5. MOLECULAR PROGNOSTIC MARKERS IN GASTRIC AND GASTRO-OESOPHAGEAL JUNCTION CANCERS

5.1 Introduction

HIF-1α is expressed in a variety of human cancers (Zhong et al. 1999), and has been linked with a poor prognosis in tumours treated by radiotherapy (Aebersold et al. 2001), chemotherapy (Sohda et al. 2004) or surgery (Kurokawa et al. 2003). As such, there is currently interest in the use of HIF-1α inhibition as a cancer therapeutic strategy. Two studies revealed encouraging results in murine models of gastric cancer (Stoeltzing et al. 2004; Yeo et al. 2003). They used either pharmacological or genetic inhibition of HIF-1α which resulted in dramatic effects on tumour vascularisation and reduced growth of xenografts derived from human gastric cancer cells. However, studies of HIF-1α expression have been conflicting in several tumour types, including cervical, lung and ovarian cancer. Conversely, some studies have related high HIF-1α expression with an improved prognosis (Beasley et al. 2002; Volm et al. 2000). Although HIF-1α expression was associated with a poor prognosis in gastrointestinal stromal tumours of the stomach (Takahashi et al. 2003), there are currently no published data on gastric or gastro-oesophageal adenocarcinoma.

The clinical relevance of different HIF proteins and variants is also of interest in oesophagogastric adenocarcinoma. Some studies have found HIF-2α to be of more prognostic significance in comparison to HIF-1α (Giatromanolaki et al. 2001; Yoshimura et al. 2004)

5.2 Aims

1) To perform HIF-1α and HIF-2α immunohistochemistry on paraffin-embedded resection specimen tissue from patients with surgically treated gastric and gastro-oesophageal adenocarcinoma.
2) To correlate the expression of HIF-1α and HIF-2α with various clinico-pathological characteristics.
3) To assess the prognostic value of HIF-1α and HIF-2 α expression in these patients.

5.3 Results

5.3.1 Study group

The study group comprised 177 patients (125 males) with a median age of 68 (range 49 – 85) years. There were 76 Siewert type II, 21 type III gastro-oesophageal junctional tumours and 80 non-cardia gastric cancers. Patients underwent either partial or subtotal gastrectomies (n=45), total gastectomy (n=44), proximal gastrectomy (n=4) or oesophago-gastrectomy (n=84). Selected patients underwent additional surgical resection of the spleen (n=21) and spleen with distal pancreas (n=5). One hundred and thirteen patients (63.8%) underwent a potentially curative resection (R0 resection), defined as complete macroscopic and microscopic removal of the tumour on intra-operative assessment and subsequent histopathological evaluation. Fifty-four (31%) patients had residual microscopic disease (R1 resection), while 10 patients (6%) had residual macroscopic disease (R2 resection).

5.3.2 Expression of HIF-1α in surgically resected specimen

The predominant staining pattern observed in adenocarcinomas was focal in nature, with small numbers of cells adjacent to each other showing positivity, rather than scattered single positive cells (Figure 5.1). Individual malignant glands showed positive staining in either the majority or none of the cells. There was increased staining within superficial malignant cells in direct contact with the gastric lumen that did not appear artefactual. In the majority of cases, there was diffuse inflammation and necrosis of variable degree throughout the tumour with an associated desmoplastic reaction, as is often the case in gastric adenocarcinoma. It was therefore not possible to assess separately staining patterns associated with inflammation and necrosis compared to non-inflammatory areas with any reliability. However it was noted that tumour cells adjacent to areas of surface ulceration that were peri-necrotic in nature showed increased levels of HIF-1α expression. Some of the tumours studied had large solid areas of malignant cells that showed no increase in HIF-1α expression within the central region of the cell groups: instead there was a tendency for increased expression in the peripheral layers of cells. Malignant cells at the invasive edge of the tumour tended to show increased staining that was more pronounced if the invasive edge was penetrating the subserosal tissue (Figure 5.1). In some cases, the only positive tumour cells were

those that had invaded through the muscularis propria into subserosal fat. It was interesting to note that macrophages and endothelial cells associated with tumour cells within the subserosal tissue also showed strong staining for HIF-1α. This feature was not seen in other layers of the gastric wall. In some cases, the adjacent non-neoplastic mucosa showed intestinal metaplasia that was associated with increased expression of HIF-1α, as seen in the biopsy specimens (Chapter 6).

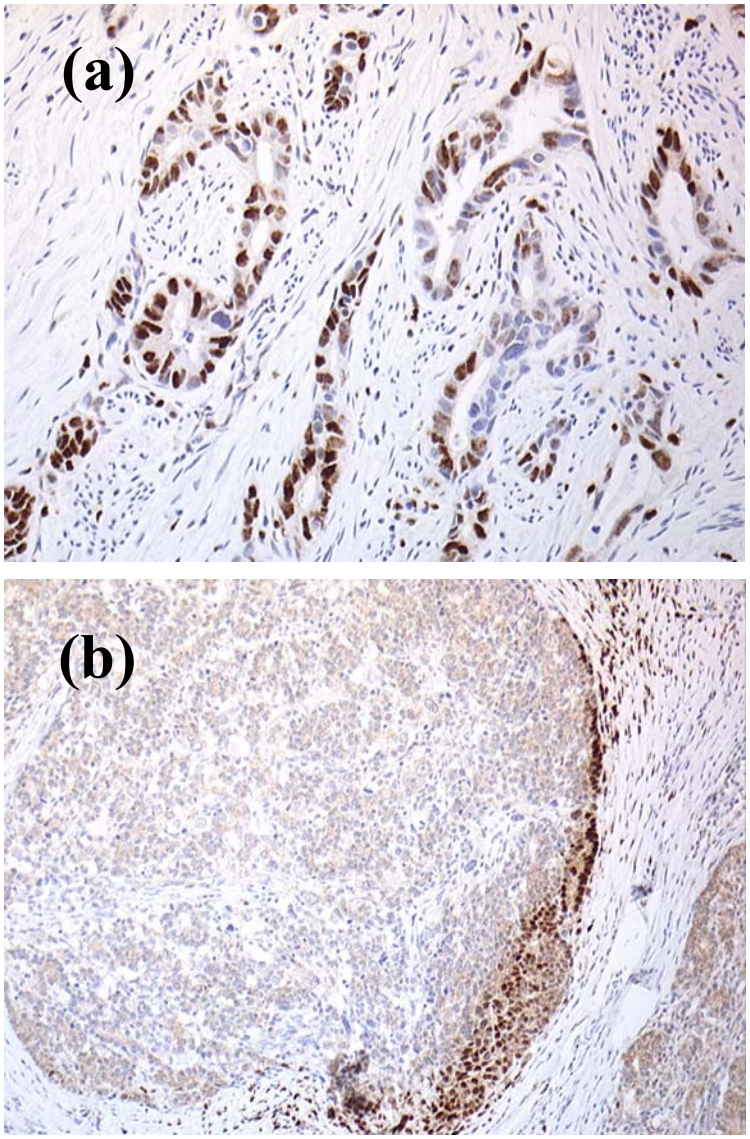

Figure 5.1 Photomicrographs of HIF-1α immunohistochemistry in resected gastric cancer specimens. (a) - HIF-1α staining focal in nature, with small numbers of cells adjacent to each other showing positivity (x200); (b) - HIF-1α staining in the invasive tumour edge (x100).

