

Utility of Genomic Analysis In Circulating Tumor DNA from Patients with Carcinoma of Unknown Primary

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Abstract

Carcinoma of unknown primary (CUP) is a rare and difficult-to-treat malignancy, the management of which might be improved by the identification of actionable driver mutations. We interrogated 54 to 70 genes in 442 patients with CUP using targeted clinical-grade, next-generation sequencing of circulating tumor DNA (ctDNA). Overall, 80% of patients exhibited ctDNA alterations; 66% (290/442) ≥ 1 characterized alteration(s), excluding variants of unknown significance. *TP53*-associated genes were most commonly altered [37.8% (167/442)], followed by genes involved in the MAPK pathway [31.2% (138/442)], PI3K signaling [18.1% (80/442)], and the cell-cycle machinery [10.4% (46/442)]. Among

290 patients harboring characterized alterations, distinct genomic profiles were observed in 87.9% (255/290) of CUP cases, with 99.7% (289/290) exhibiting potentially targetable alterations. An illustrative patient with dynamic changes in ctDNA content during therapy and a responder given a checkpoint inhibitor-based regimen because of a mismatch repair gene anomaly are presented. Our results demonstrate that ctDNA evaluation is feasible in CUP and that most patients harbor a unique somatic profile with pharmacologically actionable alterations, justifying the inclusion of noninvasive liquid biopsies in next-generation clinical trials. *Cancer Res*; 77(16); 4238–46. ©2017 AACR.

Introduction

Carcinoma of unknown primary (CUP) is a rare cancer (defined as fewer than 15 cases per 100,000 per year; ref. 1), with an incidence of 7 to 12 cases per 100,000 per year (2). CUP is defined as metastatic disease with no identifiable primary tumor despite comprehensive evaluation including serum biomarker tests, procedures such as endoscopy and colonoscopy, as well as imaging and histopathologic examination with specific immunohistochemistry (2–5). Microarray-based technology to seek the primary site of origin based on gene-expression profile has also been investigated (6). Approximately 60% of CUPs are classified as well/moderately differentiated adenocarcinomas, followed by undifferentiated/poorly differentiated adenocarcinomas (30%), and, less frequently, as squamous cell carcinoma (5%) and neuroendocrine tumor (about 1%; refs. 2, 5).

Despite its heterogeneous clinicopathologic presentation, the treatment of CUP has primarily been with platinum-based com-

ination chemotherapies. Although such regimens do demonstrate response rates of 20% to 40%, median survival remains poor at 6 to 8 months (7, 8). Novel immunotherapy approaches using pembrolizumab (anti-PD-1 antibody) in patients with rare tumors including CUP are underway (NCT02721732). Targeted therapy regimens have been investigated in the past with the combination of bevacizumab plus erlotinib in nongenomically selected CUP. However, the response rate was 10%, and the median survival was 7.4 months (9).

Among refractory malignancies, a biomarker-based (personalized) approach matching patients with drugs has shown efficacy (10–14). Further understanding of the underlying genomic alterations among patients with CUP may also prove useful. Previous studies using archival tumor tissues of CUP patients found that *TP53* (38%–55%), *KRAS* (18%–20%), *CDKN2A* (19%), *MYC* (12%), *ARID1A* (11%), and *PIK3CA* (9%–14%) were frequently altered as assessed by targeted next-generation sequencing (NGS; refs. 15, 16). Although genomic sequencing is generally done on archival cancer samples, limitations with the use of tissues include intratumor genomic heterogeneity (17) as well as the dynamic mutational processes that can occur along with therapeutic intervention (18).

One approach to overcome these challenges is to investigate circulating tumor DNA (ctDNA). ctDNA is shed into the bloodstream from the cancer cells and can be isolated from a small tube of blood (also known as a "liquid biopsy"; ref. 19). This process was first reported in 1987 (20), and, more recently, the technology has rapidly advanced and has been applied in the clinic (21–24). Analyzing multiple genes by performing NGS on ctDNA is now feasible (Supplementary Table S1; ref. 25). Here, we provide the first report of a very large series of patients with CUP ($N = 442$) whose ctDNA derived from

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-17-0628

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blood was interrogated by clinical-grade NGS, and we document illustrative cases of the results of serial ctDNA testing and of matching patients to therapy.

Patients and Methods

Patients

We investigated the genomic alteration status of 442 patients with CUP. Samples were sent to a clinical laboratory improvement amendments–licensed and College of American Pathologist–accredited clinical laboratory (Guardant Health, Inc.; <http://www.guardanthealth.com/>) for ctDNA testing using NGS (September 2014 to March 2016). Tumor types were provided by the submitting physicians. The database was deidentified. This study was performed in accordance with UCSD Institutional Review Board guidelines for deidentified databases and for any investigational treatments for which patients gave consent. The UCSD Institutional Review Board follows the Declaration of Helsinki and the Belmont Report guidelines.

NGS

ctDNA was extracted from whole blood collected in 10 mL Streck tubes, and 5 to 30 ng of ctDNA was prepared for sequencing as previously described (25). All ctDNA was sequenced, including the somatic ctDNA and the germline ctDNA that is derived from natural leukocyte lysis.

