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Rab11 in Dysplasia of Barrett's Epithelia

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Barrett's esophagus predisposes affected patients to the development of esophageal adenocarcinoma. The development of adenocarcinoma proceeds along a progression through low- and high-grade dysplasia. Surveillance of Barrett's patients requires serial endoscopic investigations and grading mucosal biopsies. Unfortunately, grading of biopsies by conventional hematoxylin and eosin staining is fraught with significant interobserver variations. We have found in both biopsy and resection specimens that immunostaining for the small GTP binding protein Rab11 is increased in low-grade dysplastic cells. This staining is lost in high-grade dysplastic cells. These results suggest that low-grade dysplastic cells undergo an apical trafficking blockade, which is released as cells progress to the less differentiated phenotype of high-grade dysplasia and adenocarcinoma. Examination of the SKGT-4 esophageal adenocarcinoma cell line demonstrated prominent mRNA and protein expression for Rab11. Rab11 immunostaining was present in SKGT-4 cells as a perinuclear nidus of punctate staining along with a more diffuse punctate pattern. Thus, Rab11 expression was present in a esophageal adenocarcinoma cells in culture. Markers of vesicle trafficking may be critical factors for grading of mucosal dysplastic transitions leading to adenocarcinoma.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is now recognized as a prevalent cause of upper gastrointestinal symptoms [17, 29]. While novel proton pump inhibitor medications and prokinetics have provided significant therapeutic interventions in the treatment of GERD, the complications of reflux disease remain of considerable concern. In particular, Barrett's esophagus or columnar metaplasia of the distal esophagus carries a 30- to 40-fold increased risk of developing esophageal adenocarcinoma [11, 16, 31, 39]. A strong relationship has been established between specialized intestinal-type Barrett's epithelium and adenocarcinoma [11, 12, 14, 26, 27, 31, 39, 41]. This association of Barrett's epithelium with adenocarcinoma has led to the recommendations that patients with Barrett's epithelium undergo a program of regular

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[b]*Abbreviations:* GERD, gastroesophageal reflux disease.

endoscopic surveillance [28, 37]. Central to this process of surveillance is the histological grading of serial endoscopic biopsies. Inherent to this protocol is the concept that adenocarcinoma develops through a progression through increasing grades of dysplasia [23, 32, 36]. When high-grade dysplasia (essentially carcinoma *in situ*) is detected, early resection of the distal esophagus is indicated, since the incidence of frank adenocarcinoma in the remaining esophagus is high [11, 23, 40].

While high-grade dysplasia and adenocarcinoma are generally easily discerned histologically, much greater interobserver variation has been reported for the grading of low-grade dysplasia [34]. These uncertainties make the surveillance of Barrett's more problematic, and lead to confusion in identifying patients who need more frequent biopsies. More importantly inconsistencies in grading likely lead to more frequent than necassary biopsy surveillance of patients with benign disease. Thus, many investigators have sought to identify more objective criteria to identify those patients with the greatest risk of developing cancer. Recent studies suggest that mutations of the p53 tumor suppressor gene, as detected by immunohistochemistry or flow cytometry, may be predictive of progression to high-grade dysplasia and adenocarcinoma [2, 3, 5, 15, 18, 25, 38, 43]. Genetic instability as determined by aneuploid or increased G2\tetraploid fractions in flow cytometric analysis and cellular ultrastructural changes have been implicated in the development of adenocarcinoma [20-22, 32, 33]. Still, the reliable identification of

