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Review

Is the hypoxia-inducible factor pathway important in gastric cancer?

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Abstract

Tumour hypoxia is well recognised in oncology to be a key factor resulting in treatment resistance and poor prognosis. Hypoxia leads to the expression of a number of gene products that are involved in tumour progression, invasion and metastasis formation. The most important of these proteins is thought to be hypoxia-inducible factor-1α (HIF-1α), which appears to be a master regulator of the cellular response to hypoxia. HIF-1α expression is associated with a poor prognosis and treatment response in a number of tumour sites. There is some evidence that the HIF-1α pathway might be involved in gastric carcinogenesis. Studies have shown reactive oxygen species from *Helicobacter pylori*, associated with the development of gastric cancer, stabilise HIF-1α. Non-steroidal anti-inflammatory drugs, shown to reduce the risk of gastric cancer, can decrease HIF-1α expression. Although a large study correlating HIF-1α expression with prognosis is lacking in gastric cancer, the immunohistochemical expression of HIF-1α target genes (*Glut-1*, *VEGF*, *CA9*, *iNOS*) is associated with a poor prognosis. In addition, the targeted inhibition of HIF-1α has been shown to inhibit the growth of gastric tumours in animals. Increased understanding of the importance of hypoxia and the HIF-1α pathways may therefore hold the key to prevention strategies, improved selection of patients for adjuvant therapy and new treatments for the disease.

Keywords: Tumour hypoxia; HIF-1α; Gastric adenocarcinoma

1. Introduction

Although the incidence and mortality of adenocarcinoma of the stomach has decreased in recent years, it remains the second most common malignancy and the second leading cause of cancer mortality worldwide [1]. In Europe, there were 130,810 new gastric cancers diagnosed in 2000 [2], with an incidence of 12–15 cases per 100,000 of the population. In the Western world, adenocarcinoma of the stomach continues to have a poor prognosis despite advances in surgical techniques and oncology treatments. The current overall 5-year survival figures for gastric cancers in Western patients are in the range 5–17% [2,3], and have not changed significantly in 30 years.

Currently, the only curative treatment for gastric cancer is surgical resection of the primary tumour with an appropriate lymphadenectomy as the disease is considered resistant to chemotherapy and radiotherapy. Patients with early gastric cancer are in the minority and the disease typically presents at an advanced stage, which often precludes curative surgical resection. Even in patients who have an apparently curative resection, about a quarter progress to develop recurrent or metastatic disease. Trials of neoadjuvant therapy have for the most part been disappointing.

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The most promising study of postoperative adjuvant treatment for gastric cancer was published in 2001 [4]. In the Intergroup 0116 trial, 556 patients with resected gastric cancer were randomly allocated to observation alone or adjuvant chemoradiotherapy. The treatment regime comprised 5-fluorouracil (5-FU), folinic acid and radiotherapy (45 Gy). The median overall survival in the surgery only group was 27 months, compared with 36 months in the adjuvant treatment group. Although this regime is popular in the United States of America (USA), it is not widely used in Europe. One major criticism of this study was heterogeneity of the surgical treatment of the patients, with only 10% having a D2 lymphadenectomy. Also, 1% of patients in the Intergroup 0116 study died as a direct result of the toxicity of chemoradiotherapy. The ability to identify those patients most likely to benefit from radiation and/or chemotherapy is of key interest to future research into multimodality treatments for gastric cancer.

Due to its limited treatment options and poor prognosis, gastric cancer therefore remains a major clinical challenge. Improvements in the treatment of the disease must arise from a better understanding of the molecular mechanisms which underlie progression, invasion and metastasis formation; processes which are resistant to most current treatments to date. To make the best use of current oncology treatments it is necessary to evaluate new biological markers that accurately predict the natural history of the disease. This will allow individualised patient therapy, targeting those patients who will derive most benefit whilst avoiding harm to those unlikely to respond. Novel therapies may also allow specific targeted treatment of invasive, angiogenic and metastatic molecules.

Hypoxia is now recognised as a key factor driving the development of malignancy, and the master regulatory protein in the response of cells to changing oxygen levels is hypoxia-inducible factor-1α (HIF-1α). Increased research and knowledge of hypoxia and the HIF-1α pathway, therefore, may hold the key to improved treatment strategies in gastric cancer. Thus, this article aims to review tumour hypoxia and the hypoxia-inducible pathways with specific relevance to gastric cancer. The prognostic and predictive relevance of HIF-1α expression (Fig. 1) and a number of its target gene products will be reviewed. The strategies for improved treatment with HIF-1α blocking agents will be discussed in addition to potential avenues for future research.

