Overexpression of Cellular Iron Import Proteins Is Associated with Malignant Progression of Esophageal Adenocarcinoma

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Overexpression of Cellular Iron Import Proteins Is Associated with Malignant Progression of Esophageal Adenocarcinoma


Abstract

Purpose: There is growing evidence that iron is important in esophageal adenocarcinoma, a cancer whose incidence is rising faster than any other in the Western world. However, how iron mediates carcinogenesis at the molecular level remains unclear. In this study, we investigated the expression of iron transport proteins involved in cellular iron import, export, and storage in the premalignant lesion Barrett’s metaplasia and esophageal adenocarcinoma.

Experimental Design: Perls’ staining was used to examine iron deposition in tissue. mRNA expression in samples of Barrett’s metaplasia matched with esophageal adenocarcinoma and samples of Barrett’s metaplasia without evidence of adenocarcinoma were examined by real-time PCR. Semi-quantitative immunohistochemistry was used to examine cellular localization and protein levels. The effect of iron loading on cellular proliferation and iron transporter expression was determined in esophageal cell lines OE33 and SEG-1 using a bromodeoxyuridine assay and real-time PCR, respectively.

Results: In the progression of Barrett’s metaplasia to adenocarcinoma, there was overexpression of divalent metal transporter 1 (DMT1), transferrin receptor 1, duodenal cytochrome b, ferroportin, and H-ferritin, and these changes were associated with increased iron deposition. Overexpression of DMT1 was further associated with metastatic adenocarcinoma. Iron loading OE33 and SEG-1 cells caused increased cellular proliferation, which was associated with increased H-ferritin and decreased transferrin receptor 1 and DMT1 expression.

Conclusions: Progression to adenocarcinoma is associated with increased expression of iron import proteins. These events culminate in increased intracellular iron and cellular proliferation. This may represent a novel mechanism of esophageal carcinogenesis.

Esophageal adenocarcinoma is a cancer whose incidence has seen an unprecedented increase in the last 30 years with no apparent evidence of a downturn (1, 2). The prognosis for patients with esophageal adenocarcinoma remains extremely poor with 5-year survival rates approximating 10% in most Western populations (3). The strongest known risk factor is Barrett’s metaplasia, a condition characterized by the replacement of native squamous esophageal mucosa with a columnar epithelium, probably as a consequence of prolonged reflux of gastric contents into the lower esophagus (1, 4). It is estimated that Barrett’s metaplasia increases the risk of developing esophageal adenocarcinoma approximately 30- to 125-fold (5, 6).

In addition to the plethora of proteins implicated in the malignant progression of Barrett’s metaplasia to adenocarcinoma, including c-MYC (7), there is evidence suggesting that iron is important in the development of esophageal cancer. The involvement of iron in esophageal carcinogenesis is exemplified by a series of studies using an animal model in which rats underwent an esophagoduodenal anastomosis to mimic reflex (10–12). Rates of esophageal adenocarcinoma 30 weeks after surgery were ~10-fold higher in animals supplemented with iron than in nonsupplemented animals. The cellular role of iron in carcinogenesis has been attributed to its potential to induce reactive oxygen species, DNA adducts, and cellular proliferation and repress cell adhesion (15–18).

Iron is principally absorbed in the duodenum where dietary ferric iron is reduced and transported into the enterocyte by the proteins duodenal cytochrome b (DCYTB) and divalent metal transporter 1 (DMT1), respectively (19–21). The iron can then be stored in ferritin, and cellular proliferation and adhesion (15–18).

Note: T. Iqbal and C. Tselepis contributed equally to this work.

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requires the ferroxidase hephaestin (23, 24) and the basolateral iron transporter ferroportin (25). Iron is transported in the serum bound to transferrin, which interacts with transferrin receptor 1 (TIR1) on the plasma membrane of cells that take up iron. The iron/transferrin complex is internalized by receptor-mediated endocytosis and iron released (26). In esophageal keratinocytes, the main route of iron acquisition is likely to be through the transferrin receptor–mediated endocytic pathway. Luminal iron absorption in the esophagus is highly unlikely.

