Short Communication

A Multiplex Tissue Immunoblotting Assay for Proteomic Profiling: A Pilot Study of the Normal to Tumor Transition of Esophageal Squamous Cell Carcinoma

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Abstract

Esophageal cancer remains a highly lethal malignancy for which the genetic and proteomic events are poorly understood. Studies have reported dysregulated proteins in esophageal carcinoma; however, the magnitude of these changes remains largely uncharacterized. Little is known about alterations early in the neoplastic pathway. Using multiplex tissue immunoblotting, we quantified the expression of seven proteins in esophageal carcinogenesis. Regions of normal, dysplasia, and invasive carcinoma of the squamous esophagus in six patients were characterized. Pan-cytokeratin (CK) was essentially unchanged across the transition (0.96 in dysplasia and 0.69 in tumor). Expression levels of annexin 1, CK-4, and CK-14 were all decreased in dysplasia and tumor compared with normal (reference, 1.00): annexin 1, 0.30 in dysplasia and 0.15 in tumor; CK-4, 0.20 in dysplasia and 0.16 in tumor; and CK-14, 0.54 in dysplasia and 0.40 in tumor. Expression of two proteins was increased in dysplasia and tumor versus normal: cyclooxygenase-2, 1.35 in dysplasia and 2.32 in tumor and p53, 1.29 in dysplasia and 2.37 in tumor. Secreted protein, acidic and rich in cysteine, which is expressed in the adjacent stroma, was 1.56-fold higher in stroma underlying dysplasia and 6.20-fold increased in dysplastic stroma surrounding invasive tumor. These findings suggest that changes in protein expression can be detected during the transition to dysplasia and may be useful biomarkers. (Cancer Epidemiol Biomarkers Prev 2006;15(7):1403–8)

Introduction

Esophageal cancer remains one of the most challenging neoplasms to control. Even with the most advanced treatments, <13% of patients survive longer than 5 years after diagnosis (1). Most tumors are asymptomatic and go undetected until patients finally present at an advanced and incurable stage. Consequently, understanding mechanisms of carcinogenesis and identifying biomarkers of increased risk would be of particularly great benefit in the early diagnosis and treatment of this disease. New biomarkers related to the transition from normal squamous epithelium to invasive cancer are desired. The molecular genetic features and gene expression profiles of esophageal cancer have been previously reported (2-6). These studies show both up-regulation and down-regulation of several genes; however, changes at the protein level are poorly characterized. Studies that examine the zone of transformation from normal epithelium to tumor are challenging, as specimens showing this zone are uncommon even in large series. Appreciating the temporal changes in protein expression during the transformation from normal epithelium to invasive tumor is essential in defining new biomarkers that will predict progression, guide therapy, and potentially be used to monitor response to chemopreventative interventions.

Proteomic profiling of tissue can provide insight into diseases and may suggest clinically applicable biomarkers. Several candidate protein biomarkers have been evaluated in immunohistochemical analyses of the neoplastic progression to esophageal cancer and found to be dysregulated in invasive carcinoma (7). These data are consistent with the hypothesis that genes involved in extracellular matrix function are overexpressed in cancer, whereas genes involved in cell cycle regulation, proliferation, cell adhesion, and cytoskeleton function are underexpressed (5). However, previous immunohistochemical studies typically have only been able to describe changes in protein expression by qualitative scoring methods.

To examine changes in protein expression in foci of dysplasia and accurately quantitate expression changes, we used a recently developed method for transferring proteins from paraffin-embedded tissue sections to a stack of membranes that can then be probed with antibodies for detection of individual epitopes (8). This approach permits quantification of multiplex markers simultaneously while preserving the morphologic structure of the tissue and normalizing to total protein content. We used this method to analyze expression levels of seven different proteins that had been previously implicated in esophageal cancer. These potential biomarkers included proteins related to cell adhesion (annexin 1), the cytoskeleton [pan-cytokeratin (CK), CK-4, and CK-14], and the cell cycle [cyclooxygenase-2 (COX-2) and p53], and a matricellular protein [secreted protein, acidic and rich in cysteine (SPARC), also known as BM-40 or osteonectin]. We

Received 8/25/05; revised 1/26/06; accepted 5/12/06.

Grant support: NIH Intramural Research Program, National Cancer Institute, Center for Cancer Research.

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doi:10.1158/1055-9965.EPI-05-0651

quantified changes in expression in regions representing the histologic spectrum of disease from normal to invasive squamous cell carcinoma of the esophagus.

