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Authors

Kato, Shumei
Alsafar, Ahmed
Walavalkar, Vighnesh
et al.

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Cancer of Unknown Primary in the Molecular Era

Shumei Kato[1,*], **Ahmed Alsafar**[1], **Vighnesh Walavalkar**[2], **John Hainsworth**[3,4], **Razelle Kurzrock**[1]

[1]Center for Personalized Cancer Therapy and Division of Hematology and Oncology, Department of Medicine, UC San Diego Moores Cancer Center, La Jolla, CA, USA

[2]Department of Pathology, University of California San Francisco, San Francisco, CA, USA

[3]Tennessee Oncology, PLLC, Nashville, TN, USA

[4]Sarah Cannon Research Institute, Nashville, TN, USA

Abstract

Cancer of unknown primary (CUP) is a rare malignancy that presents with metastatic disease and no identifiable site of origin. Most patients have unfavorable features and attempts to treat based on tissue-of-origin identification have not yielded a survival advantage compared with empiric chemotherapy. Next-generation sequencing has revealed genomic alterations that can be targeted in selected cases, suggesting that CUP represents a unique malignancy in which the genomic aberrations may be integral to the diagnosis. Recent trials focusing on tailored combination therapy matched to the genomic alterations in each cancer are providing new avenues of clinical investigation. Here, we discuss recent findings on molecular aberrations in CUP and how the genomic and immune landscape can be leveraged to optimize therapy.

Keywords

cancer of unknown primary; genomic; targeted therapy

Introduction

Cancer of unknown primary (CUP), by definition, is a metastatic syndrome with an unidentifiable primary tumor, even after extensive workup to seek the primary site. CUP constitutes 3–5% of all cancer diagnoses worldwide, with a median age at diagnosis of 65

*Correspondence: smkato@health.ucsd.edu (S. Kato).

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[iiii]https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000002034

years, and it is slightly more common in men [1,2]. Patients with CUP typically receive empiric chemotherapies such as taxanes and platinum-containing regimens [3]. In a recent study (n = 51), overall response rate (ORR) was 41.2%, with median progression-free survival (PFS) of 4.8 months for such combinations [4], but with a poor median overall survival (OS), ranging from 6 to 15 months [4–6].

Initial workup for CUP, as recommended in 2019 by the National Comprehensive Cancer Network (NCCN) guidelines[i], consists of a complete history and physical exam, basic laboratory tests, computerized tomographic scans, clinically directed endoscopy, and microsatellite instability (MSI)/mismatch repair gene testing. Breast imaging to investigate breast cancer as a primary site in women (since this cancer is both common and treatable even in the advanced setting) and serum tumor markers are also recommended in selected patients to seek the primary site of the tumor. Following a biopsy, a targeted panel of immunohistochemistry (IHC) tests is recommended. Tables 1 and 2 show examples of IHC stains that may be used along with other pathological and clinical findings. Tissue-of-origin testing, often by microarray-based gene expression tests, as well as next-generation sequencing (NGS), have also been exploited to determine a diagnosis for CUP patients.

CUP tumors can be categorized into favorable and unfavorable subsets. The prognostically favorable cases (20% of all CUP) [2] have histopathology, biomarkers, and clinical presentation consistent with specific tissues of origin and may respond to standard site-specific treatments, similar to primary tumors of the same site. Favorable presentations include axillary lymph node adenocarcinoma consistent with a breast primary, features of head and neck squamous cell carcinoma, papillary or serous peritoneal cavity tumors in women consistent with ovarian cancer, and midline nodal disease in men consistent with germ-cell cancer [8]. Outside of the subset of CUP with favorable features, understanding the tissue of origin has been of questionable value for enhancing response rates and OS [2,4,9,10]. Additionally, a meta-analysis showed that several two-drug empiric chemotherapy regimens produced similar results in 80% of CUP patients with unfavorable features; no type of chemotherapy prolonged survival in these patients [11]. Recent studies using NGS demonstrate heterogeneity and molecular distinctness amongst patients with both favorable and unfavorable CUP [12]. Herein, we review the evolution of our understanding of CUP, especially in the context of the genomic era of diagnostic tests.

Techniques for Classifying Patients with CUP

Laboratory techniques for classification of patients with CUP for the purpose of diagnosis and treatment include a variety of IHC stains and tissue-of-origin testing (Tables 1–3).

Light Microscopy and IHC

Advances in resolution and processing of images for light microscopy have been made over the last four centuries, but the basic principle of light microscopic interrogation remains unchanged [13]. In CUP, following a biopsy procedure, hematoxylin and eosin stains (H&E stains) are used to visualize the tissue material on the slide. The hematoxylin stains cell

[i] https://www.nccn.org/professionals/physician_gls/pdf/occult.pdf

nuclei blue and eosin stains the cytoplasm and extracellular matrix pink, allowing the pathologist to visualize the layout of the cells and identify the pattern in regard to a tissue-of-origin diagnosis. In CUP, while the tissue of origin is not visually apparent, approximately 50% of cases can be categorized as well-to-moderately differentiated adenocarcinomas, ~30% as poorly or undifferentiated adenocarcinomas, ~15% as squamous cell carcinomas, and ~5% as undifferentiated neoplasms under a light microscope [1]. IHC is an important tool used to further confirm the diagnoses of many types of cancerous tissues by evaluating particular proteins in a tissue sample [14]. A primary antibody is exposed to a cancer tissue sample and binds to a desired protein if it is expressed by the tissue. This binding of primary antibody to tissue is then detected by incubating the sample with secondary, labeled antibody, which may provide a visible and quantifiable protein expression pattern.

