Rosetta Genomics, Inc. -

Written comment for: -

CMS MedCAC, May 1, 2013: Genetic Tests for Cancer Diagnosis -

Submitted: March 25, 2013 (MedCACpresentations@cms.hhs.gov)

This document continues our comments to the MedCAC committee on the draft technology assessment and the **mirView® Mets[2].** In addition, we provide two additional peer-reviewed publications and one additional clinical performance abstract following our comments.

Oncologist. 2012;17(6):801-12.
A second-generation microRNA-based assay for diagnosing tumor tissue origin. -

Meiri E, Mueller WC, Rosenwald S, Zepeniuk M, Klinke E, Edmonston TB, Werner M, Lass U, Barshack I, Feinmesser M, Huszar M, Fogt F, Ashkenazi K, Sanden M, Goren E, Dromi N, Zion O, Burnstein I, Chajut A, Spector Y, Aharonov R.

BACKGROUND: Cancers of unknown primary origin (CUP) constitute 3%-5% (50,000 to 70,000 cases) of all newly diagnosed cancers per year in the United States. Including cancers of uncertain primary origin, the total number increases to 12%-15% (180,000 to 220,000 cases) of all newly diagnosed cancers per year in the United States. Cancers of unknown/uncertain primary origins present major diagnostic and clinical challenges because the tumor tissue of origin is crucial for selecting optimal treatment. MicroRNAs are a family of noncoding, regulatory RNA genes involved in carcinogenesis. MicroRNAs that are highly stable in clinical samples and tissue specific serve as ideal biomarkers for cancer diagnosis. Our first-generation assay identified the tumor of origin based on 48 microRNAs measured on a quantitative real-time polymerase chain reaction platform and differentiated 25 tumor types.
METHODS: We present here the development and validation of a second-generation assay that identifies 42 tumor types using a custom microarray. A combination of a binary decision-tree and a k-nearest-neighbor classifier was developed to identify the tumor of origin based on the expression of 64 microRNAs.
RESULTS: Overall assay sensitivity (positive agreement), measured blindly on a validation set of 509 independent samples, was 85%. The sensitivity reached 90% for cases in which the assay reported a single answer (>80% of cases). A clinical validation study on 52 true CUP patients showed 88% concordance with the clinicopathological evaluation of the patients.
CONCLUSION: The abilities of the assay to identify 42 tumor types with high accuracy and to maintain the same performance in samples from patients clinically diagnosed with CUP promise improved utility in the diagnosis of cancers of unknown/uncertain primary origins.
A novel microRNA-based Assay demonstrates 92% accuracy in classification of metastatic tumors from patients diagnosed with carcinoma of unknown primary [Abstract, ASCO, 2012].

George Pentheroudakis, Nicholas Pavlidis, Brianna St. Cyr, Anna Goussia, Yael Spector, Aikaterini Stoyianni, Alexander Faerman, George Fountzilas, Hila Benjamin, Vassiliki Malamou-Mitsi, Karin Ashkenazi, Mats Sanden

Introduction: Cancer of unknown primary (CUP) constitutes 3%-5% of all newly diagnosed cancer cases, and if cancer of uncertain origin is added, the total number increases to 12-15%. It presents a major diagnostic challenge due to the significant therapeutic management implications for the patients. Here we present miRview® mets 2 performance in a blinded study on a well annotated cohort of real CUP patients.
Results: In 77 patients (92%) the test results were fully concordant with diagnosis based on all the clinical and pathological information available including follow-up and outcome data. For 18 patients, the change from the presentation clinical diagnosis to the miRview® mets 2 assay result (which is in agreement with the final clinical diagnosis) would have resulted in administration of different

chemotherapeutic regimens.
- In 9 of them, the change in diagnosis would have resulted in different combination chemotherapies likely to be more active and associated with superior survival.
- In 16 of these 18 patients, the change in diagnosis could have been coupled to a change in targeted therapy employed.

Summary: The miRview® mets 2 assay can be successfully performed on most clinical FFPE tissue samples In the studied cohort of real CUP patients, miRview® mets 2 assay demonstrates agreement with pathological and clinical information in 92% of cases microRNA profiling can be a useful adjunct to traditional clinical and pathologic evaluation for CUP cases. For CUP patients, time is of the essence and our assay can help by both narrowing down the potential diagnostic options and increasing confidence in a suspected tissue of origin or by suggesting a different origin at presentation, resulting in earlier correct management.

Clin Exp Metastasis. 2012 Nov 4. *[Epub ahead of print]*

Global microRNA profiling in favorable prognosis subgroups of cancer of unknown primary (CUP) demonstrates no significant expression differences with metastases of matched known primary tumors.

Pentheroudakis G, Spector Y, Krikelis D, Kotoula V, Meiri E, Malamou-Mitsi V, Fountzilas G, Sanden M, Pavlidis N, Benjamin H, Aharonov R.

Abstract

No data exist on biologic differences between Cancer of unknown primary (CUP) and metastatic solid tumors of known primary site. We assigned a primary tissue of origin in 40 favorable CUP patients (A: serous peritoneal carcinomatosis n = 14, B: axillary adenocarcinoma n = 8, C: upper squamous cervical adenopathy n = 18) by means of a 64-microRNA assay. Subsequently, we profiled the expression of 733 microRNAs (miRs) in the CUP cases and compared results with metastases from 20 ovarian carcinomas, 10 breast adenocarcinomas, 20 squamous head neck or lung tumors. In the Peritoneal CUP versus Ovarian (Known Primary Metastases) KPM comparison, a total of 12 miR were significantly differentially expressed: higher than twofold expression difference in CUP was seen only for miR-513a-5p (3.7-fold upregulated) and miR-483-5p (2.5-fold upregulated), while miR-708 exhibited a twofold downregulation. In the Breast CUP versus Breast KPM comparison, only miR-29c that were downregulated in CUP by 2.7-fold satisfied the FDR threshold. miR-30e and miR-27b, downregulated in ovarian CUPs versus KPMs, were also non-significantly downregulated in breast CUP by 2.0- and 1.4-fold respectively. Six miRs, which belong to the 17-92 oncocluster showed a trend of upregulation in Breast CUP versus Breast KPM cases. A CUP signature remains elusive.

STAKEHOLDER'S COMMENTS (ROSETTA GENOMICS) -

Rosetta Genomics appreciates the chance to provide written comments for the MedCAC panel's review prior to the May 1 meeting. We intend to provide a short public presentation at the meeting, which we understand is typically limited to less than 10 minutes in length. Therefore, we are providing the appended additional peer reviewed publications for the committee's review as well as brief comments on the AHRQ assessment.

OVERVIEW

Molecular diagnostic techniques, driven forward by a number of leading academic institutions and companies over the past decade, have markedly expanded our knowledge of cancer of unknown or uncertain primary (CUP) and clinicians' abilities to diagnose these challenging oncology patients. For thirteen years, **Rosetta Genomics** has had an international leadership role in this research and in bringing the results of clinical research to oncologist and CUP patients in the form of highly innovative and accurate molecular diagnostic tests.

Of the three commercial tests reviewed by an unstated institution under AHRQ's technology assessment contract, **Rosetta Genomics** and the **mirView® Mets^2** test have a unique analytic basis, measuring the differential expression of microRNAs across tissue types. As described in our publications, microRNAs are one of biology's fundamental mechanisms of tissue differentiation. Unlike many cellular messenger RNAs, which are designed for rapid turnover and metabolism, microRNAs are designed to be relatively stable, improving the reliability of their detection under conditions such as routine surgical tissue collection, pre-fixation time delay, and paraffin embedding. The current generation of our test is based on the expression of 64 microRNAs (Meiri et al., 2012).

AHRQ TECHNOLOGY ASSESSMENT

We note that the technology assessment provided to the MedCAC panelists has not yet gone through public comment and peer review. Public comment and peer review have become key features of the modern AHRQ technology assessment process in the last several years, and may significantly impact the tone and approach of this report. Some of the comments made by the writers of the draft report suggest that they were not experts in the CUP field and had less familiarity with the totality of the large clinical and research literature in which our knowledge of CUP has developed. For example, on page 2 they cite a peripheral theory of CUP biogenesis in an obscure journal (in addition, the citation "9" is incorrect, assuming the authors intended to cite Nerash KN, Medical Hypotheses, 2002, 59:357-60) as apparent in the text.

It should also be understood, from a medical/scientific standpoint, what the concept of "FAIR" data means in an AHRQ technology assessment. For example, a 2005 AHRQ technology assessment of the BRCA gene found the statistical evidence that the BRCA gene is per se associated with increased breast cancer in populations "may not be reliable" although this association was by then universally accepted as a medical and scientific fact and onsidered causal. (For example, in addition to overwhelming human data, the BRCA mutation also causes breast cancer in mouse models). Thus, when the writers of the present assessment conclude, in their summary, that the data on CUP tests "are insufficient to confirm validity" this must be understood in the relatively arcane world of technology assessment writing style. Given the volume of data from multiple institutions, well-established researched-based commercial laboratories, and international research, the writers' prominent judgment that the field is "in its infancy" (p. ES-3) is difficult to understand and certainly has no objective definition in either science or the field of systematic technology assessments. However, we do understand that the authors likely had a large volume of papers to assess in a limited time, and we look forward to working with the authors and providing constructive comments during the public comment period for this technology assessment.

We note that the study writers did not contact authors, although this would not have been difficult, as only three commercially available tests are being reviewed.

CUP MOLECULAR ASSAYS AND THE "GOLD STANDARD"

As the writers note (p. 6) the very definition of CUP is a tumor that has defied ordinary clinical, imaging, and immunohistochemical workup and its primary tissue of origin remains unknown or uncertain.

Therefore, it is impossible to use conventional terms like "sensitivity" and "specificity" in their usual sense, since no gold standard diagnosis is possible. The writers of the technology assessment deserve credit in being aware of this, but the point also deserves emphasis for the MedCAC panelists. If there had been a gold standard diagnosis for each patient with CUP, prior to the development of oncology molecular tests, the tests would not have been necessary. Rather, there are three important ways to validate CUP tests:

> **First,** the test technologies and algorithms must be developed on a primary data set.
>
> **Second,** the test can be effectively and convincingly validated by taking large numbers of blinded human tumor samples and re-classifying them accurately, effectively, and quickly based only on the molecular test. These must be entirely new and independent samples.
>
> **Third,** the test can be applied to actual CUP samples.

In this third step, when the tests are applied to clinical CUP samples, there is now a **convincing and cumulative experience that all but a small minority of tumors will match (or "fingerprint") to a known tumor type.**

As the report concludes, some **85-90% of CUP tumors will match – will "fingerprint" – to a known cancer tumor type lineage**. We are proud to note that in the comparative clinical-pathologic studies of our test, the **miRview® Mets^2** test (and the prior **Mets** test) are well-rated by the study's authors.

Where clinical and immunohistochemical data are available, but uncertain, the resulting molecular diagnoses will generally fall within the range of possible cancers but more precisely with no subjectivity, and will, with much higher confidence, identify one tumor type. There are also hallmark cases where unusual tumors, such as testicular cancer, have actually been found only after molecular analysis, in cases where the primary tumor was very small relative to the size of metastatic site(s). It is well-established, as cited by the technology assessment writers, that only about 66% percent of metastatic cancers can be identified by immunohistochemistry alone if primary anatomical origin data is withheld for the purpose of the experiment. It is this situation that occurs naturally in the case of CUP tumors.

Although much research on CUP tumors is continuing, the overall contribution of CUP molecular diagnostics to patient diagnosis is already clear. In current data, the **miRview® Mets^2** provides an answer in 96% of clinical cases: mostly a high-sensitivity (90%) single answer in 82% of cases. In the remaining cases, **miRview® Mets^2** either produces two most-likely answers, or, produces a categorical answer (e.g. "sarcoma".) While our focus has been on direct clinical impact of CUP molecular analysis, this approach to tumor classification is also contributing to our fundamental basic science knowledge of CUP tumors. For example, in a study profiling 733 microRNAs (a research, not clinical assessment), there does not appear to be any single signature that is a marker of CUP tumors (Pentheroudakis G et al., Clin Exp Metastases, 2012, epublication ahead of print.)

SUMMARY

- In summary, the **miRview® Mets^2** test continues the exact same effort that has been underway since the development of advanced medical imaging in the 1980s and immunohistochemistry in the 1980s and 1990s: the attempt to use available technologies, such as imaging, immunohistochemistry, and molecular analysis, to identify with the greatest certainty possible possible the type of cancer that a challenging patient has.
- Whereas immunohistochemical markers have an uncertain range of positive and negative findings that vary by the specific monoclonal antibody, vary by the lab in which it is run, vary by the reading pathologist, and vary markedly from one cancer to another; centralized commercial molecular CUP assays consistently approach 90% accuracy in CUP tumor classification, a major clinical advance.

The Oncologist®

Cancer Diagnostics and Molecular Pathology

A Second-Generation MicroRNA-Based Assay for Diagnosing Tumor Tissue Origin

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Key Words. MicroRNA • Carcinoma of unknown primary origin

Disclosures: Eti Meiri: Rosetta Genomics (E, OI, I/P); **Shai Rosenwald:** Rosetta Genomics (E, OI); **Merav Zepeniuk:** Rosetta Genomics (E, OI); **Tina Bocker Edmonston:** Rosetta Genomics (E); **Meora Feinmesser:** Rosetta Genomics (RF); **Monica Huszar:** Rosetta Genomics (RF); **Mats Sanden:** Rosetta Genomics (E); **Eran Goren:** Rosetta Genomics (E, OI); **Nir Dromi:** Rosetta Genomics (E, OI, I/P); **Orit Zion:** Rosetta Genomics (E, OI); **Ilanit Burnstein:** Rosetta Genomics (E, OI); **Ayelet Chajut:** Rosetta Genomics (E, OI); **Yael Spector:** Rosetta Genomics (E, OI, I/P); **Ranit Aharonov:** Rosetta Genomics (E, OI I/P). The other authors indicated no financial relationships.

