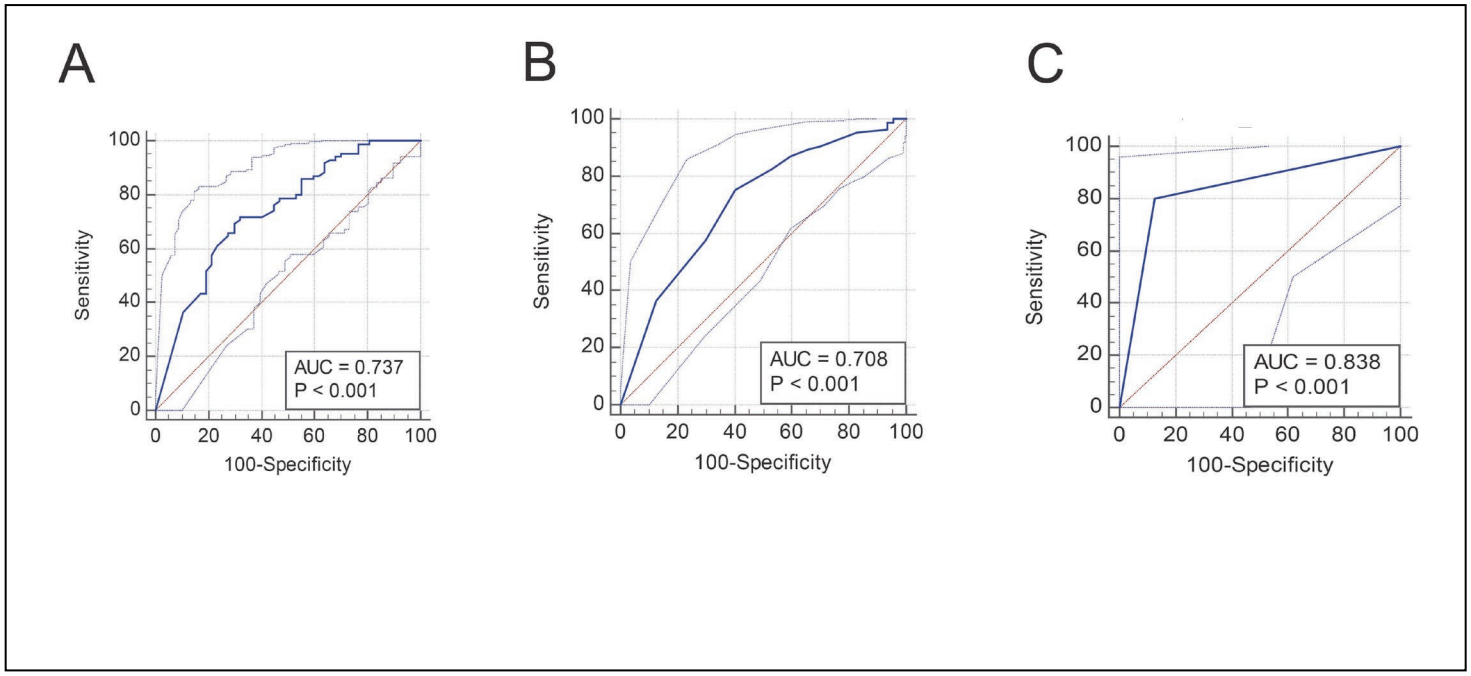
Supplementary Figure 2: Actionable genomic alterations were identified in ctDNA samples from 30% (16/53) of patients with urothelial cancer. Genomic alterations were considered actionable if labeled as levels 1-3 in OncoKB or tier 1 or 2 in PMKB. Functional annotation was based on OncoKB.



