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Molecular Tumor Profiling in the Diagnosis of Patients with Carcinoma of Unknown Primary Site: Retrospective Evaluation of Gene Microarray Assay

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Abstract

Background: Molecular tumor profiling has potential importance in identifying the tissue of origin in patients with cancer of unknown primary (CUP). We retrospectively performed the Tissue of Origin test, an FDA-cleared commercially available gene microarray assay, on biopsy specimens from patients with CUP. Assay results were correlated with clinical and pathologic features, and with previous results using the Veridex 10-gene CUP assay, a molecular RT-PCR assay designed to detect 6 primary sites.

Methods: Archival formalin-fixed, paraffin-embedded biopsy specimens from 48 patients with CUP were tested. The assay results were reported without incorporation of clinicopathologic information except biopsy site and patient gender. The assay results were correlated with clinicopathologic information, treatment results, and with results of the previously performed Veridex assay.

Results: The Tissue of Origin test was successfully performed in 45 tumor specimens. In 43 of 45 assays (96%), a specific tissue of origin was predicted. The most commonly identified tissues of origin included: lung (11), pancreas (6), sarcoma (6), ovary (5), and colon (4). Most diagnoses were compatible with the clinical features, IHC staining, and response to treatment. The finding of 6 sarcomas was unusual in this patient population and was suggested by routine pathology in only 1 patient. The Tissue of Origin test provided predictions in a higher percentage of patients than did the Veridex CUP assay (96% versus 53%). However, concordance between assay results was relatively low.

Conclusions: The Tissue of Origin test provided predictions of the primary site in 96% of patients with CUP. Predictions were generally consistent with clinicopathologic features. Agreement between the Tissue of Origin test and the Veridex CUP assay was relatively low, possibly related to the limited number of genes assessed by the Veridex CUP assay. Additional trials are necessary to confirm the value of these assays in patient management.

Carcinoma of unknown primary site (CUP) is a relatively common clinical syndrome, accounting for approximately 2-5% of all cancer diagnoses. Although several clinical subsets with specific treatment implications have been identified, the majority of patients receive empiric chemotherapy which is only modestly effective, producing median survivals of 9-11 months [1-3]. As treatments improve for specific cancer types, it becomes more important to identify the tissue of origin in patients with CUP so that site-specific treatment can be administered.

During the last several years, specific gene expression profiles based on the tissue of origin have been identified for many tumor types [4-6]. Several diagnostic assays, based on either gene microarray or reverse transcriptase polymerase chain reaction (RT-PCR) technology have been developed as an aid to tumor identification based on specific gene expression profiles. The ability to perform these assays on formalin-fixed, paraffin-embedded archival tissue specimens broadens their potential applicability in the diagnosis of patients with CUP.

The Tissue of Origin Test (Pathwork Diagnostics, Redwood City, CA) is a microarray-based gene expression assay for determining the similarity of a tumor specimen to 15 known tumor tissue types. In a group of 462 cancer patients, this assay accurately predicted the primary site in 89% when performed on biopsy specimens from metastatic sites or poorly differentiated primaries [7]. In the current study, we retrospectively performed the Tissue of Origin Test on a group of 48 patients with CUP, and correlated these results with clinical features, standard pathology results, and response to treatment. In addition, we compared the results of the Tissue of Origin Test with previous results obtained with the Veridex 10-gene assay, an RT-PCR assay also developed for this purpose [8].

Methods

Patient selection

Diagnostic biopsy specimens were available from 48 CUP patients who were diagnosed and treated at the Sarah Cannon Cancer Center in Nashville, TN, or on one of several prospective trials performed in the Sarah Cannon Research Institute Oncology Research Consortium between April 1995 and October 2005. Patients participating in these trials received various taxane/platinum-based combination chemotherapy regimens [1,2,9,10].

