Identification of Tissue of Origin in Body Fluid Specimens Using a Gene Expression Microarray Assay

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BACKGROUND: Body fluid specimens may be the first and only pathologic specimen for clinical evaluation in metastatic cancer cases. The challenge of identifying the tissue of origin in metastatic cancer has led to the emergence of molecular-based assays, such as the microarray-based Pathwork Tissue of Origin gene expression test. The ability to use body fluid specimens in this test would be valuable in providing diagnoses to cancer patients without clearly identifiable primary sites. In the current study, the authors evaluated the Tissue of Origin Test for use with malignant effusion specimens.

METHODS: A total of 27 metastasis-positive body fluid specimens from different body sites, including pleural, ascites, pericardial, and pelvic wash fluids, were obtained from patients with known diagnoses. Nine specimens from nonmalignant body fluids were included as controls. RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue and gene expression analysis was performed with the Tissue of Origin Test. RESULTS: Seventeen of 27 metastasis-positive samples were non-necrotic with ≥60% tumor and yielded sufficient RNA. Of these samples, 94.1% (16 of 17) were in agreement with the available diagnosis. Of the 9 negative control samples evaluated, 7 (77.8%) demonstrated microarray expression profiles most similar to lymphoma, which is consistent with the predominance of inflammatory cells in these specimens.

CONCLUSIONS: The results of the current study demonstrated that FFPE cell blocks from cytologic body fluid specimens yield adequate diagnostic material for the Pathwork test and can be used in the workup of patients with unknown primary tumors. Cancer (Cancer Cytopathol) 2011;000:000–000. © 2011 American Cancer Society.

KEY WORDS: metastatic cancer, unknown primary, gene expression, cytology, malignant effusion, cell block, formalin-fixed, paraffin-embedded, microarray.
Identifying the tissue of origin in patients with unknown primary tumors is essential for determining the best therapeutic approach. It has been shown that patients who receive a primary tumor diagnosis have longer survival rates when compared with those who never receive a definitive diagnosis.3

The challenges associated with identifying a tissue of origin have led to the emergence of molecular-based assays for this purpose.4 The main molecular approaches used are gene expression microarrays and polymerase chain reaction (PCR) assays, which are the bases of several commercially available assays.5-7 The Pathwork Tissue of Origin Test (Pathwork Diagnostics, Redwood City, CA) is a microarray-based gene expression test that uses the expression levels of 2000 genes to classify tumors by similarity scores (SS) into 15 sites of origin.8 In a recent study using 462 formalin-fixed, paraffin-embedded (FFPE) specimens, 88.5% agreement with available diagnosis was demonstrated.9

Cell blocks prepared from cytologic body fluid specimens such as pleural, peritoneal, or pericardial fluids are often the first pathologic specimen received for clinical evaluation. These cell blocks are often used to provide a diagnosis of malignancy and to identify the primary tumor site.10,11 In patients with metastatic disease, these body fluid specimens may be the only available pathologic specimen and thus they play a critical role in diagnosis. Because the median survival time for patients with malignant effusions is <6 months,12 the ability to use cell blocks from body fluids with molecular tests that aid in identifying the primary tumor site of metastatic carcinoma could be valuable in selecting appropriate therapies for these patients.

The purpose of the current study was to evaluate the use of cell blocks from body fluids as a specimen type for the Pathwork Tissue of Origin Test. Seventeen body fluid specimens of known origin were processed with the Tissue of Origin Test, and the accurate tissue of origin was predicted in 94.1% of cases that met specimen entry criteria.

### MATERIALS AND METHODS

#### Samples

The number of patient cases and specimens available for each case are summarized in Table 1. A total of 27 metastasis-positive cases were selected, under an Institutional Review Board-approved protocol, from patients who either had a previous tissue diagnosis from a site-specific biopsy or had a diagnosis made on tissue after malignant (metastasis-positive) cells were identified in the body fluid. Specimens from these cases were collected from different body sites including pleural, ascites, pericardial, and pelvic wash fluids. Cell blocks were generated using the formalin-fixation and thrombin (T) cell block method followed by paraffin embedding according to routine protocols. In 7 cases, a cell block was also generated using the alcohol-fixation and Cellient™ (C) method followed by paraffin embedding (Table 1).

