Fascin and ezrin expression in Barrett’s esophagus progression to adenocarcinoma

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Abstract: BACKGROUND: Esophageal adenocarcinoma can arise in Barrett’s esophagus (BE) by a sequential progression from specialized intestinal metaplasia (IM) to cancer through different grades of dysplasia. To date, the morphological identification of dysplasia represents the gold standard to stratify the risk of neoplastic progression of BE patients. However, it is not a sufficiently reliable predictor of cancer risk. For this purpose, the use of molecular biomarkers has been proposed, but none of these has yet been validated. Fascin and Ezrin, two cytoskeleton-associated proteins, have shown an altered expression in several types of human cancers but have never been assessed in the progression from BE to esophageal adenocarcinoma.

AIM: To evaluate the Fascin and Ezrin expression in relation to the sequence of BE carcinogenesis.

METHODS: Esophageal paraffin sections of 33 patients with BE, including 11 patients without dysplasia (IM), 11 patients with low-grade dysplasia (LGD) and 11 patients with high-grade dysplasia (HGD), were analyzed by immunohistochemistry to assess Fascin and Ezrin expression.

RESULTS: Fascin was prevalently not expressed in IM sections (9%), and largely expressed in both LGD sections (54.5%) and HGD sections (63.6%). Fascin expression increased significantly during the sequence of BE carcinogenesis ($p = 0.021$). In IM sections, Ezrin expression was prevalently observed in cell apical membrane; in LGD sections, Ezrin was moderately present in cell cytoplasm; in HGD sections, Ezrin was intensely distributed in cell cytoplasm. Ezrin expression increased significantly during the sequence of BE carcinogenesis ($p = 0.001$).

CONCLUSIONS: In the malignant transformation progress of BE, the increased expression of both Fascin and Ezrin correlates with such a malignant progression, and Ezrin also shows a tendency to translocate from cell membrane to cytoplasm.

Keywords: Barrett’s esophagus; GERD/GORD; fascin; ezrin; esophageal cancer

Introduction

Barrett’s esophagus (BE) is an acquired condition in which any extent of metaplastic columnar epithelium with goblet cells replaces the stratified squamous epithelium that normally lines the distal esophagus. BE is a complication observed in a subset of patients with chronic gastro-esophageal reflux disease (GERD), and clinical studies demonstrate that 5-15% of individuals with GERD develop BE [1, 2]. Population-based data have shown a marked increase in the incidence of esophageal adenocarcinoma (EAC) in
Western countries in the last decades. EAC may develop from non-dysplastic metaplasia, which can progress to increasing grades of dysplasia (low-grade dysplasia, LGD, and high-grade dysplasia, HGD) and eventually to EAC [3]. Previous studies suggested that BE could represent the main risk factor for EAC [4, 5], with an estimated 30-125 fold higher risk of developing cancer, as compared with general population [6, 7]. Of note, the risk of progression to cancer increases in proportion to increasing grades of dysplasia that is from 0.5% per year for non-dysplastic metaplasia, to 13% in LGD and 40% in HGD [8, 9]. In this regard, medical and surgical therapy of GERD seems to be able to induce regression from LGD to intestinal metaplasia (IM) [10] but timing of endoscopic surveillance is a recent matter of discussion [11].

At present, it cannot be predicted which BE patients will progress to dysplasia or EAC. Therefore, an accurate detection of Barrett’s metaplasia with high malignant potential is considered to play an important role in the surveillance of BE patients. In this respect, the morphological identification of dysplasia is regarded as the gold standard to evaluate the risk of disease progression in patients with BE [1]. Nevertheless, dysplasia is not sufficiently reliable as a predictor of cancer risk for different reasons, including poor interobserver agreement among pathologists in assessing the presence of dysplasia and in grading the severity of dysplastic change [12-14]. Although biomarkers are not recommended in clinical practice neither to confirm the histologic diagnosis of dysplasia nor to predict which patients with BE are at risk of progression, it seems reasonable to assume that biomarker validation studies are paving the way towards the search for a reliable risk stratification in BE [15].

In the present study, bioptic specimens obtained from Barrett’s epithelium were examined in order to assess the tissue expression of two proteins, Fascin and Ezrin, whose altered expression has recently been involved in several types of human cancers [16, 17] but that has never been assessed in the progression from BE to EAC. Fascin was identified in the 1970s to be a 55-kD globular protein that cross-links F-actin into well-ordered and tightly packed parallel bundles that are concentrated in cell protrusions during cell migration [18]. Therefore, Fascin is normally expressed in migrating cells, such as endothelial cells and macrophages, and also in cells which form membrane protrusions and require motility, such as neurons, glial cells and dendritic cells [19]. Fascin expression is either low or absent in adult epithelia and is often up-regulated in several types of epithelial cancers [16], such as breast [20], prostate [21], esophageal squamous cell carcinoma (ESCC) [22], and pancreatic cancers [18]. Fascin overexpression is often correlated with an invasive cancer phenotype, poor prognosis and decreased disease-free survival [18].