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The evaluation to confirm the diagnosis of CUP was standard, and included a complete medical history, physical examination, complete

Characteristic	Number of Patients (%)
Median age, years (range)	56 (31-86)
Gender – male	24 (53%)
Female	21 (47%)
Histology – adenocarcinoma	13 (29%)
poorly differentiated adenocarcinoma	12 (27%)
poorly differentiated carcinoma	15 (33%)
squamous carcinoma	5 (11%)
Number of metastatic sites – 1	9 (20%)
2	15 (33%)
≥3	21 (47%)
Metastatic sites – liver	27 (60%)
lung	23 (51%)
lymph nodes	23 (51%)
soft tissue	8 (18%)
bone	6 (13%)
peritoneum	6 (13%)
pancreas	4 (9%)
adrenal gland	2 (4%)
kidney	2 (4%)
mediastinum	2 (4%)
pelvis	2 (4%)
pleura	2 (4%)
spleen	2 (4%)
colon	1 (2%)
ovary	1 (2%)
pericardium	1 (2%)
stomach	1 (2%)
First-line therapy – taxane/platinum-based	38 (84%)
gemcitabine/irinotecan	7 (16%)
Response to first-line therapy – CR/PR	9 (20%)
Stable	18 (41%)
PD	11 (24%)
Unevaluable	6 (13%)
Unknown	1 (2%)

Table 1: Patient Characteristics (*N* = 45).

	Number of Patients (*N* = 45)	
Predicted Site of Origin	Tissue of Origin Test	Veridex 10-Gene Assay*
Lung, non-small cell	11	10
Pancreas	6	7
Sarcoma	6	-
Ovary	5	3
Colon	4	4
Kidney	3	-
Liver	3	-
Breast	2	0
Stomach	2	-
Bladder	1	-
Prostate	0	0
Other	0	19
Unsuccessful	0	2
Non-diagnostic	2	0

*The Veridex 10-gene assay allows detection of 6 sites of origin (lung, pancreas, ovary, colon, breast, prostate). Cancers with gene signatures not matching any of the 6 included types were categorized as "other."

Table 2: Summary of Predictions by the Tissue of Origin Test and the Veridex 10-Gene Assay.

blood counts, chemistry profile, computed tomography (CT) scans of the chest/abdomen/pelvis, and appropriate targeted evaluations of any specific signs or symptoms. Patients with pathologic diagnoses based only on fine needle aspiration biopsy specimens were excluded. This retrospective study was performed after approval by an institutional review board.

Specimen collection

The FFPE biopsy tissue blocks were collected for each patient and sent to Pathwork Diagnostics, Inc, where the Tissue of Origin Test was performed. The site of each biopsy and the sex of the patients were provided; otherwise, the assay was performed and test results produced without incorporation of the clinical characteristics, standard pathology results, or response to treatment.

Assay procedure

Prior to performing the Tissue of Origin Test, the biopsy specimens were examined to ensure that specimens contained at least 60% tumor. RNA was then extracted from the biopsy specimen and processed according to previously published methods [11]. Total RNA was isolated from the biopsy specimen using the FormaPure kit (Agencourt, currently Beckman-Coulter Genomics, Beverly, MA) [11]. The total RNA was processed to prepare labeled cDNA for hybridization to Patchip microarrays manufactured by Affymetrix (Santa Clara, CA) with a two-cycle amplification method using the RampUP kit (Genisphere, Hatfield, PA). A positive/negative total RNA control was run with every amplification batch. The microarrays were washed and stained using the GeneChip Hybridization Wash and Stain kit in a GeneChip Fluidics Station FS450Dx, and scanned with a Gene Chip Scanner 3000Dx (Affymetrix).

The Tissue of Origin Test algorithm transforms probe-level intensity data into gene expression values. The algorithm then performs data verification, standardization and classification in order to generate a test result [7]. Expression levels of the 2000 genes for each specimen are then compared in pairwise fashion with the pre-established gene profiles for each of the 15 tissues on the test panel. The results are reported on an electronic report as 15 separate Similarity Scores, one for each tissue on the panel. The Similarity Score (SS) is a measure of the similarity of the RNA expression pattern of the indicated tumor tissue. Similarity Scores range from 0 (very low similarity) to 100 (very high similarity) and sum to 100 across all 15 tissues on the panel. The highest SS indicates the likely tissue of origin, with one exception: in male patients, a highest SS for ovarian, followed by a second highest SS for testicular germ cell, corresponds to testicular germ cell cancer. A Similarity Score of 5 or less rules out that tissue type as the likely tissue of origin.

