On-line Supplementary Material

Mutational Landscapes of Smoking-Related Cancers in Caucasians and African Americans

Supplementary Methods

Patient Cohort

Patients in the Wake Forest Baptist Comprehensive Cancer Center are either self-referred or referred by a healthcare provider. All patients met with a new patient coordinator. After the patient consenting, the physician orders the F1 testing, a tissue request is generated. The patient then completes the clinical and research consent forms and the Financial Assistance Application (FAA). After the FAA is approved, the tissue is released from the Department of Pathology to Foundation Medicine for F1 testing, or a fresh specimen collection is requested. F1 testing results are returned to Wake Forest within 10-14 days through the F1 Portal and uploaded to the Electronic Medical Record (EPIC, Verona, WI) and the results reported to the ordering physician. Based on the F1 report, treatment options are verified. Institutionally, treatment options are categorized as 1) Clinical Trial Opportunity Only, 2) Off-Label Drug Opportunity Only, or 3) Clinical Trial and On-Label or Off-Label Drug Opportunity, 4) counter-indication for drug use (e.g. Ras mutation for EGFR inhibitors), and 45) actionable mutation. If no treatment options are identified, the subject is advised to continue Standard of Care or Palliative Care if no other therapeutic options are available. Patients with tumors harboring actionable mutations are referred to a Virtual Tumor Board (VTB), a web-based Tumor Board that includes oncologists, genetic counselors and bioinformaticians, where the treatment options are presented and discussed. Factors considered by the VTB are: 1) Patient's performance status, 2) Patient's disease burden, 3) Treatment history, 4) Previous treatment with targeted agent and whether the tissue sample analyzed was collected prior to targeted therapy administration or after, 5) Pre-existing conditions that can be exacerbated by the suggested off-label therapy, 6) Patient's ability to take medications and fluids orally, 7) Social or psychological barriers to care, and 8) Accessibility and affordability of suggested targeted therapy. The VTB makes treatment recommendations within 72 hours. Final treatment decisions are made by the treating physician in conjunction with the patient.

Genomic Profiling

Genomic alterations detected include base substitutions, insertions, deletions, copy number alterations, and selected gene fusions (http://foundationone.com/). DNA was extracted from one or more 40-μm sections of FFPE tissue using the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega) and quantified using a standardized PicoGreen fluorescence assay (Invitrogen). Library construction was performed using 50-200 ng of DNA sheared by sonication to approximately 100-400 bp before end-repair, dA addition and ligation of indexed, Illumina sequencing adaptors. Enrichment of target sequences was achieved by solution-based hybrid capture with custom biotinylated oligonucleotide bases. Enriched libraries were sequenced to an average median depth of >500X with 99% of bases covered >100X (IlluminaHiSeq 2000 platform using 49 × 49 paired-end reads) and mapped to the reference human genome (hg19) using the Burrows-Wheeler Aligner and the publicly available SAM tools, Picard, and Genome Analysis Toolkit. Point mutations were identified by a Bayesian algorithm; short insertions and deletions determined by local assembly; gene copy number alterations identified by comparison to process-matched normal controls; and gene fusions/rearrangements determined by clustering chimeric reads mapped to targeted introns[1].

Statistical and Bioinformatic Analysis

The R package "somaticSignatures" version 2.8.4 was used to distinguish mutagenic processes among tumor groups. [2] Briefly, the method extracts adjacent nucleotides from each side of the alteration and categorizes them into 96 motifs based on trinucleotide sequence. These bin counts are used to perform matrix decomposition to pre-defined number of mutation signatures and associate the contribution of each signature to a given sample. Non-negative matrix factorization (NMF) was used to create decomposition. Number of mutation signatures to be searched was set to 3 to correspond to the three aforementioned smoking categories (never, former, recent).

Case Reports

Translating Genomics into Personalized Treatment

Many of these drugs would be considered off-label if not approved by the Food and Drug Administration (FDA), for that specific cancer type. When standard treatment options are exhausted, off-label drugs provide additional options otherwise inaccessible to patients. Increasingly, pharmaceutical companies are willing to provide experimental drugs for compassionate use, and insurance companies are becoming more willing to pay or at least partially cover these expenses[3]. This is a rapidly evolving process that renders druggability or actionability a moving concept.

