

COLORECTAL CANCER

Modulation of iron transport proteins in human colorectal carcinogenesis

M J Brookes, S Hughes, F E Turner, G Reynolds, N Sharma, T Ismail, G Berx, A T McKie, N Hotchin, G J Anderson, T Iqbal, C Tselepis



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See end of article for authors' affiliations

Correspondence to:
Dr C Tselepis, Cancer
Research UK Institute for
Cancer Studies, University
of Birmingham, Vincent
Drive, Birmingham B15
2TH, UK;
c.tselepis@bham.ac.uk

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Background and aims: Total body iron and high dietary iron intake are risk factors for colorectal cancer. To date there is no comprehensive characterisation of iron transport proteins in progression to colorectal carcinoma. In this study, we examined expression of iron import (duodenal cytochrome b (DCYTB), divalent metal transporter 1 (DMT1), and transferrin receptor 1 (TfR1)) and export (hephaestin (HEPH) and ferroportin (FPN)) proteins in colorectal carcinoma.

Methods: Perl's staining was used to examine colonocyte iron content. Real time polymerase chain reaction (PCR) and western blotting were used to examine mRNA and protein levels of the molecules of interest in 11 human colorectal cancers. Semiquantitative immunohistochemistry was used to verify protein levels and information on cellular localisation. The effect of iron loading on E-cadherin expression in SW480 and Caco-2 cell lines was examined by promoter assays, real time PCR and western blotting.

Results: Perl's staining showed increased iron in colorectal cancers, and there was a corresponding overexpression of components of the intracellular iron import machinery (DCYTB, DMT1, and TfR1). The iron exporter FPN was also overexpressed, but its intracellular location, combined with reduced HEPH levels, suggests reduced iron efflux in the majority of colorectal cancers examined. Loss of HEPH and FPN expression was associated with more advanced disease. Iron loading Caco-2 and SW480 cells caused cellular proliferation and E-cadherin repression.

Conclusions: Progression to colorectal cancer is associated with increased expression in iron import proteins and a block in iron export due to decreased expression and aberrant localisation of HEPH and FPN, respectively. This results in increased intracellular iron which may induce proliferation and repress cell adhesion.

There is an emerging body of evidence implicating iron in the malignant progression of epithelial cancers, including those of the breast, liver, and colon.^{1–2} Colorectal cancer (CRC) is the third most common cancer in the US with over 106 000 new cases and 56 000 estimated deaths from colon cancer in 2004.³

Evidence of a role for iron in CRC comes from epidemiological, animal, and cellular studies.^{4–7} Numerous human epidemiological studies have examined the relationship between dietary iron, body iron stores, and CRC. One detailed analysis of 33 epidemiological studies by Nelson revealed that, among the larger studies, approximately 75% supported the association of iron with CRC risk.⁴ A more recent study suggested that a stronger association of iron and CRC risk is evident when there is elevated total body iron and a high dietary iron intake,⁸ and an important large epidemiological study from Europe has recently demonstrated a convincing link between red meat intake and CRC risk.⁹ Further epidemiological evidence for a role of iron in CRC study comes from the observation that patients with *HFE* mutations have an increased risk of CRC and this is exacerbated by high dietary iron intake.¹⁰ In a normal individual, the amount of iron required to meet metabolic needs, and hence the amount absorbed, is usually no more than 10% of the amount of iron ingested. Consequently, high levels of iron have been reported within the colonic lumen.^{10–11} It is also clear from a host of animal models that when high iron diets are administered along with a carcinogen, such as dimethylhydrazine or cyclic dextran sodium sulphate, colorectal tumour incidence and tumour multiplicity are increased.^{6–12–13}

Recently, the main proteins involved in the absorption of non-haem iron have been identified.¹⁴ Dietary ferric iron is

reduced to ferrous iron by duodenal cytochrome b (DCYTB), which is highly expressed on the brush border membrane of enterocytes.¹⁵ Ferrous iron is then transported into the enterocyte by divalent metal transporter 1 (DMT1, also known as NRAMP2/DCT1).^{16–17} Once in the enterocyte, iron has one of three fates: (i) it can be immediately utilised in the many cellular processes for which it is essential; (ii) it can be stored in an inert form bound to ferritin¹⁸; or (iii) it may be exported out of the enterocyte via a pathway which requires the ferroxidase hephaestin (HEPH),^{19–20} and the basolateral iron transporter ferroportin (FPN, also termed IREG1 and metal transporter protein 1).²¹ Following export, iron is transported in serum bound to transferrin (Tf) which interacts with transferrin receptor 1 (TfR1) on the plasma membrane of cells which take up iron. The iron/Tf complex is internalised by receptor mediated endocytosis and iron is released from transferrin by a mechanism requiring endosomal acidification.²²

As iron is a prerequisite for cell cycling, it is not surprising that neoplastic cells and other rapidly dividing cells express high levels of TfR1, and that iron withdrawal or antisense TfR1 oligonucleotide treatment causes inhibition of cell cycling.^{23–24}

It is established that TfR1 is expressed in colonocytes and overexpressed in CRC and is likely to play a role in the iron

Abbreviations: CRC, colorectal cancer; DCYTB, duodenal cytochrome b; DMT1, divalent metal transporter 1; TfR1, transferrin receptor 1; HEPH, hephaestin; FPN, ferroportin; PCR, polymerase chain reaction; CK, cytokeratin; PBS, phosphate buffered saline; DAB diaminobenzidine; LGD, low grade dysplastic adenomas, HGD, high grade dysplastic adenomas

nutrition of these cells.²⁵ Other iron transport proteins are also expressed in the colon^{26–27} but to date there is no evidence that any of these proteins are perturbed in human CRC. More recently, it has been demonstrated that elevated levels of intracellular iron in a hepatocyte cell model can modulate E-cadherin expression, an adhesion protein commonly repressed in epithelial carcinogenesis.^{28–29}

We hypothesised that, while malignant colonocytes express increased levels of the proteins required for cellular iron uptake, there is inadequate expression of the protein export machinery causing these colonocytes to accumulate iron. We believe that this resulting accumulation in cellular iron causes changes to cell behaviour, which may contribute to the malignant progression of this disease.

To test this hypothesis, the aims of this study were twofold: (i) to examine expression of proteins involved in iron uptake (DCYTB, DMT1, and Tfr1), export (FPN, HEPH), and storage (ferritin) in the progression from normal colon to CRC in human tissue samples; and (ii) to examine the effect of iron loading of colorectal cell lines on proliferation and expression of E-cadherin, an adherens junction protein which is repressed in colorectal carcinogenesis

MATERIAL AND METHODS

Ethics

This work was carried out in accordance with the declaration of Helsinki (2000) of the World Medical Association. Ethics approval for this study was approved by South Birmingham LREC No 05/Q2702/17. All patients provided informed written consent.

Patient tissue

Colorectal cancer resection specimens

Samples ($n = 11$) of colorectal carcinoma matched with normal colonic mucosa from the same resection specimen were collected during surgery and each tissue specimen was divided into three: one third for RNA extraction, one third for western blotting, and the final portion for immunohistochemistry.

Archived tissue

Paraffin sections of normal colon from patients with colorectal carcinoma ($n = 20$), low grade dysplastic adenomas (LGD) ($n = 20$), high grade dysplastic adenomas (HGD) ($n = 20$), and CRC ($n = 20$) were identified within the archived tissue bank (Department of Pathology, Queen Elizabeth's Hospital, Birmingham, UK) and processed for immunohistochemistry.

Real time polymerase chain reaction (PCR)

Real time PCR was performed, as described previously,³⁰ on CRC specimens described above. All reactions were performed using 18S ribosomal RNA as an internal standard (PE Biosystems, Roche, USA), and contained one of the sets of probes and primers listed in table 1.

Western blotting

Western blotting was performed on colorectal cancer specimens described above, as previously reported,³⁰ with a rabbit polyclonal antibody to either: (i) DMT1 (1:1000 dilution)³¹; (ii) FPN (clone 3566, 1:1000 dilution)²¹; (iii) ferritin (1:2500 dilution; Sigma UK); (iv) HEPH (1:200 dilution; HEPH11-A, ADI, USA); or (v) DCYTB (clone 834, 1:1000 dilution)¹⁵ or a monoclonal antibody to (i) Tfr1 (11000 dilution; Zymed Laboratories, San Francisco, California, USA) or (ii) E-cadherin (1:1000 dilution; BD Biosciences, Cowley, Oxford, UK). A cytokeratin 19 (CK-19) monoclonal antibody (1:2000 dilution; Oncogene Research Products, USA) was employed for normalisation of epithelial protein loading. Immunoreactive bands were then subject to densitometry using NIH Image 1.62 software.