2. Tumour hypoxia

Hypoxia, a reduction in the normal tissue oxygen tension, occurs when cellular oxygen needs are outstripped by supply. Hypoxia is associated with several pathophysiological processes including malignancy. In 1955, Tomlinson and Gray [5] showed that tumour chords, with a radius greater than 200 μm invariably contained necrosis, whereas chords with a radius less than 160 μm did not. They proposed a diffusion gradient of oxygen tension between the well-oxygenated cells adjacent to blood vessels and areas of necrosis. The presence

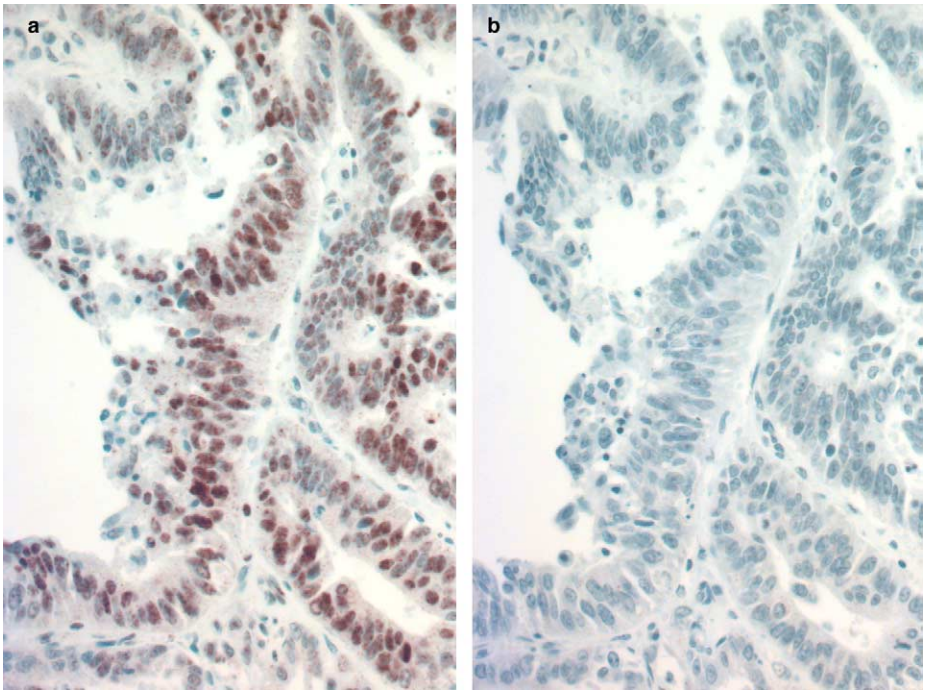

Fig. 1. (a) Immunohistochemistry for HIF-1α in gastric adenocarcinoma tissue showing moderate nuclear staining. (b) Corresponding negative control slide.

of hypoxic, but viable, cells immediately adjacent to necrosis was proposed. It is now thought that there are two principal types of tumour hypoxia: chronic diffusion-limited and acute perfusion-limited hypoxia. Diffusion-limited hypoxia occurs when cells are simply too far away from the nutrient-supplying capillaries for oxygen to diffuse. In acute/intermittent hypoxia, cells are rendered hypoxic for a variable amount of time. The intermittent type is thought to be due to abnormal tumour vasculature, such as out-pouchings, compressed vessels, arterio-venous malformations and tortuous sinusoids leading to altered and erratic blood flow [6]. Temporarily occluded vessels may re-establish flow leading to re-perfusion effects which include an increase in free-radical concentration, tissue damage and activation of stress-response genes.

3. Hypoxia and cancer treatment resistance

Hypoxic tumours are associated with a poor prognosis and resistance to cancer treatments. The response of cells to ionising radiation is dependent upon the availability of oxygen. It has been known since 1953 that well-oxygenated tumour cells have a threefold greater sensitivity to radiation than hypoxic cells [7]. The mechanism by which greater radiation damage occurs in the presence of oxygen is generally referred to as the oxygen fixation hypothesis. Briefly, the interaction between radiation and tissues produces free radicals that damage molecules, particularly DNA. The free radicals have a longer half-life in the presence of oxygen and cause more DNA damage, *i.e.* oxygen is said to 'fix' the damage produced by radiation [8].