Several IHC stains have been developed that demonstrate proteins expressed in different types of cells. Typically, a hand-picked combination of stains is tested against a tissue sample to confirm or help pinpoint the suspected primary site of a cancer[i] [2,14,15]. As shown in Table 1, the first panel used for undifferentiated neoplasms or cells of unclear lineages typically includes epithelial, lymphoid, and melanocyte antigens [14]. If the cells are believed to be of epithelial lineage, the second panel may include CK7 and CK20 to narrow down the potential organs of origin. Lastly, to pinpoint the organ, a more disease-specific group of stains is used, as shown in Table 2. The number of available IHC stains is always expanding as more specific antigens are found. More recently, IHC has also been used to detect expression of treatment-response predictors and other cancer-relevant proteins [16,17].

Tumor Markers

In general, tumor markers are serum proteins that may be elevated due to the presence of cancerous cells [18]. Tumor markers can be measured throughout treatment course to assess response to treatment or progression of cancer [19]. However, most markers such as carcinoembryonic antigen (CEA), chorionic gonadotropin (HCG), alpha-fetoprotein (AFP), carbohydrate antigen (CA) 19–9, and CA 125 have low specificity and sensitivity and therefore should not be used to pinpoint a diagnosis of CUP [18–21]. Some markers, such as CEA, can also be elevated in benign conditions, including alcoholic cirrhosis, hepatitis, and ulcerative colitis [21]. Although tumor markers are frequently elevated in patients with CUP, and occasionally could be used to monitor therapy response, clinical utility as a diagnostic tool or to predict survival has not been well established [19,20]. Moreover, serum levels of tumor markers can fluctuate [22]. Alternatively, specific tumor markers may be used to support the diagnosis of an underlying cancer. One example is hepatocellular carcinoma, in which diagnosis is based on elevated AFP in combination with radiographic findings and evidence of liver cirrhosis. A second example is in patients with a testicular mass and elevated AFP and/or HCG, where tumor markers can support the histological diagnosis of testicular cancer [18]. Overall, the low-cost and repeatability of tumor markers positions them as a complementary tool in the clinic.

Tissue-of-Origin Assays

Much of the research in CUP molecular profiling has focused on elucidating the primary site of disease. These studies generally utilize RT-PCR or microarrays to exploit differential gene expression by different tissues [4,9,10,23–27]. Comparisons with IHC results, clinical presentations, and autopsy results have been used to validate such assays.

To date, the FDA approved the use of 2000-gene microarray-based gene expression assay to predict the origin of cancer, including CUP[ii] [27]. Such assays attempt to compare RNA expression patterns in tumor tissue with a panel of 15 different tissue types with established RNA profiles to investigate the similarities. The assay was clinically validated by a study comparing its predictions of tumors with established diagnoses with an overall agreement rate of 88.5% [29].

There are several studies that assessed and reported the therapeutic impact of such assays, including the 2000-gene microarray assay on CUP patients in a prospective setting (see Table 3). A ten-gene RT-PCR assay revealed that CUP patients with a colon cancer profile had better response to colon cancer-specific regimens when compared with an empiric CUP regimen with taxane and platinum [9]. In another study, molecular profiling of genes expressed by tumor cells in a 92-gene RT-PCR assay predicted the tissue of origin in 85% of enrolled CUP patients ($n = 289$) [10]. Among those 289 patients enrolled, 194 patients received site-specific therapy according to gene expression profiling predictions. The median survival for the entire group was 12.8 months. Patients predicted to have treatment-sensitive tumor types had better median survival than did patients with treatment-resistant tumor types [13.4 months versus 7.6 months ($P = 0.04$)]. As expected, OS varied across different predicted origins, which is consistent with the heterogeneous nature of CUP. The variability in OS also mirrored that of the predicted tissues of origin, further supporting the predictions made by the assay (e.g., median OS of biliary tract tumor: 6.8 months versus ovarian cancer: 29.6 months) [10].

