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IGFBP-3 and IGFBP-10 (CYR61) up-regulation during the development of Barrett's oesophagus and associated oesophageal adenocarcinoma: potential biomarkers of disease risk

E. DI MARTINO[1], C. P. WILD[1], O. ROTIMI[2], S. J. DARNTON[3], J. R. OLLIVER[1], & L. J. HARDIE[1]

[1]*Molecular Epidemiology Unit, Centre for Epidemiology and Biostatistics, Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Leeds, UK,* [2]*Department of Histopathology, Leeds General Infirmary, Leeds, UK and* [3]*Department of Thoracic Surgery, Birmingham Heartlands Hospital, Birmingham, UK*

Abstract

Dys-regulation of the insulin-like growth factor (IGF) system increases the risk of a number of malignancies. The aim of this study was to investigate the role of members of the IGF binding protein (IGFBP) superfamily in the development of oesophageal adenocarcinoma (EAC) and their possible use as markers of disease risk. Expression of IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 was assessed using Real-Time-polymerase chain reaction (PCR) and immunohistochemistry in oesophageal tissues from Barrett's oesophagus (BE) patients with and without associated EAC, and in control subjects. IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 mRNA levels were up-regulated in Barrett's ($n=17$) and tumour tissue of EAC patients ($n=18$) compared with normal tissue of control subjects without BE or EAC ($n=18$) ($p<0.001$). Over-expression of IGFBP-3 and IGFBP-10/CYR61 proteins was observed in Barrett's, dysplastic and tumour tissue of EAC cases ($n=47$ for IGFBP-10; $n=39$ for IGFBP-3) compared with adjacent normal epithelium ($p<0.050$). Notably, IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 expression in Barrett's tissue of EAC cases ($n=17$) was significantly ($p<0.001$) higher than in Barrett's tissue of BE patients with no sign of progression to cancer ($n=15$). Overall, the results suggest that members of the IGFBP superfamily are up-regulated during oesophageal carcinogenesis and merit further investigation as markers of EAC risk.

Keywords: *IGFBP-3, IGFBP-10, CYR61, oesophageal adenocarcinoma, Barrett's oesophagus, insulin growth factor binding protein.*

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Introduction

Barrett's oesophagus (BE) is a pre-malignant lesion of the oesophageal epithelium associated with at least a 30-fold increased risk of developing oesophageal adenocarcinoma (EAC) (reviewed in Solaymani-Dodaran et al. 2004). Although BE patients have an increased risk of EAC, only 0.5–3.0% of them progress to cancer

Correspondence: L. Hardie, LIGHT Laboratories, Clarendon Way, University of Leeds, Leeds LS2 9JT, UK. Tel: +44-(0)113-343-6602. Fax: +44-(0)113-343-6603. E-mail: l.j.hardie@leeds.ac.uk

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every year (Shaheen et al. 2000). Consequently, the clinical management of these patients would greatly benefit from the identification of molecular markers and their introduction into clinical practice for stratification of cancer risk.

IGF-I and IGF-II are two potent serum growth factors with pro-proliferative and anti-apoptotic activity. Several studies have shown that dys-regulation of the IGF system increases the risk of a variety of tumours (Yu & Rohan 2000). Healthy individuals with raised IGF-I serum levels, for example, are at elevated risk of developing prostate, breast, and colon cancer (Chan et al. 1998, Hankinson et al. 1998, Giovannucci et al. 2000). The effects of these growth factors are modulated by a family of six IGF-binding proteins (IGFBP-1 to -6), which control the transport of IGFs from the circulation to the target tissue, modulate their interaction with specific receptors (IGF-IR and IGF-IIR), and influence their half-life (Rajaram et al. 1997, Firth & Baxter 2002). Furthermore, IGFBPs can exert IGF-independent effects on cell adhesion, proliferation, and apoptosis by associating with proteins of the extracellular matrix and on the cell membrane, or by interacting with nuclear receptors (Liu et al. 2000, Firth & Baxter 2002, Ricort 2004). In addition to up-regulation of IGFs, dys-regulation of IGFBPs expression is also common in a variety of malignancies. IGFBP-2 and/or IGFBP-3, for instance, are elevated in the plasma of patients with lung, colon, ovary, prostate, breast, gastric and oesophageal cancers (Kaaks et al. 2000, Hoeflich et al. 2001, Krajcik et al. 2002, Spitz et al. 2002, Franciosi et al. 2003, Sohda et al. 2004).

The IGFBP-related proteins, which comprise mac25, L56, ESM-1 and the members of the CYR61/CTGF/NOV (CCN) family (Hwa et al. 1999), share homology with IGFBPs at the N-terminus IGF-binding domain (Lau & Lam 1999, Brigstock 2003). The CCN proteins were initially proposed as divergent IGFBPs and called IGFBP-8 (CTGF), IGFBP-9 (Nov), and IGFBP-10 (CYR61) (Kim et al. 1997). However, as binding to IGF-I occurs with lower affinity than for IGFBPs, there is some debate about whether they are part of the IGFBP superfamily (Grotendorst et al. 2000). Nevertheless, in similar fashion to IGFBPs, CCN proteins can modulate cell adhesion and proliferation, and play an important role in cancer development and progression (Hwa et al. 1999). Up-regulation of IGFBP-10/CYR61, for example, is associated with expression of genes involved in the remodelling of the extracellular matrix (MMPs and TIMPs), angiogenesis (VEGF), and adhesion (collagens and integrins), and has been reported in invasive breast cancer cell lines (Xie et al. 2001, Tsai et al. 2002). IGFBP-8/CTGF, on the other hand, is over-expressed in oesophageal cancer tissue, and its levels influence overall patient survival (Koliopanos et al. 2002).