Materials and Methods

Tissue Specimens. Tissue sections were selected from esophagectomy specimens from Shanxi province in north central China. Collection and use of the tissue were approved by the Institutional Review Boards of the Shanxi Cancer Hospital (Shanxi, People's Republic of China) and the National Cancer Institute (Bethesda, MD). The tissue was fixed in 70% ethanol and processed to paraffin-embedded tissue. A series of esophagectomies was reviewed, and six individual cases that showed a zone of transition from normal epithelium to invasive cancer in a single block of tissue were chosen.

Transfer of Tissue Sections to Multilayered Membranes. Paraffin sections (5 μm) were cut, placed on standard microscope slides, and dried at 60°C for 1 hour. Sections were deparaffinized in three changes of xylene for 5 minutes each and rehydrated through a graded alcohol series to distilled water. Sections were treated with 20 μg/mL trypsin (Sigma, St. Louis, MO), 10% glycerol, and 50 mmol/L NH_4HCO_3 (pH 8.2; Fisher Scientific, Fairlawn, NJ) for 15 minutes at 37°C. The sections were washed thrice for 5 minutes each in PBS before incubation with Tris-glycine transfer buffer (50 mmol/L Tris, 380 mmol/L glycine) for 15 minutes at room temperature. Before transfer, 10 sheets of P-FILM membranes (20/20 GeneSystems, Inc., Rockville, MD) were preincubated with Tris-glycine transfer buffer for 15 minutes at room temperature. Membranes were assembled onto the slide into a transfer unit, and the transfer unit was placed in a Kapak SealPak pouch (Minneapolis, MN) and heat sealed. Finally, tissue sections were transferred under serial conditions for 1 hour at 55°C immediately followed by 0.5 hour at 65°C and 2 hours at 80°C using a heating block.

Immunoblotting. After transfer, the membranes were washed thrice with PBS for 5 minutes at room temperature. To measure total cellular protein transferred, each membrane was incubated with biotinylation solution (1 μg/mL; EZ-Link Sulfo-NHS-Biotin, Pierce, Rockford, IL) at room temperature for 10 minutes. The membranes were washed thrice with TBS containing 0.05% Tween 20 (pH 7.2) at room temperature for 5 minutes each. After biotinylation, the membranes were incubated with antibodies against pan-CK (AE1/AE3, 1:200; DAKO Cytomation, Carpinteria, CA), CK-4 (6B10, 1:100; Novocastra Laboratories, Norwell, MA), CK-14 (LL002, 1:100; NeoMarkers, Fremont, CA), annexin 1 (clone 29, 1:100; BD Biosciences, San Diego, CA), COX-2 (polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), p53 (DO-7, 1:200; DAKO Cytomation), and SPARC (15G12, 1:100; Novocastra Laboratories). All primary antibodies were incubated with membranes overnight at 4°C. The membranes were incubated with streptavidin-linked Cy5 (1:1,000; Amersham Biosciences, Uppsala, Sweden) and FITC-conjugated anti-rabbit IgG or anti-mouse IgG (1:1,000; Molecular Probes, Eugene, OR) for 45 minutes. The blots were dried, individually mounted on slides, and scanned in a ScanArray Express microarray scanner (Perkin-Elmer Life Sciences, Wellesley, MA) at 10-μm resolutions using 633-nm (Cy5) and 488-nm (FITC) lasers. Regions of interest were selected, and the signal intensity was quantified with ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA) and MARKO software version 1.0 (20/20 GeneSystems). The expression level of each antigen was normalized to the total protein amounts detected on the same membrane. For each target antigen, each case was normalized to either the expression levels in the normal epithelium or stroma.

Immunohistochemistry. To confirm finding by tissue immunoblotting, we did immunohistochemistry with same antibodies for pan-CK and COX-2. Immunohistochemistry staining was done with the LSAB+ kit from DAKO with antigen retrieval. The deparaffinized sections were incubated for 1 hour at room temperature with 20% normal goat serum in PBS containing 1% bovine serum albumin to block nonspecific antibody binding. Slides were incubated for overnight with primary antibodies. The stained slides were counterstained with hematoxylin.