For each biopsy specimen, the Tissue of Origin Test result was made available and was then correlated with available clinical, laboratory, and pathology results for each patient. In addition, biopsy specimens from these 48 patients were previously tested with the Veridex 10-gene CUP assay [12]. In this assay, 10 genes are tested using RT-PCR methods, allowing the identification of 6 tumor types (lung, breast, ovary, colon, pancreas, prostate) [8]. Although the Tissue of Origin Test and the Veridex 10-gene assay were not performed in parallel, biopsy material for both assays was obtained from the same archival tissue blocks, so that tissue and source quality was very similar.

Results

Patient characteristics

The Tissue of Origin assay was successfully performed on specimens

from 45 of 48 patients (94%). For three patients, the microarray data file did not pass data quality control, and hence reports were not generated.

Table 1 summarizes the clinical characteristics of the 45 patients assayed. In general, characteristics are typical of a patient population with CUP. The median age was 56 years; most patients had adenocarcinoma or poorly differentiated adenocarcinoma, and metastases commonly involved the liver, lung, and lymph nodes. The overall response rate to standard empiric chemotherapy regimens for CUP was 20%.

Results of molecular profiling

The sites of origin predicted by the Tissue of Origin test are listed in Table 2. Most of the common predictions (lung, pancreas, colon, and ovary) are consistent with the array of primary sites identified in historical autopsy series [11]. The identification of 6 sarcomas (all with light microscopic diagnoses of carcinoma or adenocarcinoma) was unexpected, as sarcomas are not usually identified as a substantial fraction of cancers of unknown primary. Also shown in Table 2 are the predictions made by the Veridex 10-gene assay in the same group of patients. In contrast to the Tissue of Origin test, a group of 19 patients (42%) remained undiagnosed by the Veridex assay, perhaps because this assay allowed identification of only 6 primary sites. Veridex assay predictions of lung, pancreas, and colon primaries accounted for 47% of the group, similar to the proportion identified by the Tissue of Origin test.

Comparisons of the assay results for the 24 patients given specific diagnoses by the Veridex 10-gene assay are detailed in Tables 3,4, and 5. These tables also contain the results of standard pathologic

evaluation, clinical features, and results of treatment for each patient. Non-small cell lung cancer was the most common prediction by both assays (Table 3). In 6 patients, the assays were in agreement, with both assays predicting non-small cell lung cancer. Clinical and pathologic features in these patients were generally compatible with the diagnoses of non-small cell lung cancer, although one patient had a complete response to treatment and an unusually long survival of 65 months. Non-small cell lung cancer was predicted in an additional 9 patients by either the Tissue of Origin test (5 patients) or the Veridex 10-gene assay (4 patients). In each of these cases, other predictions were made by the remaining assay (Table 3). Unfortunately, due to the retrospective nature of this study, immunohistochemical characterization of these tumors was inconsistent and often incomplete. Therefore, correlation of these mismatched assay predictions with standard IHC evaluation is not possible.

Similar inconsistencies in assay prediction were common in patients with colorectal, ovary, and pancreas diagnoses (Table 4 and Table 5). A total of 7 patients had colorectal cancer predicted by at least 1 assay. Although all 7 patients had predominantly intraabdominal metastases, only 1 patient had colon cancer predicted by both assays (IHC studies in this patient were also typical of colon cancer). As a group, these patients had poor response to empiric treatment for carcinoma of unknown primary, and none received colon cancer-specific regimens. Of the 6 patients with ovarian cancer predictions, only 2 patents had the prediction made by both assays. In general, clinical characteristics of these patients were unusual for ovarian cancer, with a predominance of lung and liver metastases.

