Diagnostic work-up of carcinoma of unknown primary: from immunohistochemistry to molecular profiling

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Carcinoma of unknown primary (CUP) remains a common and challenging clinical problem. The aim of diagnostic work-up in CUP is to classify as specifically as possible the cancer affecting the patient, according to the broad tumour type, subtype and, where possible, site of origin. This classification currently best predicts patient outcome and guides optimal treatment. A stepwise approach to diagnostic work-up is described. Although pathology is based on morphology, the assessment of tissue-specific genes through immunohistochemistry (IHC) substantially helps tumour classification at each diagnostic step. For IHC in CUP, recent improvements include more standardised approaches and marker panels plus new markers. Tissue-specific genes are also being used in CUP work-up through molecular profiling. Large-scale profiles of hundreds of tumours of different types have been generated, compared and used to generate diagnostic algorithms. Commercial tests for CUP classification have been developed at the mRNA and microRNA (miRNA) levels and validated in metastatic tumours and CUPs. While currently optimal pathology and IHC remain the ‘gold standard’ for CUP diagnostic work-up, and full clinical correlation is vital, the molecular tests appear to perform well: in the main diagnostic challenge of undifferentiated or poorly differentiated tumours, molecular profiling performs as well as or better than IHC.

Key words: cancer, immunohistochemistry, metastatic, molecular profiling, pathology, unknown primary

introduction

This paper focuses on the contribution of immunohistochemistry (IHC) and molecular profiling to the diagnostic work-up of carcinoma, or cancer, of unknown primary (CUP) in pathology. This paper is for the 2012 conference of the European Society of Medical Oncology (ESMO). It is, therefore, aimed primarily at medical oncologists, but may also be of value to pathologists, other clinicians and patients.

CUP is a common and important clinical problem as discussed in recent clinical reviews [1, 2], guidelines [3, 4] and conferences [5]. Approximately 15% of all cancers first present, that is, cause symptoms, with metastases rather than with the primary tumour. In approximately two-thirds of these, the primary tumour becomes obvious early on during investigation [6]. The remaining cases are CUP; in a minority a primary site will be identified over time. Biopsies for pathology may be taken at any stage in the diagnostic process, from initial presentation or later, once other investigations have been negative: diagnostic difficulty generally increases in the latter.

pathological aim in CUP: cancer classification by tumour site and type

Cancer classification has traditionally been based on anatomical location(s) and tumour morphology [6]; these have been the best available guide for cancer patient management. Almost all biomarkers used in pathology for CUP are aimed at establishing cancer type, subtype and site, thus are diagnostic: this includes IHC and molecular profiling. As more specific therapies emerge, prognostic and predictive biomarkers may also become important in CUP.

In CUP, the most common sites of metastasis and thus of biopsy are: solid organs including liver, lung, bone and brain; lymph nodes especially cervical, inguinal and axillary; and peritoneal and pleural serous cavities [1, 2]. In pathology, once a biopsy is obtained, the presence of malignancy must first be confirmed. Thereafter, a stepwise approach can be taken to identify the broad tumour type, then tumour subtype, and, if adenocarcinoma, the likely site of origin, as shown in Table 1. At each step, IHC can help [6–9].

The broad tumour type for almost all ‘true’ metastatic CUPs is carcinoma. CUP is a diagnosis of exclusion, since many studies exclude other tumour types including lymphoma, melanoma and sarcoma, as well as unusual primary rather than metastatic tumours [1, 2]. Because these other cancer types nevertheless often enter the clinical and pathological differential diagnosis, they must still be considered.

Within carcinoma, the most common subtypes in CUP are adenocarcinoma (60%), squamous carcinoma (5%),...
Table 1. Cancer classification by tumour type, subtype and primary site; and useful immunohistochemistry (IHC)

