Automated evaluation and normalization of immunohistochemistry on tissue microarrays with a DNA microarray scanner

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INTRODUCTION

High-throughput DNA microarray screening has led to the identification of large numbers of deregulated genes with potential utility as new diagnostic and therapeutic targets. Subsequent examination of expression patterns of the encoded proteins in normal and diseased tissues is critical for the determination of the disease relevance of these candidate targets. Immunohistochemistry localization of the deregulated gene products in human tissues can provide this information, but a conventional target-by-target, tissue-by-tissue approach is not practicable for large numbers of deregulated genes.

Tissue microarrays enable representation on one microscope slide of hundreds of tissue cores and thereby provide a corresponding high-throughput approach to this validation step (1). In addition to a broad representation of normal and/or diseased human tissues, tissue microarrays minimize depletion of precious human tissue resources (2). However, the evaluation of immunohistochemistry on each tissue microarray slide generally requires manual analysis and tracking of hundreds of samples. Automated immunohistochemistry image analysis systems may be modified for tissue microarrays, but these systems are expensive and not widely available.

There is a need for efficient methods to normalize immunohistochemistry signals for cellular content because specific protein expression levels may reflect biological relevance. This issue has particular importance with tissue microarrays because the tissue core represents a small fraction of the tissue sample employed for diagnosis. Double-label immunohistochemistry with a two-color detection system is a powerful method to achieve normalization for cellular content. DNA microarray scanners, available in core facilities at most research institutions, specifically and simultaneously detect two fluorophores, and the associated image analysis software processes the fluorescent signals. We therefore employed a dual-wavelength DNA microarray scanner and double indirect immunofluorescence to normalize target gene expression signal to tumor cell content. This approach provides signal intensity data that indicate qualitative and quantitative aspects of protein expression as evaluated with indirect immunofluorescence on several tissue microarrays.

MATERIALS AND METHODS

Tissue Microarrays

Tissues from The Institute of Pathology, University of Graz, were fixed in buffered 4% formalin. Tissue microarrays were constructed using a manual tissue-arraying instrument (Beecher Instruments, Sun Prairie, WI, USA) (1,3,4). Multiple tissue cores from each donor block were included in each tissue microarray.

Immunostaining

Five-micron tissue microarray sections on Superfrost™ Plus slides (Roth, Karlsruhe, Germany) were deparaffinized and treated with 1% H2O2 in PBS, and the antigens were retrieved with microwaves (5 min at 800 W, 20 min at 300 W, in 10 mM citrate buffer, pH 6.0). Sequential 30-min, room temperature antibody incubations were followed with standard washing steps. Stained slides were dehydrated and coverslipped with Fluoromount (Serva, Heidelberg, Germany), and digital images were obtained with a laser scanning microscope (LSM510; Zeiss, Jena, Germany).

Automated Evaluation of Tissue Microarrays

Tissue microarray immunohistochemistry.tif images from 10-µm GMS-418 scans (Affymetrix, Santa Clara, CA, USA) were analyzed with ImaGene 4.1 software. A grid adjusted to accommodate the tissue microarray tissue cores provided signals from which local background was subtracted according to the software instructions. Small tissue defects did not eliminate cores from analysis, as the normalized procedure eliminated this source of artifact. Relative signal intensity and specificity were confirmed with a fluorescence microscope. Microsoft® Excel® or SPSS® was used for statistical analysis.
RESULTS AND DISCUSSION

Indirect immunofluorescence detection of multiple antigens on several tissue microarrays using a DNA microarray scanner was sensitive and reproducible. To provide an initial validation for this approach, a well-established immunohistochemistry method for distinction of epithelial tumors was employed. Cytokeratin expression with the pan-cytokeratin antibodies AE1/AE3 (Dako, Glostrup, Denmark) on a small tumor tissue microarray demonstrated at least 10-fold higher levels of these isoforms in carcinoma tissue cores compared with sarcoma cores on the same array, as expected ($P < 0.001$, two-sided $t$ test; data not shown) (5).

Independent labeled secondary antibodies demonstrated that the apparent cytokeratin signal detected in sarcomas was background. For single-fluorophore indirect immunofluorescence, this should be taken into consideration and may be eliminated by setting the background threshold manually. For double-label indirect immunofluorescence analysis, the background was similar with both secondary antibodies and therefore did not adversely affect the data quantitation and analysis.

Normalization and Quantitative Evaluation of Tissue Microarray Indirect Immunofluorescence

The variability of a tumor marker immunohistochemistry signal due to differential tumor cell content is a limiting factor for comparison of signals from different tissue microarray tumor cores. We therefore tested the possibility that double-label indirect immunofluorescence measured with a two-color DNA microarray scanner could normalize tissue microarray tissue core signals. Sections from two lung cancer

<table>
<thead>
<tr>
<th>Tissue Core</th>
<th>Raw c-erbB2 Signal</th>
<th>Initial Rank</th>
<th>Normalized c-erbB2 Signal</th>
<th>Normalized Rank</th>
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<tbody>
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<td>A</td>
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<td>4.88</td>
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<tr>
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<td>2</td>
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<td>3.49</td>
<td>4</td>
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<tr>
<td>D</td>
<td>39,000</td>
<td>3</td>
<td>4.03</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1. Tissue Microarray Tissue Core Tumor Marker Signal Intensity Obtained with a DNA Microarray Scanner and Normalized for Tissue Epithelial Cell Content