(C/A) Consulting/advisory relationship; (RF) Research funding; (E) Employment; (H) Honoraria received; (OI) Ownership interests; (IP) Intellectual property rights/inventor/patent holder; (SAB) Scientific advisory board

ABSTRACT

Background. **Cancers of unknown primary origin (CUP) constitute 3%–5% (50,000 to 70,000 cases) of all newly diagnosed cancers per year in the United States. Including cancers of uncertain primary origin, the total number increases to 12%–15% (180,000 to 220,000 cases) of all newly diagnosed cancers per year in the United States. Cancers of unknown/uncertain primary origins present major diagnostic and clinical challenges because the tumor tissue of origin is crucial for selecting optimal treatment. MicroRNAs are a family of noncoding, regulatory RNA genes involved in carcinogenesis. MicroRNAs that are highly stable in clinical samples and tissue specific serve as ideal biomarkers for cancer diagnosis. Our first-generation assay identified the tumor of origin based on 48 microRNAs measured on a quantitative real-time polymerase chain reaction platform and differentiated 25 tumor types.**

Methods. **We present here the development and validation of a second-generation assay that identifies 42 tumor types using a custom microarray. A combination of a binary decision-tree and a k-nearest-neighbor classifier was developed to identify the tumor of origin based on the expression of 64 microRNAs.**

Results. **Overall assay sensitivity (positive agreement), measured blindly on a validation set of 509 independent samples, was 85%. The sensitivity reached 90% for cases in which the assay reported a single answer (>80% of cases). A clinical validation study on 52 true CUP patients showed 88% concordance with the clinicopathological evaluation of the patients.**

Conclusion. **The abilities of the assay to identify 42 tumor types with high accuracy and to maintain the same performance in samples from patients clinically diagnosed with CUP promise improved utility in the diagnosis of cancers of unknown/uncertain primary origins.** ***The Oncologist*** 2012;17:000–000

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Introduction

Cancers of unknown primary origin (CUP) constitute 3%–5% (50,000 to 70,000 cases) of all newly diagnosed cancers per year in the United States. Including cancers of uncertain primary origin, the total number increases to 12%–15% (180,000 to 220,000 cases) of all newly diagnosed cancers per year in the United States. The identification of the tissue of origin presents a challenge in many cases, even after a complete assessment that includes patient history, physical examination, imaging, serum markers, and pathological evaluation of tumor samples [1–4]. However, identification of tumor origin is crucial for the patient management plan because many oncology treatments are based on knowledge of the specific tumor type, especially with the growing number of cytotoxic and targeted therapies shown to be effective against specific cancers [5–9]. In addition, entry criteria into clinical trials and reimbursement strategies [10] are based on knowing the primary origin. Most importantly, it has been demonstrated that tumor-specific therapy leads to better survival [9, 11]; however, a broad-spectrum treatment approach is used when the putative site of origin cannot be assessed, which is suboptimal. Although immunohistochemistry (IHC) markers are widely used and well characterized, they are unable to determine a definitive tissue of origin in over 30% of cases [12]; in addition, they are highly subjective and dependent on many variables. Therefore, there is a substantial need to find complementary diagnostic tools for determining tissue of origin.

Currently, molecular profiling of cancers of unknown primary origin is available using expression microarrays and quantitative real-time polymerase chain reaction (qRT-PCR), targeting different molecules, namely mRNA or microRNA [13]. MicroRNAs are particularly suitable as biomarkers for identifying tumor origin as their expression levels reflect tissue differentiation and tumorigenesis [14–17]. In addition, microRNAs have been shown to be highly stable in formalin-fixed paraffin-embedded (FFPE) tissue blocks, the most common and readily available specimen type in pathology [18–20]. In fact, microRNA profiling has been described as being superior to mRNA profiling in FFPE tissue [20, 21].

We recently described the development and validation of a qRT-PCR assay that identifies the tissue of origin for FFPE tumor samples based on the expression levels of 48 microRNAs [22]. The qRT-PCR miRview mets assay was designed to discriminate between 25 possible classes corresponding to 17 distinct tissues and organs of origin; it was shown to correctly identify the tissue of origin in 85% of the cases in an independent validation study. The assay was further validated in two additional studies using samples from actual patients with CUP, demonstrating its usefulness in the more clinically relevant cases of CUP [23, 24].

Although these 25 tumor types cover the majority of tumor types seen in adults with cancer of unknown/uncertain origin [25, 26], we set forth to develop a second-generation diagnostic assay to identify a wider range of tumor types. This assay could enable physicians to resolve more cases of unknown or uncertain diagnoses and therefore enable more optimal treatment selection. The tumor panel of the second generation assay, miRview mets^2, has been expanded to include additional carcinomas and neuroendocrine tumors, as well as a variety of sarcomas and lymphoma. The 42 tumor types in its panel are described in Table 1. The main clinical need is for identifying the origin of metastases, but it is not uncommon for physicians to be uncertain whether a tumor is a metastasis or a primary tumor, such as with malignancy in the lung or liver or cases for which the clinical presentation is not consistent with the pathology. The assay is therefore designed to identify the tissue of origin of both metastases and primary tumors at the site of the biopsy/resection.

To achieve the expansion of the tumor panel and enable more efficient upscaling of sample volume, we developed the second-generation assay on custom-designed microarrays, which offer several advantages as discussed later. Here we describe the validation of the assay on 509 blinded samples of known origin, as well as results from an interlaboratory reproducibility study on 179 samples. We further extended the validation to a more challenging group of actual patients with CUP by evaluating the assay performance on 52 CUP cases from the same set studied before on the first-generation assay [23]. The results of this validation confirm the high level of accuracy of microRNA-based profiling in CUP cases and also demonstrate the importance of adding additional tumor types to the assay.

Materials and Methods

Samples and RNA Extraction

Tumor samples were obtained from several sources (see supplemental online data). Institutional review board approvals were obtained in accordance with institutes' guidelines. Samples were obtained by surgical resections and biopsies (dated 1990–2010) and included primary tumors and metastases of defined origins. An additional review of specimens confirmed the reference diagnosis as defined in the original records. In 37 cases, microdissection was performed (supplemental online data) [23]. Tumor cellular content reached at least 60% for >95% of the samples (based on hematoxylin-eosin slides). Tumors containing significant necrosis (cutoff arbitrarily set at >35%) and sections containing significant hemorrhage (cutoff arbitrarily set at >50%) were excluded. Tumors with significant fibrosis or desmoplastic reaction (>50%) were also excluded, although the fibrotic tissue is typically not very cellular.

Total RNA was extracted as previously described [17]. Briefly, FFPE sections were deparaffinized with xylene, washed in ethanol, and digested with proteinase K. RNA was extracted using acid-phenol:chloroform followed by ethanol precipitation and DNase digestion. Following a second acid-phenol:chloroform extraction, the pellet was resuspended in nuclease-free water and analyzed for its concentration and purity by spectrophotometry (NanoDrop1000).

MicroRNA Microarray, Platform, and Signal Processing

Custom-designed arrays from Agilent Technologies (Santa Clara, CA) that harbor 8 identical subarrays (8 × 15,000 for-

Table 1. Assay tumor panel

ID	Tumor organ of origin	Tumor type (as reported)	*n* of samples in training	*n* of samples in test validation
1	Adrenal	Adrenocortical carcinoma	19	11
2	Adrenal	Pheochromocytoma	15	13
3	Anus/skin	Squamous cell carcinoma of the anus or skin	28	15
4	Biliary tract	Cholangiocarcinoma or adenocarcinoma of extrahepatic biliary tract	51	15
5	Bladder/transitional cell carcinoma	Urothelial carcinoma	60	15
6	Brain	Astrocytic tumor (primary)	14	15
7	Brain	Oligodendroglioma (primary)	12	9
8	Breast	Adenocarcinoma of the breast	57	15
9	Cervix	Squamous cell carcinoma of the uterine cervix	29	14
10	Colon/rectum	Colorectal adenocarcinoma	45	15
11	Gastrointestinal	Carcinoid of the gastrointestinal tract	30	9
12	Gastrointestinal	Gastrointestinal stromal tumor	19	10
13	Kidney	Renal cell carcinoma, chromophobe	23	15
14	Kidney	Renal cell carcinoma, clear cell	38[a]	15
15	Kidney	Renal cell carcinoma, papillary	24	15
16	Liver	Hepatocellular carcinoma	24	15
17	Lung	Lung, large cell or adenocarcinoma	37	15
18	Lung	Lung, small cell carcinoma	21	13
19	Lung	Carcinoid of the lung	25	15
20	Lung, head and neck, esophagus	Squamous cell carcinoma of the lung, head and neck, or esophagus	140	15
21	Lymphoma	Lymphoma, B or T cell	108	26
22	Mesothelioma	Pleural mesothelioma[b]	32	12
23	Ovary	Ovarian carcinoma	61	15
24	Ovary	Ovarian primitive germ cell tumor	5	2
25	Pancreas	Pancreatic adenocarcinoma	25	16
26	Pancreas	Pancreatic islet cell tumor	11	2
27	Prostate	Prostatic adenocarcinoma	28	20
28	Sarcoma	Ewing sarcoma	10	2
29	Sarcoma	Chondrosarcoma	11	3
30	Sarcoma	Malignant fibrous histiocytoma or fibrosarcoma	22	9
31	Sarcoma	Osteosarcoma	11	10
32	Sarcoma	Rhabdomyosarcoma	9	2
33	Sarcoma	Synovial sarcoma	11	6
34	Sarcoma	Liposarcoma	18	10
35	Skin	Melanoma	28	15
36	Stomach/esophagus	Gastric or esophageal adenocarcinoma	47	15
37	Testis	Nonseminomatous testicular germ cell tumor	18	15
38	Testis	Seminomatous testicular germ cell tumor	27	15
39	Thymus	Thymoma/thymic carcinoma	29	11
40	Thyroid	Thyroid carcinoma, follicular	16	8
41	Thyroid	Thyroid carcinoma, papillary	25	15
42	Thyroid	Thyroid carcinoma, medullary	19	6
Total			1,282	509

In addition to these 42 tumor types, the assay can report seven tumor classes (see Results). Because metastases for primary brain tumors outside of the central nervous system (CNS) are very rare [33], the assay does not suggest brain as an origin for biopsies outside the CNS; hence these origins are noted as primary (IDs 6 and 7).
[a]Of these samples, 16 were renal cell carcinomas of unknown subtype.
[b]The reported tumor type is in the process of being changed to mesothelioma.

Table 2. Validation on patients with cancers of unknown origin

Clinicopathological data				Primary tumor type predicted by miRview mets[2]				
ID	Age/sex	Speculated tumor type at time of surgery	Speculated tumor type based on additional investigations	Suggested origin 1	Suggested origin 2	*S*	Diagnosis based on case revision	Immunohistochemistry relevant for diagnosis
1	42/F	Lung	Lung (biopsy)	Ovarian carcinoma	Lung, large cell or adenocarcinoma	1	NA	NA
2	58/F	Lung (small cell lung cancer)	Lung (imaging)	Carcinoid of the lung	Pancreatic islet cell tumor	1	Lung, atypical carcinoid	ki67 <25%; TTF1 focally positive, tumor <2 cm maximum; p53+
3	63/M	Lung	Lung (biopsy)	Squamous cell carcinoma of the lung, head and neck, or esophagus	NA	4	Lung, non-small cell lung cancer; adenocarcinoma	NA
4	75/F	Lung or thyroid	Lung (biopsy)	Lung, large cell or adenocarcinoma	NA	1	NA	NA
5	70/M	Lung	Lung (imaging)	Thyroid carcinoma, follicular	Lung, large cell or adenocarcinoma	1	Lung, non-small cell lung cancer; adenocarcinoma	NA
6	51/M	Pathology failed to identify primary	Lung (cytology)	Lung, small cell carcinoma	NA	1	NA	NA
7	61/F	Lung (small cell lung cancer)	Lung (cytology)	Lung, small cell carcinoma	NA	1	NA	NA
8	68/M	Lung	Lung (imaging)	Lung, small cell carcinoma	NA	1	NA	NA
9	43/F	Ovarian, uterus, or stomach	Stomach (biopsy)	Colorectal adenocarcinoma	Gastric or esophageal adenocarcinoma	1	NA	NA
10	53/M	Lung (small cell lung cancer)	Lung (biopsy)	Lung, large cell or adenocarcinoma	Thyroid carcinoma, follicular or papillary	2a	Lung, non-small cell lung cancer; adenocarcinoma	TTF1+, CK7+, CK20–, TG–
11	52/M	Lung (adenocarcinoma)	Lung (imaging)	Thyroid carcinoma, papillary	Lung, large cell or adenocarcinoma	1	NA	NA
12	51/M	Lung (non-small cell lung cancer/neuroendocrine)	Lung (imaging)	Urothelial carcinoma	NA	4	Lung, non-small cell lung cancer; neuroendocrine	TTF1–, CK7+, CK20–, TG–, CK5/6-, Syn+, NSE+, CD56+
13	82/F	Lung (adenocarcinoma)	Lung (imaging)	Sarcoma	Mesothelioma	2b	Non-small cell lung cancer; adenocarcinoma or biphasic synovial sarcoma	TTF1+, CK7+, CK20/ CK5/6/TG/Calretinin–, Vim strongly positive
14	58/M	Lung (adenopapillary carcinoma)	Lung/small cell lung cancer (immunohistochemistry and imaging)	Lung, small cell carcinoma	NA	1	NA	NA
15	65/M	Adenocarcinoma of unknown origin	Gastrointestinal/rectum carcinoma (biopsy)	Colorectal adenocarcinoma	NA	1	Gastrointestinal tract, adenocarcinoma	NA
16	65/F	Lung (neuroendocrine-carcinoma) or colorectal adenocarcinoma	Lung (biopsy)	Lung, small cell carcinoma	NA	1	Lung, small cell lung cancer	TTF1+, CD56+, MAP2+, Enolase+, Syn+
17	63/M	Kidney (renal cell carcinoma)	Renal cell carcinoma (biopsy)	Renal cell carcinoma, clear cell	NA	1	Kidney, renal cell carcinoma	NA
18	62/M	Kidney (renal cell carcinoma)	Renal cell carcinoma (biopsy)	Renal cell carcinoma, clear cell	NA	1	NA	NA
19	52/M	Skin (amelanotic malignant melanoma)	NA	Melanoma	NA	1	NA	NA
20	50/F	Breast or lung (squamous cell carcinoma)	Lung (imaging)	Thyroid carcinoma, papillary	Lung, large cell or adenocarcinoma	1	NA	NA
21	54/F	Lung (adenocarcinoma)	Lung (biopsy)	Thyroid carcinoma, follicular	Lung, large cell or adenocarcinoma	1	NA	NA
21	54/F	Lung (adenocarcinoma)	Lung (biopsy)	Lung, large cell or adenocarcinoma	NA	1	NA	NA
21	54/F	Lung (adenocarcinoma)	Lung (biopsy)	Thyroid carcinoma, follicular or papillary	Lung, large cell or adenocarcinoma	1	NA	NA

