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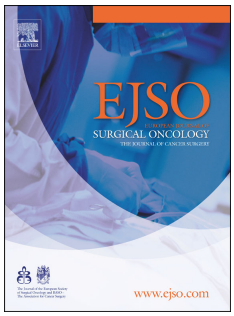

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EXPRESSION PATTERNS OF HYPOXIC MARKERS AT THE INVASIVE MARGIN OF COLORECTAL CANCERS AND LIVER METASTASES

Running Title: Colorectal cancer invasion and hypoxia

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ABSTRACT

Aim: To understand the role of hypoxia in cancer progression of primary colorectal cancer and colorectal liver metastases. To look at associations of hypoxia with more aggressive phenotypes.

Methods: Archival tissue was retrieved from 55 patients and tissue micro arrays constructed using tissue from the margin and the centre of the tumour. Hypoxia markers Hif-1α, Vegf, CA-9, VHL and Glut-1 were visualised using immunohistochemical detection and quantified using semi quantitative analysis of the digitised images. Clinical details and outcome data were retrieved by case note review and collated with hypoxia markers data in a statistical database.

Results : Significantly increased expression of all markers were found at the tumour margin compared to the tumour centre, both in primary colorectal cancer (CRC) and liver metastases. Pushing margin CRC was associated with increased Vegf expression. Positive correlations were observed between Hif-1α and Vegf ($p<0.001$), and Hif-1α and VHL ($p<0.001$) in primary CRC, but no relationship was seen between Hif-1α and either Glut-1 or CA-9. A significant trend to worse disease-free survival was also noted with increased margin expression of Hif-1α ($p<0.001$) and VHL ($p=0.02$) in primary CRC, but not for any of the other markers.

Conclusion: This study underlines the importance of the invasive margin in colorectal cancer biology. It is the area most responsive to hypoxic influences

and dependent on its ability to up-regulate Hif-1α, has a significant impact on disease-free survival.

Keywords

Colorectal cancer; hypoxia; invasion; liver metastases

Introduction

Colorectal cancer is the second most common cancer in the United Kingdom with an incidence of 17,000 new cases per annum(1). Although some 50% of patients will be cured by surgical resection, many will relapse within 2 to 3 years and the majority of these will have metastatic disease in the liver.
Most solid cancers, including colorectal cancers, exhibit upregulated glycolytic metabolism in preference to oxidative phosphorylation for the metabolism of glucose. It is debatable whether this is a consequence of a hypoxic tumour microenvironment (2)or whether it is an inherent feature of the malignant phenotype as first suggested by Otto Warburg over 70 years ago(3). Evidence is accumulating to support the latter and the existence of a pseudo-hypoxic tumour metabolic profile(4) although the molecular basis for this remains unclear.

The degree of tumour hypoxia has been associated with more aggressive phenotypes and is known to play an important role in tumour resistance to chemotherapy and radiotherapy. In soft tissue sarcoma(5) and head and neck cancer(6) tumour hypoxia has been found to correlate with distant metastatic spread and poor survival.
The oxygen status of a tumour is dependent on the local vascular supply offset by the metabolic demands of the proliferating cancer cells. This leads to heterogeneity in tumour oxygenation with focal areas of necrosis characteristically being observed towards the centre of the tumour.

In order to survive in a microenvironment of relative hypoxia, cancer cells have developed strategies that enable them to adapt to local conditions. One such mechanism involves the up-regulation of hypoxia inducible factor-1α (Hif-1α). Hif-1α has been described as the master regulator of cellular oxygen homeostasis(7), and is capable of inducing the expression of more than 30 genes involved in energy metabolism, angiogenesis, cell adhesion, proliferation and survival(8). These include various genes involved in glycolysis, such as glucose transporter-1 (Glut-1) which facilitates cellular glucose, and hexokinase II (HKII) and lactate dehydrogenase-A (LDH-A) which regulate anaerobic metabolism. In addition to up-regulating glycolysis, Hif-1α stimulates angiogenesis to increase oxygen and glucose delivery via up-regulation of vascular endothelial growth factor (Vegf) and via carbonic anhydrase-9 (CA-IX) up-regulation helps to maintain intracellular pH. Hif-1 is itself regulated by von Hippel Lindau protein (VHL) which in conditions of normoxia facilitates Hif-1 proteosomal degradation. In contrast, in hypoxic conditions VHL is down-regulated, allowing Hif-1α accumulation and a switch in cellular metabolism in favour of anaerobic glycolysis, and enhancing cell survival and promoting disease progression

The aim of the current study was to further our understanding of the role of hypoxia in cancer progression, by investigating the regional expression of a panel of surrogate markers of hypoxia within both primary and secondary colorectal cancers, exploring their potential associations, and determining their influence on patient survival.