DAB enhanced Perls' Prussian blue staining

Paraffin sections were dewaxed, washed in dH_2O , and incubated in a 1:1 solution of 4% HCl and 4% ferrous cyanate for 30 minutes. Following incubation in phosphate buffered saline (PBS) for five minutes, sections were incubated in DAB (diaminobenzidine) Chromogen Solution 50 $\times$ (Dako, Ely, Cambridgeshire, UK) (1:200) for 15 minutes followed by a further incubation for 15 minutes in DAB (1:50) in substrate buffer (Dako ChemMate). Sections were then either counterstained with haematoxylin for 30 seconds or processed for immunocytochemistry. Images were visualised using a Nikon Eclipse E600 microscope and digital image taken using a Nikon DXM1200F camera (Surrey, UK). Nikon ACT-1 version 2.62 software was used for image acquisition (Surrey, UK).

Immunocytochemistry

Briefly, sections were dewaxed and incubated in hydrogen peroxide/methanol (1:10) for five minutes followed by 15 minutes of microwave antigen retrieval using 0.1 M citric acid pH 6.0. Sections were blocked with normal goat serum for 30 minutes and then incubated for one hour with rabbit polyclonal antibodies to: (i) DCYTB (1:200, clone 834), (ii) DMT1 (1:3000), (iii) FPN (1:200 clone 3566), (iv) ferritin (1:1000, F-5012; Sigma, UK), (v) HEPH 1:50 (HEPH11-A, ADI, USA), or monoclonal antibodies to (vi) Tfr1 antibody

Table 1 Probes and primers used for real time polymerase chain reaction

	Probe (5'FAM 3'TAMRA)	Forward primer, 5'-3'	Reverse primer, 5'-3'
SLC11A2 (DMT1) IRE+ve	CTC TAT CAG GCT TAG GAT TCT TTG AAC TTA TTT CCA CTT T	CCA TAT GAA ATA TAA AAT GAA GAG ACA CCT A	CCC CTC TTA ACT TCC ACT GAG AAA
SLC11A2 IRE-ve	CCC ACC CAT AAC AGT CAT ACA CTC CCA GAG T	TGG GAA GGG TGT TTC AAA ACT G	CCA TCA GAG GCC AAT CGT TTA
SLC40A1 (FPN)	AGGATTGACCAGTTAACCAACATCT TAGCCCC	AGC AAA TAT GAA TGC CAC AAT ACG	CAA ATG TCA TAA TCT GGC CAA CAG
Ferritin	CCA ACG AGG TGG CCG AAT CTT CCT T	GGA ACA TGC TGA GAA ACT GAT GAA	CAT CAC AGT CTG GTT TCT TGA TAT CC
HEPH	ACA GTG ACA TAG TGG CTT CCA GCT TCT TAA AGT CTG	GGA AGA AAT GTC ATC ACG AAC CA	TCC CCC TAT CCG GTT CTT G
TFRC (TFR1)	AAA GAC AGC GCT CAA AAC TCG GTG ATC ATA G	CGT GAT CAA CAT TTT GTT AAG ATT CA	CCA CAT AAC CCC CAG GAT TCT
CYBRD1 (DCYTB)	CCA GGG CAT CGC CAT CAT CGT CGA GCT AGC CGC CAC GCT GG	CATGGTCACCGGCTCTCGT CTA CAT CAC CAT CCG GAG GAA	CAG GTC CAC GGC AGT CTG TA CAG ATT TTA ACC TGC CTC TCA GAG A
E-cadherin	AAA TTC ACT CTG CCC AGG ACG CGG	GGC GCC ACC TCG AGA GA	TGT CGA CCG GTG CAA TCT T

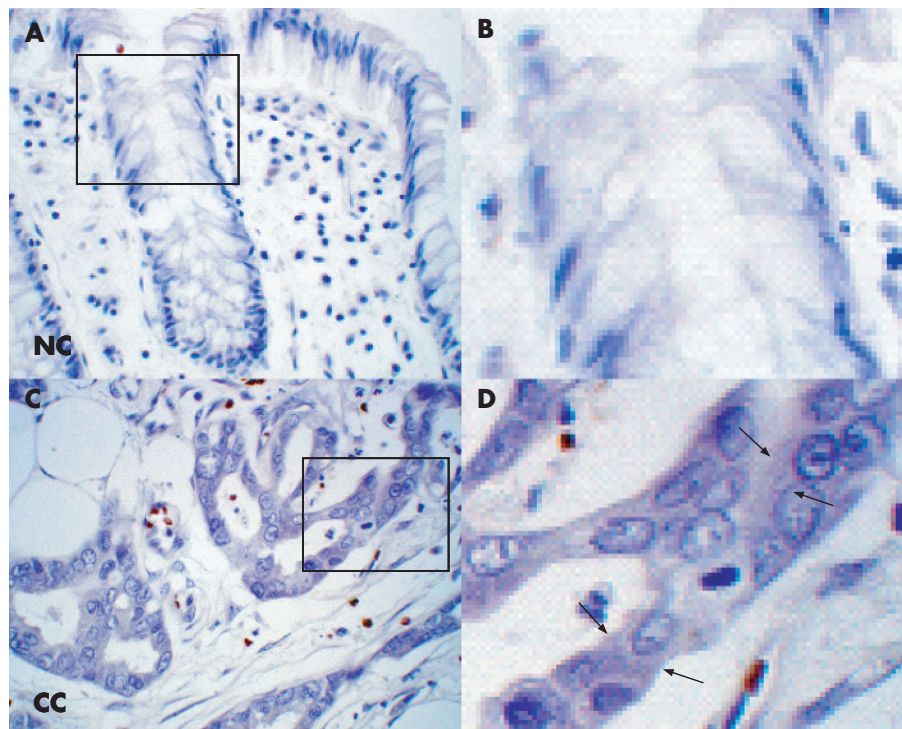


Figure 1 Elevated diaminobenzidine (DAB) enhanced Prussian blue staining in colorectal carcinoma. Sections of normal colon (n = 20) and colorectal cancer (CRC, n = 20) were subject to DAB enhanced Perl's Prussian blue staining. In normal colonic epithelium there was no detectable staining (A–B) while in CRC, diffuse cytoplasmic staining was seen (C–D). (A, C, original magnification $\times 40$; B–D original magnification $\times 100$).

(1:40, Novocastra, UK) or E-cadherin (BD Biosciences; 1:200). Following extensive washing, sections were incubated with the appropriate peroxidase linked secondary antibody and immunoreactivity was visualised using DAB reagent followed by counterstaining with haematoxylin. Small bowel mucosa was included as a positive control, and omission of primary antibody was used as a negative control.

Cellular localisation (nuclear, cytoplasmic, cell surface) and staining intensity, graded as 0 (no expression), 1 (weak), 2 (moderate), or 3 (strong) expression, were scored independently by three observers (MB, SH, and CT).

In experiments where DAB enhanced Perl's Prussian blue stain and immunocytochemistry were performed on the same section, Prussian blue staining was carried out prior to immunocytochemistry. The procedure was as described above, but a Vector VIP peroxidase substrate kit (Vector Laboratories UK, Orton Southgate, Peterborough, UK) was substituted for the DAB reagent to allow colour discrimination for visualisation purposes.

Tissue microarrays

A separate set of CRC samples (n = 250) collected between 2000 and 2005 for which complete clinical data were available were used to evaluate the association between iron transporter levels and prognostic factors. The prognostic factors included Dukes' staging (A–D), extent of vascular invasion (none, moderate, focal, or extensive), nodal involvement, sex, and age. To facilitate the screening of multiple tissue blocks from each patient, tissue microarrays were prepared containing four representative samples of tumour per patient. Sections (4 μ m) were cut from each array block onto Superfrost positively charged slides (Surgipath Europe Ltd, Bretton, Peterborough, UK) and heated for one hour at 60°C followed by processing for immunohistochemistry and scoring as described above.

Cell culture

Cell lines SW480 and Caco-2 were routinely cultured in Dulbecco's modified Eagle's medium (Invitrogen Ltd, Paisley, UK) with 10% fetal calf serum supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin. SW480 cells were iron loaded on reaching 70% confluence while Caco-2 cells were grown for 14 days after reaching confluence.

Iron loading

Cells were challenged with either growth medium alone (control) or iron loaded medium (growth medium supplemented with 100 μ M FeSO₄ and 10 mM sodium ascorbate) for between one and 24 hours. FeSO₄ (100 μ M) was chosen as this concentration has been used in other studies and was shown to be the optimal concentration for E-cadherin repression and induction of proliferation (data not shown).