Results and Discussion

Tissue Section and Protein Selection. Four of six cases contained a discrete region of dysplasia sufficient for analysis. For each case, one section was stained with H&E and one section was transferred to a stack of 10 membranes and probed with seven specific antibodies (multiplex tissue immunoblotting; ref. 8). The original H&E-stained and transferred slides were reviewed by two pathologists (T.B. and S.M.H.), and the zones of normal, dysplasia, and tumor were confirmed in each section. Antigen targets were selected from a review of the literature to include targets previously identified by microarray experiments on squamous cell carcinoma of the esophagus (5).

Each membrane was incubated with a specific primary antibody, and binding was determined by secondary FITC-conjugated antibody signal. Total protein was detected by biotinylation with a streptavidin-linked Cy5. The membranes were scanned with a microarray scanner, and signal was quantified by ImageQuant software. Signal detected for each antibody was normalized to total protein on the same membrane. The data were compiled and normalized to 1.00 for the signal from the normal epithelium or stroma as described below.

Changes in Expression of CK-4 and CK-14 Relative to Pan-CK. We first examined CK levels (pan-CK, CK-4, and CK-14) in the esophageal sections. We selected CKs because of their extensive use as a diagnostic marker for various malignancies. CKs represent an attractive candidate marker as they are expressed in a differentiation-dependent and tissue-specific manner. Several CKs have been previously shown to be up-regulated or down-regulated in tumors (5, 6, 9). Of particular interest are the conflicting results about expression levels of CK-14 mRNA in esophageal cancer. *CK-14* was identified as an underexpressed gene in a cDNA microarray experiment using an esophageal squamous cell line (6) but as an overexpressed gene in a cDNA microarray experiment using tumor and matched normal tissue from esophageal squamous cell carcinoma patients (5). We did multiplex tissue immunoblotting to further investigate these relative changes with respect to variability in protein expression across zones of dysplasia.

Figure 1 shows representative results of the multiplex tissue immunoblotting, including a H&E-stained esophageal specimen, showing the transition from normal epithelium to dysplasia and invasive tumor, with underlying stroma of soft tissue and muscle (Fig. 1A). Both pan-CK and CK-4 were highly expressed in the normal epithelium (Fig. 1B and C) along with high level expression of pan-CK in the region of tumor (Fig. 1B). Pan-CK expression was not significantly different among normal (defined as 1.00), zones of dysplasia, and invasive epithelium, although there was case-to-case variability. Regions of stroma underlying the normal epithelium showed essentially no expression of pan-CK (Table 1; Figs. 1B and 2A). Visual inspection of immunohistochemistry for pan-CK gave identical results (Fig. 3). CK-4 protein expression was decreased 80% in dysplasia and 85% in invasive squamous cell carcinoma compared with normal epithelium (Table 1; Fig. 2A). CK-14 expression was also

