Hypoxia-inducible factor-1α and -2α are expressed in most rectal cancers but only hypoxia-inducible factor-1α is associated with prognosis

S Rasheed¹, AL Harris², PP Tekkis¹-³, H Turley², A Silver⁴, PJ McDonald¹, IC Talbot⁵, R Glynn-Jones⁶, JMA Northover¹ and T Guenther¹,⁶

¹Department of Surgery, St Mark’s Hospital, Harrow, Middlesex, UK; ²Weatherall institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK; ³Department of Surgical Oncology and Technology, Royal Marsden and Chelsea and Westminster Hospitals, Imperial College, London, UK; ⁴Colorectal Cancer Genetics, St Bartholomew’s and The London Hospital, Institute of Cell and Molecular Sciences, Queen Mary’s University of London, London, UK; ⁵Academic Department of Cellular Pathology, St Mark’s Hospital, Harrow, Middlesex HA1 3UJ, UK; ⁶Department of Oncology, Mount Vernon Hospital, Northwood, Middlesex, UK

The hypoxia-mediated response of tumours is a major determining factor in growth and metastasis. Understanding tumour biology under hypoxic conditions is crucial for the development of antiangiogenic therapy. Using one of the largest cohorts of rectal adenocarcinomas to date, this study investigated hypoxia-inducible factor-1α (HIF-1α) and HIF-2α protein expression in relation to rectal cancer recurrence and cancer-specific survival. Patients (n = 90) who had undergone surgery for rectal adenocarcinoma, with no prior neoadjuvant therapy or metastatic disease, and for whom adequate follow-up data were available were selected. Microvessel density (MVD), HIF-1α and HIF-2α expressions were assessed immunohistologically with the CD34 antibody for vessel identification and the NB100-131B and NB100-132D3 antibodies for HIF-1α and HIF-2α, respectively. In a multifactorial analysis, results were correlated with tumour stage, recurrence rate and long-term survival. Microvessel density was higher across T and N stages (P < 0.001) and associated with poor survival (hazard ratio (HR) = 8.7, P < 0.005) and decreased disease-free survival (HR = 4.7, P < 0.005). hypoxia-inducible factor-1 and -2 were expressed in >50% of rectal cancers (HIF-1α, 54%; 48/90; HIF-2α, 64%, 58/90). HIF-1α positivity was associated with both TNM stage (P < 0.05) and vascular invasion (P < 0.005). In contrast, no associations were shown between HIF-2α expression and any pathological features, and HIF-1α positivity had no effect on outcome. The study showed an independent association between HIF-1α expression and advanced TNM stage with poor outcome. Our results indicate that HIF-1α, but not HIF-2α, might be used as a marker of prognosis, in addition to methods currently used, to enhance patient management.

British Journal of Cancer (2009) 100, 1666 – 1673. doi:10.1038/sj.bjc.6605026 www.bjcancer.com

Keywords: rectal cancer; angiogenesis; hypoxia; HIF-1α and HIF-2α; microvessel density

The UK incidence of colorectal carcinoma is approximately 34 000 of which a third are rectal cancers (Kmietowicz, 2004). In the United States, colorectal cancer is the second most common cancer in women and the third commonest in men with over 148 000 new cases each year (American Cancer Society, 2006). After potentially curative surgical resection, local recurrence rates of rectal cancer have been reported from 4 to 32% (Sagar and Pemberton, 1996) with overall 5 year survival of less than 40% (Lindmark et al, 1994; Dahlberg et al, 1998).

Hypoxia is one of the key stimuli for the release of angiogenic factors necessary for angiogenesis and tumour growth. Tumours outgrow their local blood supply resulting in a hypoxic microenvironment. Although lesions 1 – 2 mm in diameter receive nutrients by cellular diffusion, an increase in tumour size beyond this requires rapid adaptation to hypoxia to prevent cessation of growth and necrosis (Pugh and Ratcliffe, 2003). A component of this adaptation, increased microvessel density (MVD), has been shown in polyps and colorectal cancers (Bosci et al, 1995) and linked to increasing transmural tumour extension (Choi et al, 1998; Rasheed et al, 2009). High colorectal cancer MVD has also been associated with an increased incidence of haematogenous metastases and poor prognosis (Tomisaki et al, 1996; Gulubova and Vlaykova, 2007; Rasheed et al, 2008).

