Short communication

Gene expression profiling from formalin-fixed, paraffin-embedded tissue for tumor diagnosis ☆

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ABSTRACT

Background: Molecular profiling assays have emerged as a promising tool in tumor diagnosis. Recent advances have allowed the use of formalin-fixed, paraffin-embedded (FFPE) tissues in such assays involving the use of microarrays. The Pathwork Tissue of Origin (TOO) Test was developed for use with FFPE tissue to aid tumor diagnosis. We sought to determine the performance of the TOO test on routine specimens from an independent laboratory.
Methods: Forty-five blinded, archived clinical specimens from the UCSF Department of Pathology were tested. Total RNA was processed to prepare labeled cDNA for hybridization to Pathchip microarrays. Hybridization data was analyzed with a 2000-gene classification model to quantify similarity between RNA expression of the study specimens and the 15 tissues on the test panel.
Results: 44/45 (98%) specimens were successfully processed. 37 cases met study inclusion criteria. Of these, 35 (95%) gave results which were in agreement with the reference diagnosis. In no case was the reference diagnosis ruled out.
Conclusions: The Tissue of Origin Test gave a high agreement with the reference diagnosis when archived clinical specimens from UCSF were assessed. Molecular profiling assays are highly accurate, and can be a useful tool in cancer diagnosis.

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1. Introduction

With careful review of histology and correlation with clinical and radiographic findings, the identity of most tumors can be determined with confidence. However, when tumors are poorly-differentiated or metastatic with no clear primary, identifying the tissue of origin is difficult. Immunohistochemical stains may provide clarity, but the tissue of origin can remain uncertain after these tests [1]. Recently, molecular testing of tumors has been utilized as an additional means of characterizing these tumors [2].

The Pathwork Tissue of Origin (TOO) Test is a molecular assay developed as an aid to tumor diagnosis, and was clinically validated using tumors with known primary sites [3,4]. The TOO Test was validated for use with formalin-fixed, paraffin-embedded (FFPE) specimens [4], and is cleared by the U.S. FDA as an in vitro diagnostic.

The test uses a microarray to measure the expression of 2000 genes in a tumor specimen. The signature is compared to those of 15 known tissues representing 58 morphologies, and a report for each tissue is provided to the physician.

Pathology specimens are not handled in a uniform fashion by all laboratories. For example, there may be variation in the time before fixation, duration of fixation, and type of fixative. For some assays, including immunohistochemistry for hormone receptors and FISH for Her-2/neu, variation in the duration of formalin fixation has been shown to adversely affect reproducibility [5,6]. The TOO Test was validated with samples that reflected the range of varying handling standards employed by seven participating sites [4].

With 462 samples, the previously published validation study was powered to allow an assessment of the test performance both for overall agreement with reference diagnosis (shown to be 88.5% with 95% confidence interval 85.3% to 91.3%) and for performance of the test for each of the 15 tissue types on the panel (at least 25 samples from each tissue type were included in the study). The purpose of the current study was to assess, in a second independent study, the overall performance of the test on samples from a laboratory not involved with the clinical validation in order to confirm that it is consistent with the results of the larger controlled validation study.

2. Materials and methods

Specimens were enrolled in the study using the following entry criteria:

(i) All human tumor samples must be represented in the 15 tumor tissues on the Pathwork Tissue of Origin Test panel, namely bladder, breast, colorectal, gastric, testicular germ cell, kidney, hepatocellular, non-small cell lung, non-Hodgkin's lymphoma, melanoma, ovarian, pancreas, prostate, thyroid and sarcoma. ii) Specimens must contain enough material for one 10-μm thick curl for microarray analysis, and one 5-μm thick hematoxylin and eosin stained section for assessment of percent tumor. (iii) Specimens are required to contain ≥ 60% viable tumor tissue.

Forty-five archived (from 2000 to 2007) human tumor specimens, including high grade/poorly differentiated cases, were selected from FFPE clinical specimens from the UCSF Department of Pathology, under an IRB-approved protocol. Forty-four of 45 specimens were represented on the test panel. One specimen, a squamous cell carcinoma of the larynx, was off-panel. The UCSF reference diagnosis was made using clinical history, histological review, immunohisto-chemistry and imaging studies.