In addition, 9 metastasis-negative cases from patients who did not have a diagnosis of cancer were also selected (Table 1). In 5 of these cases, cell blocks generated using both the T and C methods were available. Among the remaining 4 cases, 3 cases only had a cell block generated using the T method whereas 1 case only had a cell block generated using the C method.

### Table 1. Numbers and Types of Patients and Specimens

<table>
<thead>
<tr>
<th>Patient Case Description</th>
<th>Total No. of Cases</th>
<th>Both Thrombin and Cellient Blocks&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Only Thrombin Block</th>
<th>Only Cellient Block</th>
<th>No. of Blocks Processed&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastasis negative</td>
<td>9</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Metastasis positive</td>
<td>27</td>
<td>7</td>
<td>20</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>12</td>
<td>23</td>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>Metastasis-positive subsets</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥60% tumor content</td>
<td>20</td>
<td>3</td>
<td>17</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>&lt;60% tumor content</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Two blocks per case processed.

<sup>b</sup>Three blocks were not processed due to low circularity on sectioning.
Cell blocks were sectioned to yield a single 5 \( \mu \text{m} \)-thick section on a slide for hematoxylin and eosin (H & E) staining and 1 to 5 manually microdissected 10 \( \mu \text{m} \)-thick tissue sections were used for RNA extraction, depending on the size of the cell button.

**Specimen Entry Criteria for the Tissue of Origin Test**

Cell blocks from 9 tissue types that are known to be commonly present in body fluid specimens and that are on the Pathwork Tissue of Origin Test panel were used for this study. The tissue types included breast, colon, gastric, kidney, lung, lymphoma, ovary, pancreas, and prostate. Specimens from nonmalignant body fluids were also included to evaluate the role of inflammatory and mesothelial cells in the Tissue of Origin Test. The H & E slide from each cell block was used to assess the percentage of tumor cells. In addition, an estimation of the inflammatory components and reactive mesothelial cells and necrosis was made. Only those specimens determined to have \( \geq 60\% \) tumor cells were deemed acceptable for the Tissue of Origin Test; however, specimens with less tumor representation were also tested to assess the effect of tumor content on test results.

**RNA Extraction, Target Preparation, and Microarray Processing**

RNA was extracted with the FormaPure Kit (Agencourt Bioscience, Beverly, MA) for nucleic acid isolation from FFPE tissue and the magnetic SPRIStand (Agencourt Bioscience) according to the manufacturer’s recommendations. Total RNA concentration and purity were assessed using the NanoDrop ND-1000 Spectrophotometer (Themo Scientific, Wilmington, DE). A total of 30 ng of total RNA at a concentration of 10 ng/\( \mu \text{L} \) was used to generate biotinylated cDNA using the Genisphere Custom RampUP and cDNA synthesis kit (Genisphere, Hatfield, PA), Superscript II (Invitrogen, Carlsbad, CA), and the RNA CleanXP Kit (Agencourt Bioscience). Biotinylated cDNA was hybridized to Pathchip microarrays (Affymetrix, Santa Clara, CA) using procedures previously described.\(^8\) Sixteen specimens (7 malignant and 9 benign) were processed at the Methodist Hospital Research Institute (Houston, Tex) and 20 malignant specimens were processed at Pathwork Diagnostics. Specimens were processed at 2 sites to assess the performance of the FFPE assay in different laboratories. The resulting raw intensity data (CEL) files for all specimens were analyzed by Pathwork Diagnostics.