A role for iron in carcinogenesis is further strengthened by our own studies showing that in the human colonic adenoma to carcinoma sequence there is a modulation in the expression of iron transport proteins, particularly an overexpression of DCYTB, DMT1, and TIR1 and a reduction in hephaestin (15). These changes culminate in increased intracellular iron import and reduced iron efflux leading to increased intracellular iron, which we believe is crucial in driving Wnt signaling, a common oncogenic signaling pathway (27).

Our hypothesis is that the modulation of iron transport proteins, culminating in cellular iron accumulation, represents a mechanism of carcinogenesis central to all cancers. Thus, the primary aim of this study was to characterize the expression of the iron import (DCYTB, DMT1, and TIR1), export (ferroportin and hephaestin), and storage (H-ferritin) proteins in normal esophagus, Barrett’s metaplasia, and esophageal adenocarcinoma and to further determine the effect of increased intracellular iron on esophageal cellular proliferation and the expression of iron transport proteins.

Materials and Methods

Ethics
This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Ethical approval for this study was approved by University Hospital Birmingham Trust (LREC 2002/166). All patients provided informed written consent. Immunohistochemical studies using the tissue microarrays were approved by the South Sheffield Research Ethics Committee (02/155).

Patient tissue
Esophageal adenocarcinoma resection specimens. Samples of esophageal adenocarcinoma (n = 29), some of which were matched with Barrett’s metaplasia (n = 11) from the same resection specimen, were collected during surgery and each tissue specimen was divided in two for RNA extraction and pathologic confirmation.

Endoscopic specimens. Samples of long-segment (≥2 cm) Barrett’s metaplasia (n = 13), defined as columnar mucosa with intestinal-type goblet cells, with matched normal gastric fundal mucosa and esophageal squamous mucosa from the same patient were collected during endoscopy. Each tissue specimen was divided in two for RNA extraction and immunohistochemistry. Any patients with Barrett’s metaplasia with evidence of dysplasia or adenocarcinoma were excluded from this study.

Archived tissue. Paraffin sections of normal esophagus (n = 10), Barrett’s metaplasia from patients with no evidence of esophageal dysplasia or adenocarcinoma (BM-ve; n = 20), Barrett’s metaplasia with adenocarcinoma in the same section (BM+ve; n = 20), Barrett’s with low-grade dysplasia (n = 10), Barrett’s with high-grade dysplasia (n = 20), and esophageal adenocarcinoma (n = 20) were identified within the archived tissue bank, Department of Pathology, Queen Elizabeth’s Hospital Birmingham, and processed for immunohistochemistry.

Real-time PCR
Real-time PCRs were done using 18S rRNA as an internal standard (PE Biosystems, Roche) and primers to DMT1 [iron response element (IRE)-containing isoform], TIR1, DCYTB, ferroportin, hephaestin, H-ferritin, and MYC as previously described (7, 15). All analyses were done on the same pool of CDNA samples.

Immunohistochemistry
Immunohistochemistry was done as previously described (15) using microwave antigen retrieval and rabbit polyclonal antibodies to (a) DCYTB (1:200, clone 834; a kind gift from Prof. A. McKie), (b) DMT1 (1:3,000; ADI), (c) ferroportin (1:200, clone 3566; a kind gift from Prof. A. McKie), (d) H-ferritin (1:1,000, F-5012; Sigma), and (e) hephaestin (1:50, HEPH11-A; ADI) or a mouse monoclonal antibody to TIR1 (1:40, clone 10F11; Novocastra). Small bowel mucosa was included as a positive control, and omission of primary antibody and the use of rabbit serum alone and isotype controls were all used as negative controls. In addition, cellular iron localization was assessed by 3,3′-diaminobenzidine–enhanced Prussian blue staining as previously described (15). The slides were scored by a previously described method for intensity of staining and percentage of epithelial cell staining (28). All sections were scored independently by three observers (S.H., K.R., and C.T.).

