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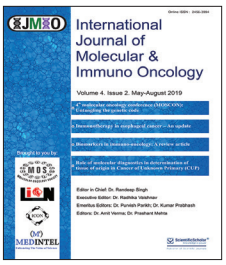
International Journal of Molecular and Immuno Oncology



Review Article

Role of molecular diagnostics in determination of tissue of origin in Cancer of Unknown Primary (CUP)

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Received : 14 March 19
Accepted : 17 March 19
Published : 20 May 19

DOI
10.25259/IJMIO-4-050

Quick Response Code:

ABSTRACT

Cancer of unknown primary (CUP) is a condition seen in around 2–10% of patients with malignancy where the standard diagnostic workup fails to identify a specific tissue from which the tumor has arisen. As the survival of these patients is short, around 6–10 months, it becomes important to adopt newer technologies which aid in identifying the type of primary tumor so that the treatment can be guided based on the cancer type. Commonly used immunohistochemistry (IHC) panels may sometimes fail in providing a diagnosis as the CUP tissues contain usually either undifferentiated or dedifferentiate cells. Molecular cancer-classifier assay (MCCA) is an emerging diagnostic modality which is based on either gene expression profiling of the tumors or identification of epigenetic pattern of the tissues to make a probable diagnosis of the tissue from which the primary tumor may have arisen. Studies have shown that when tailored site-specific treatment was administered based on the outcomes of the MCCAs; an improvement in survival of the patients was seen. Recent NCCN guidelines suggest that the MCCAs should be used judiciously and on a case-to-case basis. The 2018 consensus statement from the Spanish Society of Pathology and the Spanish Society of Medical Oncology recognizes that the MCCAs are helpful when used to complement IHC, allowing for more accurate diagnosis of the primary site of tumor.

Keywords: Cancer of unknown primary, Molecular diagnostics, Tissue of origin.

INTRODUCTION

Majority of patients with Cancer of unknown primary (CUP) receive empiric antineoplastic regimens (most often a platinum combined with either paclitaxel or gemcitabine). The empiric regimens do provide benefit to some patients but the overall median survival for this group has remained approximately 9 months. Search is on to find reliable diagnostics which would give insight to the treating physician about possible origin of these CUPs. Molecular diagnostics in particular have evolved in the recent years as a way to identify tissue origin of the CUPs. This article discusses the relevance of the molecular diagnostics in CUPs.

OVERVIEW OF CANCER OF UNKNOWN PRIMARY (CUP)

Identification of the primary site of tumor can be challenging in around 2–10% of malignancies, especially in poorly differentiated and undifferentiated cancers, which form a diagnostic dilemma for histopathologists.[1] When the routine diagnostic workup fails to identify the site of origin at the time of diagnosis, these occult malignancies are termed as "CUP."[2] Around 80% of difficult

to diagnose cancer patients present with commonly affected metastatic sites - liver, lymph nodes, bone, and lung do not fit into a favorable subset.[3] These patients have an average survival ranging from 6 to 10 months.[4]

IMPORTANCE OF IDENTIFYING TISSUE OF ORIGIN (TOO) IN MANAGEMENT OF CUP

Institution of tumor-specific therapy

Evidence suggests that patients who receive a primary tumor diagnosis have longer survival rates compared to those never get a definite identification of the primary site.[5] It is this expectation of improved outcome with tumor-specific therapy that motivates the search for the primary site. The search has recently intensified with availability of new targeted drugs introduced as therapy for specifically indicated tumor tissue types. Furthermore, a definitive primary site relieves patient's anxiety over uncertain diagnosis. Hence, it is important to diagnose the cancer type in patients with difficult to diagnose cancers to further guide the choice of site-specific therapy.[6]

CHALLENGES IN MANAGEMENT OF CUP

The rationale for identifying the primary tumor has been to provide patients with cancer-specific treatment recommendations.[7] Evidence suggests that relatively site-specific regimens used in approximately 20% of patients belonging to favorable subsets of difficult to diagnose cancer patients have better survival than the patients belonging to the unfavorable subset.[3] With optimal management, only 30–60% of cases of the CUP patients demonstrate long-term disease control.[7]

Majority (80%) of patients with CUP receive empiric antineoplastic regimens (most often platinum combined with either paclitaxel or gemcitabine). The empiric regimens do provide benefit to some patients (with a few long-term survivors documented), but the overall median survival for this group has remained approximately 9 months.[4]

METHODS FOR DETERMINING THE TOO

Besides the histopathological assessments and full body imaging, two widely used approaches are available for determining the primary tumor:[7]

- Immunohistochemistry (IHC)
- MCCAs.