Immunofluorescence

Cells were fixed in methanol/acetone, blocked (20% goat serum in 1% bovine serum albumin/PBS) and incubated with a monoclonal anti-E-cadherin antibody (BD Biosciences 1:500) for one hour prior to labelling with FITC goat antimouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA; 1:500). Cells were then washed and incubated in DAPI (1:10 000) for one minute prior to visualisation. Omission of the primary antibody was employed as a negative control.

E-cadherin promoter assay

The wild-type human E-cadherin promoter sequence (–301/+21) cloned into pGL3basic luciferase reporter (EproWT) was a kind gift from Prof Frans van Roy.³² pGL3 basic, which contains the firefly luciferase gene without a promoter sequence, was used as a control, with *Renilla* luciferase plasmid (pRL TK) used as a transfection control. Caco-2 cells were transiently transfected with 1 μ g EproWT or pGL3 basic

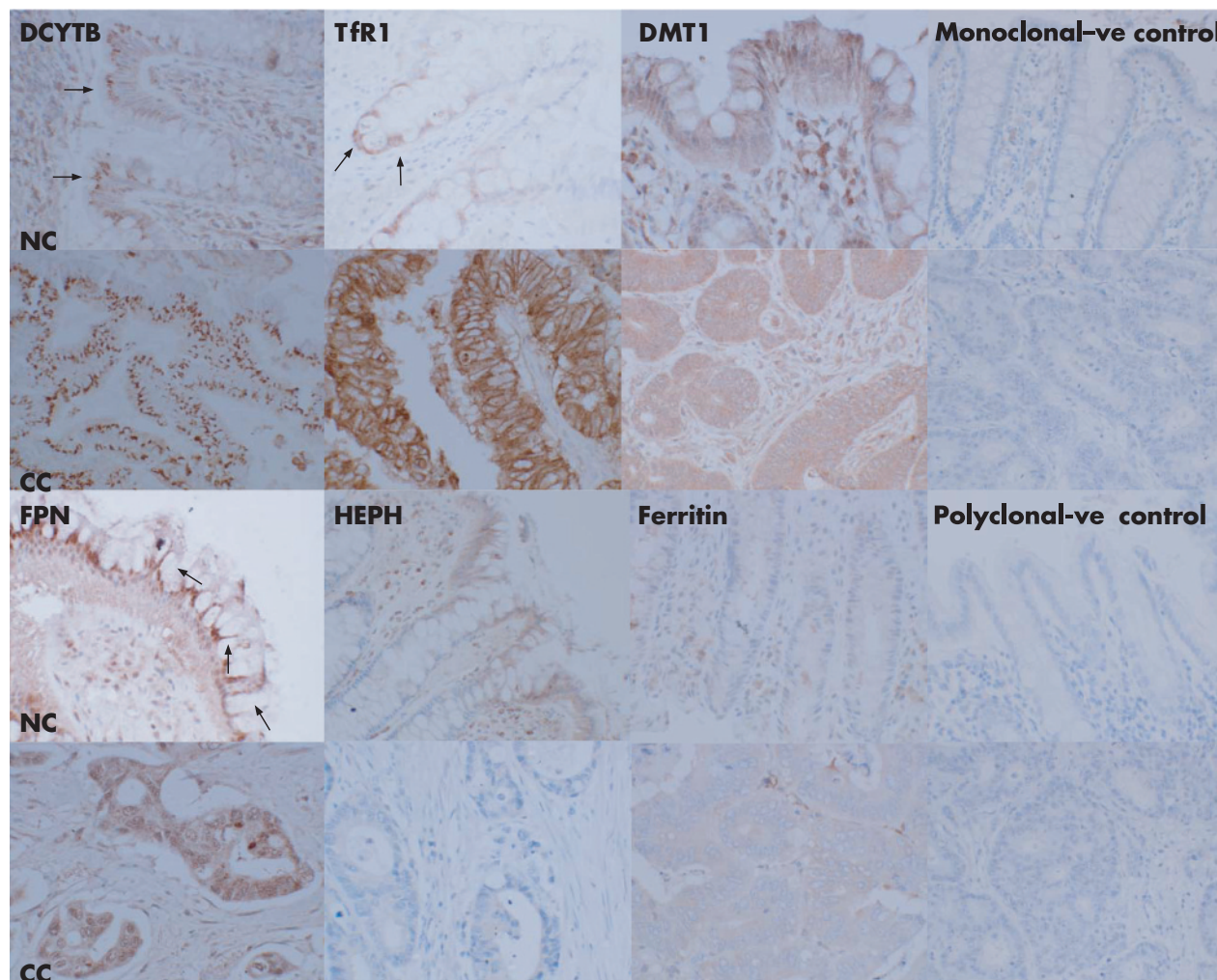


Figure 2 Immunolocalisation of iron transport proteins in normal colon and colorectal cancer. Paraffin sections of normal colon associated with colorectal cancer (NC) and colorectal cancer (CC) were subjected to immunohistochemistry using antibodies to various proteins of iron metabolism. Duodenal cytochrome b (DCYTb), divalent metal transporter 1 (DMT1), ferroportin (FPN), ferritin, transferrin receptor 1 (TfR1), and hephaestin (HEPH) were all weakly expressed in NC. With the exception of ferritin and HEPH, there was strong immunoreactivity in CC. As antibodies used were either mouse monoclonal or rabbit polyclonal antibodies, negative controls included omission of primary antibody followed by processing with either a rabbit secondary (polyclonal -ve control) or mouse secondary (monoclonal -ve control) antibody, as described in material and methods. Arrows denote areas of positivity (original magnification $\times 60$).

Table 2 Semiquantitative analysis of immunoreactivity of iron transport proteins in progression from normal colon to colorectal carcinoma

	NC	LGD	HGD	CRC
DCYTb	1.05	1.84	1.7	2.25*
TfR1	1.30	0.40	0.50	2.08*
DMT1	1.20	0.80	1.45	2.10*
FPN	1.36	1.64	2.55*	2.10*
HEPH	1.23	1.50	1.54	0.82*
Ferritin	0.68	0.20*	0.40*	0.80

Paraffin sections of normal colon (NC) (n=20), low grade dysplastic adenomas (LGD) (n=20), high grade dysplastic adenoma (HGD) (n=20), and colorectal carcinoma (CRC) (n=20) were all subject to immunohistochemistry with antibodies to duodenal cytochrome b (DCYTb), transferrin receptor 1 (TfR1), divalent metal transporter 1 (DMT1), ferroportin (FPN), hephaestin (HEPH), and ferritin. Staining intensity was graded as 0 (no expression), 1 (weak), 2 (moderate), or 3 (strong) expression. The mean of each group is presented and numbers in bold denote a value that is significantly different when compared with NC (*p<0.05).

in the presence of 0.1 μ g pRL TK using standard calcium phosphate transfection methods. Sixteen hours post transfection, the culture medium was replaced with either fresh medium or iron loaded medium for 24 hours and then cells were assayed for firefly and *Renilla* luciferase activities using the dual reporter assay kit Stop 'N' Glow (Promega, Southampton Science Park, Southampton, UK) according to the manufacturer's instruction. Firefly luciferase activity was normalised to *Renilla* luciferase activity as a transfection control. Promoter activity was expressed as a fold change in relative luciferase units compared with that obtained in pGL3basic control transfected cells. Results shown represent the means (SEM) of six independent experiments.

Proliferation assay

The effect of cellular iron loading on proliferation was examined using a bioluminescent technique to measure changes in cellular ATP (ViaLight HS; LumiTech, Nottingham, UK) as previously described.³³ ATP levels were recorded in relative luciferase units and proliferation was expressed as a percentage of control.