Tissue microarrays
A separate set of esophageal carcinoma specimens (n = 76) collected between 2000 and 2005, for which clinicopathologic data were available, was used to evaluate the association between iron transporter levels and prognostic factors. The prognostic factors included T stage, differentiation status, vascular invasion, and nodal involvement. Each tumor is represented on the microarray in triplicate to provide representative sampling of the source tissue. Immunohistochemistry and scoring were done as described above.

Western blotting
Western blotting was done as described previously with antibodies to either ferroportin (1:1,000) or H-ferritin (1:2,500; ref. 15). A mouse monoclonal antibody to cytokeratin 19 (1:2,000, cloneA53-B/A2.26; Merck Chemicals Ltd.) was used for normalization of epithelial protein

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean fold change in mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BM-ve vs G</td>
</tr>
<tr>
<td>TIR1</td>
<td>1.87</td>
</tr>
<tr>
<td>DMT1(IRE+ve)</td>
<td>1.79*</td>
</tr>
<tr>
<td>DCYTB</td>
<td>1.09</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>2.95</td>
</tr>
<tr>
<td>Hephastin</td>
<td>1.96</td>
</tr>
<tr>
<td>H-ferritin</td>
<td>2.37</td>
</tr>
</tbody>
</table>

NOTE: Mean fold changes in mRNA levels of TIR1, DMT1, DCYTB, ferroportin, hephaestin, and H-ferritin in samples of (a) intestinal-type Barrett’s metaplasia (with no evidence of associated adenocarcinoma) compared with matched gastric mucosa, (b) intestinal-type Barrett’s metaplasia compared with matched squamous esophageal mucosa, and (c) esophageal adenocarcinoma matched with Barrett’s metaplasia obtained from the same resection specimen as the comparative control were determined. Abbreviations: ADC, adenocarcinoma; G, gastric mucosa; S, normal squamous esophagus.

*Significant differences in mean mRNA expression between the two mucosa (P < 0.05).

Table 1. Analysis of mRNAs encoding iron transport proteins in Barrett’s metaplasia and adenocarcinoma

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loading. Immunoreactive bands were then subjected to densitometry using NIH Image 1.62 software.

**Cell culture**

Cell lines derived from esophageal adenocarcinomas [OE33 (29) and SEG-1 (30)] were routinely cultured in DMEM (Life Technologies) with 10% FCS supplemented with 100 units/mL penicillin and 0.1 mg/mL streptomycin. On reaching 70% confluence, lines were challenged with either growth medium alone (control) or iron loading medium (growth medium supplemented with 100 μmol/L FeSO₄ and 10 μmol/L sodium ascorbate) for between 1 and 24 h. FeSO₄ (100 μmol/L) was chosen as this source of iron and concentration has been used in previous studies (15, 27). Furthermore, this concentration of iron was shown to be optimal for esophageal cellular proliferation (data not shown). At the end of the time period, cells were used for intracellular iron determination, RNA extraction, or cell proliferation assays. All experiments were done in triplicate on three separate occasions.

**Bromodeoxyuridine incorporation assay**

A colorimetric cell proliferation ELISA was done according to the manufacturer's instructions (Roche Applied Science). Briefly, cells were labeled with bromodeoxyuridine followed by fixation and incubation with anti-bromodeoxyuridine peroxidase, and the immune complex was then detected using a 3,3',5,5'-tetramethylbenzidine substrate with the reaction product assessed at 370 nm.

**Ferrozine assay**

Nonhaem iron was assayed as previously described (15) and cellular iron content was expressed as nmoles of iron per mg protein. Protein concentrations were assessed by the bicinchoninic acid protein assay according to the manufacturer's instructions (Pierce).

**Statistics**

All data are presented as mean ± 1 SE. Statistical significance was calculated using Mann-Whitney tests for analysis of immunohistochemical staining, paired t tests for mRNA analysis where multiple samples were obtained from individual patients, and unpaired Student’s t tests for analysis of the in vitro experiments. To assess the association between c-MYC and iron transport proteins, linear regression analysis was done on log-transformed mRNA fold change values, and the $R^2$ value was determined to show the extent to which expression of TfR1 and H-ferritin was correlated to MYC (31). A Bonferroni adjustment was applied to $P$ values attained from multiple comparisons during the analysis of prognostic factors (32).
univariate analyses, significance was accepted at $P \leq 0.05$ following Bonferroni adjustment, and $P \leq 0.0083$ was considered significant. All analyses were done using Statistical Package for the Social Sciences version 10.0 (SPSS, Inc.).