Hypoxia-inducible factor (HIF) is a heterodimeric basic helix-loop-helix transcription factor involved in the regulation of cellular adaptation to hypoxia by upregulating genes directly responsible for angiogenesis. HIF consists of an α- and β-subunit (HIF-α and HIF-β/ARNT; Conway et al, 2001), which can be further subdivided into distinct isoforms, including HIF-1, HIF-2 and HIF-3. The first two of these exhibit conserved amino-acid sequences, but different mRNA expression patterns (Conway et al, 2001; Aprilekova et al, 2004). The HIF-β/ARNT subunit is constitutively expressed and involved in a number of
non-hypoxia-related processes, whereas the α-subunit is the hypoxia-regulating component (Carmeliet et al, 1998; Talks et al, 2000). Both HIF-1α and HIF-2α are subject to post-transcriptional regulation mediated by the von Hippel–Lindau protein (VHL), but only HIF-1α regulates the induction of the glycolytic enzymes essential for cell proliferation and survival under hypoxic stress (Aprilekova et al, 2004). Under physiological normoxic conditions, HIF-2α is propyl hydroxylated after activation of VHL and then prepared for degradation through mitochondrial ubiquitination. However, under hypoxic conditions, this degradation cannot take place due to a reduction in propyl hydroxylation, which leads to HIF-1α accumulation; levels of HIF-1α correlate with the oxygen status of the cell (Iyer et al, 1998; Blancher et al, 2000). HIF-α upregulation causes activation of target genes and expression of various growth factors, including VEGF, which induce endothelial cell proliferation and migration resulting in new vessel growth (Ratliffe et al, 1997; Talks et al, 2000; Giatromanolaki and Harris, 2001).

Patients with mutations in the VHL tumour suppressor gene develop highly vascular tumours, including renal cell carcinomas, phaeochromocytomas and retinal haemangioblastomas (Semenza, 2001; Wykoff et al, 2001). Hypoxia-inducible factor has been shown to play a major role in the angiogenesis and growth of various tumours, including breast (Leek et al, 2002), bladder (Palit et al, 2005), renal (Klatte et al, 2005; Maynard and Ohh, 2007; Trastour et al, 2007) and cervical (Birner et al, 2000) cancers. Indeed, over-expression of, in particular, HIF-1α, has been correlated with unfavourable prognosis in a number of malignancies (Theodoropoulos et al, 2005; Maynard and Ohh, 2007; Trastour et al, 2007).

In colorectal cancer, Jiang et al (2003a, b) showed that HIF-1α mRNA was present in a significant number of colorectal adenoma and carcinoma specimens. They also found an increase in HIF-1α expression concordant with more advanced Dukes’ stage. Kuwai et al (2003) associated HIF-1α expression with tumour invasion, venous invasion, liver metastasis and vascular endothelial growth factor (VEGF) expression. Similarly, Lu et al (2006) found a strong association between HIF-1α expression, high VEGF expression, nodal metastasis and Dukes’ stage in rectal cancer and, in contrast to Kuwai et al (2003) showed an overall reduction in survival in patients with high HIF-1α expression. These findings were supported by an investigation in patients with locally advanced rectal cancer where an association was shown between HIF-1α expression, lymph node metastasis and poor outcome. Antiangiogenic drugs have been shown to be effective both experimentally and in clinical trials in various malignancies, including colorectal cancer. The monoclonal IgG1 antibody, Bevacizumab (Avastin), which binds VEGF preventing receptor binding, has been evaluated in the first- and second-line treatment in patients with colorectal cancer (Willett et al, 2004, 2006; Chua and Cunningham, 2006; Rasheed et al, 2008). Interestingly, novel antiangiogenic compounds have been developed that decrease HIF-1α and other HIFs (Mackay et al, 2005; Zhu et al, 2007; Singh et al, 2008).

We are evaluating whether detection of hypoxic factor expression in rectal cancer can be used to identify patient subgroups at increased risk of recurrences and poorer outcome. Recognising positive factor expression will identify patients most likely to benefit from specific antiangiogenic therapies, including HIF inhibitors. In this study, we investigate one of the largest cohorts of patients to date with rectal adenocarcinoma across all stages for HIF-1α and HIF-2α expressions and have appraised associations between expression and a number of clinicopathological variables.

**MATERIALS AND METHODS**

Patients with rectal adenocarcinoma operated on between 1991 and 1997 at St Mark’s Hospital were selected for this study provided they had curable, local disease before surgery. Those with metastatic disease and/or had received neoadjuvant chemoradiotherapy were excluded. Complete follow-up data and sufficient paraffin-embedded tissue to stain with antibodies for HIF-1α, HIF-2α and CD34 (for MVD) were available in 90 cases. Normal proximal adjacent bowel obtained from 25 randomly selected patients was used as internal control tissue.

