New Strategies for Carcinoma of Unknown Primary: The Role of Tissue-of-Origin Molecular Profiling

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Abstract

The taxonomy and management of carcinoma of unknown primary (CUP) has matured over the past decade with the use of sophisticated imaging and pathologic tools. In the era of tailored therapeutics, this presents both an opportunity and a challenge. Tissue-of-origin (ToO) molecular profiling has an important role in the diagnostic armamentarium of CUP cancers, and its niche continues to evolve with ongoing prospective studies. Despite the inability to conduct direct validation (i.e., primary tumor), the use of the indirect validation methods with immunohistochemistry (IHC), imaging, and treatment response has allowed us to evaluate the performance accuracy of ToO profiling assays in CUP cancers. Despite advances, we struggle with the undifferentiated neoplasms, which often remain unclassifiable after an exhaustive use of IHC and ToO profiling assays. Genomic characterization of these and other select CUP cancers using next-generation sequencing techniques may reveal actionable biomarkers outside the (tissue specific) cellular framework. Also, going forward, using data from comparative effectiveness research, one could envision using a streamlined, cost-effective algorithm that integrates IHC and ToO molecular profiling in patients with limited (or difficult-to-access) biopsies and difficult-to-diagnose cancers.

Background

CUP classification

Carcinoma of unknown primary (CUP) is a diverse group of cancers with poor prognosis and an unmet research need. The reported incidence of CUP varies with the practice setting, and averages 2% to 4% of all cancers. Not unlike other solid tumors, the classification of CUP continues to evolve.

The past four decades encompass several periods. CUP first came to be defined when improved imaging suggested, with some confidence, the absence of a primary tumor. This was followed by diagnostic histopathology and robust immunohistochemistry (IHC), which allowed definition of select CUP subsets (1–4). The current era of molecular profiling describes tissue-of-origin (ToO) profiles, which assign putative primary sites to CUP cancers. In addition, robust IHC and profiling together (5–6) inform actionable targets (e.g., Her-2, KRAS, ALK, others).

As the genomic and proteomic characterization of CUP malignancies are refined, the assigned "unknown" designation is being challenged. Furthermore, ambiguity is created by the "uncertain primary," which can present in one of three settings. First, patients who have been diagnosed and treated in the past for a specific known cancer can present with metastatic cancer and an atypical, usually poorly differentiated, pathology that does not match that of the previously treated primary. This creates the ambiguity around whether it is a recurrence or a new CUP cancer. Second is an unclassifiable cancer, which is usually a poorly differentiated or undifferentiated neoplasm. Last, and perhaps the most common of all, is metastatic cholangiocarcinoma in the presence of an intrahepatic lesion(s), masquerading as CUP cancer. An academic CUP program evaluates an assortment of these presentations, and each presentation poses a different diagnostic and therapeutic challenge (Fig. 1).

Imaging in the presence of sophisticated pathology

Optimal and focused imaging is critical in the management of a suspected CUP even in the setting of ToO molecular assays. Expensive profiling tests should not compensate for a substandard work-up. In the absence of contraindications, a baseline i.v. contrast computed tomography (CT) scan of the chest, abdomen, and pelvis is the standard of care in all CUP patients (7). Several studies have been conducted to determine the value of [18F]fluorodeoxyglucose–positron emission tomography (FDG-PET) imaging in detecting occult primary tumors after unsuccessful conventional diagnostic evaluation. Most of the studies consist of a small number of evaluable patients and focus primarily on patients with cervical lymphadenopathy with squamous cell pathology (8–12). In this group of patients, PET-CT is useful in about a third of patients and helps with...
search of the primary, radiation planning, and surveillance. It is important to note that in the CUP setting, a baseline PET-CT scan may miss a small renal or urothelial primary cancer. Larger studies to evaluate the utility and cost-effectiveness of PET-CT in CUP are warranted, although they can be challenging. There are some practical applications in which PET-CT scan may be useful, although this approach has not been studied prospectively—first, in evaluating select CUP patients presenting with solitary metastatic disease who are candidates for definitive locoregional therapy, and second, in patients with osseous predominant CUP. With the latter, a single study may suffice and can replace multiple scans to image response of osseous metastases to therapy.