5.3.3 Expression of HIF-2α in surgically resected specimen

Tumours tended to show diffuse staining for HIF-2α in almost all nuclei or negative staining (Figure 5.2). Unlike the HIF-1α staining pattern, there was no obvious association with inflammation, ulceration or infiltrative edge and location of HIF-2α positive staining. In a small number of cases, cytoplasmic staining was present; this was not scored (Figure 5.2). Focal staining was identified in some inflammatory cells that acted as an internal positive control.

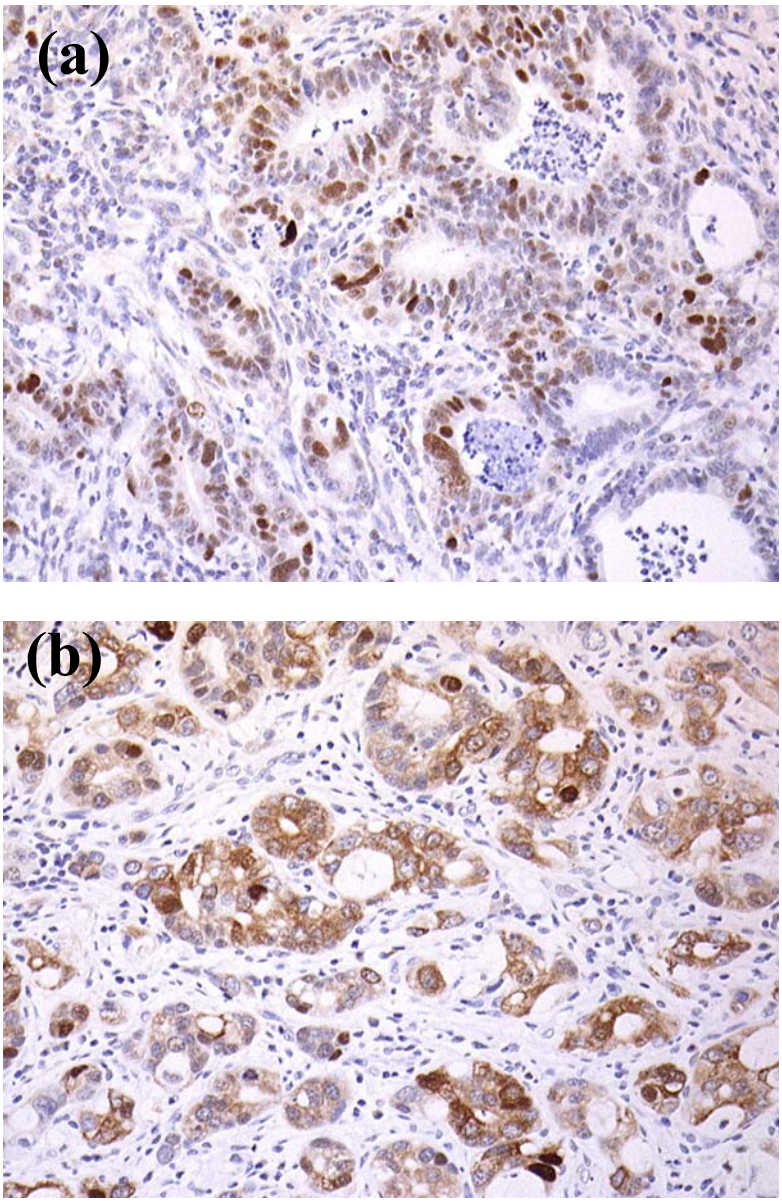

Figure 5.2 Photomicrographs of HIF-2α staining in gastric cancer (x200). (a) – Typical nuclear staining observed. (b) – Nuclear and cytoplasmic staining was observed in some sections [cytoplasmic staining was not scored].

5.3.4 Marker scoring

HIF-1α scoring

In addition to the predominantly nuclear expression, cytoplasmic staining was also observed, but was not scored. In 83 tumour sections (46.9%), no HIF-1α nuclear immunostaining was observed. Positive nuclear staining was as follows: <2 % staining in 62 sections (35%), 2–10% staining in 21 sections (12%), 11–30% staining 7 sections (4%) and >30% staining in 3 sections (2%). Staining pattern was focally positive in 49 (28%), at the invasive tumour edge in 41 (23%) and diffusely positive in 3 (2%). One slide was lost after staining. All negative controls showed no immunoreactivity.

HIF-2α scoring

Cytoplasmic staining was occasionally observed, but was not scored. Five sections (2.8%) had insufficient tissue for HIF-2α scoring. In 66 tumour sections (37.3%), no HIF-2α immunostaining was observed. Positive nuclear staining was as follows: <2 % staining in 9 sections (5.1%), 2-10% staining in 13 sections (7.3%), 11-30% staining 6 sections (3.4%) and >30% staining in 78 sections (44.1%). All negative controls showed no immunoreactivity.

Inter-observer correlation of HIF-1α and HIF-2α scoring

Both HIF-1α and HIF-2α scoring were repeatable with good inter-observer correlations. Table 5.1 shows the inter-observer agreement between Scorer 1 and Scorer 2 in the allocation of HIF-1α and HIF-2α biopsy scores.

Table 5.1 Inter-observer agreement between Scorer 1 and Scorer 2 for assessment of HIF-1α and HIF-2α score

Marker	n	r*	p*
HIF-1α	177	0.90	0.0001
HIF-2α	171	0.97	0.0001

* Spearman's rank correlation
Score: (0), negative; (1), <2%; (2), 2-10%; (3), 11-30%; (4), >30%

Comparison of HIF-1α and HIF-2α expression in resected surgical specimens

The expression of HIF-1α and HIF-2α were compared (Table 5.2). No statistically significant relationships were found between the expression of both factors (p=0.31, χ^2 test).

Table 5.2. Comparison between HIF-1α and HIF-2α scores in 171 resection specimens.