Tissue sections (4 μm) of archival paraffin-embedded block specimens were mounted onto slides. The antibodies used were for identification of CD34 blood vessels (Novus Biologicals, LLC, Littleton, CO, USA, QBend10, dilution 1 : 100, no pre-treatment), HIF-1α (NB 100-131B, Novus Biologicals, dilution 1 : 500, pressure cooked for 2 min at full pressure, pre-treated with citrate buffer, pH 6.0) and HIF-2α (NB100-132D3, Novus Biologicals, dilution 1 : 100, pressure cooked for 2 min at full pressure, pre-treated with citrate buffer, pH 6.0; Talks et al, 2000; Koukourakis et al, 2002; Leek et al, 2002). Immunohistochemistry was undertaken in a DAKO Immuno Autostainer Plus (Glostrup, Denmark) with ‘Chemmate Envision’ after standard dewaxing with xylene and ethanol and pre-treatment. Microvessel density was assessed by counting vessels per power field (23-mm eye piece width) at × 200 magnification following identification of the three most vessel-dense ‘hotspots’ at × 40 magnification (Figures 1 and 2;...
Bossi et al., 1995). Vessels were counted in the three most vessel-dense areas within the central part of the tumour and at the invasive tumour front. A mean score of all areas was calculated. The mean value of 60 vessels per high power field was used as the cut-off between high and low MVDs.

Hypoxia-inducible factor-1α (nuclear and cytoplasmic) stainings (Figures 3 – 5) were reviewed by two experienced assessors (SR and TG) in terms of percentage of positive tumour cells. The predominant site of distribution, for example, intratumoral, invasive edge of tumour was noted. The following clinicopathological factors were analysed: age at the time of surgery; gender; surgical procedure categorised as abdomino-perineal excision of rectum or anterior resection of rectum; tumour grading; staging according to the Dukes’ and tumour node metastasis (TNM) classifications; venous invasion (intramural and extramural); perineural invasion, number of regional lymph nodes and number of involved lymph nodes.

RESULTS

Patients and clinicopathological characteristics

A total of 90 patients comprising 56 males (62%), 34 females (38%) were studied with a mean age of 59 (standard deviation, s.d. ± 12) years and a median follow-up of 78 months (range: 2 – 228 months). There was no significant difference between male and female patients in terms of mean age or follow-up. Clinicopathological characteristics are described in Table 1.

Microvessel density differs between tumour and normal tissue, across T and N stages and is associated with poor survival

For the cases (n = 90), the mean MVD was 63 vessels per high-power field (HPF; s.d. ± 20) and for controls (n = 23), the MVD was 22 vessels per HPF. The MVD was significantly higher in tumour versus non-neoplastic mucosa (63 vs 22; P < 0.01, Fisher’s exact) and across the various T stages: T1 = 44 (s.d. ± 3); T2 = 45 (s.d. ± 13); T3 = 67 (s.d. ± 21) and T4 = 68 (s.d. ± 9; post hoc, ANOVA, P < 0.001); and N stages: N0 = 56 (s.d. ± 16), N1 = 74 (s.d. ± 17), N2 = 76 (s.d. ± 26; post hoc, ANOVA, P < 0.001). Survival analysis showed a significant difference in cancer-specific
survival in the high versus low MVD groups divided by the median MVD of 60 vessels per HPF (Figure 6A; log-rank test \( \chi^2 = 12.5, \text{d.f.} = 1 \) (degree of freedom); HR = 8.7, 95% confidence interval (CI): 2.0–37.3, \( P < 0.005 \)) and disease-free survival (Figure 6B; log-rank test \( \chi^2 = 11.1, \text{d.f.} = 1 \), HR = 4.7, 95% CI: 1.6–13.7, \( P < 0.005 \)).

HIF-1α but not HIF-2α protein expression is associated with advanced pathological features and poor survival

Hypoxia-inducible factor-1α and -2α stains were present within the epithelial and stromal compartments in over half of rectal cancers (54%, 48/90 and 64%, 58/90, respectively). In contrast, HIF-1α stain was only present in a small fraction of control sections (8%, 2/25; HIF-1α staining in cases vs controls, \( P < 0.005 \)) and HIF-2α stain was not seen in any of the control sections. The following features were observed to be associated with HIF-1α positivity (Table 2): lymph node stage \( (P < 0.02) \); TNM stage \( (P < 0.05) \); and vascular invasion \( (P < 0.005) \).

