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# ESOPHAGIN AND PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) ARE BIOMARKERS OF HUMAN ESOPHAGEAL NEOPLASTIC PROGRESSION

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**PCNA and esophagin have been implicated in the multistep process of carcinogenesis, but simultaneous characterization of these proteins in the early stages of esophageal neoplastic progression has yet to be undertaken. In morphologically normal esophageal epithelium, esophagin stains the granular layer cells, principally in their cell membrane portions. PCNA, in contrast, stains the nuclei of cells in the parabasal and basal layers. We examined 201 regions from 47 patients that represented different stages of esophageal neoplasia, comprising 34 areas of normal mucosa, 18 of dysplasia in squamous epithelium (DYS/SC), 39 squamous cell carcinoma (SCCA), 29 areas of Barrett's esophagus, 48 of Barrett's dysplasia (DYS/BAR) and 33 areas of adenocarcinoma (AC). The immunostaining patterns of esophagin and PCNA were evaluated and graded for level of expression. There was loss of esophagin expression in the high- and low-grade dysplasias compared to normal epithelia. In the squamous dysplasias, there was more intense staining (of esophagin) in the atypical nuclei and superficial squamous epithelial cells than in the basal cells. PCNA staining was increased in intensity in the high-grade dysplasias relative to normal basal layer cells. Combined analysis of esophagin and PCNA appears to reveal an inverse relationship between proliferation and differentiation during esophageal neoplastic progression. Moreover, this combined staining approach also offers promise for detecting esophageal cancer in early, precancerous stages.**
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Phenotypic evidence of impaired cellular differentiation is a defining characteristic of cancer cells. Many genes have been identified whose protein products are markers for cellular differentiation in specific cell types or tissues. Loss of expression of these genes in human cancers can be assessed and may afford diagnostic and/or prognostic significance. Altered expression of cellular differentiation genes, however, is merely an indicator of a more fundamental abnormality present in neoplastic cells: disruption of signal transduction pathways controlling the balance between cellular differentiation and proliferation. In stratified squamous epithelia such as the epidermis and esophagus, terminal differentiation involves a highly coordinated sequence of events that is reflected in the histological appearance of several distinct layers of epithelial cells.[1,2] As part of the normal growth of stratified squamous tissues, terminally differentiated cells are continuously sloughed off.[3] In this context, epithelial cell renewal is dependent on a small population of stem cells possessing an intrinsic ability to divide and produce committed progenitor cells to repopulate the mature cell layers.[4] Failure of a progenitor cell to fully differentiate as a result of genetic or epigenetic influences may preserve a state of enhance cell proliferation that may predispose to the accumulation of mutations in cancer genes and thus to eventual neoplastic transformation.[5]

The esophagin gene (also known as SPRR3) is a member of the multigene family encoding small proline-rich proteins (SPRRs), which are expressed in stratified squamous epithelia during differentiation. The SPRRs are components of the cell envelope, which is a specialized structure that forms in terminally differentiated epithelial cells and provides a barrier against mechanical and chemical stress.[6–8] Thus far, 11 human SPRRs (2 SPRR1s, 8 SPRR2s and 1 SPRR3) have been identified.[9]

Previously, we reported that loss of esophagin expression is observed in primary esophageal squamous cell carcinomas and adenocarcinomas and esophageal cancer cell lines.[10] In our previous study, loss of esophagin expression was seen in areas of low- and high-grade dysplasia, as well as in normal mucosa adjacent to cancers, suggesting that loss of esophagin expression occurs early in neoplastic transformation.[10] In the current work, we describe a detailed histologic study of the tissues at several stages of esophageal carcinogenesis. Our study focused on 2 potential biomarkers of neoplasia: esophagin and PCNA. We provide further evidence that esophagin is a marker of differentiation, while PCNA is a marker of cellular proliferation in the esophagus. Combined use of these markers offers promise in detecting early stages of esophageal neoplastic progression.