<table>
<thead>
<tr>
<th>Stepwise approach to CUP work-up</th>
<th>Useful immunohistochemical biomarkers</th>
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<tbody>
<tr>
<td><strong>Step 1: identify broad cancer type</strong></td>
<td>Cytokeratins and other epithelial markers, e.g. AE1/3, CK7, CK20, CK5, EMA S100, Melan-A, HMB45</td>
</tr>
<tr>
<td>Carcinoma</td>
<td><strong>CLAR, CD20, CD3, CD138, CD30 etc.</strong></td>
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<tr>
<td>Melanoma</td>
<td><strong>Vimentin, actin, desmin, S100, c-kit etc.</strong></td>
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<tr>
<td>Lymphoma/leukaemia</td>
<td><strong>(Specific markers)</strong></td>
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<tr>
<td>Sarcoma</td>
<td><strong>(Neuro-glial tumours)</strong></td>
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<tr>
<td>(Neuro-glial tumours)</td>
<td><strong>(Sarcoma)</strong></td>
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<tr>
<td><strong>Step 2: if carcinoma or related, then identify its subtype</strong></td>
<td><strong>CK7, CK20, PSA</strong> plus other adenocarcinoma markers</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td><strong>CK3, p63</strong></td>
</tr>
<tr>
<td>Squamous carcinoma</td>
<td><strong>(TCC: CK7&amp;20, urothelin)</strong></td>
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<tr>
<td>(Transitional cell carcinoma)</td>
<td><strong>Chromogranin, CD56, synaptophysin, TTF-1</strong></td>
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<tr>
<td>Neuroendocrine carcinoma</td>
<td><strong>RCC, CD10, PAX8, Napsin A</strong></td>
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<tr>
<td>Solid organ carcinoma: kidney (renal)</td>
<td><strong>Hepar1, CD10, glypican-3</strong></td>
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<tr>
<td>Solid organ carcinoma: liver (hepatocellular)</td>
<td><strong>TTF-1, thyroglobulin, PAX8</strong></td>
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<td>Solid organ carcinoma: thyroid</td>
<td><strong>Melan-A, inhibin</strong></td>
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<td>Solid organ carcinoma: adrenal</td>
<td><strong>OCT4, PLAP, HCG, AFP</strong></td>
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<tr>
<td>(Germ cell tumour)</td>
<td><strong>Calretinin, mesothelin, WT1, D2-40</strong></td>
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<tr>
<td>(Mesothelioma)</td>
<td><strong>See separate table</strong></td>
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<tr>
<td><strong>Step 3: If adenocarcinoma, then predict possible primary site(s)</strong></td>
<td><strong>e.g. lung, pancreas, colon, stomach, breast, ovary, prostate</strong></td>
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<tr>
<td>e.g. lung, pancreas, colon, stomach, breast, ovary, prostate</td>
<td><strong>AFP, alpha fetoprotein; CLA, common leucocyte antigen; EMA, epithelial membrane antigen; HCG, human chorionic gonadotrophin; PAX8, paired box gene 8; RCC, renal cell carcinoma marker; WT1, Wilms’ tumour protein.</strong></td>
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</table>

Based on [6–9]. The markers in bold are especially useful. For tumour diagnosis, IHC results must be integrated with the morphology, other IHC markers and clinical findings: for example, CK20 is positive in colorectal adenocarcinoma but also in transitional cell carcinoma and Merkel cell carcinoma.

Cancer classi
cation by tumour type, subtype and primary site

CUP biomarkers: specific tissues and issues

Before applying biomarkers in CUP, let us consider what these biomarkers represent. Cancer classification is based on the differences in the appearance of different cancers and on their resemblance to the corresponding normal tissues. Tissues are aggregates of cells of similar type and function. Differences between tissues, normal or malignant, are based on the differences in their gene expression. The human genome contains ~30 000 protein-coding genes [10]. Approximately 12 000 genes are active, i.e. expressed at mRNA and protein levels, in each tissue [10]. Gene expression depends on the underlying DNA sequence and is regulated at multiple levels including epigenetic and via microRNAs (miRNAs).

Of the 12 000 genes active in each tissue, 8000 are widely expressed and involved in basic cellular functions, e.g. protein and energy production or cell proliferation. A subset of active genes is specific to one or a few tissue types, related to its mature function, i.e. its differentiation. Such tissue-specific or tissue-restricted genes are often regulatory genes or protein products [10]. Regulatory genes include transcription factors, especially homeobox genes controlling tissue development and maintenance, e.g. thyroid transcription factor-1 (TTF-1) in lung and thyroid. Protein products may be secreted or expressed in or on the cell and include cytokeratin, e.g. CK7 and CK20 and prostate-specific antigen (PSA) [6].