In our patient population, 56 drugs comprising 15 different drug classes were available for therapeutic consideration based on Foundation Medicine's threshold for biological and clinical evidence. Of 431 patients, 325 (75.4%) had tumors harboring one or more gene mutations potentially sensitive to one or more targeted drugs, while the tumors of 31 patients (7.2%) possessed one or more mutations likely to confer resistance to one or more applicable drugs. Among patients with druggable tumors, the median number of drugs per patient was 3.0. The drugs most frequently suggested were the MEK inhibitor trametinib (33.9% of patients), the mTOR inhibitors everolimus and temsirolimus (31.3% of patients) and the multi-targeted tyrosine kinase inhibitor sorafenib (13.2% of patients). For 57.8% of patients in the cohort, one or more of these 3 drugs were available.

Case Report 1

Nivolumab for a Hypermutator Metastatic Colorectal Cancer

One of the responsive patients is a white male current smoker with metastatic colorectal cancer to the lung, peritoneum and omentum. This patient failed FOLFIRI with bevacizumab and cetuximab treatments. His FM sequencing revealed that this cancer had wild-type RAS genes but 397 protein-coding mutations (a hypermutator). He began receiving nivolumab in February 2016 due to his high tumor mutation burden. A CT scan 4 months later showed stable disease. He had a steady decrease of his CEA from 226.7 to 14.1 on last evaluation.

Case Report S2

Nivolumab for a Metastatic Colorectal Cancer with *PD-L1/PD-L2* Gene Amplification

NGS analysis revealed a unique case of metastatic colorectal cancer (CRC) with a nonsmoking AA woman of 83 years. She was diagnosed with stage III CRC at the end of 2014. After exploratory laparotomy and En bloc resection of right colon tumor, the patient received FOLFOX plus celecoxib on a clinical trial. The cancer progressed to Stage IV with biopsy proven metastasis to the retroperitoneum in July 2015. The tumors did not respond to radiation treatment (5040 cGy) and metastasized to the chest wall and skin. Given the patient's age and comorbidities, the treating physician felt that further chemotherapy would not be well tolerated and sought an alternative strategy based on NGS results. Results from tumor DNA sequencing showed mutations were present in genes *TP53*, *APC, FLT3* and *KRAS*. The tumor did not have mutations in mismatch repair genes and was not hypermutated. However, sequencing results also revealed amplification of *PD-L1* (*CD274*) and *PD-L2* (*PDCD1LG2*). The patient was given compassionate immunotherapy with nivolumab at the end of January in 2016. After 3 months of therapy, all but one of the metastatic skin tumors had disappeared with no autoimmune symptoms. After 6 months of treatment, her CT scan showed remarkable reduction and "melt-away" of metastatic nodules in chest walls, peritoneal space, and skin (Figure S6A). The patient is living independently at home and is essentially tumor free 8 months after nivolumab was initiated.

Case Report 3

Regorafenib for BRAF Mutated Appendiceal Cancer

A 63-year-old white female never-smoker developed metastatic appendiceal adenocarcinoma with peritoneal carcinomatosis. She initially underwent an appendectomy and right hemicolectomy, but 1 year later she developed peritoneal recurrence. She underwent cytoreductive surgery and hyperthermic intraperitoneal chemotherapy followed by 6 months of adjuvant chemotherapy with capecitabine and oxaliplatin. Ten months later she developed progression of disease and started receiving FOLFIRI. FOLFIRI was stopped 1 year later secondary to progression. She was switched to FOLFOX for 6 months but again the disease progressed. NGS results showed mutations in *BRAF, TP53, SMAD4, DNMT3A*, as well as equivocal amplification of *CCND3*. Due to the uncommon nature of appendiceal cancer, treatment is often extrapolated from colorectal cancer data. Because the patient had already progressed on FOLFOX and FOLFIRI and was found to have a *BRAF* mutation, she was treated with regorafenib, which has activity against BRAF. After 3 months on regorafenib, CT imaging showed significant decrease in the abdominopelvic mucinous fluid (Figure S6B). She required dose reductions due to GI side effects, but continues on regorafenib with stable disease after 8 months of treatment.

Case Report 4

FGFR inhibition in urothelial carcinoma

At age 69, a white former smoker underwent a cystoprostatectomy for stage II invasive high-grade urothelial carcinoma of the bladder. He developed biopsy-proven lung metastases 4 months later. He was treated initially with 6 cycles of carboplatin and gemcitabine followed by single-agent paclitaxel for 14 cycles. A growing lung nodule was biopsied, and F1 testing revealed an *FGFR3* activating mutation (G370C, a missense mutation in the extracellular region of FGFR3 resulting in constitutive activation of the receptor). Other genetic changes from that sample included a *TSC1* mutation, *CDKN2A/B* loss, *MDM4* mutation, and functional loss of *TP53*. Twenty months after developing metastatic disease, the treatment was changed to the multi-kinase inhibitor pazopanib, which has activity against FGFR3 and has documented activity in advanced bladder cancer patients.[4] Within 6 weeks of treatment initiation, CT imaging showed a decrease in size of multiple lung nodules. After four months on pazopanib, all nodules were responding and the index lung lesion had decreased in size from 3.3 x 2.2 cm to 1.6 x 0.4 cm (Figure S6C). He continues on oral pazopanib 600mg daily, initiated at 25% dose reduction for hypertension, and is currently in his 8th month of treatment.