IHC

Numerous panels of immunohistochemical stains are used by histopathologists to identify the primary site of origin for metastatic cancers, particularly when limited diagnostic information is obtained with morphologic characteristics. IHC has been proven to be tool for differential diagnosis and to effectively rule out one or the other candidate tissues.[8]

Advantages

- IHC evaluations complement the pathologist's diagnostic review of the hematoxylin and eosin-stained slide by testing a series of markers in a systematic approach to determine the likely primary site.[7]
- The IHC tests are cheap and easily available in most parts of the country.

Disadvantages

- The IHC assays are performed on a serial 5-μm thick section from a formalin-fixed, paraffin-embedded (FFPE) tumor block, which is challenging when the tumor resection, cytology sample, or biopsy sample are limited.[7]
- Only a minority (about 30%) of patients with difficult to diagnose cancers receive an accurate single cancer type diagnosis based on IHC analyses.[6]
- The choice and usage of IHC stains vary from institution to institution. In addition, interpretation and reporting of IHC results remain highly subjective.[7]
- Deficient performance by the IHC panels is observed in determining the TOO in metastatic tumors. Consequently, the use of IHC panels for site determination of metastatic tumors poses greater challenge.[8]

MCCAs

MCCA is an emerging diagnostic method that empowers prediction of the tissue of tumor origin by identifying site-specific gene expression profiles.[7]

MCCA in combination with histopathology and IHC empowers clinical evaluation of tissue-of-origin diagnosis in >90% of difficult to diagnose patients.[9] The gene expression tests have been developed as an adjunct to morphological evaluation and IHC analysis in the assessment of patients with dubious primary cancer.

These molecular profiling assays either use microarrays, reverse transcription polymerase chain reaction (RT-PCR), or epigenetic to quantify mRNA or microRNA. The microarray-based assays can measure the expression levels of thousands of gene markers, whereas the RT-PCR-based assays focus on a smaller subset of 10–100 gene markers.[9]

GENE EXPRESSION PROFILE IN THE DIAGNOSIS OF PRIMARY SITE OF TUMOR

Gene expression profiling (GEP) tests aid in the diagnosis of difficult to diagnose tumors. These tests use an algorithm-based approach to predict the most probable primary site for

a particular sample.[1] Advances in microarray technology have enabled the development of gene expression signatures of known tumor types for predicting the primary tumor site.[9] TOO test discussed below is a good example of GEP test.

TOO test

The TOO test by cancer genetics incorporated (CGI, USA) is a microarray-based gene expression test that uses the expression levels of 2000 genes to classify tumors by similarity scores (SS) into 15 sites of origin.[4] The US FDA for the 1st time approved the TOO test for GEP of tumors in June 2010.[10]

The TOO test includes 15 tumors in the panel such as tumors of prostate, ovary, thyroid, breast, lung (non-small cell), gastric, colorectal, pancreatic, liver, urinary bladder, kidney as well as melanoma, non-Hodgkin's lymphoma, sarcoma, and testicular germ cell tumor.[11]

Methodology of TOO test

The CGI laboratory receives FFPE blocks or unstained slides and extracts, and amplifies the messenger RNA from the tumor tissue. The RNA profile generated from the microarray analysis is compared to profiles from known cancers in the database, and a similarity score is generated. A similarity score is a measure of the similarity of the RNA expression pattern of the specimen to the RNA expression pattern of the indicated tissue. SSs range from 0 (low) to 100 (high) and sum to 100 across 15 tissues on the panel. A list of markers and a set of coefficients are combined to produce 15 SSs, each corresponding to the probability that the input specimen has a molecular signature of the corresponding TOO.[9]

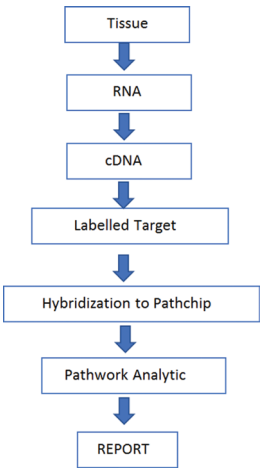
TOO test identifies primary site with:[10]

- 89% positive percent agreement (akin to sensitivity)
- 99% negative percent agreement (akin to specificity).