(continued)

Table 2. (continued)

	Clinicopathological data			Primary tumor type predicted by miRview mets[2]				
ID	Age/sex	Speculated tumor type at time of surgery	Speculated tumor type based on additional investigations	Suggested origin 1	Suggested origin 2	*S*	Diagnosis based on case revision	Immunohistochemistry relevant for diagnosis
22	65/M	Lung (small cell lung cancer)	Lung (imaging)	Lung, small cell carcinoma	NA	1	NA	NA
23	78/M	Lung	NA	No result generated	NA	NA	NA	NA
24	72/F	Lung	Breast (biopsy)	Thyroid carcinoma, papillary	Adenocarcinoma of the breast	1	Breast cancer	TTF1–, CK7+, TG–, CK20–, CD56–, NY-BR-1+
25	59/F	Lung (non-small cell lung cancer)	NA	Lung, large cell or adenocarcinoma	NA	2a	NA	NA
26	69/M	Lung (small cell lung cancer)	NA	Lung, small cell carcinoma	NA	2a	NA	NA
27	61/F	Lung (adenocarcinoma)	NA	Lung, large cell or adenocarcinoma	NA	2a	NA	NA
28	64/M	Kidney (renal cell carcinoma)	NA	Renal cell carcinoma	Malignant fibrous histiocytoma or fibrosarcoma	2a	NA	NA
29	72/F	Gastrointestinal tract (adenocarcinoma)	NA	Colorectal adenocarcinoma	NA	2a	Gastrointestinal tract, adenocarcinoma	CK20+, CDX2+, CK7–, TTF1–
30	61/F	Pathology failed to identify primary	NA	Lung, small cell carcinoma	NA	2a	Lung, possible small cell lung cancer	Lu5+, Syn–, Chromogranin–
31	73/F	Kidney (renal cell carcinoma)	NA	Renal cell carcinoma, clear cell	NA	2a	NA	NA
32	60/M	Lung (squamous cell carcinoma)	Lung/small cell lung cancer (immunohistochemistry)	No result generated	NA	NA	NA	NA
33	50/M	Lung (adenocarcinoma)	NA	Lung, large cell or adenocarcinoma	NA	2a	NA	NA
34	61/M	Lung (adenocarcinoma or squamous cell carcinoma)	NA	Lung, large cell or adenocarcinoma	NA	2a	NA	NA
35	48/M	Stomach (cardia carcinoma)	NA	Lung, small cell carcinoma or carcinoid	NA	3	Lung, non-small cell lung cancer; adenocarcinoma	TTF1+, CK7+, Chromo/NSE/Syn/CK20/CD56/TG/CDX2 NA
37	46/F	Pathology failed to identify primary	NA	Ovarian carcinoma	NA	2b	NA	NA
38	67/F	Pathology failed to identify primary	NA	Sarcoma	Astrocytic or oligodendroglial tumor (primary)	2a	Sarcoma possible	AE1/3–, CK7–, CK20–, Ber-EP4–, Claretinin–, CD99–, S100–, Vim+, SMA+
39	27/M	Unidentified site (malignant neuroendocrine tumor)	NA	Carcinoid of the lung	Carcinoid of the gastrointestinal tract	2a	Carcinoid possible	KL1+, NSE/Syn/CD56+, p53+, ki67: 15%–20%, TTF1/CK7/CK20–
39	27/M	Unidentified site (malignant neuroendocrine tumor)	NA	Carcinoid of the lung	Carcinoid of the gastrointestinal tract	2a	Carcinoid possible	KL1+, NSE/Syn/CD56+, p53+, ki67: 15%–20%, TTF1/CK7/CK20–
40	56/F	Stomach (signet-ring cell carcinoma)	NA	Gastric or esophageal adenocarcinoma	NA	2a	NA	NA
41	61/M	Lung	NA	Adenocarcinoma of the breast	Squamous cell carcinoma of the lung, head and neck, or esophagus	3	Lung, non-small cell lung cancer; adenocarcinoma	TTF1+, CK7+, CK5/6–, CK20–, TG–
42	75/F	Kidney (renal cell carcinoma)	NA	Lung, large cell or adenocarcinoma	NA	2b	Lung, non-small cell lung cancer	AE1/3+, KL1+, TTF1/TG/CK20/CD10/ER/PR–, CK7+, Vim–, CA125+
43	53/F	Gastrointestinal tract (adenocarcinoma)	Lung/adenocarcinoma (immunohistochemistry)	Ovarian carcinoma	Lung, large cell or adenocarcinoma	1	Lung, non-small cell lung cancer; adenocarcinoma	AE1/3+, CK7+, TTF1+, ki67: 30%, MG/NY-BR-1/ER/PR/TG/CK20–
45	75/M	Gastrointestinal tract or lung	Lung (imaging)	Urothelial carcinoma	Squamous cell carcinoma of the lung, head and neck, or esophagus	4	Lung, non-small cell lung cancer; adenocarcinoma	TTF1–, TG–, CK7+, CEA+, CK20+, CK5/6–, CDX2–

(continued)

Table 2. (continued)

	Clinicopathological data			Primary tumor type predicted by miRview mets[2]				
ID	Age/sex	Speculated tumor type at time of surgery	Speculated tumor type based on additional investigations	Suggested origin 1	Suggested origin 2	*S*	Diagnosis based on case revision	Immunohistochemistry relevant for diagnosis
46	62/M	Squamous cell carcinoma	Lung (biopsy)	Squamous cell carcinoma of the lung, head and neck, or esophagus	NA	1	NA	NA
47	72/M	Lung (adenocarcinoma squamous cell carcinoma)	Lung (imaging)	Adenocarcinoma of the breast	Squamous cell carcinoma of the lung, head and neck, or esophagus	1	Lung, squamous cell carcinoma	TTF1+, TG–, CK20+, CK5/6+
48	73/M	Urothelial carcinoma (prostate carcinoma ruled out)	Prostate (biopsy)	Prostatic adenocarcinoma	Adenocarcinoma of the breast	1	Prostate carcinoma	TTF1–, PSA–, PSAP+, CK5/6–, CK20+, CK7/TG–
49	70/M	Lung (non-small cell lung cancer)	Lung (imaging)	Sarcoma	NA	2b	Lung associated sarcoma possible	TTF1+, CK14+, CK7/TG/S100/CD99–, Vim+, CK20+, SMA+, Calretinin+
50	68/F	Lung (squamous cell carcinoma)	Lung (imaging)	Squamous cell carcinoma of the lung, head and neck, or esophagus	NA	1	NA	NA
51	59/M	Pathology failed to identify primary	NA	No result generated	NA	NA	NA	NA
52	75/M	Pathology failed to identify primary	Lung (imaging)	Urothelial carcinoma	Squamous cell carcinoma of the lung, head and neck, or esophagus	2a	Lung, squamous cell carcinoma	CK5/6+, TTF1–, CK7–, CD56–, CK20–
53	62/M	Pathology failed to identify primary	NA	Failed quality assurance		NA	NA	NA
54	66/F	Lung or ovarian (adenocarcinoma)	NA	Carcinoid of the gastrointestinal tract	Squamous cell carcinoma of the lung, head and neck, or esophagus	3	Lung, non-small cell lung cancer; adenocarcinoma	TTF1+, TG–, CK7+, CK5/6–, CK20–

The table contains 52 patients. Patient ID numbers match the patient ID numbers from a previously published study [23] (patients 36 and 44 did not have enough RNA left and, hence, were excluded from this study). Further detailed clinical data can be obtained elsewhere [23].
Abbreviations: ID, patient identification; NA, not available; S, consensus score (see Results); TTF, thyroid transcription factor; CK, cytokeratin; TG, thyreoglobulin; Syn, synaptophysin; NSE, neuronspecific enolase; Vim, vimentin; MAP, microtubule-associated protein; Chromo, cromogranin; SMA, smooth muscle actin; ER, estrogen receptor; PR, progesterone receptor; CA, cancer antigen; MG, mammaglobin; CEA, carcino embryonic antigen; PSA, prostate specific antigen; PSAP, prostatic specific acid phosphatase.

mat) were used. Then 0.25–1 μg of total RNA was labeled by ligation of an RNA linker, p-rCrU-Cy/dye (BioSpring, Frankfurt, Germany; Cy3 or Cy5) to the 3′ end. Synthetic small RNA controls were spiked before labeling. Slides were incubated with the labeled RNA for 12–16 hours at 55°C and washed according to the Agilent protocol. Arrays were scanned using the Agilent DNA Microarray Scanner Bundle at a resolution of 5 μm, dual pass at 100%, and 10% laser power.

Array images were analyzed using Agilent Feature Extraction software version 10.7.1.1. Triplicate spots were combined to produce one signal by taking the logarithmic mean of reliable spots. Analysis was performed in log space ($\log_2$). Normalization was performed for each sample with respect to a reference vector (R), calculated by taking the median expression level over the training set. For each sample data vector S, a second-degree polynomial F was found so as to provide the best fit between S and R, such that $R \approx F(S)$. This was performed on a set of invariant microRNAs; remote datapoints (outliers) were not used for fitting the polynomial. For each probe in the sample (element Si in the vector S), the normalized value (in $\log_2$) Mi is calculated from the initial value Si by transforming it with the polynomial function F, so that $Mi = F(Si)$.

Assay Protocol

Following extraction, seven RNA samples together with a positive control (PC) underwent labeling and hybridization to one array. The PC is an RNA sample that was set as a reference and met defined quality assurance (QA) criteria: Pearson correlation to the reference hybridization, median of differences from reference, and the number of the expressed microRNAs in the dynamic range (expression >300). QA for each sample was based on several parameters, such as the number of microRNAs in the dynamic range, the 98th percentile expression level of the microRNA, the Pearson correlation between the hybridization spikes and the reference, the expression of the negative control probes, and the number of microRNAs with consistent triplicate signals. The signal values of the 64 assay

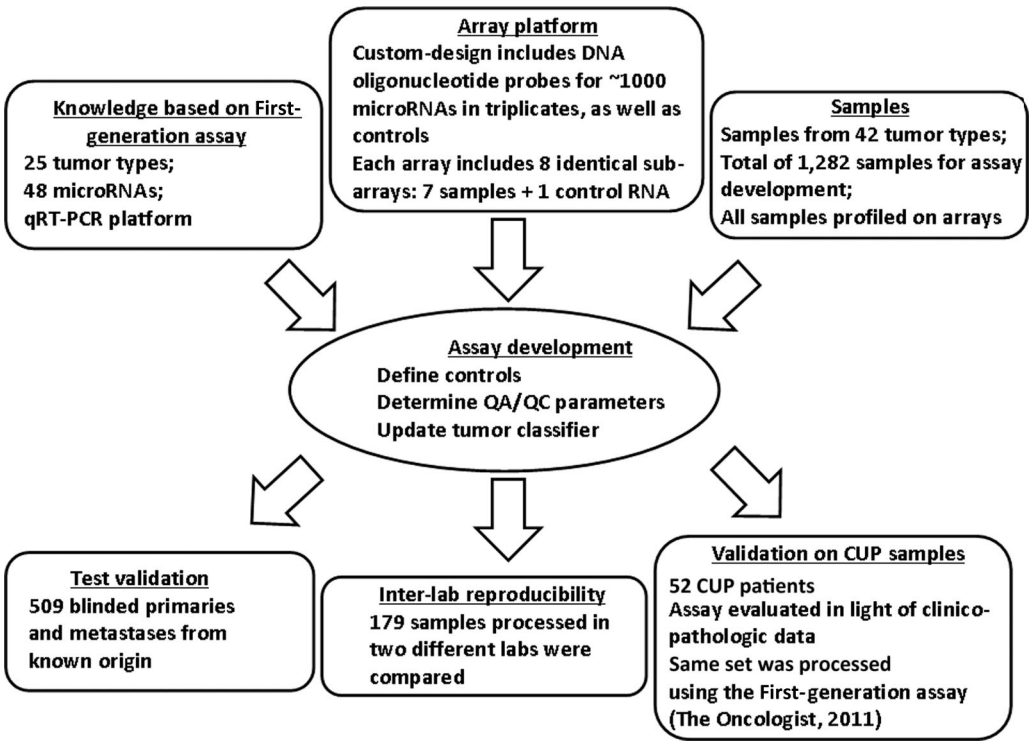

Figure 1. Development and validation of miRview mets[2], a second-generation diagnostic assay for determining tissue-of-origin. Assay development was based on knowledge collected during the development of the first-generation assay and profiling of additional samples. Assay was validated on primary tumors and metastases of known origin as well as patients with cancers of unknown primary origin.