Two small, randomized trials have also addressed the utility of site-specific therapy (based on gene expression profiling versus empiric chemotherapy). The first study is a randomized Phase II study that evaluated 130 CUP patients randomized to receive either empiric chemotherapy (paclitaxel/carboplatin) or site-specific therapy based on gene expression profiling [4] (clinical trial identification: UMIN000001919[iii]). The primary endpoint of this trial was the 1-year survival rate among evaluable patients. Overall, there was no survival difference between patients who received empirical carboplatin plus paclitaxel versus site-specific treatment. Regarding adverse events, one sudden death was reported in the site-specific treatment arm, where the patient received cisplatin and S-1 (tegafur, gimeracil, and oteracil). Hematologic toxicities were common in both groups, especially with a decrease in white blood cell count (70–80% of patients with any grade). More recently, the GEFCAPI 04 Phase III trial (clinical trial identification: NCT01540058[iv]) randomized 243 patients with unfavorable CUP to receive either empiric cisplatin plus gemcitabine or gene expression-based treatment, according to the suspected primary tumor site [30]. The primary

[ii] www.cancergenetics.com/laboratory-services/specialty-tests/too-tissue-of-origin-test/
[iv] https://clinicaltrials.gov/ct2/show/NCT01540058

endpoint of the study was PFS. Preliminary results from this study also failed to show a PFS and OS difference between treatment groups. Moreover, meta-analysis suggested that site-specific treatments in CUP had no significant survival benefit when compared with patients managed with empiric chemotherapy [31]. Although the results of the two randomized trials are consistent, they are somewhat difficult to interpret since the majority of patients in both studies were those predicted to have cancer types (e.g., pancreas, biliary) with poor outcomes, in which site-specific treatment makes little impact on survival. The value of site-specific treatment in patients predicted to have treatment-sensitive tumor types was not adequately addressed in either study, due to the small numbers of patients included. In the GEFCAPI 04 study, a small subset of patients with predicted cancer types unlikely to benefit from empiric chemotherapy (e.g., renal, melanoma) may have benefited from site-specific therapy. Further investigation is needed among cancers types in which standard treatment differs substantially from the empiric chemotherapy regimens used in CUP.

As treatment improves rapidly for many cancer types, the continued management of the heterogeneous group of CUP patients with a single empiric chemotherapy regimen is far from ideal. Furthermore, recent advances in oncology indicate that some effective new cancer treatments target specific molecular alterations. Therefore, comprehensive molecular profiling using NGS is rapidly becoming an integral part of the management of advanced cancers, including CUP.

Molecular Profile-Based Management of Patients with CUP

The value of molecular profiling data in CUP remains inconclusive, due to the lack of large-scale prospective studies examining personalized biomarker-directed therapy in this population. However, several retrospective studies have suggested the need for further investigation of this approach [12,32–37]. For example, between 85% and 91% of patients with CUP harbored ≥1 oncogenic driver mutation, as determined by tissue-based NGS, according to two studies totaling 350 patients [32,37]. In a more recent study (n = 442 patients), approximately 65% of individuals with CUP harbored ≥1 potentially actionable mutations, as detected by liquid biopsy-based cell-free circulating-tumor DNA (cfDNA) [12]. Importantly, no two patients with ≥2 alterations had identical molecular portfolios [12], consistent with the known heterogeneity of CUP. These data suggest the feasibility of investigating matched targeted therapy for patients with CUP.

According to previous reports, the most common alterations found in patients with CUP are in the *TP53* gene (37–55% of cases) [12,32,36,37] followed by *KRAS* (18–20%), *PIK3CA* (9–15.4%), *ARID1A* (~11%), and *EGFR* (~6–17%) genes. Some of these gene alterations are considered difficult to target, but others (e.g., aberrant *EGFR* in lung cancer and altered *PIK3CA* in breast cancer) are clearly druggable. However, most data on therapeutic matching to genomic alterations in CUP comes from case reports [12,36,38–44]. A prospective, Phase II, randomized study is underway to elucidate if tailored treatment based on genomic profiling is beneficial when compared with the standard chemotherapy approach for patients with CUP (CUPISCO trial, NCT03498521[v] [45]). Several classes of targeted

[v] https://clinicaltrials.gov/ct2/show/NCT03498521

therapies have the potential to improve outcomes in this population, including immunotherapeutic agents and receptor tyrosine kinase inhibitors (Figure 1).

Immune Checkpoint Inhibitors (CPI)

Over the past decade, new immune CPI, especially anticytotoxic T lymphocyte-associated protein 4 (CTLA-4), anti-programmed cell death protein 1 (PD-1), and anti-programmed death-ligand 1 (PD-L1), have led to a paradigm shift in cancer management and have become the standard treatment option in several types of cancers, including melanoma, non-small cell lung cancer (NSCLC), kidney, and bladder cancers. Subsequently, several biomarkers to predict response to CPI were identified. Indeed, pembrolizumab earned a histology-agnostic, biomarker-based FDA approval for MSI-high tumors and for tumors with tumor mutation burden (TMB) ≥10 mutations/Mb [46]. Other biomarkers of interest for CPIs in general are PD-L1 overexpression or amplification [47,48] and the aforementioned high TMB [49,50]. Within the CUP population, tumor PD-L1 expression was seen in 22% of 362 patients [34]. Meanwhile, 11.8% of 389 patients harbored a high TMB, defined as ≥17 mutations/Mb in the report, and 1.8% of 384 patients' tumors were MSI-high [34], both of which have been, as mentioned, implicated as predictors of CPI responsiveness [47,49,50]. Of equal importance, genomic alterations associated with lack of response and/or hyperprogression [51] to CPI were also detected, including *MDM2* amplification in 2% of patients [34].