Hypoxic cells in tumours can also be resistant to chemotherapy. Reasons for this are multifactorial, but include the impaired drug diffusion [9], reduced cell proliferation [10], decreased cytotoxic drug activity [11] and induction of stress proteins [12] that occur under hypoxia and can result in resistance to some chemotherapeutic agents. 5-FU, doxorubicin, bleomycin, procarbazine, etoposide and vincristine are examples of drugs which are dependent on cellular oxygenation for their maximal efficacy [13,14].

Hypoxia is important not only in the prognosis of patients undergoing chemotherapy and/or radiotherapy, but also for those treated with surgery alone. Studies in patients with uterine cervix carcinoma [15] or soft tissue sarcoma [16] showed that hypoxia, measured using Eppendorf polarographic electrodes, predicted a poor outcome in patients who had primary surgery with or without radiation. The papers hypothesised that the poor prognosis of surgically treated hypoxic cancers was related to hypoxia-induced changes that made the tumour intrinsically more aggressive. Emerging evidence has supported this suggestion and shown that hypoxia plays a key role in promoting tumour progression by stimulating angiogenesis, invasion and metastasis formation [17]. In order to increase oxygen availability and decrease oxygen consumption, hypoxic cells exhibit an adaptive response by increasing the transcription of a wide range of genes including those involved in the control of angiogenesis, pH, glucose transport, oxygen transport and cellular proliferation [18]. The key factor involved in the adaptive response of a tumour to cellular hypoxia is HIF-1α.

4. Hypoxia-inducible factor-1α (HIF-1α)

HIF-1 is a heterodimer consisting of α and β subunits. HIF-1α expression is related to cellular oxygen status, whereas the HIF-1β subunit is constitutively expressed independent of cellular hypoxia [19]. HIF-1α dimerises with HIF-1β in the nucleus and transcriptionally activates a number of genes through binding to hypoxia-responsive elements (HREs). The HIF-1α subunit is stabilised under hypoxia but degrades rapidly in normoxia via the ubiquitin pathway (Fig. 2) [20]. This process is primarily regulated by proline hydroxylation. The presence of functional von Hippel-Lindau (VHL) protein is required for ubiquitination. In tumours with VHL mutations, such as renal cell carcinoma and cerebellar haemangioblastomas, accumulation of HIF-1α is found [21].

HIF-1α expression is common in human cancers. Zhong *et al.* [22] studied immunohistochemical HIF-1α expression in 179 tumour specimens and found that it was overexpressed in 13 out of 19 common tumour types. These included colon, breast, lung, skin, ovarian, pancreatic, prostate, renal and gastric carcinomas. Positive HIF-1α staining was found in pre-malignant tissue, such as colonic adenoma, breast ductal carcinoma *in situ* and prostate intraepithelial neoplasia. In contrast, most benign tissue showed no evidence of HIF-1α expression, although weak HIF-1α staining was reported in some tissue, such as adrenal cortical cells and pancreatic acinar cells. The localisation of HIF-1α expression in the malignant tissue was predominantly nuclear. Areas immediately adjacent to necrotic tumour and the invading tumour margins revealed the most intense staining. Occasionally, other localised or more diffuse staining patterns were found, and considered to be the result of genetic alterations and local microenvironmental factors other than hypoxia.

Although hypoxia is the main regulator of HIF-1α, there is emerging evidence that it is stabilised by several non-oxygen dependent mechanisms. Various tumour specific genetic alterations involving oncogenes (*PAS* and *MYC*) and tumour suppressor genes (*p*53, *PTEN* and *VHL*) have been associated with HIF-1α stabilisation [23]. Cytokines, such as insulin, insulin-like growth