### Results

**mRNA expression of iron metabolism molecules in Barrett’s metaplasia and esophageal adenocarcinoma specimens**

The expression of mRNAs encoding TR1, DMT1, DCYTB, ferroportin, hephaestin, and H-ferritin in benign Barrett’s metaplasia (BM-ve) was initially assessed and compared with internal matched gastric and esophageal squamous control tissue. Relative to squamous mucosa, the mRNAs encoding all iron metabolism proteins studied were increased in Barrett’s metaplasia (Table 1). When comparing intestinal Barrett’s metaplasia with matched gastric mucosa, only DMT1 was significantly altered (Table 1). To determine if there was any further change in these transcripts in esophageal adenocarcinoma, matched internal Barrett’s metaplasia (BM+ve) obtained from the same resection specimen was used as the comparative control. We show that the expression levels of all transcripts are statistically higher in esophageal adenocarcinoma than the associated Barrett’s metaplasia, with the exception of hephaestin (Table 1). In terms of transcript levels in adenocarcinoma, the highest to lowest were H-ferritin (dCt = 9.08 ± 0.13) > ferroportin (dCt = 14.06 ± 0.31) > TR1 (dCt = 15.18 ± 0.19) > DCYTB (dCt = 16.28 ± 0.31) > hephaestin (dCt = 16.35 ± 0.32), with the least expressed being DMT1 (dCt = 17.46 ± 0.25).

Because we had previously shown that the oncogene c-MYC was overexpressed in esophageal adenocarcinoma and that previous reports have suggested that c-MYC can induce TR1 and repress H-ferritin, we chose to examine the transcriptional relationship between MYC and TR1, and MYC and H-ferritin expression in all the esophageal adenocarcinoma samples ($n = 29$). Using linear regression analysis on log-transformed mRNA expression values, we were able to show that MYC mRNA expression in esophageal adenocarcinoma specimens was positively associated with TR1 ($R^2 = 0.203; P = 0.014$) and also with H-ferritin mRNA expression ($R^2 = 0.144; P = 0.042$).

![Fig. 2. Immunolocalization of proteins implicated in cellular iron export and storage in normal esophagus, Barrett’s metaplasia, and esophageal adenocarcinoma. Paraffin sections of normal squamous esophagus, Barrett’s metaplasia, and esophageal adenocarcinoma were subjected to immunohistochemistry using antibodies to ferroportin (FPN), hephaestin (HEPH), and H-ferritin. Dashed line, basal squamous layer-connective tissue interface; arrows, areas of positivity. Original magnification, ×40.](image-url)
However, there was no association between MYC and TJR1 or between MYC and H-ferritin in Barrett’s metaplasia specimens (n = 24).

**Immunolocalization of iron metabolism proteins in archived tissue**

To complement the mRNA analyses, we did semiquantitative immunohistochemistry on archived paraffin tissue specimens from patients with a range of esophageal pathologies (Figs. 1 and 2). In addition to cellular localization being determined, all staining was scored (Table 2) as described above.

**Iron import proteins.** In normal esophagus, DCYTB was strongly expressed in the basolateral layer of the stratified epithelium, with immunoreactivity in both the cytoplasm and on the plasma membrane. In Barrett’s metaplasia, DCYTB immunoreactivity was predominantly localized on the villous tips with decreasing reactivity in the deeper lying crypts. Immunoreactivity was predominantly supranuclear and vesicular. This pattern of cellular localization was retained in low- and high-grade dysplasia and in esophageal adenocarcinoma (Fig. 1). However, there was significantly higher DCYTB immunoreactivity in high-grade dysplastic Barrett’s metaplasia and also in adenocarcinoma compared with nondysplastic Barrett’s metaplasia (P = 0.045 and 0.0001, respectively; Table 2).