Several ongoing trials are investigating CPI across various tumor types, including CUP. For example, one of the Phase II trials assessed pembrolizumab in a broad range of unresectable or metastatic rare tumors, including a cohort for CUP (NCT02721732[vi]). Early results showed an ORR of 23% (3 of the 13 evaluable patients with CUP) [52]. Another trial of the ipilimumab/nivolumab combination in rare tumors is currently ongoing with a cohort for CUP [dual anti-CTLA-4 and anti-PD-1 blockade in rare tumors (DART); NCT02834013[vii]]. Both trials mentioned above treat all eligible patients with their respective CPI, regardless of biomarker status, but biomarker correlatives will be examined.

Receptor Tyrosine Kinases

Several tyrosine kinase family members are frequently altered in CUP. A subgroup of patients with CUP tumors harbor genomic rearrangements, fusions, or other alterations in genes, including *ALK*, *EGFR*, *RET*, *FGFR1*, and *NTRK1*, and may have substantial benefit from targeted therapy [37,53].

Epidermal Growth Factor Receptor (EGFR)—EGFR forms homodimer or heterodimer units when bound by specific extracellular ligands. Downstream, activation enhances cell proliferation and survival [54]. Although no comprehensive trial has been done for *EGFR*-altered CUP patients, prior molecular profiling studies reported that *EGFR* is amplified in 17% [36], mutated in ~6% [12], and EGFR protein overexpressed in 55% of cases [36], all of which can potentially be targets of interest. There have been at least six

[vi] https://clinicaltrials.gov/ct2/show/NCT02721732
[vii] https://clinicaltrials.gov/ct2/show/NCT02834013

reports of patients with CUP who harbored *EGFR* alterations and were treated with EGFR inhibitors, either as monotherapy or in combination, and showed tumor regression and clinical benefit lasting 4+ to 24+ months [36,38–41,54]. Additionally, we have also observed remarkable tumor regression (Figure 2) in a patient with CUP harboring *EGFR* amplification managed with a matched targeted therapy approach [54] using the anti-EGFR antibody cetuximab and the EGFR small molecule inhibitor erlotinib (for *EGFR* amplification) along with the CDK4/6 inhibitor palbociclib (for *CDKN2A* H83Y).

Human Epidermal Growth Factor Receptor 2 (HER2/ERBB2)—Another tyrosine kinase receptor in the ERBB family that is capable of homodimerization or heterodimerization and is catalytically active is *HER2/ERBB2*. This protein is frequently altered in CUP (~4–8%). Indeed, *ERBB2* amplification or overexpression is an FDA-approved target for breast and gastric cancers. Recent data suggest *ERBB2* is also an actionable target among colorectal and lung carcinomas [55,56]. To our knowledge, very little response data has been published in *ERBB2*-altered CUP. One study reported a patient with *ERBB2* gene amplification receiving targeted treatment for 5 months before discontinuation and another patient with a S310F/Y mutation receiving targeted treatment for only 2 months [37]. Of interest, however, multiple *ERBB2*-altered tumor types respond to HER2-targeting agents [55]. Further treatment studies are required for *ERBB2*-altered CUP.

ROS Proto-oncogene 1 (ROS1)—The ROS1 receptor has been shown to be a key regulator of physiological cellular processes as well as tumorigenesis and growth [57]. Crizotinib, a multikinase inhibitor including ROS1, first gained FDA approval in 2016 for the small fraction of NSCLC harboring *ROS1* alterations [58]. Of interest is a case report that demonstrated response in a patient with CUP harboring *ROS1* rearrangement [42].

Neutropenic Tyrosine Kinases (NTRK)—*NTRK1*, *NTRK2*, and *NTRK3* gene fusions have recently gained recognition as important biomarkers, due to remarkable responses observed with administration of targeted therapies [59]. In normal cell function, they code for neutrotrophin receptors TrkA, TrkB, and TrkC, which are known to participate in a variety of functions, including neuronal development and differentiation [60]. Both larotrectinib and entrectinib (NTRK inhibitors) are now FDA approved for refractory, metastatic, and/or unresectable solid tumors with *NTRK* fusions [60–62]. Although a prior study reported the presence of *NTRK* fusions in only ~0.9% of CUP samples [63], patients can demonstrate remarkable response from NTRK inhibitors regardless of tissue of origin. Furthermore, the rate of NTRK fusions across solid tumors is actually lower than in CUP: 0.31% [59]. One patient with CUP harboring an *IRF2BP2-NTRK1* fusion was reported to benefit from NTRK inhibitor for 14 months and was still receiving treatment as of the data cutoff [37].