The association between Dukes’ stage and HIF-1α positivity almost reached statistical significance \( (P = 0.05) \), although combining Dukes’ A and B cancers together and C1 with C2 cancers resulted in a statistically significant difference in HIF-1α positivity between Dukes’ A/B and C (Dukes’ A/B, 44% positive vs 56% negative; Dukes’ C, 70% positive vs 30% negative; \( P < 0.02 \)). In contrast to results for HIF-1α, there were no observed associations between HIF-2α positivity and any of the pathological features under investigation (Table 3). Cox regression univariate analysis revealed a significant effect of HIF-1α positivity on cancer-specific survival (Figure 7A; log-rank test \( \chi^2 = 12.2, \text{d.f.} = 1 \), HR = 5.47 95% CI: 1.96–16.03, \( P < 0.002 \)) and disease-free survival (Figure 7B; log-rank test \( \chi^2 = 10.85, \text{d.f.} = 1 \), HR = 4.47, 95% CI: 1.68–11.89, \( P = 0.003 \)). On multivariate analysis, HIF-1α positivity retained a significant effect on cancer-specific survival independent of TNM stage and vascular invasion \( (HR = 4.11, 95\% \text{ CI} = 1.37–12.35, P = 0.012; \text{Table 4}) \). Again, in direct contrast, there was no effect of HIF-2α positivity on cancer-specific survival (log-rank test \( \chi^2 = 2.275, \text{d.f.} = 1 \), HR = 0.545, 95% CI: 0.244–1.217, \( P = 0.139 \); Figure 8A), although, contrary to HIF-1α, a trend towards decreased recurrence (log-rank test \( \chi^2 = 2.51, \text{d.f.} = 1 \), HR = 0.542, 95% CI: 0.25–1.17, \( P = 0.12 \)) was observed for HIF-2α positivity (Figure 8B).

**DISCUSSION**

Our study comprises one of the largest groups of rectal cancer cases across all stages and we have determined that both HIF-1α

---

**Table 1** Clinicopathological features of a cohort of rectal cancer patients

| Clinicopathological variables | N (%) |
|------------------------------|------|
| **T stage**                  |      |
| T1                           | 2 (2) |
| T2                           | 16 (18)|
| T3                           | 68 (76)|
| T4                           | 4 (4) |
| N stage                      |      |
| N0                           | 57 (63)|
| N1                           | 21 (23)|
| N2                           | 12 (13)|
| Operation                    |      |
| Anterior resection           | 75 (83)|
| Abdominopereineal excision   | 15 (17)|
| **Histological grade**       |      |
| Well differentiated           | 4 (4) |
| Moderately differentiated     | 73 (81)|
| Poorly differentiated         | 13 (14)|
| **Dukes’ stage**             |      |
| Dukes’ A                      | 17 (19)|
| Dukes’ B                      | 40 (44)|
| Dukes’ C1                     | 28 (31)|
| Dukes’ C2                     | 5 (6)  |
| **TNM stage**                |      |
| Stage I                      | 17 (19)|
| Stage II                     | 40 (44)|
| Stage III                    | 33 (37)|
| **Vascular invasion**        |      |
| Not seen                     | 63 (70)|
| Intramural                   | 11 (12)|
| Extramural                   | 16 (18)|

TMN = tumour node metastasis.
and HIF-2α were widely expressed in rectal cancer compared with normal large bowel mucosa (controls). Over half of all cases showed HIF-1α expression (54%) and nearly two-thirds were found to have positive HIF-2α expression (64%). As discussed earlier by Talks et al (2000), as HIF-α-subunits are affected by cellular oxygenation, it is uncertain as to whether the findings in paraffin-embedded-fixed tissue accurately reflect cellular status in vivo. However, our observed findings do show clear differences between the two groups and, as cases and controls have been processed in a consistent manner, this does represent a genuine finding of increased hypoxic factor expression in rectal cancer cases when compared with normal rectal tissue.

We have shown an association between increasing depth of tumour invasion and MVD and have shown a relationship between increased MVD and poor prognosis, a finding consistent with earlier studies investigating colorectal cancers (Tomisaki et al., 1996; Choi et al., 2003). We have shown a direct relationship between increasing MVD and cancer-specific survival. In our study, an MVD of above the cutoff of 60 microvessels per HPF was a clear determinant of poor outcome. As such, we would propose that high MVD (above 60 HPF) can be used as a factor to determine surveillance and adjuvant therapy. As yet, to our knowledge, the effect of specific chemotherapeutic or antiangiogenic agents has not been assessed in a randomised controlled trial with groups determined by MVD.

Our data show an association between HIF-1α expression and TNM stage, nodal stage, vascular invasion and Dukes’ stage, on subdivision between node-positive and -negative cases, suggesting a direct role for HIF-1α in disease progression. Interestingly, we found no association between HIF-1α and MVD in our study, and -catenin in colon cancer cell

