# IGFBP7 is associated with poor prognosis in oesophageal adenocarcinoma and is regulated by promoter DNA methylation

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**Background:** We examined whether silencing of IGFBP7 was associated with survival in patients with oesophageal adenocarcinoma.

**Methods:** Protein expression of IGFBP7 was determined using immunohistochemistry in a tissue microarray representing tumours from 65 patients with oesophageal adenocarcinoma who had not had neoadjuvant therapy. DNA methylation of the IGFBP7 promoter was determined with the melt curve analysis in cell lines and patient tissues.

**Results:** Expression of IGFBP7 was observed in the oesophageal adenocarcinoma of 34 out of 65 (52%) patients and was associated with significantly reduced median (11 vs 92 months) and 5-year survival (25% vs 52%). Multivariate analysis identified expression as an independent prognostic indicator for survival (hazard ratio = 3.24, 95% confidence interval = 1.58–6.67, $P$-value = 0.0014). Hypermethylation of IGFBP7 was associated with silencing of gene expression in cell lines and patient tissues ($P$-value = 0.0225). Methylation was observed in the squamous mucosa of 2 out of 15 (13%) patients with Barrett's oesophagus and 3 out of 17 (18%) with oesophageal adenocarcinoma. Methylation was observed in 14 out of 18 (78%) of biopsies of Barrett's mucosa and 23 out of 34 (68%) patients with oesophageal adenocarcinoma.

**Conclusion:** Reduced IGFBP7 protein expression was associated with longer survival in patients with oesophageal adenocarcinoma. Methylation of the IGFBP7 promoter was associated with silencing of gene expression and was frequent in Barrett's oesophagus and oesophageal adenocarcinoma.

Oesophageal adenocarcinoma has increased rapidly in incidence in the Western world over recent decades and continues to have a poor prognosis, with 5-year survival rates of <10% (Umar and Fleischer, 2008). Approximately 0.1–0.3% of patients with the premalignant lesion Barrett's oesophagus will develop oesophageal adenocarcinoma each year (Bhat et al, 2011; Hvid-Jensen et al, 2011). The majority of patients present with late-stage disease that is not amenable to resection and have a poor response to non-surgical therapy.

The insulin-like growth factor (IGF) signalling pathway has a key role in mediating normal growth and development, and in the malignant progression of many cancers including oesophageal adenocarcinoma (Doyle et al, 2012). The IGF system involves the complex coordination of growth factors (IGF1 and IGF2), cell surface receptors (IGF1R, IGF2R and INSR), high- and low-affinity IGF-binding proteins (IGFBPs), as well as the IGFBP-degrading proteases. The IGFBPs serve as carrier proteins for IGFs, prolonging their half-life and modulating their binding to the

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IGF receptors. Some IGFBP proteases cleave IGFBPs, release IGFs, and increase signalling. IGFBP7 is one of 16 members of the IGFBP superfamily of proteins. It binds IGFs with relatively low affinity but binds insulin with very high affinity (500-fold higher than other IGFBPs) (Burger *et al*, 2005).

Changes in DNA methylation are frequent, early events in carcinogenesis, widely present even in premalignant lesions such as Barrett's oesophagus (Smith *et al*, 2008, 2010). Hypermethylation in promoter regions is associated with gene silencing, whereas hypomethylation in other regions is associated with genomic instability. DNA methylation is of clinical interest because it may lead to biomarkers for early detection, diagnosis, prognosis, therapeutic stratification, and post-therapeutic monitoring. Down-regulation of IGFBP7 with promoter DNA methylation has been demonstrated in various cancers (Komatsu *et al*, 2000; Ruan *et al*, 2006; Lin *et al*, 2007; Jiang *et al*, 2008; Lin *et al*, 2008; Hinoue *et al*, 2009; Suzuki *et al*, 2010; Heesch *et al*, 2011; Chen *et al*, 2011b; Sullivan *et al*, 2012; Dimberg *et al*, 2013; Suzuki *et al*, 2013) but has not been previously reported in Barrett's oesophagus or oesophageal adenocarcinoma.

Here we report the correlation between IGFBP7 expression and clinicopathological parameters, including survival, in patients with oesophageal adenocarcinoma, and the association between IGFBP7 expression and DNA methylation in oesophageal adenocarcinoma and Barrett's oesophageal cell lines and tissues.