Anaplastic Lymphoma Kinases (ALK)—ALK is another rare, yet important biomarker due to the availability of effective targeted therapies. Clinical activity has been demonstrated in patients with ALK-rearranged lung cancer [64]. While the prevalence of altered *ALK* gene or its products is unclear in CUP, there have been three reports indicating a therapeutic

role of ALK inhibitors in this setting (one patient with 30+ month treatment response) [37,43,44].

Mitogen-Activated Protein Kinase (MAPK) Pathway—The MAPK signaling pathway is also frequently altered in patients with CUP [12]. Although more complex than initially thought, the main known function is pathway activation that promotes cell growth, proliferation, and survival. Intracellular signaling may involve interactions of different RAS, RAF, MEK, and ERK proteins, in that order [65,66].

In prior studies of CUP profiling, *KRAS* was altered in 18–20% of patients [12,32,36] and *BRAF* was altered in up to 7.5% [12]. Although there is no FDA-approved regimen that directly targets *KRAS* mutations, there are ongoing studies using a novel *KRAS* inhibitor, specifically against *KRAS* G12C (AMG 510; NCT03600883[viii]); 48% of patients with NSCLC patients achieved an objective response [67,68]. Another study with *KRAS* G12C inhibitor, MRTX849, is ongoing and await further clinical outcomes [69]. However, results in other tumor types, such as colorectal cancer, may be lower [70]. Despite the fact MEK inhibitors previously failed to be beneficial for *RAS* mutated patients [71,72], there is a case with *KRAS* G12R mutated Rosai-Dorfman disease who responded to single agent MEK inhibitor; this patient had no co-alterations suggesting that molecular context may be important [73]. Further investigation is warranted.

BRAF alterations, in particular V600 mutations, are effectively inhibited by BRAF inhibitors and MEK inhibitors, such as vemurafenib or dabrafenib, given alone or together [74–76]. Very little data regarding targeting BRAF in CUP is available.

Concluding Remarks

Although rapid advances have been made in some areas of oncology, especially via the use of NGS and exploitation of immunotherapy, management of CUP continues to be challenging, with a poor prognosis even with a variety of cytotoxic chemotherapy regimens. Determining site of origin for selecting treatment for patients with CUP has shown inconsistent salutary effects. Current data suggest that patients with CUP have complex molecular portfolios [12,32]. CUP harbors a median of four characterized genomic alterations per tumor [32] and nearly 90% of patients have a unique pattern of molecular abnormalities that differ from each other [12,77]. These observations suggest that a customized approach with potentially more than one matched therapy is required to better manage CUP patients, if genomic biomarkers are to be used (see Outstanding Questions). Consistent with this notion, a trial where patients with treatment-refractory solid tumors were managed with individualized (N-of-1) combinations based on their underlying genomic profiles has been ongoing [Investigation of Profile-Related Evidence Determining Individualized Cancer Therapy (I-PREDICT study; NCT02534675[ix])]. The study demonstrated that patients who received matched therapy that impacted more than half of their genomic alterations (higher match; targeted >50% of genomic alterations)

[viii] https://clinicaltrials.gov/ct2/show/NCT03600883
[ix] https://clinicaltrials.gov/ct2/show/NCT02534675

demonstrated significantly better clinical outcomes compared with patients in lower match group (targeted ≤50% of genomic alterations): rate of complete response (CR)/PR/SD ≥ 6 months, 50% versus 22.4% ($P = 0.028$); median PFS, 6.5 months versus 3.1 months ($P = 0.001$); median OS, not reached versus 10.2 months ($P = 0.046$) [78]. The I-PREDICT study is ongoing with additional patients in various subgroups being accrued, including CUP. Utilizing genomic interrogation to navigate patients with CUP to matched gene- and immune-targeted therapy merits further exploration [79].

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Highlights

Cancer of unknown primary (CUP), by definition, is metastatic disease with an unidentifiable primary tumor.

Patients with CUP are generally treated with empiric chemotherapies, such as taxanes and platinum-containing regimens; however, clinical outcomes remain poor.

Recent studies with next-generation sequencing revealed that most CUP tumors harbored unique and complex genomic portfolios, with a mean of four to five alterations per tumor.

CUP represents a unique cancer in which the genomic alterations may be the cornerstone of the diagnosis. Matched individualized combination therapy in CUP merits prospective clinical investigation.

Outstanding Questions

- Should we be integrating multiple potential biomarkers, including those derived from genomics, transcriptomics, proteomics, and immunomics, in order to optimize the CUP treatment strategy?
- Since CUPs are clinically and molecularly heterogeneous, can we use master protocols to enhance the clinical trial strategy and navigate patients to individually tailored treatments?
- Since CUPs each harbor four to five pathogenic alterations, should we be treating with customized matched combination therapy, rather than matched single agents?