All sequence-based mutations were evaluated for allele frequency. Allele frequencies were typically at approximately 100% (homozygous single-nucleotide polymorphism), approximately 50% (heterozygous germline), and <5% (somatic fraction; Supplementary Fig. S1). In addition to the allele frequency, the specific alteration was also evaluated using the Database of Short Genetic Variation (dbSNP) and COSMIC database to differentiate germline from somatic mutation (25). Germline alterations were filtered out and not reported. The fractional concentration or variant allele fraction for a given somatic mutation is calculated as the fraction of ctDNA harboring that mutation in a background of wild-type ctDNA fragments at the same nucleotide position (25). The analytic sensitivity reaches detection of one to two single-mutant fragments from a 10 mL blood sample (0.1% limit of detection), and analytic specificity is greater than 99.9999% (25). Throughout the timeframe of this study, the ctDNA assay expanded from 54 to 68 to 70 genes (Supplementary Table S1): 13 patients were tested with the 54-gene panel; 207 with the 68-gene panel; and 222 with the 70-gene panel. The assay reports single-nucleotide variants in all genes and select copy-number amplifications, fusions, and indel events (Supplementary Table S1). We analyzed only nonsynonymous alterations throughout the article, except in the case report with serial sampling wherein all alterations were followed serially.

Endpoints, statistical methods, and case studies

Demographic information such as age and gender was extracted from the deidentified database. Descriptive statistics were used to summarize the genomic alterations identified in this study. Two case studies (from outside time range of deidentified database) are presented: (i) a patient with five serial ctDNA samples and (ii) a patient who was successfully matched to an immunotherapy/targeted treatment combination [consent obtained according to the University of California San Diego

Internal Review Board guidelines; protocol: I-PREDICT study (NCT02534675)].

Results

Genomic alterations among CUP

Among all patients with CUP ($N = 442$), the median age was 65 years (range, 20–94); 231 cases were women (52.3%; Table 1; Figs. 1 and 2; Supplementary Table S2; and Supplementary Fig. S2). The total number of alterations found among 442 patients were 1,368, and of these, 768 (56.1%) were characterized alterations including substitutions ($N = 507$, 37.1%), amplifications ($N = 257$, 18.8%), fusions ($N = 3$, 0.22%), and indels ($N = 1$, 0.07%). Six hundred (43.9%) alterations were variants of unknown significance. Eighty percent of patients (353/442) had ctDNA alterations detected, with 66% (290/442) having at least one characterized alteration and 43.9% (194/442) harboring ≥ 2 characterized alterations. Among all patients ($N = 442$), the median number of alterations per patient was 2 (range, 0–20) and the median number of characterized alterations was 1 (range, 0–10). Among patients found to have alterations ($N = 353$), the median number of alterations was 3 (range, 1–20) and the median number of characterized alterations was 2 (range, 1–10; Table 1). Focusing on characterized alterations, the most commonly altered gene was *TP53* (37.1%, 164/442) followed by *KRAS* (18.6%, 82/442), *PIK3CA* (15.4%, 68/442), *BRAF* (7.5%, 33/442), and *MYC* (7.5%, 33/442; Figs. 1 and 2; Supplementary Fig. S2).

Number of alterations with possible cognate-targeted therapies

Of the 768 characterized alterations, 89.6% (688/768) were potentially targetable with FDA-approved agents as off-label use, and an additional 8.5% (65/768) were theoretically

Table 1. Clinical characteristics and number of genomic alterations in 442 patients with CUP

Basic characteristics ($N = 442$)	
Age, median (range), year	65 (20–94)
Female, N (%)	231 (52.3%)
Male, N (%)	211 (47.7%)
Genomic alterations in 442 cases of CUP	
Number of alterations ^a	1368
Mean number of alterations per patient (range) (includes characterized alterations and VUS)	3.1 (0–20)
Median number of alterations per patient (range) (includes characterized alterations and VUS)	2 (0–20)
Number of characterized alterations	768 (56.1%)
Mean number of characterized alterations per patient (range)	1.7 (0–10)
Median number of characterized alterations per patient (range)	1 (0–10)
Substitution, N (%)	507 (37.1%)
Amplification, N (%)	257 (18.8%)
Fusion, N (%)	3 (0.22%)
Indel, N (%)	1 (0.07%)
Variant of unknown significance, N (%)	600 (43.9%)
Number of patients with ≥ 1 alteration (%) ^a	353 (79.9%)
Number of patients with ≥ 1 characterized alteration (%)	290 (65.6%)
Number of patients with ≥ 2 characterized alterations (%)	194 (43.9%)

^aIncludes both characterized alterations and variants of unknown significance (VUS).