### Table 2

| Patient and tumour characteristics and HIF-1α staining | HIF-1α, number of patients (%) |  
|--------------------------------------------------------|-------------------------------|
| Gender                                                 | Negative | Positive | P-value |
| Male                                                   | 24 (43)  | 32 (57)  | P=0.35  |
| Female                                                 | 18 (53)  | 16 (47)  |         |
| T stage                                                |          |          | P=0.48  |
| T1/2                                                   | 9 (50)   | 9 (50)   |         |
| T3/4                                                   | 33 (46)  | 39 (54)  |         |
| N stage                                                |          |          | P<0.02* |
| N0                                                     | 32 (56)  | 25 (44)  |         |
| N1                                                     | 6 (29)   | 15 (71)  |         |
| N2                                                     | 4 (33)   | 8 (67)   |         |
| Differentiation                                        |          |          | P=0.38  |
| Well                                                   | 3 (75)   | 1 (25)   |         |
| Moderate                                               | 33 (45)  | 40 (55)  |         |
| Poor                                                   | 6 (46)   | 7 (54)   |         |
| Dukes’ stage                                           |          |          | P=0.05  |
| Dukes’ A                                               | 9 (53)   | 8 (47)   |         |
| Dukes’ B                                               | 23 (58)  | 17 (42)  |         |
| Dukes C1                                               | 8 (29)   | 20 (71)  |         |
| Dukes C2                                               | 2 (40)   | 3 (60)   |         |
| Dukes’ stages combined                                 |          |          | P<0.02* |
| A/B                                                    | 32       | 25       |         |
| C                                                       | 10       | 23       |         |
| TNM stage                                              |          |          | P<0.05* |
| Stage I                                               | 9 (53)   | 8 (47)   |         |
| Stage II                                              | 23 (58)  | 17 (42)  |         |
| Stage III                                             | 10 (30)  | 23 (70)  |         |
| Vascular invasion                                      |          |          | P<0.005*|
| Absent                                                | 35 (56)  | 28 (44)  |         |
| Intramural                                             | 4 (36)   | 7 (64)   |         |
| Extramural                                             | 3 (19)   | 13 (81)  |         |
| Microvessel density                                    |          |          | P=0.32  |
| <60 vessels                                            | 20 (53)  | 18 (47)  |         |
| ≥60 vessels                                            | 21 (42)  | 29 (58)  |         |

HIF = hypoxia-inducible factor; TNM = tumour node metastasis. *Statistically significant at 5% level.

### Table 3

| Patient and tumour characteristics and HIF-2α staining | HIF-2α, number of patients (%) |  
|--------------------------------------------------------|-------------------------------|
| Gender                                                 | Negative | Positive | P-value |
| Male                                                   | 19 (34)  | 37 (66)  | P=0.68  |
| Female                                                 | 13 (38)  | 21 (62)  |         |
| T stage                                                |          |          | P=0.53  |
| T1/2                                                   | 6 (33)   | 12 (67)  |         |
| T3/4                                                   | 26 (36)  | 46 (64)  |         |
| N stage                                                |          |          | P=0.10  |
| N0                                                     | 17 (30)  | 40 (70)  |         |
| N1                                                     | 8 (38)   | 13 (62)  |         |
| N2                                                     | 7 (58)   | 5 (42)   |         |
| Differentiation                                        |          |          | P=0.94  |
| Well                                                   | 2 (50)   | 2 (50)   |         |
| Moderate                                               | 25 (34)  | 48 (66)  |         |
| Poor                                                   | 5 (39)   | 8 (61)   |         |
| Dukes’ stage                                           |          |          | P=0.25  |
| Dukes’ A                                               | 6 (35)   | 11 (65)  |         |
| Dukes’ B                                               | 11 (28)  | 29 (72)  |         |
| Dukes C1                                               | 12 (43)  | 16 (57)  |         |
| Dukes C2                                               | 3 (60)   | 2 (40)   |         |
| TNM stage                                              |          |          | P=0.28  |
| Stage I                                               | 6 (35)   | 11 (65)  |         |
| Stage II                                              | 11 (28)  | 29 (72)  |         |
| Stage III                                             | 15 (46)  | 18 (54)  |         |
| Vascular invasion                                      |          |          | P=0.74  |
| Absent                                                | 22 (35)  | 41 (65)  |         |
| Intramural                                             | 3 (27)   | 8 (73)   |         |
| Extramural                                             | 7 (44)   | 9 (56)   |         |
| Microvessel density                                    |          |          | P=0.86  |
| <60 vessels                                            | 13 (34)  | 25 (66)  |         |
| ≥60 vessels                                            | 18 (36)  | 32 (64)  |         |

HIF = hypoxia-inducible factor; TNM = tumour node metastasis.
expression, although this did not reach statistical significance. Both Jiang et al (2003b), in colorectal cancer, and Lu et al (2006), in rectal cancer, showed increased positivity for HIF-1α expression with increasing Dukes’ stage. The latter group also found an overall reduction in survival in patients with high HIF-1α expression. Theodoropoulos et al (2006) showed a significant association between HIF-1α and lymph node metastasis, low rectal location and advanced tumour grade. Although HIF-1α expression, infiltrative tumour growth pattern, positive lymph node status and VEGF upregulation were associated with decreased disease-free and overall survival, multivariate analysis only revealed high HIF-1α reactivity and lymph node positivity as predictors of poor outcome. This is the more notable of the earlier rectal cancer studies due to the higher numbers, and it is interesting to note the similarities in terms of percentage of expression in these studies to our own.