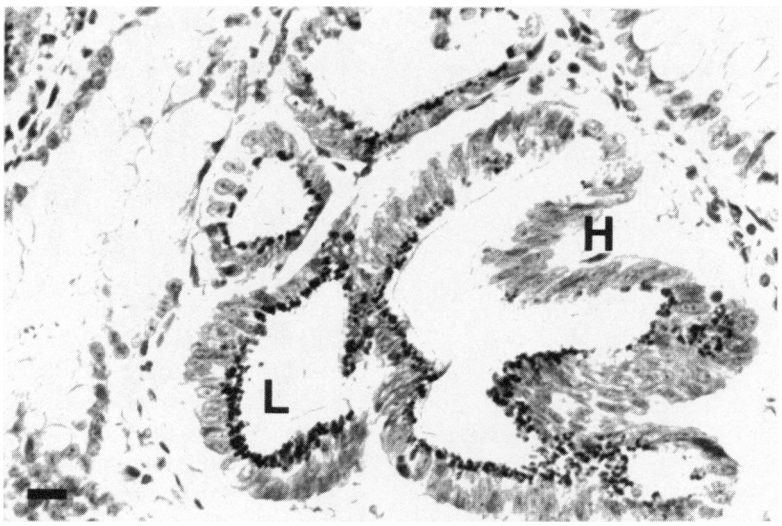

Figure 1. Rab11 immunostaining in low-grade dysplasia within Barrett's mucosa. A five μm section of archival paraffin embedded tissue from an esophageal resection of a patient with adenocarcinoma arising within Barrett's epithelium was stained with Rab11 monoclonal antibody (8H10 ascites, 1:300, for 2 hours at room temperature). Specific staining was visualized with alkaline phosphatase conjugated goat-anti-mouse IgG secondary and Vector Red chromagen. Sections were lightly counterstained with Mayer's hematoxylin. Non-dysplastic Barrett's epithelium (at left) showed no significant staining. Low-grade dysplastic cells (L) showed a prominent perinuclear vacuolar staining for Rab11. However, cells demonstrating high-grade dysplastic phenotypes (H) showed diminished or absent staining. Bar = 20 μm.

low-grade dysplasia versus readings of indeterminant or reactive changes remains a problem for biopsy grading.

INCREASED RAB11 IMMUNOSTAINING IS ASSOCIATED WITH LOW-GRADE DYSPLASIA

The small GTP-binding protein, Rab 11, is a ubiquitous protein in non-polarized and polarized cells which is associated with plasma membrane vesicle recycling systems [42]. In epithelial cells, Rab11 is associated with sub-apical vesicles in a number of cells including the gastric fundic, ileal, and colonic epithelia as well as the squamous epthelium of the esophagus [8]. In the gastric parietal cell, Rab11, as well as its closely related molecular cousin Rab25, are located on the tubulovesicular system which is responsible for the regulated recycling of the H/K-ATPase to the secretory canaliculus [4,9]. Since dysplasia in Barrett's epithelia appeared to correlate with a loss of apical secretory phenotype, we sought to investigate whether dysplasia alterred the expression and distribution of Rab11 [30]. We first investigated the staining for Rab11 in esophageal adencarcinoma resection specimens. These resections are especially appropriate for this analysis since they usually contain the full range of phenotypes from non-dysplastic Barrett's columnar epithelia through low and high-grade dysplasia into frank adenocarcinoma. In these resection specimens, little staining for Rab11 was oberved in the specialized intestinal cells of Barrett's mucosa. However, we observed strong staining in low-grade dysplastic cells (Figure 1). Rab11 antibodies stained large supranuclear vesicles in low-grade dysplastic cells (Figure 1). Interestingly, Rab11 staining became more diffuse or non-detectable in high-grade dysplastic cells and frank adenocarcinoma. Similar results were obtained when specimens were stained for Rab25. In contrast with Rab11 staining, staining for p53 showed significant increases in high-grade dysplasia and frank adenocarcinoma. While our data therefore suggest that elevated p53 expression (a reflection of p53 mutation) is a late finding and are similar to three previous studies [6, 13, 35], it shouldbe noted that other investigators have reported elevations in p53 in low-grade dysplasia [15, 18, 19, 24, 43]. Whether these differences reflect differences in staining technique or variations in histological grading of biopsies remains to be determined. Nevertheless, these results do indicate that Rab11 staining reflects the presence of low-grade dysplasia.