Materials and methods

Patients with metastatic colorectal cancer, who had undergone potentially curative surgical resection of the primary cancer and liver metastases, were identified from databases held at St James's University Hospital, Leeds and the Royal Liverpool University Hospital, Liverpool between June 1994 and June 2000. Patients with conditions known to predispose to colorectal cancer (e.g. inflammatory bowel disease, familial adenomatous polyposis), and those who had undergone preoperative chemo/radiotherapy were excluded. Patients who died as a direct complication of their surgery were excluded as their death was unrelated to tumour biology. In total 55 patients were available to study, 18 of which underwent surgical resection for synchronous liver metastases and 37 where operated for metachronous disease detected on follow-up. Colorectal resections included right hemicolectomy (19), Left hemi colectomy/ sigmoid colectomy (13) and anterior resection (23) with median duration of surgery being 183 minutes (range 165-242). Liver resections carried out were segmentectomy (17), hemihepatectomy (30) and trisectionectomy (8) with a median duration of 218 minutes (range 134-315). The same operative methods were applied to patients in both hospitals with tissue samples processed in the same way following resection.

Local ethics committee approval was obtained for the study (Leeds West Research Ethics Committee, RL04/6669).

Analysis of the invasive growth pattern of colorectal cancer and liver metastases

The invasive growth patterns of both primary cancers and liver metastases were classified by an experienced histopathologist (NS) with an interest in colorectal pathology, using Haematoxylin and Eosin (H&E) histochemistry. The invasive growth pattern of the primary cancers was classified as either pushing or infiltrative as defined by Jass *et.al* (9). A modified classification based on Lunevicus *et.a*l(10), was used for liver metastases; cancers encompassed by a fibrous capsule over >50% of the invasive margin being defined as capsulated, and those with <50% fibrosis defined as non-encapsulated. (11)

Tissue Microarray (TMA) construction

Haematoxylin and eosin (H&E) whole tumour sections were obtained from paraffin embedded tissue blocks to enable accurate identification of the target areas. Cores from the tumour margin and tumour centre with a core size of at least 0.6mm were taken. Four cores from each tumour region were used in the study to account for core loss during processing and because this number has previously been shown to be representative of the tumour as a whole (12). Cores taken from the two areas were transferred to pre-constructed holes in a recipient paraffin block at precise 'XY' co-ordinates and the position and tissue reference recorded in a data sheet. Human placenta, kidney, breast, normal colon and normal liver tissue were used as internal controls and helped in the orientation of the block.

Immunohistochemistry protocol for TMAs

Tissue sections of 4 μm thickness were prepared from the TMAs, de-waxed in xylene (3 x 5min immersions) and rehydrated in graded alcohol (3 x 5 min immersions). Slides were washed in running tap water for 5 min, endogenous peroxidase activity blocked by incubation in 0.3% (v/v) H_20_2/methanol for 10 min, followed by rinsing in running tap water for 5 min.

Antigen retrieval was performed as follows: Vegf, pressure cooking in 0.01% unmasking solution (Vector Laboratories Inc, Peterborough, UK) for 2 min; Hif-1α, pressuring coking in citrate buffer (pH6.0) for 2 min; CA-9, Glut-1, and VHL, microwave treatment in citrate buffer (pH6.0) for 10 min at 800w. Sections were incubated in 100 μl avidin for 15 min, washed in TBS, incubated with 100 μl biotin solution (Vector Laboratories) for 15 min, and washed in TBS again. Incubation in 5% swine serum (DAKO Cytomation, Ely, UK) for 30 min was used to prevent non-specific binding for Glut-1, CA-9, and Vegf, and 5% rabbit serum (DAKO Cytomation) for Hif-1α and VHL. Sections were incubated in 100μl of primary antibody/TBS under the following conditions: Hif-1α (mouse monoclonal, 1:100 dilution, Abcam), Glut-1 (rabbit polyclonal, 1:200 dilution, Chemicon International), and VHL (mouse monoclonal, 1:50 dilution, Abcam) for 1 hour at room temperature; CA-9 (rabbit polyclonal, 1:1000 dilution, R&D systems) and Vegf (rabbit polyclonal, 1:50 dilution, Zymed laboratories) overnight at 4°C. Slides were rinsed in TBS (2 x 5min incubations) and incubated with the appropriate secondary antibody. All secondary antibodies were obtained from DAKO Cytomation and incubated for 1 hour at room temperature diluted in TBS with 5% swine or

rabbit serum at the following concentrations: Hif-1α (rabbit anti-mouse, 1:200), Glut-1 (swine anti-rabbit, 1:200), CA-9 (swine anti-rabbit, 1:200), Vegf (swine anti-rabbit, 1:200), VHL (rabbit anti-mouse, 1:50). Antigen visualisation was performed using a peroxidase conjugated streptavidin-biotin complex (ABC kit, Dako Cytomation): 100µl ABC complex was applied for 30min, slides were washed with TBS, and colour developed using DAB (diaminobenzidine tetrahydrochloride) diluted 1:50 with HRP substrate buffer for 10 min. Slides were rinsed in tap water, counterstained with haematoxylin for 1 min, dehydrated in alcohol (3 x 5 min incubations), cleared in xylene (3 x 5 min incubations, and mounted in DePex with cover slips. A variety of tissues, included in the TMA construction, served as positive controls: normal placenta and breast tissue for Hif-1α, Vegf, CA-9 and Glut-1, and normal kidney for VHL. Negative controls omitted the primary antibody from the first incubation step.