Abbreviations: CUP, cancer of unknown primary origin; QA/QC, quality assurance/quality control; qRT-PCR, qualitative real-time polymerase chain reaction.

microRNAs for each sample were obtained following normalization and used as input to the assay classifier.

Results

Figure 1 describes the development and validation of the second-generation assay, which was developed using the same principles and statistical methods as the first-generation assay, with several improvements allowing for the expansion of the tumor panel. The assay development was mainly based on expression profiles of 1282 primary and metastatic FFPE samples from the 42 tumor types described in Table 1. The assay was first validated on 509 samples (Table 1) of known origin in a blinded manner, for which it demonstrated 85% accuracy; the vast majority of samples resulted in a single reported origin, which was accurate in 90% of these cases.

We then extended the validation to address CUP, which is the most challenging diagnostic dilemma for a test designed to identify tumor origin. Because by definition no definitive reference diagnosis exists in CUP, these cases present a challenge for test validation. We performed validation of the assay on CUP cases, assessing the performance of the assay using clinicopathologic evaluation, and demonstrated that the performance of the assay remains the same for these challenging cases.

Array Platform

We have developed an array platform that measures the expression level of almost 1,000 microRNAs. This platform was the basis for the development of the miRview mets[2] assay. The custom-made array is designed to harbor eight identical subarrays allowing for the simultaneous hybridization of seven samples plus a PC. To increase the measurement precision, each microRNA-related DNA oligonucleotide probe was spotted in triplicate and the logarithmic mean signal intensity was calculated.

To determine the performance of these subarrays, several parameters were studied. A reference sample was labeled and rehybridized to the array on different days. When either an RNA sample extracted from a fresh-frozen sample or RNA extracted from a FFPE sample was measured dozens of times, the overall mean correlation coefficient of both was 0.99, demonstrating the high reproducibility of the process (supplemental online Figs. S1A and S1B). Reproducibility was also demonstrated by comparing 179 RNA samples hybridized in two different laboratories (supplemental online Fig. S2). The sensitivity and dynamic range of the platform were measured using five artificial RNAs (similar in length and composition to endogenous microRNAs) in different concentrations. The lowest sensitivity was 0.1 fmol with a linear dynamic range of 10^3. The specificity was measured by hybridizing five members of the hsa-let-7 family (with 1–4 nucleotide mismatches) and comparing the signal of the relevant probe to the other probes. As seen in supplemental online Fig. S1C, specificity of 10- to 100-fold is achieved (except for let-7c when hybridized

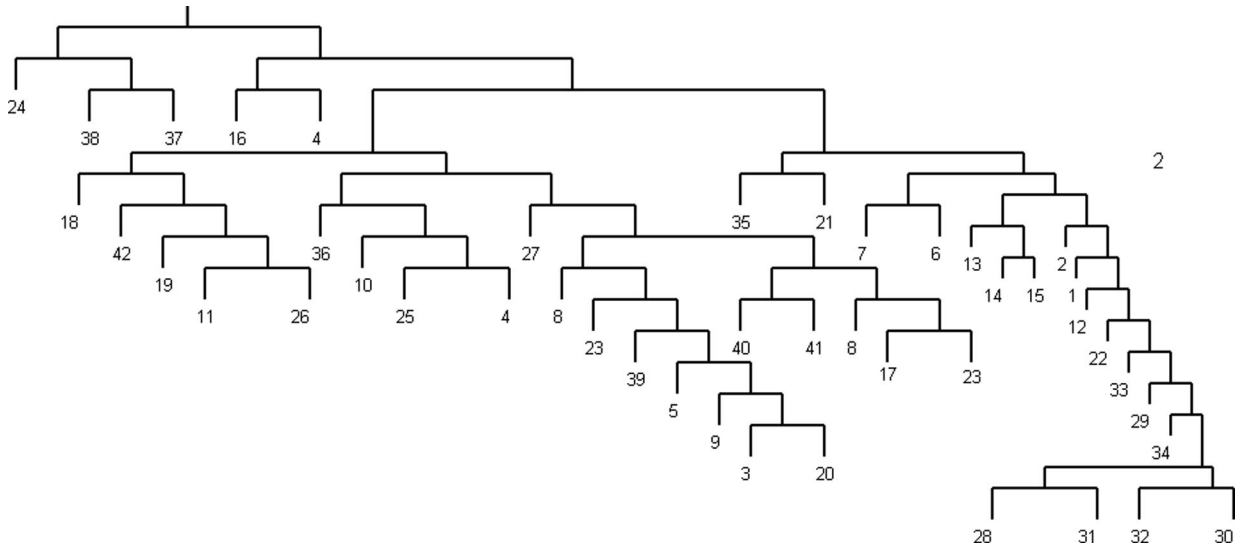

Figure 2. Structure of the binary decision tree. Decisions are made at consecutive nodes using expression levels of 1–3 microRNAs. The leaves of the tree are numbered by the identifications representing the tumor types in Table 1. The structure and logic of the tree are similar to the decision tree of the first-generation assay [22].

to labeled let-7b), demonstrating a high level of specificity for as little as a single nucleotide mismatch.

Tumor Classification

The assay relies on two classifiers to determine the tissue of origin, a binary decision tree and a k-nearest-neighbor (KNN). Both classifiers assign a tissue of origin based on the normalized expression of 64 microRNAs as measured by the array. The decision tree predicts the tissue of origin by following the branches and choosing the left or right branch at each node (Fig. 2). This binary decision is made at each node by comparing a combination of microRNA expression levels to a preset threshold. This approach is described in detail elsewhere [17].

The prediction of the tree is accompanied by a confidence measure, *p*, which is the cumulative probability (between 0 and 1) over all individual probabilities in the decisions taken in the nodes of the path taken to the tree result. The KNN approach compares the expression across all 64 microRNAs to the dataset of the 1,282 training samples and selects the majority vote among the nearest five samples, measured by Pearson correlation (see Rosenwald et al. [22] for more details on this approach). The KNN prediction is also accompanied by a confidence measure, *V*, which is the number of neighbors (between 1 and 5) agreeing with the KNN reported result.

Each of the two classifiers predicts one of the 42 tumor types listed in Table 1 or one of the following seven tumor classes:

1. Sarcoma (any of case identifications [IDs] 28–34).
2. Renal cell carcinoma (IDs 13–15).
3. Lung, small cell carcinoma or carcinoid (IDs 18 and 19).
4. Testicular germ cell tumor, seminomatous or nonseminomatous (IDs 37 and 38).
5. Astrocytic or oligodendroglial tumor, primary (IDs 6 and 7).
6. Thyroid carcinoma, follicular or papillary (IDs 40 and 41).
7. Adenocarcinoma of biliary tract or pancreas (IDs 4 and 25).

These additional possible diagnoses are reported when the classifier has high certainty regarding the tumor class (e.g., sarcoma) but low certainty regarding the specific tumor type (e.g., which type of sarcoma). Importantly, for these tumor classes, knowledge of the specific subtypes does not have major therapeutic implications, or the subtypes can be determined by further investigation. The two predictions are then combined into a single predicted tissue of origin or two different predictions, based on whether the two classifiers agree (either on tumor type or on one of the seven tumor classes) and on their confidence measures (*p* and *V*). When two predictions are reported, they are ordered by the likelihood as estimated by the positive predictive value of each of the answers. When both classifiers exhibit very low confidence in their result (low *p* and *V*), the assay does not generate a result and reports that the microRNA expression pattern of the sample does not match any of the expression patterns in the panel closely enough.

We estimated the performance of the assay by cross-validating the training set data and then by additional validation sets as detailed later. Cross-validation of the training data showed that the estimated overall accuracy of the assay is 87%, and that in 86% of the cases a single origin is reported, with an accuracy of 89%.

Assay Validation and Interlaboratory Reproducibility

The assay performance was assessed using an independent set of 509 validation samples (Fig. 1; Table 1). These archival samples included primary as well as metastatic tumor samples, whose original clinical diagnosis (reference diagnosis) was one of the 42 tumor types on which the classifier was trained. The samples were processed according to the appropriate standard operating procedures by personnel blinded to the original reference diagnosis of the samples, and classifications were automatically generated by dedicated software. In all, 11 of the

509 samples (2%) failed QA and an additional 9 samples (2%) completed processing but did not generate a result. For 489 samples (96%), including 146 metastatic tumor samples (30% of the samples), the assay was completed successfully and produced tissue-of-origin predictions. For 418 of the 489 samples, the reference diagnosis was predicted by at least one of the two classifiers, resulting in an overall sensitivity (positive agreement) of 85%. Specificity (negative agreement) was >99%. One of the seven tumor classes, rather than a specific tumor type, was reported for 54 (11%) of the cases. For 403 samples (82%), the assay reported a single tissue of origin (supplemental online Fig. S5). For these single-prediction cases, the sensitivity was 90% (361 of 403). Reassuringly, these performance values are very similar to the results obtained by cross-validation on the training data.

We further analyzed the assay validation data set by different divisions to subgroups. The performance of the assay in metastatic and primary tumors showed no significant difference for all origins except prostate, which was previously discussed as a special challenging case [23]. Tumor percentage in the acceptable range for the assay (>60%) also had no effect on the assay performance, regardless of whether the sample underwent microdissection. To test the performance of the assay according to biopsy site, we calculated for each biopsy site the expected performance based on the distribution of the origins of the metastases to this site (in the validation set) and checked whether there was any site with a significant performance difference from the expected performance. No biopsy site showed any significant difference, attesting to the assay's performance being insensitive to the biopsy site.

Interlaboratory reproducibility was assessed by processing RNA from the training and validation samples independently and blindly in two Rosetta Genomics laboratories (Philadelphia and Israel). Data and classifications for 179 samples that produced results in both laboratories were compared. A Pearson correlation on the expression of the 64 assay microRNAs of >0.95 was achieved in 160 (89%) samples (supplemental online Fig. S3). In addition, the two laboratories agreed on the diagnosis in 175 (98%) of the cases, demonstrating the robustness of the assay.

Validation on Patients with CUP

Assay performance to correctly identify the primary tumor type in patients with brain metastases from unknown origin was tested in a cohort of 55 CUP samples (52 patients) published previously [23]. One sample (<2%) failed QA. For 3 of the remaining 54 samples (6%), the assay did not generate a result. For the remaining 51 samples (48 patients), the assay was completed successfully and produced results (Table 2). Three different brain metastases from one patient (ID 21), later found to have lung adenocarcinoma, were correctly identified by the assay. For another patient (ID 39), two metachronous metastases were studied and resulted in a classification of a carcinoid tumor either in the lung or the intestine. Clinical evaluation of the patient determined a neuroendocrine tumor of unknown primary—a diagnosis that is compatible with both assay predictions. For performance evaluation, we use only one sample per patient.

To evaluate the performance of the assay, we implemented the same concordance score as published previously [23], based on the clinicopathological data available at the time of diagnosis, additional information gathered during patient follow-up, and in some cases data resulting from investigations following the assay result.

The score divides the results into four main categories:

- Type 1: clinical match, in which the diagnosis obtained with the assay is clinically confirmed by imaging or surgery of the primary tumor and pathological findings are compatible.
- Type 2: pathological match (no clinically verified primary tumor), which is subdivided into type 2a (pathology findings are consistent with the assay results) and type 2b (pathology findings cannot rule out the assay results).
- Type 3: pathology mismatch (no clinically verified primary tumor), in which pathology workup is not typical for the assay diagnosis (when the assay predicts two possible origins, the pathology workup is not typical of both).
- Type 4: clinical mismatch, in which the clinical diagnosis is discordant with the assay result.

The assay result predicted a convincing suggested origin (i.e., score type 1 or type 2) in 42 (88%) of the 48 cases that had a suggested origin based on clinical and/or pathological data. For 23 (48%) out of 48 cases, the assay generated a single answer. A clinical and/or pathological match was achieved in 21 (91%) of these samples.