Supplementary Figures

Figure S1. POI Process Flowchart.

POI participants, either self-referred or referred by a healthcare provider, are consented by a new patient coordinator. After the patient consenting, the physician orders the F1 testing, a tissue request is generated. The patient then completes the clinical and research consent forms and the Financial Assistance Application (FAA). After the FAA is approved, the tissue is released from the Department of Pathology to Foundation Medicine for F1 testing, or a fresh specimen collection is requested. F1 testing results are returned to Wake Forest within 10-14 days through the F1 Portal and uploaded to the Electronic Medical Record (EPIC, Verona, WI) and the results reported to the ordering physician. Based on the F1 report, treatment options are verified. Institutionally, treatment options are categorized as 1) Clinical Trial Opportunity Only, 2) Off-Label Drug Opportunity Only, or 3) Clinical Trial and On-Label or Off-Label Drug Opportunity, 4) counter-indication for drug use (e.g. Ras mutation for EGFR inhibitors), and 5) actionable mutation. If no treatment options are identified, the subject is advised to continue standard of care or palliative care if no other therapeutic options are available. Patients with tumors harboring actionable mutations are referred to a Virtual Tumor Board (VTB), a web-based Tumor Board that includes oncologists, genetic counselors and bioinformaticians, where the treatment options are presented and discussed. Factors considered by the VTB are: 1) Patient's performance status, 2) Patient's disease burden, 3) Treatment history, 4) Previous treatment with targeted agent and whether the tissue sample analyzed was collected prior to targeted therapy administration or after, 5) Pre-existing conditions that can be exacerbated by the suggested off-label therapy, 6) Patient's ability to take medications and fluids orally, 7) Social or psychological barriers to care, and 8) Accessibility and affordability of suggested targeted therapy. The VTB makes treatment recommendations within 72 hours. Final treatment decisions are made by the treating physician in conjunction with the patient.