HIF-2α score	**HIF-1α score**					
	0%	**<2%**	**2-10%**	**11-30%**	**>30%**	**Total**
0%	31	26	6	2	1	66
<2%	5	1	3	0	0	9
2-10%	5	3	4	0	1	13
11-30%	2	4	0	0	0	6
>30%	37	28	7	4	1	77
Total	80	62	20	6	3	171*

* 6 sections staining for HIF-2α had insufficient tissue for scoring

5.3.5 Relation between HIF-1α expression and clinico-pathological features

For correlation with various clinico-pathological features HIF-1α expression was sub-categorized into negative (Score 0) and positive (Scores 1/2/3/4) staining. The distributions of patients according to their tumour expression of HIF-1α (positive vs. negative) and staining pattern (HIF-1α negative, HIF-1α focal expression and expression in the invasive tumour edge) compared with various clinical characteristics are shown in Tables 5.3 and 5.4, respectively.

HIF-1α positive tumours were of a higher overall TNM stage than HIF-1α negative tumours (p=0.045). There were no statistically significant differences between HIF-1α positive and negative tumours in differentiation, Lauren type, T stage, N stage, M stage or R classification (Table 5.3). However, there were differences when the staining pattern of HIF-1α was correlated with clinico-pathological factors (Table 5.4). HIF-1α expression at the invasive edge was associated with aggressive tumour characteristics such as a trend for more advanced T Stage (p=0.087), lymph node

metastases (p=0.034), advanced TNM stage (p=0.001) and incomplete surgical resection (p=0.014).

Table 5.3 The distribution of patients according to their tumour expression of HIF-1α (negative versus positive) and clinico-pathological characteristics (n=176)[†]

Factor		Pattern of HIF-1α staining		P[*]
		HIF-1α negative	HIF-1α positive	
Differentiation	Well	12	6	
	Mod	33	34	
	Poor	38	53	0.14
Lauren type	Diffuse	41	50	
	Intestinal	42	43	0.56
T Stage	T in-situ	2	1	
	T1	6	10	
	T2	29	25	
	T3	43	56	
	T4	3	1	0.44
N Stage	N0	23	29	
	N1	51	49	
	N2	7	13	
	N3	2	2	0.58
M Stage	M0	81	91	
	M1	2	2	0.91
Overall TNM Stage	0	2	1	
	I	11	20	
	II	33	21	
	III	31	48	
	IV	6	3	0.045
R Class	R0	49	63	
	R1	30	24	
	R2	4	6	0.32

[*] Chi-squared p value
[†] 1 patient with missing slide was excluded

Table 5.4 The distribution of patients according to their tumour expression of HIF-1α (according to staining pattern) and clinico-pathological characteristic (n=173)[†]

Factor		Pattern of HIF-1α staining			p[*]
		HIF-1α focal positivity	HIF-1α negative	HIF-1α at the invasive edge	
Differentiation	Well	4	12	2	
	Mod	20	33	14	
	Poor	25	38	25	0.37
Lauren type	Diffuse	24	41	23	
	Intestinal	25	42	18	0.74
T Stage	T in-situ	1	2	0	
	T1	7	6	3	
	T2	18	29	6	
	T3	22	43	32	
	T4	1	3	0	0.087
N Stage	N0	22	23	5	
	N1	20	51	28	
	N2	6	7	7	
	N3	1	2	1	0.034
M Stage	M0	48	81	40	
	M1	1	2	1	0.99
Overall TNM Stage	0	1	2	0	
	I	16	11	3	
	II	13	33	7	
	III	17	31	30	
	IV	2	6	1	0.001
R Class	R0	40	49	21	
	R1	8	30	15	
	R2	1	4	5	0.014

[*] Chi-squared p value
[†] 3 patients with diffusely positive HIF-1α staining and 1 patient with a missing slide were excluded

5.3.6 Relation between HIF-2α expression and clinico-pathological features

For correlation with various clinico-pathological features HIF-2α expression was sub-categorized into negative (Score 0) and positive (Scores 1/2/3/4) staining. The distributions of patients according to their tumour expression of HIF-2α (positive vs. negative) are shown in Table 5.5. HIF-2α positive tumours were more likely to be diffuse (p=0.025). There was a trend for HIF-2α tumours to have a more advanced T

stage (p=0.058). No statistically significant correlations were found between HIF-2α and differentiation, N stage, M stage, overall TNM stage or R Classification (Table 5.5).

Table 5.5 The distribution of patients according to their tumour expression of HIF-2α (negative versus positive) and clinico-pathological characteristics (n=172)

Factor		HIF-2α negative	HIF-2α positive	p*
Differentiation	Well	8	10	
	Mod	24	41	
	Poor	34	55	0.84
Lauren type	Diffuse	27	62	
	Intestinal	39	44	0.025
T Stage	T in-situ	0	3	
	T1	7	9	
	T2	27	26	
	T3	32	64	
	T4	0	4	0.058
N Stage	N0	20	29	
	N1	37	62	
	N2	7	13	
	N3	2	2	0.921
M Stage	M0	65	103	
	M1	1	3	0.58
Overall TNM Stage	0	0	3	
	I	14	15	
	II	23	31	
	III	27	50	
	IV	2	7	0.31
R Class	R0	46	64	
	R1	19	33	
	R2	1	9	0.13

* Chi-squared p value

5.3.7 Relationship between HIF-1α and HIF-2α expression and patient survival

At the time of analysis, 51 patients were alive with a median follow-up of 48 months (range 13–118) months, whilst 107 had died of disease with a median time to death of 14 (range 2–74) months. There were 16 inter-current deaths from other causes. HIF-1α expression was examined in relation to overall and disease-specific survival by Kaplan-Meier and log-rank analysis (Figure 5.3).

HIF-1α expression and patient survival

There was no difference in survival in patients with HIF-1α expression (categories 1/2/3/4) compared with negative staining (category 0), either for the group as a whole or when gastric and GOJ tumours were analysed separately (Figure 5.3 and Table 5.6). However, HIF-1α expression pattern was a significant predictor of survival on univariate analysis (p=0.019). The median survival for patients with HIF-1α expression at the invasive edge was 18 (95% CI 11.1 to 24.9) months, HIF-1α negative tumours 33 (95% CI 22.3 to 43.7) months and focally positive HIF-1α expression was 46 months (Figure 5.3). Similar, although non-statistically significant, trends were observed when survival was sub-stratified by tumour location as gastric (p=0.16) and GOJ (p=0.092).

HIF-2α expression and patient survival

Positive (categories 1/2/3/4) HIF-2α expression was a statistically significant poor prognostic factor on univariate analysis (Figure 5.4 and Table 5.6). The median overall survival for patients with HIF-2α expression was 22 (95% CI 18 to 26) months, whereas HIF-2α negative patients had a median survival of 37 (95% CI 29 to 44) months (p=0.015). HIF-2α expression was more prognostic for gastric cancers (p=0.032) compared with GOJ (p=0.26) tumours (Figure 5.4). Other significant factors on univariate survival analyses were tumour differentiation, T stage, N stage, overall TNM stage and R classification (Table 5.6).