Case ID 38 illustrates the power of the assay in a patient in whom extensive clinical and pathological workup failed to provide a convincing tissue of origin. miRview mets^2 suggested a sarcoma as the origin, although sarcoma was not part of the original differential. Following this result, IHC evaluation of the sample was extended by numerous panepithelial markers as well as lymphoma- and melanoma-markers. In line with the assay result, of all markers tested, the tumor cells revealed a robust and strong expression only for smooth muscle actin and focal robust tumor cell expression of vimentin, both of which are mesenchymal antigens frequently encountered in sarcomas (Fig. 3).

Discussion

We present here an improved assay for prediction of the tissue of origin in metastatic samples. The second-generation assay employs the expression of 64 microRNAs to predict 42 tumor types, covering >92% of all solid tumors [27]. The assay uses a custom-designed microarray and the results were highly reproducible when the assay was performed in two laboratories. The overall accuracy of the assay, based on an independent validation set of 509 samples, was high (85%), with 82% of the samples producing a single predicted origin with 90% accuracy. The assay was also validated on a set of CNS metastatic samples of patients with CUP, resulting in 88% concordance with the clinicopathological evaluation of the patients—an extremely high concordance compared with published studies

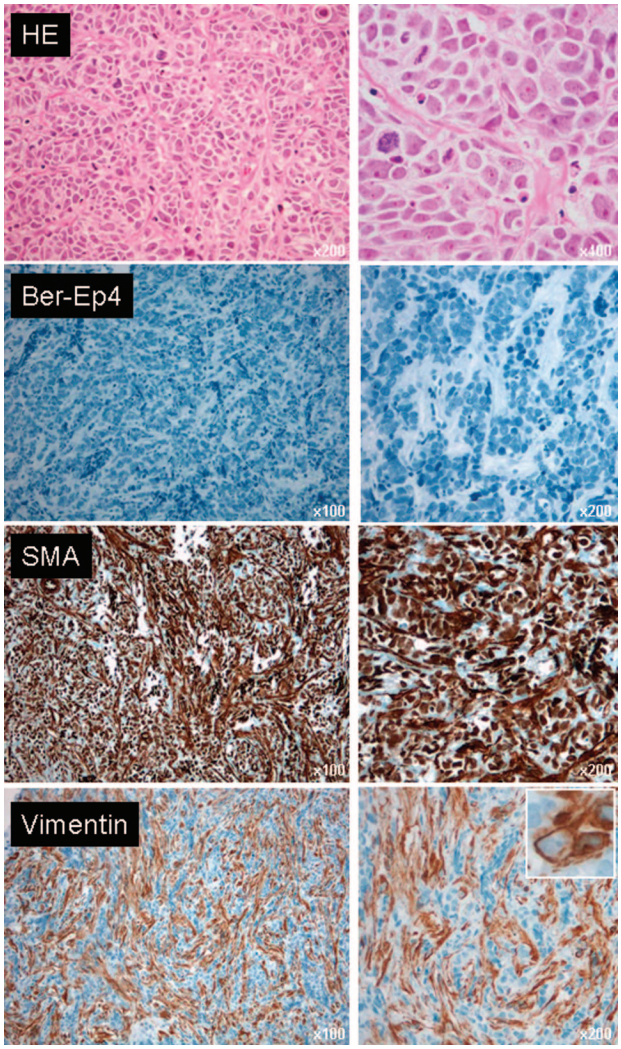

Figure 3. Histomorphology and immunohistochemistry of case identification 38. Despite epitheloid appearance, tumor cells were tested negative for numerous pancytokeratin markers. In contrast, tumor cells revealed a robust and strong expression for smooth muscle actin and focal robust tumor cell expression of vimentin, both of which are mesenchymal antigens frequently encountered in sarcomas.

Abbreviations: HE, hematoxylin and eosin; SMA, smooth muscle actin.

looking at different genomic profiling approaches for diagnosing the tumor of origin in patients with CUP [9, 28, 29].

Our previous experience developing a clinical assay for the identification of the origin of metastatic tumors, which identifies the tissue of origin from 17 organs with a total of 25 histologic subtypes [22], has shown proven usefulness in clinical studies that demonstrate the high accuracy of the molecular profiling results [23, 24]. Even though the most common primary tissues of origin for CUP were represented in our first-generation assay, there was a desire to improve its clinical utility by including other carcinomas, such as urothelial carcinoma, carcinoma of the uterine cervix, additional histological subtypes for renal cell carcinoma, adrenocortical carcinoma and pheochromocytoma, and different types of sarcoma, mesothelioma, lymphoma, and primitive germ cell tumors of the ovary.

One of the challenges for the development of FFPE-based assays with retrospective samples is that older archival blocks may not provide RNA of sufficient quality to obtain meaningful results. Penland et al. reported successful mRNA expression analysis using microarrays in only one-quarter of unselected FFPE blocks that were between 2 and 8 years old [30]. More recently, much higher failure rates have been described for mRNA-based expression used for clinical commercial assays: 22% for Pathwork CUP assay [10] and 6%–29% for BioTheranostics CUP assay [28, 31]. The QA failure rate for the microRNA-based assay presented here is 2% (11 of 509 cases of known origin and 1 of 55 patients with CUP) for specimens 1–20 years old without reduction in the quality of the RNA extraction or the accuracy of the assay results (supplemental online Fig. S4).

Potential issues were recently raised [28] about the use of microarray platform as compared to qRT-PCR, claiming lower sensitivity, batch effects, and a limited dynamic range of 10^2. These limitations were all indeed demonstrated for mRNA microarray measurement. In contrast, our microRNA microarray platform (supplemental online Fig. S1) demonstrated an extremely high reproducibility (at least 10 different batches of microarrays were used), sensitivity, specificity, and a dynamic range of $>10^3$, thereby demonstrating the validity of this platform for use in a clinical setting.

Another potential issue often raised regarding the development and validation of molecular profiling assays is the number of specimens used. The miRview mets^2 assay presented here was developed based on 1,282 tumor samples and validated on a cohort of 509 tissue specimens that was independent of the discovery and training cohort. The size of the validation cohort is similar to the 547 tissue samples used for the validation of the Pathwork CUP [10] assay that uses an Affymetrix microarray platform [32] and significantly more than the 187 samples used for the validation of the bioTheranostics CUP assay that uses a qRT-PCR platform [28]. The number of specimens per tumor class that were used in the training phase of the assay ranged from 5 to 140 (median 24); in the validation cohort, the range was 2–26 samples per tumor class (median 15; Table 1). The tumors with the smallest numbers were typically subgroups of larger categories (e.g., different types of sarcomas). The validation panel included primaries and metastases from different differentiation levels, including poorly and undifferentiated tumors.

The assay was further validated on a cohort of actual CUP patients, previously studied on the first version of the microRNA-based assay [23]. This validation confirms the high level of accuracy of microRNA-based profiling in CUP cases that we have seen in the earlier study and also demonstrates the improvement of the new assay with an overall concordance to the clinicopathological evaluation in 88% of the samples compared with 80% concordance in the previous study. This high level of concordance can be compared to other commercial tests, which have similar performance in validations based on

known primaries but show marked deterioration in performance when testing real patients with CUP. Pathwork reported 62% concordance [33] and bioTheranostics reported 75%–76% concordance [28, 31], compared with the 88% concordance when using the miRview mets^2 assay.

Molecular profiling in CUP should be considered in the context of IHC, which is a standard diagnostic method used to determine tissue origin. IHC is a powerful tool in CUP [32, 33] cases, but even with the use of IHC, there remains a need for additional diagnostic methods. The choice of the IHC panel itself is a subjective decision that may be biased by the clinical history and presentation of the patient. Interpretation of the IHC results is also subjective, resulting in high interobserver and intraobserver variability. The objective and unbiased approach of this assay is a major advantage, as well as its high reproducibility demonstrated in the interlaboratory results comparison. Moreover, in >30% of the cases, the staining pattern of IHC does not result in a conclusive diagnosis [12]. This may be the case for tumor locations for which no specific markers are available or dedifferentiated tumors which have lost expression of characteristic markers. The fact that we found no deterioration of performance of our assay between cases of known primary and CUP cases that are more difficult to diagnose suggests that this molecular assay adds information to that obtained by IHC. Thus, the miRview mets^2 assay may complement IHC and guide diagnosis in difficult or uncertain cases, especially when IHC studies are inconclusive or incompatible with clinical findings.

Finally, any given assay able to predict tissue of origin with high sensitivity and specificity is potentially interesting for clinical oncologists. It is the more practical issues, however, that determine its definite clinical implementation in day-to-day practice. One major issue with expression platform-based analyses is time. Ideally, the timeframe from obtaining the tissue to the decision to process the tissue on the platform to the result of the platform analysis guiding all further clinical decisions should not exceed the time usually needed for a standard pathology workup of a surgically obtained specimen. The total turnaround time for the miRview mets^2 assay is 7–10 days, which is a timeframe well suited to meet clinical needs. In addition, in the case of patients with cancers of unknown or uncertain primary origin, this short processing time allows unguided tumor evaluation and staging investigations to be put on hold until the analysis data are available. Besides better guiding patient management and therapy, this might also help reduce constantly growing evaluation costs in patients with cancers of unknown or uncertain primary origin.

In summary, this improved second-generation microRNA-based assay can serve as a reliable diagnostic tool to aid physicians with challenging diagnostic dilemmas.

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E.M., W.C.M., and S.R. contributed equally to this work. T.B.E. is currently affiliated with the Department of Pathology, Cooper University Hospital, Camden, NJ.

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A Second-Generation MicroRNA-Based Assay for Diagnosing Tumor Tissue Origin

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A novel microRNA-based Assay demonstrates 92% accuracy in classification of metastatic tumors from patients diagnosed with carcinoma of unknown primary

ASCO (2012)

American Society of Clinical Oncologists

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Introduction

- Cancer of unknown primary (CUP) constitutes 3%-5% of all newly diagnosed cancer cases, and if cancer of uncertain origin is added, the total number increases to 12-15%. It presents a major diagnostic challenge due to the significant therapeutic management implications for the patients.
- Here we present miRview® mets 2 performance in a blinded study on a well annotated cohort of real CUP patients.

Summary

- miRview® mets 2 assay can be successfully performed on most clinical FFPE tissue samples
- In the studied cohort of real CUP patients, miRview® mets 2 assay demonstrates agreement with pathological and clinical information in 92% of cases microRNA profiling can be a useful adjunct to traditional clinical and pathologic evaluation for CUP cases.
- For CUP patients, time is of the essence and our assay can help by both narrowing down the potential diagnostic options and increasing confidence in a suspected tissue of origin or by suggesting a different origin at presentation, resulting in earlier correct management.

Introduction

- Cancer of unknown primary (CUP) constitutes 3%-5% of all newly diagnosed cancer cases, and if cancer of uncertain origin is added, the total number increases to 12-15%. It presents a major diagnostic challenge due to the significant therapeutic management implications for the patients.
- Here we present miRview® mets^2 performance in a blinded study on a well annotated cohort of real CUP patients.

miRview® mets^2

- miRview® mets^2 assay is capable of identifying the tissue of origin using a set of 64 microRNAs and a custom array platform.
- The assay was trained on a total of 1282 primary and metastatic samples from 42 tumor types (all from known origins). The assay returns either a single tissue of origin or two possible origins
- The assay was validated on an independent set of 509 samples, and demonstrated high level of accuracy: sensitivity for a single answer prediction of 90%, overall sensitivity of 85% and overall specificity of up to 99%
- An inter-lab reproducibility study of 179 samples verified high technical concordance of 98% agreement between labs
- A separate validation study on 52 true CUP cases from CNS origin showed 88% concordance with clinical presentation and pathology

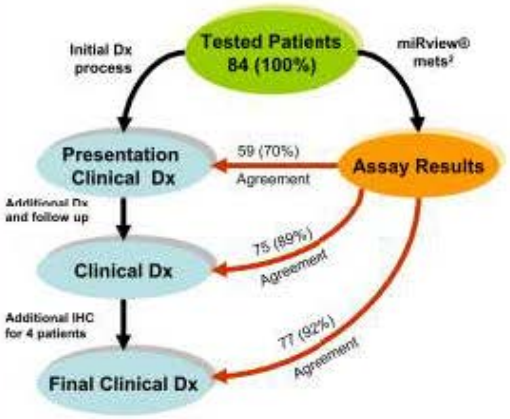
Test Protocol and Tumor Classification

- High quality RNA is extracted from FFPE sections of a tissue or a cell block from resection, biopsy or FNA procedure
- RNA is labeled and hybridized onto custom microarrays with triplicate probes to measure the expression of each of the 64 test microRNAs in the specific specimen, as well as hundreds of microRNAs for normalization and control
- The test relies on two classifiers to determine the tissue of origin: a binary decision-tree classifier and a k-nearest neighbors (KNN) classifier. Each of the two classifiers predicts one of the 42 tumor types or one of 7 combined tumor classes (e.g. Adenocarcinoma of Biliary Tract or Pancreas), and assigns a confidence measure to its prediction
- The two predictions are then combined into a single predicted tissue origin or two different predictions, based on whether the two classifiers agree and on their confidence measures. When two predictions are reported, they are ranked by the PPV of each answer

Validation on a cohort of real CUP cases

- FFPE CUP blocks were collected retrospectively from patients diagnosed with CUP according to a standardised clinicopathologic diagnostic algorithm and managed in HeCOG-affiliated centers from 2001 until 2009. Most patients were males (59), and belonged to visceral (34), squamous head neck (18), nodal (11), peritoneal carcinomatosis (20) and axillary nodal (9) CUP subgroups.
- Samples from 92 CUP patients were tested blindly on the miRview® mets^2 assay
- 8 samples failed the process due to inadequate RNA quality
- Samples from 84 patients were processed successfully and were assigned assay results