Although the dys-regulation of members of the IGFBPs superfamily has been linked to a number of malignancies, their role in the neoplastic transformation of the oesophageal epithelium has not been thoroughly investigated. IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 were recently identified within our laboratory as genes often dys-regulated in EAC cell lines, by using cDNA arrays (di Martino et al. 2005). The present study has examined the expression of these genes in oesophageal adenocarcinoma (EAC) and its precursor lesion, Barrett's oesophagus (BE), with the aim of assessing their utility as markers of EAC risk. Our results suggest that members of the IGFBP and CCN families are dys-regulated in pre-neoplastic and neoplastic oesophageal tissues and deserve further investigation as molecular markers of cancer risk.

Materials and methods

Tissues for RNA analysis

EAC cases. Oesophageal tissue samples were collected from 18 patients (14 males and four females; mean age 68 years, range 42–82 years) undergoing oesophageal resection for EAC at Birmingham Heartlands Hospital, Birmingham, UK, between 1990 and 1998. Resected specimens were examined pathologically and representative sections of primary tumour tissue ($n=18$), Barrett's epithelium ($n=17$) and, where possible, matched normal oesophageal mucosa ($n=5$) were collected for RNA analysis. Additional sections were collected for histological confirmation of Barrett's epithelium and tumour. Barrett's epithelium was defined as the presence of intestinal metaplasia containing goblet cells. All tumours were confirmed to contain more than 80% of neoplastic cells. Notably, none of the patients received chemo- or radiotherapy before surgery.

Barrett's cases without malignancy. Oesophageal biopsies were collected from the Barrett's epithelium of 15 BE patients (ten males and five females; mean age 60 years, range 40–74 years) with no evidence of cancer, recruited from Barrett's surveillance clinics at Leeds General Infirmary, Leeds, UK, between 2001 and 2003. Additional biopsies were taken in adjacent positions, and histological examination confirmed the presence of specialized intestinal metaplasia including goblet cells, with the absence of any evidence for dysplasia or malignant transformation.

Control subjects without BE or EAC. Squamous oesophageal biopsies were obtained from 18 patients (five males and 13 females; mean age 52 years, range 36–77 years) undergoing endoscopy who had no evidence for oesophagitis, BE or EAC. Indication for endoscopy in these patients was primarily dyspepsia and epigastric abdominal pain but also included dysphagia, diarrhoea, weight loss, nausea, and suspected coeliac disease. Histological analysis of biopsies from these patients confirmed normal squamous epithelium and the absence of intestinal metaplasia, dysplasia, or cancer in these individuals.

RNA extraction

Samples for RNA analysis were immediately snap-frozen after collection and maintained in liquid nitrogen until RNA extraction, which was performed using the RNA/DNA Midi kit (Qiagen Ltd, Crawley, UK) (EAC resections) or the TriReagent™ kit (Sigma-Aldrich Ltd, Gillingham, UK) (biopsies). Samples were DNAse treated using the DNA-free™ kit (Ambion Ltd, Huntingdon, UK). RNA integrity was assessed by verifying the presence of the 18S and 28S ribosomal bands via electrophoresis on a 1.5% agarose gel.

Real-Time-polymerase chain reaction (PCR)

cDNA was synthesized from 1–5 μg of DNAse-treated RNA using 50 ng of Oligo(dT) primers and the other reagents included in the RT-Thermoscript™ RT-PCR System kit (Invitrogen Ltd, Paisley, UK), according to the manufacturer's instructions. A total of 1 μl of cDNA was then amplified with a GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Warrington, UK), using 300 nM specific primers

(Invitrogen Ltd) and 1 × Sybr®Green PCR Master Mix (Applied Biosystems), in a total volume of 20 µl. Polymerase chain reaction (PCR) conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Primers for Real-Time PCR were designed using Primer Express® 2.0 Software (Applied Biosystems) to span exon–exon boundaries and had sequences: 5′-ATGGGCGAGGGCACTTG-3′ and 5′-GTCATCGCCATTGTCTGCAA-3′ for *IGFBP2* (GenBank Accession Number = NM_000597), 5′-CTCTACGGCAGGGACCATATTC-3′ and 5′-ACTACGAGTCTCAGAGCACAGATACC-3′ for *IGFBP3* (NM_000598), 5′-CCCACGAGGACCTCTACATCA-3′ and 5′-GAGCTGGGTGACACTGCTTG-3′ for *IGFBP4* (NM_001552), 5′-CTGAAGCGGCTCCCTGTTT-3′ and 5′-TGAACAATACATTTCTGGCCTTGT-3′ for *CYR61* (NM_001554), 5′-GGGCTGGCAAGCATGTG-3′, and 5′-GGTAGGTGCCAGTGCGAACT-3′ for *TUBA3* (NM_006009). Primer specificity for the target of interest was verified using the Blast program from the National Center for Biotechnology Information (Altschul et al. 1990). Each amplification reaction was performed in duplicate or triplicate. Negative controls, for which the cDNA was omitted, were included in each plate. A positive control sample was included to account for variability across different plates. Lack of primer dimers or other non-specific products was verified for each reaction by examining the dissociation curve. Expression of the genes of interest was normalized for the expression of tubulin alpha-3 chain (*TUBA3*). This gene was selected as an internal control after evaluation of a number of other genes (*GAPDH*, *HPRT*, *ACTB*), as it showed the least variability across patients. Gene expression was calculated using the $\Delta\Delta C_t$ method, although for a subset of samples levels were also calculated using the standard curve method, without significant differences in the results.