Digitised image analysis

All slides were scanned at x40 magnification using an Aperio™ scanner, with images stored on a CD. Scanned images were visualised and analysed using Aperio™ software. A single observer performed semi-quantitative analysis of immunoreactivity of the five markers on two separate occasions. The average score of the cores was used in data analysis. Scores were assigned based on both the intensity of staining in the cytoplasmic and nuclear compartments and the percentage of positively stained cells according to previously for published systems: Vegf, CA-9 and Glut-1 expression were calculated by combining the intensity of stained cells (0-3) with the percentage of positive

cells (0-4) to give a score within the range 0 – 12(13) VHL expression was based on cytoplasmic staining only (range, 0-3)(14) and Hif-1α expression on the combined cytoplasmic and nuclear staining (range, 0-4)(6). Twenty tissue samples were re-analysed one month following the initial analysis by the first author and another independent observer to assess intra and inter-observer variation.

Statistical analysis

Statistical analysis was performed using SPSS for windows (SPSS v12.0, California, USA). The relationship between the invasive growth patterns of primary colorectal cancers, their liver metastases and the markers of hypoxia, were tested using the chi-squared test. Pearson's correlation was used to test the relationship between marker expressions in the primary cancer with that in the liver metastases. The influence of marker expression on disease-free and overall survival was analysed using Kaplan- Meier survival curves and the log rank test. Cohen's kappa was calculated to investigate inter and intra observer variation. In all analyses, a p-value of <0.05 was taken to indicate statistical significance.

RESULTS

The demographics of patients included in the study together with the routine histological analyses of their resected primary colorectal cancers are shown in Table 1. 18 patients were found to have synchronous liver metastases whilst 37 patients developed metachronous liver disease. None of the patients had synchronous primary colorectal cancers. The tumour size was 4.6cm (range 3.1-5.9). No significant difference in tumour size was seen between the pushing and infiltrative margins.

The mean length of follow-up for all patients was 47 months (range 6 – 171 months). 28/55 patients were alive on completion of the study. The mean overall survival following resection of the primary cancers was 47 months (range: 6 – 171 months) and 36.3 months (range: 0 – 118 months) following liver resection. The mean disease free survival following resection of primary cancers was 30 months (3 - 88 months) and 28 months (4 – 118 months) following liver resection. 17/55 patients developed disease recurrence following liver resection: recurrence at the primary site (2), liver recurrence (7), lung recurrence (5), and multi-site recurrence (3).

The scoring system for hypoxia markers were shown to be reproducible with fair to moderate correlations between inter and intra observer variation when 20 sections were re-scored (Table-2). This might be due to quantification variation or maybe there is heterogenous expression of the markers which is

not accounted for by just averaging the scores. A larger cohort of matched samples would be needed to validate these findings further.

Regional expression of hypoxic markers in primary cancers and liver metastases

The high and low expression patterns of the individual markers of hypoxia in primary CRC and liver metastases are shown in Figure 1. The majority of the necrotic areas were found in the centre of the tumour. Colorectal liver metastases expression scores were found to be higher compared to the primary colorectal cancer in Vegf (p=0.005), CA-9 (p=0.07) and Glut-1 (p=0.04). Hif-1α (p=0.17) and VHL (p=0.02) showed higher levels of scores in primary colorectal cancers compared to the liver metastases.

Vascular invasion and perineural invasion were found to be associated with Increased expression of Hif-1α (p=0.001), Vegf (p=0.018) and VHL (p=0.016). The advanced Dukes' stage was associated with increased expression of Hif-1α (p=0.003) and VHL (p=0.001), but not Vegf (p=0.11) (Figure 2). No relationship was found between any of these histological variables and either Glut-1 or CA-9 expression.

Correlation between markers of hypoxia at the margin and centre of primary cancers and matched liver metastases

Significantly increased expression of all the markers was noted at the margin as compared to the centre in the primary cancers: Hif-1α, p<0.001; Vegf, p<0.001; VHL, p<0.001; Glut-1, p=0.015; CA-9, p=0.013. Similar findings were observed in the liver metastases with the exception of Vegf which

reached borderline significance: Hif-1α, p<0.001; VHL, p<0.001; Glut-1, p=0.002; CA-9, p=0.05; Vegf, p=0.53 (Table 3)

Given the prominence of the margin expression for all markers of hypoxia, subsequent analysis concentrated on this region of the cancers rather than data derived from the tumour centre.

Correlation between the invasive growth pattern and markers of hypoxia

Primary colorectal cancers and liver metastases were each divided into high and low groups based on the mean expression of the hypoxia markers at the tumour margin for the whole cohort (Table-4). The high and low expression groups were used for further analysis of the invasive margin and survival.