Figure 1. Double immunostaining on tissue microarrays. (a) Double-label indirect immunofluorescence of a lung adenocarcinoma tissue microarray stained with antibodies to the pan cytokeratin marker AE1/AE3 (green) and to the tumor marker c-erbB2 (red), scanned with a DNA microarray scanner. (b) Double-label indirect immunofluorescence of a lung squamous cell carcinoma tissue microarray stained and scanned as in panel a. (c) The same squamous cell carcinoma array as in panel b, stained with the c-erbB2 diagnostic kit HercepTest™ (Dako). The weakly positive cores in panel b are negative with this diagnostic immunohistochemistry procedure because of the lower sensitivity of the test.
tissue microarrays were double-stained with antibodies to the tumor markers c-erbB2 (Dako), epidermal growth factor receptor (EGFR) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or CD44 variant 6 (CD44v6; Bender MedSystems, Vienna, Austria) and anti-cytokeratin (5,6). Cytokeratin signal normalized each tissue core for the amount of epithelial carcinoma cells represented. Lung adenocarcinoma TMA (75 cases, 476 cores) and lung squamous cell carcinoma tissue microarray (67 cases, 479 cores) sections were labeled with c-erbB2 or EGFR polyclonal and cytokeratin monoclonal antibodies, and the lung adenocarcinoma tissue microarray sections were labeled with the CD44v6 monoclonal and cytokeratin 8/18 polyclonal antibodies (Figure 1).

Each tumor marker signal detected with a Cy3-conjugated secondary antibody was normalized by division with the keratin signal detected with a Cy5-conjugated secondary antibody. Normalization of the 5–7 cores from each tumor reduced variability in tumor marker expression between cores from the same tumor. Distinct c-erbB2 expression on the lung squamous cell carcinoma TMA was confined to just one of the 76 cases (Figure 1, b and c) (7,8), and normalization reduced the variability by 33% [average coefficient of variation (CV) reduced from 0.3 to 0.2; \( P < 0.01 \), Student’s \( t \) test].

Conventional EGFR immunohistochemistry (graded negative/weakly positive/strongly positive) revealed 18 weakly positive and 26 strongly positive cores on the adenocarcinoma array (44 of 479), and 12 weakly positive cores on the squamous cell carcinoma array (of 476). Compared with DNA microarray scanner-derived normalized values for these cores, the weakly positive samples were significantly stronger than those judged as negative, and the strongly positive were significantly different from those judged weakly positive (each \( P < 0.01 \), Student’s \( t \) test).

Table 1 provides the raw and normalized c-erbB2 signal intensity from four adjacent cores on the lung adenocarcinoma tissue microarray (two of these cores are shown in Figure 2, a and b). The intensity of c-erbB2 expression is greatest in core A tumor cells, despite the lowest absolute tumor marker signal for this core. The DNA microarray scanner analysis approach does not preclude detailed histological examination of immunostaining results, as indicated with histochemical and indirect immunofluorescence staining of these cores (Figure 2, c and d).

### Evaluation of Correlations with Clinicopathologic Parameters

Correlation with clinical and diagnostic parameters would be a major indication for the automated evaluation of immunostaining on tissue microarrays.
Therefore, we compared tumor marker indirect immunofluorescence signals obtained from the DNA array scanner on the lung adenocarcinoma tissue microarray to the diagnostic pathology tumor, lymph node, distant metastasis staging and grading system (9).

Normalized CD44v6 expression correlated with node metastasis, but not with tumor size or differentiation \((P < 0.05; \text{Figure 3})\); consistent with the finding that CD44v6 expression levels correlate with the metastatic potential of cell lines (10) and higher recurrent distant metastases in CD44v6-positive non-small-cell lung carcinoma (11). However, a lack of correlation between metastatic stage and CD44v6 expression has also been reported (12).

Normalized c-erbB2 expression levels determined with indirect immunofluorescence on tissue microarrays did not correlate with tumor lymph node metastatic stage, as previously reported (13). On this lung adenocarcinoma tissue microarray, poorly differentiated (G3) tumors expressed significantly lower c-erbB2 levels than moderately and well-differentiated tumors (G1 and G2), and levels of this antigen were higher in stage T1 tumors than in T2 tumors \((P < 0.05)\) (data not shown). Although c-erbB2 expression in non-small cell lung carcinoma has been correlated with a higher tumor stage and positive lymph node status, this study assessed only membranous staining, semiquantitatively (14). A clinicopathologic role of c-erbB2 in lung cancer remains controversial both with regard to potential disease involvement and to assessment methodology.

The expression of EGFR on this tissue microarray did not show any significant correlation with tumor, lymph node, distant metastasis parameters. Furthermore, expression of EGFR in these tissues correlated with neither c-erbB2 nor CD44v6 expression (data not shown), which is in agreement with previous reports (15).

In conclusion, application of a DNA microarray scanner and analysis software for tissue microarray indirect immunofluorescence data provides an efficient and versatile tool for initial automated analysis of this protein expression profiling approach. Double-label indirect immunofluorescence normalization extends the power of this approach by reducing the effects of tissue variability.

ACKNOWLEDGMENTS

The technical expertise of Andrea Fuchsbiicher, Iris Halbwedl, Peggy Samitch, and Andrea Sommersacher is gratefully acknowledged. Supported by the Austrian FFF fund (Oridis) and by the Austrian Science Fund (14759 to H.P. and S7401-MOB to K.Z.).

REFERENCES


Received 20 November 2002; accepted 26 March 2003.

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