Sample sections were coded prior to processing in order to blind the laboratory to tissue type. The specimens were processed as described previously [4]. Total RNA was isolated at UCSF from one 10-μm thick curl using the FormaPure kit (Agencourt, currently Beckman–Coulter Genomics, Beverly, MA). The total RNA was processed at Pathwork Diagnostics Laboratory to prepare labeled cDNA for hybridization to Pathchip 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 Hybridi-zation Wash and Stain kit in a GeneChip Fluidics Station FS450Dx, and scanned with a GeneChip Scanner 3000Dx (Affymetrix).

Microarray data files (CEL) that passed data verification [4] were analyzed using the TOO Test algorithm, a 2000-gene classification model which quantifies the similarity between RNA expression patterns of a study specimen and the 15 tissues on the test panel. A TOO Test report is generated for each case (see Fig. 1 for an example).

The Test report is interpreted and results are analyzed using the following guide to report interpretation: "The Similarity Score (SS) is a measure of the similarity of the RNA expression pattern of the specimen to the RNA expression pattern of the indicated 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. An SS less than or equal to 5 rules out that tissue type as the likely tissue of origin." The TOO Test result was automatically generated by the computer algorithm with no consideration of the reference diagnosis.

The TOO Test results were compared with the reference diagnosis of primary site to compute sensitivity (positive percent agreement [PPA]) with 95% confidence intervals (CI), overall specificity (negative percent agreement [NPA]), and diagnostic odds ratio [7], as previously described [4].

3. Results

Of the 45 cases that were screened, 37 met entry criteria. One case was off-panel, six had inadequate tumor purity to meet inclusion criteria, and RNA from one specimen was lost in transport.

Of the 37, 6 are high grade/poorly differentiated tumors, and 5 are metastatic tumors. Patients were 22 to 74 in age, with a median age of 55. There are roughly equal numbers of male (17) and female (20) patients.

The total RNA yield ranged from 900 ng to 49 μg averaging 10.6 μg. The A260/A280 ratios ranged from 1.74 to 2.05. Specimens were amplified in 7 batches. An RNA control was run with every batch, and in every batch gave the correct tissue call (positive control) and a Similarity Score of ≤ 5 for at least 9 tissues (negative control). All specimens were successfully amplified and hybridized to Pathchip microarrays, and all arrays passed array quality metrics [4].

The distribution of the TOO results by tissue is shown in the confusion matrix (Table 1). Thirty-five of 37 results (95%) agreed with the reference diagnosis. The sensitivity (PPA) was 95% [81.8, 99.3] and the overall specificity (NPA) was 99.6%. The diagnostic odds ratio [7] a

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UCSF FFPE Block ID: S00-845 A4
Age: 58
Gender: F
Biopsy Site: RT lung, upper lobe

Pathwork Sample ID: HR25_00845P99Z
File ID: 22132
Processed Date: 05/07/10 10:55:19

Data Quality: Acceptable

TISSUE	SIMILARITY SCORE	LOW 0 5	HIGH 100
Non-Small Cell Lung	86.6		◆
Breast	3.3	◆	
Bladder	2.4	◆	
Ovarian	1.4	◆	
Kidney	1.2	◆	
Sarcoma	1.2	◆	
Colorectal	1.1	◆	
Gastric	0.7	◆	
Thyroid	0.5	◆	
Pancreatic	0.5	◆	
Non-Hodgkin's Lymphoma	0.3	◆	
Hepatocellular	0.3	◆	
Germ Cell	0.2	◆	
Prostate	0.2	◆	
Melanoma	0.1	◆	

Fig. 1. Tissue of Origin Test Report. The specimen was from a right lung upper lobe biopsy from a 58 year-old female, showing a high grade, poorly differentiated squamous cell carcinoma morphology. A strong call for non-small cell lung was made (Similarity Score 86.6) and the 14 other tissues were ruled out as possible primary sites, with scores of <5.