No discernible DMT1 immunoreactivity was observed in normal esophageal epithelium unlike in Barrett’s metaplasia where staining was evident on the apical surface epithelium. However, no immunoreactivity was observed in the deeper lying crypts. In the majority of columnar cells with low- and high-grade dysplasia, there was cytoplasmic staining, which became more widespread in adenocarcinoma (Fig. 1). Semiquantitative analysis showed a statistical increase in DMT1 immunoreactivity in esophageal adenocarcinoma compared with nondysplastic Barrett’s metaplasia (P = 0.001; Table 2).

TIR1, like DMT1, was not detected in normal esophagus. In Barrett’s metaplasia, there was lateral membranous immunoreactivity in all columnar cells, whereas in high-grade dysplastic Barrett’s metaplasia and in adenocarcinoma there was an abundance of both membranous and cytoplasmic immunoreactivity (Fig. 1). Semiquantitative analysis revealed that there was significantly higher TIR1 immunoreactivity in high-grade dysplastic Barrett’s metaplasia and also in adenocarcinoma compared with nondysplastic Barrett’s metaplasia (P = 0.0001 and 0.0001, respectively; Table 2).

**Iron export and storage proteins.** Neither ferroportin nor hephaestin was detected in normal squamous mucosa but there was evidence of immunoreactivity for both proteins in Barrett’s metaplasia. Localization of ferroportin was mostly supranuclear on the surface epithelium with diminishing reactivity down into the crypts. Hephastaen was localized in a diffuse cytoplasmic manner throughout all columnar cells with immunoreactivity more intense toward the basal pole of the columnar cells. In adenocarcinoma, whereas there was an abundance of discrete supranuclear and vesicular ferroportin immunoreactivity, there was only weak diffuse immunoreactivity for hephaestin (Fig. 2). Semiquantitative analysis showed that ferroportin was overexpressed in adenocarcinoma compared with nondysplastic

### Table 2. Semiquantitative analysis of iron transport proteins in the malignant progression of Barrett’s metaplasia to adenocarcinoma

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mean fold change in protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADC vs BM+ve</td>
</tr>
<tr>
<td>TIR1</td>
<td>5.07*</td>
</tr>
<tr>
<td>DMT1 (IR+ve)</td>
<td>7.06*</td>
</tr>
<tr>
<td>DCYTB</td>
<td>7.72*</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>2.80*</td>
</tr>
<tr>
<td>H-ferritin</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>3.38*</td>
</tr>
</tbody>
</table>

NOTE: Immunohistochemistry for TIR1, DMT1, DCYTB, ferroportin, hephaestin, and H-ferritin was done on archived paraffin tissue specimens of (a) intestinal-type Barrett’s metaplasia associated with adenocarcinoma (n = 20), (b) Barrett’s metaplasia with low-grade dysplasia (n = 10), (c) Barrett’s metaplasia with high-grade dysplasia (n = 20), and (d) esophageal adenocarcinoma (n = 20). All slides were quantitated as described in Materials and Methods. The mean staining scores were calculated for each type of mucosa and the mean fold change in protein expression was calculated. Abbreviations: LGD, low-grade dysplasia; HGD, high-grade dysplasia.

*Significant differences between the two groups being compared (P < 0.05).

### Table 3. Semiquantitative analysis of immunoreactivity of iron transport proteins in an esophageal adenocarcinoma tissue array

<table>
<thead>
<tr>
<th>Protein</th>
<th>T stage</th>
<th>Differentiation</th>
<th>Vascular invasion</th>
<th>Nodal involvement</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>T2</td>
<td>T3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Well</td>
<td>Moderate</td>
<td>Poor</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMT1</td>
<td>2.5</td>
<td>3.02</td>
<td>2.38</td>
<td>2.77</td>
</tr>
<tr>
<td>DCYTB</td>
<td>4.97</td>
<td></td>
<td></td>
<td>4.78</td>
</tr>
<tr>
<td>TIR1</td>
<td>5.06</td>
<td>9.04</td>
<td>7.4</td>
<td>7.84</td>
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<tr>
<td>H-ferritin</td>
<td>1.65</td>
<td>2.75</td>
<td>1.66</td>
<td>2.43</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>2.99</td>
<td>2.66</td>
<td>2.33</td>
<td>2.7</td>
</tr>
<tr>
<td>H-phaestin</td>
<td>0.14</td>
<td>0.3</td>
<td>0.11</td>
<td>0.24</td>
</tr>
</tbody>
</table>