Table 6 includes details of the 6 patients who were given sarcoma

Assay Prediction		Pathology		Clinical Features				
Tissue of Origin	Veridex	Histology	IHC	Age/Sex	Sites of Metastasis	Intrathoracic Location (Y/N)	Response to Treatment	Survival (mo)
NSCLC	NSCLC	PD adenocarcinoma	TTF1+, CEA+, Pan-CK+, S100-	45/F	bone, soft tissue	N	PD	8
NSCLC	NSCLC	PD carcinoma	CK AE1/AE3+, HMB45-, CK20-, CK7+, AFP-, BhCG-	54/F	lymph nodes	Y	CR	65
NSCLC	NSCLC	PD carcinoma	CK7+, CEA+, B14/18+, S100-, PSA-, PSAP-, HMB45-	60/M	kidney, spleen	N	PD	9
NSCLC	NSCLC	PD carcinoma	CK+, EMA+, CD30-, LCA-	74/M	liver, lung, lymph nodes	Y	PR	24
NSCLC	NSCLC	PD carcinoma	AE1/AE3+, chromogranin-, synaptophysis-, S100-, HMB45-, CA-	64/M	liver, lung, lymph nodes, kidney	Y	PR	8
NSCLC	NSCLC	PD carcinoma	None	68/M	pancreas, lymph nodes, lung	Y	SD	14
NSCLC	Colon	Squamous	None	59/M	lymph nodes, soft tissue	Y	PD	6
NSCLC	Other	Squamous	None	43/M	liver, lung, bone, lymph nodes	Y	PR	11
NSCLC	Other	PD adenocarcinoma	None	72/F	lung	Y	PD	30
NSCLC	Pancreas	PD adenocarcinoma	None	65/M	abdomen, lung, bone	Y	UE	<1
NSCLC	Pancreas	PD adenocarcinoma	None	54/M	lymph nodes	Y	PR	33+
Ovarian	NSCLC	PD adenocarcinoma	CK+, CEA+, CK20+, ER-, PR-, HMB45-, S100-, CK7-, Her2/neu-	51/F	lung, liver, lymph nodes	Y	PD	5
Colorectal	NSCLC	PD adenocarcinoma	CK20+, CK+, PLAP-, PSA/PSAP-, melanin A-	71/M	abdomen, soft tissue	N	SD	21
Bladder	NSCLC	Squamous	None	60/M	Soft tissue	N	UE	32
Non-diagnostic	NSCLC	Squamous	TTF1-	55/M	lung, soft tissue	Y	SD	9

Table 3: Clinicopathologic Characteristics of Patients with NSCLC Predictions (*n* = 15).

Assay Prediction		Pathology		Clinical Features				
Tissue of Origin	Veridex	Histology	IHC	Age/Sex	Sites of Metastasis	Intra-abdominal Location (Y/N)	Response to Treatment	Survival (mo)
Colorectal	Colon	Adenocarcinoma	CK20+, CK7-, TTF1-, CEA-	73/F	Pleura, left adrenal	Y	SD	23
Colorectal	Other	Adenocarcinoma	None	64/F	Liver, omentum, peritoneum, pancreas	Y	UE	1
Colorectal	Pancreas	Adenocarcinoma	CK7+, CK20-, CA125-, TTF1-, CEA-	66/F	Liver	Y	UE	4
Colorectal	NSCLC	PD adenocarcinoma	CK+, CK20+, PLAP-, PSA/PSAP-, melanin A-	71/M	Abdomen, Soft tissue	Y	SD	21
Pancreas	Colon	Adenocarcinoma	None	44/F	Liver, abdominal wall, peritoneum	Y	UE	1
Kidney	Colon	PD carcinoma	CK+, calretinin+, vimentin+, chromogranin-, CD15-, CD10-, AFP-, CEA-	64/M	Liver, peritoneum	Y	SD	6
NSCLC	Colon	Squamous	None	59/M	Lymph nodes, internal jugular vein mass	N	PD	7
Ovary	Ovary	PD carcinoma	CK7+, ER+, PR+, GCDFP15-, HER2-	69/F	Lung, liver, lymph nodes, pelvis, abdomen	Y	PR	57
Ovary	Ovary	PD carcinoma	AE1/AE3+, CK7+, EMA+, CK5/6+, calretinin+, pan keratin+, CEA-, CK20-, LeuM1-, thyroglobulin-, ER-, PR-, Her2/neu-, HMB45-, S100-,chromogranin-, synaptophysin-, NSE-	45/F	Lung, bone, pelvis, lymph nodes, mediastinum	Y	SD	6
Ovary	Other	Adenocarcinoma	CK7+, CD15+, CEA+, calretinin-, CK20-, GCDFP15-, S100-, TTF1-	70/F	Lung	N	PR	19
Ovary	Other	PD adenocarcinoma	None	48/F	Lung, liver, mediastinum	Y	SD	18
Ovary	NSCLC	PD adenocarcinoma	CK+, CK7+, CEA+, ER-, PR-, HER2-, CK20-, HMB45-, S100-	51/F	Lung, liver	Y	PD	5
Sarcoma	Ovary	PD carcinoma	vimentin+, NSE+, CD117+, synaptophysin+, S100-, HMB45-, LCA-, chromogranin-, actin-, desmin-, pan CK-, EMA-	40/F	Lung, liver, pelvis	Y	PD	9