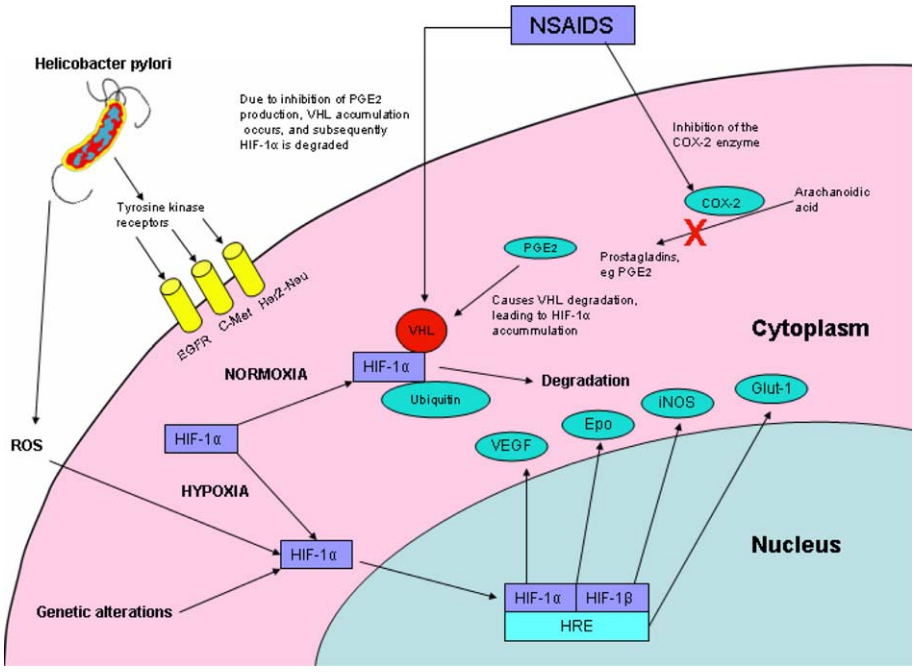

Fig. 2. Hypoxia-inducible factor (HIF) pathway. Hypoxia, reactive oxygen species from *Helicobacter pylori* and specific genetic alterations (such as *p*53, *PTEN* and *pVHL* mutations) can cause HIF-1α to be stabilised. When activated, HIF-1α forms a dimer with HIF-1β and binds to hypoxia-responsive elements within the nucleus. This initiates the transcription of a number of hypoxia-inducible gene products, such as VEGF, Epo, iNOS, and Glut-1. During normoxia, HIF-1α is rapidly degraded by the ubiquitin pathway in conjunction with VHL. This is a process that involves prolyl hydroxylation. This degradation pathway may be disrupted by PGE2, which is a product of the COX-2 enzyme. Non-steroidal anti-inflammatory drugs inhibit the production of prostaglandins, including PGE2, and are known to result in increased HIF-1α degradation. c-MET, receptor for hepatocyte growth factor; COX-2, cyclo-oxygenase enzyme-2; EGFR, epidermal growth factor receptor; Epo, erythropoietin; Glut-1, glucose transporter 1; HIF-1α, hypoxia-inducible factor-1α; HRE, hypoxia responsive element; iNOS, inducible nitric oxide synthase; NSAIDS, non-steroidal anti-inflammatory drugs; PGE2, prostaglandin-E2; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau protein.

factor (IGF), epidermal growth factor (EGF) and interleukin-1 stimulate receptor tyrosine kinases which also influence HIF-1α levels [18].

4.1. HIF-1α expression as a prognostic factor

Table 1 summarises the findings from studies examining the prognostic significance of HIF-1α expression in tumours. Most have shown that the immunohistochemical expression of HIF-1α is associated with a poor prognosis, however others have only shown a trend for poor prognosis and some have been not been statistically significant. Differences in the scoring systems used along with the small number of patients included may explain the lack of significance in some of the studies. In three studies, in head and neck, oral cavity and non-small cell lung cancer, HIF-1α expression predicted a good prognosis [24–26]. Evidence is emerging that suggests that HIF-1α has both pro- and anti-tumour properties, especially with regard to apoptosis [27]. In support of this suggestion one of the studies where HIF-1α expression was a good prognostic factor showed a strong correlation between HIF-1α and the expression of pro-apoptotic factors such as caspase-3, Fas, and Fas ligand [25]. The balance between the pro- or anti-apoptotic effects of HIF-1α are likely to be determined by the associated genetic alterations, such as *p*53 or members of the *Bcl-2* family.

In addition to prognosis, HIF-1α expression has also been shown in some studies to predict response to chemoradiotherapy [28,29]. For example, in a study of 65 patients with oesophageal cancer undergoing chemoradiotherapy with 5-FU and cisplatin, HIF-1α expression predicted a poor treatment response [29].