Pushing margin CRC were associated with increased Vegf expression (p=0.01), while the infiltrative margin was associated with decreased Vegf and increased VHL (p=0.019) expression. Hif-1α expression on the other hand was reversed with more pushing margin patients having low expression patterns while the infiltrative tumours had increased Hif-1α expression. Even though a trend to increased expression of Hif-1α was seen with the infiltrative margin this did not reach statistical significance (p=0.07). Infiltrative margin expression of VHL (p=0.002) and CA-9 (p=0.024) were also found to be up regulated. No such differences were seen with Glut-1 expression either in the primary or the liver metastases.

The invasive growth pattern of liver metastases showed a positive correlation, with non-encapsulated tumours being associated with increased expression of CA-9 ($p=0.004$) and Hif-1α ($p=0.03$).

Correlation between individual markers of hypoxia

Significant positive correlations were observed between Hif-1α and Vegf ($p<0.001$), and Hif-1α and VHL ($p<0.001$) in the primary cancers, but no relationship was seen between Hif-1α and either Glut-1 or CA-9. No significant correlation was found between any of the markers in the liver metastases.

Markers of hypoxia and survival

Kaplan- Meier curves for disease free and overall survival were plotted for primary cancers and liver metastases. A significant trend to worse disease-free survival was seen with increased margin expression of Hif-1α ($p<0.001$) and VHL ($p=0.02$) in primary CRC, but not for any of the other markers. None of the markers had a significant influence on overall survival.

DISCUSSION

The aim of this study was to investigate the regional expression of markers of hypoxia in primary colorectal cancers and their liver metastases, their potential correlations, and influence on survival. To achieve this we selected a cohort of patients who had undergone surgical resection of both primary colorectal cancer and liver metastases. The patients were operated in two different hospitals. This may influence the level of hypoxia with different operation methods(6), operation time and differences in processing the specimen. The operation time was calculated from the time patient enters theatre to leaving it and therfore is not a true reflection of the time patient was operated on. We have tried to limit this bias by choosing patients who underwent standard surgical procedures and also all specimen were fixed in formal saline as soon as they were resected.

This cohort inevitably represents the most advanced cases of colorectal cancer and is reflected in the large number of T3/T4 cancers and cancers with nodal disease. A larger proportion of patients also had synchronous metastases. Care should therefore be taken when extrapolating the results of this study to earlier stage disease.

The cohort studied only consisted of 55 paired samples. This was only achieved following collaboration between two tertiary referral centres. Reasons for not having a larger cohort included, being unable to locate all the relevant tissue samples with the invasive margin and the centre being

represented. Consent for the use of tissue in research was also unable to be verified. This study has been able to report important findings to previous studies and identify two invasive phenotypes which have shown differential expression patterns of hypoxic markers. A larger cohort of matched samples would be needed to validate these findings further.

TMA technology was employed to facilitate efficient, high-throughput tissue analysis. Four cores from each cancer margin and centre were included to improve sample representation of the whole cancer (12) and to compensate for core disintegration during tissue processing. By using TMA technology it was hoped to reduce variations during processing.

Markers of hypoxia at the margin and centre of primary cancers and matched liver metastases

One of the most notable findings in this study is the differential regional expression of all hypoxic markers with increased expression at the invasive margin compared to the tumour centre. This finding has previously been reported for primary cancers, but to our knowledge this is the first study to document a similar differential expression in liver metastases. Kuwai *et.al,* in their study observed a significant correlation between Hif-1α, Vegf and MVD in primary CRC, but the expression pattern failed to significantly correlate with prognosis (6;15). Increased Glut-1 and CA-9 expression have also been shown to correlate with poor prognosis in a variety of tumours including CRC(16;17) .

The universal up-regulation of hypoxic markers at the tumour margin suggests that it is the margin rather than the centre that is most responsive to the hypoxic influences of the tumour microenvironment. Our findings also support the critical role of Hif-1α in orchestrating tumour adaptation to hypoxia. All the markers studied, with the exception of VHL, are known targets for Hif-1α transcription. In the present study, a positive correlation was only observed between Hif-1α and Vegf expression, but not CA-9 or Glut-1, in primary cancers. This might suggest that other mechanisms, in addition to Hif-1α, are important in their expression. Glut-1 has been shown to be responsive to hormonal influences(18), alkaline pH(19), and the supply of glucose(20). CA-9 is also thought to be influenced by the tumour pH(21). No such correlation between Hif-1α and Vegf was observed in liver metastases, which might reflect a differing tumour requirement for angiogenesis in the highly vascular liver.

Correlation between individual markers of hypoxia

Correlation between Hif-1α and VHL was found when the expression patterns of the hypoxic markers were looked at. Conventional theory suggests that up-regulation of Hif-1α in response to hypoxia is a consequence of diminished VHL orchestrated Hif-1α proteosomal degradation(22). Thus, one might expect Hif-1α expression to show a negative correlation with VHL. A possible explanation for the contrary positive correlation observed may be due to inactivating mutations within the VHL gene. Loss of heterozygosity in the VHL gene has been previously documented in sporadic colorectal cancer(23) and it is possible that a second hit mutation results in non-functioning VHL, which

despite up-regulated expression is unable to deactivate Hif-1α activity. Further functional *in vitro* studies will be necessary to test this hypothesis.