It is quite likely that numerous factors act separately to cause tumour growth and spread, and pathways may interact in an as yet undefined manner. A number of these factors have been described to be hypoxia and non-hypoxia dependent, including VHL, COX-2, CA-9, CHOP and ATF4, among many others (Yoshimura et al, 2004; Kivela et al, 2005; Carracedo et al, 2006; Cleven et al, 2007).

Table 4  Multivariate analysis of survival for HIF-1α

| HIF-1α  | HR   | 95% CI       | P-value |
|---------|------|--------------|---------|
| No      | 1    |              |         |
| Yes     | 4.108| 1.366-12.352 | 0.012   |

Vascular invasion

|        | HR   | 95% CI       | P-value |
|--------|------|--------------|---------|
| No     | 1    |              |         |
| Intramural | 1.483| 0.400-5.495 | 0.555   |
| Extramural | 2.284| 0.917-5.688 | 0.016   |

TNM stage

|        | HR   | 95% CI       | P-value |
|--------|------|--------------|---------|
| I      | 1    |              |         |
| II     | 1.724| 0.197-15.069 | 0.622   |
| III    | 10.048| 1.302-77.553 | 0.027   |

CI = confidence interval; HIF = hypoxia-inducible factor; HR = hazard ratio; TNM = tumour node metastasis. *Reference values.

with increasing Dukes’ stage. The latter group also found an overall reduction in survival in patients with high HIF-1α expression. Theodoropoulos et al (2006) showed a significant association between HIF-1α and lymph node metastasis, low rectal location and advanced tumour grade. Although HIF-1α expression, infiltrative tumour growth pattern, positive lymph node status and VEGF upregulation were associated with decreased disease-free and overall survival, multivariate analysis only revealed high HIF-1α reactivity and lymph node positivity as predictors of poor outcome. This is the more notable of the earlier rectal cancer studies due to the higher numbers, and it is interesting to note the similarities in terms of percentage of expression in these studies to our own.

It is quite likely that numerous factors act separately to cause tumour growth and spread, and pathways may interact in an as yet undefined manner. A number of these factors have been described to be hypoxia and non-hypoxia dependent, including VHL, COX-2, CA-9, CHOP and ATF4, among many others (Yoshimura et al, 2004; Kivela et al, 2005; Carracedo et al, 2006; Cleven et al, 2007).

Table 4  Multivariate analysis of survival for HIF-1α

| HIF-1α  | HR   | 95% CI       | P-value |
|---------|------|--------------|---------|
| No      | 1    |              |         |
| Yes     | 4.108| 1.366-12.352 | 0.012   |

Vascular invasion

|        | HR   | 95% CI       | P-value |
|--------|------|--------------|---------|
| No     | 1    |              |         |
| Intramural | 1.483| 0.400-5.495 | 0.555   |
| Extramural | 2.284| 0.917-5.688 | 0.016   |

TNM stage

|        | HR   | 95% CI       | P-value |
|--------|------|--------------|---------|
| I      | 1    |              |         |
| II     | 1.724| 0.197-15.069 | 0.622   |
| III    | 10.048| 1.302-77.553 | 0.027   |

CI = confidence interval; HIF = hypoxia-inducible factor; HR = hazard ratio; TNM = tumour node metastasis. *Reference values.
REFERENCES

American Cancer Society (2006) Cancer Facts and Figures. American Cancer Society: Atlanta

Aprelikova O, Chandramouli GV, Wood M, Vasselli JR, Riss J, Maranchie JK, Linehan WM, Barrett JC (2004) Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J Cell Biochem 92: 491 – 501. Binrner P, Schindl M, Obermair A, Plank C, Breiteneker G, Oberhuber G (2000) Overexpression of hypoxia-inducible factor-1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res 60: 4693 – 4696

Blancher C, Moore FW, Talks KL, Houlbrook S, Harris AL (2000) Relationship of hypoxia-inducible factor (HIF-1alpha) and HIF-2alpha expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. Cancer Res 60: 7106 – 7113

Bosi P, Viale G, Lee AK, Alfano R, Coggi G, Bosari S (1995) Angiogenesis in colorectal tumors: microvessel quantification in adenomas and carcinomas with clinicopathological correlations. Cancer Res 55: 5049 – 5053

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch C, Ratcliffe P, Moons L, Jain RK, Collen D, Keshet E, Keshet E (1998) Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394: 485 – 490

Carracedo A, Lorente M, Egia A, Blazquez C, Garcia S, Giroux V, Malfait F, Viluendas R, Girondel M, Gonzalez-Feria L, Piris MA, Iovanna JL, Guzman M, Velasco G (2006) The stress-regulated protein p8 mediates cannabinoids-induced apoptosis of tumor cells. Cancer Cell 9: 301 – 312