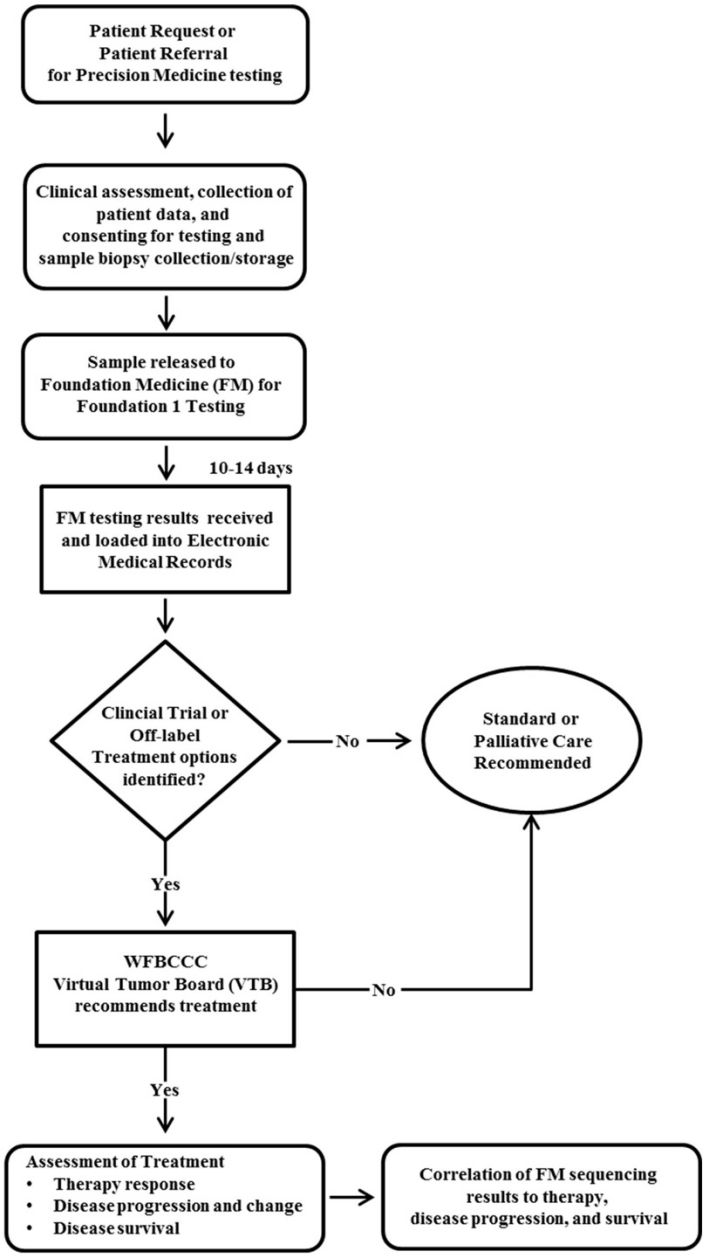

Patient Request or
Patient Referral
for Precision Medicine testing
Clinical assessment, collection of
patient data, and
consenting for testing and
sample biopsy collection/storage
Sample released to
Foundation Medicine (FM) for
Foundation 1 Testing
10-14 days
FM testing results received
and loaded into Electronic
Medical Records
Clincial Trial or
Off-label
Treatment options
identified?
No
Standard or
Palliative Care
Recommended
Yes
WFBCCC
Virtual Tumor Board (VTB)
recommends treatment
No
Yes
Assessment of Treatment
• Therapy response
• Disease progression and change
• Disease survival
Correlation of FM sequencing
results to therapy,
disease progression, and survival

Figure S2. Comparison of Somatic Mutations between Wake Forest Lung Cancers and TCGA Lung Cancers (Adenocarcinoma and squamous)

Twenty most frequently mutated genes in Wake Forest lung cancer cohort (Upper panel) and their mutation rates in TCGA lung cancer in corresponding order.