## MATERIALS AND METHODS

**Tissue microarrays and immunohistochemistry.** Tissue microarrays (TMAs) were constructed as previously described (Thompson *et al*, 2011). Sections (4 μm) were mounted on polylysine-coated slides, dewaxed and rehydrated. Antigen retrieval was performed by heating the sections for 5 min in $10 \, \mathrm{mmol} \, l^{-1}$ citrate buffer (pH 6) in a microwave pressure cooker. After cooling to room temperature, sections were immunostained using an Autostainer Plus (Dako, Glostrup, Denmark). Sections were incubated for 60 min with a 1 : 75 dilution of rabbit anti-human IGFBP7 polyclonal IgG antibody (H-102; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and then with MACH 4 Universal Horseradish Peroxidase-Polymer (Biocare Medical, Concord, CA, USA). Liquid 3,3-diaminobenzidine (Dako) was used as the chromogen, and sections were counterstained with Meyer's haematoxylin. The expression of IGFBP7 was scored as positive (present) or negative (absent) by an experienced gastrointestinal pathologist (ARR).

**Cell lines.** Human oesophageal adenocarcinoma (OE33, OE19, and JH-EsoAd1 (Alvarez *et al*, 2008)) and squamous cell carcinoma (OE21 and TE7 (Nishihira *et al*, 1993)) cell lines were maintained in RPMI-1640 supplemented with 10% foetal bovine serum, $4 \, \mathrm{mmol} \, l^{-1}$ L-glutamine, $200 \, \mathrm{U} \, \mathrm{ml}^{-1}$ penicillin, and $200 \, \mu g \, \mathrm{ml}^{-1}$ streptomycin at 37 °C in a humidified incubator with 5% $CO_2$ in air. The human telomerase immortalised Barrett's oesophagus epithelial cells, CP-A, CP-B, and CP-D were maintained as previously described (Palanca-Wessels *et al*, 1998). The nontumourigenic SV40T-transformed squamous oesophageal cell line HET1A (Stoner *et al*, 1991) was maintained as previously described (Underwood *et al*, 2010). All cell lines were cultured at 37 °C in a humidified incubator with 5% $CO_2$ in air. To study the effects of demethylation, triplicate cultures were treated with either $1 \, \mu \mathrm{mol} \, l^{-1}$ 5-aza-2'-deoxycytidine (aza, Sigma-Aldrich, St Louis, MO, USA) or vehicle (veh; 0.0027% v/v final concentration acetic acid), as described previously (Smith *et al*, 2005, 2008).

**Patient samples.** Multiple biopsies every 2 cm from within circumferential columnar lined oesophagus (*n* = 40) and a single biopsy of squamous mucosa proximal to the squamocolumnar

junction (*n* = 15) from 18 patients with Barrett's oesophagus were collected into RNAlater. For a diagnosis of Barrett's oesophagus, goblet cells had to be observed in at least one biopsy from the columnar-lined oesophagus. All patients with Barrett's oesophagus were being treated for gastro-oesophageal reflux disease with anti-secretory therapy. Single samples of primary tumour oesophageal adenocarcinoma (*n* = 34), adjacent high-grade dysplasia (*n* = 7), and histologically normal squamous mucosa from the proximal resection margin (*n* = 17) from 34 patients with oesophageal adenocarcinoma were collected into liquid nitrogen or into RNAlater (Ambion, Austin, TX, USA). The study complied with the appropriate institutional guidelines.

**Isolation of RNA and DNA from cell lines and patient samples.** Patient tissues were disrupted in either disposable pestles (Edwards Instruments, Narellan, NSW, Australia) or a TissueLyser with 5-mm stainless steel beads (Qiagen, Hilden, Germany). The RNA and DNA were isolated from cell lines and oesophageal adenocarcinoma patient tissues using Trizol (Invitrogen, Carlsbad, CA, USA) and from all other biopsies using either the RNA/DNA Kit or the AllPrep DNA/RNA Mini Kit (Qiagen).