The combination of HIF-1α and HIF-2α and patient survival

The combined effect of HIF-1α and HIF-2α were analysed with regard to patient outcome. No statistically significant effect was found (Figure 5.5). Although, there were trends for patients who were HIF-1α and HIF-2α negative to have better prognosis in all subgroups, the data should be interpreted with caution because of the small number of patients in each group.

Table 5.6 Univariate survival analysis of prognostic factors following surgical resection in gastric and gastro-oesophageal cancer

Parameter		Overall Survival			Disease-specific Survival		
		HR	95% CI	p*	HR	95% CI	p*
HIF-1α	**0**	1	-	-	1	-	
	1/2/3/4	1.1	0.8 – 1.4	0.62	1.0	0.7 – 1.5	0.82
HIF-1α	**Negative**	1	-	-	1	-	-
	Focal	0.9	0.5 – 1.3	0.49	0.7	0.5 – 1.2	0.26
	Invasive edge	1.6	1.0 – 2.4	0.042	1.6	1.0 – 2.5	0.047
HIF-2α	**0**	1	-	-	1	-	-
	1/2/3/4	1.6	1.1 – 2.4	0.018	1.6	1.0 – 2.4	0.038
Diff	**Well**	1	-	-	1	-	-
	Mod	2.9	1.4 – 6.2	0.005	3.4	1.3 – 8.5	0.011
	Poor	3.7	1.8 – 7.8	0.001	5.3	2.1 – 13.3	0.001
Lauren	**Intestinal**	1	-	-	1	-	-
type	**Diffuse**	1.4	1.0 – 2.0	0.052	1.8	1.2 – 2.6	0.003
Location	**Non-GOJ**	1	-	-	1	-	-
	GOJ	1.4	1.0 – 2.0	0.083	1.5	1.0 – 2.2	0.059
T Stage	**T0/1**	1	-	-	1	-	-
	T2	2.6	1.0 – 6.7	0.052	5.2	1.2 – 22.0	0.023
	T3	4.8	1.9 – 12.0	0.001	9.6	2.3 – 39.0	0.002
	T4	16.8	4.4 – 64.2	0.0001	37.5	6.8 – 207.6	0.0001
N Stage	**N0**	1	-	-	1	-	-
	N1	2.0	1.3 – 3.0	0.003	2.5	1.5 – 4.1	0.001
	N2	3.5	1.9 – 6.4	0.0001	4.8	2.5 – 9.2	0.0001
	N3	4.2	1.5 – 12.0	0.008	5.7	1.9 – 16.9	0.002
M Stage	**M0**	1	-	-	1	-	-
	M1	2.6	1.0 – 7.1	0.062	2.9	1.1 – 7.9	0.037
Overall	**0/1**	1	-	-	1	-	-
TNM	**2**	1.4	0.8 – 2.6	0.25	1.8	0.9 – 3.6	0.12
Stage	**3**	3.3	1.9 – 5.9	0.0001	4.5	2.3 – 8.8	0.0001
	4	7.6	3.3 – 17.5	0.0001	10.9	4.4 – 27.1	0.0001
R Class	**R0**	1	-	-	1	-	-
	R1	2.3	1.6 – 3.3	0.0001	2.7	1.8 – 4.0	0.0001
	R2	5.8	2.9 – 11.6	0.0001	7.2	3.6 – 14.5	0.0001

HR = Hazard ratio, CI = Confidence Interval, * obtained using a univariate Cox-proportional hazards model