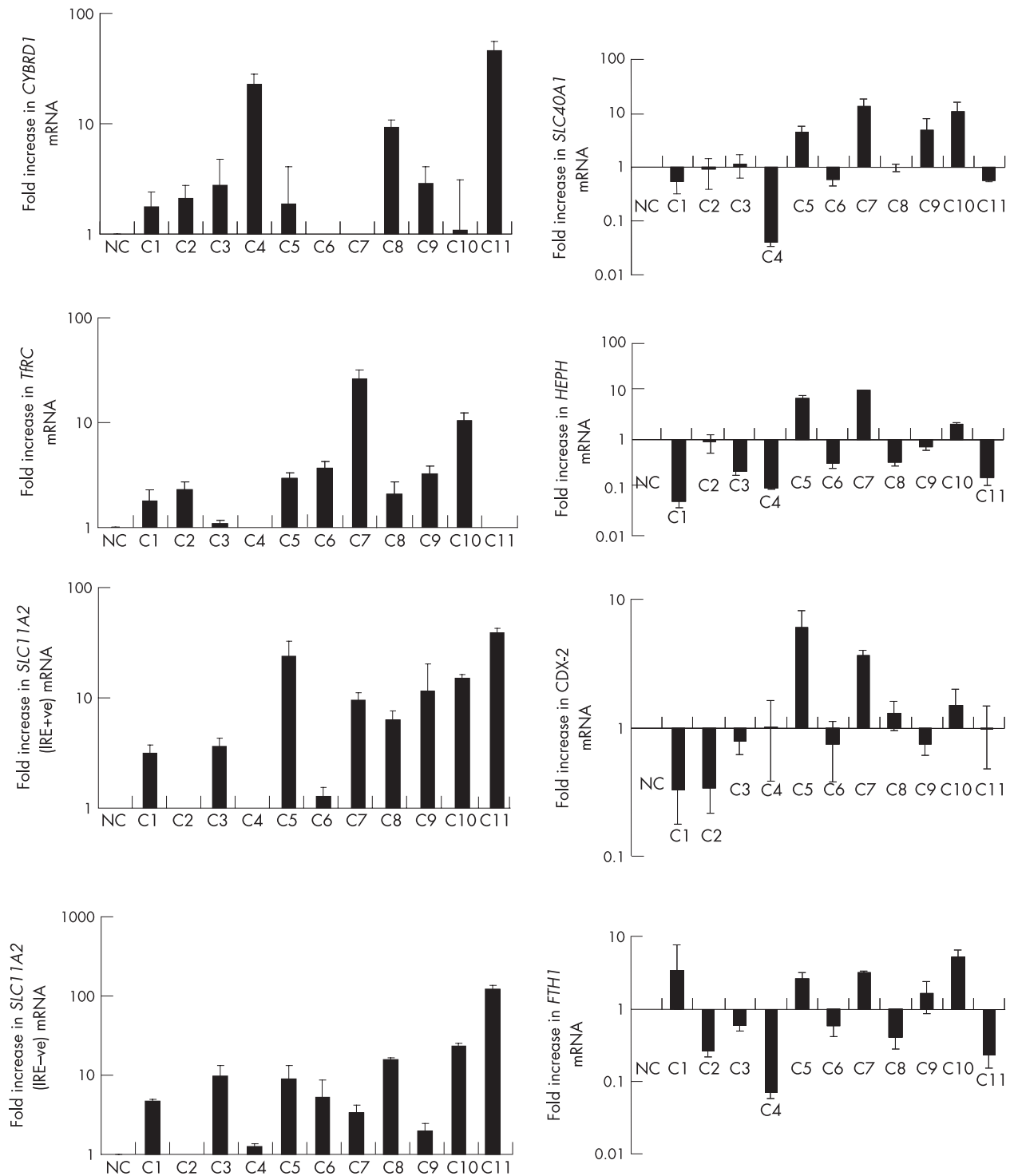


Figure 3 mRNA expression of iron transport genes in normal colon and colorectal carcinoma. Real Time polymerase chain reaction was used to examine expression of genes encoding various iron transport proteins in colorectal carcinoma specimens 1–11 (C1–C11) compared with their own normal tissue control (NC), normalised to 1. Transferrin receptor 1 (*TfR*), *CYBRD1* (duodenal cytochrome b), and *SLC11A2* (divalent metal transporter 1; both IRE containing and non-IRE variants) mRNAs were overexpressed in over 73% of cancers examined compared with their normal colonic control. *SLC40A1* (ferroportin) and *FTH1* (ferritin) mRNA expression were variable within the 11 samples. Hephaestin (*HEPH*) and *CDX-2* mRNA expression were both mostly reduced. Values are mean (2 SEM).

Ferrozine assay

Non-haem iron was assayed, as previously described,³⁴ and cellular iron content was expressed as nmol of iron per mg protein. Protein concentrations were assessed by Bradford assay (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK).

Statistics

All experimental errors are shown as two standard error of the mean (representing 95% confidence intervals). Statistical significance was calculated by use of the unpaired Student's *t* test and the Mann-Whitney test where appropriate. Correlation was evaluated using Pearson's correlation

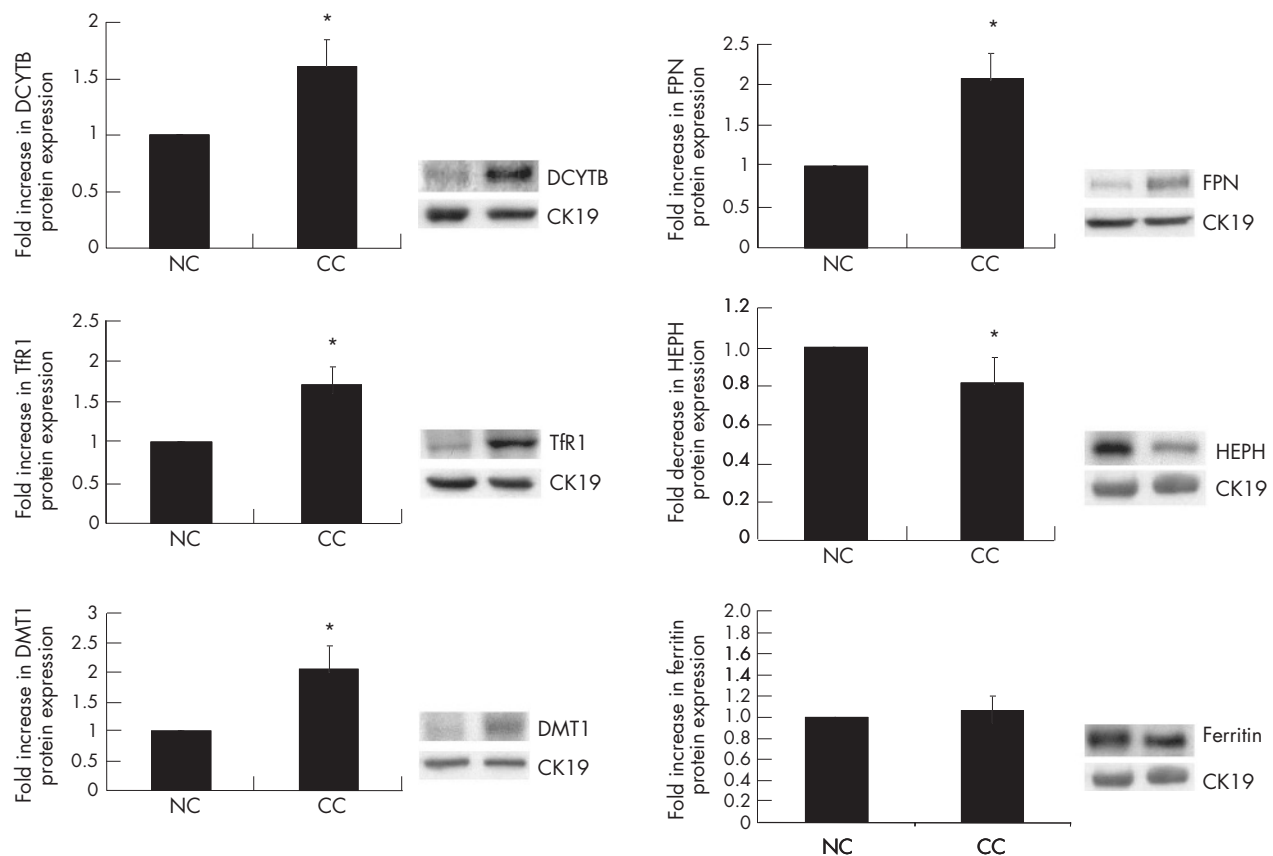


Figure 4 Protein expression of iron transporters in normal colon and colorectal carcinoma. Expression of divalent metal transporter 1 (DMT1), transferrin receptor 1 (TfR1), duodenal cytochrome b (DCYTB), ferritin, hephaestin (HEPH), and ferroportin (FPN) protein was studied in normal colon (NC) and colorectal cancer (CC) by western blotting and quantitated by densitometry. A representative western blot for each protein is shown and cytokeratin 19 (CK19) employed for normalisation. Relative protein expression was normalised to 1.0 (100%) of controls. Values are mean (2 SEM). * $p < 0.05$, Student's *t* test.

coefficient and a one way ANOVA test. Comparison between categorical variables was made using a χ^2 analysis. All analyses were performed using SPSS version 10.0 (SPSS Inc, North Carolina, USA). Significance was accepted at $p < 0.05$.

RESULTS

Iron content of colorectal cancer tissue

Whether colonocytes in CRC are relatively iron deficient or iron rich is unclear at present. To answer this question, we initially stained sections of CRC and matched normal colonic mucosa ($n = 20$) for iron by DAB enhanced Perls' Prussian staining.