Diagnostic Process



Results

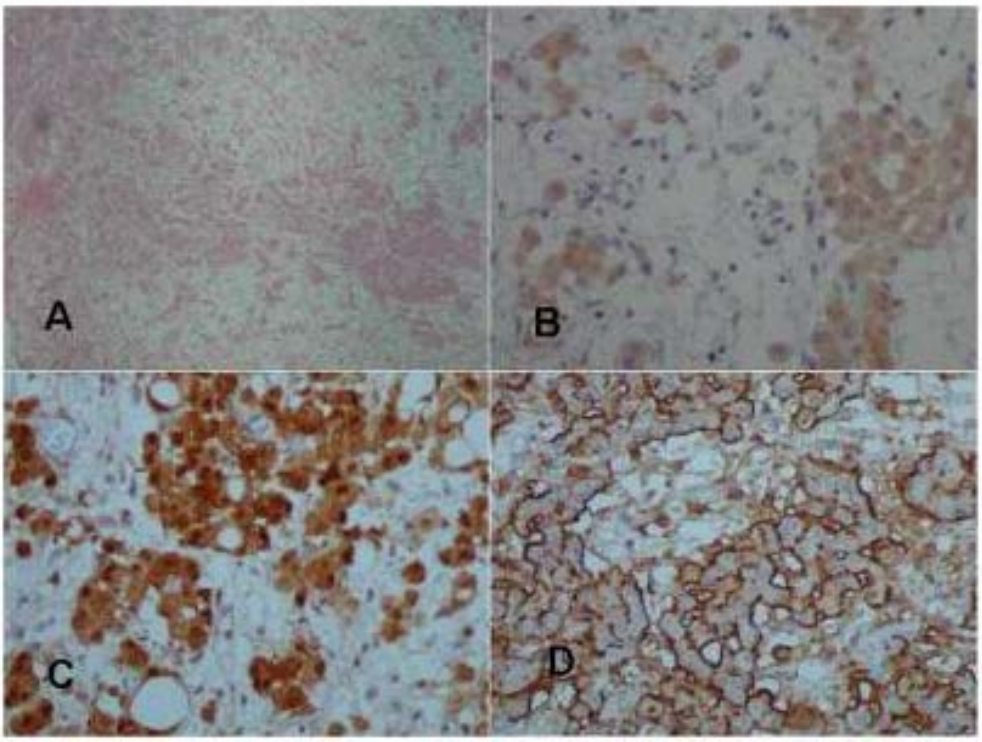
- In 77 patients (92%) the test results were fully concordant with diagnosis based on all the clinical and pathological information available including follow-up and outcome data
- The diagnosis based on the clinical and pathological data available at presentation, and without additional data gathered throughout patient management, had agreement with 59 patients (70%) with the assay results
- For 18 patients, the change from the presentation clinical diagnosis to the miRview® mets^2 assay result (which is in agreement with the final clinical diagnosis) would have resulted in administration of different chemotherapeutic regimens.
  - In 9 of them, the change in diagnosis would have resulted in different combination chemotherapies likely to be more active and associated with superior survival.
  - In 16 of these 18 patients, the change in diagnosis could have been coupled to a change in targeted therapy employed.
- The assay may have also proved useful for the other 59 patients (groups 2 and 3 in the table), by providing objective information useful for narrowing down the spectrum of differential diagnoses and supportive for increasing the confidence level of the diagnosis

Agreement to miRview® mets^2

Patient Group #	Group Description	n (%)	High confidence of Presentation Clinical Dx	Presentation Clinical Dx & miRview mes2 agreement	Final Clinical Dx & miRview mets2 1st result agreement	Final Clinical Dx & miRview mets2 2nd result agreement
1	miRview mets2 results agree with Final Clinical Dx but not with Presentation Clinical Dx	18 (21.5)	3/18	0/18	16/18	2/18
2	miRview mets2 reported a single result which agreed with Final Clinical Dx and with Presentation Clinical Dx	33 (39.5)	22/33	33/33	33/33	0/33
3	miRview mets2 reported two results which agreed with Final Clinical Dx and with Presentation Clinical Dx	26 (31)	13/26	26/26	25/26	1/26
4	miRview mets2 reaults are not consistent with Clinical Dx	7 (8)	0/7	0/7	0/7	0/7

A case report

- A 61-year-old female patient, suffering from peritoneal carcinomatosis and ascites, diagnosed with CUP. Ovaries were normal, but the adnexal biopsy disclosed an adenocarcinoma positive for CK7, CK8, CK19, CA125, and PR and negative for CK20, CEA, TTF1, CA19-9 and vimentin
- Initial clinical and pathological information directed to a diagnosis of primary peritoneal or ovarian carcinoma
- The patient did not respond to first line taxane-platinum therapy as anticipated but exhibited a rather indolent disease course, reaching an overall survival of 30 months on 2nd line oral vinorelbine, followed by best supportive care only
- miRview® mets2 resulted with a single answer of pleural mesothelioma
- This result triggered more IHC tests to be performed (see figure below)
- The reviewed pathologic diagnosis agreed with the diagnosis of mesothelioma
- The revised diagnosis would have implicated a change of therapy from platinum/taxane to pemetrexed/platinum salts



Results of further IHC performed following miRview® mets^2 test results:

A H and E, **B** CK5/6 X400, **C** Calretinin X400, **D** Mesothelin X400

Clin Exp Metastasis
DOI 10.1007/s10585-012-9548-3

RESEARCH PAPER

Note to MedCAC: We include this to provide complete copies of additional 2012 publications; however, this is primarily a tumor biology study and not a clinical utility publication.

Global microRNA profiling in favorable prognosis subgroups of cancer of unknown primary (CUP) demonstrates no significant expression differences with metastases of matched known primary tumors

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Received: 13 September 2012 / Accepted: 26 October 2012

Abstract No data exist on biologic differences between Cancer of unknown primary (CUP) and metastatic solid tumors of known primary site. We assigned a primary tissue of origin in 40 favorable CUP patients (A: serous peritoneal carcinomatosis n = 14, B: axillary adenocarcinoma n = 8, C: upper squamous cervical adenopathy n = 18) by means of a 64-microRNA assay. Subsequently, we profiled the expression of 733 microRNAs (miRs) in the CUP cases and compared results with metastases from 20 ovarian carcinomas, 10 breast adenocarcinomas, 20 squamous head neck or lung tumors. In the Peritoneal CUP versus Ovarian (Known Primary Metastases) KPM comparison, a total of 12 miR were significantly differentially expressed: higher than twofold expression difference in CUP was seen only for miR-513a-5p (3.7-fold upregulated) and miR-483-5p (2.5-fold upregulated), while miR-708 exhibited a twofold downregulation. In the Breast CUP versus Breast KPM comparison, only miR-29c that were downregulated in CUP by 2.7-fold satisfied the FDR threshold. miR-30e and miR-27b, downregulated in ovarian CUPs versus KPMs, were also non-significantly downregulated in breast CUP by 2.0- and 1.4-fold respectively. Six miRs, which belong to the 17–92 oncocluster showed a trend of upregulation in Breast CUP versus Breast KPM cases. A CUP signature remains elusive.

Keywords Cancer of unknown primary · MicroRNA · Gene expression

Introduction

Cancer of unknown primary (CUP) is defined as histologically verified metastatic malignant deposits in the absence of an identifiable primary tumor despite a standardized diagnostic work up based on physical examination, imaging and pathologic studies. It represents 3–5 % of newly diagnosed malignancies per year and ranks as the 5th–7th most common cause of cancer death [1]. Epidemiologic and clinicopathologic studies provide ample evidence for the heterogeneity of CUP, which is made up from several unfavorable (poor) prognosis and favorable (good) prognosis subgroups. However, to date there is no consensus on the true biology or pathogenesis of CUP or of CUP subgroups. Some investigators consider it an artifactual grouping of tumors for which the primary could simply not be identified due to limitations of bioimaging/sampling technologies, while others regard it as a distinct clinical entity bearing a CUP-specific biological signature resulting in the hallmark features of early systemic spread and

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primary tumor dormancy [2]. Sound data on the nature of CUP would guide therapeutic management of the patients on divergent philosophies: assignment of a primary tissue of origin and administration of primary-specific therapy versus targeting biomolecules important for the prometastatic phenotype irrespective of the primary.

High-throughput gene expression platforms have emerged over the last decade and are used in a universal manner: they profile gene expression of solid tumors, identify gene sets differentially expressed between them and are applied in CUP cases in order to "molecularly" assign them to a tissue of origin [3]. This would allow administration of optimal, primary tissue of origin-specific cytotoxic and targeted therapy. However such a therapeutic strategy is based on the unproven assumption that CUP that is biologically classified will behave and respond to therapy similarly to metastatic solid tumors of known primary. In other words, the hypothesis that CUP harbors no major genetic differences compared to metastatic solid tumors not only lacks data, but lacks studies to generate the data as well. Epidemiologic data support the similar biology and natural history of subgroups of CUP with favorable prognosis to that of equivalent metastatic tumors of known primary, but uncertainty exists for visceral CUP [4–7], which has unfavorable prognosis.

MicroRNAs (miRs) are short RNA genes that regulate a variety of biologic and pathologic processes, and are emerging as highly tissue-specific biomarkers which are well preserved in formalin-fixed tissue. Their tissue-specific expression and their ability to control protein synthesis via regulation of hundreds of messenger RNA (mRNA) molecules makes them excellent biomarkers for profiling studies that look at tumor biological behavior [8]. In order to screen for miR differences between CUP metastases with favorable prognosis and metastases of known primaries (Known Primary Metastases, KPM) we embarked on a two-stage profiling project. First, we biologically assigned CUP cases to primary tissues of origin using the miRview® mets2 64-microRNA microarray assay [9] from Rosetta Genomics (Philadelphia PA). In the second stage, we profiled the expression of 733 miRs in those CUP tumors biologically classified as breast, serous ovarian and upper squamous cancer as well as in matched KPM of metastatic breast, ovarian and upper squamous cancer. Comparison of the global miR expression profile in biologically classified CUPs versus tissue-of-origin matched KPM was performed in order to screen for a distinct, CUP-specific miR signature in CUP subgroups with favorable prognosis.

Materials and methods

Tissue blocks were collected retrospectively from patients diagnosed with CUP according to a standardized clinicopathologic diagnostic algorithm (history, physical examination, CT of chest/abdomen/pelvis, pelvic examination, mammography in women, full blood counts, full biochemistry, serum PSA, AFP, bHCG). These patients were managed in HeCOG-affiliated centers from 2001 to 2009 and provided written informed consent for research use of their biologic material. The research project was approved by the Scientific and Ethics Committee of the Ioannina University Hospital (16-1-2007/15) and by the Bioethics Committee of the Medical School of the Aristotle University of Thessaloniki (8-9-2010/A254).

The CUP samples were assigned up to three clinical diagnoses of the most probable primary by the treating physicians based on history, presentation and other clinical and pathologic criteria, including immunohistochemistry. They were also assigned a molecular diagnosis (tissue of origin of malignancy) provided by the 64-microRNA assay, as previously described [10]. We chose samples for which the 64-microRNA assay results were in agreement with the origin suspected by the HeCOG-affiliated centers and that belonged to three main groups of tumor types: serous ovarian/peritoneal carcinoma, breast adenocarcinoma and squamous cell carcinoma of the lung and head/neck. We compared their global miR expression to their expression in KPMs, matched from one of the three main groups of tumor types: serous ovarian carcinoma, breast adenocarcinoma and squamous cell carcinoma of the lung and head/neck of known primary tumors. These KPM samples were previously used for the development and validation of the 64-microRNA assay [10].

Microdissection and RNA extraction

Total RNA was isolated from three to ten 10 μm-thick tissue sections per case. Hematoxylin Eosin (H&E) slides were reviewed by a certified pathologist to estimate the tumor cellular content of the block. Blocks containing > 60 % tumor cellular content were sliced into Eppendorf tubes and sections from blocks containing lower tumor content were mounted onto glass slides for microdissection. RNA was extracted as previously described [11]. Briefly, the sample was deparaffinized in xylene. Proteins were degraded by proteinase K followed by phenol/chloroform and precipitation using ethanol; DNAses were introduced to digest DNA. For microdissection, sections on glass slides were deparaffinized in xylene and rehydrated through descending ethanols. Sections were stained in .01 % methylene blue, washed in double distilled water and dipped into 10 % glycerol. Area containing > 60 % tumour content was scrapped off the slide with sterile scalpel blade under the upright microscope. Dissected tissue fragments were collected into Eppendorf tubes with proteinase K buffer. RNA was then extracted.