## MATERIAL AND METHODS

### *Material*

We examined 201 regions from 47 patients containing areas that presented different stages of esophageal neoplasia. These regions consisted of 34 areas of normal mucosa, 18 of dysplasia in squamous epithelium (DYS/SC), 39 squamous cell carcinoma (SCCA), 29 areas of Barrett's esophagus, 48 of Barrett's dysplasia (DYS/BAR) and 33 areas of adenocarcinoma (AC). Each block contained a combination of cell types, but no patient possessed all tissue types studied. Tissues were obtained from endoscopic biopsies and were previously fixed in formalin and embedded in paraffin at the Baltimore VA Hospital Pathology Department and at the University of Maryland Hospital.

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*Methods*

*Histopathological analysis.* A hematoxylin and eosin-stained slide was produced from each block for analysis. Based on pathologic criteria, tissue regions were classified as follows: high-grade dysplasia, low-grade dysplasia, normal esophageal epithelium and adenocarcinoma. Normal epithelium and adenocarcinoma were included not only as part of our study of neoplastic progression but also as positive (normal) and negative (adenocarcinoma) controls for esophagin staining. The stained sections were reviewed and scored independently by a single pathologist (A.B.). The slides were assigned a score of 0–2 for both esophagin and PCNA staining as follows: 0 for no staining, 1 for weakly positive staining (number of positive cells less than 10%), 2 for moderately positive staining (number of positive cells between 10 and 50%) and 3 for strongly positive staining (number of positive cells more than 50%. For summary comparisons, scores of 0 or 1 were counted as negative (0 in Table I), scores of 2+ (1 in Table I) and 3+ (2 in Table I) were counted as positive.

*Immunohistochemical analyses.* A monoclonal antibody against PCNA was purchased from DAKO Corporation (Carpinteria, CA). The previously described rabbit polyclonal anti-esophagin antibody was made by Invitrogen (Carlsbad, CA).[13] Immunostaining of esophagin and PCNA were performed on serial sections cut from each paraffin block. Slides were baked at 65°C for 1 hr and washed in xylene 3 times for 5 min each to dewax. After dewaxing, the slides were dehydrated in an ethanol series and rinsed 3 times in 1× phosphate-buffered saline (PBS). The DAKO LSAB+ kit components were used for the remainder of this process. Slides were incubated at room temperature for 5 min with 3% hydrogen peroxide to neutralize endogenous peroxidases. No antigen retrieval was performed for these samples (antigen retrieval was tested once but the tissues, because of their glandular delicacy, were destroyed and staining was impossible to score; data not shown). Next, slides were incubated with the primary antibody (1:100 dilution for PCNA and 1:15,000 dilution for esophagin) overnight at 4°C. Slides were sequentially incubated with DAKO protein block, serum-free for 10 min at room temperature, the linker antibody from the kit for 30 min at room temperature, rinsed in 1× Tris-buffered saline with 1% Tween-20, in streptavidin-peroxidase for 30 min at room temperature and rinsed as before. Diaminobenzine (DAKO LSAB+ kit) was used as the chromagen and the counterstain was hematoxylin. The slides were then dehydrated and mounted with Permount (Fisher Chemicals, Piscataway, NJ).

## RESULTS

*Immunohistochemistry results*

To more clearly define the typical staining pattern for each histological subtype, we stained a variety of tissues with both anti-esophagin and anti-PCNA antibodies. Figure 1 illustrates Esophagin and PCNA staining for squamous esophageal epithelium with low-grade dysplasia, Barrett's esophagus with high-grade dysplasia, esophageal adenocarcinoma and esophageal squamous cell carcinoma. Figure 1 clearly shows negative staining of malignant cells and strongly positive staining of normal cells by esophagin, along with strongly positive nuclear staining of malignant cells by PCNA. There is a gradual progression of both of these staining trends in the transition from normal to dysplastic to cancerous cells.

To aid in correlative analyses, a list was compiled of all tissues according to histological type and staining score (Table I). The observed trends in esophagin and PCNA staining intensities were apparent when the data was viewed by graphing the staining index for each tissue type (Fig. 2). This average score was calculated by taking the number of 2+ stained areas and multiplying them by 2 and the number of 1+ regions by 1. These totals were then divided by the total number of areas for each histological type. Esophagin staining was lost as the lesions progressed from normal to higher grades of dysplasia and cancer. The trend was the opposite for PCNA. Staining was highest for adenocarcinomas and leveled off as the lesions were examined from high-grade to low-grade dysplasia to normal epithelium. Some normal samples received a PCNA staining score of 3+ because basal layers were highly proliferative and had dark PCNA staining, rather than because of the volume of cells stained.