In normal colonic epithelium there was no detectable DAB enhanced Perl's Prussian blue staining (fig 1A–B), and only very low levels of iron were apparent in LGD and HGD. However, in all of the CRCs there was evidence of more intense staining which was diffuse throughout the cell cytoplasm (fig 1C–D).

To test whether the observed colonocyte iron accumulation could be attributed to changes in iron transport proteins, immunohistochemistry with semiquantitative analysis was performed.

Immunolocalisation of iron metabolism proteins in archived tissue

Localisation of the proteins involved in cellular iron uptake (DCYTB, DMT1, and TfR1), iron export (FPN and HEPH), and iron storage (ferritin) was examined in archived tissue specimens of: (i) normal colonic mucosa (adjacent to colorectal carcinoma) (normal colon); (ii) LGD; (iii) HGD;

and (iv) CRC (fig 2). Staining intensity for all specimens was also scored and the data are presented in table 2.

In normal colonic mucosa, DCYTB was weakly expressed on the colonic surface epithelium with no expression at crypt bases. Expression was predominantly apical and vesicular. This pattern of immunoreactivity was retained in LGD and HGD, and in CRC. However, there was significantly higher DCYTB immunoreactivity in CRC compared with normal colon ($p < 0.05$). DMT1 was expressed weakly on the surface epithelium of normal mucosa and in the top third of the crypts with predominantly apical diffuse cytoplasmic staining. No staining was seen in crypt bases. Similar immunoreactivity was observed in LGD and HGD. However, stronger cytoplasmic staining for DMT1 was seen in CRC specimens compared with normal colon ($p < 0.05$). TfR1 was predominantly localised at the basolateral membrane of crypt colonocytes, strongest immunoreactivity being at the crypt base with a gradation to weak immunoreactivity on surface epithelium. In CRC there was marked overexpression of TfR1 compared with normal colon ($p < 0.05$), with immunoreactivity predominantly on the plasma membrane.

FPN was predominantly localised on the basolateral membrane of the colonocyte with a gradation of immunoreactivity from high to low from the surface epithelium to the base of the crypt. A similar profile of expression was observed in dysplastic adenomas. Interestingly, there was strong cytoplasmic immunoreactivity in CRC compared with normal colon ($p < 0.05$) although poorly differentiated cells at invasive fronts showed weaker immunoreactivity (data not shown). HEPH was observed only on the surface epithelium,

Table 3 Semiquantitative analysis of immunoreactivity of iron transport proteins in a colorectal carcinoma tissue array

	All samples (mean (SD))	Female (mean (SD))	Male (mean (SD))
HEPH			
Duke's stage			
A	1.08 (0.60)	1.09 (0.7)	1.08 (0.53)
B	0.95 (0.66)	0.95 (0.59)	0.91 (0.72)
C	0.91 (0.69)	0.89 (0.68)	0.93 (0.71)
Vascular invasion			
None	0.97 (0.66)	0.94 (0.63)	0.99 (0.67)
Moderate	0.94 (0.64)	1.28 (0.56)	0.65 (0.35)
Focal	0.97 (0.71)	0.94 (0.68)	1.04 (0.77)
Extensive	0.50* (0.43)	0.50 (0.07)	0.50* (0.54)
Nodal involvement			
Negative	0.98 (0.7)	0.95 (0.61)	1.00 (0.68)
Positive	0.91 (0.64)	0.94 (0.69)	0.91 (0.7)
FPN			
Duke's stage			
A	2.08 (0.87)	2.18 (1.0)	2.01 (0.76)
B	2.46 (0.85)	2.52 (0.98)	2.36 (0.83)
C	1.70* (0.75)	1.84 (0.77)	1.59* (0.72)
Vascular invasion			
None	2.01 (0.84)	2.14 (0.92)	1.97 (0.78)
Moderate	1.96 (0.8)	2.10 (0.82)	1.73 (0.77)
Focal	1.86 (0.94)	1.78 (1.11)	2.06 (0.87)
Extensive	2.14 (0.85)	2.34 (0.76)	1.34 (0.82)
Nodal involvement			
Negative	2.28 (0.87)	2.36 (0.97)	2.22 (0.75)
Positive	1.70* (0.85)	1.90 (0.8)	1.44* (0.78)

Colorectal cancers (n = 250) for which complete clinical data were available (extent of vascular invasion, nodal involvement, Dukes' stage, sex, and age) were used to evaluate the association between iron transporter levels and these prognostic factors.

Immunohistochemistry was performed with antibodies to duodenal cytochrome b (DCYTB), divalent metal transporter 1 (DMT1), ferroportin (FPN), ferritin, transferrin receptor 1 (TfR1), and hephaestin (HEPH). Staining intensity was graded as 0 (no expression), 1 (weak), 2 (moderate), and 3 (strong) expression. As there were no statistically significant associations between DCYTB, DMT1, ferritin, and TfR1 expression and any prognostic factor, these values have not been presented.

However, both FPN and HEPH significantly correlated with vascular invasion, Dukes' stage, and nodal involvement. The mean of each group is presented and numbers in bold denote values significantly different from control (*p < 0.05; Student's *t* test).

not in crypts. Intracellular localisation of HEPH was predominantly basal with some weak supranuclear staining. A similar pattern of expression was observed in both LGD and HGD while in CRC there was only weak diffuse cytoplasmic staining (p < 0.05).

Very weak immunoreactivity for ferritin was observed in normal colonic mucosa with most positivity observed in non-epithelial cells within the lamina propria. In LGD and HGD, ferritin immunoreactivity was almost completely absent within colonocytes and was shown to be significantly lower compared with normal colon, while ferritin expression was unchanged in colorectal carcinomas compared with normal colon (table 2).

These results suggest that the cellular iron import machinery is overexpressed in CRC while the capacity of cells to export iron is reduced.

mRNA and protein expression of iron metabolism molecules in colorectal cancer resection specimens

To extend the immunohistochemistry results described above, we examined mRNA and protein expression of iron transport proteins in CRC resection specimens (CRC) matched with normal colonic mucosa (n = 11) by real time PCR (fig 3) and western blotting (fig 4), respectively.

TfRC, *CYBRD1*, and *SLC11A2* mRNAs were overexpressed in over 73% of cancers examined compared with their internal matched normal colonic controls (fig 3). As four *SLC11A2* splice variants have been described,³⁵ (5' variants (exon 1A or 1B) and 3'UTR variants (IRE+ve or IRE-ve)) we investigated mRNA expression of these variants and showed that both

IRE+ve and IRE-ve variants were overexpressed in these cancers (fig 3). Additionally, we were able to show overexpression of the 5' 1B variant in tumours which showed overexpression of the 3' variants but we were unable to detect any 1A variant in any sample (results not shown).

To complement the mRNA analyses, we examined all 11 cancer samples with their matched normal colonic mucosa control for protein expression (fig 4). DCYTB, DMT1, and TfR1 were overexpressed with mean fold increases of 1.6, 2.05, and 1.7 (p < 0.05), respectively (fig 4), consistent with the immunohistochemistry data.

Analysis of *SLC40A1* mRNA expression demonstrated overexpression in 4/11 and reduced expression in 2/11 CRCs, the remainder being unchanged. *HEPH* mRNA was overexpressed in only 3/11 cancers while the majority of cancer specimens (7/11) showed reduced expression (fig 3). Moreover, a correlation between *SLC40A1* and *HEPH* was observed (Pearson correlation coefficient $r^2 = 0.57$, $p = 0.007$, ANOVA). As it has recently been reported that hephaestin can be regulated by the transcription factor CDX-2, we examined CDX-2 mRNA and compared the findings to hephaestin (fig 3).³⁶ A significant correlation between *HEPH* and CDX-2 mRNA expression was observed ($r^2 = 0.72$, $p = 0.001$) although no significant correlation between CDX-2 and *SLC40A1* mRNA expression ($r^2 = 0.23$, $p = 0.22$) was apparent, consistent with previous findings.³⁶ FPN protein was overexpressed with a mean fold increase of 2.07 (p < 0.05) while HEPH protein was decreased (mean 0.81 fold; p < 0.05) (fig 4). In summary, HEPH is repressed at both the mRNA and protein levels while FPN protein data suggest