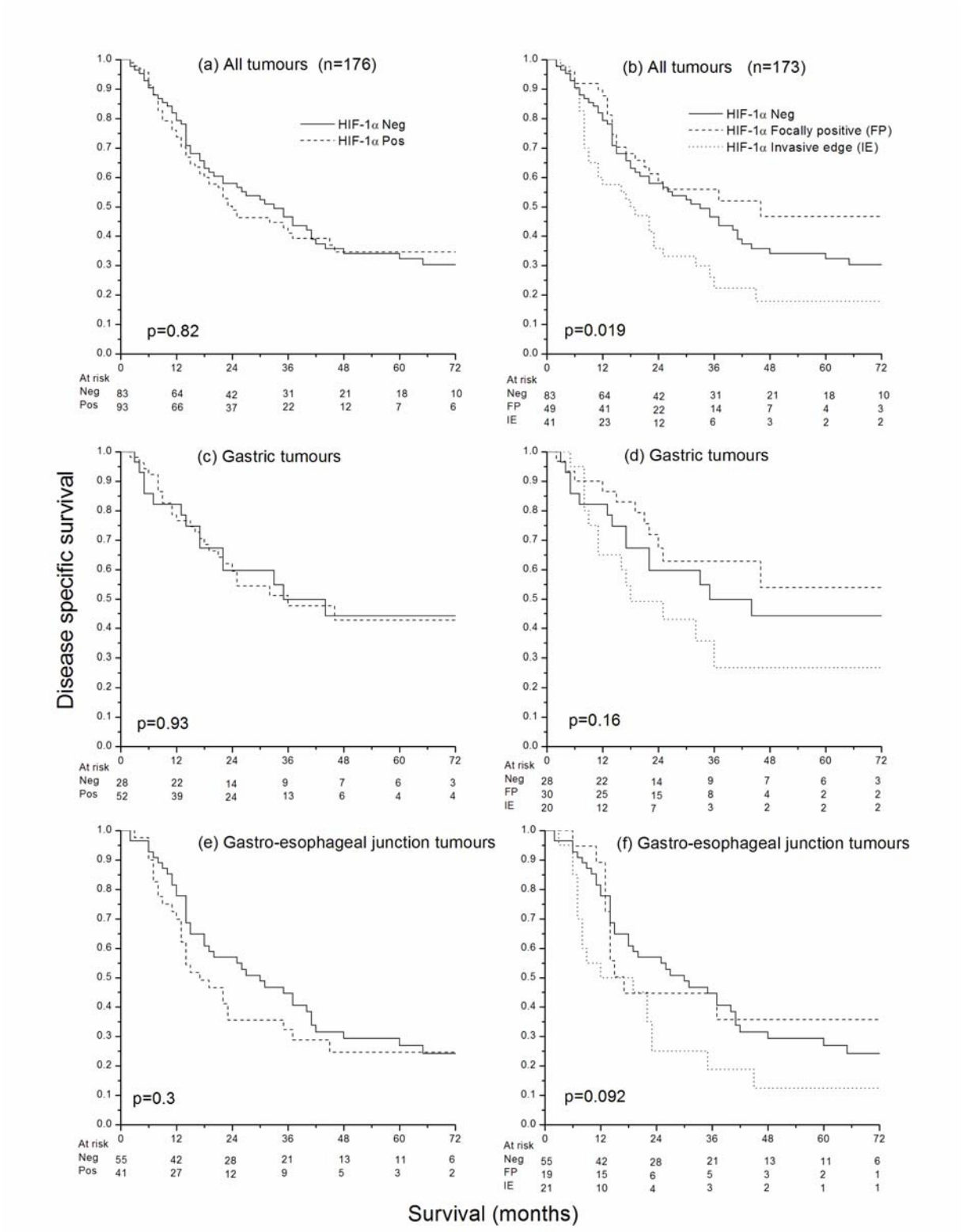

Figure 5.3 HIF-1α expression and patient outcome in all 177 tumours and those with non-cardia gastric cancers (n=80) or gastro-oesophageal junction tumours (n=97). First column shows HIF-1α negative (Score 0) versus positive (Scores 1/2/3/4) expression and prognosis. Second column shows HIF-1α expression categorised as HIF-1α at the invasive edge, HIF-1α negative and HIF-1α focally positive.

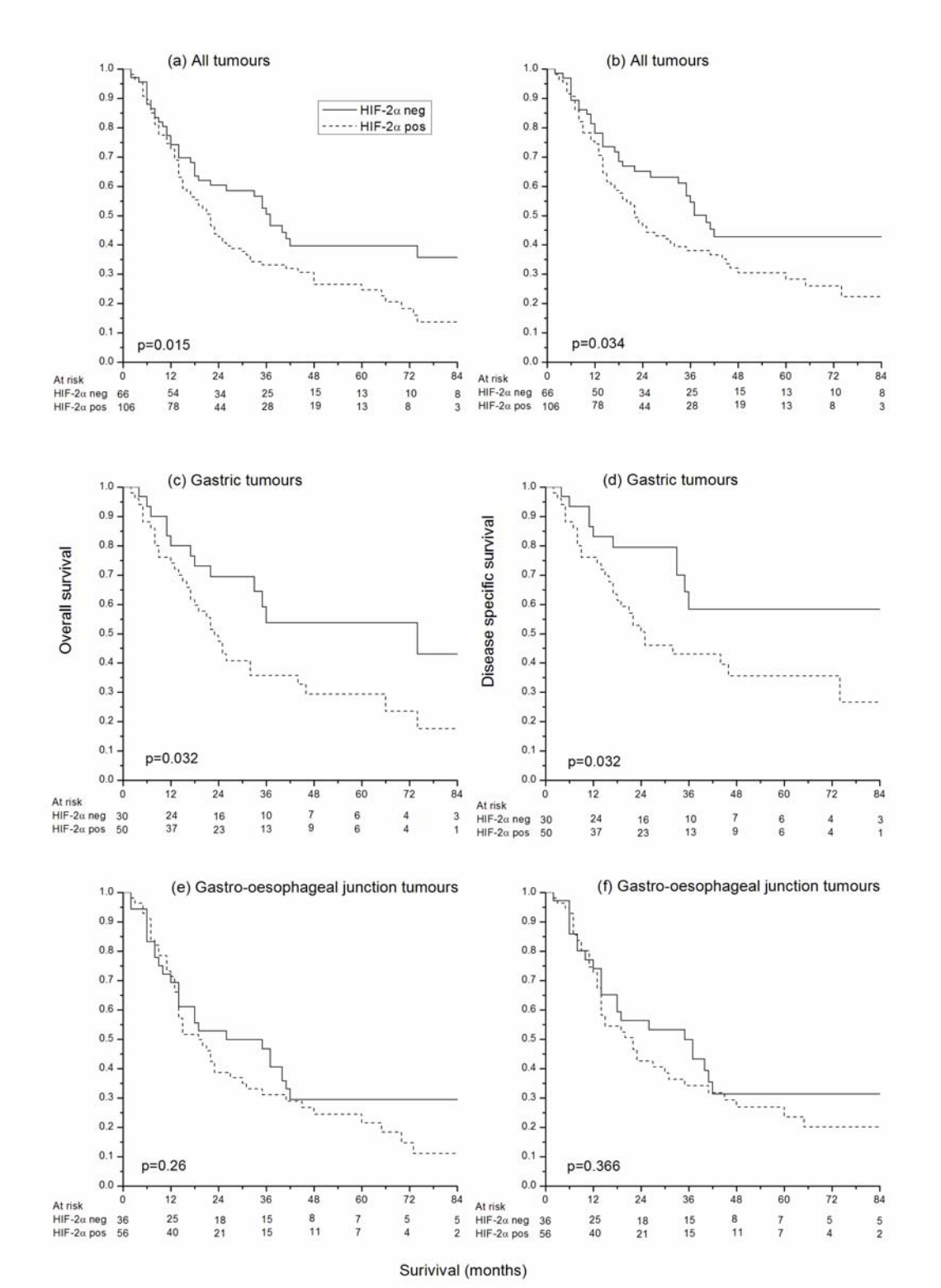

Figure 5.4 HIF-2α expression and patient outcome in 172 tumours including non-cardia gastric cancers (n=80) and gastro-oesophageal junction tumours (n=92). First column shows HIF-2α negative (Score 0) versus positive (Scores 1/2/3/4) expression and overall survival. Second column shows disease-specific survival.