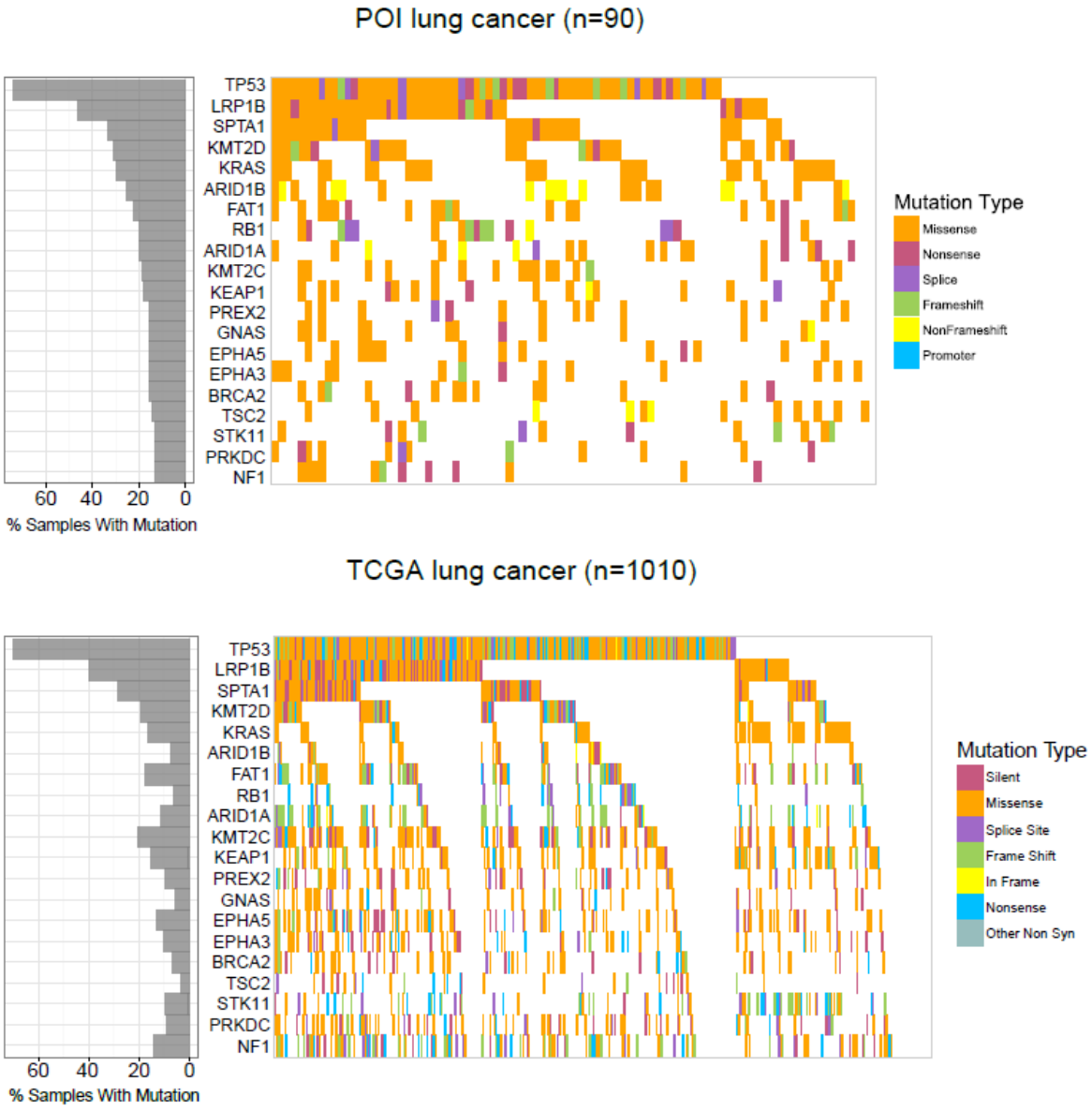


Figure S3. Structural copy number alterations in cancer types.

(A) Total numbers of structural alterations in all patients for each cancer type are shown as counts on the Y-axis. (B) Most amplified and deleted genes.