**Framework of ToO molecular profiling: effort in known cancers**

Most agree that an accurate diagnosis of the putative primary would support the use of site-specific therapy and affect the prognosis and management of CUP patients. The premise for studying ToO molecular profiling assays in CUP cancers is that, when a large number of genes are examined using tools such as DNA microarray or quantitative real-time PCR (qRT-PCR) assay, metastatic tumors have molecular signatures that match their primary origin (13).

Ramaswamy and colleagues (14) studied 218 tumor tissues across 14 common tumor types and constructed a predictive support vector machine (SVM) algorithm. This algorithm, tested on an independent group of 54 tumors, yielded an overall prediction accuracy of 78%, and the accuracy rate stayed above 70% with fewer than 50 genes. Su and colleagues extracted mRNA from 100 primary carcinomas in 10 common cancers and then used an Affymetrix oligonucleotide microarray to identify genes that were differentially expressed (15). The 110-gene based algorithm was tested against 75 blinded samples and accurately predicted the tumor of origin in over 90% of the cases. Bloom and colleagues (16) combined multiple tumor datasets to obtain a large collection of tumors (21 tumor types) and built a neural network-based classifier with 85% accuracy. Tothill and colleagues (17) constructed a support vector machine classifier, showing 89% accuracy using a 13-class model. They also showed the translation of a five-class classifier to a quantitative PCR-based platform and studied a few CUP cancers.

Because a microarray as a tool is complex and time consuming, there was a move toward converting the microarray database to a clinic-ready qRT-PCR platform using formalin-fixed, paraffin-embedded (FFPE) specimens. Ma and colleagues (18) developed a qRT-PCR assay involving 92 genes and used it on FFPE samples. A validation set of 119 FFPE tumor samples from 32 different tumor classes showed an 87% accuracy rate. More recently, a multisite validation study of the same assay was done using 790 specimens from more than 50 subtypes. The 92-gene assay showed overall sensitivities of 87% for tumor type and 82% for subtype (19).
Monzon and colleagues (20) described a multicenter validation of a 1,550-gene expression profile for identification of tumor ToO. Four institutions processed 547 frozen specimens, representing 15 ToO using oligonucleotide microarrays. The study revealed an overall sensitivity of 88% and an overall specificity of 99%. A validation study for this assay using 462 FFPE specimens maintained an accuracy rate of 89% (21).

At least two groups have evaluated the role of microRNA (miRNA) in ToO profiling. Ferracin and colleagues (22) identified a 47-miRNA signature representing 10 cancer types (in 40 samples) and reported an accuracy rate of 100% for primary cancers and 78% for metastases. This signature was applied to an independent published dataset of 170 samples, and prediction was found within the first two options (differential) in 86% of the metastasis cases (first prediction was correct in 68% of cases). Rosenwald and colleagues (23) measured expression levels of 48 miRNAs by qRT-PCR and predicted the ToO among 25 possible classes, corresponding to 17 distinct tissues and organs. The biologically motivated classifier combined the predictions generated by a binary decision tree and K-nearest neighbors (KNN). The classifier was validated on an independent, blinded set of 204 FFPE tumor samples; the test predictions correctly identified the reference diagnosis in 85% of the cases. In 66% of the cases, the two algorithm predictions (tree and KNN) were in agreement on a single-tissue origin, which was identical to the reference diagnosis in 90% of cases. A second-generation assay (24) that identified 42 tumor types using expression of 64 miRNAs was reported to have an overall assay sensitivity on a validation set of 509 independent samples of 85%.

Retrospective and prospective molecular profiling studies: effort in CUP

The challenge with validating a ToO test for CUP is that, by definition, the diagnosis of the primary cancer cannot be verified. Thus, estimates of ToO test accuracy have depended on indirect metrics or the later appearance of latent primaries.