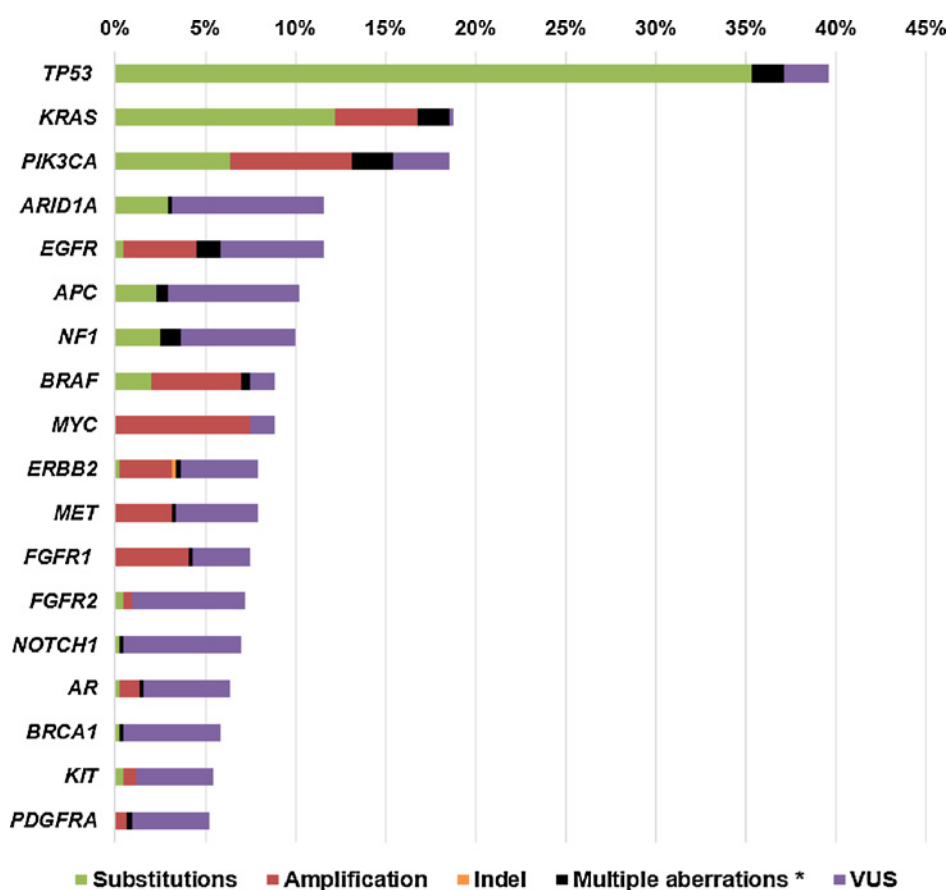


Figure 1.

Frequency of genomic alterations among patients with CUP ($N = 442$). It includes alterations with >5% frequency. Please see Supplementary Table S1 for complete list of alterations found in this study. *, multiple alterations indicate that the patient had >1 type of alteration in the same gene (substitutions, amplification, indel, VUS, etc.). VUS, variants of unknown significance.

targetable with therapies that are currently in clinical trials (Tables 1 and 2; Supplementary Tables S3 and S4; and Supplementary Figs. S3 and S4). Tyrosine kinase families were altered in 17.9% (79/442); the MAPK signaling pathway was altered in 31.2% (138/442); the PI3K pathway was altered in 18.1% (80/442); cell-cycle associated genes were altered in 10.4% (46/442); and alterations in *TP53*-associated genes were seen in 37.8% (167/442) of patients with CUP (Table 2). Among the 768 characterized alterations, 335 were molecularly distinct alterations (e.g., *KRAS* and *PIK3CA* alterations were considered genomically distinct; *KRAS* G12C and *KRAS* G12V were considered genomically identical but molecularly distinct). Although infrequent, classically targetable alterations were observed in this CUP cohort, including nine occurrences of *BRAF* V600E, four occurrences of *EGFR* L858R, three *KIF5B-RET* fusions, and one *ERBB2* exon 20 insertion. Altogether, 98.0% (753/768) were theoretically actionable either with agents that are approved by the FDA (albeit off-label) or with agents that are in clinical trials (Supplementary Tables S3 and S4).

Among all 442 patients with CUP, 63.8% (282/442) had theoretically actionable alterations by an FDA-approved agent, and an additional 1.6% (7/442) patients had alterations targetable with investigational agents in clinical trial. The mean number of actionable alterations per patient was 1.7 (range, 0–10). Altogether, 65.4% (289/442) of patients had theoretically actionable alterations either with FDA-approved or with investigational agents (Supplementary Tables S3 and S4; Supplementary Figs. S3 and S4). The majority of the patients (34.4%) who were deemed

to have no targetable alterations had no characterized ctDNA alterations detected.

Distinctness of genomic alterations among 442 patients with CUP

Among the 290 patients with at least one characterized genomic alteration, 12.1% (35/290) had identical molecular portfolios [*KRAS* G12C as a single characterized alteration (ID# 52, 94, 214, 335, 280, 404); *KRAS* G12V (ID# 54, 271, 277); *KRAS* G12D (ID# 206, 416); *KRAS* G13C (ID# 81, 225); *TP53* R110P (ID# 30, 248); *TP53* V173L (ID# 318, 386), *TP53* R175H (ID# 116, 317, 418); *TP53* C176Y (ID# 275, 305); *TP53* C275Y (ID# 98, 166); *TP53* Y220C (ID# 22, 32, 385); *CCNE1* amplification (ID# 395, 127); *GNAS* R201H (ID# 46, 105, 178); and *IDH1* R132C (ID# 118, 120, 336); Supplementary Table S4]. However, among the 194 patients who had more than one characterized alteration, no 2 patients had identical molecular profiles (Supplementary Table S4).

Comparative quantitation of ctDNA in the circulation

The current study calculated the fractional concentration of mutant allele by comparing the concentration of wild-type ctDNA fragments at the same nucleotide position (Supplementary Fig. S5). Among frequently altered genes (Fig. 1; Supplementary Table S1), all characterized mutations had a median ctDNA fraction of less than 5%. There was no clear association between the frequencies of gene alterations and the fraction of ctDNA detected in the blood (Supplementary Fig. S5).