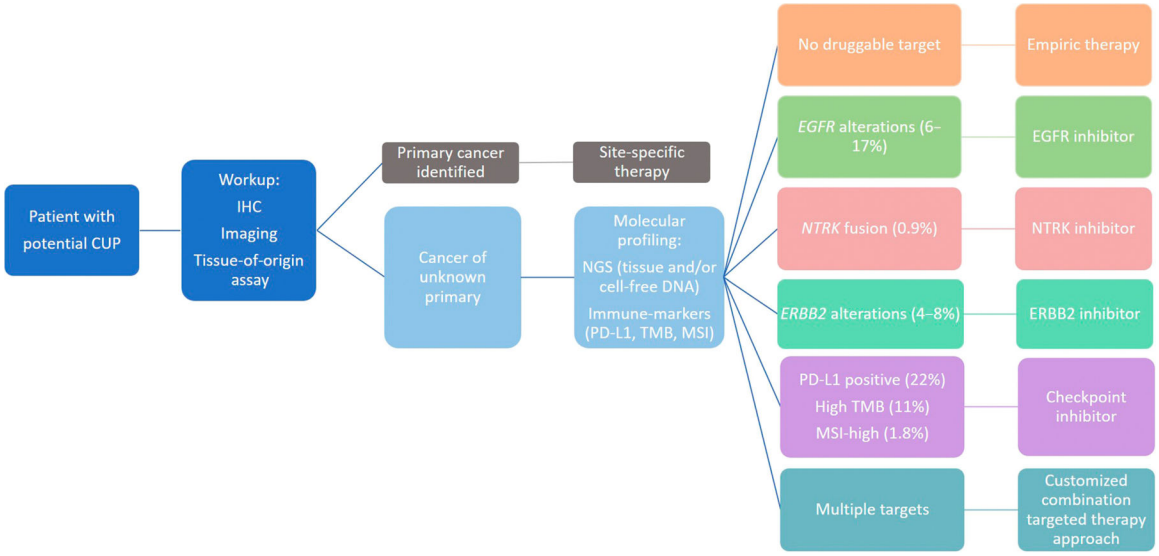

Figure 1.

Proposed Strategy for Patients with CUP. Patients with potential CUP should undergo standard workup (including IHC, imaging, and tissue-of-origin assay) to seek a primary cancer diagnosis. If a primary cancer is identified, patients should seek site-specific therapy. Once a patient is determined to have CUP, we propose obtaining molecular profiling (including NGS from tissue and/or from cell-free DNA) and immune-profiling (including PD-L1, TMB, and MSI testing) to seek actionable targets. If there is no druggable target, the patient may be managed with empiric therapy. However, if there are potentially targetable alterations, the use of a targeted therapy approach based on the underlying molecular features may be considered. Percent indicate the frequency of cognate target among CUP patients. Note: *EGFR* alterations have been most frequently associated with lung cancer; *ERBB2* alterations with breast and gastric cancer; and *NTRK* alterations, PDL1 expression, and high TMB are tissue agnostic; however, any one of these alterations may occur across a variety of tumor types. Only a few examples of potential genomic alterations are shown. Abbreviations: CUP, Cancer of unknown primary; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; MSI, microsatellite instability; NGS, next-generation sequencing; NTRK, neutropenic tyrosine kinases; PD-L1, programmed death ligand 1; TMB, tumor-mutation burden.

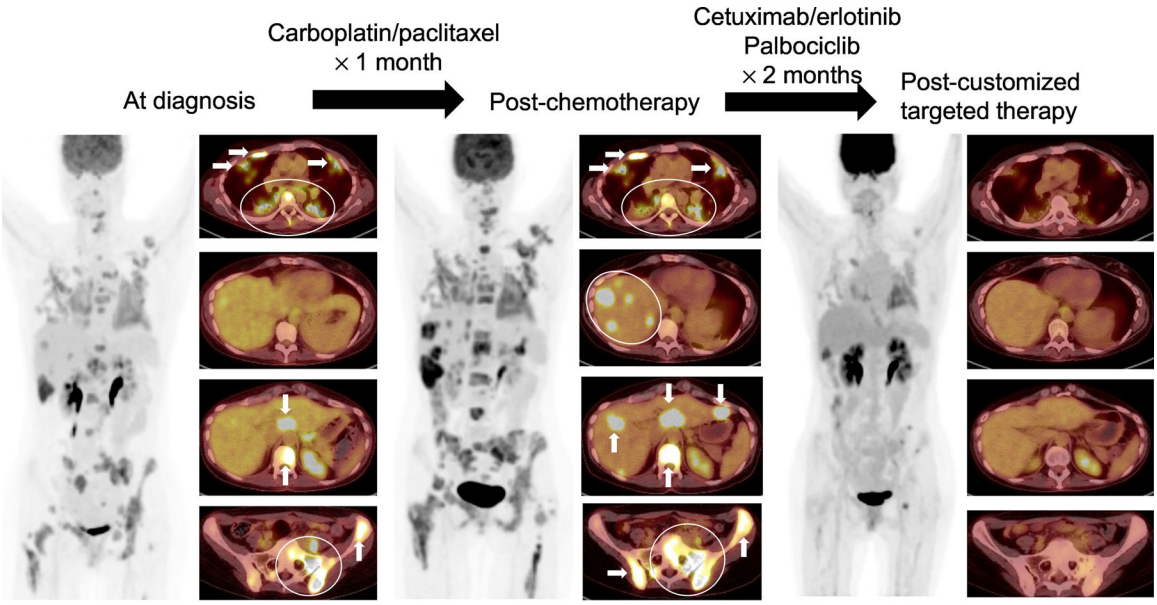

Figure 2.