NOTE: Esophageal adenocarcinomas (n = 76) for which complete clinical data were available (T stage, differentiation, extent of vascular invasion, and nodal involvement) were used to evaluate the association between iron transporter levels and these prognostic factors. Immunohistochemistry was done with antibodies to DCYTB, DMT1, ferroportin, H-ferritin, TIR1, and hephaestin. Staining scores were calculated as described in Materials and Methods. Increased expression of DMT1 was associated with positive nodal involvement.

*Following application of the Bonferroni adjustment for multiple comparisons, differences in staining intensity between tumors with or without the specified characteristic were considered significant at P ≤ 0.0083.
Barrett’s metaplasia \( (P = 0.0002) \), whereas hephaestin expression showed no statistical difference in expression when compared with nondysplastic Barrett’s metaplasia.

The storage protein H-ferritin was expressed in normal squamous esophagus in a distribution similar to that of DCYTB. Immunoreactivity was mostly confined to the basal layer and was predominantly cytoplasmic in all columnar cells. In nondysplastic and dysplastic Barrett’s metaplasia, weak diffuse immunoreactivity was observed in all cellular compartments, whereas in adenocarcinoma there was an abundance of cytoplasmic immunoreactivity (Fig. 2). Semiquantitative analysis showed that H-ferritin was overexpressed in adenocarcinoma compared with nondysplastic Barrett’s metaplasia \( (P = 0.04; \text{Table 2}) \).

Thus, in the progression from normal esophagus through Barrett’s metaplasia to adenocarcinoma, there is an overexpression of iron import proteins \( (\text{DCYTB, DMT1, and TfR1}) \), iron export protein \( (\text{ferroportin}) \), and iron storage protein \( (\text{H-ferritin}) \). However, there was no alteration in hephaestin expression.

**Tissue microarray.** We further sought to determine whether changes in protein expression were associated with prognostic factors, such as stage of disease and nodal involvement, by using a tissue array of esophageal adenocarcinomas with known outcome (Table 3). The tissue microarray analysis showed no significant changes in staining intensity for any of the iron transport proteins analyzed with respect to T stage, tumor differentiation status, presence of vascular invasion, age, or gender. However, DMT1 expression was significantly associated with lymph node involvement \( (P = 0.0068) \).

**Enhanced Perls’ Prussian blue staining.** To determine whether these changes in expression and localization of the iron metabolism proteins were associated with increased intracellular iron, we did enhanced Perls’ Prussian blue staining on tissue sections. In normal squamous esophagus \( (n = 20) \), there was no discernible staining other than in occasional basal keratinocytes, whereas in Barrett’s metaplasia specimens \( (n = 20) \) there was evidence of diffuse cytoplasmic staining in approximately half of the specimens examined. In the majority of esophageal adenocarcinoma specimens \( (n = 20) \), there were discrete areas of iron staining that seemed to be both nuclear and cytoplasmic in localization (Fig. 3).

**The effect of increased intracellular iron levels on cellular proliferation.** To determine the effect of iron loading on cell behavior, we experimentally iron loaded the esophageal adenocarcinoma–derived cell lines OE33 and SEG-1. Cells were exposed to iron for 24 h and cellular iron content and cellular proliferation were assessed using standard ferrozine and bromodeoxyuridine assays, respectively (Fig. 4A and B). Following 24 h of culture under iron-loading conditions, both OE33 and SEG-1 showed significantly increased intracellular iron \( (P = 0.001; \text{mean, 0.45 and 0.20 nmol/mg protein}) \).