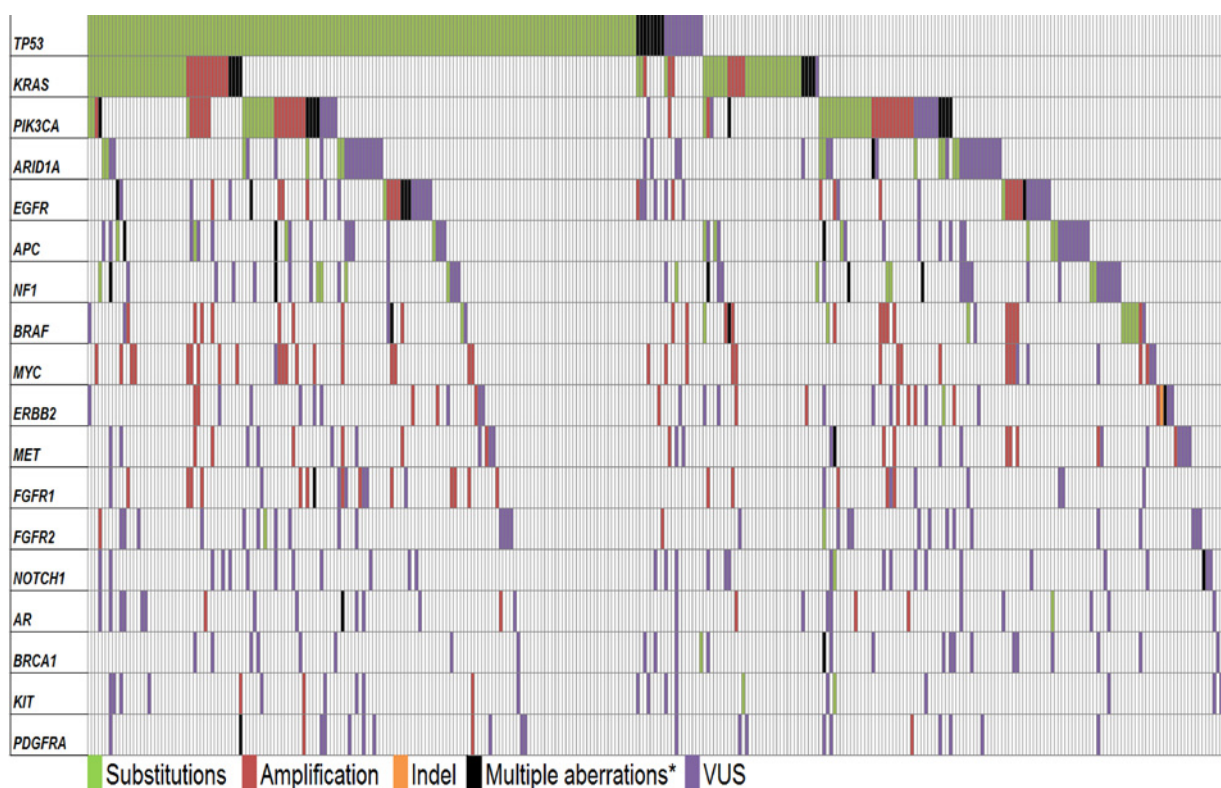


Figure 2.

Oncoprint of frequently altered genes in patients with CUP. Of the 442 cases studied, 325 patients had at least one alteration with >5% frequency, which were included in the figure. Each vertical line represents one patient, and the horizontal lines indicate specific genes. Please see Supplementary Fig. S2 for the complete oncoprint for 442 cases evaluated in this report. *, multiple alterations indicate that the patient had >1 type of alteration in the same gene (substitutions, amplification, indel, VUS, etc.). VUS, variants of unknown significance.

Clinical observation of dynamic change in ctDNA along with therapeutic intervention

To demonstrate the dynamic change seen in ctDNA that accompanies therapeutic intervention, we report a case of 60-year-old woman with squamous CUP who had five ctDNA analyses during the disease course (one ctDNA test before initiation of therapy and four additional analyses during the therapy; Fig. 3A). The tumor initially demonstrated *MYC* N402N and *JAK2* E621K alterations. Following treatment initiation with cisplatin and gemcitabine, tumor reduction was observed, and the *MYC* and *JAK2* alterations diminished/disappeared from circulation at the second timepoint (Fig. 3B). However, multiple new alterations emerged including *APC*, *NF1*, *KIT*, *AR*, and *STK11* anomalies. Pazopanib was added to the chemotherapy (based on the molecular profiling from archival tissue that revealed *FGFR2-DDX21* fusion), and cisplatin was held due to toxicity. Interestingly, after holding cisplatin, the originally observed *MYC* alteration re-emerged (Fig. 3).

Patient with CUP managed with matched combination of immunotherapy and targeted therapy based on ctDNA

An 82 year-old-man with adenocarcinoma of unknown primary with liver and abdominal lymph node metastases harbored *KRAS* G12D and *MLH1* R389W mutations detected by ctDNA (and also by tissue NGS; Fig. 4). Matched therapy with a combination of trametinib (a MEK inhibitor targeting downstream of

KRAS) and nivolumab (checkpoint inhibitor associated with activity in colorectal cancer with mismatch repair gene anomalies such as *MLH1*) was initiated (Protocol: I-PREDICT study, NCT02534675). The patient achieved a partial response (PR; 36.4% reduction per RECIST 1.1; Fig. 4A) at 8 weeks, along with rapid decline in the carbohydrate antigen 19-9 tumor marker (Fig. 4B). Treatment is ongoing.

Discussion

CUP is a rare malignancy with an incidence of 7 to 12 cases per 100,000 individuals per year (2). In order to establish the primary site, there is often extensive evaluation, including laboratory, imaging, gene expression profiling, and immunohistochemical testing. Most recently, an algorithm that quantifies the similarity between RNA expression patterns of specimens and tissues on a test panel has shown high reliability in identifying tissue of origin (6). Even so, a single putative primary origin may only be assigned in about 25% of cases of CUP (5). Although empiric platinum-based regimens are generally used in CUP patients with modest response (7, 8), overall median survival is poor at 6 to 8 months, and a meta-analysis comparing different chemotherapy regimens demonstrated no statistical difference in overall survival for any treatment group over others (26). Thus, there is an unmet need for novel treatment approaches for patients with CUP.