Just as tumours resemble morphologically the tissue from which they were derived, so tumours generally still express some tissue-specific genes, not only in primary cancers but also in metastases [11]. This is demonstrated using the
bioinformatics technique of unsupervised clustering applied to cancer mRNA or miRNA gene expression profiles. Samples group (cluster) together by similarity; those with the most similar gene expression cluster most closely. In such experiments, cancers of one histological type or subtype cluster together and with the corresponding normal tissue; likewise paired primary and metastatic tumours usually cluster together [11]. Clustering is due at least partly to tissue-specific genes, which explains their utility as diagnostic cancer biomarkers.

This raises two general issues for diagnostic work-up in CUP. First, tissue-specific gene expression is better retained in well-differentiated than in poorly differentiated cancers [11]; the latter are thus harder to diagnose. Second, metastatic tumours are usually harder to diagnose than the corresponding primaries [12, 13]; metastases might have lower expression of tissue-specific genes than the primary tumour.

**IHC for CUP**

IHC is fundamental to cancer classification in pathology. Most IHC biomarkers have been identified on a candidate basis, as single genes involved in a particular process. For most diagnostic purposes, however, IHC antibodies are used in a panel, including markers expected to be positive and negative in different tumours. This should mean that no single aberrant IHC stain causes incorrect diagnosis. IHC results depend on both the staining technique and microscopic interpretation: both may vary and their optimal performance is crucial.

Table 1 presents IHC markers used in tumour typing and carcinoma subtyping [7, 14, 15]. Most markers are familiar [6]; newer ones include OCT4, a transcription factor expressed in germ cell tumours, and D2-40, found in mesothelioma and other tumours [6].

Table 2 presents IHC markers for the prediction of primary site in adenocarcinoma. These include the classic CK7 and CK20, and newcomers such as Napsin A, a lung aspartic protease [9]; paired box gene 8 (PAX8), a Paired-box transcription factor regulating gynaecological tissues, kidney and thyroid [8]; and NKX3.1, a prostatic tumour suppressor gene [16]. The ESMO guidelines for CUP suggest a similar IHC work-up [3]. The NICE (National Institute for Clinical Excellence) guidelines from the UK suggest a minimum of CK7, CK20, PSA or oestrogen receptor (ER), placental alkaline phosphatase (PLAP) and TTF-1 [4].

Primary site can also be predicted in well-differentiated neuroendocrine tumours, in which TTF-1, CDX2 or PDX1 positivity suggests lung, gastrointestinal or pancreatic origin, respectively [9]. TTF-1 positivity is not site-specific in poorly differentiated neuroendocrine carcinoma. No IHC biomarkers are established to predict the primary site in squamous carcinoma although some have been suggested [17].

**diagnostic work-up for CUP including IHC: performance and practice**

Pathology is an interpretative, thus subjective, discipline. In practice, the stepwise diagnostic approach is often subconscious. Not all steps may be needed; at each step, the pathologist chooses whether to use IHC and, if so, which markers; and different pathologists vary in approach. Faced with a biopsy from multiple liver tumours, one pathologist may report metastatic adenocarcinoma based solely on the H&E appearance of epithelioid cells with focal glandular structures. Another may confirm carcinoma using IHC with positive cytokeratin and negative S100 (Table 1); then may, in the same or a second ‘round’ of IHC, use differential cytokeratins to confirm adenocarcinoma and exclude other carcinomas; and finally may apply markers of primary site (Table 2).

CUP biopsy or cytology specimens are usually small: it may be difficult to cut 25 sections from a tissue core. Each ‘round’ of IHC takes 1–2 days. Therefore, only limited IHC markers can be tested: the average in CUP is 7–8 [18, 19] (range 0–27) [20]. Marker selection is thus crucial; one barrier to correct tumour classification is simply not considering and applying the most appropriate markers.