This trend was also evident in the esophagin and PCNA immunohistochemical staining itself. In Figure 1, esophagin staining for each tissue type is shown. It is clear that esophagin staining in normal squamous epithelium is much darker and more intense than in the other 2 tissue types, yielding a score of 3+. Interestingly, some staining occurred in low-grade but not in high-grade Barrett's dysplasia, consistent with the high-grade lesion being advanced; frank adenocarcinomas stained completely negative for esophagin. PCNA staining progressed in the opposite direction, with high-grade dysplastic lesions staining the most intensely and low-grade dysplasias and normal mucosa least intensely (Fig. 1). As stated above, some normal glands received a score of 3+ for PCNA staining, because while the uppermost layers of the epithelia were negative, the basal layers were positive (Fig. 1).

Not only was there a pattern for esophagin and PCNA staining, but also when the 2 were compared, a dramatic effect was seen (Fig. 2). For example, there was a marked difference between the index scores between normal esophageal samples and adenocarcinomas. There was also a marked difference between Barrett's metaplasia samples and adenocarcinomas. Finally, there was a strong, but not significant, trend within dysplastic samples compared to squamous cell carcinomas (Fig. 2).

**TABLE I.** – SUMMARY OF TISSUE TYPES AND THEIR RESPECTIVE INDEX SCORES[1]

| | 1 | 2 | 3 | 4 | 5 | 6 | |
|---|---|---|---|---|---|---|---|
| Esophagin | Ne | Dys/SC | SCCA | Barrett's | Dys/Bar | ADEN | |
| 2 | 18 | 3 | 4 | 0 | 0 | 0 | |
| 1 | 12 | 4 | 17 | 6 | 6 | 2 | |
| 0 | 4 | 11 | 18 | 23 | 42 | 31 | |
| Total areas | 34 | 18 | 39 | 29 | 48 | 33 | 201 |
| PCNA | | | | | | | |
| 2 | 14 | 5 | 20 | 3 | 21 | 13 | |
| 1 | 5 | 5 | 6 | 12 | 19 | 9 | |
| 0 | 13 | 8 | 10 | 14 | 9 | 11 | |
| Total areas | 32 | 18 | 36 | 29 | 49 | 33 | 197 |

[1]This Table shows the scores for each of the tissue types studied: normal esophagus (Ne), dysplasia arising in squamous epithelium (DYS/SC), squamous cell carcinoma (SCCA), Barrett's metaplasia (Barrett's), dysplasia arising in Barrett's esophagus (DYS/Bar) and adenocarcinoma (ADEN). A score of 0 and 1+ was counted as 0; a score of 2+ was counted as 1 and 3+ as 2. The total number of areas shown given for each histological type.