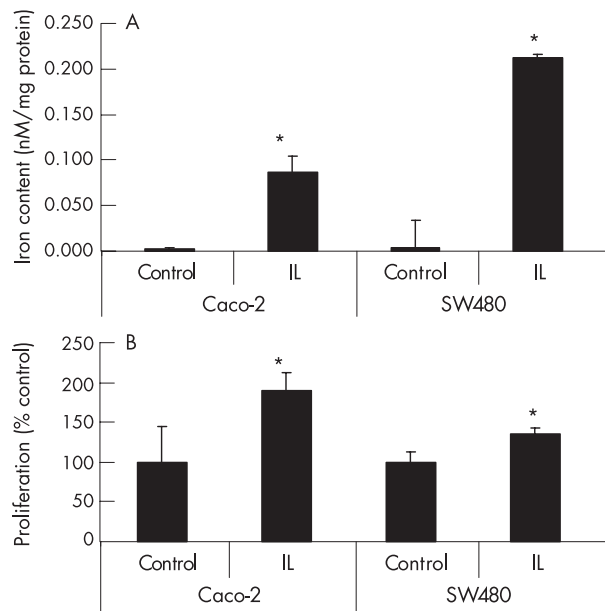


Figure 5 Iron loading of Caco-2 and SW480 cells causes increased proliferation. (A) Caco-2 and SW480 cells were either cultured in growth medium (Control) or growth medium supplemented with 100 μ M FeSO_4 for 24 hours (IL). Iron loading for 24 hours resulted in iron accumulation in both Caco-2 and SW480 cells. Values are mean (2 SEM). * $p < 0.05$ using the Student's t test. These data are the mean of three independent experiments, each performed in triplicate. (B) Iron loading of Caco-2 and SW480 cells resulted in a marked induction in cellular proliferation. Values are mean (2 SEM). * $p < 0.05$ using Student's t test. These data are the mean of three independent experiments.

overexpression consistent with the immunohistochemistry data.

FTH1 mRNA expression varied within the 11 samples studied with roughly an equal number showing over- and reduced expression (fig 3). Furthermore, on western blotting there was no change in ferritin protein levels between normal colon and CRC specimens (fig 4), consistent with the immunohistochemistry data.

For DCYTB, DMT1, TfR1, and HEPH, a positive correlation between mRNA and protein expression was observed for each CRC specimen ($p < 0.05$). No such correlation was seen for FPN.

These data support the hypothesis that in CRCs there is overexpression of the proteins implicated in cellular iron uptake and that levels of HEPH are repressed while those of FPN are elevated.

Tissue microarray

This study provides evidence that iron transport proteins are modulated in the malignant progression of disease. We further sought to determine whether any changes in protein expression were associated with prognostic factors such as stage of disease, extent of vascular invasion, and or metastasis by utilising a tissue array of 250 CRCs with known outcome.

The tissue microarray analysis showed no significant changes in staining intensity for DMT1, TfR1, DCYTB, or ferritin with respect to clinical outcome. However, HEPH expression was reduced in cancers with extensive vascular invasion compared with cancers with no vascular invasion ($p < 0.05$) and this finding was observed in both males and females ($p < 0.05$) (table 3). Similarly, FPN was repressed in Dukes' stage C CRCs compared with earlier stage cancers ($p < 0.05$). Not surprisingly, as Dukes' stage C is classified as positive nodal involvement, statistical repression in FPN was also observed in all patients with nodal involvement ($p < 0.05$). Interestingly, this was confined to males only, with no significant change observed for females (table 3).

Modulation of cellular iron levels and its effect on cellular proliferation

To determine whether iron loading exerted any effects on cellular proliferation, we experimentally iron loaded the well and poorly differentiated colorectal cell lines Caco-2 and SW480, respectively.

Caco-2 and SW480 cells were iron loaded and iron content was measured using a ferrozine assay (fig 5). Control non-iron loaded cells showed low iron levels (means 0.002 and 0.004 nmol/mg protein for Caco-2 and SW480 cells, respectively) while on iron loading there was a significant increase in iron levels ($p < 0.05$, means 0.086 and 0.212 nmol/mg protein, respectively) in both Caco-2 and SW480 cells (fig 5A). Increased cellular iron was associated with a significant increase in cellular proliferation compared with controls ($p < 0.05$) (fig 5B).

Modulation of cellular iron levels and its effect on the cell adhesion molecule E-cadherin

As previous studies have shown that a common event in nearly all epithelial malignancies, including CRC is repression of the cell adhesion molecule E-cadherin, we examined whether loading cells with iron could reduce expression of E-cadherin.

Iron loading of Caco-2 and SW480 cells for 24 hours resulted in a 37% ($p < 0.05$) and 48% ($p < 0.05$) decrease in E-cadherin mRNA, respectively (fig 6A, B). To ensure that this observation was not a global mRNA repression, we also

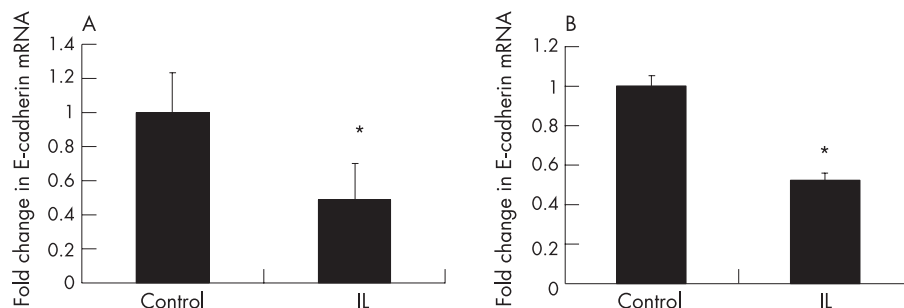


Figure 6 Iron loading decreases E-cadherin mRNA expression. To determine whether iron loading modulated expression of E-cadherin mRNA, control and 24 hour iron loaded (IL) Caco-2 (A) and SW480 (B) cells were assessed by real time polymerase chain reaction. Iron loading of both cell lines resulted in a marked decrease in E-cadherin mRNA expression. Relative gene expression was normalised to 1.0 (100%) of controls. Values are mean (2 SEM). * $p < 0.05$ using Student's t test. These data are the mean of three independent experiments.