Two common diagnostic difficulties in CUP work-up are poorly differentiated or undifferentiated cancer and better differentiated carcinoma, especially adenocarcinoma, without an obvious primary site. In either, there may be one likely diagnosis, multiple differential diagnoses [20] or the diagnosis may be truly unknown. For poorly differentiated carcinoma, a conscious stepwise approach helps to ensure that all appropriate tumour classes are considered; and therefore that

<table>
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<tr>
<th>Table 2. Prediction of the primary site of adenocarcinoma using selected immunohistochemistry (IHC)</th>
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<tr>
<td><strong>PSA or NKX3.1</strong></td>
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<tr>
<td>Prostate</td>
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<tr>
<td>Lung</td>
</tr>
<tr>
<td>Breast</td>
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<tr>
<td>Ovary serous</td>
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<td>Ovary mucinous</td>
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<td>Pancreas</td>
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<td>Stomach</td>
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<td>Colon</td>
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</table>

GCDFP-15, gross cystic disease protein 15; WT1, Wilms’ tumour protein.

+ ≥ 90%, ± = 50%–90%, + = 10%–50%, – = ≤ 10%.

Based on [6, 8, 9, 16].
the IHC panel(s) selected will enable their diagnosis. For adenocarcinoma, pancreatic and gastric origins are especially difficult to establish because their morphology and IHC are suggestive but not specific, and diagnostic dilemmas about the primary site are often pairwise, including pancreatico-biliary versus gastric, gastric versus colorectal and pancreatico-biliary versus ovarian [6]. Diagnostic difficulties exist when no specific, or a few non-specific, IHC markers are positive; when IHC is hard to interpret due to insufficient tissue, necrosis or poor staining; or when IHC results conflict with the morphology or clinical scenario. The ‘unmet clinical need’ for molecular profiling is to achieve better classification in these difficult-to-diagnose tumours, especially where treatment options include tumour-specific versus empiric CUP therapy [21].

How does IHC currently perform in cancer classification? A recent meta-analysis identified only five to six large, ‘blinded’ studies of multiple (>3) IHC markers across many (>50) different tumours [12]. For an individual diagnostic marker, specificity should be over 96% and ideally 99% [6, 22]; sensitivity should be as high as possible, but at least over 50%. Across the meta-analysis, IHC sensitivity was remarkably consistent, ~82% in mixed primary and metastatic tumours and 66% in metastases alone [12]. This confirms that metastatic tumours are more difficult to classify than primaries by IHC and provides a baseline above which molecular profiling should perform to be potentially clinically useful.

**molecular profiling for CUP: development and characteristics of tests**

Large-scale molecular profiling applicable to CUP has been achieved at mRNA, miRNA, DNA and epigenetic levels. Three tests, based on mRNA or miRNA, are commercially available: Pathwork Tissue of Origin (TOO) test [23], bioTheranostics’ Cancer Type ID (CTID) [20] and miRview mets2 [24]. Their development, characteristics, validation and clinical impact will be described and compared [25–29].

The principle underlying these molecular CUP tests is that ‘different tissue types have distinct RNA profiles’ [23]. To develop each test, gene expression profiles were generated for hundreds of different tumours. Using bioinformatics, a subset of discriminatory genes was identified and diagnostic algorithms built for cancer classification. Knowledge of the biopsy site enables distinction of genes expressed by the tumour and by the organ biopsied [30]. All three tests have evolved significantly since their initial development and are now termed ‘second generation’.

The TOO test analyses 2000 mRNAs by microarray [23]. Its database contains 2140 tumours of 58 types and subtypes, grouped into 15 classes: breast, bladder, colorectal, gastric, testicular germ cell, hepatocellular, kidney, non-small-cell lung, non-Hodgkin’s lymphoma, melanoma, ovarian, pancreatic, prostate, sarcoma and thyroid [23]. The TOO test reports similarity scores (SS) compared with each of the 15 tumour classes: for one sample, the 15 SS total 100. The higher the SS, the more likely that diagnosis is: thus a prediction with SS > 60 agrees with 90% of reference diagnoses; as SS falls, agreement declines until SS < 5 rules out that tumour class with >99% confidence [23, 31]. Most reports provide one highly likely diagnosis and rule out at least 12 tumour classes. Additional tests, based on 10 more tumour types and subtypes, enable the separation of ovary from endometrium, and lung from head and neck cancer: primary site of squamous carcinoma is thus also being predicted by molecular profiling.