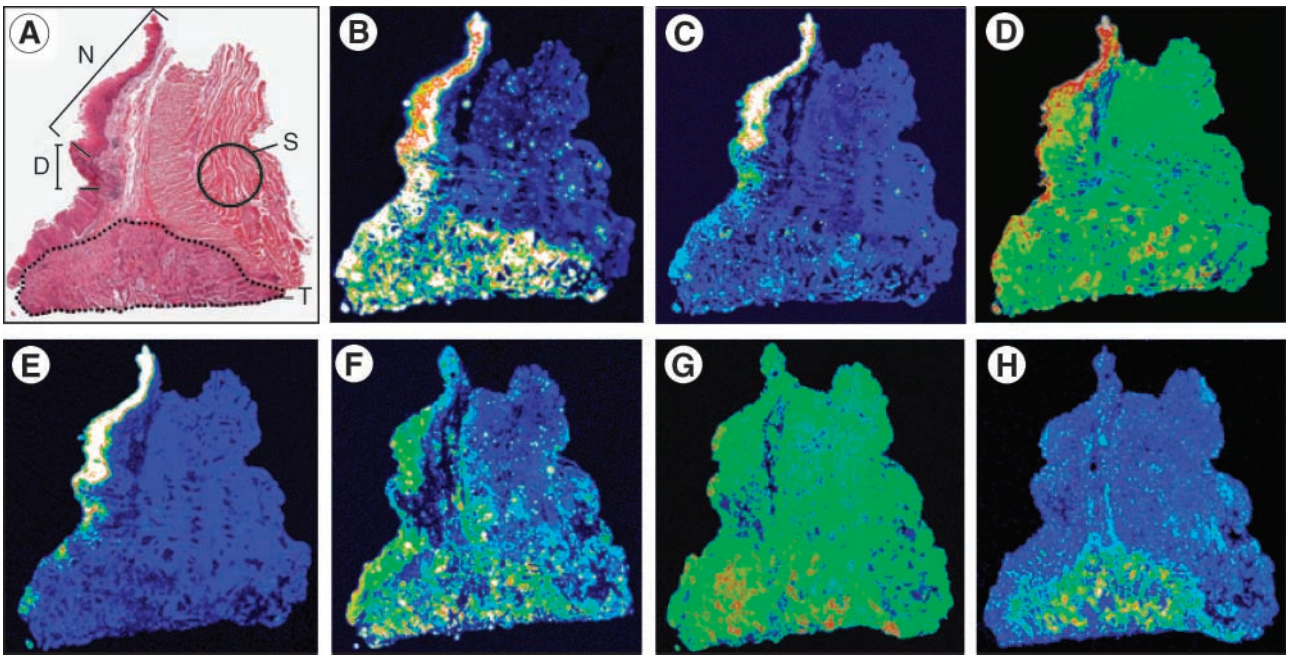

Figure 1. Biomarker expression profiling by multiplex tissue immunoblotting in human esophagus tissue. **A.** H&E-stained human esophagus. *N,* normal; *S,* stroma; *D,* dysplasia; *T,* tumor. Transferred membranes were incubated with antibodies against pan-CK (**B**), CK-4 (**C**), CK-14 (**D**), annexin 1 (**E**), COX-2 (**F**), p53 (**G**), and SPARC (**H**). After incubation of primary antibodies, the membranes were detected with FITC. Membranes were imaged with a microarray scanner. Fluorescent scans represented with pseudocolor, where signal intensity is white-red-yellow-green-blue-black from maximum to minimum signal.

decreased in dysplastic epithelium and invasive tumor, 46% and 60%, respectively (Table 1; Figs. 1D and 2). In summary, these results show that CK-4 and CK-14 protein expressions are progressively reduced in the transition from normal epithelium to invasive tumor. The greater degree of reduction in CK-4 expression compared with CK-14 makes it a more promising biomarker.

Annexin 1 Dysregulation during Invasion. Previous studies have shown variable reduction of annexin 1 protein expression in invasive esophageal carcinoma with Western blot analysis of laser capture microdissected tumor (10, 11). However, the exact amount of this decrease is poorly appreciated, and variable expression of annexin 1 was observed by immunohistochemistry (12). We showed marked reduction of annexin 1 expression in dysplasia, 70% compared with normal epithelium, and in invasive tumor, 85% compared with normal (Table 1; Figs. 1E and 2B).

Overexpression of COX-2 in Tumor Invasion. Increased COX-2 expression is well described in numerous squamous epithelial cancer systems (13), and these findings have led to clinical trials with COX-2 inhibitors (14). Overexpression of COX-2 has been detected by cDNA microarrays and *in situ* hybridization approaches (5, 15), but quantification of changes in the expression of COX-2 protein by a traditional immunohistochemical approach in paraffin-embedded tissue remains problematic. Using multiplex tissue immunoblotting, we showed that COX-2 was only slightly up-regulated in dysplastic lesions and invasive carcinomas (1.35- and 2.37-fold, respectively; Table 1; Figs. 1F and 2B). These findings are consistent with the immunohistochemical staining pattern (Fig. 3).

Profile of p53 Expression in the Transition from Normal Epithelium to Invasive Tumor. Overexpression of p53 is well documented in esophageal carcinoma (16, 17); however, in this study we sought to characterize the extent of this up-regulation of protein more accurately. Using multiplex tissue immunoblotting, normal epithelium and stroma showed similar levels of p53 expression. In one case, there was a small increase in p53 expression in the normal epithelium when compared with stroma (1.35-fold increase), which was confirmed by immunohistochemistry (data not shown). Aberrant expression of p53 in normal epithelium adjacent to tumor is frequently described and may indicate that p53 dysregulation is an early event in carcinogenesis (18). We used patient samples from Shanxi province in north central China, which were previously observed with highly frequent p53 mutation (19). Although we do not have p53 mutation status data on these patients, it is anticipated that 96% of cases will have at least one genetic alteration, including p53 mutation (77%), allelic loss within the *p53* gene (73%), and/or loss of heterozygosity at the p53 microsatellite marker (80%). In both the zones of dysplasia and invasive carcinoma, p53 expression was increased (1.29- and 2.37-fold, respectively; Table 1; Figs. 1G and 3). This increase was modest but compatible with the potent role of p53 in tumorigenesis.