The invasive growth pattern and markers of hypoxia

Both primary colorectal cancers and liver metastases demonstrate different invasive growth patterns, with primary cancers exhibiting either a pushing or infiltrative pattern and liver metastases a capsulated or non-encapsulated morphology at the invasive margin. The type of growth pattern is of importance in predicting outcome, with infiltrative primary cancers and non-encapsulated metastases being associated with worse survival. We have previously shown a correlation between the primary margin with that of the liver metastases(11). The pushing primary CRC tumours were unexpectedly found to have increased micro vessel density compared to the infiltrative margin. Vegf was found to follow a similar pattern to MVD in this study, with pushing margin having a higher expression of Vegf. Infiltrative margin on the other hand was found to have higher levels of Hif-1α and CA-9. Thus, it would appear that the variations in invasive growth can be explained, at least in part on the basis of differing tumour response to hypoxia.

The differential expression patterns seen in the two invasive phenotypes could be due to the sample size or the advanced nature of disease in the study population. The finding could also signify a real difference between the two phenotypes. Calvani *et.al* in their recent paper showed different Vegf mediated induction of Hif-1α within different colorectal cancer cell lines(24). Other studies have also looked at Hif-1 independent pathways in tumour

angiogenesis(25). Further studies will need to be carried out to validate our finding, but with monoclonal antibodies like Bevacizumab (humanized anti-Vegf), being approved for the treatment of patients with metastatic colorectal cancer, it is important to identify which patients might benefit from this new treatment. The invasive margins of primary CRC and liver metastases can be a potential target to identify patients who might be sensitive or resistant to anti-Vegf strategies.

Hypoxia and survival

The importance of tumour hypoxia and Hif-1α expression in predicting survival outcome in colorectal cancer has been confirmed in this study. Hif-1α has been shown to affect the adaptive processes to hypoxia through key apoptotic regulators such as Bcl-2 and p53. p53 is a tumour suppressor gene which normally has a short half- life, but is stabilised in hypoxia. Low expression of p53 is thought to compete with Hif-1α for the transcriptional co-activator p300 which attenuates Hif-1α transactivation, whilst a high rate expression of p53 is thought to facilitate destruction of Hif-1α via Mdm2- targeted proteosomal degradation(26;27). This mechanism of Hif-1α could serve to protect cells from apoptotic death, in the early phase of hypoxia. Another mechanism could be genetic alterations that results in increased Hif-1α expression by either activating oncogenes or inactivating tumour suppressor genes, which in turn promotes pro-survival conditions and contributes to clonal selection and cancer progression. This view is supported by the association of increased Hif-1α expression with increased mortality in many cancers including colorectal cancer(6).

We have shown that up-regulated Hif-1α expression in primary cancers to be associated with significantly worse disease-free survival. This did not translate into a difference in overall survival, which is probably attributable to a number of non-cancer related deaths in this predominantly elderly colorectal cancer population. VHL also had a significant influence on disease-free survival, with higher expression associated with worse outcome. It is unlikely that this effect is independent of Hif-1α and serves to highlight the importance of Hif-1α regulated expression on predicted survival. In this study the pushing and infiltrative tumours were assessed together. Subgroup analysis was not carried out due to the small sample size in the study.

This study underlines the importance of the invasive margin in colorectal cancer biology. It is the area most responsive to hypoxic influences and dependent on its ability to up-regulate Hif-1α has a significant impact on disease-free survival. Larger studies are needed to look further into our preliminary findings.

Clinicopathological details of patients (n=55)	Frequency
Patient age mean	63
range	(41-80)
Patient sex:	
Male	26
Female	29
Dukes stage at first presentation	
A	1
B	18
C	18
D	18
T stage:	
1	1
2	3
3	44
4	7
N stage:	
0	27
1	19
2	9
M stage:	
0	37
1	18
Tumour size mean (range)	4.6cm(3.1cm-5.9cm)
Tumour site:	
Right-sided tumour	19
Left-sided tumour	13
Rectal tumour	23

Table 1 Histological and clinical demographics of patients with CRC included in the study. Variables relate to the time of surgical resection for primary CRC.

Marker	Hif-1α	Vegf	VHL	CA-9	Glut-1
Inter observer variation (Cohen's Kappa)	0.89	0.59	0.49	0.49	0.60
Intra observer variation (Cohen's Kappa)	0.49	0.59	0.58	0.51	0.40