![Fig. 3. Elevated 3,3’-diaminobenzidine – enhanced Prussian blue staining in esophageal adenocarcinoma. Sections of normal squamous esophagus, intestinal-type Barrett’s metaplasia, and esophageal adenocarcinoma were subjected to 3,3’-diaminobenzidine – enhanced Perls’ Prussian blue staining. A section of liver from a patient with hereditary hemochromatosis was used as a positive control. Original magnifications, ×20 and ×40.](https://www.aacrjournals.org/clin-cancer-res/article-pdf/14/2/384/872476/clin-cancer-res-2008-1054.pdf)
respectively; Fig. 4A). This increased intracellular iron was associated with a significant increase in cellular proliferation compared with control non–iron-loaded OE33 and SEG-1 cells (P = 0.001; Fig. 4B).

**The effect of increased intracellular iron levels on iron transporter expression.** The effect of increased cellular iron on the mRNAs encoding the iron metabolism proteins was also examined in esophageal adenocarcinoma cell lines (Fig. 5). In both OE33 and SEG-1 cells, the profile of transcript expression in untreated cells was comparable with that observed in adenocarcinoma tissue, with the most highly expressed transcript being H-ferritin. Iron loading both OE33 and SEG-1 cells resulted in significant reductions in both Tfr1 and DMT1 mRNA expression (Fig. 5A) as expected because both mRNAs contain IREs within their 3′ untranslated regions (33, 34). No change in DCYTB, DMT1, hephaestin, or H-ferritin mRNA levels was observed (Fig. 5A and B). At the protein level, there was a significant increase in H-ferritin expression following iron loading in both cell lines (Fig. 6A). Interestingly, ferroportin was also significantly repressed in SEG-1 cells (Fig. 6B).

**Discussion**

In this study, we investigated, for the first time, the expression of iron metabolism proteins in the evolution of esophageal adenocarcinoma. In the examination of Barrett’s metaplastic tissue, it was evident that there was expression of these iron metabolism proteins consistent with an intestine-like phenotype. When comparing Barrett’s metaplasia with esophageal squamous control, significant expression changes in all the iron metabolism proteins were observed, which we believe reflect the fact that we are comparing different types of mucosa, intestinal columnar mucosa with a stratified squamous mucosa.

During the progression from Barrett’s metaplasia to adenocarcinoma, we showed that components of the main cellular iron import machinery (Tfr1, DMT1, and DCYTB) were overexpressed, as was the iron storage protein H-ferritin. Surprisingly, the major iron export protein ferroportin was also overexpressed under these conditions, although it seemed to be cytoplasmic in localization, suggesting a lack of functionality. These data are comparable with our previous findings in colorectal carcinogenesis (15).

Using a semiquantitative method, we further showed that these changes in iron transport proteins were not just evident in late-stage disease but that two of the proteins, Tfr1 and DCYTB, were induced in the transition from low- to high-grade Barrett’s dysplasia. Akin to our previous data in colon carcinogenesis, DMT1 overexpression was associated with more aggressive disease, as shown by the correlation of DMT1 expression and...
nodal metastasis. Whether changes in iron transport proteins can drive metastasis is unclear; however, it is possible that elevated intracellular iron caused by increased expression of iron import protein DMT1 mediates a repression in the cell adhesion molecule E-cadherin, a molecule that is commonly silenced in cellular invasion and metastasis. In support of this, we have previously shown iron-mediated E-cadherin repression in a colorectal model system (15).

We believe that it is highly unlikely that the overexpression of these iron transport proteins represents increased luminal iron uptake. Rather, we predict that their overexpression, coupled to their described localization, in adenocarcinoma suggests that the main route of iron acquisition is through transferrin receptor–mediated endocytosis. The only anomaly to this is DCYTB, which has no role in transferrin receptor–mediated endocytosis. We describe it to be cytoplasmic in localization, suggesting that neither is it involved in luminal iron uptake in adenocarcinoma. However, the fact that we observed DCYTB in the very basal layer of the stratified squamous esophagus suggests that it may have a function other than in iron absorption, such as cellular proliferation.