Table 1. Analysis expression levels of several biomarkers in esophageal normal, dysplastic and cancerous epithelium, and associated normal stroma

Biomarkers*	Type of tissue			
	Normal (n = 6)	Stroma (n = 6)	Dysplasia (n = 4)	Tumor (n = 6)
Pan-CK	***1.00***	0.05	1.04	1.06
CK-4	***1.00***	0.05	0.27	0.16
CK-14	***1.00***	0.21	0.56	0.43
Annexin 1	***1.00***	0.08	0.31	0.18
COX-2	***1.00***	0.26	1.44	2.92
p53	***1.00***	0.97	1.29	2.75
SPARC	0.88	***1.00***	1.67	6.67

*The expression level of each antigen was normalized to expression either in the normal epithelium or in the stroma (bold italics), which was defined as 1.00.

Overexpression of SPARC in Desmoplastic Stroma in Response to Tumor Invasion. SPARC mRNA was overexpressed in esophageal cancers in one report (5); however, immunohistochemistry for SPARC in another study showed that the overexpression of the protein was not within the tumor cells but, rather, in the desmoplastic stroma response to tumor (20). After normalizing SPARC expression to the stroma underlying the normal epithelium, we found overexpression of SPARC in invasive tumor (6.20-fold); expression was modestly up-regulated (1.56-fold) even before the tumor breached the basement membrane (Table 1; Figs. 1H and 3). Because SPARC is up-regulated and can be detected in serum (21), it holds particular promise as a potential biomarker for the early detection and screening of esophageal carcinoma.

We showed a progressive change in protein expression with histologic evolution from normal through dysplasia to invasive cancerous epithelia. Although it has been previously appreciated that some biomarkers are dysregulated early in the carcinogenesis pathway, the magnitude of these changes in expression has been previously challenging to quantitate and impossible to visualize using an *in situ* approach (22). A multiplex tissue immunoblotting method permits rigorous comparisons of protein expression across geographic zones of histology as well as interrogation of multiple antigens from a single section of tissue. An added feature is the normalization by total protein present on the same membrane in which the antigen is quantified. This approach does not require microdissection or Western blotting yet retains the histopathologic relationship during protein profiling. Essential to success with this method is the quality and optimization of the antibody to achieve adequate signal for measurement. We believe this method will further expand the use of using formalin-fixed, paraffin-embedded tissue for proteomic analysis by expanding the spectrum of detectable antibodies.

The capacity to define the level of dysregulation of potential biomarkers in zones of dysplasia is essential toward defining