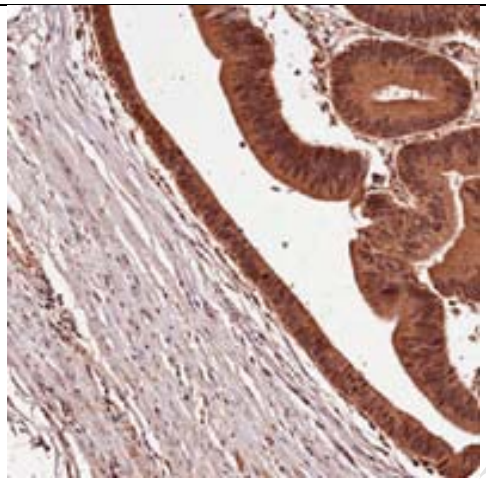
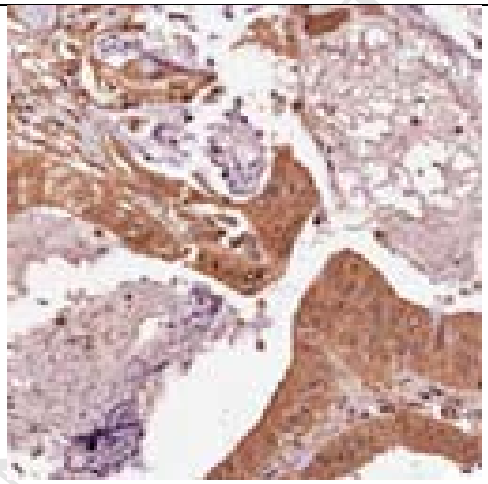
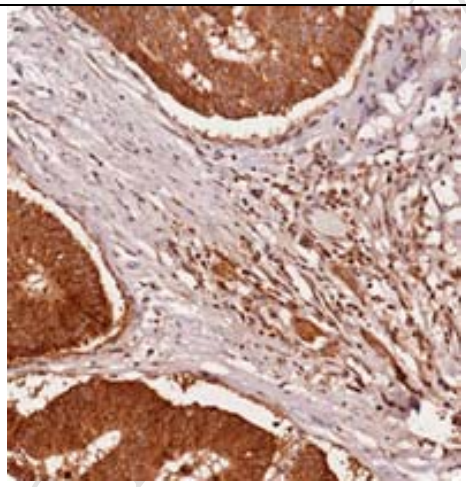
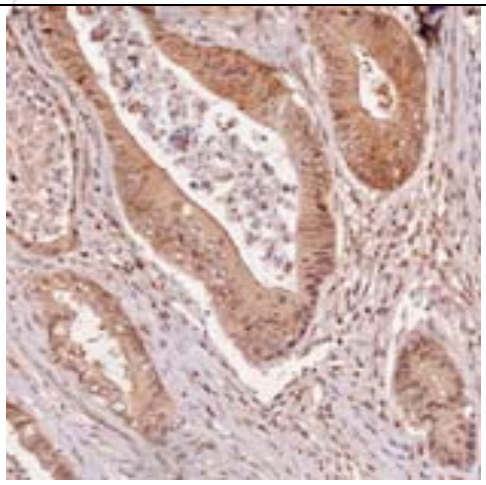
Table-2 Inter and intra observer variations were assessed by twenty of the specimen being reanalysed by the same and different observer respectively. The scores were used to calculate Cohen's kappa as above. Fair to moderate agreement was found between the inter and the intra observer variations.

	Hif-1α	Vegf	VHL	CA-9	Glut-1
Colon tumour margin mean score (range)	3.27(1-4)	4.6(0-7)	1.93(0-3)	4.22(0-7)	4.22(0-7)
Colon tumour centre mean score (range)	2.27(0-4)	3.4(0-6)	1.51(0-3)	2.76(0-7)	1.42(0-3)
Comparison of the margin vs. centre marker expression of primary CRC (Chi square test)	p<0.001	P<0.001	P<0.001	P=0.013	P=0.015
Liver tumour margin mean score (range)	2.04(0-4)	4.49(1-7)	1.53(0-3)	3.8(0-7)	3.04(0-7)
Liver tumour centre mean score (range)	1.64(0-4)	4.38(0-7)	1.25(0-3)	2.78(0-7)	2.24(0-3)
Comparison of the margin vs. centre marker expression Of liver metastases (Chi square test))	p<0.001	p=0.53	P<0.001	p=0.05	p=0.002

Table-3 Expression of markers of hypoxia at the tumour margin and the tumour centre of primary CRC and colorectal liver metastases. Increased expression of all markers were found at the tumour margin compared to the tumour centre.

Marker	Low expression group cut off value (number of patients in this group)	High expression group cut off value (number of patients in this group)	Maximum total score of marker
Vegf	<5(27)	>=5(28)	7
Hif-1α	<3(30)	>=3(25)	4
VHL	<2(44)	>=2(11)	3
Glut-1	<3(30)	>=3(25)	7
CA-9	<3(34)	>=3(21)	7

Table-4 Each hypoxic marker was divided into high and low expression groups using the median value. The cut off values have been shown in the table above and also the total number of patients allocated to each group.