A 42-Year-Old Woman with Metastatic Adenocarcinoma of Unknown Primary. A 42-year-old woman initially presented with a seizure. Further workup showed multiple brain masses along with lymphadenopathy and bone and liver metastases. Biopsy was consistent with poorly differentiated adenocarcinoma. Immunohistochemistry was positive for CK7 and CDX-2, while CK20 was negative, which was suggestive for upper gastrointestinal primary; however, upper endoscopy was unremarkable without underlying mass. Random biopsy of distal esophagus, stomach, and duodenum were negative for malignancy. Overall, the patient was determined to have cancer of unknown primary and started on carboplatin and paclitaxel. Unfortunately, tumor rapidly progressed on cytotoxic chemotherapies (left to middle). During this time, genomic profiling revealed *EGFR* amplification and *CDKN2A* H83Y. Based on the molecular profiling, patient was started on cetuximab (anti-EGFR antibody) and erlotinib (EGFR small molecule inhibitor) (for *EGFR* amplification) along with palbociclib (CDK4/6 inhibitor) (for *CDKN2A* H83Y) and initially demonstrated remarkable response (middle to right) [patient consented to profile related evidence determining individualized cancer therapy (PREDICT); NCT02478931]. Unfortunately, after 4 months, the patient progressed and a new alteration, *EGFR* T790M, a known EGFR resistance mutation, appeared.

Table 1.

Immunohistochemistry Flowchart for Patients with CUP

Step 1: Determining the lineage	**Step 2: Determining the subtype**[a]		**Refs**
Positive pancytokeratin (AE1/AE3, Cam5.2, OSCAR, etc.) → Carcinoma	CK7+/CK20− →	Breast carcinoma Cholangiocarcinoma Endometrial adenocarcinoma Endocervical adenocarcinoma Gastric adenocarcinoma Lung adenocarcinoma Mesothelioma Ovarian (serous) carcinoma Pancreatic adenocarcinoma Renal (papillary) Salivary gland tumors Small cell lung carcinoma Thyroid carcinoma Urothelial carcinoma (subset)	[14,80,81]
	CK7+/CK20+ →	Bladder adenocarcinoma Cholangiocarcinoma Gastric adenocarcinoma Ovarian mucinous carcinoma Pancreatic adenocarcinoma Urothelial carcinoma	
	CK7−/CK20+ →	Colorectal adenocarcinoma Gastric adenocarcinoma Merkel cell carcinoma	
	CK7−/CK20− →	Adrenocortical carcinoma Gastric adenocarcinoma Hepatocellular carcinoma Mesothelioma Non-seminoma germ cell tumors Prostate adenocarcinoma Renal (clear cell types) Small cell lung carcinoma	
Positive HMB-45/Melanin-A/S100/ SOX10 → Melanoma	More testing may be needed to determine subtype		
Positive CD45/CD20/CD3 → Lymphoma	More testing may be needed to determine subtype		
Other[b] → Sarcoma	More testing may be needed to determine subtype		

[a] See also Table 2 for organ-specific stains.

[b] If epithelial, melanocytic, and lymphoproliferative lineages are ruled out then sarcomas may be considered using specific IHC stains based on morphology, location of tumor, and clinical characteristics.

Abbreviations: CD, Cluster of differentiation; CK, cytokeratin; HMB-45, Human Melanoma Black 45; SOX10, Sex-determining region Y box 10.

Table 2.

nohistochemistry Stains and Associated Cancer Diagnoses[a,b]

	Bladder	Breast	Colorectal	Gastric	Lung	Mullerian	Pancreas/ biliary	Prostate	Germ cell	Hepatocellular	Neuroendocrine	Renal (clear cell)	Renal (papillary)	Small cell lung	Squamous cell	Thyroid	Undifferentiated
marker	Adenocarcinoma								Carcinoma								
AE3, 5.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+/−
ase/ ar1										+							
/RCC												+	+				
2		−	+	+	−		+/−	−	−		−					−	−
CK6															+		
	+	+	−	+/−	+	+/−	+	−		−	+/−	−	+	+/−	+/−	+	−
	+	−	+	+/−	−	−	−	−		−	−	−	−	−	−	−	
nogranin, tophysin		−	−		−			−	−		+			+		+/−	−
		+/−	−		−	+		−	−		−					−	−
3	+	+													+		
FP-15, naglobin		+															
n A					+	+/−		−	−		−					−	−
3–1	−	+/−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
, PLAP, 4									+								
63	+														+		
						+						+	+			+	
PSA[c]	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
2			+														
globulin		−	−		−			−	−		+/−					+	−
d		−	−		+			−	−	+	+/−					+	−