Table 1
Confusion matrix for 37 specimens, that met entry criteria. The rows indicate the reference diagnosis, and the columns indicate the Tissue of Origin Test result.

Result/specimen	No.	BL	BR	CO	GA	GC	KI	LI	LU	LY	ME	OV	PA	PR	SC	TH
Bladder (BL)	3	3														
Breast (BR)	2		2													
Colorectal (CO)	3			3												
Gastric (GA)	2				2											
Testicular Germ Cell (GC)	3					2						1				
Kidney (KI)	4						4									
Hepatocellular (LI)	3							3								
Non-small Cell Lung (LU)	2								2							
Non-Hodgkin's Lymphoma (LY)	3									3						
Melanoma (ME)	2										2					
Ovarian (OV)	3			1								2				
Pancreas (PA)	2												2			
Prostate (PR)	1													1		
Sarcoma (SC)	3														3	
Thyroid (TH)	1															1
Total	37	3	2	4	2	2	4	3	2	3	2	3	2	1	3	1

single measure of test performance, ranged from 66 to 483 indicating that all 15 tests are highly informative.

With inclusion of the off-panel specimen in the analysis, 35/38 (92.1%) were in agreement. Of the two non-agreements, one was a seminoma (called ovarian), and the other was an ovarian mucinous cystadenocarcinoma (called colon). In both cases, the TOO test did not rule out the reference diagnosis as a possibility. For the off-panel case, a squamous carcinoma of the larynx, the TOO Test result was non-small cell lung cancer.

4. Discussion/conclusions

As new molecular tests are introduced into clinical practice, it is important to confirm that the performance in controlled validation studies continues to be observed when the test is applied to samples derived from a wide variety of practice settings. In this study, FFPE samples stored for three to ten years in the archives of a large academic hospital produced results equivalent or superior to those previously reported in a larger validation study conducted by the manufacturer.

The larger validation study [4] with 462 samples showed overall agreement with reference diagnosis was 88.5% (95% CI 85.3% to 91.3%). This study, where the test results showed 95% overall agreement with reference diagnosis (95% CI 81.8% to 99.3%), confirmed the overall performance as being consistent with the first validation study. Although the current study included samples from all of the tissue types represented on the test panel, the sample size does not allow an estimate of the performance of the test on individual tissues.

These samples included five metastatic tumors and six high-grade/poorly-differentiated primary tumors. While infrequent, the results not in agreement with the reference diagnosis (3/38) demonstrate the areas in which gene expression profiles may overlap and the need for expert interpretation as a component of test utilization. One off-panel tumor, a laryngeal squamous cell carcinoma, gave a result of lung non-small cell carcinoma. This is not unexpected, since lung squamous cell carcinoma is an included histology on the panel, and lung bronchial mucosa is anatomically continuous with laryngeal mucosa and may undergo metaplasia to resemble the squamous mucosa of the larynx. In a second example, a mucinous cystadenocarcinoma provided a test result for colorectal carcinoma. Mucinous ovarian tumors have been shown to have both histologic and immunohistochemical features that overlap with intestinal adenocarcinoma, including expression of CDX-2, a marker of intestinal differentiation [8]. These results emphasize the importance of correlating the TOO test results with histopathologic, radiologic and clinical findings.

In routine pathologic evaluation of tumors, most tissues of origin can readily be excluded by histologic or clinical means. This leaves a limited number of remaining options, and additional testing often is focused on ruling out possibilities. For example, ruling out colon cancer in a patient with a history of colon cancer and a new lung tumor would help confirm a new primary tumor, rather than high-stage, metastatic disease. The TOO Test can be very helpful in this type of role. In none of the results was the correct diagnosis ruled out. Thus, a tissue ruled out by the TOO Test makes a strong argument against that tissue as the origin of the tumor. In summary, we have confirmed that the Tissue of Origin molecular profiling assay is highly accurate, works with formalin-fixed clinical specimens without special handling, and can be a useful tool in cancer diagnosis.

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