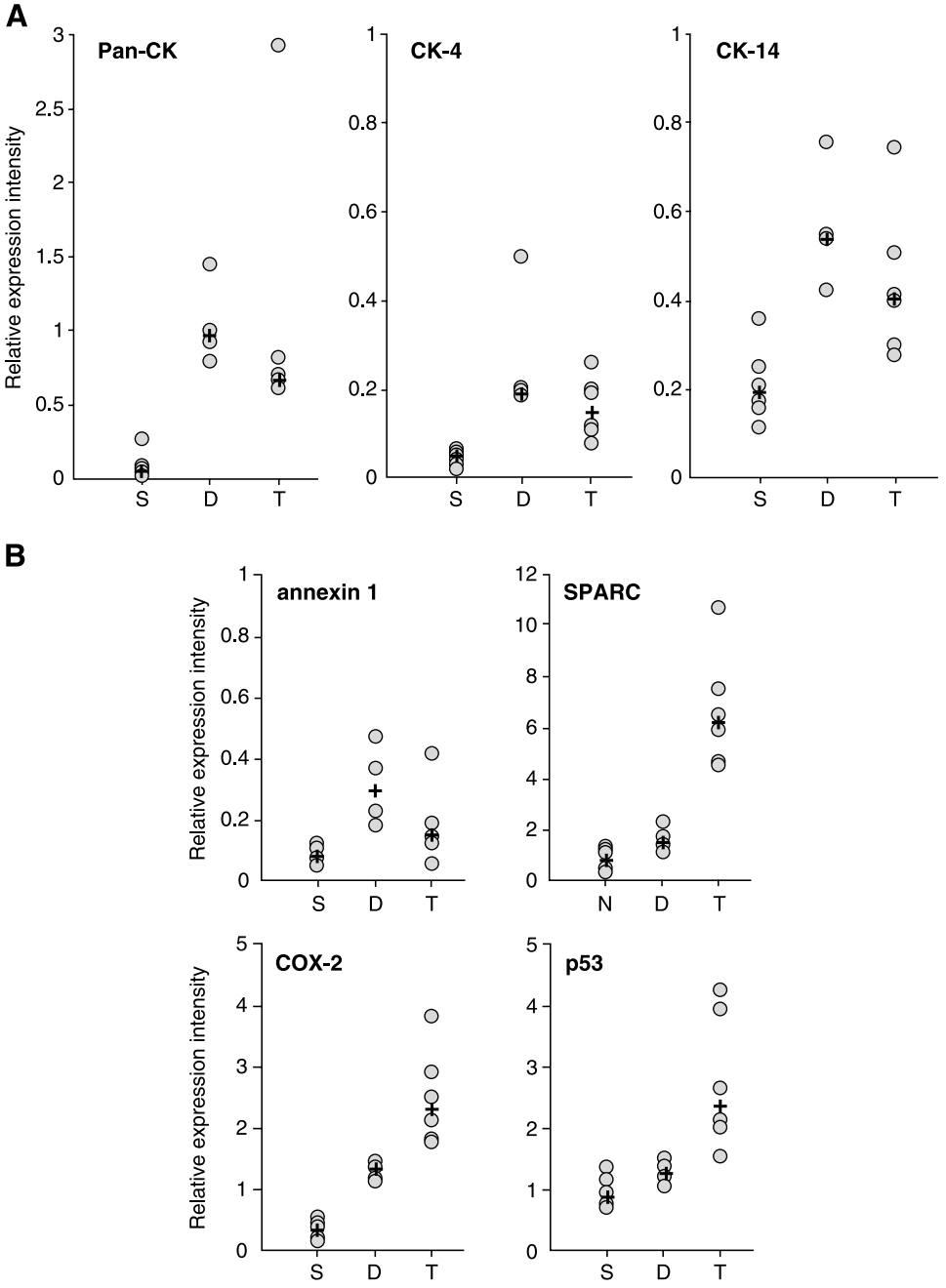

Figure 2. Quantification of proteomic expression profiling of different biomarkers in squamous cell carcinoma of the esophagus. **A.** Pan-CK, CK-4, and CK-14. **B.** Annexin 1, COX-2, p53, and SPARC. After immunoblotting, membranes were scanned and then analyzed using ImageQuant 5.2 software program. Briefly, five equivalent circular areas from each region were defined and the mean value of fluorescence was determined. Finally, specific antibody signal was calculated based on expression levels of total cellular protein. *Symbols,* relative expression level of an individual case in normal epithelium (n = 6), stroma (n = 6), dysplastic epithelium (n = 4), and invasive tumor (n = 6); +, median value. *Points,* average of two independent experiments. Relative expression intensity of each entity is normalized to normal epithelium or stroma.