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	Bladder	Breast	Colorectal	Gastric	Lung	Mullerian	Pancreas/ biliary	Prostate	Germ cell	Hepatocellular	Neuroendocrine	Renal (clear cell)	Renal (papillary)	Small cell lung	Squamous cell	Thyroid	Undifferentiated
			+		+/−		+/−										

4,15,80,81].

cells are those not routinely used for the differential diagnosis.

nale breast cancers and salivary cancers are PSA positive [82].

staining is typically cytoplasmic in the liver and nuclear in lung and thyroid specimens. WT-1 positivity may also be observed in mesothelioma (favors mesothelioma in males).

iations: CD, Cluster of differentiation; CDX2, caudal type homeobox 2; CK, cytokeratin; ER, estrogen receptor; GATA3, GATA binding protein 3; GCDFP-15, gross cystic disease fluid protein-15; 1, hepatocyte paraffin 1; NKX3–1, Homeobox protein Nkx-3.1; NSE, neuron specific enolase; OCT4, octamer-binding transcription factor 4; p40 /p63, protein/tumor protein 40/63; PAP, prostatic osphatase; PAX, paired box gene; PLAP, placental alkaline phosphatase; PSA, prostate-specific antigen; RCC, renal-cell carcinoma; SALL4, spalt like transcription factor 4; SATB2, special AT-rich e-binding protein 2; TTF1, thyroid transcription factor 1.

Table 3.

Selected Studies That Utilized CUP Tissue-of-Origin Testing

Year published (study setup)	Method/panel	CUP sample (*n*)	Assignment of tissue of origin	Therapeutic benefit/comment	Refs
2008 (Retrospective and prospective cohorts)	Ten-gene RT-PCR assay	78 in retrospective cohort	A putative tissue of origin was assigned to 63 of 120 patients (52.5%)	In retrospective cohort, 19 of 68 patients (28%) responded to empiric CUP first-line therapies (e.g., taxanes, platinum, or gemcitabine-based therapy).	[9]
		42 in prospective cohort		In prospective cohort, 12 of 36 patients (33%) had response to first-line therapy.	
2008 (Retrospective)	Microarray assay	84	Assay classified the primary site correctly in 70 (83%) of 84 patients with tumors of known origin	No therapeutic intervention.	[26]
2008 (Retrospective)	495-Gene microarray assay	21	In 16 of 21 patients, site of origin was confirmed	No therapeutic intervention.	[27]
2010 (Retrospective)	87-Gene RT-PCR assay	38	In 15 of 38 patients (39%), assay predicted latent primary site identified after initial CUP diagnosis	No therapeutic intervention.	[25]
2011 (Prospective)	Assay with 48 microRNAs	104	A tissue of origin was assigned to 74 of 104 patients (71%), 62 of which (60%) were compatible with clinicopathologic presentation	No therapeutic intervention.	[23]
2012 (Retrospective)	2000-Gene microarray assay	107	After the assay, physicians changed the working diagnosis for 50% of patients and the management for 65% of patients	Not assessed.	[83]
2013 (Prospective)	92-Gene RT-PCR assay	289	A tissue of origin was predicted in 247 patients (85%); 194 patients received assay-based site-directed therapy	Patients who received assay-directed site-specific therapy had longer median OS compared with non-site-specific therapy [13.4 versus 7.6 months (P = 0.04)]. Predicted ovarian and breast cancers had longest median survivals (29.6 and 24+ months, respectively).	[10]
2016 (Retrospective)	Microarray DNA methylation signatures	216	The assay predicted tissue of origin in 188 (87%) of patients	Retrospectively, therapies received by patients were divided into site-targeted verses empiric therapies. Patients who received a tumor type-specific therapy had improved OS compared with patients who received empiric therapy (hazard ratio: 3.24, P = 0.0051).	[24]
2018 (Prospective, randomized)	Microarray assay	130	All patients had successful assays; 50 patients received site-specific therapy based on the assay and 51 received empiric therapy with carboplatin and paclitaxel	No survival difference between site-specific treatment versus empirical carboplatin/paclitaxel. Median OS and PFS were 9.8 and 5.1 months, respectively (site-specific treatment) versus 12.5 and 4.8 months (carboplatin/paclitaxel) (P = 0.9 and 0.6, respectively)	[4] Clinical trial identification: UMIN000001919[ii]
2019 (Prospective, randomized)	92-Gene RT-PCR assay	243	123 patients received site-specific therapy based on the assay and 120 received empiric therapy with cisplatin and gemcitabine.	No survival difference between site-specific treatment versus empirical cisplatin/gemcitabine. Median OS and PFS were 10.7 and 4.6 months, respectively (site-specific treatment) versus 9.99 and	[30] Clinical trial identification: NCT01540058[iv]

Year published (study setup)	Method/panel	CUP sample (*n*)	Assignment of tissue of origin	Therapeutic benefit/comment	Refs
				5.3 months (cisplatin/gemcitabine) ($P = 0.92$ and 0.95, respectively)	