Thus, increased transferrin receptor–mediated endocytosis in the face of a potential functional loss of iron export, as suggested by cytoplasmic localization of ferroportin, might represent a mechanism by which iron could accumulate within transferrin receptor–mediated endocytosis. The only anomaly to this is DCYTB, which has no role in transferrin receptor–mediated endocytosis. We describe it to be cytoplasmic in localization, suggesting that neither is it involved in luminal iron uptake in adenocarcinoma. However, the fact that we observed DCYTB in the very basal layer of the stratified squamous esophagus suggests that it may have a function other than in iron absorption, such as cellular proliferation.

Despite normal IRP/IRE iron sensing, the balance is skewed toward induced expression. One such factor could be c-MYC, an oncogene reported to induce TFR1 and IRP2, while repressing H-ferritin expression (31, 35). Interestingly, we previously showed c-MYC to be induced by acidified bile acids and overexpressed in the esophageal metaplasia-dysplasia-adenocarcinoma sequence (7). We compared the mRNA expression of MYC and TFR1 in both Barrett’s metaplasia and adenocarcinoma tissue and showed a positive correlation in adenocarcinoma but not in Barrett’s metaplasia. This may suggest that in adenocarcinoma the balance is skewed toward MYC-mediated TFR1 induction rather than an IRE/IRP-mediated TFR1 repression.

Iron loading esophageal adenocarcinoma cell lines resulted in the predicted reduction in TFR1 and DMT1 mRNA and increase in H-ferritin protein expression. Iron loading SEG-1 cells also resulted in a significant reduction in ferroportin protein expression. Because DMT1 has a 5’ IRE, increasing cellular iron would be expected to result in increased ferroportin expression as reported in some cell types, such as macrophages (36). However, our data are consistent with other studies that suggest that iron does not regulate intestinal ferroportin expression in an IRE-predicted manner. This is exemplified by several studies showing elevated ferroportin expression in the duodenum of iron-deficient mice (25, 37).

Irrespective of the molecular mechanism by which adenocarcinoma cells become iron loaded, the cellular downstream effects are likely to be multiple. In this and previous studies, iron loading is important in cellular proliferation (15, 17, 27, 36). Historically, this has been attributed to iron being crucial in the activity of ribonucleotide reductase, an iron-containing enzyme responsible for the synthesis of deoxyribonucleotides (38). However, more recently, the phosphatase CDC14A, which has been shown to directly regulate the activity of several cell cycle proteins, is an IRE-containing protein.
and thus regulated by iron (39). In addition, we have recently shown that iron can induce Wnt signaling, which culminates in oncogene activation and cellular proliferation (27). In support of a role for iron in proliferation, studies by Le and Richardson (40) have shown that reducing intracellular iron levels by chelation blocks cellular proliferation and induces differentiation through several cell cycle proteins, including NDRG1.

As well as modulating cellular proliferation, iron is likely to induce, through Fenton reaction chemistry, reactive oxygen species and oxidative stress (16). In vitro studies have clearly shown that iron can induce oxidative damage to DNA, protein, and lipid. Furthermore, murine models of esophageal adenocarcinoma suggest that iron-mediated oxidative stress in concert with reflux is central to esophageal carcinogenesis, as exemplified by marked overexpression of oxidative stress–responsive genes (9–12).

What remains unclear following this study is how these cellular and molecular alterations are linked to the well-characterized risk factors for esophageal adenocarcinoma (male sex, obesity, gastroesophageal reflux, and the presence of Barrett’s metaplasia; ref. 1). Our previous studies have shown that c-MYC can be induced by components of the refluxate (i.e., acidified bile acids; ref. 7). Thus, because c-MYC is a trigger for changes in TIR1, H-ferritin, and IRP2, this would suggest that ablating reflux may represent a strategy for reducing intracellular iron and malignant progression.

In summary, we have shown changes in iron transport proteins in the malignant progression of Barrett’s metaplasia to adenocarcinoma and speculate that this may act to increase cellular iron acquisition. Whether this represents an essential molecular aberration in esophageal carcinogenesis or whether it reflects the increased metabolic rate and mitotic activity of cancer tissue requires delineation.

References
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