Tissues for immunohistochemistry (IHC)

EAC cases. Paraffin-embedded tissues from 47 EAC patients (37 males and ten females; mean age 66 years, range 46–87 years) who underwent oesophageal resection at the Leeds General Infirmary between 1985 and 2000 were retrieved from the archive of the Institute of Pathology. None of the patients selected for the study had received pre-operative chemo- or radiotherapy. For each case, all available haematoxylin- and eosin-stained slides were reviewed by a histopathologist (OR) and a representative block was selected for immunohistochemical analysis. A total of 47 tumour samples were stained for IGFBP-10/CYR61 and 39 for IGFBP-3. Beside tumour tissue, the majority of the specimens also contained adjacent normal squamous ($n = 33$ for IGFBP-10/CYR61 and $n = 23$ for IGFBP-3), Barrett's ($n = 40$ for IGFBP-10/CYR61 and $n = 31$ for IGFBP-3), and dysplastic tissue ($n = 22$ for IGFBP-10/CYR61 and $n = 12$ for IGFBP-3). For each patient, information was collected regarding sex, date of birth, age at surgery, depth of invasion of the oesophageal wall (T_1 to T_4), lymph node positivity, and involvement of the surgical circumferential resection margin (CRM). The CRM was considered as positive if tumour cells were detected at or within 1 mm of the resection margin.

Barrett's cases. Matched Barrett's and normal squamous oesophageal biopsies were collected from 38 BE patients (26 males and 12 females; mean age 58 years, range 32–79 years) recruited within the Barrett's surveillance clinics at the Leeds General Infirmary, Leeds between 2001 and 2003. A total of 38 (IGFBP-3) or 36 (IGFBP-10/

CYR61) normal squamous tissues and 35 (IGFBP-3) or 34 (IGFBP-10/CYR61) matched adjacent BE tissues were used for the IHC study.

IHC

Tissue sections (3 μm) were de-waxed with xylene (3 × 5 min) (Genta Medical, York, UK), rehydrated, and treated with 1.5% H_2O_2 (VWR International Ltd, Poole, UK) in methanol (Rathburn Chemicals Ltd, Peebles, UK) for 30 min to block endogenous peroxidases. Antigen retrieval was performed by heating for 2 min in 0.01 M sodium citrate buffer, pH 6.0, in a pressure cooker. Endogenous biotin was blocked using the Avidin-Biotin Blocking kit (Vector Laboratories Ltd, Peterborough, UK). Slides were incubated overnight at 4°C with 1:100 murine monoclonal antibody directed against recombinant full-length human IGFBP-3 (AbCam Ltd, Cambridge, UK; code no. ab10733), or for 1 h at room temperature with 1:200 rabbit polyclonal antibody directed against amino acids 163–240 of IGFBP-10/CYR61 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany; code no. sc-13100). Detection of the primary antibody was performed using a mixture of biotinylated anti-mouse and anti-rabbit IgG and with HRP-streptavidin, both from the IHC Select Kit (Chemicon Europe Ltd, Hampshire UK). Staining was developed by exposing the slides to 3,3′-diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories Ltd). Negative control slides, for which the primary antibody was omitted, were included in each staining batch. Positive control slides were included in each batch to assess reproducibility of the staining. Slides were scored for percentage of positive cells (0 = 0–5%, 1 = 6–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%), intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong), and localization of staining by a histopathologist (OR), blinded to patient clinicopathological data and the results of the mRNA study. The total IHC value was calculated as the score for percentage of positive cells multiplied by the score for intensity of staining, as described previously (Heideman et al. 2001).

Ethical approval

Tissues for RNA and protein analysis were collected following informed consent and ethical approval from the Research Ethics Committee of the United Leeds Teaching Hospitals Trust (biopsies) or the Birmingham Heartlands Hospital (EAC resections). The paraffin-embedded EAC resections stored in the archive of the Institute of Pathology of the Leeds General Infirmary were retrieved and utilized for protein analysis after obtaining permission from the Research Ethics Committee of the United Leeds Teaching Hospitals Trust.

Statistical analysis

Average values for mRNA and protein expression in different groups were compared using the Mann–Whitney *U*-test, while the frequency of discrete variables across two different groups was compared using the Pearson χ^2-test. Statistical analysis was performed using the SPSS 12.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA). A $p \leq 0.05$ was accepted as being statistically significant.