The cytoskeleton protein Ezrin is a member of the Ezrin-Radixin-Moesin (ERM) family, which is required for epithelial cell integrity and participates in several actin-based functions such as cell adhesion [23], cell motility and morphogenesis, and also organization of the apical surface of epithelial cells [24]. Ezrin is linked to aggressive cancer behaviour by involving all stages of cancer metastasis including cell adhesion, survival, motility and signal transduction [25]. Recent studies showed that Ezrin is strongly expressed in a variety of invasive cancers, including ESCC [26], rectal cancer [27], pancreatic carcinoma [28], gastric [29] and breast cancers [30].

Based on the above consideration, the aim of this study was to evaluate the tissue expression of Fascin and Ezrin in BE biotic specimens with increasing grades of dysplasia.

Materials and methods

Patients and sample processing

A group of patients with BE, diagnosed between September 2011 and December 2011, were selected from the Endoscopic Center database of the Gastroenterology Unit in Pisa University. This approach has allowed us to select three numerically equivalent groups of patients divided on the basis of the presence and degree of dysplasia.

For all the screened patients, a medical history was recorded in order to assess their global health status. All patients signed an informed consent. All endoscopies were performed and recorded by two senior endoscopists, and videos were reviewed to reduce inter-operator variability. Endoscopies were performed with standard devices.

BE was defined as a detectable upward displacement of the squamocolumnar junction (SCJ) at endoscopy, and it was histologically confirmed by the presence of IM. SCJ was defined as the point where the normal squamous epithelium joined the red mucosa of columnar-lined esophagus. Esophageal-gastric junction (EGJ) was defined as the level at which tubular esophagus joined saccular stomach. In patients with hiatal hernia, this junction was defined by the proximal margin of gastric folds. The length of BE was measured from EGJ to the most proximal extension of columnar epithelium. Systematic 4-quadrant biopsy specimens were collected with standard-size forceps at 2-cm intervals along the whole length of the Barrett’s segment, starting from the EGJ. Agreement on the presence and extension of BE mucosa and the degree of esophagitis was obtained in all cases. BE was defined as long segment when the length of BE was greater than 3 cm, otherwise it was defined as short segment. The esophagitis, if present, was classified into four grades (from A to D) according to Los Angeles Classification [31].

Biopsies were formalin-fixed and paraffin-embedded. All biopsies were then stained with hematoxylin-eosin and evaluated twice by the same expert pathologists, blinded to all clinical data, in order to reduce the intra-observer variability. In the case of disagreement between the two
In relation to the presence and degree of dysplasia, according to the Vienna Classification [33], BE biopsic specimens were divided into three groups: IM without dysplasia, low-grade dysplasia (LGD), and high-grade dysplasia (HGD). IM was identified in case of preserved surface maturation (regenerating epithelial cells, with progressive increase in mucin content and reduction in nuclear/cytoplasmic ratio, from the bases of the glands to the mucosal surface) and absence of atypical cytological or architectural features that are characteristic of dysplasia. Slight baseline architectural distortion, such as occasional branching and budding of crypts, atrophy, irregularity, and mitotic activity (limited to the basal “regenerative” zone of the crypts and not to the surface epithelium), was considered part of this diagnostic category. LGD was characterized by enlarged, elongated, hyper-chromatic and stratified nuclei, generally confined to the basal half of the cell cytoplasm. The cytoplasm was mucin-depleted and showed an increased nuclear/cytoplasmic ratio; goblet cells were generally inconspicuous. These changes involved the crypts and surface epithelium (lack of surface maturation). Glands might demonstrate slight crowding and show other mild architectural abnormalities, such as atrophy, dilatation, and branching. HGD exhibited a greater degree of cytological and/or architectural aberration. Characteristic architectural changes included increased budding, branching, and crowding, villiform surface configuration, and the presence of intraluminal bridges or papillae. Cytological features of HGD included marked nuclear pleomorphism (variation in nuclear size and shape), loss of polarity (loss of normal nuclear orientation, in which the long axis of the nucleus is perpendicular to the basement membrane and basally oriented), and full-thickness nuclear stratification. Mitotic figures, especially atypical ones, were often present and might involve the surface epithelium.