Performance based. Over the past several years, several groups (22, 25–30) have reported on performance-based studies for CUP. One of the first studies evaluated the feasibility of a 10-gene RT-PCR assay to identify the ToO in CUP patients (25). The assay was successfully done in 104 patients (87%), and a ToO was assigned in 63 patients (61%). The ToO most commonly identified were lung, pancreas, and colon; most of these patients had clinical and pathologic features consistent with these diagnoses. Our group conducted a miRNA study in 87 patients that quantitated 48 miRNAs and assigned one of 25 tumor diagnoses by using a biologically motivated binary decision tree and KNN. The assay result was consistent or compatible with the clinicopathologic features in 84% of CUP cases (28). The second-generation assay using 64 miRNAs identifying 42 tumor types has been studied in 52 CUP patients with an 88% concordance with clinicopathologic evaluation (24).

Greco and colleagues (29) presented a retrospective study that compared the 92-gene assay results to the latent primary cancer diagnosed over the course of the patient’s life/treatment. Fifteen of the 20 assay predictions (75%) were correct, corresponding to the actual latent primary sites identified after the initial diagnosis of CUP.

Outcomes based. Hainsworth and colleagues (30) recently published a prospective single-arm study evaluating the role of the 92-gene assay to predict the ToO and assay-directed site-specific therapy in CUP patients. The authors concluded that the median overall survival (OS) of 12.5 months (95% confidence interval, 9.1–15.4 months) for patients who received assay-directed site-specific therapy compares favorably with previous studies using empiric therapy. Patients with more responsive tumor types had a longer survival duration compared with those with less responsive tumor types. Biliary and urothelial profiles comprised 33% of the predictions.

Although these findings are intriguing, a firm conclusion cannot be drawn from this study. As with many other cancers, the OS in CUP patients receiving empiric therapy has improved over the past decade, and similar survival durations have been noted in other studies with empiric modern chemotherapy combinations (31). Furthermore, it is difficult to evaluate the exact role of molecular profiling in any study without the important components, namely, clinicopathologic evaluation. Finally, the challenges of confounding variables, including use of subsequent lines of (empiric) therapy and the heterogeneity of these cancers, make it difficult to interpret the survival data in the absence of randomization.

Emerging themes with ToO molecular profiling and CUP cancers

CUP researchers are enthusiastic about the ToO profiling technology, and several years of study have answered many questions and left some unanswered (Table 1, Box 1). Even in the absence of direct validation (i.e., primary tumor), the indirect validation metrics using IHC, imaging, and treatment response have allowed us to extrapolate the performance accuracy of profiling studies from known to CUP cancers. What remains unclear at the present time is how best to manage substantial discordancy between clinicopathologic features and molecular profiling.

Outcomes-based studies using OS as an endpoint present a challenge. The traditional prospective randomized trial design to evaluate impact on OS is difficult because an adequately powered trial would require more than 500 patients and still run the risk of ambiguous results due to of the very heterogeneous presentations of CUP cancers. In practice, profiling assays aid diagnosis where IHC fails and are especially helpful when diverse treatment choices are planned based on the differential diagnosis.

Insufficient tumor for mRNA or miRNA retrieval is not an uncommon circumstance, especially if exhaustive IHCs have been done and in the setting of limited biopsies.
and fine-needle aspirations (~15% in clinical practice). Emerging studies comparing IHC and molecular profiling may give way to novel cost-effective algorithms.

The commercial ToO profiling companies conduct their proprietary profiling tests in their Clinical Laboratory Improvement Act certified, CAP-accredited diagnostic services laboratories. These molecular assays are currently labeled as laboratory-developed tests (LDT) and previously were categorized as in vitro diagnostic multivariate index assays products (IVD-MIA). One of these assays was cleared by the U.S. Food and Drug Administration (FDA) as an IVD-MIA.

ToO profiling assays are generally reimbursed by Medicare, although major insurance companies have not yet established a formal coverage policy for these tests. The current version of the National Comprehensive Cancer Network guidelines suggests judicious use of profiling on a case by case basis pending outcomes and comparative effectiveness data (7).