Table 2. Selected actionable genomic alterations and examples of possible targeted therapies in patients with CUP (*N* = 442)

Genomic alteration	<i>N</i> (%)	Example of possible targeted therapies ^a
Tyrosine kinase families (<i>N</i> = 79, 17.9%)		
<i>EGFR</i> substitutions/amplification	26 (5.9%)	Afatinib, cetuximab, erlotinib
<i>ERBB2</i> substitutions/amplification/indel	16 (3.6%)	Afatinib, trastuzumab, lapatinib
<i>FGFR1</i> amplification	19 (4.3%)	Lenvatinib
<i>FGFR2</i> substitutions/amplification	4 (0.9%)	
<i>FGFR3</i> substitutions	3 (0.7%)	
<i>JAK2</i> substitutions	1 (0.2%)	Ruxolitinib
<i>KIT</i> substitutions/amplification	5 (1.1%)	Dasatinib, imatinib, sunitinib
<i>MET</i> amplification	15 (3.4%)	Cabozantinib, crizotinib
<i>PDGFRA</i> amplification	4 (0.9%)	Dasatinib, imatinib, sunitinib
<i>RET</i> fusion	3 (0.7%)	Cabozantinib, lenvatinib, vandetanib
MAPK signaling (<i>N</i> = 138, 31.2%)		
<i>HRAS</i> substitution	3 (0.7%)	MEK inhibitor (e.g., trametinib or cobimetinib)
<i>KRAS</i> substitution/amplification	82 (18.6%)	
<i>NRAS</i> substitution	8 (1.8%)	
<i>NF1</i> substitution	16 (3.6%)	
<i>GNAS</i> substitution	10 (2.3%)	
<i>RAF1</i> substitution/amplification	8 (1.8%)	
<i>MAP2K1</i> substitution	2 (0.5%)	
<i>BRAF</i> substitution/amplification	33 (7.5%)	BRAF inhibitor (e.g., dabrafenib, vemurafenib), MEK inhibitor (e.g., trametinib or cobimetinib)
PI3K signaling (<i>N</i> = 80, 18.1%)		
<i>PIK3CA</i> substitution/amplification	68 (15.4%)	mTOR inhibitor (e.g., everolimus, temsirolimus)
<i>PTEN</i> substitution	10 (2.3%)	
<i>AKT1</i> substitution	2 (0.5%)	
<i>STK11</i> substitution	4 (0.9%)	
<i>TSC1</i> substitution	1 (0.2%)	
Cell-cycle-associated genes (<i>N</i> = 46, 10.4%)		
<i>CDKN2A</i> substitution	8 (1.8%)	Cyclin-dependent kinase inhibitor (e.g., Palbociclib)
<i>CCND1</i> substitution/amplification	3 (0.7%)	
<i>CCND2</i> substitution/amplification	2 (0.5%)	
<i>CDK4</i> amplification	5 (1.1%)	
<i>CDK6</i> amplification	18 (4.1%)	
<i>CCNE1</i> amplification	16 (3.6%)	Proteasome inhibitor (e.g., bortezomib)
<i>TP53</i> -associated genes (<i>N</i> = 167, 37.8%)		
<i>TP53</i> substitution	164 (37.1%)	Anti-VEGF (e.g., bevacizumab), WEE1 inhibitor (e.g., AZ1775, NCT01748825)
<i>ATM</i> substitution	4 (0.9%)	PARP inhibitor (e.g., olaparib)
Mismatch repair gene alterations (<i>N</i> = 7, 1.6%)		
<i>MLH1</i> gene ^b	7 (1.6%)	Immunotherapy with checkpoint inhibitors (50)

^aSee Supplementary Table S3 for the rationale for possible targeted therapies.

^b*MLH1* was the only mismatch repair gene tested in the ctDNA assay used.

Accumulating evidence suggests that, among patients with refractory cancers, a biomarker-based, personalized approach that matches patients with therapies based on specific genomic or protein markers may be able to improve clinical outcome (10–14). Herein, we report the genomic alterations identified in 442 patients with CUP using targeted NGS that evaluated ctDNA from patient plasma. Overall, 79.9% of CUP patients (353/442) had ctDNA alterations detected, and 65.6% (290/442) harbored at least one characterized genomic alteration (Table 1). The most frequent alterations were in the *TP53* gene; these alterations were detected in 37.1% of cases, followed by aberrations in *KRAS* (18.6%) and *PIK3CA* (15.4%). These results are consistent with previous reports of approximate frequencies of gene alterations in tissue samples from CUP patients (Supplementary Fig. S6; refs. 15, 16).

There are several advantages to evaluating ctDNA from patient plasma as compared with tissue. First, interrogating ctDNA requires only a small blood sample. ctDNA sampling is therefore safe, easy, and inexpensive when compared with more invasive tissue biopsies. Further, some cancers may involve a difficult-to-biopsy location that limits the availability of tissue samples for

molecular profiling (19). Second, because ctDNAs are shed into the bloodstream from multiple sites of metastases, theoretically, ctDNA may attenuate the issues related to assessing tumor heterogeneity (17). This is especially important for patients with CUP, because these individuals often harbor numerous metastases. Third, serial liquid biopsies to identify emergence of resistance mutations that can occur along with therapeutic intervention are feasible (27). Indeed, we have described a patient with CUP who underwent ctDNA analysis at five different time points. This patient demonstrated dynamic changes in ctDNA levels while receiving various therapies (Fig. 3). Further understanding of the correlation between genomic alterations and treatment outcomes may help pinpoint response and resistant mechanisms.