**Methylation analysis.** Genomic DNA (500 ng) was bisulphite-modified using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). The bisulphite-modified DNA was amplified using primer sets, which did not discriminate between methylated and unmethylated sequences. The primer sequences were (forward) 5'-GGGTTGTTTGTTGGGYGAGATT-3' and (reverse) 5'-ACTTTACCCTTCCRCCTCTTAC-3'. The primers and PCR conditions were specific for bisulphite-modified DNA and did not amplify unmodified DNA. All methylation analysis PCRs were performed using the QuantiTect SYBR Green PCR Kit (Qiagen) in a final volume of 15 μl, containing 1 μl of bisulphite-modified DNA and a final concentration of $0.5 \, \mu \mathrm{mol} \, l^{-1}$ of each forward and reverse primer. Bisulphite-modified lymphocyte DNA, CpG methyltransferase (M.SssI) (New England Biolabs Inc, Ipswich, MA, USA) treated lymphocyte DNA, and unmodified DNA were included in each PCR run and served as unmethylated, methylated, and negative controls, respectively. Reactions were run in a Rotor-Gene 3000 (RG-3000) (Corbett Life Science, Sydney, NSW, Australia) at 95 °C for 15 min, then 45 cycles of 95 °C for 30 s and 59 °C for 60 s, followed by a final extension of 72 °C for 4 min. At the end of the amplification cycle, the PCR products were melted by increasing the temperature from 60 to 95 °C, rising 0.5 °C at each step, waiting 30 s on the first step and then 5 s for each step thereafter. The raw fluorescence data were normalised as previously described (Smith *et al*, 2009). Methylation was also measured using an Infinium HumanMethylation450 BeadChip Kit (Illumina, San Diego, CA, USA), as previously described (Lim *et al*, 2013).

**Measurement of gene expression by quantitative real-time reverse-transcription PCR.** To measure gene expression, cDNA was synthesised using SuperScript II (Invitrogen) from 2 μg of total RNA that had been treated with the TURBO DNA-free Kit (Ambion). Quantitative real-time reverse-transcription polymerase chain reaction (QPCR) was performed using the QuantiTect SYBR Green PCR Kit in a final volume of 10 μl, containing 0.2 μl of cDNA and a final concentration of $0.5 \, \mu \mathrm{mol} \, l^{-1}$ of each forward and reverse primer. The primer sequences were (forward) 5'-AGCTGTGAGGTCATCGGAAT-3' and (reverse) 5'-ACCAGCACCCAGCCAGTTA-3'. Triplicate reactions were run in an RG-3000 at 95 °C for 15 min, then 45 cycles of 95 °C for 30 s and 57 °C for 60, followed by a final extension of 72 °C for 4 min. Data were collected and analysed using the RG-3000 Application Software. Threshold cycle $C_t$ values were determined on auto-threshold settings with reference to a standard dilution curve. All mRNA quantitation data were normalised to hydroxymethylbilane

synthase (HMBS). Following the PCR, the products were melted to confirm specificity and electrophoresed on 1.5% (w/v) agarose gels stained with ethidium bromide to confirm expected product size.

**Statistical analyses.** Statistics were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA) and Prism version 6.0b for Macintosh (GraphPad Software, San Diego CA, USA; www.graphpad.com). The median overall survival and the 5-year survival were calculated using the Kaplan–Meier estimator. Kaplan–Meier survival curves were compared using the Log-rank (Mantel–Cox) test. Hazard ratios (HRs), 95% confidence intervals (CIs), and P-values were calculated from univariate and multivariate Cox proportional hazards models. Gene expression in cell lines comparing treatment with aza to veh was performed using multiple t-tests and significance determined using the Holm–Sidak method, with alpha of 5%. Methylation frequency in tissues and the correlation between methylation and expression was compared using Fisher's exact test. All statistics were considered significant when the two-tailed P-value was $\leqslant 0.05$.

## RESULTS

**Expression of IGFBP7 in oesophageal adenocarcinoma and Barrett's oesophagus.** The protein expression of IGFBP7 was measured using immunohistochemistry in oesophageal adenocarcinoma resections from 65 patients. None of the patients had preoperative chemotherapy or radiotherapy. Follow-up data were available from all patients, with a median follow-up time of 33 months (range 1–149). The median patient age at surgery was 63 years (range 35–80). The overall 5-year survival rate was 28%, and the overall median survival was 27 months (range 1–129).

Positive staining for IGFBP7 in tumour cells was observed in 34 of 65 (52%) patients (Figure 1). In positive tumour cells, staining was localised to the cytoplasm and was uniformly of moderate intensity. Sporadic staining of positive stromal cells, including endothelial cells, was localised to the cytoplasm and ranged in intensity from weak to intense.