### Immunohistochemistry

Fascin expression was assessed by immunostaining tissue sections with mouse monoclonal primary antibody directed against Fascin (55k-2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Moreover, a separate series of tissue samples was immunostained with mouse monoclonal primary antibody directed against Ezrin (3C12) (Thermo Fisher Scientific, Fremont, CA, USA). For both series of tissue sections, incubation with the primary antibody was followed by biotinylated secondary antibodies and avidin-streptavidin peroxidase complex, according to manufacturer’s protocols (i-VIEW-DAB, Ventana Medical Systems, Arizona, USA). Briefly, 4-μm paraffin-embedded sections were deparaffinized and rehydrated by standard methods. After endogenous peroxidase blocking in H2O2 at 3% and several washings with phosphate-buffered saline (PBS, pH 7.4), slices were incubated with anti-Fascin (1:100 dilution) or anti-Ezrin (1:100 dilution) primary antibody at 37°C for 32 min. Slices were washed twice in PBS, incubated with biotinylated secondary antibody for 8 min, washed and then incubated with streptavidin-horseradish peroxidase complex for 8 min. After washing, the slices were put in 3,3′-diaminobenzidine (DAB), activated with hydrogen peroxide for 8 min, then washed once again and counterstained with hematoxylin. Sections were then dehydrated and coverslipped.

### Statistical Analysis

### Table 1. Fascin expression in BE sections with increasing grades of dysplasia

<table>
<thead>
<tr>
<th>Positive cells</th>
<th>IM</th>
<th>LGD</th>
<th>HGD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10% (not-expressed)</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>0.021</td>
</tr>
<tr>
<td>&gt;10% (expressed)</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

IM: intestinal metaplasia; LGD: low-grade dysplasia; HGD: high-grade dysplasia.

### Table 2. Semi-quantitative Ezrin expression in BE sections with increasing grades of dysplasia

<table>
<thead>
<tr>
<th>Positive cells</th>
<th>Staining intensity</th>
<th>IM</th>
<th>LGD</th>
<th>HGD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10% (not-expressed)</td>
<td>0 – absent</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&gt;10% (expressed)</td>
<td>1 – faint (&lt;25%)</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>2 – weak or moderate (≥25%&lt;50%)</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 – strong (≥50%)</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

IM: intestinal metaplasia; LGD: low-grade dysplasia; HGD: high-grade dysplasia.
Results

Thirty-three patients, 23 males and 10 females, with a mean age (±sd) of 61.4 (±12.8), were enrolled in the study. Twenty-nine out of 33 patients (87.9%) were negative for esophagitis, and 4/33 patients (12.1%) were positive for it. All cases of esophagitis were grade A or B according to Los Angeles Classification. Short segment BE was detected in 15/33 patients (45.5%) and long segment BE in 18/33 (54.5%). IM was detected in 11/33 patients (33.3%), LGD in 11/33 patients (33.3%) and HGD in 11/33 patients (33.3%). No one presented EAC.

Fascin expression in tissue sections

Fascin expression (Table 1) was scored, according to the percentage of Fascin-positive cells, as follows: < 10% of positive cells was considered as a negative Fascin expression; > 10% of positive cells was considered as a positive Fascin expression.

Fascin was expressed in 1 out of 11 IM patients (9%). In particular, Fascin positivity was present only in the squamous epithelium and in some mesenchymal structures (i.e. endothelial cells, leukocytes) (Figure 1a), except that in a case in which Fascin expression resulted lightly increased in the metaplastic epithelium. In LGD sections, Fascin expression was positive in 6/11 patients (54.5%), and its positivity was never intense covering a small proportion of cell/glandular unit (Figure 1b). In HGD sections, Fascin expression was positive in 7/11 patients (63.6%), and Fascin cytoplasmic positivity was, at least focally, intense and present in a considerable proportion of cell/glandular units. (Figure 1c). Comparing the three subgroups of patients,
Fascin expression increased significantly during the sequence of BE carcinogenesis ($p=0.021$).

**Ezrin expression in tissue sections**

Ezrin cytoplasmic immunoreactivity (Table 2) was scored, according to the intensity of cytoplasm staining, as follows: 0, in case of absence of visible staining or appreciable staining in $<10\%$ of cells; 1, when a faint staining ($<25\%$) was detected in $>10\%$ of cells; 2, when weak or moderate staining ($\geq25<50\%$) was detected in $>10\%$ of cells; 3, when a strong staining ($\geq50\%$) was detected in $>10\%$ of cells.