Table 4: Clinicopathologic Characteristics of Patients with Colorectal (*n* = 7) or Ovary (*n* = 6) Prediction.

Assay Prediction		Pathology		Clinical Features			
Tissue of Origin	Veridex	Histology	IHC	Age/Sex	Sites of Metastasis	Response to Treatment	Survival (mo)
Pancreas	Pancreas	Adenocarcinoma	None	50/F	lung, liver, spleen	PD	5
Pancreas	Pancreas	Adenocarcinoma	CK7+, CK20+	64/F	abdominal soft tissue, liver	SD	6
Pancreas	Pancreas	PD adenocarcinoma	None	52/M	Liver, lung, lymph nodes	PR	11
Pancreas	Pancreas	Adenocarcioma	CEA+, Moc31 (EpCAM)+, B723+, HepPar1-	56/F	Lung, GE junction, liver, bone, lymph nodes	SD	7
Pancreas	Colon	PD adenocarcinoma	None	44/F	Liver, peritoneum, pancreas	NE	1
Pancreas	Other	PD carcinoma	CK7+, CK20-, AFP-	35/M	Liver, omentum	PD	2
Colorectal	Pancreas	Adenocarcinoma	CK7+, CK20-, CA125-, TTF1-, CEA-	66/F	Liver	NE	4
NSCLC	Pancreas	PD adenocarcinoma	None	65/M	Lung, lymph nodes, abdominal wall	NE	<1
NSCLC	Pancreas	PD adenocarcinoma	None	44/F	Lymph nodes	PR	21

Table 5: Clinicopathologic Characteristics of Patients with Pancreas Predictions (*n* = 9).

diagnoses by the Tissue of Origin test. In these patients, histologic diagnoses included poorly differentiated carcinoma (4 patients) and adenocarcinoma (2 patients). The 4 patients with histologic diagnoses of poorly differentiated carcinoma had IHC stains performed; 3 of 4 had one or more cytokeratin markers, while the fourth had staining suggestive of sarcoma (vimentin+, synaptophysin+, CD117+). The Veridex assay predicted ovarian cancer in 1 patient, but in the other 5 patients predicted either "other" (4 patients) or was unsuccessful (1 patient).

Discussion

Recognition of tissue-specific patterns of gene expression provides a potential new diagnostic method for determining the tissue of origin in patients with carcinoma of unknown primary site. Several assays have been developed to predict the tissue of origin by assaying the expression of varying numbers of key genes in a tumor biopsy specimen. When applied to metastatic tumor tissue in patients with cancers of known primary, these assays can correctly identify the tissue of origin in 76-89% of cases [7,8,13-16]. When performed retrospectively in several

Assay Prediction		Pathology		Clinical Features			
Tissue of Origin	Veridex	Histology	IHC	Age/Sex	Sites of Metastasis	Response to reatment	Survival (mo)
Sarcoma	Ovarian	PD carcinoma	vimentin+, NSE+, synaptophysin+, CD117+, HMB45-, LCA-, chromogranin-, actin-, desmin-, Pan CK-, EMA-, S100-	40/F	abdomen, pelvis, liver	PD	9
Sarcoma	Other	Adenocarcinoma	None	56/M	abdomen, liver	SD	24
Sarcoma	Other	PD carcinoma	CK7+, EMA+, CK20-, TTF1-, PLAP-	61/M	lung, lymph nodes	PD	12
Sarcoma	Other	PD carcinoma	AE1/AE3+, CK20+, NSE+, CK7-, CD30-, desmin-, synaptophysin-, EMA-	35/M	lung, liver, lymph nodes	PR	6
Sarcoma	Other	PD carcinoma	CK+, S100-, HMB45-	86/F	pericardial effusion, adrenal gland, liver	SD	5
Sarcoma	Unsuccessful	Adenocarcinoma	None	65/M	liver	SD	9