Expression of IGFBP7 protein was associated with decreased patient survival (Figure 2). With univariate analysis, IGFBP7 positivity, advanced pT stage, lymph node metastases, a positive resection margin, and vascular and perineural invasion were significantly associated with decreased survival (Table 1). Expression of IGFBP7 was associated with a significant decrease in the median overall survival (11 vs 92 months; P-value = 0.0140) and decreased 5-year survival (24.5% vs 51.6%), (HR = 2.16; 95% CI = 1.14–4.09; P-value = 0.0177).

Owing to the limited number of patients, only IGFBP7 expression, pT, pN, and positive resection margin were retained in the multivariate model (Table 1). The prognostic value of IGFBP7 expression, pT stage, and a positive resection margin were maintained even after adjustment for the other covariates, supporting the independent role of these markers in predicting survival. Survival was significantly reduced for patients who were
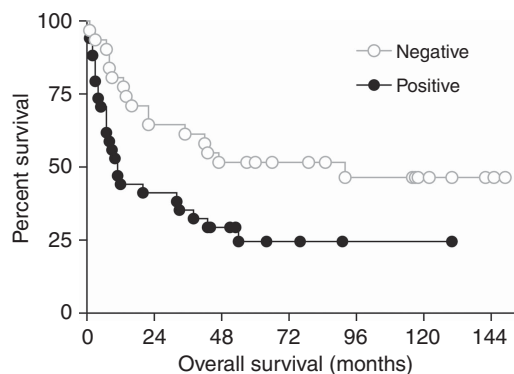


**Figure 2. Kaplan–Meier analysis of overall survival of patients with oesophageal adenocarcinoma in relation to IGFBP7 expression.** Expression of IGFBP7 in tumour cells, determined by immunohistochemistry, scored as negative (n = 31) or positive (n = 34). Log-rank (Mantel–Cox) test P-value = 0.0140.
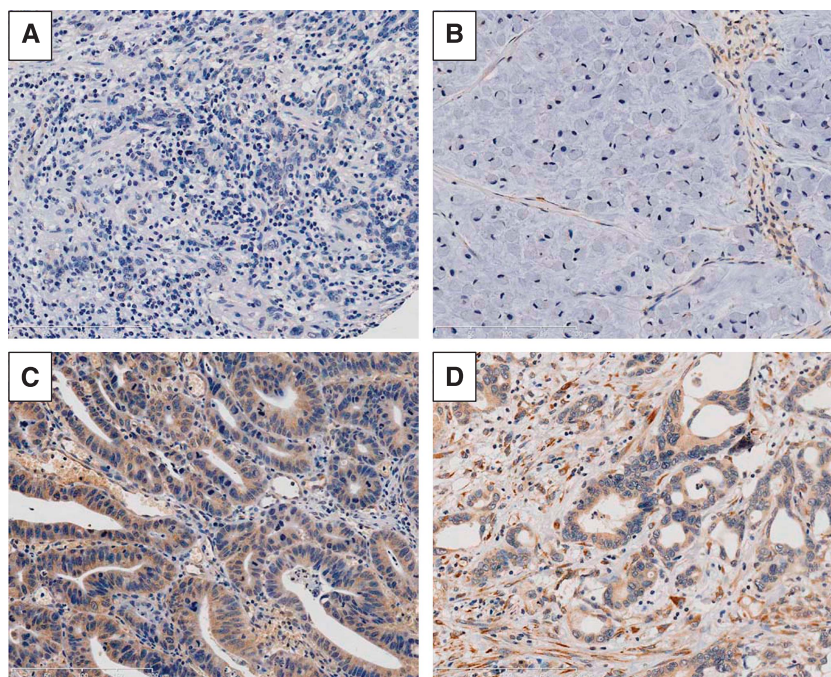


**Figure 1. Immunohistochemistry of IGFBP7 in human oesophageal adenocarcinoma.** Representative images of IGFBP7 immunohistochemistry displaying (**A**) negative expression in tumour cells and stroma; (**B**) negative expression in tumour cells, and positive in stroma; (**C**) positive expression in tumour cells, but negative in stroma; (**D**) positive expression in tumour cells and stroma.