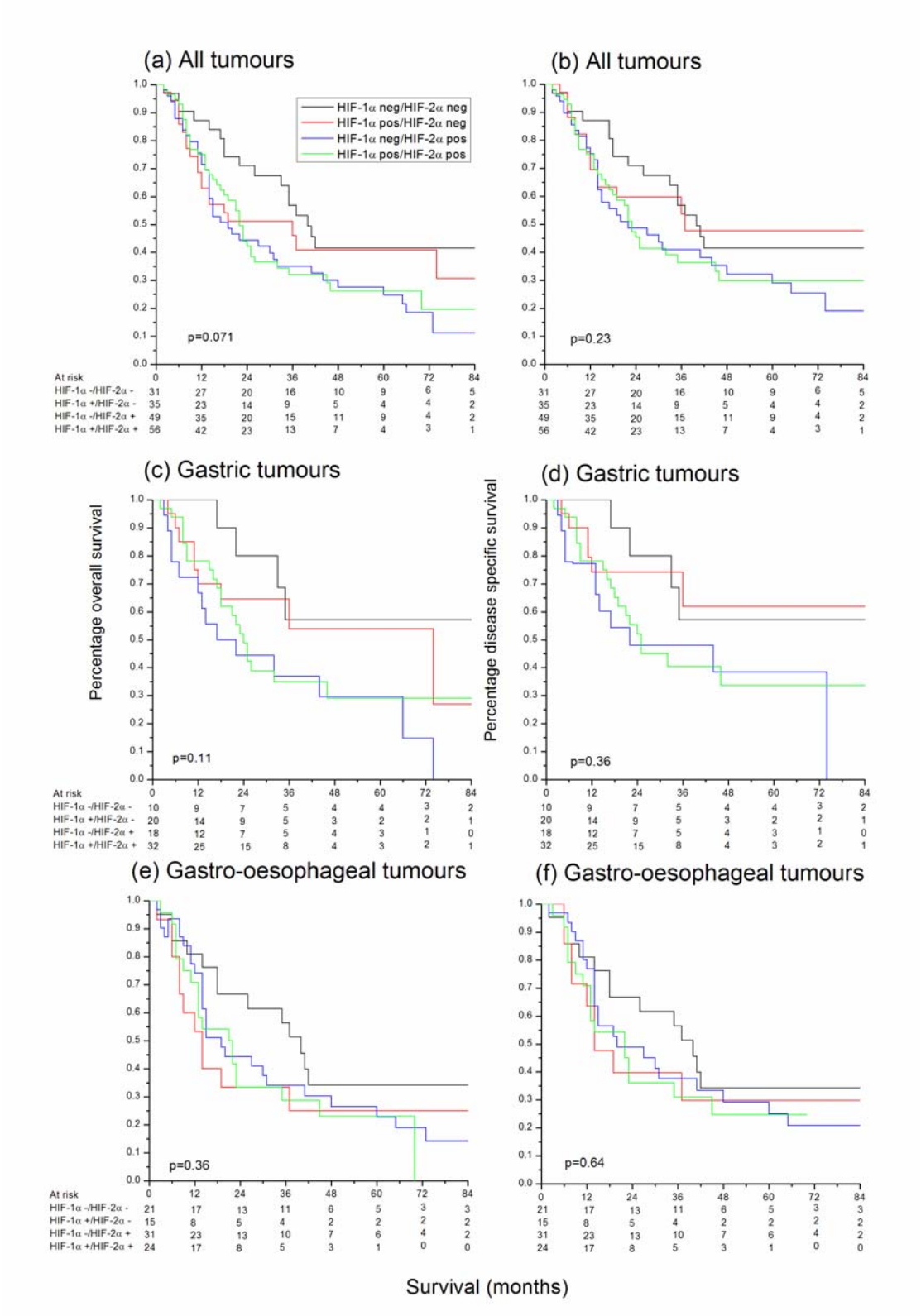

Figure 5.5 The combination of HIF-1α and HIF-2α in relation to patient outcome in 172 tumours including non-cardia gastric cancers (n=80) and gastro-oesophageal junction tumours (n=92). First column shows overall survival and the second column shows disease-specific survival.

Multivariate survival analysis

A multivariate survival analysis was performed using the Cox proportional hazard model (Table 5.7). All factors that achieved statistical significance ($p<0.05$) on the univariate survival analysis were entered into the multivariate model. Neither HIF-1α nor HIF-2α expression were independent predictors of prognosis. Only tumour differentiation, overall TNM stage and R classification were significant on multivariate survival analysis (Table 5.7).

Table 5.7 Multivariate survival analysis of prognostic factors following surgical resection in gastric and gastro-oesophageal cancer

Parameter		**Overall Survival**			**Disease-specific Survival**		
		HR	**95% CI**	**p**	**HR**	**95%**	**p**
Differentiation	**Well**	1	-	-	1	-	-
	Mod	2.6	1.2 – 5.9	0.02	2.5	1.0 – 6.5	0.055
	Poor	2.8	1.2 – 6.4	0.013	3.2	1.2 – 8.3	0.014
Overall TNM stage	**0/1**	1	-	-	1	-	-
	2	1.2	0.6 – 2.2	0.61	1.2	0.6 – 2.4	0.7
	3	2.8	1.5 – 5.2	0.001	3.0	1.5 – 6.0	0.002
	4	5.0	2.0 – 12.6	0.001	5.4	2.0 – 14.3	0.001
R Class	**R0**	1	-	-	1	-	-
	R1	1.4	0.9 – 2.2	0.095	1.5	1.0 – 2.4	0.07
	R2	4.9	2.4 – 10.0	0.0001	6.1	2.9 – 12.5	0.001

HR = Hazard ratio, CI = Confidence Interval

5.4 Discussion

This is the first study to assess the prognostic impact of both HIF-1α and HIF-2α expression in patients with surgically treated gastric and GOJ adenocarcinoma.

HIF-1α

Urano et al recently assessed HIF-1α expression in 146 patients with distal gastric adenocarcinoma treated with surgery (Urano et al. 2006). They found a higher level of expression of HIF-1α compared with this chapters results; 61% had >10% HIF-1α expression. HIF-1α expression was not associated with histological sub-type, depth of tumour invasion, lymphatic invasion and overall stage. Interestingly, negative HIF-1α expression was associated with the development of distant metastases (p=0.014). This is more indication of the dual nature of HIF-1α expression which will be discussed further later in this Chapter. Like the initial analysis performed in this chapter, HIF-1α expression was not associated with patient outcome (p=0.62). However, expression pattern was not considered in this study. Urano et al also found that HIF-1α expression could not predict clinical response to chemotherapy in 31 patients with gastric cancer (Urano et al. 2006).

Several other HIF-1α regulated products have been shown to be markers of a poor prognosis in gastric cancer, including VEGF, CA-9, Glut-1 and iNOS (Griffiths et al. 2005). These findings are consistent with experimental studies demonstrating that pharmacological or genetic inhibition of HIF-1α reduces tumour growth and vascularity in xenografts derived from human gastric cancer cells (Stoeltzing et al. 2004; Yeo et al. 2003). However, the situation appears to be complex as, in the study reported here, HIF-1α expression had no prognostic significance when analysed as percentage of expression only. This finding is in keeping with other studies in gastric (Urano et al. 2006), cervical (Haugland et al. 2002; Hutchison et al. 2004), colorectal (Yoshimura et al. 2004) and ovarian (Birner et al. 2001b) cancers. However, when staining pattern was considered it was observed that there were two predominant types of HIF-1α expression: staining of the tumour's invasive edge and focally positive staining. HIF-1α staining of the invasive edge was associated with aggressive tumour characteristics, such as incomplete surgical resection, T stage, lymph node metastases and worse overall stage. Other studies have noted HIF-1α expression at the invasive edge of tumours (Zagzag et al. 2000; Zhong et al. 1999), but have not performed survival analyses. In the survival analysis performed here, tumours with invasive edge staining

had a worse prognosis compared with either HIF-1α negative or focal HIF-1α expression. Similarly the HIF-1α regulated CA-9 was expressed at the invasive tumour edge in a subset of gastric cancer (Chen et al. 2005a) where it was associated with tumour invasion, advanced disease and a poorer prognosis.