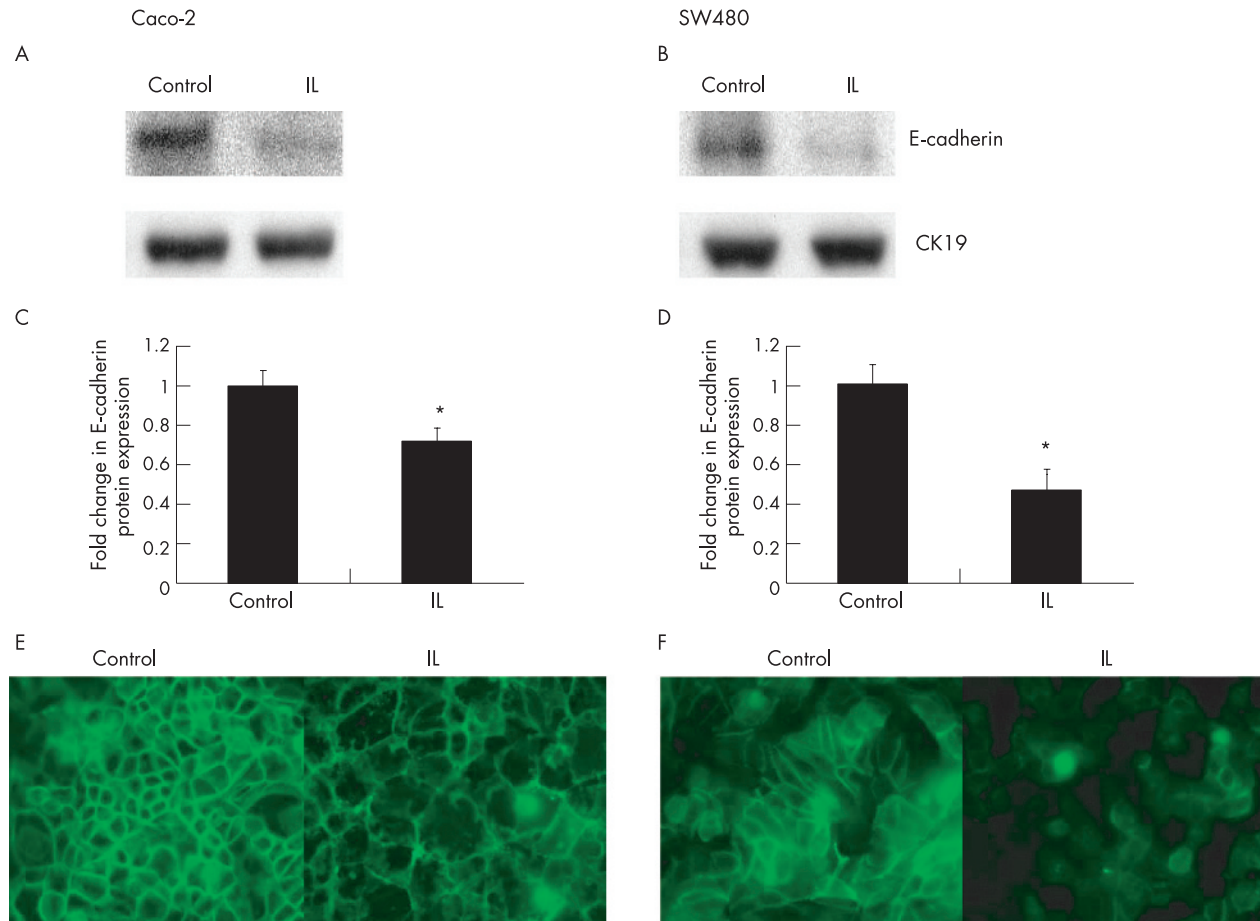


Figure 7 Iron loading reduces E-cadherin protein expression. Extracts from control and 24 hour iron loaded (IL) Caco-2 (A) and SW480 (B) cells were subjected to western blotting. (A, B) Representative E-cadherin immunoblots with cytokeratin 19 (CK-19) employed for normalisation. The E-cadherin immunoreactive band (M_r 120 000) was quantitated by densitometry and controls normalised to 1 (C, D). There was a marked decrease in E-cadherin protein expression following iron loading. Values are mean (2 SEM). * $p < 0.05$ using Student's *t* test. Data are the mean of three independent experiments. Additionally, immunofluorescence staining demonstrated that while control Caco-2 and SW480 cells showed the expected cobblestone network pattern of cell surface staining indicative of E-cadherin, expression of E-cadherin in iron loaded cells was much reduced (E, F) (original magnification $\times 40$).

examined other transcripts, including actin, and β - and γ catenin, and these showed no changes (results not shown).

Iron loading caused a significant decrease in E-cadherin protein expression compared with non-iron loaded controls in both Caco-2 and SW480 cells (30% and 54%, respectively; $p < 0.05$) (fig 7A–D). Immunofluorescence for E-cadherin in

Caco-2 and SW480 cells showed that while there was preserved E-cadherin immunoreactivity on the cell surface in both control and iron loaded cells, the intensity of the staining in iron loaded cells was markedly lower. This was more pronounced in SW480 cells than in Caco-2 cells (fig 7E–F).

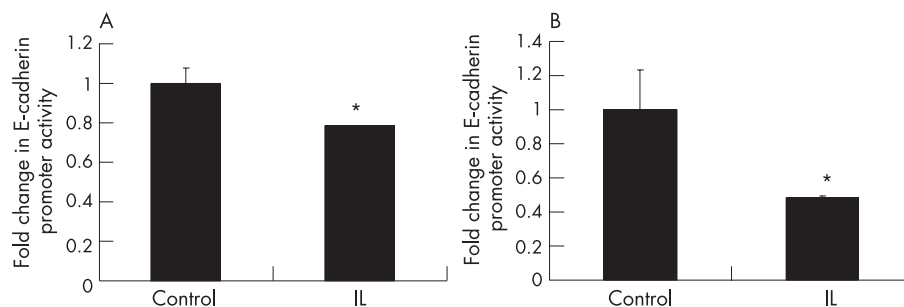


Figure 8 Iron loading causes a decrease in E-cadherin promoter activity. Caco-2 (A) and SW480 (C) cells were transiently transfected with 1 μ g EproWT (containing the wild-type human E-cadherin promoter sequence linked to a firefly luciferase reporter gene) and 0.1 μ g pRL-TK (*Renilla* luciferase plasmid used as a transfection control). Sixteen hours post transfection, culture medium was replaced with either control or iron loaded (IL) medium. After 24 hours cells were harvested, lysed, and luciferase activity analysed. The control value was set at 100% for normalisation purposes. Iron loading resulted in marked repression in E-cadherin promoter activity. Data shown represent the mean (2 SEM) of six independent experiments. * $p < 0.05$ using Student's *t* test.

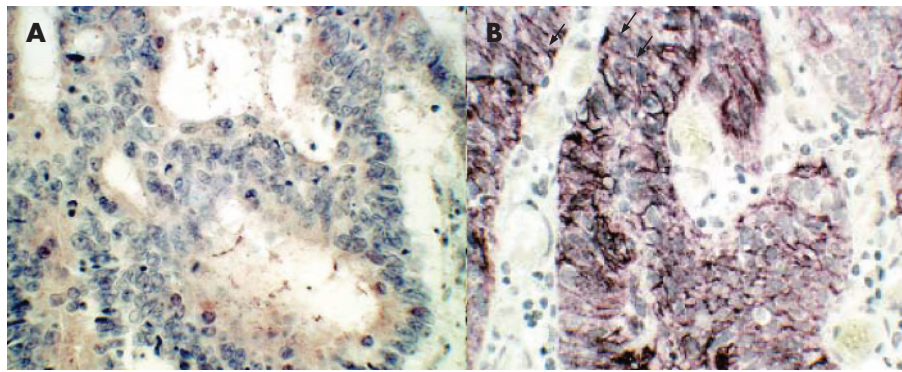


Figure 9 Enhanced Prussian blue staining and E-cadherin immunoreactivity in colorectal carcinomas. Paraffin sections of colorectal carcinomas were subjected to Prussian blue staining followed by immunohistochemistry for E-cadherin. (A) In discrete areas of the colorectal carcinoma where there was positive diaminobenzidine (DAB) enhanced Perls' Prussian blue staining (brown) there was little or no evidence of E-cadherin staining. (B) Conversely, in areas of E-cadherin plasma membrane staining (purple) little if no DAB enhanced Perls' Prussian blue staining was observed. Arrows denote areas of positivity (original magnification $\times 40$).

To ascertain whether the changes in E-cadherin mRNA levels were due to changes in E-cadherin promoter activity, we performed an E-cadherin promoter assay using the wild-type human E-cadherin promoter sequence ($-301/+21$) cloned into the luciferase reporter vector pGL3basic (fig 8).³² We demonstrated a significant reduction in E-cadherin promoter activity in response to 24 hours of iron loading in both Caco-2 and SW-480 cells (22% and 48% reduction, respectively; $p < 0.05$) (fig 8A, B).

E-cadherin expression and cellular iron loading in colorectal carcinoma specimens

To support the observation that in vitro cellular iron loading causes a decrease in E-cadherin expression, we sought to determine if this was also the case in vivo. This was addressed by staining paraffin sections of CRC ($n = 20$) using the DAB enhanced Perls' Prussian blue procedure immediately followed by immunohistochemistry for E-cadherin on the same section (fig 9). In all CRCs there was a reciprocal relationship between iron staining and E-cadherin expression. Where there was positive DAB enhanced Perls' Prussian blue staining there was no E-cadherin immunoreactivity observed (fig 9A). Conversely, in areas of strong E-cadherin immunoreactivity, little if any iron staining was apparent within those colonocytes (fig 9B).

DISCUSSION

Accumulated data suggest that high dietary iron intake is a major risk factor for CRC.^{4 37 38} In particular, while colonic luminal iron is likely to cause free radical damage leading to inflammation and ultimately cell damage, luminal iron appears to exert a more potent effect in the presence of a background molecular lesion.^{10 13} This is supported by the observation of an increased risk of CRC in individuals with an existing HFE mutation.¹⁰ Furthermore, data from animal models indicate that iron exacerbates colorectal tumorigenesis induced by carcinogens such as DMH or DSS, although the mechanism behind this remains obscure.^{6 12 13}

While this evidence suggests a role for iron in colorectal carcinogenesis, how iron is implicated at the cellular and molecular level is not known. We hypothesised that changes in expression of the proteins involved in epithelial iron transport could cause accumulation of iron in colonocytes and this could potentiate malignant transformation and tumour progression.