| Table 1. Univariate and multivariate survival analyses (Cox proportional hazards model) | | | | | | | |
|---|---|---|---|---|---|---|---|
| | *n* | Median survival (mo)[a,b] | 5-year survival (%)[a] | Univariate HR (95% CI) | *P*-value | Multivariate HR (95% CI) | *P*-value |
| **IGFBP7 expression** | | | | | | | |
| Negative | 31 | 92 | 51.61 | 1 | 1 | | |
| Positive | 34 | 11 | 24.51 | 2.163 (1.143–4.093) | 0.0177 | 3.247 (1.580–6.673) | 0.0014 |
| **Gender** | | | | | | | |
| Female | 9 | 10 | 11.11 | 1 | | | |
| Male | 56 | 40 | 42.34 | 0.514 (0.235–1.126) | NS | | |
| **Age** | | | | | | | |
| <70 | 46 | 38.5 | 40.82 | 1 | | | |
| ≥70 | 19 | 22 | 31.58 | 1.471 (0.771–2.807) | NS | | — |
| **pT stage** | | | | | | | |
| T1a/1b | 15 | — | 86.67 | 1 | | | |
| T2 | 10 | 65 | 50.00 | 6.107 (1.227–30.395) | 0.0271 | 7.068 (1.411–35.407) | 0.0174 |
| T3 | 31 | 20 | 22.12 | 9.304 (2.185–39.621) | 0.0026 | 19.423 (2.478–152.243) | 0.0047 |
| T4 | 9 | 5 | 0.00 | 24.894 (5.223–118.637) | <0.0001 | 27.357 (3.143–238.134) | 0.0027 |
| **pN** | | | | | | | |
| 0 | 28 | — | 67.86 | 1 | | 1 | |
| 1 | 37 | — | 15.77 | 3.603 (1.754–7.402) | 0.0005 | 3.951 (1.182–13.208) | 0.0256 |
| **Grade of differentiation** | | | | | | | |
| Well/moderate | 19 | — | 51.46 | 1 | | | |
| Poor/undifferentiated | 46 | 20 | 30.95 | 1.975 (0.941–4.147) | NS | | |
| **Resection margin** | | | | | | | |
| Negative | 34 | — | 63.89 | 1 | | 1 | |
| Positive | 31 | 9 | 9.68 | 4.503 (2.301–8.810) | <0.0001 | 3.951 (1.182–13.208) | 0.0256 |
| **Vascular invasion** | | | | | | | |
| No | 18 | — | 71.30 | 1 | | | |
| Yes | 47 | 16 | 25.35 | 4.171 (1.625–10.708) | 0.0030 | | |
| **Perineural invasion** | | | | | | | |
| No | 35 | — | 57.14 | 1 | | | |
| Yes | 30 | 10.5 | 15.56 | 2.503 (1.329–4.715) | 0.0045 | | |

Abbreviations: CI = confidence interval; HR = hazard ratio; NS = not significant; pT stage = pathological tumour stage.
[a]Median survival/5-year survival calculated using the Kaplan–Meier estimator.
[b]Median survival could not always be estimated as <50% death in some subgroups.

positive compared with those who were negative for IGFBP7 expression (HR = 3.25; 95% CI = 1.58–6.67; *P*-value = 0.0014).

Next, we analysed IGFBP7 mRNA expression in normal oesophagus, Barrett's oesophagus, and oesophageal adenocarcinoma in published transcription microarray data sets. There was a significant increase in IGFBP7 mRNA in adenocarcinoma (four of four data sets) and Barrett's oesophagus (three of four), compared with normal oesophageal tissue (Figure 3). Despite the significant increase in expression in oesophageal adenocarcinoma, it was clear that there was a wide range of expression from low to high, consistent with our finding that IGFBP7 was only expressed in a subset of patients. Some clinical data were available for one of the public data sets (Kim *et al*, 2010), and the expression of IGFBP7 was significantly increased in stage 3 tumours compared with stage 0; however, no other significant differences were observed.