Marker	Strong expression Pattern	Weak expression pattern
Hif-1$^{\alpha}$		
Vegf		

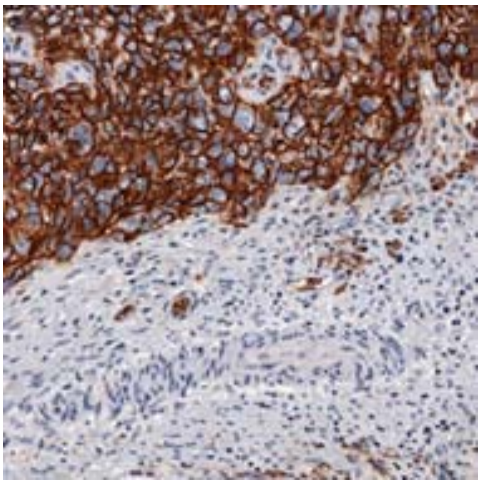
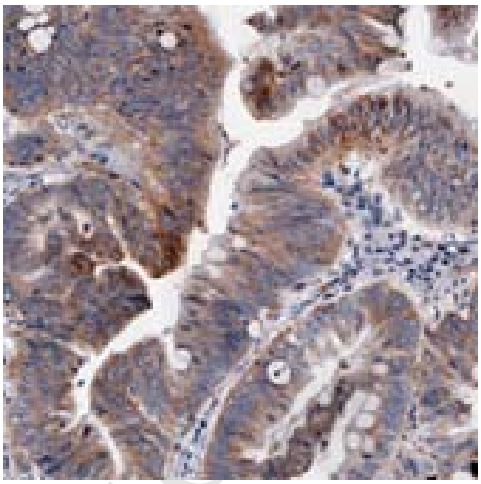
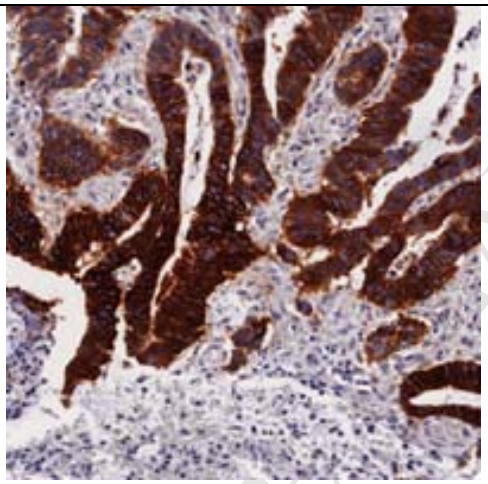
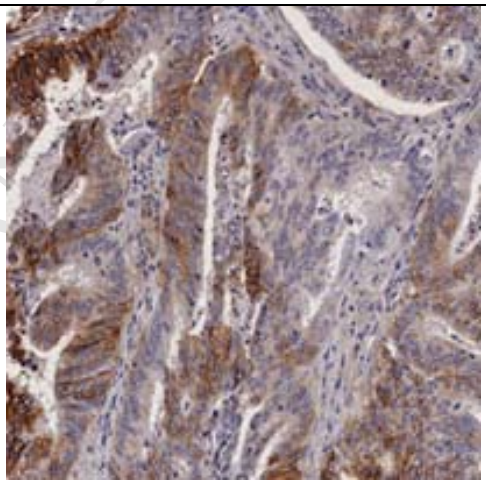
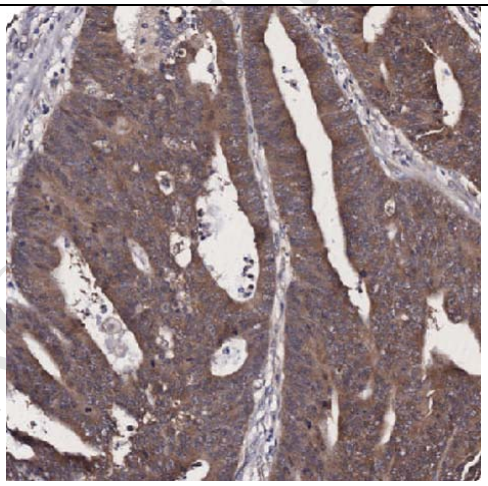
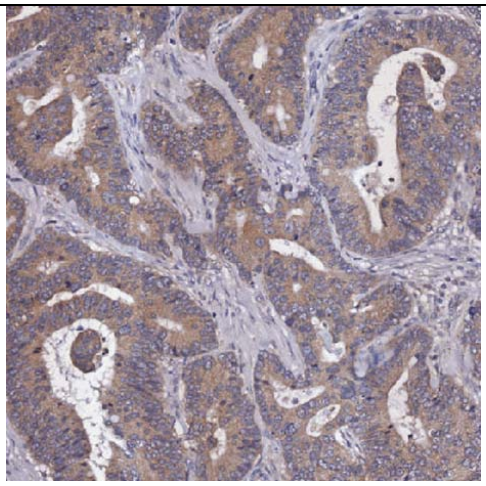
Glut-1		
CA-9		
VHL		