To identify better the association of Rab11 mmunoreactivity with low-grade dysplasia, 60 endoscopic biopsies were examined retrospectively for histological grading and Rab11 immunoreactivity [30]. Thirty of the cases had previously been graded as no dysplasia while 30 had been graded as low-grade dysplasia. Eighty-four percent ofbiopsies graded as low-grade dysplastic mucosae stained for Rab11 with characteristic preinuclear immunoreactivity. Twenty-four percent of non-dysplastic biopsy samples displayed perinuclear staining. While the staining in non-dysplastic cells was less intense than that in low-grade dysplastic regions, definite punctate perinuclear immunoreactivity could be detected in histologically non-dysplastic biopsies. Interestingly, the three blinded coders disagreed on the histological grading of 17% of the biopsies, while they disagreed on the Rab11 staining interpretation of only one out of the 60 biopsies. Thus, Rab11 staining appears to be less susceptible to interobserver interpretation. It is also impertant that 2 biopsies that were graded as low-grade dysplasia by all three graders did not display Rab11 immunostaining. Our results therefore suggest that Rab11 immunostaining may provide a reliable indicator of low-grade dysplasia.

DISTRIBUTION OF RAB11 IN LOW-GRADE DYSPLASIA: IMPLICATIONS FOR TRAFFICKING

The pattern of Rab11 immunostaining in low-grade dysplastic cells was considerably different from the subapical staining pattern that we had observed in most epithelial cells [8]. Haggitt and colleagues [20] have previously noted significant ultrastructural changes in low-grade dysplastic cells. Notably, using electron microscopy, they identified the presence of large perinuclear vacuoles in low grade dysplastic cells. Importantly, high-grade dysplastic cell and adenocarcinoma cells appeared to lose this perinuclear vacuole [20]. The electron microscopic description of a perinuclear vacuole was similar to the pattern which we observed for both Rab11 and Rab25 immunostaining. We therefore sought to identify the compartment involved in the formation of the perinuclear vacuole by staining with antibodies against two proteins associated with the trans-Golgi apparatus, the mannose-6-