Results

IGFBPs mRNA expression

In EAC patients, an increase in the average mRNA expression of IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 but not IGFBP-2 was measured in resected Barrett's and tumour tissue when compared with matched normal squamous tissue (Figure 1). However, with the exception of the over-expression of IGFBP-3 in the tumour tissue ($p = 0.025$), none of these differences reached statistical significance, possibly due to the small number of normal squamous samples available for the cancer patients and the wide heterogeneity in mRNA expression observed across subjects. When average expression in Barrett's and tumour tissue of EAC cases was compared with levels in normal oesophageal epithelium of control subjects without BE or EAC, a statistically significant increase was observed for IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 ($p < 0.001$) but not IGFBP-2 ($p > 0.050$) (Figure 1). The highest IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 mRNA expression was detected in Barrett's tissue, while the levels in tumour tissue were slightly decreased but still significantly above those in squamous epithelium of control subjects ($p < 0.001$). To assess whether the observed increase of IGFBP-3, IGFBP-4 and IGFBP-10/CYR61 in pre-malignant epithelium of cancer patients was specific and indicative of BE patients who had developed EAC, we investigated gene expression in Barrett's epithelium of 15 patients who did not have any evidence of malignancy. It was found that expression of these three genes was significantly higher in the Barrett's tissue of EAC cases compared with levels in Barrett's tissue of patients without EAC ($p < 0.001$) (Figure 2).

IGFBP-3 and IGFBP-10/CYR61 protein expression

IGFBP-3 and IGFBP-10/CYR61 protein expression were further investigated in oesophageal tissue of BE patients with and without associated EAC. Immunohistochemical analysis of IGFBP-4 in oesophageal tissues is not reported as attempts to detect this protein by immunohistochemistry generated indistinct staining patterns.

In EAC cases, analogous to the mRNA results, IGFBP-3 protein expression was significantly up-regulated in Barrett's, dysplastic, and tumour tissue of EAC cases compared with adjacent normal squamous tissue ($p < 0.050$) (Figure 3). Intense and widespread IGFBP-3 staining was commonly detected in pre-malignant and malignant tissue while staining in the adjacent squamous mucosa was weaker and mainly localized in the basal epithelial layers (Table I and Figure 4a–c). Mean IGFBP-3 tumour expression did not vary with T-stage, differentiation, or lymph node positivity ($p > 0.050$).

IGFBP-3 protein up-regulation in pre-malignant epithelium was not exclusive to BE patients with EAC, as it was also observed in the Barrett's tissue of patients without EAC when compared with their normal squamous tissue ($p = 0.012$) (Figure 3). In contrast with the mRNA results, no differences in the average IGFBP-3 protein levels were detected between Barrett's tissue of individuals with or without associated EAC ($p > 0.050$). However, a significant difference in protein localization was observed. Specifically, 50% (12/24) of BE of EAC cases showed nuclear IGFBP-3 staining in Barrett's tissue, compared with only 23% (6/27) of BE in non-cancer patients ($p = 0.038$, Pearson χ^2) (Table I and Figure 4b). In the other tissue types IGFBP-3 staining was mainly localized in the cytoplasm and nuclear localization was

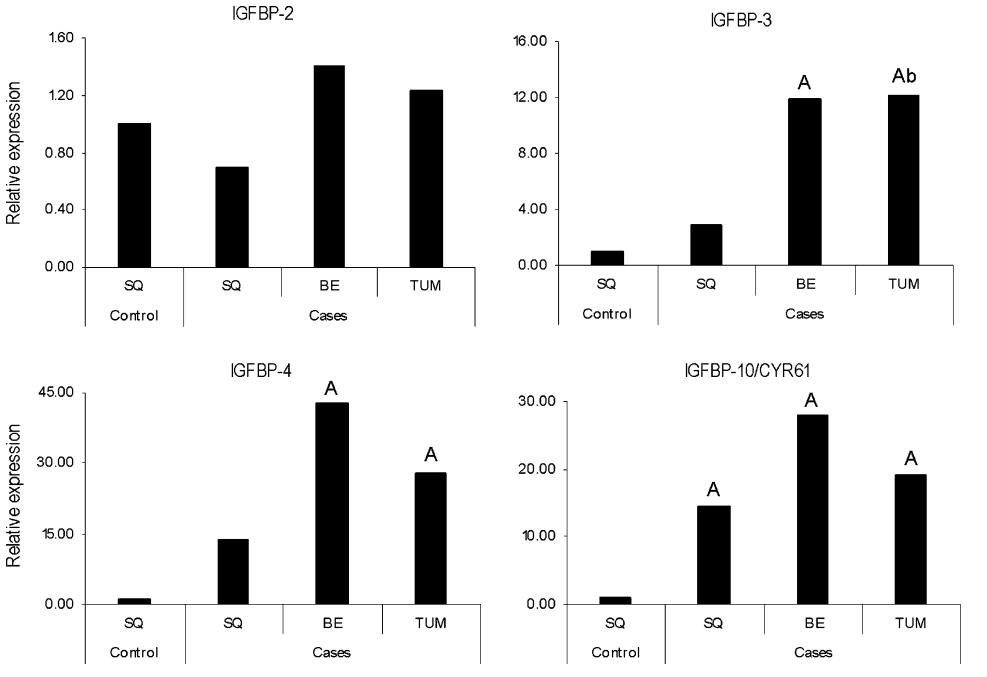

Figure 1. Average expression of IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-10 mRNA in resected normal squamous (SQ), Barrett's (BE), and tumour (TUM) tissue of cancer cases, expressed as a ratio to the average expression in normal tissue of controls. Statistically significant differences compared with SQ of control and SQ of cases are indicated as A and B, respectively, in upper case ($p < 0.01$) and lower case ($p < 0.05$).

observed only occasionally in positive tumours (7%, 2/30) or dysplastic tissue (10%, 1/10) (Table I).