Staining of HIF-1α at the invasive edge appeared to be associated with an incomplete surgical resection. However, the residual disease after surgical resection or involvement of the surgical resection margins may or may not be a biological indicator of aggressive disease. These may be related to other factors; for example the adequacy of surgical resection or the surgical operation performed. This result should therefore be interpreted with caution.

Other authors showed that different patterns of HIF-1α expression were associated with different survival characteristics (Vleugel et al. 2005). In a study in breast cancer, peri-necrotic HIF-1α was associated with the expression of CA-9 and Glut-1 and was associated with a poor prognosis (Vleugel et al. 2005). However, the diffuse staining type had a more favourable prognosis and was not associated with CA-9 or Glut-1 expression.

Focally positive HIF-1α expression was associated with a less aggressive tumour phenotype and an improved prognosis. Some studies have found that HIF-1α expression in head and neck (Beasley et al. 2002), non-small cell lung (Volm et al. 2000) and renal cell (Lidgren et al. 2005) cancer is associated with an improved survival (Table 5.8). However, as described in the Introduction, most studies have shown HIF-1α expression is associated with a poor prognosis. There are a number of possible explanations for these apparently contradictory findings, such as differences in the staining and scoring methods used and in the treatment patients received. For example, it has been suggested that HIF-1α expression may be a less important prognostic factor in surgically treated patients as the major influence of hypoxia-induced radiation resistance is lacking (Beasley et al. 2002). However, as more reports are published this seems less likely. For example, studies in patients with cervical cancer who underwent radiotherapy showed either a trend towards improved prognosis (Mayer et al. 2004) or an improved prognosis in a subgroup of patients (Hutchison et al. 2004). Although differences in staining and scoring methods cannot be ruled out completely, it seems more likely that the differences in prognostic outcome observed in numerous studies may reflect the differential regulation by HIF-1α of a range of downstream target molecules (Figure 5.7). This differential regulation might also be determined in

individual tumours by the different processes leading to HIF-1α stabilisation (eg, hypoxia/oncogene/ROS).

HIF-1α can have both pro- and anti-apoptotic effects (Piret et al. 2002), and can also both stimulate (Carmeliet et al. 1998) and inhibit (Bacon et al. 2004) proliferation. There is evidence for communication between HIF-1α and p53: p53 can stabilize HIF-1α and vice versa (Greijer et al. 2004; Schmid et al. 2004). Of interest, recent data showed HIF-1α phosphorylation status may determine whether it acts to promote or check tumour cell survival. Dephosphorylated HIF-1α stabilized p53 and induced apoptosis, whereas phosphorylated HIF-1α bound to HIF-1β to form the HIF-1 transcription factor thereby promoting tumour growth (Suzuki et al. 2001). There is likely to be an intricate balance between the different roles of HIF-1α, which might be determined by the cumulative effect of multiple interactions within a cell. In the series of gastric cancer patients studied here, therefore, the beneficial effect of a focal pattern of HIF-1α expression on prognosis may relate to its pro-apoptotic and anti-proliferative effects. It would be interesting to test these proposed mechanisms by assessing apoptosis and proliferation in the same cohort of patients.

A final consideration that might play a role in determining whether HIF-1α expression is a good or bad prognostic factor is any contribution from other members of the HIF family. This will be discussed further later in the Chapter.

Tumour biology of the invasive tumour edge

Prominent HIF-1α expression was observed at the tumour's invasive edge. In this area important interactions occur between cancer cells, endothelial cells and the tumour's supporting stroma (Sivridis et al. 2005). Previous studies in gastric cancer have shown that immunohistochemical markers of proliferation (proliferating cell nuclear antigen [PCNA] and argyrophilic nucleolar organizer regions [AgNOR]) in this area are associated with a poor prognosis or lymph node metastases (Ikeguchi et al. 1996; Nishida et al. 1995). A recent study from colorectal cancer has shown that host fibroblasts at the invasive edge develop malignant features; such as high proliferative index and overexpression of various angiogenesis markers (VEGF and thymidine phosphorylase [TP]) (Sivridis et al. 2005). This study also showed that this intense fibroblast activity was associated with HIF-1α and a marker of oxidative stress (lactate dehydrogenase-5). This would fit with the HIF-1α expression that was observed in macrophages and endothelial cells associated with tumours that penetrated through the subserosal layer.

Table 5.8 Other studies which have shown HIF-1α expression to be associated with an improved prognosis

Author, Year	No.	Cancer	Treatment	Cut-off	Technique	Survival (Univariate)	Survival (Multivariate)
(Volm et al. 2000)	96	NSCLC	Surgery	N/A	IHC	Improved overall survival (p=0.05)[†]	N/A
(Beasley et al. 2002)	79	Head and neck (SCC)	Surgery ± radiotherapy	Pos vs Neg	IHC	Improved disease free (p=0.016) and overall survival (p=0.027)	N/A
(Hutchison et al. 2004)	99	Cervix	Radical radiotherapy	>10%	IHC	Improved disease free (p=0.02), metastasis free (p=0.02) and local recurrence free (p=0.03) survival [*]	N/A
(Mayer et al. 2004)	38	Cervix	Surgery ± radiotherapy ± chemotherapy	Median (computer image analysis)	IHC	Trends for improved prognosis in overall (p=0.11) and recurrence-free (p=0.19) survival	N/A
(Lidgren et al. 2005)	92	Renal Cell Carcinoma	Surgery	Median	Western blot	Improved overall (p=0.024) survival	Overall (p=0.024) survival
(Fillies et al. 2005)	85	T1/T2 SCC Oral floor	Surgery	< or > 5%	IHC/TMA	Improved overall (p=<0.01) and disease-free (p=0.01)survival	Improved overall (p=0.001) and disease-free (p=0.01) survival

[*]only in larger tumours (> 4 cm);[†] statistically associated with the expression of apoptosis and pro-apoptotic molecules such as caspase-3, Fas, and Fas ligand; NSCLC = non-small cell lung cancer; SCC = squamous cell carcinoma; IHC = immunohistochemistry; TMA = tissue micro-array; N/A = not applicable