Benefits of using TOO test

1. It can help in diagnosis of cases in which the location, histology, or IHC results make it difficult to dependably diagnose the primary site.[4]
2. In patients with unidentified primary sites, the recommended first-line chemotherapy for most patients is likely to change with the utilization of the TOO test results.[12]
3. Decreased requirement for repeated testing, examinations, and imaging/biopsy procedures.[13]
4. Prospects to enter appropriate clinical trials.[13]
5. Vital information in evaluating one's familial risks for cancer[13]
6. Knowing the primary tissue type with greater certainty helps physicians choose the most appropriate treatment regimens.[13]

TOO test workflow is below:[9]



Clinical utility of TOO test

For predicting primary tumor site in challenging situations as mentioned below:[13]

- The tumor is poorly differentiated or undifferentiated
- There is an unanswered differential diagnosis of two or more cancer types
- The specimen is small, constraining the diagnostic workup and limiting prognostic studies
- The patient has a history of multiple cancers
- IHC is inconclusive or conflicting after the few rounds
- Histology and clinical history differ on the diagnosis
- There is atypical distribution of metastases
- When the patient fails to respond to treatment and diagnosis is questioned.

Validation of the TOO test[9]

A total of 462 tumor specimens consisting of metastatic and poorly differentiated primary tumors which had a reference diagnosis were included in a blinded validation study conducted in three different laboratories. The accuracy was determined by comparing the TOO test to the reference diagnosis. The positive percent agreement of the test with reference diagnosis was 88.5 (akin to sensitivity) and the negative percent agreement (akin to specificity) was 99.1.

Limitations of the TOO test[13]

- It is not intended to subclassify or modify the classification of tumors that can be diagnosed by current clinical and pathological practice, or to predict disease course or survival or treatment efficacy, or to distinguish primary from metastatic tumor.
- Tumor types not in the test database may have RNA expression patterns that are similar to patterns in the database. Therefore, results cannot be used to distinguish

tumor types in the database from tumor types not in the database.

Epigenetic test to identify tumors of unknown origin

Epigenetics refers to heritable changes in gene expression without an alteration in the DNA sequence.[14] DNA methylation, of the carbon-5 of cytosine moieties, is the most recognized epigenetic mark in the human genome.[15] Cancer-specific signatures have been identified by DNA-methylation profiling of hypermethylated CpG islands which enable differentiation of tumor types.[16,17] A clear advantage of an epigenetic-based approach is that DNA remains stable overtime.

EPICUP is a test based on microarray technology and identifies the tumor-specific methylation profiles. Bisulfite-converted DNA from FFPE samples will be processed on Infinium MethylationEPIC BeadChip (850K DNA methylation microarray) for the analysis of DNA methylation.[15]

It can identify 38 cancer types covering 90% of the most frequent solid tumors. The test has a sensitivity of 99.7%, specificity of 99.6%, and a positive predictive value of 88.6%.[15]

The test was validated using an initial training set of 2790 samples of known origin (primary tumors or metastases) from 38 cancer types [Table 1] to interrogate more than 485,577 CpG sites and a classifier based on cancer type was established.[17] This classification was validated in 7691 samples.[2,15]

Table 1: List of 38 cancers covered in EPICUP test[15]

Acute lymphoblastic leukemia	Papillary renal cell carcinoma
Non-small cell lung carcinoma	Endometrial carcinoma
Bladder urothelial carcinoma	Retinoblastoma
Pancreatic carcinoma	Esophageal carcinoma
Acute myeloid leukemia	Sarcoma
Non-seminomatous germ cell tumors	Head and neck squamous cell carcinoma
Adrenocortical carcinoma	Seminoma
Ovarian carcinoma	Hepatocellular carcinoma
Brain lower grade glioma	Skin cutaneous melanoma
Pheochromocytoma	Lymphoid neoplasm (diffuse large B-cell lymphoma)
Breast carcinoma	Small cell lung carcinoma
Prostate carcinoma	Meningioma
Cervical squamous carcinoma	Stomach carcinoma
Chronic lymphocytic leukemia	Mesothelioma
Rectal adenocarcinoma	Thymoma
Renal tumor chromophobe	Multiple myeloma
Colon carcinoma	Thyroid carcinoma
Renal tumor clear cell	Neuroendocrine carcinoma
Cutaneous lymphoma	Uveal melanoma

Implications on survival

The administration of treatment based on the diagnosis provided by the validated molecular and GEP platforms has shown survival benefits in the patients with CUP.