CTID analyses 92 mRNAs by RT-PCR [20]. Its database contains 2206 tumours of 30 main types and 54 subtypes [20]. (This database was also used by Agenda in the Netherlands to develop a microarray-based test, CUPPrint, which is published but no longer marketed [28].) CTID reports one main cancer class, e.g. ovary, and subclass, e.g. ovary serous, with its probability. CTID also reports any other main cancer classes with >5% probability and those with <5% probability, which are ruled out [20].

miRview mets2 analyses 64 miRNAs by microarray [24]. Its dataset contains 1282 tumours of 42 types and subtypes [24]. Each test miRNA profile is subjected to two classification algorithms, with separate outputs: if these diagnoses agree, confidence is higher. miRview mets2 reports one or two of either the 42 tumour types or seven combined classes: sarcoma, kidney, thyroid, neuroendocrine lung, germ cell, astrocytic or oligodendrogial, and pancreatico-biliary adenocarcinoma [24].

All three tests can use formalin-fixed, paraffin-embedded tissue or cytology specimens [20, 24, 31]. Minimal tumour is required: only a few histological sections, less than for most CUP IHC. Most (>60%) of the sample should comprise viable tumour and cytology [31] and 95% sensitivity in 37 different tumours [23]. The TOO test showed 94% sensitivity in 17 malignant effusions by cytology [23]. In further studies, the TOO test showed 87% sensitivity in 283 poorly differentiated cases [23]. Its database contains 790 known primary and metastatic cancers [33]. Sensitivity of the TOO test declines until SS < 5 rules out that tumour class with >99% confidence [23, 31]. Most reports provide one highly likely diagnosis and rule out at least 12 tumour classes. Additional tests, based on 10 more tumour types and subtypes, enable the separation of ovary from endometrium, and lung from head and neck cancer: primary site of squamous carcinoma is thus also being predicted by molecular profiling.

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**molecular profiling for CUP: validation in known tumours**

Molecular tests thus developed require validation in independent tumour sets, containing primary tumours and/or metastases from known primaries; poorly differentiated tumours are more realistic. Only a few validation studies have been published for the second generation tests, some as abstracts only. Overall, all three tests have a high specificity of ≥99%. Their sensitivity in tumours of known origin ranges from 72% to 95%; sensitivity is often but not always lower in metastases than in primary tumours [25].

The TOO test showed 87% sensitivity in 283 poorly differentiated or undifferentiated primary tumours and 91% sensitivity in 179 known metastases [23]. In further studies, the TOO test showed 94% sensitivity in 17 malignant effusions by cytology [31] and 95% sensitivity in 37 difficult primary and metastatic cases [32].

CTID showed 83% sensitivity in 187 known primary tumours [20]. In an independent study, CTID showed sensitivities of 87% for tumour typing and 82% for subtyping in 790 known primary and metastatic cancers [33]. Sensitivity
was similar in primary and metastatic cancers, in tumours across the range of differentiation and with limited tissue [33]. In further studies, CTID showed sensitivities of 78% for tumour typing and 72% for subtyping in 132 high-grade known metastatic cancers [18] and predicted the primary site in 95% of 75 primary and metastatic neuroendocrine tumours [34].

miRview mets2 showed 85% sensitivity in 509 known primary and metastatic tumours [24]; in the 82% of samples in which both algorithms predicted the same diagnosis, sensitivity was 90%.

molecular profiling for CUP: application in CUP

We will now consider the performance of these second generation tests in unknown cases. By definition, CUP generally lacks a definitive diagnosis, but the molecular prediction may be compared with the initial clinical diagnosis, with the pathology and IHC prediction and with the eventual diagnosis after follow-up and/or autopsy.

The TOO test yielded a prediction in 96% of 45 CUPs [21], including 11 lung, 6 pancreas, 6 sarcomas, 5 ovary and 4 colon. Most appeared clinically appropriate; those which did not include five sarcoma predictions, in which the tumours showed cytokeratin positivity by IHC, suggesting carcinoma.