Our initial data indicated that normal human colon expresses DCYTB, DMT1, FPN, ferritin, HEPH, and TfR1, consistent with previous investigations which have shown

DCYTB, FPN, and HEPH expression in rodent colon.^{19 26 36} We observed DCYTB, DMT1, HEPH, and FPN expression predominantly on the luminal surface of the colonic epithelium, with decreasing expression towards the crypts. Conversely, and consistent with previous observations, TfR1 expression was mainly at the bases of the crypts.³⁹ This localisation in the proliferative compartment suggests a role for TfR1 in the supply of iron for cell growth and proliferation.⁴⁰ These data add weight to existing literature showing that the colon is capable of absorbing iron.^{27 41–43}

Of particular interest is modulation of these proteins in the malignant progression of normal colon to cancer. Data from our characterisation of iron transporter expression in CRC specimens indicate that DMT1 is overexpressed and immunohistochemical results suggest that this is a late feature of colorectal tumorigenesis as no induction is seen in LGD or HGD specimens. As four splice variants of DMT1 have been described,³⁵ we examined the expression profile of each variant and found only the exon 1B 5' variant. Both the 3' IRE- and non-IRE variants were detected in most of the 11 cancers investigated. This, in conjunction with the observation of increased intracellular iron accumulation in CRC specimens compared with normal samples, mitigates against a predominant IRE/IRP mediated induction of DMT1. We suggest that there is likely to be at least one other regulatory mechanism operating to modulate expression of DMT1.

Interestingly, we also found a dramatic induction of TfR1 in CRC specimens. This is well recognised in the literature both in colorectal and other cancers, and indeed TfR1 has been suggested as a target for tumour chemotherapy in the past.^{25 44} However, as we have demonstrated that colonocytes in CRC are iron rich, one might expect to see reduced TfR1 expression. As this is not the case, TfR1, like DMT1, may also be regulated by other intracellular and possibly extracellular signals which increase its expression independent of cellular iron status.

Our findings on expression of the putative ferric reductase, DCYTB, were surprising. DCYTB has been suggested to play a pivotal role in small intestinal iron absorption in the reduction of ferric to ferrous iron. Although we found overexpression of DCYTB in the majority of CRC specimens, the protein appears to be localised in cytoplasmic vesicles rather than at the cell membrane. Furthermore, DCYTB staining was observed at sites of invasion far from luminal surfaces. This would mitigate against a role for DCYTB in DMT1 mediated iron uptake in the human colon. Thus DCYTB may have a cellular function separate from its postulated role in ferric iron reduction.⁴⁵

Thus consistent with our hypothesis, there is indeed overexpression of the proteins involved in iron uptake. This would clearly cause increased entry of iron into cells. If these cells are indeed highly proliferative and have adequate iron export machinery, why is it that we observe increased intracellular iron in CRCs? To address this we assessed levels of the main proteins involved in iron export, HEPH and FPN, to determine whether their expression was altered.

Expression of HEPH was lower in CRCs relative to normal colon at both the mRNA and protein levels. In particular, when utilising a tissue microarray containing 250 CRCs, we were able to show that reduced HEPH expression was associated with extensive vascular invasion. Consistent with the recent finding that the transcription factor CDX-2 is able to regulate HEPH expression,³⁶ we found coordinate expression of CDX-2 and *HEPH* in the majority of cancers examined. Hinoi *et al* proposed that elevated intracellular iron induces CDX-2 which in turn promotes HEPH expression.³⁶ In concert with FPN, this would promote iron export. We suggest that loss of CDX-2 in CRCs prevents high intracellular iron from inducing HEPH expression thus sustaining the high iron levels in colonocytes.⁴⁶ However, this does not explain the decrease in HEPH expression observed in CRCs.

Expression of the mRNA encoding the iron export protein FPN largely paralleled expression of *HEPH* mRNA, although paradoxically FPN protein levels increased while those of HEPH decreased. Of particular interest, FPN was the only iron transport protein to show overexpression in adenomas with HGD. Despite this overexpression of FPN, as the protein FPN was predominantly localised intracellularly, we propose that it is non-functional in cellular iron export. Data from analysis of the colon cancer tissue array indicate that there was loss of cytoplasmic FPN expression in advanced (Dukes' C) cancers. In tumours with nodal involvement, this loss of FPN appeared to be restricted to men. Why specimens from women with such advanced CRC retain FPN is unexplained.

Thus in CRCs there is overexpression of the iron uptake proteins DMT1 and TfR1 and likely reduced iron export with HEPH expression reduced and FPN probably non-functional due to inappropriate cytoplasmic localisation. Furthermore, in advanced cancers, as well as a reduction in HEPH there was also a decline in FPN expression. This suggests that while intracellular iron entry is likely to be accelerated, iron export is abrogated creating accumulation of cellular iron. This is supported by the increased staining for iron observed in these cancers. Apparently inconsistent with this hypothesis is the observation that ferritin expression is unchanged in CRCs. In fact, in LGD and HGD lesions, ferritin staining was reduced compared with controls. It is possible that the lack of elevation of ferritin in the face of increased intracellular iron content in cancers reflects other competing pathways modulating ferritin expression. For example, it is well recognised that inflammatory cytokines such as tumour necrosis factor α can modulate ferritin expression,¹⁸ and there is also evidence that c-myc, an oncogene overexpressed in CRCs, can repress ferritin.⁴⁷

To investigate what effect this increased intracellular iron has on cell phenotype, we studied experimentally iron loaded colorectal tumour cell lines. We were able to load SW480 and Caco-2 cells with iron to levels similar to those detected in the 11 CRC specimens studied (data not shown) and both lines showed increased proliferation in response to loading. Interestingly, Caco-2 cells, which were iron loaded to a lesser extent than SW480 cells, showed a more dramatic increase in cellular proliferation. As Caco-2 cells are well differentiated while SW480 cells represent a poorly differentiated lineage, our observations may indicate that the cellular response to iron is dependent on differentiation status. Whether iron

mediates cellular proliferation or cell toxicity is likely to be due to a number of intracellular signalling mechanisms and this is likely to be cell dependent. This clearly requires further investigation.

We then investigated the effect of iron loading on E-cadherin, an adherens junction protein commonly repressed in epithelial malignancies including those of the colon.²⁸⁻²⁹ This protein has been reported to be repressed by iron in a hepatocellular model system.⁴⁸ In this study, we showed that iron loading of both cell lines resulted in decreased E-cadherin promoter activity, and mRNA and protein expression. This inverse relationship between E-cadherin expression and iron was also observed in stained CRC tissue sections. Thus a possible consequence of iron loading is increased motility, invasiveness, and ultimately metastasis through reduced E-cadherin expression.⁴⁹ Consistent with this interpretation is the finding that reduced HEPH and FPN expression was associated with extensive vascular invasion and metastasis, respectively. How iron might alter E-cadherin transcriptional repression remains unclear although a candidate molecule could be the transcription factor Snail, a member of the Slug/Snail superfamily. Snail has been shown to repress E-cadherin expression through the E-box sequences in the proximal *E-cadherin* promoter and has been shown to be overexpressed in CRC.⁵⁰

In summary, the human colon expresses all of the proteins necessary to absorb inorganic iron. In colorectal carcinoma there was overexpression of components of the iron import machinery while components of the iron export protein machinery were either decreased in expression or mislocalised, suggesting a block on iron export. The net effect of these changes is to render the colonocytes iron rich. Despite this increased intracellular iron, expression of molecules such as DMT1 and TfR1 remains paradoxically high, suggesting that there may be inadequate sensing of intracellular iron or that there may be mechanisms modulating the expression of these proteins other than iron.

Our data would suggest that these changes in iron transport proteins are likely to impact on late stage disease with little evidence of a stepwise progression through the adenoma-carcinoma sequence. This lends support to existing evidence that iron mediates carcinogenesis in a background of existing genetic aberrations.⁶⁻¹⁰⁻¹²⁻¹³ However, this does not rule out an early role for iron in CRC as there are studies implicating iron in colonocyte proliferation and aberrant crypt foci development.¹³⁻⁵¹ Clearly performing experiments with primary and adenoma derived cell lines would be informative in addressing any potential early effects of iron in colorectal neoplasia.

The observed accumulation of iron in colonocytes could drive cell proliferation through modulation of cell cycle proteins and induction of reactive oxygen species culminating in DNA adduct formation and further mutagenesis, especially on a background of loss of DNA surveillance proteins. Moreover, as evidenced in the current study, iron is likely to cause repression of the cell adhesion protein E-cadherin, increasing cell motility and invasiveness and impacting on other intracellular pathways, including Wnt signalling.

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Authors' affiliations

M J Brookes, S Hughes, G Reynolds, N Sharma, T Ismail, C Tselepis*, Cancer Research UK Institute for Cancer Studies, University of Birmingham, UK

F E Turner, N Hotchin, School of Biosciences, University of Birmingham, UK
G Berx, Department for Molecular Biomedical Research, VIB-Ghent University, Belgium
A T McKie, Division of Nutritional Sciences, Kings College London, UK
G J Anderson, Iron Metabolism Laboratory, Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Brisbane, Queensland, Australia
T Iqbal*, Gastroenterology Unit, Walsgrave Hospital, UK

*C Tselepis and T Iqbal contributed equally to this work.

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Modulation of iron transport proteins in human colorectal carcinogenesis

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