**Analysis**

Only CEL files that had an overall signal \( \geq 10 \), a percentage present value \( \geq 5 \), and a regional discontinuity value \( \leq 0.84 \) were used for further analysis as per the Tissue of Origin Test guidelines. The overall signal is the mean of the summarized expression values of all probesets on the Pathchip microarray, the percentage present value is the percentage of probesets on the Pathchip microarray that are assigned a present call, and the regional discontinuity is a measure of the correlation between the intensity of a probe and the mean intensity of the 2 vertically adjacent probes on the chip surface, computed over all Pathchip probes. These quality metrics were established when the Tissue of Origin Test was developed and are a part of this US Food and Drug Administration-cleared test.\(^9\) CEL files are processed through the Tissue of Origin Test algorithm, and a test report with 15 SS is automatically generated independent of the reference diagnosis. The positive percentage agreement with the reference diagnosis was calculated as previously described.\(^6\)

**Immunohistochemistry**

Samples with Tissue of Origin Test results that were discordant with the reported diagnosis were analyzed using routine immunohistochemistry (IHC) for the following markers: for colon origin, cytokeratin (CK) 7, CK20, and CDX2; for breast versus ovarian origin, mammaglobin, prolactin-induced protein (PIP, also known as BRST2 and GCDFP15), paired box gene 8 (PAX-8), and Wilms tumor gene 1 (WT1); for gastric origin, CK7+, CK20+, CDX2, and carcinoembryonic antigen (CEA); and for mesothelial cells, calretinin and D2-40.

**RESULTS**

**Processing of Body Fluid Cell Blocks**

Twenty of the 27 metastasis-positive cases were estimated to contain \( \geq 60\% \) tumor and were processed through the
Tissue of Origin Test (Table 1). In addition, 4 metastasis-positive cases containing <60% tumor were processed to assess the effect of body fluid specimens that do not meet selection criteria for the Tissue of Origin Test. Including metastasis-negative cases, a total of 45 specimens were evaluated (Table 1). At the time of processing, the age of these cell blocks ranged from <1 year to 7 years.

RNA yields of ≥30 ng are necessary to proceed to the amplification step. RNA extraction yielded adequate amounts of RNA in 44 of 45 (97.8%) of the specimens extracted, and yields ranged from 49 ng to 4.3 µg (Fig. 1). Although the range of RNA yields was wider for the T blocks compared with the C blocks, the median RNA yield for both cell block types was similar (Fig. 1). The 1 specimen that yielded insufficient RNA, 137T, was a metastasis-positive case with >60% estimated tumor content.

No differences in cDNA yields and overall signal, percentage present value, and regional discontinuity value were observed between T and C blocks after hybridization to the microarrays (Fig. 1). A metastasis-negative specimen, 183T, failed the overall signal threshold of 10. The remaining 43 specimens gave valid Tissue of Origin Test results.

**Performance of the Tissue of Origin Test in Metastasis-Positive Samples**

Among the 20 metastasis-positive cases that were estimated to contain ≥60% tumor cells, 19 specimens (95%) passed all Tissue of Origin Test quality metrics, generating ≥3 µg of biotinylated cDNA and yielding CEL files that passed quality array parameters as described earlier. These 19 cases were used to report the assay performance.

The Tissue of Origin Test results are summarized in Table 2. Cell blocks were derived from pleural fluids (10 of 19 cases), peritoneal/ascitic fluids (8 of 19 cases), and pericardial fluids (1 of 19 cases). The positive percentage agreement with the available diagnosis was 78.9% (15 of 19 cases). An average of 12 tissue types of the 15 tissues on
Discordant Samples

We investigated the 4 specimens that had discordant Tissue of Origin Test results and clinical diagnoses by retrospectively performing IHC studies on additional slides obtained from the same cell blocks that were tested to confirm the original diagnosis (Table 3). Two of the discordant samples were shown to have <60% tumor content on retrospective IHC staining, despite the initial morphologic evaluation indicating >60% tumor content (Fig. 2). One of these cases (1268T) had the available diagnosis as the second highest score (lung), and the other case (213T) had the available diagnosis as the third highest score (colorectal). For both of these cases, the clinical history and IHC profiles confirmed the available diagnosis as originally reported. Case 1268T demonstrated a predominance of mesothelial cells that was recognized only after IHC staining (Fig. 2). This case had a test result that was most similar to ovarian tissue, although the patient was male.