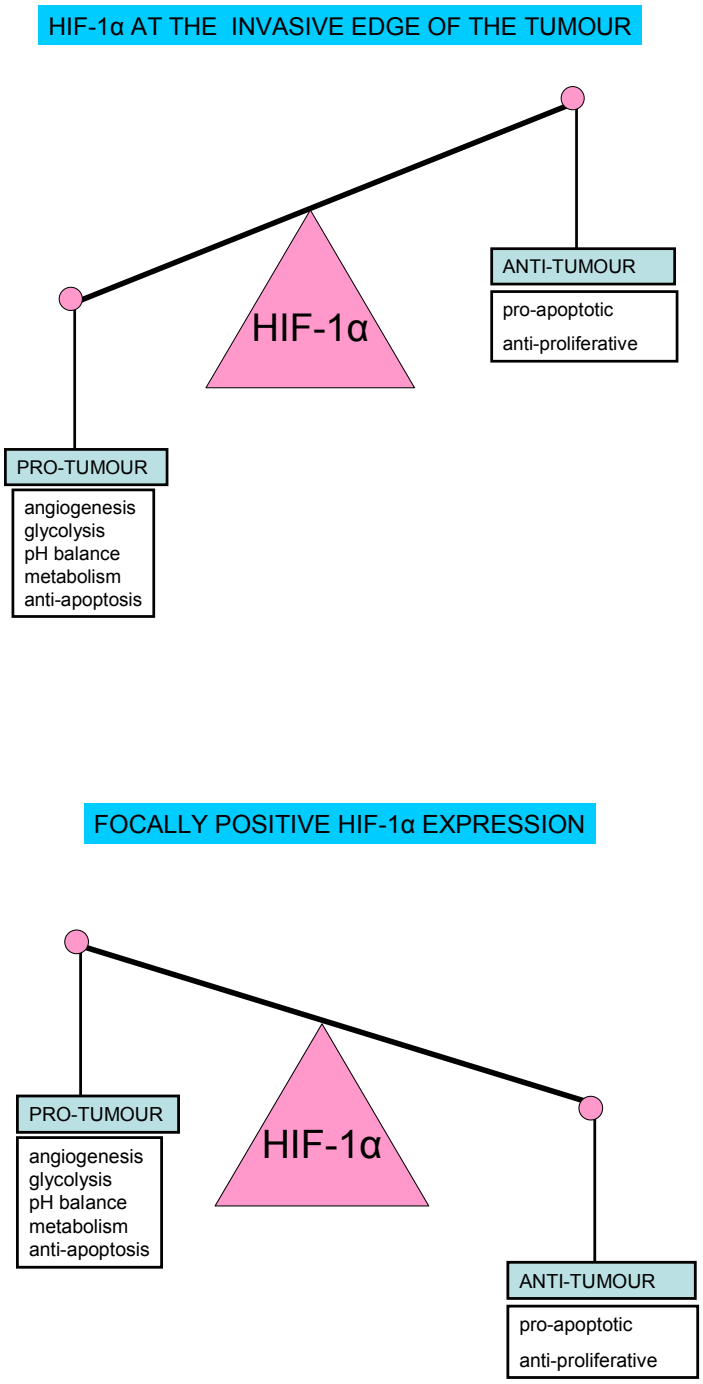

Figure 5.6 Hypothesis: the different prognostic outcomes of the different pattern of HIF-1α expression may be related to induction of different downstream HIF-1α target molecules

Potential problems of assessing HIF-1α by tissue micro-array method (TMA)

Immunohistochemistry was used to stain for HIF-1α and HIF-2α in resection surgical specimens using conventional whole tissue sections. Although the use of TMAs was considered it has several disadvantages in relation to HIF-1α staining (van Diest et al. 2005). A recent study using TMA to assess the expression of HIF-1α revealed that only 5% of ductal adenocarcinomas of the breast had positive nuclear staining (Jubb et al. 2004), whereas other studies using conventional immunohistochemistry observed expression rates of between 44 to 80% (Bos et al. 2001; Vleugel et al. 2005). The use of TMA would not have allowed detailed pathological description of the location of HIF-1α (in particular with regard to expression at the tumours invasive edge). It may be possible to overcome this by taking multiple cores of tissue and noting the location of the punch. A recent study suggested that four is the minimum number of core biopsies that should be taken, but the degree of heterogeneity of the antibody expression should also be considered to avoid sampling error (Goethals et al. 2006b).

HIF-2α

Like HIF-1α, HIF-2α accumulates in the presence of hypoxia, forms a heterodimer with HIF-1β, and binds to HREs. HIF-2α has been shown to regulate a number of the same hypoxia-inducible genes as HIF-1α (Hu et al. 2003). However, it is now known that the various hypoxia inducible genes vary in their sensitivity to HIF-1α and HIF-2α (Wang et al. 2005) and therefore different downstream pathways can be preferentially activated (Hu et al. 2003; Sowter et al. 2003).

The classical 'hypoxic' expression of HIF-2α in tumour sections was not found as there was no association with necrosis or distance from blood vessels. This may suggest non-hypoxic activation. However, unlike HIF-1α, the non-hypoxic activation of HIF-2α has not been confirmed. In a study in breast adenocarcinoma, a strong correlation between HIF-2α and c-erbB-2 was found and it was suggested this was due to oncogenic rather than hypoxic activation (Giatromanolaki et al. 2006).

Few other studies have assessed both the expression of HIF-1α and HIF-2α and patient outcome. Yoskimura et al examined 87 surgically treated patients with colorectal cancer and found that HIF-2α but not HIF-1α expression predicted prognosis on univariate analysis (Yoshimura et al. 2004). Interestingly, the combined expression of HIF-1α and HIF-2α was a significant prognostic factor on multivariate analysis. Other studies in non-small cell lung cancer and malignant melanomas showed that HIF-

2α expression was related to a poor outcome when HIF-1α was not (Giatromanolaki et al. 2001; Giatromanolaki et al. 2003). These studies confirm the likely tissue specific differences in the relative importance of HIF proteins in determining tumour progression and prognosis.

Conclusion

The prognostic impact of HIF-1α expression in gastric cancer appears to be dependent on the staining pattern; with HIF-1α expression at the invasive tumour edge having a poor prognosis. However, focal HIF-1α expression was associated with a less aggressive tumour phenotype and a better prognosis. This is perhaps related to the pro-apoptotic, anti-proliferative and cell cycle inhibitory effects of HIF-1α. It is hypothesised that the differences in clinico-pathological and survival characteristics may be related to the differential regulation by HIF-1α of a range of downstream target molecules.

The expression of HIF-2α was significant on univariate analysis; however, it was not an independent predictor of prognosis. The combination of HIF-1α and HIF-2α did not provide any extra prognostic information. There was a trend, however, for HIF-1α/ HIF-2α negative patients to have an improved survival.

In view of the lack of independent prognostic significance of these factors, they are unlikely to impact on clinical management. However, the work described here increases the understanding of the biology of gastric and GOJ tumours. The high expression of HIF-2α suggests that it may be of value as a potential therapeutic target. Additional work assessing down-stream HIF target molecules in relation to apoptosis and proliferation will hopefully increase our understanding of the biology of oesophagogastric cancer, and aid in the possible future development of prognostic molecular marker profiles.