**DNA methylation and expression of IGFBP7 in oesophageal cell lines.** The melt curve analysis was used to measure the extent

of DNA methylation, and QPCR to measure expression, of IGFBP7 in three oesophageal cancer cell lines. OE21 and TE7 were unmethylated and expressed IGFBP7, whereas OE33 was methylated and did not express the gene (Figure 4). When OE33 was grown in the presence of aza, the extent of DNA methylation decreased, and the expression of IGFBP7 significantly increased (*P*-value <0.0001). In contrast, aza did not significantly alter IGFBP7 DNA methylation or expression in either OE21 and TE7. Methylation of IGFBP7 in OE33 was also assessed on an Infinium HumanMethylation450 BeadChip Kit (Figure 4C). All CpG probes assessed within the promoter region ( − 500 to + 1000 nt from the transcription start site) were heavily methylated, with beta-values >0.8. Together, these results are consistent with methylation of the region of IGFBP7 assessed with the melt curve analysis being associated with silencing of its expression in oesophageal cell lines.

Methylation was also assessed in the following oesophageal cell lines: oesophageal adenocarcinoma (OE19, JH-EsoAd1), telomerase immortalised non-dysplastic (CP-A), and high-grade
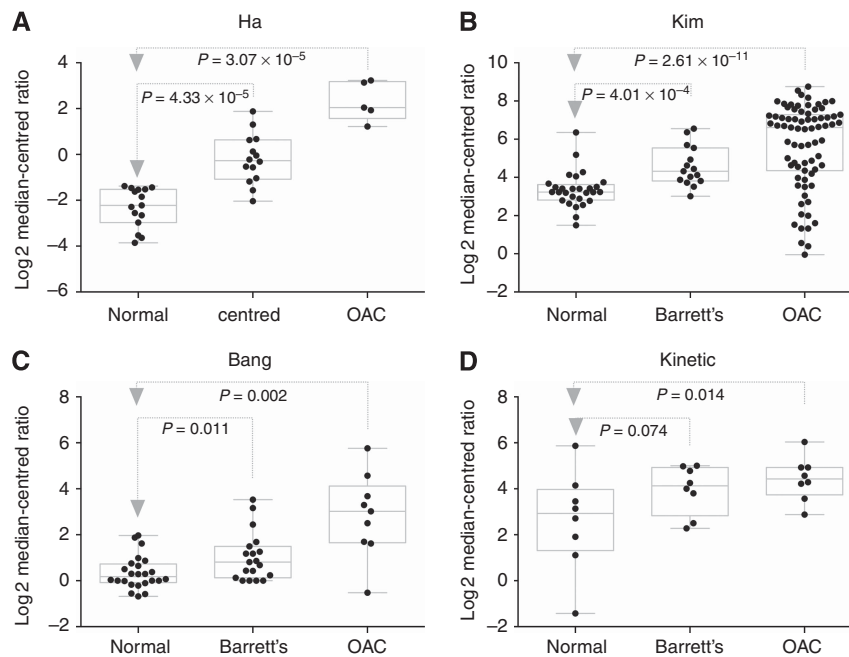
**Figure 3. Expression of IGFBP7 in oesophageal tissues.** Expression of IGFBP7 in histologically normal squamous mucosa (Normal), Barrett's oesophagus (Barrett's), and oesophageal adenocarcinoma (OAC) tissues was determined using transcriptional microarrays in four different data sets as named and published on Oncomine.

dysplastic (CP-B, CP-D) Barrett's oesophagus, and immortalised squamous epithelium (HET1A). Methylation was detected in OE19, JH-EsoAd1, and CP-A, but not in CP-B, CP-D nor HET1A (Figure 4D). We found that methylation of IGFBP7 is a common feature of Barrett's and oesophageal adenocarcinoma cell lines.

**DNA methylation of IGFBP7 in Barrett's oesophagus and oesophageal adenocarcinoma tissues.** Methylation was determined using the melt curve analysis in biopsies of squamous mucosa and columnar lined oesophagus from 18 patients with Barrett's oesophagus. Methylation was detected in significantly more biopsies of columnar lined oesophagus (27 of 40; 68%) than squamous mucosa (2 of 15; 13%) (P-value = 0.0006). Methylation was detected in at least one biopsy of columnar lined oesophagus in 14 of 18 (78%) patients with Barrett's oesophagus.

Methylation was determined in biopsies of tumour, and when available, adjacent high-grade dysplasia, and histologically normal squamous proximal resection margin from 34 patients with oesophageal adenocarcinoma. Compared with squamous mucosa (3 of 17; 18%), methylation was detected in significantly more biopsies of high-grade dysplasia (5 of 7; 71%) (P-value = 0.0207) and oesophageal adenocarcinoma (23 of 34; 68%) (P-value = 0.0010). Together, these results demonstrate that IGFBP7 methylation is infrequent in squamous mucosa but is common in Barrett's oesophagus and oesophageal adenocarcinoma tissue.