The Institute of Medicine highlighted the need for additional regulation of LDTs in the era of "-omics"-based research (32). Current FDA guidance on in vitro diagnostic devices suggests that an investigational drug exemption may be required for an investigational diagnostic procedure that is the basis for a treatment decision. The FDA is developing more specific guidance (33) for LDTs after considering input from a broad range of stakeholders.

On the Horizon

Evolving CUP definition—left with the unclassifiable?

As the CUP classification and diagnostic tools continue to develop, CUP academicians may see a shift toward the unclassifiable cancers in their academic practice. My working definition of "unclassifiable" CUP cancers includes tumors in which neither comprehensive IHC nor molecular ToO have plausible answers (often referred to as unclassified or undifferentiated neoplasms on the standard pathology and molecular profiling report) or in which there is a significant unresolvable discrepancy in the clinicopathologic and molecular profiling results.

### Table 1. Limitations of pathologic tools used for CUP diagnosis

<table>
<thead>
<tr>
<th>Considerations</th>
<th>IHC performance</th>
<th>ToO profiling performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct validation</td>
<td>Difficult in the absence of a primary (ToO validates IHC)</td>
<td>Difficult in the absence of a primary (IHC validates ToO)</td>
</tr>
<tr>
<td>Cost</td>
<td>Moderate (when overuse avoided)</td>
<td>Higher than IHC but may drop over time</td>
</tr>
<tr>
<td>Sensitivity and specificity</td>
<td>Moderate—depends on IHC antibodies and execution</td>
<td>Moderate to high for cancers in training set</td>
</tr>
<tr>
<td>Intra-/intertumoral heterogeneity</td>
<td>Not well studied in CUP</td>
<td>Not well studied in CUP</td>
</tr>
<tr>
<td>Clinical impact/utility</td>
<td>Forms the backbone of CUP diagnosis; tiered approach preferred albeit poorly implemented</td>
<td>Role is evolving; no strategy in place if results are discordant with clinicopathologic diagnosis Prospective randomized studies using OS as endpoint are a challenge</td>
</tr>
<tr>
<td>Execution challenges</td>
<td>Intra- and interoperator variability; factors affecting tissue antigenicity and stain interpretation</td>
<td>Requires special molecular pathology expertise</td>
</tr>
<tr>
<td>Diagnosis of rare cancers</td>
<td>Limited by availability of validated antibodies</td>
<td>Limited by assay training set</td>
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### Box 1. CUP and ToO assays: key points

- More confident assignment of ToO in CUP can guide management by suggesting the use of therapies shown to be effective in a class of cancers.
- Emerging data suggest that molecular profiling ToO assays have a place in the management of CUP patients. Additional trials are required to determine their role in different settings.
- Molecular profiling tools have a role in ToO determination when standard IHC is nondiagnostic.
- Their role may be limited in rarer cancer because accuracy is limited by the number and type of cancers selected for the training sets of the assays.
- Going forward, molecular profiling assays should be integrated into the pathologist’s diagnostic armamentarium with a combination/sequence of IHC and molecular profiling to optimize accuracy, reliability, and cost of ToO predictions.
- Beyond ToO determination, newer genomic methods drawn from non-CUP cancer experience attempt to use individual tumor data to help guide a personalized cancer therapy. The use of these tools in CUP is currently being investigated.
The "unclassifiable" designation on molecularly profiling does not necessarily reflect on the robustness of the assay but rather on the indistinct genetic signature of these cancers, which is unmatched in the profiling training tests. These are perhaps the best candidates for next-generation deep sequencing to unravel the biology and actionable targets.

The primary focus of this discussion is determining the CUP ToO to guide selection of a therapy that has shown effectiveness in a class of cancers. However, the other goal of molecular profiling, shared with tumors of known origin, is using individual tumor data to help guide a personalized cancer therapy. This applies to unclassifiable tumors as well as to those for which a ToO has been assigned.