Table 6: Clinicopathologic Characteristics of Patients with Sarcoma Predictions (*n* = 6).

groups of patients with carcinoma of unknown primary site, molecular assays provided a putative diagnosis in the majority of patients [7,12, 17-19]. In general, these predictions were consistent with clinical features and with the results of standard pathologic evaluation. However, confirmation that the assay predictions were "correct" has been impossible in the majority of patients, since the actual primary site usually remains unidentified. In one small series, the correct primary site was predicted by assay of the original biopsy in 15 of 20 patients with an initial CUP diagnosis who subsequently had primary sites detected during their clinical course [20].

In spite of these promising results from early studies, the role of molecular tumor profiling in the diagnosis of patients with CUP remains undefined. The relative merits of molecular profiling as compared to standard pathologic evaluation have not been completely defined. Should molecular profiling be performed in the evaluation of every patient with CUP, or are there certain subsets particularly likely to benefit? Should it replace part of the standard pathologic evaluation, such as IHC staining? Is one of the currently available molecular assays more accurate, and therefore "better" than others? The other important unanswered questions relate to the impact of molecular diagnosis on outcome of patient treatment. Can treatment be selected based on molecular profiling predictions? Will that treatment be more effective than empiric CUP regimens or regimens selected based on IHC predictions?

In the study reported here, the Tissue of Origin test was performed on biopsies from a large group of patients with CUP. The important results of this study are as follows: 1) the assay was successfully performed on these archival paraffinized, formalin-fixed biopsies in 45 of 48 cases (94%), 2) in 43 of 45 successfully performed assays, a tissue of origin was predicted; 3) the most common predictions (non-small cell lung, pancreas) are consistent with the most common occult primary sites identified in previous autopsy series, and 4) clinical features and routine pathology results in most cases were consistent with the molecular assay predictions. Patients given the diagnosis of ovarian cancer were an exception: in these 5 patients, clinical features were atypical with prominent metastases in the lungs and liver. Another unexpected result was the prediction of 6 patents with sarcoma, most of whom did not have this diagnosis suggested by routine pathology.

Since all biopsies in this series had been previously tested with the Veridex 10-gene assay, this study provided the first opportunity to compare the predictions from 2 molecular profiling assays in a group of CUP patients. As expected, the Tissue of Origin test was able to make a prediction in a higher percentage of cases (96% versus 53%), due at least in part to the Veridex assay's ability to recognize only 6 tumor types. However, agreement between the assays was relatively poor, even when both rendered a prediction (Tables 3-6). When

lung, colon, or ovarian cancer was predicted, the two assays were in agreement less than 50% of the time. While the methodology of this retrospective study does not allow a determination of which assay was "correct" more frequently, the large number of genes assayed by the Tissue of Origin test suggests that identification of complex patterns of tissue-specific gene expression would be much more likely identified. Conversely, the assay of only 10 genes by the Veridex assay (with the detection of some tumor types reliant on only one gene assayed) is less likely to be specific.

Additional evaluation of the Tissue of Origin test and other "second generation" molecular assays currently available is necessary to define their role in the management of patients with CUP. Although retrospective studies and anecdotal cases suggest the value of these assays in diagnosis and treatment planning, additional clinical studies are urgently needed. The impact of these assays on treatment results is most likely to be detectable in subsets of patients for whom standard site-specific treatment differs from the empiric CUP regimens currently in use (e.g. colorectal cancer, renal cell carcinoma). Demonstration of superior outcome with site-specific treatment in patients given these diagnoses by molecular assay would provide strong rationale for their incorporation into the standard diagnostic evaluations of CUP. Although immediate improvement in treatment results for other subsets (e.g. pancreas, non-small cell lung cancer) is unlikely, even if correctly identified by assay, accurate identification would allow these groups to benefit as therapy for these malignancies improves in the future.

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