Microarray platform

Custom-designed arrays from Agilent Technologies (Santa Clara, CA) which harbour 8 identical sub-arrays (8 × 15,000 format) were used for array platform. .37–1 μg of total-RNA was labeled by ligation of an RNA-linker, p-rCrU-Cy/dye (BioSpring GmbH, Frankfurt, Germany; Cy3 or Cy5) to the 3′end. Synthetic small RNA controls were spiked before labeling. Slides were incubated with the labeled RNA for 12–16 h at 55 C and washed according to the Agilent's protocol. Arrays are scanned using the Agilent DNA Microarray Scanner Bundle at a resolution of 5 μm, dual pass at 100 and 10 % laser power. Array images were analyzed using Agilent Feature Extraction software (FE) version 10.7.1.1. Triplicate spots were combined to produce one signal by taking the logarithmic mean of reliable spots. Analysis was performed in log2-space. Normalization was performed for each sample with respect to a reference vector (R), calculated by taking the median expression level over the training set. For each sample data vector S, a 2nd degree polynomial F was found so as to provide the best fit between S and R, such that $R \approx F(S)$. This was performed on a set of invariant miRs; remote data points ("outliers") were not used for fitting the polynomial. For each probe in the sample (element Si in the vector S), the normalized value (in log2-space) Mi was calculated from the initial value Si by transforming it with the polynomial function F, so that $Mi = F(Si)$.

Data analysis

The expression of 733 miRs was studied in each CUP and KPM sample in the array platform described above. Since the different groups of tumor types present different miR expression profiles, we analyzed each group in a separate analysis, with the aim of identifying miRs differentiating CUP from matched KPM in each group, as well as those in common, if such exist.

For each CUP–KPM comparison, only miRs that expressed above the background level in one of the groups were compared. *P* values were calculated using a two-sided unpaired *t* test on the log-transformed normalized signal, and significance level was adjusted using False Discovery Rate of .1 to deal with the multiple hypothesis issue [12]. In other words, the *P* value threshold for identifying significantly differential miRs is set to the level as calculated using the FDR, which takes into account the number of hypotheses (miRs tested). An FDR threshold of .1 means that it is expected that 10 % of the miRs identified as significant are randomly differentially expressed. Fold-change for each miR was calculated by the change in the median values of the normalized fluorescence signal between the groups.

Results

We chose 40 CUP samples for which the primary assigned by the 64-microRNA assay results were in agreement with the clinicopathologic diagnosis, extracted RNA was of good quality/quantity, and which represented the three most commonly diagnosed CUP subgroups with favorable prognosis: serous ovarian/peritoneal carcinoma (N = 14), breast adenocarcinoma (N = 8) and squamous cell carcinoma of the lung and head/neck (N = 18). A CONSORT flow diagram is provided in Fig. S1. We compared their global miR expression to 50 KPM samples of the same origins: serous ovarian/peritoneal carcinoma (n = 20), breast adenocarcinoma (n = 10) and squamous cell carcinoma of the lung and head/neck (n = 20). The KPM samples were randomly chosen in similar size groups out of hundreds of samples used to develop and validate the mets2 assay on the basis of availability of enough tumour, extracted RNA, matched primary and histology.

Clinicopathologic characteristics

The characteristics of CUP cases analyzed are summarized in Table 1. Fourteen female patients with serous peritoneal

Table 1 Clinicopathologic characteristics of favorable prognosis CUP cases analyzed

Characteristic	Ovarian/ peritoneal CUP N = 14	Breast CUP N = 8	Upper squamous CUP N = 18
Histology	Serous in all	Adenocarcinoma in all	Squamous in all
Median age	62	56	68
(Range)	(44–78)	(35–71)	(54–77)
Gender	All female	All female	Male 14
			Female 4
Histological grade			
1	4	1	2
2–3	10	7	16
Deposit biopsied			
	Omental 3	Axillary node 7	Cervical nodes 15
	Peritoneal 7	Inguinal node 1	Inguinal node 1
	Lymph nodes 3		Peritoneal 1
	Liver 1		Liver 1
Metastatic sites			
	Peritoneal deposits 14	Axillary nodal 7	Cervical nodes 18
	Lymph nodes 8	Other lymph nodes 1	Visceral 2
	Visceral 4	Visceral 1	

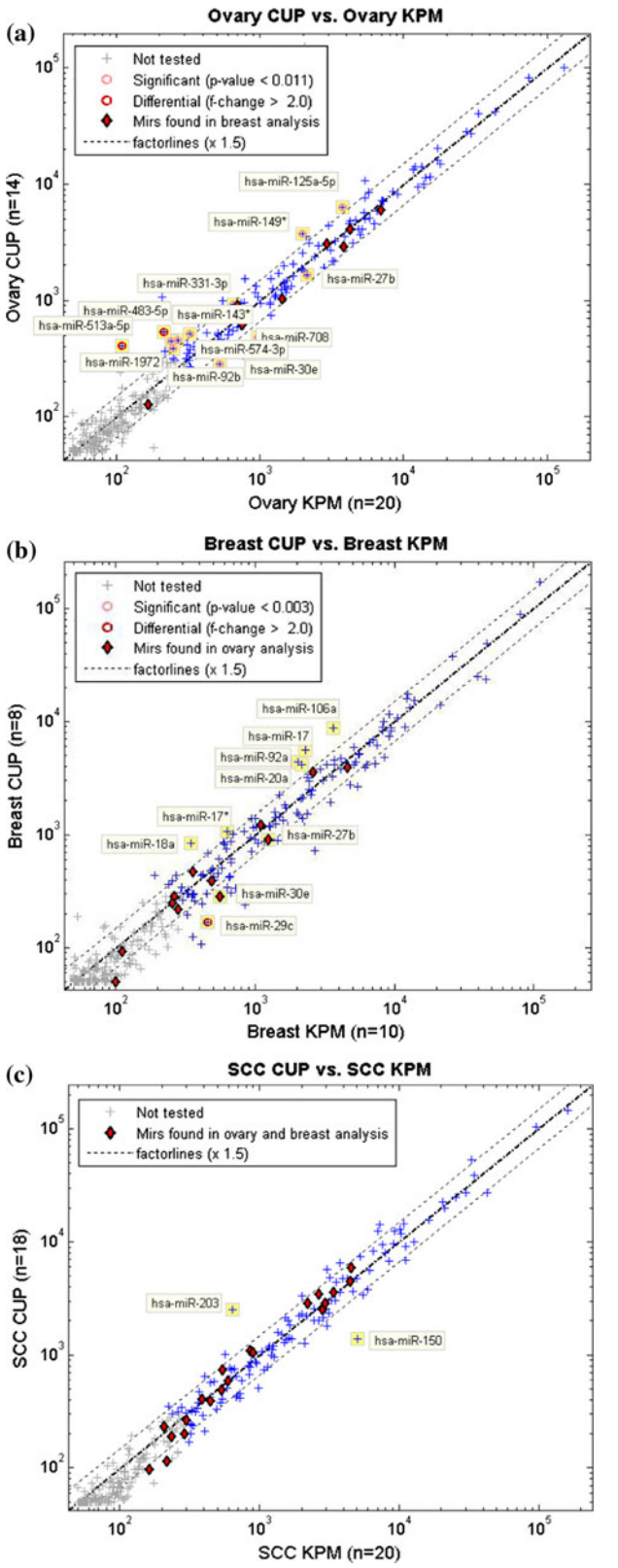

Fig. 1 Differences in expression levels of microRNAs between serous ovarian/peritoneal CUP and ovarian KPM (**a**); Breast CUP and Breast KPM (**b**) and lung and Head Neck squamous cell carcinoma CUP and KPM (**c**): *Scatter-plot* showing the median microRNA expression levels (normalized fluorescence signals by microarray, shown in log-scale) in CUP and KPM. *Grey crosses* show microRNAs whose expression level was at background levels in both groups. All other microRNAs were tested for statistical differences by two-sided unpaired *t* test, with significance corrected by False Discovery Rate (FDR) of .1 (Methods). microRNAs which had a *P* value lower than the FDR threshold *P* value of .011 (**a**) or .003 (**b**) are marked by *pink circles*, and those that in addition had fold-change of medians > 2, are marked by *red circles*. MicroRNAs of interest discussed in the Results section are highlighted in *yellow* and *labeled*. (Color figure online)

CUP and no pathologic evidence of ovarian primary had a median age of 62 and harbored peritoneal deposits, mostly high-grade, occasionally accompanied by nodal (8) and visceral (4) metastases. In the KPM group, 20 female patients of a median age of 65 had moderately to poorly differentiated KPMs from ovarian origin. Metastatic sites were omentum (9), peritoneum (9) and lymph node (2).

Eight female patients with breast CUP and no clinicopathologic evidence of a breast primary had a median age of 56 years and presented with high-grade adenocarcinomatous deposits in axillary lymph nodes, visceral metastases being present in one case only. The 10 KPM cases were female patients of a median age of 60 years with moderately to poorly differentiated breast adenocarcinomatous metastases in axillary lymph nodes (8) or lung (2).

In the upper squamous CUP group, 14 males and 4 females of a median age of 68 were affected by moderately to poorly differentiated squamous carcinoma in cervical lymph nodes with distant nodal and visceral deposits in only one and two cases respectively. Imaging studies and ENT panendoscopy/bronchoscopy failed to document a primary in head, neck or lung. The matched KPM group consisted of 14 males and 6 female patients with squamous KPMs from head to neck (17) or lung (3), presenting with metastases in neck lymph nodes (15), brain/spine (3) and lung parenchyma (2). Their median age was 62.

Peritoneal CUP versus Ovarian KPM

14 serous peritoneal CUP were classified as ovarian cancer by the 64-microRNA assay and were compared to 20 serous ovarian KPM. A total of 12 miR were differentially expressed between ovarian CUP and KPM, nine overexpressed and three downregulated in CUP (Fig. 1a). These 12 differential miRs (Table 2) were statistically significant for a False Discovery Rate (FDR)-adjusted $P < .011$. Of miRs upregulated in CUP, a higher than twofold difference in relative quantification was seen only for miR-513a-5p (3.7-fold difference) and miR-483-5p (2.5-fold). miR-149* had a 1.9-fold expression difference, while miR-92b a 1.8-fold difference. Of the miRs that were repressed in CUP, only miR-708 exhibited a twofold downregulation, while miR-30e and miR-27b were downregulated by 1.8- and 1.3-fold respectively.

Table 2 MicroRNA expression differences of between CUP and matched KPM for microRNAs discussed in the results

MicroRNA	CUP	Median fold change in expression	*P* value	Satisfied FDR criterion
Serous ovarian/peritoneal CUP				
n = 14 vs. KPM n = 20				
513a-5p	Up	3.7	.00012	Yes
483-5p	Up	2.5	.00162	Yes
149*	Up	1.9	.00061	Yes
92b	Up	1.8	.00802	Yes
1972	Up	1.7	.00014	Yes
125a-5p	Up	1.7	.00820	Yes
143*	Up	1.6	.00359	Yes
574-3p	Up	1.5	.00294	Yes
331-3p	Up	1.4	.00001	Yes
708	Down	2.0	.00934	Yes
30e	Down	1.8	.00022	Yes
27b	Down	1.3	.00958	Yes
Breast CUP				
n = 8 vs. KPM n = 10				
17	Up	2.5	.0074	No
18a	Up	2.5	.01771	No
106a	Up	2.4	.01024	No
92a	Up	2.2	.02318	No
20a	Up	1.9	.01985	No
17*	Up	1.7	.01623	No
29c	Down	2.7	.00050	Yes
30e	Down	2.0	.01337	No
27b	Down	1.4	.03035	No
Upper squamous CUP				
n = 18 vs. KPM n = 20				
203	Up	4.0	.34730	No
150	Down	3.6	.01179	No

Breast CUP versus Breast KPM

Eight CUP classified as breast cancer by the 64-microRNA assay were compared to 10 breast KPM (Fig. 1b). Only miR-29c that was downregulated in CUP versus KPM by 2.7-fold satisfied the FDR-adjusted $P < .003$ criterion (Table 2). However, several other miRs showed a difference in median expression that should be noted: miR-30e and miR-27b that were downregulated in ovarian CUPs versus KPMs were also downregulated here by 2.0-fold and 1.4-fold in CUP respectively but did not pass the FDR criterion (Table 2). Interestingly, six miRs which belong to the 17–92 microRNA oncocluster (miR 17, 18a, 106a, 92a, 20a, 17*) showed a trend of upregulation in CUP cases, four of them by more than twofold (miR 17, 18a, 106a, 92a). However, none of them qualified as significant by the FDR criterion.

Upper squamous CUP versus upper squamous KPM

We compared global miR expression between 18 64-micro RNA assay-assigned upper squamous (Lung–Head–Neck) CUP and 20 upper squamous KPM samples. We did not find any miR that was differentially expressed in a statistically significant manner (Table 2, Fig. 1c). miR-203 had a fourfold increased expression in CUP cases, however the individual cases exhibited large variation in expression levels resulting in a non-significant *P* value. miR-150 was downregulated in upper squamous CUP by 3.6-fold, however not satisfying the FDR criterion.

MicroRNA with unidirectional expression differences in all CUP subgroups

A number of miR exhibited consistent differential expression toward the same direction in all three comparisons (Ovarian/Peritoneal CUP versus KPM, Breast CUP versus KPM, upper squamous CUP versus KPM). Universally upregulated in CUP were miR-331-3p, and miR-17* (Fig. 2) while miR-708, miR-30e and miR-29c were universally downregulated in all CUP subgroups studied (Fig. 3). Of note, no miR exhibited difference in expression levels that was statistically significant across all three CUP subgroup comparisons. Accordingly, this observation may well be due to chance and can only be used for generation of hypotheses to be tested in larger patient cohorts.