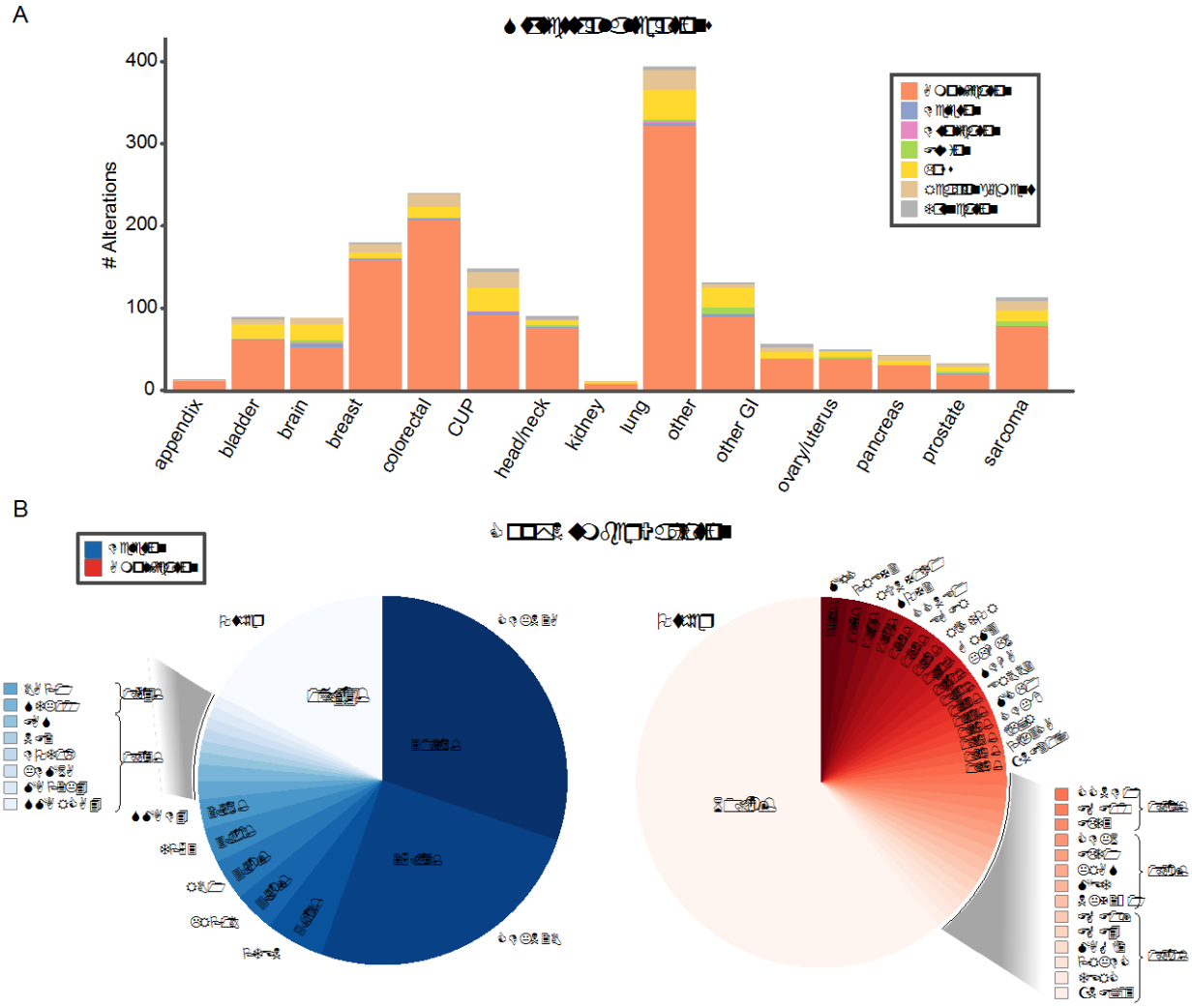


Figure S4. Mutation Loads in Different Cancer Groups.

Each point represents a sample and the Y-axis, shown in logarithmic scale, the total number of somatic alterations. Black horizontal lines indicate median of number of mutations for each cancer type. Within cancer type the data points have been spread over the column X-axis for better readability. Two outliers have been marked on top with their position not relative to Figure Y-axis scale.

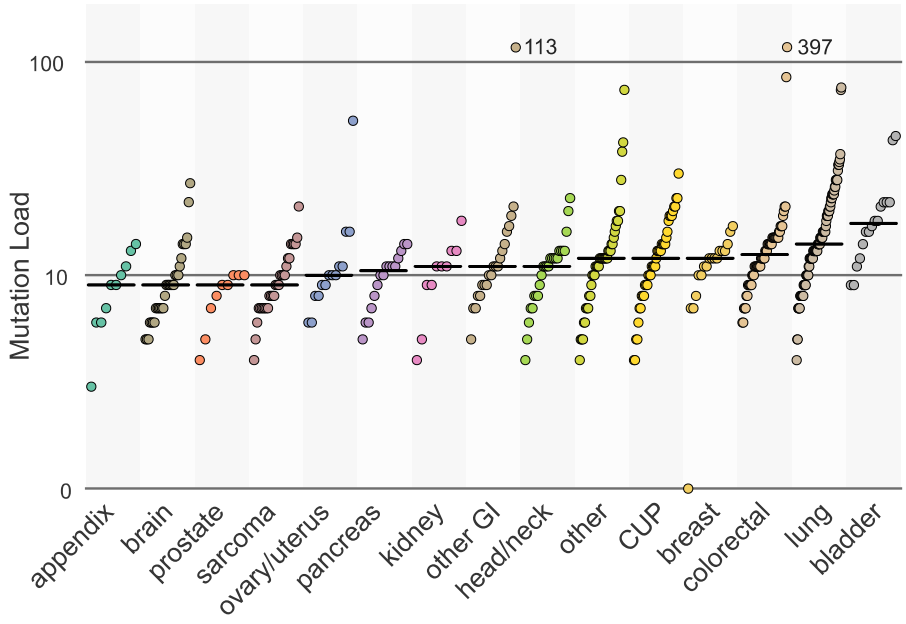


Figure S5. Clonality Analysis Based on Somatic Mutation Variant Allele Frequencies (VAFs).

(A) and (B) Two examples of high clonality patients who had undergone multiple sequential treatments. (C) All predicted clonalities larger than 2 were summarized into one category. Samples with few somatic mutations were not analyzed due to unreliability of the prediction in such cases and collected into low mutation count category.