Matched DNA methylation and expression data were available for 22 of the 65 patients. Methylation was detected in seven out of seven (100%) patients who did not express IGFBP7, compared with 47% (7 of 15) patients who did (P-value = 0.0225). Survival was reduced in patients with unmethylated IGFBP7 (9 vs 56 months; Figure 5); however, this difference was not significant (log-rank test, P-value = 0.071; Cox proportional hazards model, HR = 0.46, 95% CI = 0.19–1.10).

## DISCUSSION

We have demonstrated that, in patients with oesophageal adenocarcinoma, protein expression of IGFBP7 was associated

with significantly reduced survival, independent of other clinico-pathological features. In both oesophageal adenocarcinoma and Barrett's cell lines, DNA methylation within the IGFBP7 promoter was associated with transcriptional silencing. In patient samples, DNA methylation of IGFBP7 was infrequent in squamous mucosa, but a common feature in Barrett's and oesophageal adenocarcinoma, and it correlated with silencing of protein expression.

The levels of expression of IGFBP7 differ among different cancer types. Increased expression has been observed in glioblastoma multiforme (Jiang et al, 2008), oesophageal squamous cell (Kashyap et al, 2012), and colorectal carcinoma (Umeda et al, 1998). In Barrett's and oesophageal adenocarcinoma samples we found, from an analysis of four independent expression array data sets in public repositories, that IGFBP7 transcript expression was increased in a proportion of patient samples compared with their squamous mucosa, consistent with our immunohistochemical findings. In contrast, decreased expression has been reported in cancers of the breast (Burger et al, 1998), lung (Chen et al, 2007), prostate (Hwa et al, 1998), pancreas (An et al, 2012), liver (Chen et al, 2011a), and thyroid (Vizioli et al, 2010), raising the possibility that, at least in some types of cancer, IGFBP7 may function as a tumour suppressor. A limitation of our correlative study is that the data do not allow any conclusions to be reached regarding the possible biological role of IGFBP7 in oesophageal adenocarcinoma.