IGFBP-10/CYR61 protein was also significantly over-expressed in Barrett's, dysplastic, and tumour tissue of EAC cases compared with their normal squamous tissue ($p < 0.001$) (Figure 3). Moderate to strong and widespread IGFBP-10/CYR61 staining was detected more frequently in Barrett's, dysplastic and tumour tissues than in adjacent squamous tissues (Table II and Figure 4d–f). Notably, as observed for the mRNA, the highest protein levels were detected in Barrett's and dysplastic tissue. Expression was decreased in tumour tissue compared with dysplastic tissue ($p = 0.025$), although still significantly above levels of the normal tissue ($p = 0.008$) (Figure 3). Similar to the observations with IGFBP-3, IGFBP-10/CYR61 protein up-regulation was also detected in Barrett's tissue of patients with no sign of cancer compared with adjacent normal squamous epithelium ($p < 0.001$) (Figure 3 and Table II). In contrast with the mRNA results, however, down-regulation of IGFBP-10/CYR61 was observed in Barrett's tissue of cancer cases compared with BE epithelium of patients without cancer ($p = 0.012$).

As expected, IGFBP-10/CYR61 staining was mainly observed in the cytoplasm, although in a small proportion of samples IGFBP-10/CYR61 was also located in the nucleus (Figure 4e). Interestingly, the highest occurrence of nuclear staining was seen in the dysplastic and tumour tissue (14 and 13%, respectively), followed by Barrett's tissue (5%) and normal squamous epithelium (3%) of EAC cases, while no nuclear

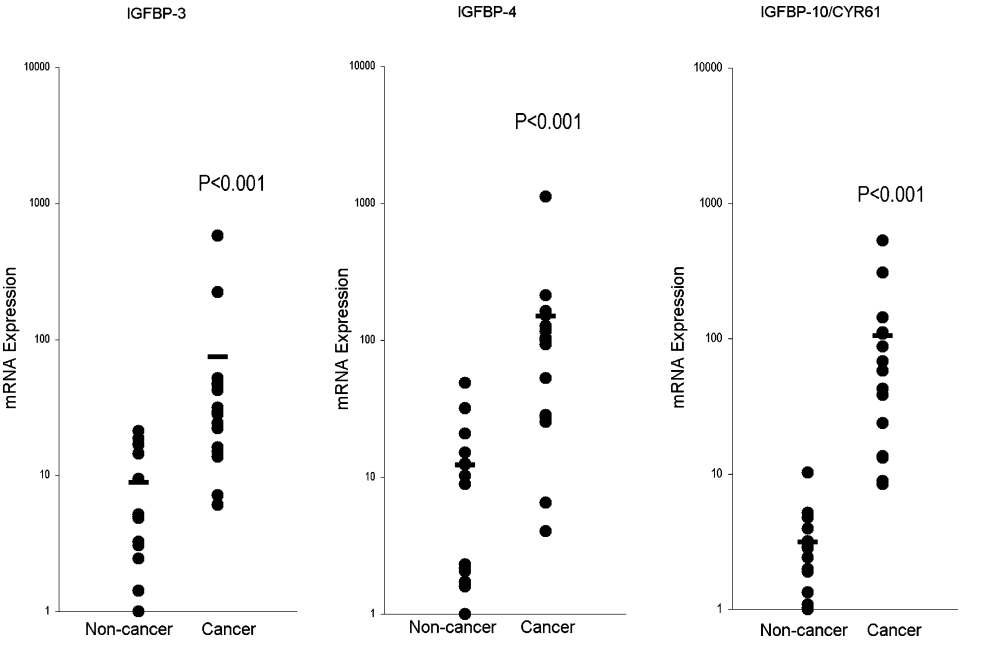

Figure 2. Expression of IGFBP-3, IGFBP-4 and IGFBP-10/CYR61 mRNA in Barrett's tissues of EAC cancer and BE patients with no histological evidence of progression. Mean values are indicated by horizontal lines.

staining was observed in the normal and Barrett's tissue of non-cancer patients (Table II). Notably, in half of the cases (23/47) a stronger IGFBP-10/CYR61 expression was observed at the invasive front of the tumour or in areas of vascularization. However, no differences in lymph node status, CRM status, or depth of invasion were observed between tumours showing a stronger IGFBP-10/CYR61 staining at the invasive edge and the tumours with a more homogeneous staining pattern ($p > 0.050$, Pearson χ^2). Additionally, no association was found between IGFBP-10/CYR61 IHC score and lymph node status, invasion, tumour differentiation, CRM positivity, or age at on-set ($p > 0.050$) (data not shown).