Chen YB, Yan DS, Zhan YQ, Zhou ZW, Li W, Chen G (2004) Correlation of tumor microvessel density to metastasis and recurrence of rectal cancer. Ai Zhong 25: 1203 – 1206

Choi HJ, Hyun MS, Jung GJ, Kim SS, Hong SH (1998) Tumor angiogenesis as a prognostic predictor in colorectal carcinoma with special reference to mode of metastasis and recurrence. Oncology 55: 575 – 581

Chua YJ, Cunningham D (2006) Emerging therapies for rectal cancer. Colorectal Dis (Suppl 3): 18 – 20

Cleven AH, van Engeland M, Wouters BG, de Bruine AP (2007) Stromal expression of hypoxia-regulated proteins is an adverse prognostic factor in colorectal carcinomas. Cell Oncol 29: 229 – 240

Conway EM, Collen D, Carmeliet P (2001) Molecular mechanisms of blood vessel growth. Cardiovasc Res 49: 507 – 521

Dahleberg M, Palhman L, Bergstrom R, Glimelius B (1998) Improved survival in patients with rectal cancer: a population-based register study. Br J Surg 85: 515 – 520

Giatromanolaki A, Harris AL (2001) Tumour hypoxia, hypoxia signaling pathways and hypoxia inducible factor expression in human cancer. Anticancer Res 21: 4317 – 4324

Gulubova M, Vlakyova T (2007) Prognostic significance of mast cell suppressor and tumor-associated carbonic anhydrases IX and XII in normal and neoplastic colorectal mucosa. World J Gastroenterol 9: 491 – 494

Kaidi A, Williams GC, Parakeeva C (2007) Interaction between beta-catenin and HIF-1 promotes cellular adaption to hypoxia. Nat Cell Biol 9: 210 – 217

Kivela AJ, Parkkila S, Saarnio J, Karttunen TJ, Kivelä J, Parkkila AK, Bartosova M, Mucha V, Novak M, Waheed A, Sly WS, Rajaniemi H, Pastorekova S, Pastorek J (2005) Expression of von Hippel-Lindau tumor suppressor and tumor-associated carbonic anhydrases IX and XII in normal and neoplastic colorectal mucosa. World J Gastroenterol 11: 2050 – 2055

Klatte T, Seligson DB, Biggs SB, Leppert JT, Berkman MK, Kleid MD, Yu H, Kabbinavar FF, Pantuck AJ, Beldegrun AS (2007) Hypoxia-inducible factor 1 alpha in clear cell renal cell carcinoma. Clin Cancer Res 13: 7388 – 7393

Kmitowicz Z (2004) British cancer death rates fell by 12% between 1972 and 2002. BMJ 328: 303

Koukourakis MI, Giatromanolaki A, Sivridis E, Simopoulos C, Turley H, Talks K, Gatter KC, Harris AL (2002) Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. Int J Radiat Oncol Biol Phys 53: 1192 – 1202

Kuwai T, Kitadai Y, Tanaka S, Onogawa S, Matsutani N, Kaio E, Ito M, Chayama K (2003) Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma. Int J Cancer 105: 171 – 181

Leek RD, Talks KL, Pezzella F, Turley H, Campo L, Brown NS, Bicknell R, Taylor M, Gatter KC, Harris AL (2002) Relation of hypoxia-inducible factor-2 alpha (HIF-2 alpha) expression in tumor-infiltrative macrophages to tumor angiogenesis and the oxidative thromboperoxidase pathway in Human breast cancer. Cancer Res 62: 1326 – 1329

Li C, Gardy R, Seon BK, Duff SE, Abdalla S, Renehan A, O’Dwyer ST, Haboubi N, Kumar S (2003) Both high intratumoral microvessel density determined using CD105 antibody and elevated plasma levels of CD105 in colorectal cancer patients correlate with poor prognosis. Br J Cancer 88: 1424 – 1431

Lindmark G, Gerdin B, Pahlman L, Bergstrom R, Glimelius B (1994) Prognostic predictors in colorectal cancer. Dis Colon Rectum 37: 1219 – 1227

Lu XG, Xing CG, Feng YZ, Chen J, Deng C (2006) Clinical significance of immunohistochemical expression of hypoxia-inducible factor-1alpha as a prognostic marker in rectal adenocarcinoma. Clin Colorectal Cancer 5: 350 – 353

Mackay H, Hedley D, Major P, Townsley C, Mackenzie M, Vincent M, Degendorfer P, Tsaao MS, Nicklee T, Birle D, Wright J, Siu L, Moore M, Oza A (2005) A phase II trial with pharmacodynamic endpoints of the proteasome inhibitor bortezomib in patients with metastatic colorectal cancer. Clin Cancer Res 11: 5526 – 5533