One case, 1533T, was diagnosed as a metastatic breast cancer, and the Tissue of Origin Test predicted the tumor as being ovarian in origin with an SS of 94.5, indicating a very strong similarity to ovarian cancer. It also excluded the breast as a primary tumor site. Review of the clinical history revealed that the patient had breast cancer before the development of a malignant pleural effusion, which was diagnosed as being of breast origin. The patient subsequently was identified as having ovarian cancer. IHC staining identified the metastatic tumor as being ovarian in origin (Fig. 3) (Table 3). Given this IHC profile and the clinical history, it is likely that the pleural effusion in this patient was of ovarian origin. The fourth discordant case, 3067T, had a diagnosis of gastric carcinoma with a supportive clinical history and IHC profile (CK7+, scant CK20+, CDX2-, and CEA+); however, the test result indicated the colon as the origin, albeit with a relatively low SS (SS, 45.4).

Thus, after retrospective evaluation, 2 cases did not meet tumor content criteria for the Tissue of Origin Test, 1 case was confirmed to be consistent with the test result, and 1 case remained discordant. Taking these results into account, the corrected positive percentage agreement of the Tissue of Origin Test was 94.1% (16 of 17 cases).
Table 3. IHC Staining and Clinical Profile of Discordant Cases

<table>
<thead>
<tr>
<th>Specimen ID</th>
<th>Reference Diagnosis</th>
<th>IHC Profile</th>
<th>Tissue of Origin Test Result (SS)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>213T</td>
<td>Colon</td>
<td>CK7(-), CK20 (-), CDX2 (-)</td>
<td>Lymphoma (57.6)</td>
<td>&lt;60% tumor cells after IHC staining, approximately 10% mesothelial cells. Mucinous colonic primary with metastatic deposits in omentum.</td>
</tr>
<tr>
<td>1268T</td>
<td>Lung</td>
<td>CK7(+), CK20 rare (+), CDX2 (-)</td>
<td>Ovary (59.3)</td>
<td>&lt;60% tumor cells after IHC staining. History of non-small cell carcinoma of the lung. Abundant mesothelial cells confirmed by positivity for calretinin and D2-40.</td>
</tr>
<tr>
<td>1533T</td>
<td>Breast</td>
<td>Mammaglobin(-), GCDFP15, PAX-8 (+), WT1 rare (+)</td>
<td>Ovary (94.5)</td>
<td>Patient diagnosed with ovarian cancer at a later date, metastatic to peritoneal cavity.</td>
</tr>
<tr>
<td>3067T</td>
<td>Gastric</td>
<td>CK7(+), CK20 rare (+), CDX2(-), CEA (-)</td>
<td>Colon (45.4)</td>
<td>Supportive clinical history for gastric cancer.</td>
</tr>
</tbody>
</table>

Abbreviations: -, negative; +, positive; CEA, carcinoembryonic antigen; CK, cytokeratin; IHC, immunohistochemical; PAX-8, paired box gene 8; SS, similarity scores; WT1, Wilms tumor gene 1.

FIGURE 2. Examples of specimens that were found to be discordant because of tumor representation are shown. (A-C) Case 213T, demonstrating metastatic colon cancer, is shown (A: H & E, interpreted as >60% tumor nuclei; B: pancytokeratin stain showing <60% tumor cells; and C: calretinin stain demonstrating a paucity of mesothelial cells). (D-F) Case 1268T, demonstrating metastatic lung cancer, is shown (D: H & E, interpreted as >60% tumor nuclei; E: cytokeratin 7 stain; and F: calretinin stain). These stains demonstrated approximately equal numbers of tumor and mesothelial cells.
Specimens With <60% Tumor Content

Four of the metastasis-positive specimens evaluated had a tumor content of <60% (range, 5%-40%) and thus did not fulfill entry criteria for the Tissue of Origin Test. We decided to perform the analysis to evaluate performance in cases with low tumor content. Results for the Tissue of Origin Test were in agreement with the reference diagnosis in only 1 of these cases with low tumor content; the remainder had a top SS indicative of lymphoma, which is consistent with the predominant lymphocytic population in these specimens.