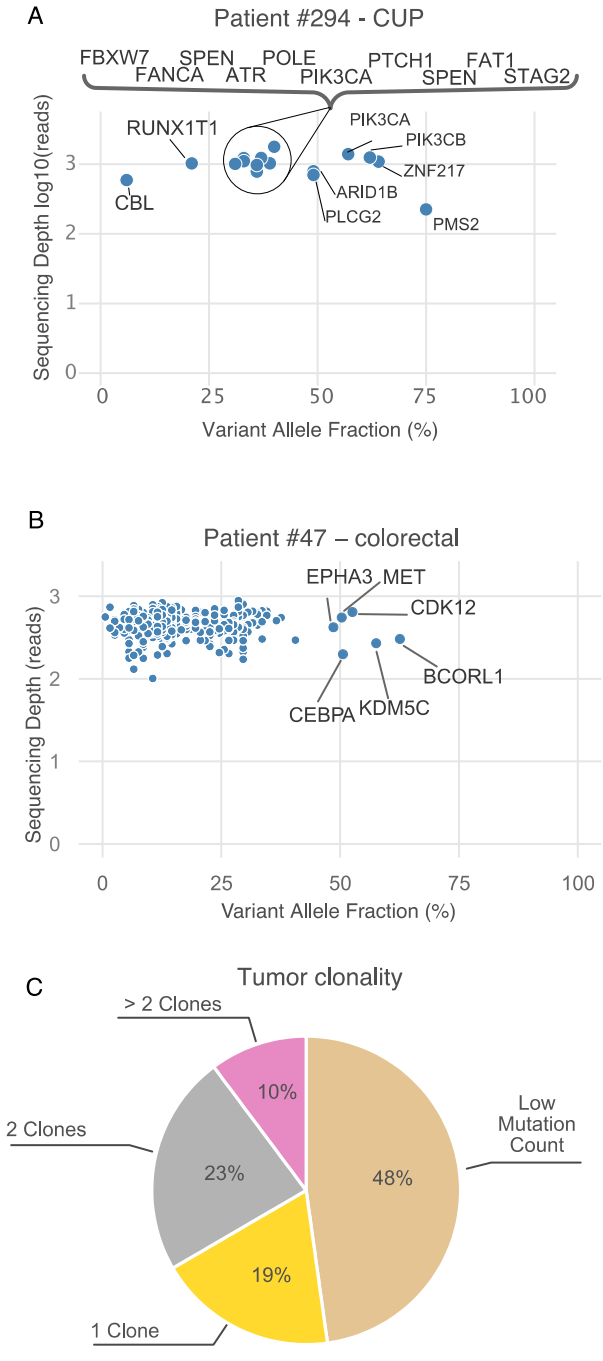
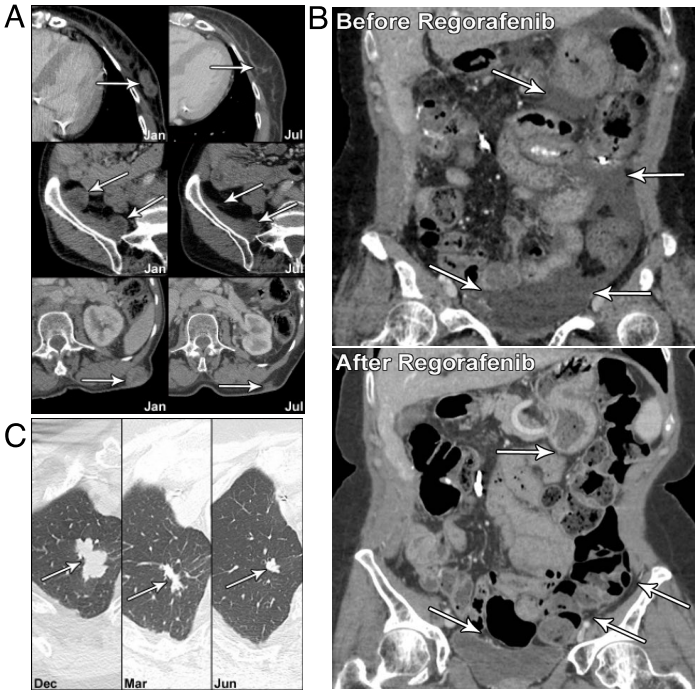


Figure S6. Response to Targeted Therapies Based on Genomics Profiling Results.

(A) Composite contrast enhanced CT axial images of chest wall (top row), retroperitoneal (middle row) and dorsal lumbar subcutaneous (bottom row) metastatic lesions from colorectal primary demonstrate near complete resolution with only minimally nodularity/scarring remaining after 6 months of treatment with Nivolumab. (B) Significant decrease in the abdominopelvic mucisnous fluid of RAF mutation carrier of metastatic appendiceal cancer after Regorafenib treatment. (C) Composite image of axial NECT in lung windows demonstrates progressive decrease in size of a lobulated right upper lobe mass (white arrow) over 6 months in a patient with metastatic high-grade urothelial carcinoma of the bladder with an FGFR3 activating mutation while on oral pazopanib.