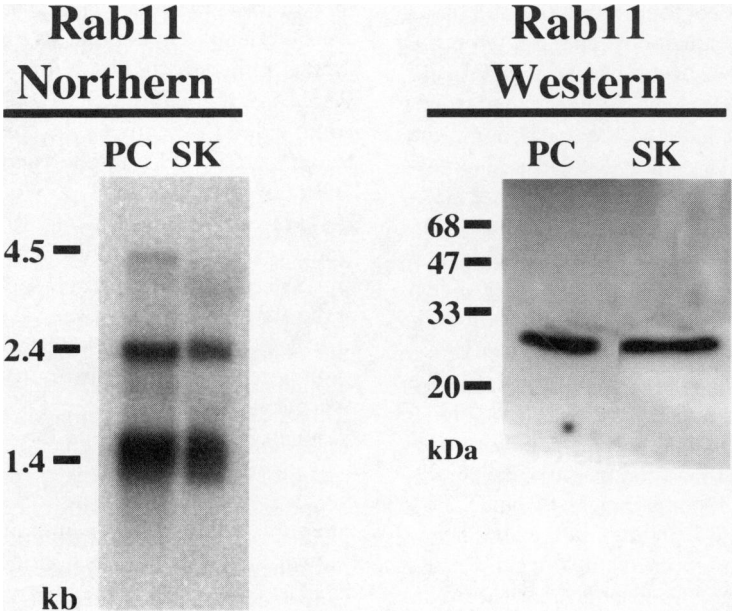

Figure 2. Rab11 expression in SKGT-4 cells. SKGT-4 cells (a kind gift of Dr. A. Albino, Memorial Sloan-Kettering Medical Center) were cultured in 20 percent fetal calf serum in DMEM-F12 on tissue culture plastic for 14 days. Cells from two T75 flasks were used to prepare either total RNA by standard RNAZOL extraction or protein following dissolution in 1.5 percent SDS. At left 10 µg of rabbit parietal cell total RNA (PC) and 20 µg of SKGT-4 total RNA were resolved on 1.2 percent agarose formaldehyde gels and transferred to Magnagraph membrane. The northern blot was then probed under moderate stringency (1X SSPE, 55 C) with full length rabbit Rab11 cDNA probe. Labeled bands were visualized on a Phosphorimaging screen (Molecular Dynamics). The image demonstrates the presence of Rab11 mRNA in SKGT-4 cells. At right, 25 µg of rabbit parietal cell protein and 100 µg of SKGT-4 protein were resolved on 15 percent SDS-PAGE gels and transferred to Immobilon-P membrane. The western blot was then probed with anti-Rab11 antibodies (1:1 dilution of monoclonal culture supernate with 5 percent non-fat dry milk in Tris-buffered saline) overnight at room temperature (8). Specific labeling was visualized by incubation with horseradish peroxidase-conjugated goat-anti-mouse-IgG and enhanced chemiluminescence detection with Supersignal reagent (Pierce). These results demonstrate the presence of prominent amounts of Rab11 protein in SKGT-4 cells.

phosphate receptor and γ-adaptin, a component of the AP-1 adapter complex. Both mannose-6-phosphate receptor and γ-adaptin colocalized with Rab11 in the perinucelar vacuoles associated with low-grade dysplasia. Importantly, immunostaining with both of the Golgi markers was also markedly increased in the perinuclear vacuole compared with both non-dysplastic and high-grade dysplastic cells. These results suggested that the vacuolar compartment in low-grade dysplasia was likely a swollen trans-Golgi compartment. Thus, the observed increase in staining for both Rab11 and Rab25 as well as the Golgi markers could result from an apical secretory blockade. Alternatively, since Rab11 is associated with apical and plasma membrane recycling systems [7, 10, 42], the expanded vacuole could represent an expanded apical recycling system. Such a blockade of either apical trafficking or apical recycling might be expected for a cell which is in the process of transition from an apically secreting mucous cell to a less differentiated dysplastic cell. The decrease in staining with these markers in high-grade dysplasia would then reflect of release of blockade at the trans-Golgi or recycling system level in a cell that is now trafficking membrane in a less polar fashion, reflective of dysplasia.

RAB11 EXPRESSION IN SKGT-4 ESOPHAGEAL ADENOCARCINOMA CELLS

Our investigations in archival tissue samples suggested that low-grade dysplastic cells within Barrett's epithelia demonstrated a trafficking defect causing an accumulation of Rab11 within a perinuclear vacuolar compartment, rather than a frank increase in protein expression. This suggested that the transition between low and high-grade dysplasia released a trafficking blockade, rather than decreasing expression. Unfortunately it is difficult to address questions of Rab11 distribution and expression in archival tissue samples. Furthermore, few cell lines have ever been developed from esophageal adenocarcinomas. Albino and colleagues developed the SKGT-4 cell line from an esophageal adenocarcinoma arising from a segment of Barrett's esophagus [1]. The cell line grows as islands of cells with heterogeneous phenotypes ranging from poorly to moderately differentiated. We studied the expression of Rab11 in SKGT-4 cells grown on tissue culture plastic to near confluence. Total mRNA from SKGT-4 cells was probed under moderate stringency with cDNA probes for the human Rab11 sequences. Figure 2 demonstrates that SKGT-4 cells express prominent levels of mRNA for Rab11. SKGT-4 cells also expressed significant amounts of Rab25 mRNA (data not shown). In addition, SKGT-4 cells demonstrated prominent amounts of immunoreactive protein for Rab11 (Figure 2). These results demonstrate that esophageal adenocarcinoma cells do express Rab11.