Decreased IGFBP7 expression has been associated with DNA methylation in a range of cancers including T-lymphoblastic leukaemia (Heesch et al, 2011), liver (Komatsu et al, 2000), lung (Chen et al, 2011b; Suzuki et al, 2013), prostate (Sullivan et al, 2012), and colorectal (Ruan et al, 2006; Lin et al, 2007, 2008; Hinoue et al, 2009; Suzuki et al, 2010; Dimberg et al, 2013), as well as premalignant colorectal adenomas (Kaji et al, 2012). In our study, methylation was detected in oesophageal adenocarcinoma and Barrett's oesophagus cell lines. The methylation was associated with decreased transcript expression, and demethylation with 5-aza-2′-deoxycytidine resulted in IGFBP7 re-expression. Methylation was more frequently observed in primary Barrett's and oesophageal adenocarcinoma tissues than in adjacent histologically normal squamous mucosa. Finally, methylation in adenocarcinoma was more likely to be associated with a lack of protein expression.
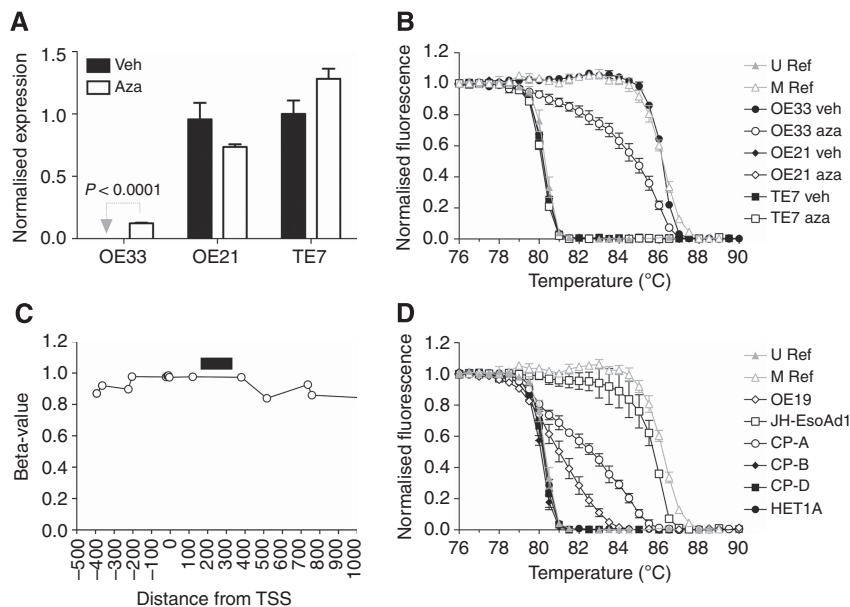
Figure 4. Expression and DNA methylation of IGFBP7 in oesophageal cell lines. The Barrett's associated oesophageal adenocarcinoma (OE33) and oesophageal squamous cell carcinoma (OE21 and TE7) cell lines were treated with either vehicle (veh) or $1\,\mu mol\,l^{-1}$ 5-aza-2'-deoxycytidine (aza) and analysed for (A) expression of IGFBP7, measured by QPCR (mean ± s.e.m. of three replicates), and (B) DNA methylation, measured with the melt curve analysis (mean ± s.e.m. of two to three replicates). (C) Methylation of individual CpGs in the IGFBP7 promoter was measured in OE33 using an Infinium HumanMethylation450 BeadChip Kit. The position of individual CpGs was determined relative to the transcription start site (TSS) of IGFBP7 (assembly GRCh37/hg19, chr4:57898404-57976551). The black horizontal bar denotes the region analysed with the melt curve analysis. (D) Methylation, measured with the melt curve analysis, in oesophageal adenocarcinoma (OE19 and JH-EsoAd1), Barrett's oesophagus (CP-A, CP-B, and CP-D) and squamous epithelial cell lines (mean ± s.e.m. of two to three replicates).
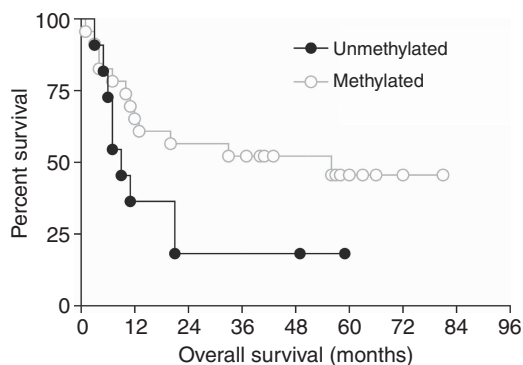


Figure 5. Kaplan–Meier analysis of overall survival of patients with oesophageal adenocarcinoma in relation to IGFBP7 methylation. Methylation of the IGFBP7 promoter, determined with the melt curve analysis, was recorded as unmethylated ($n = 11$) or methylated ($n = 23$). Log-rank (Mantel–Cox) test $P$-value $= 0.0707$.

Taken together, these data demonstrate that DNA methylation of the IGFBP7 promoter is associated with the silencing of its expression and is common in Barrett's and adenocarcinoma of the oesophagus.

Reports on the prognostic significance of IGFBP7 expression in cancer are conflicting. In accordance with our findings, increased expression of IGFPB7 was associated with decreased patient survival in colorectal (Adachi et al, 2001), T-acute lymphoblastic leukaemia (Heesch et al, 2010), and head and neck squamous cell carcinoma (Sepiashvili et al, 2012). In contrast, increased expression correlated with increased survival in pancreatic ductal (An et al, 2012), breast (Landberg et al, 2001), hepatocellular (Tomimaru et al, 2012), and colorectal carcinoma (Ruan et al, 2007). These conflicting observations may be because of heterogeneous expression within the cancer. For example, Adachi et al (2001) reported that expression was greatest in the cells located at

the invasive front of tumour nests, and that it was the expression in these cells that correlated with poor patient prognosis, independent of other clinicopathological covariates. In our study, we could not be confident that the increased expression that we observed was restricted to the cells at the invasive front because we stained TMAs that were constructed using 1 mm cores taken from representative regions of the tumour, which did not always include the invasive front.

In conclusion, expression of IGFBP7 was an independent prognostic indicator for decreased patient survival in oesophageal adenocarcinoma. DNA methylation within the IGFBP7 promoter was associated with transcriptional silencing in oesophageal adenocarcinoma and Barrett's cell lines, was a common feature in Barrett's and oesophageal adenocarcinoma tissues, and correlated with silencing of protein expression.

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