Discussion

Although dys-regulation of the IGF system has been associated with a predisposition to a number of malignancies (Yu & Rohan 2000, Juul 2003), the role of the IGFBP superfamily in oesophageal carcinogenesis has not been thoroughly investigated and little information is available regarding the possible use of these proteins as markers of EAC risk. This study is, therefore, the first comprehensive investigation describing the patterns of IGFBPs expression in the oesophagus. The results show that IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 are expressed in oesophageal tissue, and that their levels tend to increase during malignant transformation. Specifically, a significant IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 mRNA up-regulation was detected during the progression from normal oesophageal epithelium to adenocarcinoma. Over-expression of IGFBP-3 and IGFBP-10/CYR61 in metaplastic, dysplastic, and tumour tissue of EAC cases was also confirmed at the protein level.

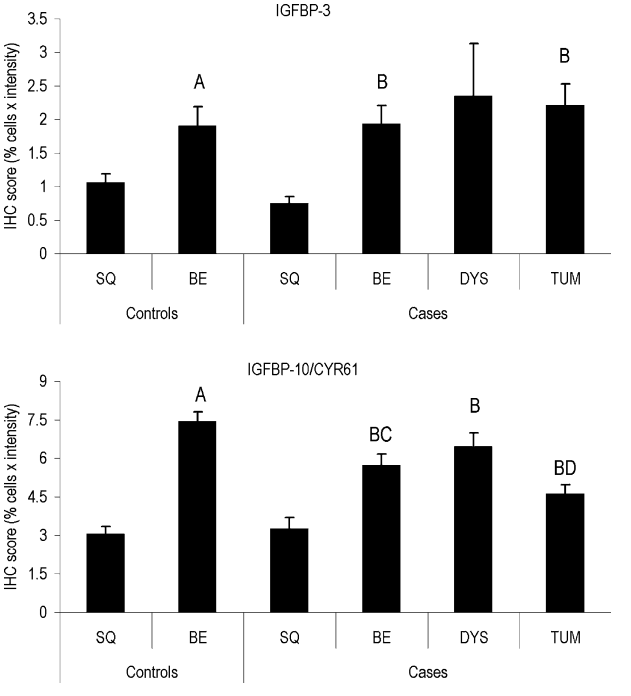

Figure 3. IGFBP-3 and IGFBP-10/CYR61 protein expression in squamous (SQ), Barrett's (BE), dysplastic (DYS), and tumour (TUM) tissue of EAC cases and in squamous and Barrett's tissue of controls expressed as the mean ± standard error. Significant differences from SQ of control, SQ of cases, BE of control, and DYS are indicated by A, B, C and D, respectively.

Table I. Intensity and cellular localization of IGFBP-3 protein staining.

	Tissue type†					
	SQ controls‡	*BE controls*	*SQ cases*	*BE cases*	*DYS cases*	*TUM*
Staining intensity						
negative	19% (7/38)	23% (8/35)	30% (7/23)	23% (7/31)	17% (2/12)	23% (9/39)
weak	76% (29/38)	40% (14/35)	65% (15/23)	45% (14/31)	58% (7/12)	41% (16/39)
moderate-strong	5% (2/38)	37% (13/35)	5% (1/23)	32% (10/31)	25% (3/12)	36% (14/39)
Positive cells						
negative	18% (7/38)	23% (8/35)	30% (7/23)	23% (7/31)	17% (2/12)	23% (9/39)
1–50%	79% (30/38)	63% (22/35)	70% (16/23)	68% (21/31)	58% (7/12)	57% (22/39)
51–100%	3% (1/38)	14% (5/35)	0% (0/23)	9% (3/31)	25% (3/12)	20% (8/39)
Localization						
cytoplasmic	100% (31/31)	78% (21/27)	100% (16/16)	50% (12/24)	90% (9/10)	93% (28/30)
cytoplamic and/or nuclear	0% (0/31)	22% (6/27)	0% (0/16)	50% (12/24)	10% (1/10)	7% (2/30)
Average IHC score ± SE	1.05 ± 0.14	1.89 ± 0.30	0.74 ± 0.11	1.92 ± 0.29	2.34 ± 0.79	2.20 ± 0.33

†SQ, squamous tissue; BE, Barrett's oesophagus tissue; DYS, dysplastic tissue; TUM, tumour tissue.
‡Controls are defined as BE patients with no evidence of progression to cancer.