Negative Specimens

Of the 9 tumor-negative samples evaluated, 7 (77.8%) demonstrated microarray expression profiles most similar to those of lymphoma, which is again consistent with the predominance of lymphocytes in these specimens. Of these 7 samples with a “lymphoma” profile, the second top SS included gastric, kidney, ovarian, and sarcoma expression profiles. However, none of these second top SS were >15 (range, 2.6-14.4). One negative paracentesis sample demonstrated a top score consistent with a sarcoma only on the C block extraction. The T block resulted in a lymphoma prediction. A negative pelvic wash sample demonstrated a gene expression profile indicating an ovarian origin. Both of these samples had a lymphoma signature as the second top score.

Effect of Cell Block Preparation

No statistically significant difference was observed with regard to the total RNA yield and intermediate labeling products between the C and T cell block methods (using the Student t test). Overall, including specimens with <60% tumor content, the Tissue of Origin Test results were concordant in 10 of 12 cases in which both blocks were tested. In paired analysis, top scores were significantly higher in T cell blocks compared with C blocks ($P = .047$, Student $t$ test). However, when comparing only blocks with >60% tumor content, the difference was not significant. In the 3 cases with >60% tumor content for which the C blocks were also available, test results were always concordant (Table 4). One of the discordant samples was a metastasis-negative case in which the C block gave a result of sarcoma (SS, 2.28), and the other was a metastasis-negative specimen with a result indicating gastric origin in the C block analysis (SS, 18.4).

DISCUSSION

Gene expression profiles have been shown to be useful for identifying the primary tumor site in solid tumor specimens. In the current study, we evaluated whether the Pathwork Tissue of Origin Test could be used with cell blocks generated from cytologic fluid specimens. The Tissue of Origin Test correctly identified the reference diagnosis, with a high degree of accuracy of 94.1%. Furthermore, we found that in order to obtain an accurate result with cytologic specimens on the Pathwork test, it is important for the analyzed specimen to be non-necrotic, with abundant tumor content (>60%) and few inflammatory infiltrates or mesothelial cells.
These findings are consistent with reported testing requirements for this assay. Indeed, of the 4 cases with results that were discrepant with the reference diagnosis, 2 cases of metastatic carcinoma initially believed to contain abundant tumor cells on H & E staining actually demonstrated very few tumor cells when assessed by IHC staining. This highlights the importance of correctly qualifying the percentage of tumor cells in a given specimen before performing gene expression analysis with microarrays. Using IHC stains to differentiate tumor from reactive mesothelial cells may be helpful in this situation. Conversely, the majority of tumor-negative specimens and those with a low tumor content (<30%) demonstrated expression profiles most similar to lymphoma, which is consistent with the predominant lymphocytic population in these specimens. Although a “false-positive” result for lymphoma may represent a potential problem in the workup of a patient with metastatic cancer, it is unlikely that the Tissue of Origin Test would be performed if a lymphoid neoplasm is suspected on a body fluid specimen; flow cytometry would be better suited in that scenario. This underscores the role of pathologists in selecting the most appropriate test for each specific case.

One of the interesting findings from the current study was a specimen with predominantly reactive mesothelial cells (originally interpreted as predominantly tumor cells) that demonstrated a tissue prediction of an ovarian primary tumor, even though the patient was male. Given that ovarian and mesothelial carcinomas are considered to originate from the same cell type (either the mesothelial lining of the ovary or fallopian tube epithelium) it is certainly plausible that their expression profiles demonstrate some degree of similarity. This constitutes an important caveat to consider when interpreting results with abundant mesothelial cells.

SS for the Tissue of Origin Test result for the metastasis-positive cases had a broad range of 24.5 to 97. As demonstrated by Pillai et al in the validation study for the FFPE version of the Tissue of Origin Test, higher SS values have a better correlation with the reference diagnosis. Thus, confidence in the result is certainly affected by the SS value, which reflects the degree of molecular similarity to a tissue of origin. In that study, the great majority of cases were found to have SS >60 with >90% agreement with the tissue of origin, whereas cases with SS between 30 and 60 had approximately 75% agreement. Nonetheless, in the current study, 2 results with top SS in the range of 20 to 30 were found to be in agreement with the available diagnosis. Although in the current study all SS values were reported, in clinical practice Pathwork Diagnostics does not report scores >5 and <20 because concordance with the tissue of origin in specimens with those scores is poor and thus scores within this range should not be used for clinical management. However, values <5 have been reported to be useful for ruling out possible sites of origin.