In IM sections, Ezrin expression was absent in 3/11 patients, faint in 6/11, moderate in 2/11. Ezrin expression was never strongly present in IM sections. In LGD sections, Ezrin expression was faint in 2/11 patients, moderate in 7/11, strong in 2/11. In HGD sections, Ezrin expression was faint in 1/11, moderate in 4/11, strong in 6/11. Comparing the three subgroups of patients, Ezrin expression increased significantly during the sequence of BE carcinogenesis ($p=0.001$).

Regarding the cellular localization of Ezrin, in IM sections its expression was prevalently observed in cell apical membrane (Figure 1d); in LGD sections, Ezrin was moderately present in cell cytoplasm (Figure 1e); in HGD sections, Ezrin was intensely distributed in cell cytoplasm (Figure 1f).

**Discussion**

BE represents the only identified precursor lesion and most important risk factor for EAC [34]. The progression of BE to EAC occurs by a metaplasia-LGD-HGD-carcinoma sequence and, to date, dysplasia remains the only validated marker for risk stratification of BE patients. On the other hand, monitoring of dysplasia by endoscopic surveillance biopsies is not proven to reduce population mortality, mainly because of inter and intra-observer errors in diagnosing dysplasia [35]. In this regard, the role of biomarkers as predictors of BE progression, in adjuncts or to replace the current surveillance program for the detection of dysplasia, as well as predictors of prognosis has been assessed [36]. A recent study showed that biomarkers could represent reliable indicators of tissue homeostasis and reflect the real effectiveness of medical therapy [37]. However, their concrete validation is still needed.

The present study is the first single-centre analysis in which BE tissue samples were examined in order to assess the tissue expression of two proteins, Fascin and Ezrin, whose altered expression has been involved in several types of tumors but that has never been assessed neither in EAC nor in the progression from BE to EAC. Moreover, their expression has always been investigated separately.

Fascin expression is up-regulated in several types of epithelial cancers, and its overexpression correlates with tumor aggressiveness [16]. Based on this knowledge, we compared biopsic samples obtained from three groups of patients representing three consecutive steps of BE progression to EAC, and showed that Fascin expression increased significantly during the sequence of BE carcinogenesis. Similar to our results, Takikita et al. [22], demonstrates that Fascin was overexpressed in ESCC and its expression progressively increased across the transition from normal tissue to dysplasia to ESCC, increased Fascin expression was also associated with increased risk of both dysplasia and invasive cancer of the esophagus. Moreover, Alam et al. [38], demonstrates that Fascin overexpression in oral squamous cell carcinoma (OSCC) is correlated with stage, lymph node metastasis and poor patient survival, as a negative prognostic value, and could be also a therapeutic target in the prevention or treatment of higher grade and metastatic OSCC.

In parallel to evaluating Fascin expression, the same three groups of patients were compared to assess Ezrin expression. In this regard, our data showed that Ezrin expression increased significantly during the sequence of BE carcinogenesis. In addition, Ezrin showed a tendency to translocate from cell membrane to cytoplasm during the malignant transformation progress of BE. In keeping with our data, Ezrin has been linked to aggressive cancer behaviour by involving all stages of cancer metastasis including cell adhesion, survival, motility and signal transduction [25]. Clinical studies [39, 40] have shown that Ezrin overexpression was also correlated with adult tumor metastasis; conversely, Ezrin silencing or inhibition contributed to decrease cell motility. In a study about gastric adenocarcinoma, Jin et al. [29] found that Ezrin expression was significantly up-regulated in gastric cancers and dysplasia compared with normal gastric mucosa, although no difference was found between gastric cancer and dysplasia. In the same study, by means of immunohistochemistry, the diffusely and strongly positive signals for Ezrin protein were detected in the cytoplasm of gastric cancer cells.

In the present study, immunohistochemistry, that is considered a reliable indicator of cellular homeostasis [37, 41], has been performed to detect Fascin and Ezrin expression. Overall, for the first time, this study suggests a relevant role of both Fascin and Ezrin expression in BE malignant progression. Considering the increased expression of the two proteins during the sequence of BE carcinogenesis, their assessment could be helpful in the BE surveillance program. However, future prospective studies, by means of a proper endoscopic follow-up, are required to assess the altered expression of Fascin and Ezrin in the same patient and to validate their use for BE risk stratification. In the last year, a large group of cellular biomarkers has been evaluated to describe a step by step cancer progression [37, 41] from IM to EAC. In this context, our findings could contribute, at least in part, to better understand the
pathophysiology of BE carcinogenesis, paving the way for more accurate pathophysiological studies.

**Conflict of interests**

The authors declare no conflict of interest.

**References**