Discussion

Several investigators have used proteomic or mRNA profiling platforms in order to identify pro-metastatic gene signatures in solid tumors such as breast, colorectal and lung cancer [13–17]. However these signatures reflect genetic differences between solid tumors that metastasize and those that do not, and are restricted to a primary tumour type. In contrast, Ramaswamy et al. [18] used mRNA microarrays to identify a 17-gene metastatic signature common across several tumor types, suggesting for the first time that systemic spread is a complex tumor feature transcending the tissue-of-origin biologic characteristics. Finally, several multi-gene platforms that probe [measure?] protein, mRNA or miR differential expression in various carcinomas are able to identify, with higher than 80 % accuracy, the primary tumor type of malignancies of unknown or uncertain origin [3]. The goal of our research was distinct from the three approaches described above:

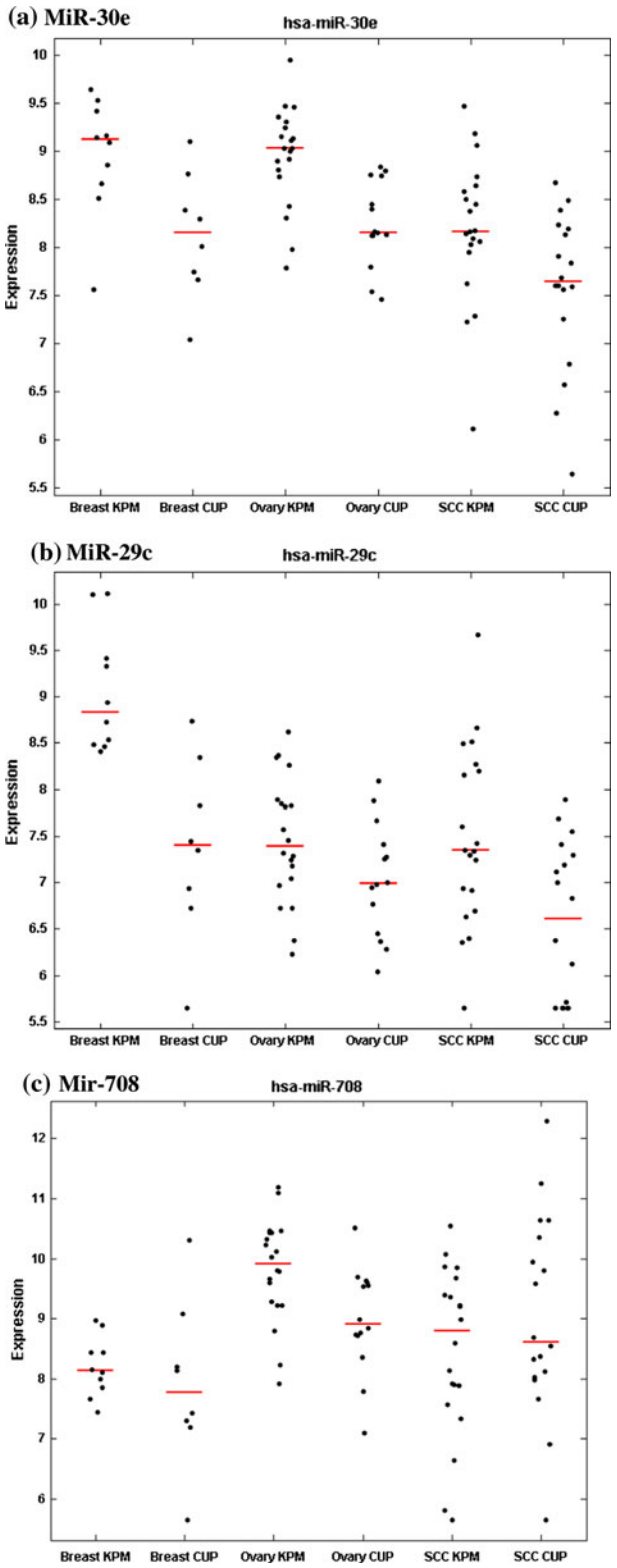

Fig. 2 *Legend* Relative expression levels of annotated microRNAs upregulated in CUP: *Dot plot* representation of the expression of miR-30e (**a**), miR-29c (**b**) and miR-708 (**c**) in CUP vs. KPM in all three groups. *Horizontal red lines* represent the median expression in each group. Units show log2 of the normalized fluorescence signal. miR-30e presented fold changes of median of 1.95, 1.84 and *P* values of .081, .013 and .0002 in breast and ovary respectively. Fold change and *P* value were not calculated for SCC as expression is within the background level. miR-29c presented fold change of 2.7 and *P* value of .0005 in breast. Fold changes and *P* values were not calculated for ovary and SCC as expression is within the background level. miR-708 presented fold changes of median of 2.0, 1.13 and *P* values of .009, .26 ovary and SCC respectively. Fold change and *P* value were not calculated for breast as expression is within the background level. (Color figure online)

We chose to look for differences in miR expression between metastases of unknown primary and metastases from known primary tumors in order to screen for an aggressive, pro-metastatic, CUP-specific biologic signature. In order to exclude miR differences that were simply due to distinct tissues of origin, we used the 64-microRNA assay to assign a primary to CUP cases and proceed to CUP versus KPM comparisons matched for primary site. We acknowledge the reduction in power resulting from the breaking up of our cohort to subgroups, however we consider this primary tissue match relevant and biologically necessary, since miR expression is primary tissue-dependent.

The decision for what to look for or the knowledge of what is important in CUP is of pivotal importance. If indeed the biology of CUP is defined by the biology of the primary tumor or the primary tissue-of-origin, we should focus our efforts towards molecular identification of the primary and administration of primary tissue-optimized chemotherapy along with targeted therapy. On the other hand, if CUP biology is mostly defined by its odd metastatic tropism irrespective of the primary, we should strive to identify biomolecules responsible for the systemic dissemination and therapeutically target them [2]. In a large registry analysis, Bishop et al. [19] suggested that patients with metastatic adenocarcinomas of unknown primary fare significantly worse (median survival of 3 months) than patients with metastases of known solid tumors (median survival of 9 months) [20]. More recently, Greco et al. [21] reported on the outcomes of CUP patients who had their tumors biologically assigned to a primary by means of a multi-gene mRNA expression array and were treated with primary-specific, optimal chemoimmunotherapy. Although survival was superior compared to historical controls receiving empiric chemotherapy, a hint emerges from rather small tumor subgroups that it may not be as high as reported survival times of patients with matched metastases of known primaries (breast cancer, colorectal cancer) [22].

We observed no miRs, differentially expressed between CUP and KPM, that were common in all three subgroups (ovary, breast, upper squamous) with either statistical significance or biological relevance (more than twofold difference in expression). However, in tissue-of-origin matched comparisons some hints of uncertain significance

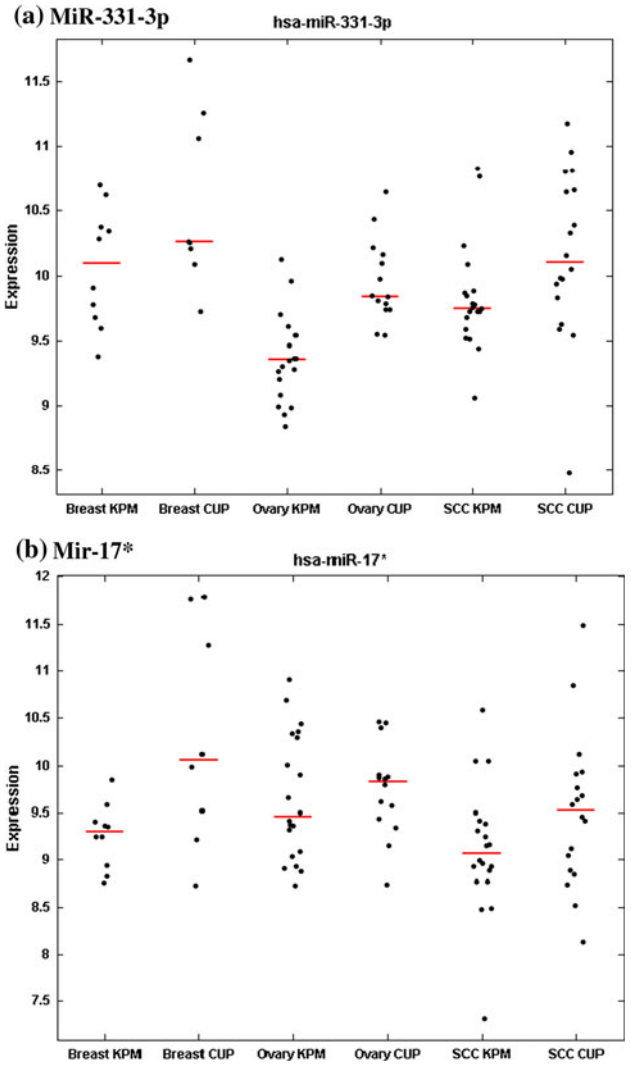

Fig. 3 *Legend* Relative expression levels of annotated microRNAs upregulated in CUP: *Dot plot* representation of the expression of miR-331-3p (**a**) and miR-17* (**b**) in CUP vs. KPM in all three groups. *Horizontal red lines* represent the median expression in each group. Units show log2 of the normalized fluorescence signal. miR-331-3p presented fold changes of median of 1.12, 1.4, 1.28 and *P* values of .081, .00001 and .062 in breast, ovary and SCC respectively. miR-17* presented fold changes of 1.69, 1.29, 1.37 and *P* values of .016, .67 and .12 in breast, ovary and SCC respectively. (Color figure online)

did emerge. In the ovarian CUP versus KPM comparison, miR-513a-5p and miR-483-5p were overexpressed in CUP, while miR-708, miR-30e and miR-27b were downregulated, though the latter two by less than twofold. In the breast CUP versus KPM comparison, miR-30e and miR-27b were also (marginally) downregulated along with miR-29c, while several miRs belonging to the 17–92 oncocluster were upregulated by more than twofold. CUP-downregulated miRs (708, 29c, 30e, 27b) were shown to repress proliferation, invasion and inflammation, to freeze the cell cycle and activate apoptosis in cancer cell cultures and xenografts of several solid tumors by regulating tumor suppressor gene methylation, PPARγ, growth factor receptors and extracellular matrix proteins [23–27]. On the other hand, CUP-overexpressed miRs (513a-5p, 483-5p, 17, 18a, 106a, 92a) are oncomiRs that increase angiogenesis, detachment from epithelial niches and dissemination, cellular proliferation, invasion and inhibit apoptosis in most known solid tumors [28–34]. In vitro data suggest extreme variation of the biological pathways through which these actions are effected. Still, the biological significance of these preliminary findings is uncertain for several reasons. Firstly, the sample size is small. Second, the miRs that had differential expression by more than twofold and satisfied criteria of statistical significance were only three: miR-513a-5p, miR-483-5p in the ovary subgroup and miR-29c in the breast subgroup. Third, for most miRs the medical literature contains reports on opposing effects in regulation of cell cycle, invasion, apoptosis and angiogenesis [35, 36]. This suggests that fine-tuning of miR effects by complex molecular factors is critical and makes interpretation of the biological impact of level fluctuations very difficult. Of note, a global miR profile comparison between all CUP cases (including the 22 CUP with various molecular diagnoses) and KPM resulted in low-power comparisons between several small tumor subgroups with no miR differences emerging (data not shown).

CUP is classified to favorable prognosis and unfavorable prognosis subgroups according to clinicopathologic criteria [37]. Patients with favorable prognosis CUP (axillary nodal adenocarcinoma, squamous cervical or inguinal lymphadenopathy, serous peritoneal carcinomatosis) are managed similarly to those with equivalent metastatic tumors of known primary and frequently enjoy long-term disease control. On the contrary, patients with unfavorable prognosis CUP harbor high-volume visceral metastases to several, often atypical sites, experience rapid disease progression despite multi-agent chemotherapy and have a poor outcome. Systematic reviews of all published cases suggest that patients with favorable prognosis CUP subgroups have a presentation, epidemiology, response to therapy and outcome no different from the stage-matched metastatic tumors of matched primaries and should be treated accordingly [4–7]. We confirm this indirect epidemiologic evidence by finding no marked differences in the miR expression profile of favorable prognosis CUP patients with serous peritoneal, axillary adenocarcinoma and squamous cervical adenopathy compared to patients with metastatic ovarian, breast and squamous head/neck/lung tumours respectively.

Our research is the first to look for characteristics in metastases of unknown primary that differentiate them from primary tissue-matched metastases of known primary. We establish the absence of a CUP miR signature that transcends tissue of origin and the absence of marked miR expression differences within each tissue of origin subgroup

comparison. However, our findings do not exclude the presence of a distinct CUP biological signature that may exist in parallel to a primary tissue-of-origin signature. Such a signature may reside in the realm of epigenetics (DNA methylation, histone acetylation) or proteomics. Most importantly, this signature should be looked for in the clinicopathologic group of unfavorable prognosis, visceral CUP patients for whom there is accumulated epidemiological evidence of early, high-volume systemic spread of malignant deposits in parallel to dormancy of the primary, resistance to therapy and dismal outcome.

Conflicts of interest George Pentheroudakis, Dimitrios Krikelis, Vassiliki Kotoula, Vassiliki Malamou-Mitsi, George Fountzilas, Nicholas Pavlidis have no conflicts of interest to disclose. Yael Spector, Eti Meiri, Mats Sanden, Hila Benjamin and Ranit Aharonov are employees of Rosetta Genomics. YS, EM, HB and RH hold stock options of the company, which develops miR based diagnostic products and may stand to gain by publications of these findings. Each of the authors is willing to complete an individual, electronic conflict-of-interest form.

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