Among 290 patients whose tumors carried characterized alterations, most individuals (97.2%, 282/290) had at least one potentially actionable alteration that could be affected by an FDA-approved agent (albeit off-label); an additional 7 patients had targetable alterations with agents that are available through clinical trials. Altogether, among patients with characterized genomic alterations, 99.7% (289/290) had

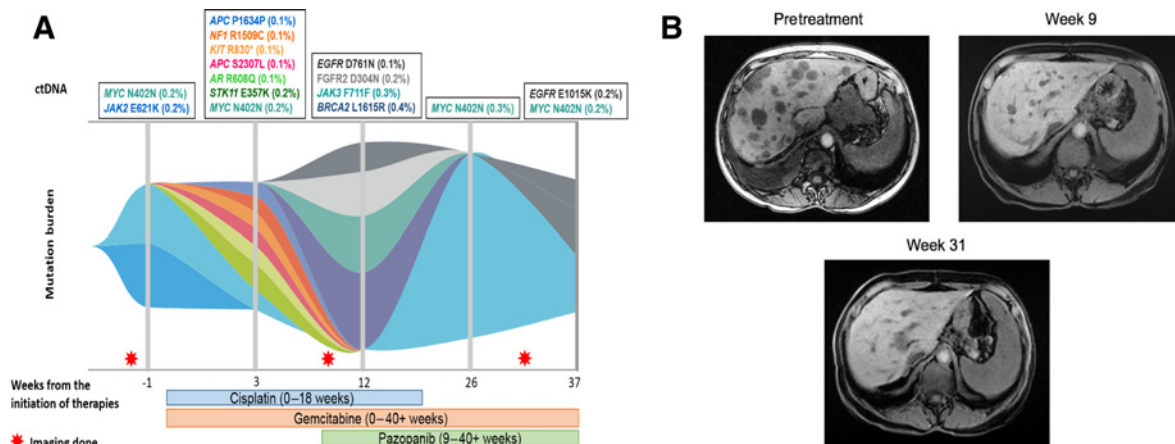


Figure 3.

Case report of patient with CUP illustrating dynamic changes in ctDNA that accompanied therapeutic intervention. **A**, Dynamic change of ctDNA along with therapeutic intervention. **B**, Serial imaging with MRI of the abdomen pre- and posttherapeutic intervention. A 60-year-old woman presented with abdominal bloating. Imaging showed extensive thoracic and retroperitoneal adenopathy and liver metastases. Because the patient had a history of cholangiocarcinoma that was surgically managed 8 years prior to the presentation, recurrence of cholangiocarcinoma was suspected. However, biopsy of the liver mass revealed poorly differentiated squamous cell carcinoma that was not consistent with recurrent cholangiocarcinoma. Diagnosis was determined to be squamous cell CUP. Molecular profiling from archival tissue revealed *FGFR2-DDX21* fusion, *NF2 E270D*, *CDKN2A/B* loss, and *PBRM1 Q478** alterations, and ctDNA at the time of diagnosis revealed *MYC N402N* and *JAK2 E621K* alterations (*FGFR2* fusions were not in the panel when the ctDNA analysis was performed; **A**). The patient was started on chemotherapy with cisplatin and gemcitabine and achieved an excellent response (**B**). During the course of therapy, pazopanib was added for the *FGFR2-DDX21* fusion, and cisplatin was held after 18 weeks due to toxicity. The patient continued on gemcitabine and pazopanib and has attained an ongoing PR at 40+ weeks (**B**). During treatment, the patient underwent ctDNA analysis at five different time points, including the one prior to the initiation of therapy (**A**). The patient initially had *MYC N402N* and *JAK2 E621K* alterations (week -1). After starting cisplatin and gemcitabine, the *MYC* and *JAK2* alterations disappeared, but multiple new alterations started to emerge at week 3, including those in the *APC*, *NFI*, *KIT*, *AR*, and *STK11* genes. However, after adding pazopanib, all the previously detected alterations disappeared; even so, new alterations in the *EGFR*, *FGFR2*, *JAK3*, and *BRCA2* genes emerged (week 12). Interestingly, after holding the cisplatin, the *MYC N402N* alteration, which was originally seen in pretreatment ctDNA and disappeared while on cisplatin, reappeared (**A**). Among the ctDNA alterations seen in this patient, the following were characterized: *AR R608Q*, *KIT R830**, *EGFR D761N*, and *EGFR E1015K*. The following alterations were variants of unknown significance: *JAK2 E621K*, *STK11 E357K*, *APC S2307L*, *NFI R1509C*, *BRCA2 L1615R*, and *FGFR2 D304N*. The following alterations were synonymous substitution: *MYC N402N* and *APC P1634P*. This illustrates mutation % based on maximum ctDNA% detected at each time point. Therefore, if two alterations were each detected at 0.2%, the percentage of total ctDNA detected is 0.2%.

anomalies that might be pharmacologically tractable with either FDA-approved or investigational agents (Fig. 3; Supplementary Tables S3 and S4; and Supplementary Fig. S4).

Interestingly, among these 290 patients, only 12.1% (35/290) had identical molecular portfolios. Among patients harboring more than one genomic alteration, no 2 patients had an identical molecular portfolio (Supplementary Table S4). Malignant "snowflakes" in advanced cancer are commonly reported in other cancers as well (28, 29). Therefore, individualized cotargeting of multiple genomic alterations may be necessary to improve clinical outcome. A prospective observation trial with matched targeted therapy based on NGS results from archival tissue is ongoing for patients with CUP (NCT02628379).