Figure 1

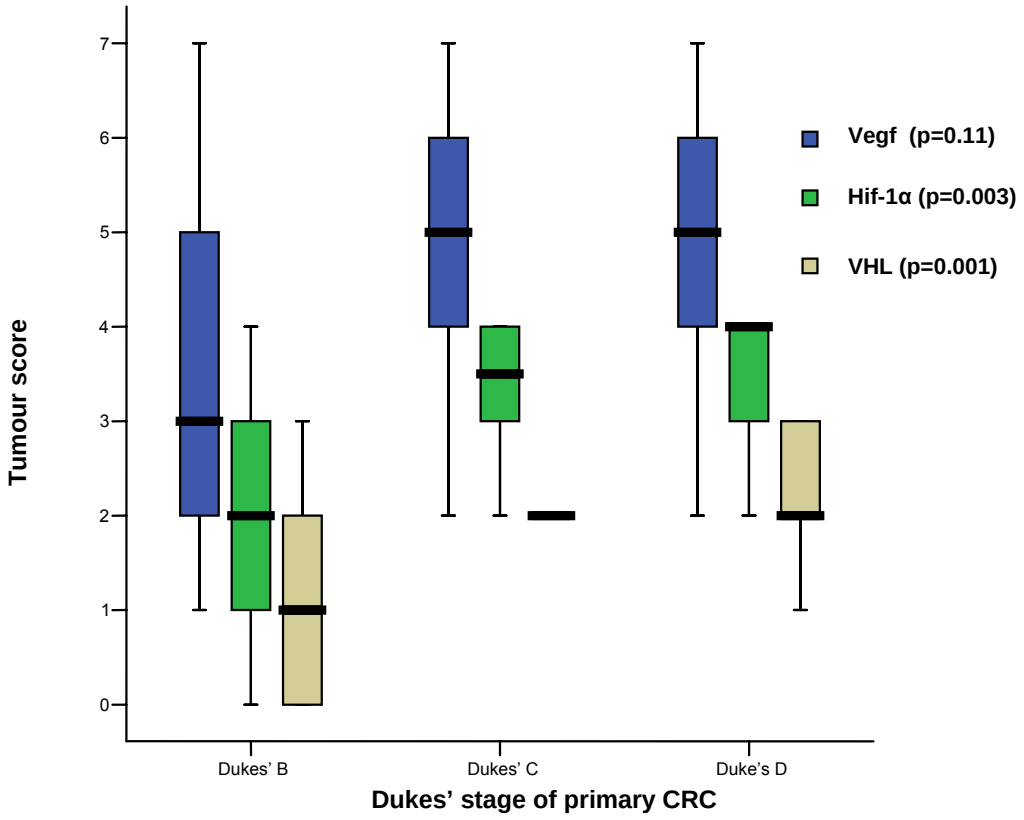
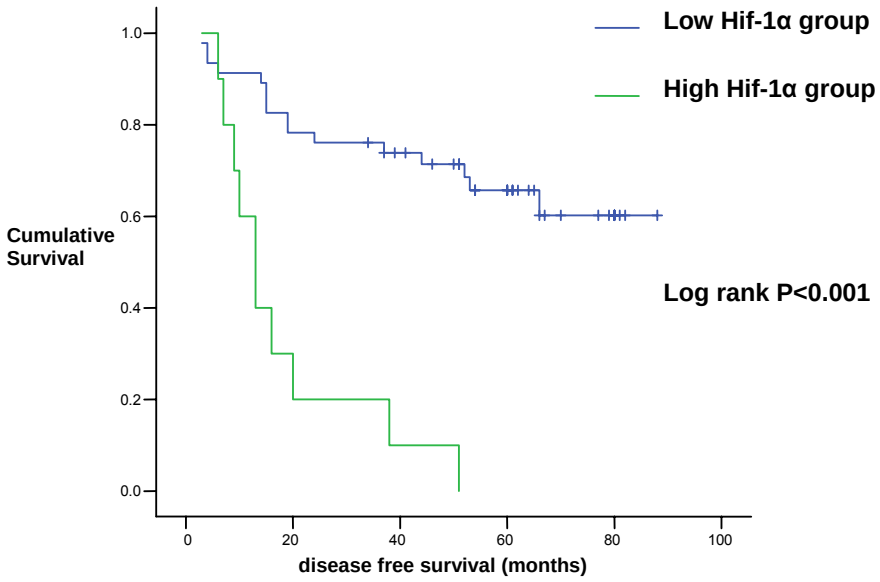


Figure 2



Number at risk

High Hif-1α	**35**	**26**	**22**	**14**	**3**
Low Hif-1α	**25**	**17**	**14**	**7**	**3**

Figure 3

FIGURE LEGENDS

Figure 1 Primary colorectal cancer and colorectal liver metastases showing high and low expression patterns of the different hypoxic markers. Higher expression patterns at the invasive margin were seen compared to the tumour centre with the exception of Vegf in liver metastases. Magnification at x20

Figure 2 Box plot graph to show the difference in distribution of Vegf, Hif-1α and VHL staining with the Dukes' stage of primary CRC. Chi square test was used to calculate the significance. The graph shows the increasing expression of Vegf, Hif-1α and VHL with the change in the Dukes' stages.

Figure-3 Kaplan-Meier curve for disease-free survival following resection of primary colorectal cancer (CRC) vs. Hif-1α divided into groups of high and low expression. Log rank test used to calculate significance.

Conflict of interest

The authors state that they have no conflict of interest.

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