Supplementary Tables

Table S1. Gender and Age Distribution of Patients by Cancer Groups

Disease Group	Gender: Female No (%)	Gender: Male No %	Age: < 31 No (%)	Age: 31-40 No (%)	Age: 41-50 No (%)	Age: 51-60 No (%)	Age: 61-70 No (%)	Age: >70 No (%)
Lung	39 (43.3)	51 (56.7)	0 (0.0)	0 (0.0)	8 (8.9)	21 (23.3)	32 (35.6)	29 (32.2)
Colorectal	22 (39.3)	34 (60.7)	1 (1.8)	2 (3.6)	15 (26.8)	7 (12.5)	18 (32.1)	13 (23.2)
Other	16 (37.2)	27 (62.8)	2 (4.7)	3 (7.0)	9 (20.9)	11 (25.6)	7 (16.3)	11 (25.6)
Cup	17 (41.5)	24 (58.5)	0 (0.0)	3 (7.3)	7 (17.1)	9 (22.0)	14 (34.1)	8 (19.5)
Brain	10 (32.3)	21 (67.7)	6 (19.4)	2 (6.5)	8 (25.8)	11 (35.5)	2 (6.5)	2 (6.5)
Sarcoma	11 (36.7)	19 (63.3)	2 (6.7)	3 (10.0)	1 (3.3)	10 (33.3)	6 (20.0)	8 (26.7)
Head/Neck	6 (23.1)	20 (76.9)	0 (0.0)	0 (0.0)	5 (19.2)	6 (23.1)	13 (50.0)	2 (7.7)
Other GI	8 (38.1)	13 (61.9)	1 (4.8)	3 (14.3)	3 (14.3)	4 (19.0)	6 (28.6)	4 (19.0)
Breast	18 (100.0)	0 (0.0)	0 (0.0)	1 (5.6)	6 (33.3)	2 (11.1)	9 (50.0)	0 (0.0)
Bladder	3 (18.8)	13 (81.3)	0 (0.0)	0 (0.0)	1 (6.3)	1 (6.3)	8 (50.0)	6 (37.5)
Pancreas	7 (43.8)	9 (56.3)	0 (0.0)	0 (0.0)	1 (6.3)	5 (31.3)	8 (50.0)	2 (12.5)
Ovary/Uterus	14 (100.0)	0 (0.0)	0 (0.0)	1 (7.1)	1 (7.1)	7 (50.0)	2 (14.3)	3 (21.4)
Appendix	3 (30.0)	7 (70.0)	0 (0.0)	1 (10.0)	2 (20.0)	2 (20.0)	2 (20.0)	3 (30.0)
Kidney	4 (40.0)	6 (60.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (20.0)	6 (60.0)	2 (20.0)
Prostate	0 (0.0)	9(100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (55.6)	4 (44.4)

Table S2. DNA Damage Repair (DDR) and Chromatin Remodeling Genes and their Association with Smoking.

DDR and chromatin remodeling genes measured by the targeted sequencing panel were collected from literature and tested for association between somatic alterations and smoking categories (current/recent, former, non-smoker). Cochran-Mantel-Haenzel test was used for the association and resulting p-values were multiple testing corrected within their category and summarized to this table.

DDR Gene	P-value
CDK12	0.0069
BRCA2	0.016
RAD50	0.072
BRIP1	0.075
BARD1	0.08
PMS2	0.2
STAG2	0.21
MSH6	0.26
BRCA1	0.27
FANCA	0.27
ATR	0.28
MLH1	0.54
ATM	0.55
PALB2	0.55
PTEN	0.78
FANCC	0.78
MSH2	0.78

Chromatin Remodeling Gene	P-value
KMT2D	0.0087
KDM6A	0.026
SMARCA4	0.032
EZH2	0.07
CHD2	0.075
EP300	0.1
KDM5A	0.1
NCOR2	0.1
ARID1A	0.11
ATRX	0.19
KMT2A	0.2
KDM5C	0.21
HDAC4	0.25
CHD4	0.29
KMT2C	0.32
CREBBP	0.38
DAXX	0.5
DNMT3A	0.52
MEN1	0.52
SETD2	0.53
SMARC6B1	0.53
HDAC7	0.56
KDM2B	0.56
TOP2A	0.75
TOP1	0.86
ARID2	0.92
ARID1B	0.93
KDM4C	0.94

Supplementary References:

1. Frampton GM, Fichtenholtz A, Otto GA, et al. Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. *Nature biotechnology.* 2013;31(11):1023-1031.
2. Gehring JS, Fischer B, Lawrence M, Huber W. SomaticSignatures: inferring mutational signatures from single-nucleotide variants. *Bioinformatics (Oxford, England).* 2015;31(22):3673-3675.
3. Lawler M, French D, Henderson R, Aggarwal A, Sullivan R. Shooting for the Moon or Flying Too Near the Sun? Crossing the Value Rubicon in Precision Cancer Care. *Public health genomics.* 2016;19(3):132-136.
4. Necchi A, Mariani L, Zaffaroni N, et al. Pazopanib in advanced and platinum-resistant urothelial cancer: an open-label, single group, phase 2 trial. *The Lancet Oncology.* 2012;13(8):810-816.