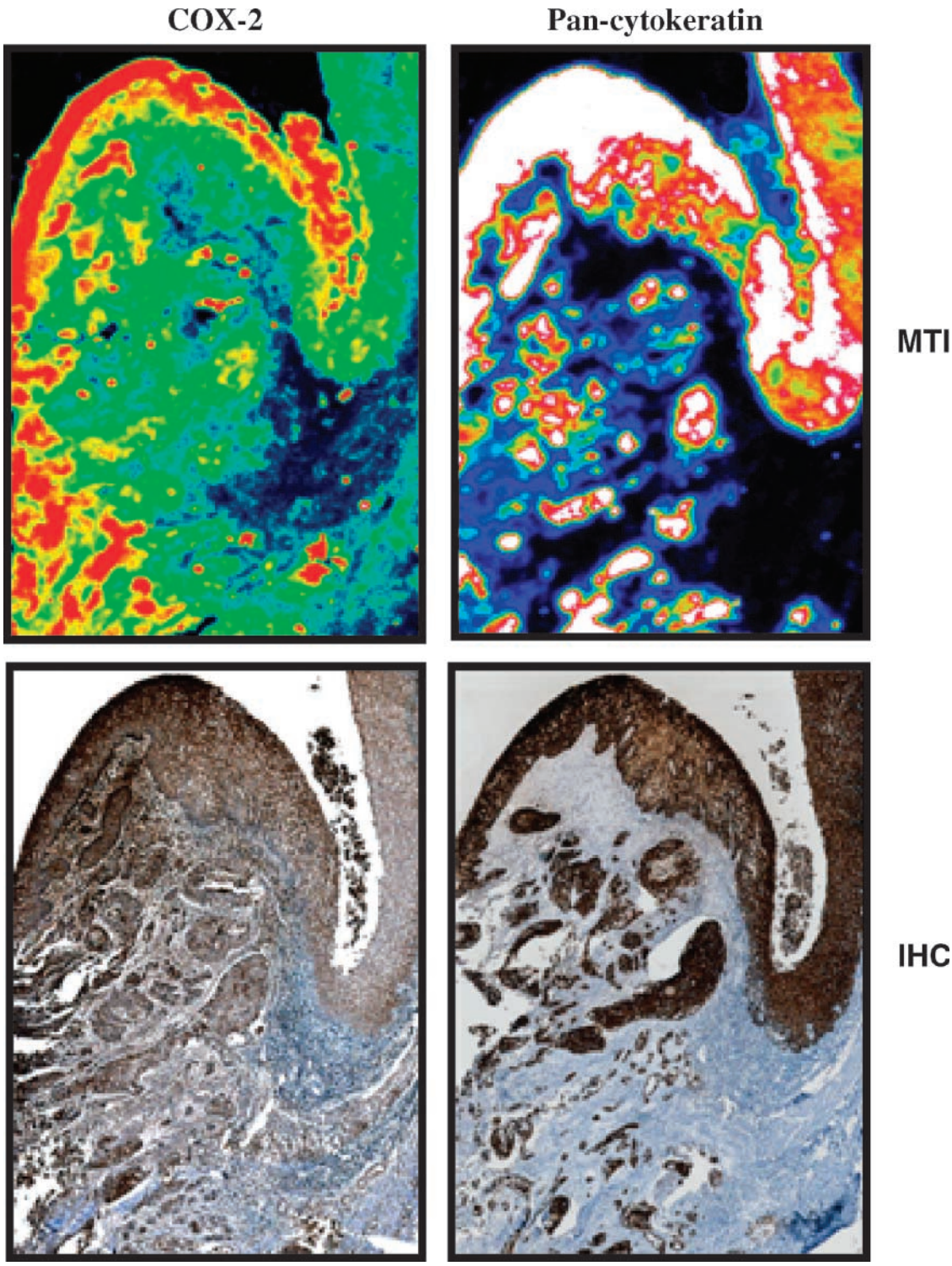

Figure 3. Comparison between multiplex tissue immunoblotting (*MTI*) and immunohistochemistry (*IHC*). We did both experiments using anti-rabbit COX-2 antibody and anti-mouse pan-CK antibody. The positive signal correlates with intensity of immunohistochemistry staining. Fluorescent images of multiplex tissue immunoblot represented with pseudocolor, where signal intensity is white-red-yellow-green-blue-black from maximum to minimum signal.

robust biomarkers that will play a role in prediction of progression of dysplasia. Demonstration of dysregulation of CK-4 and CK-14 in invasive tumor is of little use in diagnostic pathology. However, showing that CK-4 and CK-14 are consistently and significantly altered in zones of dysplasia suggests they may have some value as prognostic markers in patients with dysplastic epithelium (23). In contrast, p53 and COX-2, although significant at the biological level in the progression to tumor, are poor candidate biomarkers for progression. Obtaining adequate specimens with zones of dysplasia contiguous with both normal epithelium and invasive tumor is challenging and limited the number of cases that we could examine.

In conclusion, we were able to quantify the change in seven proteins in the transition from normal epithelium to invasive tumor using a multilayered proteomic profiling approach. Our data suggest that decreased expression of CK-4, CK-14, and annexin 1 are early events. In contrast, the modest increase observed in COX-2 and p53 protein expression with progression from normal to dysplasia suggests that these markers may be most informative in more advanced neoplasias. The increase in SPARC even in stroma underlying dysplasia, as well as the potential to measure the protein in serum, makes it a potential biomarker of early disease. This approach of quantifying the changes in expression of potential biomarkers before invasive disease will be a useful tool in biomarker development for early detection of cancer.

Acknowledgments

We thank Kimberly Tuttle and Yvonne Gathright for providing technical expertise.

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Cancer Epidemiol Biomarkers Prev 2006;15:1403-1408.