Our group recently used the Sequenom (SQM) MassARRAY platform in an all-comers CUP population (34) and found that the overall mutational rate in a 25-gene hotspot assay was surprisingly low (18%). No "new" low-frequency mutations were found using a panel of mutations involving the PI3K/AKT pathway, the MEK pathway, receptors, and downstream effectors. Focusing on CUP subsets and minimizing heterogeneity where possible may provide a higher mutational yield. Mutational profiling and other tumor-specific pathway profiling tools should be evaluated in additional clinical trials, drawing on experience with non-CUP cancers. Currently, as with tumors of known origin, the incremental value of the SQM platform or other genomic tools is not well established. The optimal timing of some types of testing is also not clear (e.g., on presentation vs. progression). An initial focus could be with patients lacking a standard therapeutic option, which may include those in the two "extremes" of presentations—the good prognosis, "adenopathy" dominant, chemo-responsive patients who have already received multiple lines of therapy and those with "unclassifiable" tumors for which there are no good directed or empiric therapy options.

Molecular profiling: oncologist's or pathologist's domain?

Today, molecular profiling assays are typically requested by an oncologist based on a patient's clinicopathologic presentation (including IHC) and therapy options. Going forward, it is reasonable to ask if molecular profiling assays should be used by pathologists as part of their armamentarium of diagnostic tools to diagnose cancers. Studies have reported on the comparative effectiveness of IHC and ToO molecular profiling (35–37) in diagnosing the primary site. Diagnostically challenging metastatic samples (reference diagnosis) were evaluated in prospectively defined, blinded studies. Blinded FFPE sections were sent to pathologists involved with the study and independently evaluated by either morphology (H&E) and IHC or the ToO assay(s). The primary endpoint was concordance with the reference diagnosis. The authors concluded that the molecular profiling assay was more accurate than IHC-based diagnosis, including in patients with poorly differentiated and undifferentiated (non-CUP) samples. In one study, pathologists' accuracy and confidence increased significantly from H&E to round one of IHC stains but not much from additional rounds of stains. In the other study, in cases requiring more than nine IHC stains, the assay conducted better than IHC (69% vs. 46%, respectively).

Cost-effectiveness models with profiling and IHC

Over the past few years, the primary focus of the ToO molecular profiling field has been to evaluate its accuracy and reliability. Attempts to show the independent value of ToO profiling by measuring its impact on management decisions or survival have been less than convincing in the face of challenges relating to trial design and CUP heterogeneity.

We now have increasing evidence that ToO profiling can provide an acceptable level of accuracy in some settings and has a high degree of concordance with IHC, especially with well-defined tumors. The next step in the evolution of this tool is to integrate its use into the broader diagnostic armamentarium used in CUP, defining its role vis-à-vis other tools such as IHC, rather than viewing it as an add-on tool after other investigations are complete. Such an approach would also meet the needs of our current health-care economic environment (and bundled payments).

One could envision using an algorithm that integrates IHC and ToO molecular profiling to maximize accuracy and minimize costs, especially in patients with limited tissue, difficult-to-access tumors, malignant effusions, and poorly differentiated/undifferentiated neoplasms. In such a model, the timing and role for ToO profiling would need to be defined. For example, pathologists could use a profiling assay earlier in their tissue diagnosis, ideally after the first round of pertinent five to six IHC stains, as in the algorithm presented in Fig. 2. An algorithm such as this would continue to evolve as additional experience is gained with ToO profiling and the trade-off in costs and accuracy becomes clearer.

To be successful, such an effort would require a collaborative approach from all the relevant stakeholders including practitioners and the relevant agencies and industry groups including the FDA, the Centers for Medicare & Medicaid Services, and the College of American Pathologists. This approach may also require rationalization of the cost structure involving molecular profiling assays and IHC costs (professional and technical fees). Our ultimate goal should be to provide a cost-effective and clinically effective algorithm that leverages genomics and proteomics techniques to deliver validated new approaches to our patients. The emergence of new tools provides an occasion to greatly strengthen our diagnostics and management approaches in CUP, and we should seize this opportunity by being innovative in our thinking.
Disclosure of Potential Conflicts of Interest

G.R. Varadhachary has a commercial research grant from Rosetta Genomics and is a consultant/advisory board member of Biotheranostics and Pathwork Diagnostics.

Received January 3, 2013; revised February 25, 2013; accepted March 10, 2013; published OnlineFirst March 21, 2013.

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