CTID yielded a prediction in 91% of 815 submitted cancers of indeterminate or unknown primary [19]. CTID agreed with submitted diagnoses in 74%–78% of 300 mainly poorly differentiated and metastatic submitted cancers [20]. Predictions included more rare tumours than expected, including cholangiocarcinoma, small intestinal adenocarcinoma, ovarian mucinous tumour, neuroendocrine carcinoma and Merkel cell carcinoma [20]. In a third study of CUP patients in whom the primary site became known, CTID provided 20 predictions: 75% were correct; 10% were indeterminate, truly being lung and 15% appeared incorrect with predictions being sarcoma, germ cell and intestinal adenocarcinoma and true diagnoses being lung, pancreatic and gastro-esophageal cancers [35].

miRview mets2 agreed with clinico-pathological data in 88% of 55 brain CUPs [24]. In a second study of 92 CUP patients, miRview mets2 agreed in 92% of 84 assessable cases: the seven incorrect predictions had a final diagnosis of lung, head and neck or pancreas [36].

molecular profiling for CUP: limitations and strengths compared with IHC

Each molecular test may have diagnostic difficulties made evident through careful validation studies, of which few have yet been published for miRview mets2. The TOO test has two specific potential issues. First, its results are reported as one of 15 classes; the tumour subtype at a given site does not appear to be described, although the original database included most subtypes. Second, the latter lacked certain tumour types (termed ‘off-panel’), including neuroendocrine tumours, cholangiocarcinoma and mesothelioma [23], which is important because neuroendocrine tumours are ‘good prognosis’ CUPs and cholangiocarcinoma enters the differential diagnosis for liver metastases. As the test information states [37], ‘It is not possible to distinguish a tumour type if not included in the original diagnostic panel.’ One cannot know in advance whether a tumour is off-panel; and off-panel tumours yield indeterminate or incorrect results [37].

Certain diagnoses may be difficult. For the TOO test, sarcoma predictions appeared incorrect in five of six CUPs [21] and 16 of 40 known tumours [23]. For all three tests, but perhaps most for CTID and least for miRview mets2, common incorrect diagnoses include pancreatic, colonic and gastro-oesophageal cancer and their separation, and lung cancer [33, 35]. The problem appears less in second than in first generation tests [13, 23, 30, 35, 37, 38]. Diagnostic difficulty is greater when the tumour is poorly differentiated or has atypical morphology for its site; and the test may be confounded by insufficient tissue, ‘off-panel’ cases or normal tissue from the biopsy site [30, 37].

The strengths of molecular profiling are shown by comparison with IHC in two of the above studies. CTID was more sensitive than optimal IHC in 132 high-grade metastases, for tumour typing (78% versus 68%) and subtyping (72% versus 61%) [18]. CTID was more sensitive than IHC for lung, bladder and breast, and of similar sensitivity for gastrointestinal and kidney tumours [18]. miRview mets2 agreed with the final diagnosis in 92% of 84 CUPs, compared with 70% for IHC [36]. These IHC sensitivities are similar to those in the IHC meta-analysis [12]. In these studies, molecular profiling was more accurate than IHC in metastases, confirming its potential clinical utility.

molecular profiling for CUP: clinical impact

What difference might these molecular tests make to diagnosis and management [19, 20, 39, 40]? One good example is a retrospective analysis of 107 patients with poorly differentiated or metastatic cancer who had undergone extensive clinico-pathological evaluation [39]. TOO testing affected the working diagnosis as follows: 14 patients still had site unspecified; 30 changed from unspecified to a specified site; 36 still had the same specified site; 24 had a change of the specified site; and 3 had a specified site changed to unspecified. Overall, the diagnosis changed in 57 (53%) and clinical management changed in 72 patients (65%), including: ruling in or out further investigations; changing specific treatment; and facilitating hospice referral. Confirming a diagnosis can be as helpful as changing it [39]. The figures appear similar for CTID which yielded a confirmatory prediction in 47%–55% and a new diagnosis in 36%–37% of up to 815 submitted cases [19, 20].

The impact of molecular tests on the patient outcome is less clear: studies are few and not randomised. For CTID, CUP patients whose molecular profiles suggested colorectal tumour, and who then received colorectal-specific therapy, had survival times longer than historical CUP controls but similar to
patients with known metastatic colorectal cancer [41]. In a further study, CTID was used to direct site-specific therapy in CUP patients, yielding a median overall survival (12.2 mo) better than survival for historical controls receiving empirical CUP therapy [42]. These studies suggest that molecular profiling may lead to improved survival in those treated accordingly; but further studies are needed to confirm and clarify the benefit.