Benefits with TOO test

- In a clinical utility study of GEP platforms in 107 patients, 65% of patients were administered treatment based on guidelines compared to 42% before the GEP tests. The median survival in these patients was 14.2 months, and at the end of two years, 30% of the patients were alive.[10]

Benefits with EPICUP test

- The effectiveness of EPICUP in predicting the site of primary was demonstrated retrospectively in a 216 patients international cohort with CUP.[13] Treatment based on the tumor type predicted EPICUP test was associated with significantly longer overall survival of 13.6 months (95% CI 4.1–55.4) compared to 9 months in those who received empirical therapy ($P = 0.0029$).[15]

Conditions where TOO test and EPICUP test are useful

The molecular cancer classifier assays are useful in identification of the primary site in patients with CUP are as follows:

Table 2: Summary of the molecular diagnostic tests for CUP

	Tissue of origin test	Cancer type ID	Cancer origin test	EPICUP
FDA cleared/CE marked	FDA cleared	None	None	CE marked
Number of genes/DNA methylation sites (EPICUP) measured	2000[7]	92[20]	64[21]	485,577 CpG sites
Biomarker	mRNA	mRNA	miRNA	DNA methylation
Tumor types covered	15	50	42	38
Positive percentage agreement (PPA) (akin to sensitivity)	89%	85%	74 or 85%	99.7%
Negative percentage agreement (NPA) (Akin to specificity)	>99%	95%	Not provided	99.6%

CUP: Cancer of unknown primary

1. The tumor is poorly differentiated or undifferentiated.
2. There is an unresolved differential diagnosis of two or more cancer types
3. The specimen is small, constraining the diagnostic workup and limiting prognostic studies
4. The patient has a history of multiple cancers
5. IHC is inconclusive or conflicting after the first round
6. Clinical history and histology differ on the diagnosis
7. There is atypical distribution of metastases
8. Oncology and pathology differ on the diagnosis
9. The diagnosis is questioned when the patient fails to respond to treatment.

Clinical guidelines on molecular diagnostics to identify primary site in CUP

Guidelines recommend that patients with CUP undergo a thorough evaluation, including a complete history, physical examination, complete blood count, urine analysis, pathological evaluation, histologic evaluation, chest radiograph, computed tomography, magnetic resonance imaging, and IHC studies.

The Ad-Hoc Committee on IHC Standardization 2007 highlighted some potential "deficiencies" in the consistency, reproducibility, quality assurance, concordance, validation, and results reporting of IHC studies. Although the recommendations are being adopted, full characterization of the tumor-site origin by IHC may not be precisely possible.[10]

The recent NCCN guidelines agree that GEP is comparable to the accuracy of IHC for poorly differentiated/undifferentiated carcinomas. NCCN guidelines suggest the collaboration of pathologists and oncologists on the judicious use of GEP profiling on a case to case basis with the best possible individualized patient outcome in mind.[18]

As per the recent ESMO guidelines on CUP, GEP tests may aid in the diagnosis of the putative primary tumor site in some patients. The guidelines also highlight that survival may be improved by site-specific therapy determined by a gene expression profile assay of the biopsy specimen, particularly for patients with a TOO diagnosis of more responsive tumor types.[2] There are few tests available that could be of use in CUP, a summary of the tests is mentioned in Table 2.

The recent Spanish Society of Pathology and the Spanish Society of Medical Oncology consensus statement on the diagnosis and treatment of CUP recognize that the molecular diagnostics and gene expression platforms are helpful when they are used to complement the IHC, allowing for more accurate diagnosis of tumor origin. It recommends that, based on clinical and histopathological features, a limited number of basic and specific advanced IHC tests should be performed initially to ensure the availability of sufficient quantity of tissue for molecular platforms.[19]

CONCLUSION

CUP represents heterogeneous metastatic tumors where the site of primary is not identified by the standard diagnostic workup.[2] Gene expression profiling assays identify the primary site or TOO accurately and clinical studies have supported the value of site-directed diagnosis.[3] Recognition of the primary tumor and administration of a tailored site-specific therapy have improved the survival of these patients.[15]

Molecular platforms are considered as complement to IHC and beneficial when a reasonable number of IHC stains have failed to predict tumor origin, particularly in poorly differentiated tumors.[22] NCCN guidelines recommend judicious use of the TOO tests on a case-to-case basis.[18]

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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How to cite this article: Verma A, Goswami C, Maniar V. Role of molecular diagnostics in the determination of tissue of origin in cancer of unknown primary, Int J Mol Immuno Oncol 2019;4(2):50-5.