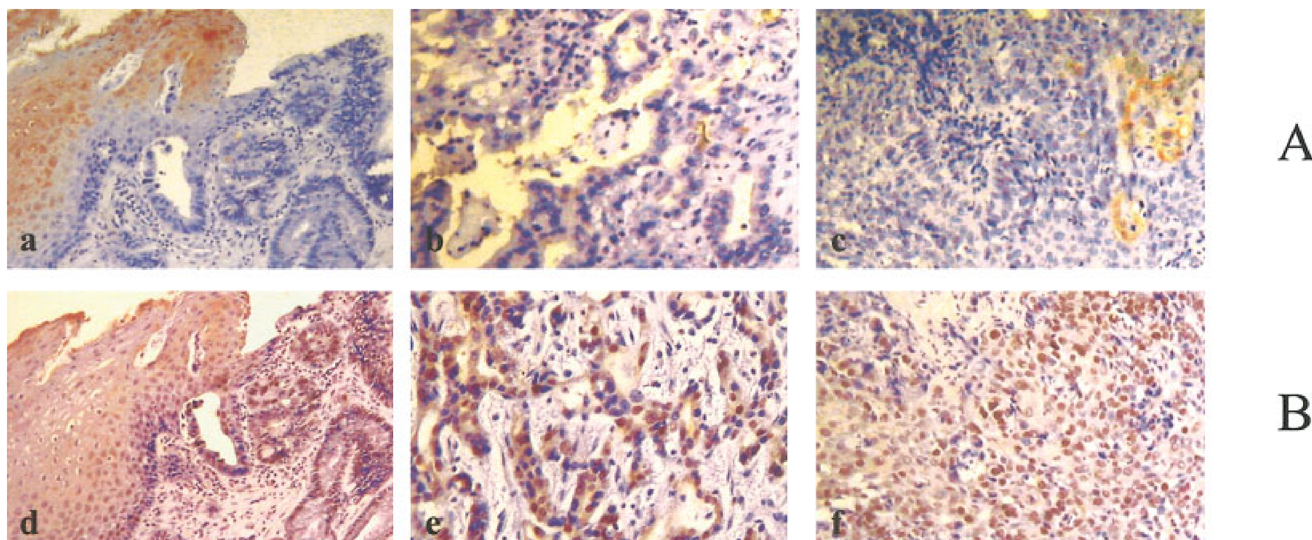


**Figure 1** – A: Esophageal staining: esophageal squamous epithelium with low-grade dysplasia and Barrett's esophagus with high-grade dysplasia (*a*), esophageal adenocarcinoma (*b*) and esophageal squamous carcinoma (*c*). B: PCNA staining: esophageal squamous epithelium with low-grade dysplasia and Barrett's esophagus with high-grade dysplasia (*d*), esophageal squamous cell carcinoma (*e*) and adenocarcinoma (*f*).

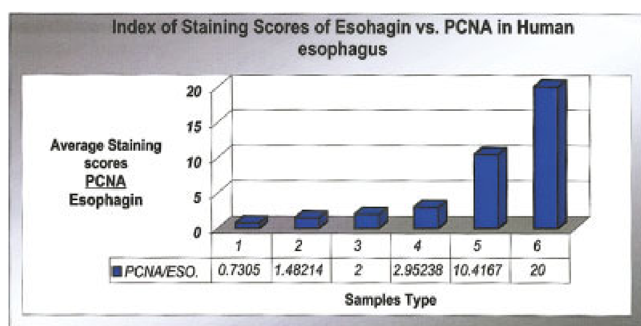


**Figure 2** – Bar graph of index staining scores for esophagin and PCNA. Index scores were calculated by dividing the average score for PCNA by the average score for esophagin. Bar 1 represents normal esophageal epithelium. Bar 2 represents dysplasias present in squamous epithelium. Bar 3 represents squamous cell carcinoma. Bar 4 represents Barrett's metaplasia. Bar 5 represents dysplasias arising in Barrett's esophagus. Bar 6 represents adenocarcinomas.

## DISCUSSION

The current study was performed to establish esophagin as a biomarker of esophageal neoplastic progression and to couple it to proliferation. The proliferation marker used was proliferating cell nuclear antigen (PCNA). PCNA was employed, rather than a cell division marker such as Ki-67, because it corresponds to a known protein-encoding gene. In contrast, Ki-67 is a nongene marker.

The current findings suggest that combined staining with esophagin and PCNA offers potential as a biomarker of esophageal cellular differentiation and proliferation. This combined staining approach appears to identify the transition between normal esophageal epithelium and early dysplastic esophageal transformation. In our study, there was clear loss of staining as lesions with further progression along the neoplastic cascade were studied. Furthermore, the current data suggest that PCNA is a marker of esophageal cellular proliferation.

Immunostaining for PCNA clearly demarcated proliferating areas, such as the basal layers of normal mucosa and all layers of dysplastic esophageal mucosa. Thus, combined analysis of esophagin and PCNA appears to represent a useful biomarker-based approach to improved detection of esophageal cancer in its early, precancerous stages and suggests that a prospective, longitudinal study of combined staining in patients at increased risk of developing esophageal cancer would be of value.

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