There were several specific pathway alterations that were potentially attractive for matched targeted therapy (Table 2). *TP53*-associated genes (*TP53* and *ATM*) were the most commonly altered pathway in patients with CUP (37.8%, 167/442). Although there is no agent that directly targets *TP53* alterations, mutations in this tumor suppressor are associated with high VEGF-A levels (30). Consistent with this association, clinical data suggest that patients with *TP53* alterations had longer progression-free survival (PFS; median, 11.0 vs. 4.0 months; $P < 0.0001$; ref. 31) and improved clinical outcome (rate of stable disease 6 months/PR/complete response; 31% vs. 7%) with anti-VEGF-

containing regimens (32). However, the same effect was not seen among patients with *TP53* wild-type malignancies. In addition, another recent study documents that patients with sarcomas who respond to the VEGFR inhibitor pazopanib harbored *TP53* mutations (33). *TP53* is also potentially targetable with the *WEE1* inhibitor currently in clinical development (AZ1775, NCT01748825; ref. 34).

Tyrosine kinase family members were altered in 17.9% (79/442) of CUP cases, and, in this pathway, *EGFR* abnormalities were the most common (5.9% of cases, 26/442; Table 2). Cancers with *EGFR* alterations, especially in patients with non-small cell lung cancer, respond well to *EGFR* inhibitors including afatinib (35) or erlotinib (36). Although previous studies that investigated erlotinib-containing regimens in patients with nongenomically selected CUP only had modest response rates (~10%), further investigations that are focused on CUP with *EGFR* alterations are required. The *ERBB2* gene was altered in 3.6% of our patients (16/442; Table 2), which is consistent with previous reports (Supplementary Fig. S6; refs. 15, 16). To our knowledge, there is no report evaluating CUP patients with anti-HER2 therapies. Because *ERBB2* alterations are targetable with therapies such as afatinib, lapatinib, and trastuzumab (37–39), a clinical trial is warranted.

Alterations in MAPK signaling were also commonly seen (31.2%, 138/442; Table 2 and Fig. 1). *KRAS* was the most

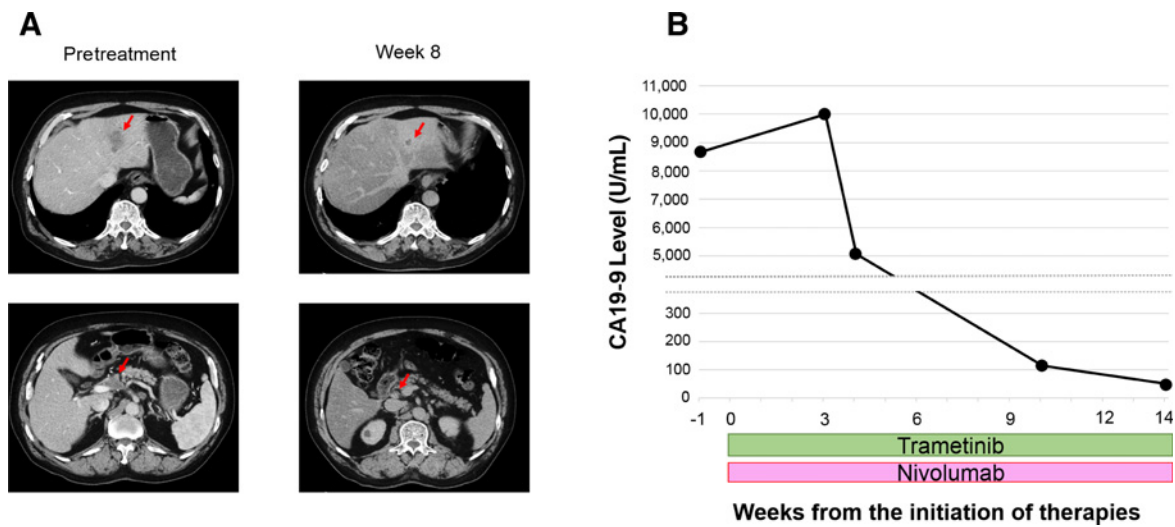


Figure 4.

Case report of patient with CUP harboring *MLH1* and *KRAS* mutations managed with matched targeted therapy approach. **A**, Serial imaging with CT of the abdomen pre-and posttherapeutic intervention. **B**, Change in serum tumor marker along with targeted therapeutics. A 82-year-old-man presented with 3-month history of worsening right upper quadrant abdominal pain. Imaging showed liver mass along with enlarged abdominal lymph nodes. Tumor marker was positive for CA19-9 (8,667 U/mL; reference range, 30–42 U/mL) but negative for carcinoembryonic antigen (3.9 ng/mL; reference range, <5.5 ng/mL) and α -fetoprotein (3 ng/mL; reference range, 0–15 ng/mL). Biopsy of liver mass was consistent with adenocarcinoma. Further analysis with immunohistochemistry was negative for CK7 and CK20, but positive for CK19 and CDX-2. Work up including upper gastrointestinal endoscopy, endoscopic ultrasound of pancreas, and colonoscopy was unremarkable. The patient was diagnosed as adenocarcinoma of unknown primary. Molecular profiling from archival tissue revealed *KRAS* G12D, *MLH1* splice site 1989+1G>T, and *TP53* R248Q mutations, and ctDNA at the time of diagnosis also revealed *KRAS* G12D, *MLH1* R389W, *TP53* R248Q, as well as *CCND2* C46*. Based on the mutation in mismatch repair gene *MLH1* and *KRAS* G12D mutations, patient was initiated on nivolumab (for *MLH1*) and trametinib (for *KRAS*). Four weeks after the initiation of nivolumab and trametinib, patient reported less abdominal pain and stopped his pain medication. Eight weeks after starting treatment, patient achieved PR (36.4% reduction per RECIST 1.1) by CAT scan imaging (**A**). Tumor marker CA19-9 fell markedly (**B**). Treatment is ongoing.