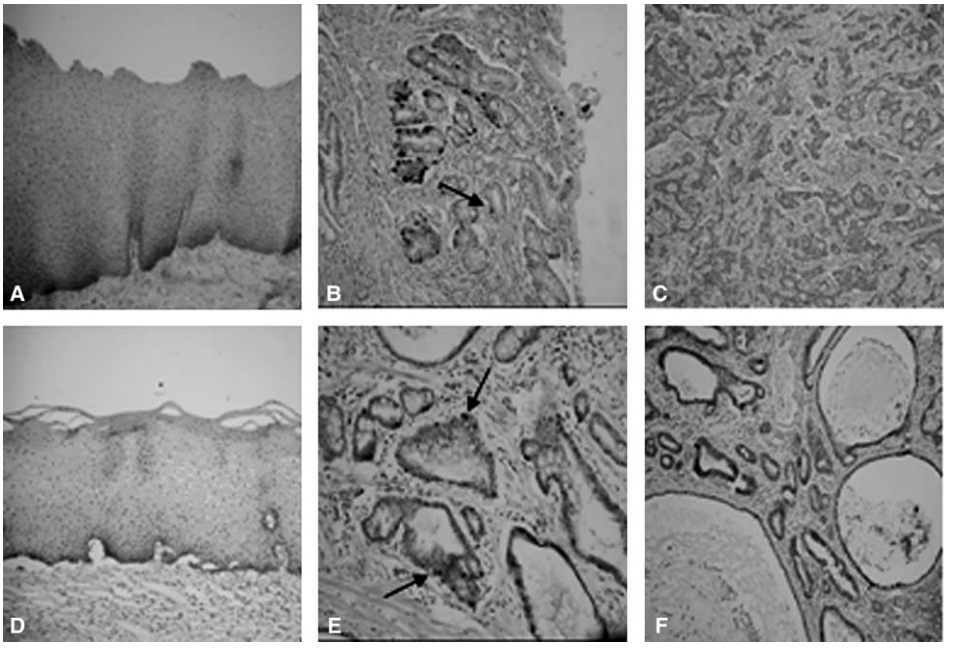

Figure 4. IGFBP-3 and IGFBP-10/CYR61 protein localization in oesophageal tissue. (A) Weak IGFBP-3 staining in normal squamous mucosa of a BE patient without cancer (original magnification ×32); (B) intense IGFBP-3 nuclear (arrow) and cytoplasmic staining in Barrett's tissue of an EAC case (original magnification ×80); (C) widespread IGFBP-3 staining in a tumour specimen (original magnification ×80); (D) IGFBP-10 staining in basal layers of normal squamous epithelium (original magnification ×32); (E) IGFBP-10 nuclear (arrows) and cytoplasmic staining in Barrett's tissue of a patient with EAC (original magnification ×80); and (F) widespread IGFBP-10 staining in tumour tissue (original magnification ×80).

Table II. Intensity and cellular localization of IGFBP-10/CYR61 protein staining.

	Tissue type[†]					
	SQ controls‡	*BE controls*	*SQ cases*	*BE cases*	*DYS cases*	*TUM*
Staining intensity						
negative	0% (0/36)	0% (0/34)	0% (0/33)	0% (0/40)	0% (0/22)	4% (2/47)
weak	69% (25/36)	12% (4/34)	73% (24/33)	45% (18/40)	27% (6/22)	41% (19/47)
moderate-strong	31% (11/36)	88% (30/34)	27% (9/33)	55% (22/40)	73% (16/22)	55% (26/47)
Positive cells						
negative	0% (0/36)	0% (0/34)	0% (0/33)	0% (0/40)	0% (0/22)	4% (2/47)
1–50%	66% (24/36)	6% (2/34)	58% (19/33)	17% (7/40)	0% (0/22)	22% (10/47)
51–100%	34% (12/36)	94% (32/34)	42% (14/33)	83% (33/40)	100% (22/22)	74% (35/47)
Localization						
cytoplasmic	100% (36/36)	100% (34/34)	97% (32/33)	95% (38/40)	86% (19/22)	87% (39/45)
cytoplasmic and nuclear	0% (0/36)	0% (0/34)	3% (1/33)	5% (2/40)	14% (3/22)	13% (6/45)
Average IHC score ± SE	3.03 ± 0.32	7.41 ± 0.40	3.24 ± 0.45	5.57 ± 0.46	6.43 ± 0.56	4.60 ± 0.37

[†]SQ: squamous tissue; BE: Barrett's esophagus tissue; DYS: dysplastic tissue; TUM: tumour tissue.
[‡]Controls are defined as BE patients with no evidence of progression to cancer.

To our knowledge, IGFBP-2 and IGFBP-4 have not been previously examined in relation to EAC. IGFBP-10/CYR61 was one of 38 genes found to be altered in Barrett's epithelium using microarray analysis (Barrett et al. 2002), consistent with the early dys-regulation of this gene observed herein. With respect to IGFBP-3, the results are in agreement with two previous reports detailing the up-regulation of this gene in oesophageal tumorigenesis (Takaoka et al. 2004, Hansel et al. 2005). The first study (Takaoka et al. 2004) focused mainly on squamous cell carcinoma, but IGFBP-3 over-expression was also detected in a small number of EAC ($n=7$). A subsequent independent investigation (Hansel et al. 2005) described up-regulation of IGFBP-3 in dysplastic and tumour tissue of EAC; this study also reported IGFBP-3 expression pattern in Barrett's tissue of EAC cases, but included only a limited number of specimens ($n=10$). The present study, therefore, not only confirms these earlier findings, but also adds novel information by analysing IGFBP-3 expression in a larger series of Barrett's tissue samples from EAC patients and in normal and Barrett's epithelium of non-cancer cases.