To investigate the localization of Rab11 within SKGT-4 cells, we studied the distribution of Rab11 using a monoclonal antibody which is specific for Rab11 [8]. We compared Rab11 immunoreactivity with that for the EGF receptor which is overexpressed in esophageal adenocarcinoma cells. In islands of moderately differentiated cells, prominent lateral membrane staining for EGF receptor was observed (Figure 3). A perinuclear nidus of punctate Rab11 staining was observed in most cells along with a more diffuse punctate pattern throughout the cytoplasm. This pattern of perinucelar staining is typical for most Rab11-expressing non-polarized cells [4, 10]. No prominent perinuclear vacuolar staining was observed. Thus, given that the SKGT-4 cell line likely is reflective of at least high-grade dysplastic cells, the expression pattern for Rab11 supports the lack of a secretory blockade in high-grade dysplastic cells. It remains to be determined whether the vacuolar staining in low-grade dysplastic cells is due to a defect in either

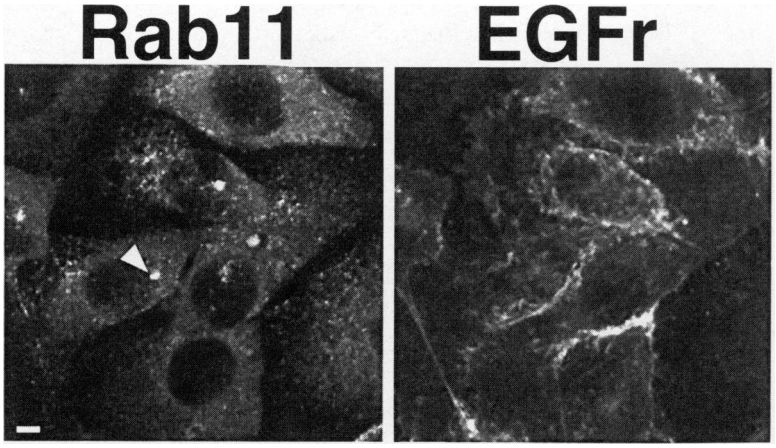

Figure 3. Rab11 immunostaining in SKGT-4 cells. SKGT-4 cells were cultured in 20% fetal calf serum in DMEM-F12 on permeable supports (Transwell-clear) for 10 to 14 days. Cells were fixed in 4% paraformaldehyde for 15 min at 4 C. For immunostaining, cells were permeabilized with 0.3% Triton X-100/5% donkey serum in phosphate buffered saline for 30 min and then incubated simultaneously with monoclonal anti-Rab11 (1:100) and goat anti-EGF receptor (EGFr; 1:100) for two hours at room temperature. Specific labeling was visualized with incubation with Cy5-donkey anti-mouse IgG and Bodipy-rabbit anti-goat IgG. Dual immunofluorescence labeling was observed on a Molecular Dynamics confocal fluorescence microscope. Images represent maximum intensity projection reconstructions of forty 0.29 μm optical sections. EGFr staining is observed primarily along the ruffled lateral borders of the cells. Most cells demonstrated a central perinuclear nidus of Rab11 staining (e.g. arrowhead) as well as a more diffuse punctate staining pattern in the cytoplasm. Bar = 2 μm.

trans-Golgi to plasma membrane trafficking or apical recycling.

THE FUTURE OF BARRETT'S BIOPSY SURVEILLANCE.

While several previous reports have highlighted the variability of histological grading based on hematoxylin and eosin staining, our results along with those of others indicate that more functional markers may provide a better method for grading Barrett's biopsies. Thus, Rab11 immunostaining may provide a functional assay of the changes in trafficking that are inherent to the changes involved in the transition to low-grade dysplasia. This functional aspect would also explain why some increases in staining can be observed in histologically non-dysplastic cells. Similarly, staining for p53 immunoreactivity may provide a functional assessment of the mutational status of higher grade dysplastic cells. Unfortunately, no large prospective study has attempted to compare all of the various markers that are available. Such a comparison is needed and might reveal important algorithms for assessing the Barrett's mucosa with multiple stains. It seems likely that such a study might be able identify parameters for the large group of patients who never show progression of their mucosa through dysplasia. Increases in the certainty of biopsy grading might therefore allow significant curtailment in the frequency of endoscopic surveillance for many patients.

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