Maynard MA, Ooh M (2007) The role of hypoxia-inducible factors in cancer. Cell Mol Life Sci 64: 2170 – 2180

Palit V, Phillips RM, Puri R, Shah T, Bibby MC (2005) Expression of HIF-1alpha and Glut-1 in human bladder cancer. Oncol Rep 14: 909 – 913

Pugh CW, Ratcliffe PJ (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9: 677 – 684

Rasheed S, Harris AL, Tekkis PP, Turley H, Silver A, McDonald PJ, Talbot IC, Glynne-Jones R, Northover JM, Guenther T (2009) Assessment of microvessel density and carboxy anhydrase-9 (CA-9) expression in rectal cancer. Pathol Res Pract 205(1): 1 – 9

Rasheed S, McDonald PJ, Northover JM, Guenther T (2008) Angiogenesis and tumor-associated factors HIF-1alpha and HIF-2alpha in human colorectal carcinoma. Clin Cancer Res 14: 575 – 581

Rasheed S, Harris AL, Tekkis PP, Stryker H, Silver A, McDonald PJ, Talbot IC, Glynne-Jones R, Northover JM, Guenther T (2009) Assessment of microvessel density and carboxy anhydrase-9 (CA-9) expression in rectal cancer. Pathol Res Pract 205(1): 1 – 9

Sagar P, Pemberton J (1996) Surgical management of locally recurrent rectal cancer. Br J Surg 83: 293 – 304

Semenza GL (2001) Hypoxia-inducible factor 1: control of oxygen homeostasis in health and disease. Pediatr Res 49: 614 – 617

Shibaji T, Nagao M, Ikeda N, Kanehiro H, Hisanaga M, Ko S, Fukumoto A, Nakajima Y (2003) Prognostic significance of HIF-1 alpha overexpression in human pancreatic cancer. Anticancer Res 23: 4721 – 4727

Singh RP, Gu M, Agarwal R (2008) Silibinin inhibits colorectal cancer growth by inhibiting tumor cell proliferation and angiogenesis. Cancer Res 68: 2043 – 2050

Skeels CL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL (2000) The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol 157: 411 – 421

Theodoropoulos GE, Lazaris AC, Theodoropoulos VE, Papathesiosiu K, Gazoulakis M, Brannis J, Patsouris E, Panoussopoulos D (2006) Hypoxia, angiogenesis and apoptosis markers in locally advanced rectal cancer. Int J Colorectal Dis 21: 248 – 257

Theodoropoulos VE, Lazaris AC, Kastriotis I, Spiladi C, Theodoropoulos GE, Tsoukala V, Patsouris E, Sofras F (2005) Evaluation of hypoxia-inducible factor-1alpha overexpression as a predictor of tumour recurrence and progression in superficial urothelial bladder carcinoma. BJU Int 95: 425 – 431
Tomisaki S, Ohno S, Ichiyoshi Y, Kuwano H, Maehara Y, Sugimachi K (1996) Microvessel quantification and its possible relation with liver metastasis in colorectal cancer. *Cancer* 77: 1722–1728

Trastour C, Benizri E, Ettoire F, Ramaiali A, Chamorey E, Pouyssegur J, Berra E (2007) HIF-1alpha and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome. *Int J Cancer* 120: 1451–1458

Vermeulen PB, Van den Eynden GG, Huget P, Goovaerts G, Weyler J, Lardon F, Van Marck E, Hubens G, Dirix LY (1999) Prospective study of intratumoral microvessel density, p53 expression and survival in colorectal cancer. *Br J Cancer* 79: 316–322

Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Koizin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 10: 145–147

Willett CG, Koizin SV, Duda DG, di Tomaso E, Kozak KR, Boucher Y, Jain RR (2006) Combined vascular endothelial growth factor-targeted therapy and radiotherapy for rectal cancer: theory and clinical practice. *Semin Oncol* 33: S35–S40

Wykoff CC, Pugh CW, Harris AL, Maxwell PH, Ratcliffe PJ (2001) The HIF pathway: implications for patterns of gene expression in cancer. *Novartis Found Symp* 240: 212–225; discussion 225–231

Yoshimura H, Dhar DK, Kohno H, Kubota H, Fujii T, Ueda S, Kinugasa S, Tachibana M, Nagasue N (2004) Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. *Clin Cancer Res* 10: 8554–8560

Zhu B, Lu L, Cai W, Yang X, Li C, Yang Z, Zhan W, Ma JX, Gao G (2007) Kallikrein-binding protein inhibits growth of gastric carcinoma by reducing vascular endothelial growth factor production and angiogenesis. *Mol Cancer Ther* 6: 3297–3306