Notably, up-regulation of IGFBP-3 and IGFBP-10/CYR61 was detected in Barrett's tissue of both EAC and BE patients without cancer, suggesting that over-expression of IGFBPs may represent an early molecular change during the intestinal metaplasia–dysplasia–adenocarcinoma sequence. As the IGF system has been implicated in wound and oesophageal mucosa healing (Tchorzewski et al. 1998, Chen et al. 2001), it is possible that the early up-regulation of IGFBPs may be triggered by the reflux-induced injury of the oesophageal mucosa. Interestingly, a recent study has suggested that IGFBP-3 up-regulation in oesophageal cells may be mediated by the epithelial growth factor receptor (EGFR) (Takaoka et al. 2004). Epithelial growth factor (EGF) signalling is known to play a crucial role in oesophageal healing (Marcinkiewicz et al. 1998), supporting, therefore, our hypothesis that IGFBPs expression may be part of the physiological response to epithelial damage.

Interestingly, the *EGFR* and *IGFBP3* genes lie in close proximity on chromosome 7p, a region often amplified in pre-neoplastic and neoplastic oesophageal tissue (Riegman et al. 2001). Polysomy or amplifications of chromosome region 17q, to which *IGFBP-4* maps, are also common in dysplastic and EAC tissue (Riegman et al. 2001, Walch et al. 2001). Therefore, an increase in gene copy number could drive the over-expression of both IGFBP-3 and IGFBP-4 observed during oesophageal carcinogenesis. It could be argued that the up-regulation of IGFBPs may possibly occur as an indirect effect of the selective advantage offered by the amplification of other oncogenes located in close chromosomal proximity. For example, the *ERBB2* oncogene, which is often up-regulated in EAC tissue, similar to *IGFBP4* also maps to 17q (Dahlberg et al. 2004). Such an argument, however, would not account for the over-expression of IGFBP-10, as aneuploidy or amplification of the chromosomal region 1p, to which IGFBP10/CYR61 maps, are uncommon in EAC (Jenkins et al. 2002). Furthermore, the fact that three members of the same gene family showed consistent up-regulation during tumour development supports an important and direct role of IGFBPs in oesophageal malignant transformation.

Up-regulation of IGFBPs could confer a selective advantage to pre-malignant oesophageal cells by enhancing IGF pro-proliferative and anti-apoptotic effects. Indeed, IGF-I has been shown to induce the proliferation of oesophageal cells *in vitro* (Qureshi et al. 1997). Alternatively, IGFBPs may support cancer development via

IGF-independent mechanisms. The fact that IGFBP-3 nuclear staining was significantly more common in Barrett's tissue of cancer cases than in subjects without cancer suggests that IGFBP-3 may favour BE and/or EAC development through its ability to bind to nuclear receptors, such as RXR-α and RAR-α (Liu et al. 2000, Schedlich et al. 2004), which mediate the expression of a variety of genes involved in cell proliferation, differentiation and apoptosis (Chambon 1996). Interestingly, dys-regulation of RXR-α and RAR-α is common in BE and associated EAC tissue (Lord et al. 2001, Brabender et al. 2004), suggesting that abnormalities in these pathways could indeed contribute to oesophageal transformation. Apart from the frequent nuclear localization of IGFBP-3, we also noted a progressive increase in the occurrence of nuclear IGFBP-10/CYR61 in oesophageal tissue during the intestinal metaplasia–dysplasia–EAC sequence, suggesting that the nuclear localization of this protein may also have a role during cancer development. This result is intriguing, as localization of IGFBP-10/CYR61 in the nucleus has not been previously described.

Interestingly, the comparison between BE tissue of patients with and without cancer indicated that IGFBP-3, IGFBP-4, and IGFBP-10/CYR61 mRNA levels may be a good indicator of cancer risk. Specifically, IGFBPs mRNA levels in BE tissue of cancer cases were significantly higher than levels measured in uncomplicated BE. These differences, however, were not apparent at a protein level. The discrepancy between mRNA and protein results could be because IGFBPs are secreted by the liver into the circulation as well as being produced locally by a variety of tissues (Rajaram et al. 1997, Juul 2003). Serum IGFBPs may, therefore, have influenced the overall levels of protein detected in the oesophageal tissues by IHC and caused the inconsistencies observed between the results of the protein and mRNA parts of the study. Local production of IGFBPs by oesophageal cells, as indicated by the mRNA level, may be a more appropriate marker of cancer risk, because it could directly reflect molecular events occurring in pre-malignant oesophageal cells, such as the dys-regulation of the EGF pathway or the accumulation of chromosome aberrations. Therefore, these preliminary results suggest that IGFBPs mRNA levels deserve further investigation as putative markers of EAC risk.

One of the strengths of this investigation is the fact that all samples for mRNA and protein analysis were collected from patients who had not undergone any chemo- or radiotherapy, eliminating potential confounding effects due to molecular changes that could have been induced by the therapeutic treatment. Since the current management of EAC patients frequently includes the use of pre-operative adjuvant therapy, untreated matched oesophageal specimens such as those used in this study are particularly rare and difficult to obtain.

In conclusion, this study provides novel information concerning the expression of members of the IGFBPs superfamily in oesophageal tissue and their role in the development of EAC. The over-expression of these genes during the early and later stages of progression to cancer was demonstrated at both the mRNA and protein level. IGFBP-3 nuclear localization was found to be significantly more frequent in BE tissue of patients with EAC than in BE patients without cancer, suggesting that IGFBPs over-expression may favour EAC development through interaction with nuclear receptors. Our results also indicate that IGFBP mRNA levels are interesting candidate markers of EAC risk and in this context merit further investigation.

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