Cost and logistics also need consideration: commercial molecular profiling is an external test, which is more expensive than IHC but less expensive than many therapies.

**molecular profiling for CUP: further tests**

Most other molecular profiling approaches have been described in a single paper, with initial validation, often including CUP specimens. Some are broad-based assays, classifying all likely tumours, like the commercial tests [43–45]; others are more specific, classifying tumour subsets [14, 46, 47]. Most assays involve pre-specified gene sets [14, 43, 44, 46, 47], like the commercial tests, and have been developed similarly; a few are more flexible [48, 49]. Most have sensitivities of 78%–90% [43, 44, 48], similar to the commercial tests.

mRNA-based assays include a broad 79-gene assay with 13 classes and 89% accuracy [43]; and a specific 10-gene RT-PCR assay, for six primary sites of adenocarcinoma [46, 47]. Other assays are based on miRNA [44] and methylation [45]. Two more papers describe *de novo* prediction of tumour class by comparison with a tumour database but without using predefined gene sets, based on miRNA [48] or high-throughput sequencing [49].

Lastly, Centeno et al. described an integrated stepwise approach with diagnostic steps involving: identification of carcinoma; subtyping into adenocarcinoma, neuroendocrine carcinoma, squamous carcinoma and urothelial carcinoma; then prediction of primary site in each subtype. Each step is based on the morphology and IHC plus, where necessary, mRNA profiling using separate gene sets: at each step, accuracy was 87%–90%. This paper demonstrated the integration of (subset) molecular profiling into a standard stepwise pathology approach [14].

**further integration of tests, conclusions and future**

It can be seen that pathology with IHC and molecular profiling are not completely independent, but represent ‘different sides of the same coin’. First, IHC and molecular profiles use many of the same tissue-specific genes, including PAX8, CDX2, PSA and S100 [6, 13, 38]. Second, they perform in parallel: tumour types easy to distinguish on morphology and IHC are also distinct on molecular testing; tumours hard to diagnose on morphology and IHC may also be challenging for molecular tests, e.g. pancreatic adenocarcinomas. Given these similarities, where can molecular profiling make an impact?

Pathology, with IHC where necessary, is likely to retain its place as gold standard in tumour classification, especially where the tumour is primary and/or is well or moderately differentiated and/or has classical IHC results and appropriate clinical findings; no publications have suggested otherwise. However, in already well worked-up poorly differentiated and/or metastatic tumours, including CUP, molecular profiling performs well, with sensitivities of 72%–95% and may outperform optimal IHC by 10%–20%, according to initial studies. For CUP patients, molecular profiling may change the diagnosis in around half, and affects management in most.

Molecular profiling could thus contribute to diagnosis of poorly differentiated and/or metastatic tumours. In these, the diagnosis may be truly unknown or there may be an intractable differential diagnosis of two or more possibilities [20]. It is not clear whether profiling could be a valuable first-line option where tumour tissue or turnaround time is limited. Profiling appears unhelpful in necrotic tumour. Of the CUP molecular tests, a few address specific differential diagnoses and are, therefore, directly integrated with pathology. Most, including the commercial tests, are ‘stand-alone’, for diagnosis across almost all likely tumour types; their integration involves comparison of the molecular prediction with the morphology and IHC and a careful overall interpretation to ensure that the diagnosis fits the clinical context [37].

For the future, further studies are needed on the performance and comparison of molecular profiling and IHC in large cohorts of poorly differentiated and/or metastatic tumours, including CUPs; and the impact of molecular profiling and subsequent specific therapy on survival and other outcome measures in patients with CUP. IHC and molecular classifiers should be flexible to enable tumour classes to change as new therapies emerge; ideally, further development should include better classification of the remaining difficult-to-diagnose tumours, e.g. pancreatico-biliary and gastric.

Recent developments in molecular profiling and IHC mean that the unknown primary is gradually becoming more known; we hope that these improvements will continue over the next decade in parallel with advances in treatment and outcomes for patients with CUP.

**disclosure**

KAO has no financial interests in any company mentioned in this paper; she is conducting a research study, which includes collaborations with the company BioTheranostics and with the research group of Professor David Bowtell, which are mentioned in this paper; their contribution is intellectual and to provide molecular analyses but not otherwise financial. JLD declares no conflicts of interest.

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