commonly affected gene (18.6% of cases, 82/442; Table 2 and Supplementary Table S2). Due to limited therapeutic options, RAS-driven cancers are considered to be among the most difficult to treat (40); however, they are potentially targetable with MEK inhibitors (e.g., trametinib and cobimetinib; refs. 41, 42). Although less frequent, *BRAF* V600E mutations were found in 1.6% (7/442) of our CUP patients (Supplementary Table S3). Recent reports suggest that many cancer types, including CUP, with *BRAF* V600 mutations are targetable with vemurafenib (*BRAF* inhibitor; refs. 43, 44). *BRAF* alterations are also targetable with trametinib (MEK inhibitor; ref. 42) or with dual suppression by both *BRAF* and MEK inhibitors (e.g., dabrafenib plus trametinib; ref. 45).

PI3K signaling pathway anomalies involving *PIK3CA*, *PTEN*, *AKT1*, *STK11*, and *TSC1* genes were seen in 18.1% (80/442) of our patients (Table 2). Biomarker analysis of two phase III trials (BOLERO-1 and BOLERO-3) that randomized Her2-positive breast cancer patients to receiving a chemotherapy with and without everolimus revealed that participants with an altered PI3K pathway had a statistically improved PFS with an everolimus-containing regimen [HR, 0.67; 95% confidence interval (CI), 0.48–0.93], whereas there was no benefit from everolimus among patients with wild-type PI3K pathway (HR, 1.19; 95% CI, 0.87–1.62; ref. 46). Janku and colleagues have also reported that, among diverse malignancies, *PIK3CA* or *PTEN* alterations were the independent factors predicting response to inhibitors targeting PI3K pathway, including mTor inhibitors, albeit when used in combination therapy rather than as single agents (47).

In the current report, cell-cycle-associated genes were altered in 10.4% of patients (46/442; Table 2). This rate is lower than that in a previous publication that showed 41.6% (112/269) of patients with CUP had alterations in cell-cycle-associated genes when archival tumor tissue was interrogated by NGS (48). The difference in frequencies among the studies is possibly due to the different techniques used [e.g., in the previous publication, *CDKN2A/B* loss was reported in 18% of cases of CUP (48); however, loss of this gene was not assessed in the current study]. In terms of therapeutic approach, although there are conflicting data, *CDKN2A*, *CCND1/2* alterations, and *CDK4/6* amplification may potentially be targetable with a *CDK4/6* inhibitor such as palbociclib (49).

Rare alterations may also be important. For instance, *MLH1* (mismatch repair gene) abnormalities were found in only 1.6% (7/442; Supplementary Table S2) of our CUP patients. However, these alterations have been shown to correlate with high tumor mutational burden and response to checkpoint inhibitors in diseases such as colorectal cancer (50). Indeed, our elderly patient with CUP and liver metastases had a rapid response after therapy with a checkpoint inhibitor nivolumab-based regimen (Fig. 4).

There are several limitations to the current report. Since the database was deidentified, we were not able to evaluate clinical characteristics such as those related to outcome. Moreover, the cancer diagnosis was reported by the referring physician. Despite these limitations, the current report provides the largest dataset of clinical-grade NGS in ctDNA among patients with CUP.

In conclusion, we have evaluated the genomic landscape of ctDNA in 442 patients with CUP. *TP53*-associated genes were most commonly altered followed by abnormalities in the MAPK pathway, PI3K signaling, and cell-cycle-associated genes. Overall, 80% of CUP patients (353/442) had detectable ctDNA alterations and 66% (290/442) had at least one characterized alteration. Serial ctDNA sampling in a patient revealed significant evolution with therapy (Fig. 3). Among the patients in this report, 99.7% (289/290) had an alteration hypothetically targetable with either an FDA-approved or investigational agents. Therefore, patients could potentially be matched to genomically targeted therapy or to immunotherapy or both, as demonstrated by our responding patient who received both a MEK and a checkpoint inhibitor because of the presence of a *KRAS* and a mismatch repair gene alteration (Fig. 4). Previous literature suggests that biomarker-based, matched targeted therapy can improve clinical outcome (10–14). Our current report indicates that the noninvasive liquid biopsy approach merits investigation in next generation clinical trials of CUP.

Disclosure of Potential Conflicts of Interest

K.C. Banks has an ownership interest (including patents) in Guardant Health. B. Leyland-Jones has received honoraria from the speakers bureau of Genentech and is a consultant/advisory board member for Amgen and Genentech. R.B. Lanman is Chief Medical Officer at, and has an ownership interest (including patents) in, Guardant Health, Inc. R. Kurzrock has an ownership interest in CureMatch, Inc., reports receiving commercial research

grant from Foundation Medicine, Genentech, Guardant, Merck, Pfizer, Sequenom, and Serono, and is a consultant/advisory board member for Actuate Therapeutics and Xbiotech. No potential conflicts of interest were disclosed by the other authors.

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Other (targeted drug matching): S. Kato, P. De, B. Leyland-Jones, R. Kurzrock

Acknowledgments

This study was funded in part by the Joan and Irwin Jacobs fund and by the NCI grant P30 CA016672 (R. Kurzrock).

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Received March 7, 2017; revised April 19, 2017; accepted June 9, 2017; published OnlineFirst June 22, 2017.

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