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

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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.

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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;17:801–812

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 mets², 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,

Table 1. Assay tumor panel

ID	Tumor organ of origin	Tumor type (as reported)	<i>n</i> of samples in training	<i>n</i> 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).

^aOf these samples, 16 were renal cell carcinomas of unknown subtype.

^bThe 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		S	Diagnosis based on case revision	Immunohistochemistry relevant for diagnosis
ID	Age/sex	Speculated tumor type at time of surgery	Speculated tumor type based on additional investigations	Suggested origin 1	Suggested origin 2			
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, thyroglobulin; Syn, synaptophysin; NSE, neuron-specific enolase; Vim, vimentin; MAP, microtubule-associated protein; Chromo, chromogranin; 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.

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 format) 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 (log₂). 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 *S_i* in the vector *S*), the normalized value (in log₂) *M_i* is calculated from the initial value *S_i* by transforming it with the polynomial function *F*, so that $M_i = F(S_i)$.

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

Figure 1. Development and validation of miRview mets², 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.

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 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 mets² 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 demon-

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].

strated 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 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 mets² 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 re-

Figure 3. Histomorphology and immunohistochemistry of case identification 38. Despite epithelioid 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.

producible 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 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 his-

tologic 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 BioTheragnostics 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 mets² 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 bioTheragnostics 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 mi-

croRNA-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 bioTheragnostics reported 75%–76% concordance [28, 31], compared with the 88% concordance when using the miRview mets² 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 mets² 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 mets² 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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