The results of the current study indicate that cell blocks from cytology specimens yield RNA of acceptable quantity and quality with which to perform a microarray assay such as the Tissue of Origin Test. Our samples were stored from <1 to 7 years, indicating that sample age does not adversely affect the results of this assay. In addition, we compared the C and T cell block preparations in a subset of our specimens. From samples with adequate cellularity, both cell block types demonstrated similar efficiencies for RNA extraction, cDNA amplification, and Tissue of Origin Test results. This suggests that both types of cell blocks are adequate for tissue of origin identification by molecular methods.

### Table 4. Comparison of Tissue of Origin Test Results Using the Thrombin and Cellient Methods to Generate Cell Blocks From Body Fluids

<table>
<thead>
<tr>
<th>Specimen ID</th>
<th>Body Fluid Type</th>
<th>Sex</th>
<th>Available Diagnosis</th>
<th>Thrombin Block</th>
<th>Cellient Block</th>
</tr>
</thead>
<tbody>
<tr>
<td>2213</td>
<td>Peritoneal</td>
<td>Female</td>
<td>Gastric</td>
<td>46.7 Gastric</td>
<td>38.5 Gastric</td>
</tr>
<tr>
<td>2216</td>
<td>Ascites</td>
<td>Male</td>
<td>Ovary</td>
<td>76.5 Ovary</td>
<td>76.9 Ovary</td>
</tr>
<tr>
<td>2716</td>
<td>Pleural</td>
<td>Female</td>
<td>Ovary</td>
<td>97 Ovary</td>
<td>97 Ovary</td>
</tr>
<tr>
<td>180</td>
<td>Ascites</td>
<td>Male</td>
<td>Normal</td>
<td>84 Lymphoma</td>
<td>70 Lymphoma</td>
</tr>
<tr>
<td>185</td>
<td>Paracentesis</td>
<td>Male</td>
<td>Normal</td>
<td>56.7 Lymphoma</td>
<td>22.8 Sarcoma</td>
</tr>
<tr>
<td>2059</td>
<td>Pleural</td>
<td>Female</td>
<td>Normal</td>
<td>81 Lymphoma</td>
<td>77.7 Lymphoma</td>
</tr>
<tr>
<td>2310</td>
<td>Pleural</td>
<td>Female</td>
<td>Normal</td>
<td>60.8 Lymphoma</td>
<td>52.2 Lymphoma</td>
</tr>
</tbody>
</table>

aOnly positive cases with >60% tumor content are shown.
Although the current study involved a small sample size with only a few tumor types, the results indicate promise for the use of microarray-based molecular tests with cytologic body fluids. Although the positive percentage agreement of 94.1% reported in the current study could be an overestimate because of the small sample size, it is consistent with the previously reported agreement (89%).

Further studies with larger sample sizes are needed to confirm performance with cytology samples. The results of the current study underscore the finding that > 60% tumor cellularity is an important parameter for obtaining reliable Tissue of Origin Test results, as well as the role of pathologists in adequate specimen and test selection. Potential pitfalls with the use of specimens that fail to meet quality control criteria for specimen entry into the Tissue of Origin Test include both false-negative diagnoses and incorrectly identified primary tumor sites, emphasizing the need for suitable specimens that fit testing requirements.

In conclusion, the results of the current study demonstrate that cell blocks from cytology body fluid specimens yield adequate diagnostic material for the Pathwork Tissue of Origin Test (and likely other molecular assays) and can be used in the workup of patients with unknown primary tumors who present with a malignant effusion.

**FUNDING SOURCES**

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**CONFLICT OF INTEREST DISCLOSURES**

Dr. Monzon has received research support and honoraria for speaking engagements from Pathwork Diagnostics. Drs. Lal and Halks-Miller are employees of Pathwork Diagnostics.

**REFERENCES**