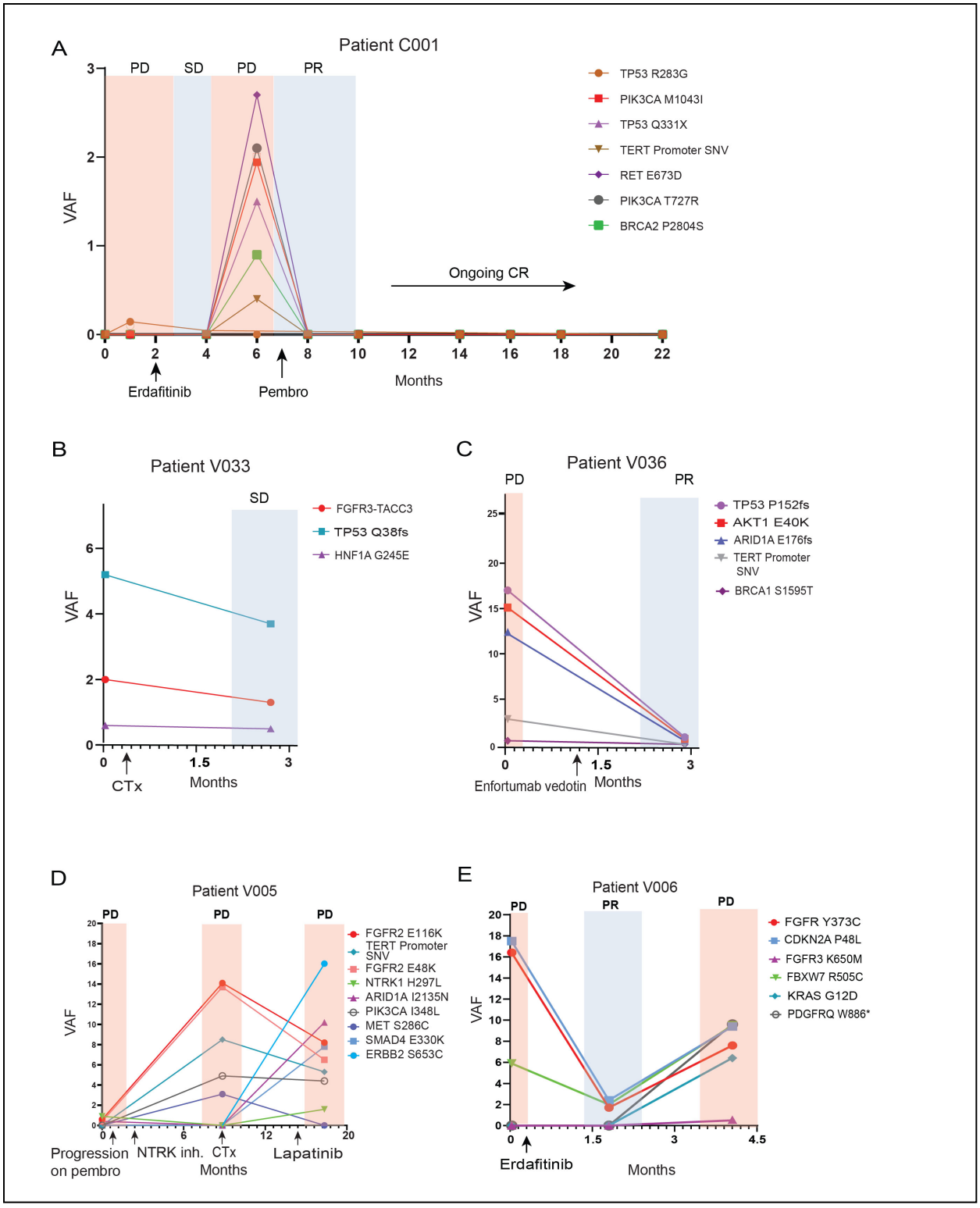
Supplementary Figure 3: Low abundance genomic alterations clonally expand significantly with UC progression events.



Supplementary Figure 4: The area under the curve for the performance of ctDNA derived parameters**:** A) aggregate ctDNA VAF, B) the number of genomic alterations, C) ctDNA delta VAF for more than two successive ctDNA samples.



Supplementary Figure 5: Serial ctDNA measurements trace the clonal evolutionary trajectories of UC patients as illustrated in five representative patient serial ctDNA samples. Pembro: Pembrolizumab, CTx: chemotherapy, CR/PR: complete/partial response, SD/PD: Stable/Progressive disease.



Supplementary References

1. Robertson AG, Kim J, Al-Ahmadie H, Bellmunt J, Guo G, Cherniack AD, et al. Comprehensive Molecular Characterization of Muscle-Invasive Bladder Cancer. Cell. 2017;171(3):540-556.e25.
2. Faltas BM, Prandi D, Tagawa ST, Molina AM, Nanus DM, Sternberg C, et al. Clonal evolution of chemotherapy-resistant urothelial carcinoma. Nat Genet. 2016 Dec 17;48(12):1490–9.
3. Barata PC, Koshkin VS, Funchain P, Sohal D, Pritchard A, Klek S, et al. Next-generation sequencing (NGS) of cell-free circulating tumor DNA and tumor tissue in patients with advanced urothelial cancer: a pilot assessment of concordance. Ann Oncol. 2017 Oct 1;28(10):2458–63.
4. Vasan N, Razavi P, Johnson JL, Shao H, Shah H, Antoine A, et al. Double PIK3CA mutations in cis increase oncogenicity and sensitivity to PI3Ka inhibitors. Science (80-). 2019;366(6466):714–23.
5. Gorelick AN, Sánchez-Rivera FJ, Cai Y, Bielski CM, Biederstedt E, Jonsson P, et al. Phase and context shape the function of composite oncogenic mutations. Nature. 2020 Jun 27;582(7810):100–3.
6. Saito Y, Koya J, Araki M, Kogure Y, Shingaki S, Tabata M, et al. Landscape and function of multiple mutations within individual oncogenes. Nature. 2020 Jun 8;582(7810):95–9.
7. Pahuja KB, Nguyen TT, Jaiswal BS, Prabhash K, Thaker TM, Senger K, et al. Actionable Activating Oncogenic ERBB2/HER2 Transmembrane and Juxtamembrane Domain Mutations. Cancer Cell. 2018 Nov;34(5):792-806.e5.

List of Supplementary Tables

Supplementary Table 1: The Guardant360 targeted NGS platform gene list

Supplementary Table 2: The baseline clinical characteristics of the study patients.

Supplementary Table 3: The frequency of unique genomic alterations per sample

Supplementary Table 4: The frequency of genomic alterations per patient and actionable targets.

Supplementary Table 5: Co-occurrence of actionable targets identified in ctDNA samples in the study patients.

Supplementary Table 6: Multivariate Cox regression models of the number of genomic alterations, *PIK3CA*, and overall survival

Supplementary Table 7: Genomic alterations identified in a subgroup of patients with matched plasma and tissue-based NGS testing