4.2. HIF-1α regulated products

Around 50 genes with HREs have been identified [30,31]. Known HIF-1 inducible proteins which might be important in gastric cancer include carbonic anhydrase IX (CA IX), glucose transporter 1 (Glut-1), erythropoietin (Epo), inducible nitric oxide synthase (iNOS), and vascular endothelial growth factor (VEGF). CA IX is a transmembrane glycoprotein which is involved in the maintenance of intracellular pH in the hypoxic environment [32]. Glut-1 plays an important role in the survival of tumour cells by ensuring an adequate energy supply [33]. Epo is the principal regulator of red blood

Table 1
Hypoxia-inducible factor (HIF) expression and prognosis in different tumour sites

Cancer site	Subtype	Stage	Treatment	Patients (*n*)	HIF expression (%)	Exp. pat.	Cut-offs	Surv.	*P*-value Uni.	*P*-value Multi.	Author, Reference
Astrocytomas	N/A	Operable	Surgery ± ART	83	92	N	< or >30%	Poor	0.0227	0.03[a]	Korkolopoulou *et al.* [103]
Bladder	TCC	All	TUR ± BCG ± CRT	93	75	N	Quartiles	Poor	0.009	0.02	Theodoropoulos *et al.* [104]
Bladder	TCC	Ta/T1	TUR ± BCG	140	47	N + C	Low *vs.* high	TP	0.058	N/S	Theodoropoulos *et al.* [105]
Breast	A	Lymph node+	Surgery ± CT ± tamoxifen	206	76	N + C	Quartiles	Poor	0.0454	0.003	Schindl *et al.* [106]
Breast	A	Lymph node+	Surgery ± CT ± tamoxifen	77	56	N	Quartiles	Poor	0.04	NS	Gruber *et al.* [107]
Breast	A	All	Surgery ± CT ± tamoxifen	150	75	N	< or ⩾5%	Poor[b]	0.008[b]	0.021[b]	Bos *et al.* [108]
Breast	A	Early	Surgery	745	100	N + C	< or >10%	Poor	0.019	0.03	Dales *et al.* [109]
Cervix	S (63) A (15)	Advanced	Radical RT	78	94	N	<1%, 10–50, >50%	Poor	0.04	0.02	Burri *et al.* [110]
Cervix	S	Early	Surgery ± ART	91	81	N	>4 points	Poor	<0.0001	0.0129	Birner *et al.* [111]
Cervix	S (33) A (3) AS (7) O (2)	Locally advanced	RT	45	Unknown	N	Median (2%)	NS	NS	N/A	Haugland *et al.* [112]
Cervix	S	Ib–IVa	Radical RT	99	67	N + C	< or >10%	NS[c]	0.56	N/A	Hutchison *et al.* [113]
Cervix	S	Ib–IVb	Surgery ± RT ± CT	38	Unknown	N + C	Median	TG	0.11	N/A	Mayer *et al.* [114]
Colorectal	A	All	Surgery ± CT	139	58	N + C	< or >10%	TP	0.077	N/A	Kuwai *et al.* [115]
Colorectal	A	Curatively treated	Surgery	87	45	N + C	< or >5%	NS[d]	>0.05	N/A	Yoshimura *et al.* [116]
Endometrial	A	All	Surgery ± RT	81	49	N + C	Low *vs.* high	Poor	0.03	0.01	Sivridis *et al.* [117]
GIST	N/A	All	Surgery	53	32	N	< or >10%	Poor	<0.05	NS	Takahashi *et al.* [56]
GIST	N/A	All	Surgery	62	56	N + C	< or >10%	Poor	0.009	N/A	Chen *et al.* [57]
Head and neck	S	Locally advanced	CRT	75	48	N + C	Mean value	Poor	0.05	NS[e]	Koukourakis *et al.* [118]
Head and neck	S	Early	Surgery	79	87	N	Positive or negative	Good	0.027	N/A	Beasley *et al.* [24]
Oesophageal	S	All	Surgery ± CRT	82	39	N + C	Mean %	TP	0.078	N/A	Kimura *et al.* [119]
Oesophageal	S	Early	PDT ± RT	37	65	N + C	Unknown	TP	0.08	N/A	Koukourakis *et al.* [120]
Oesophageal	S	All	Surgery	130	68	N	< or >10%	Poor	0.0007	NS	Kurokawa *et al.* [121]
Oligodendroma	N/A	Operable	Surgery ± CRT	51	80	N	>4 points	Poor	0.0434	0.0187	Birner *et al.* [122]
Oral cavity	S	Early	Surgery ± RT	85	36	N	< or >5%	Good	<0.01	0.01	Fillies *et al.* [26]
Oropharygneal	S	All	RT and CT	98	98	N + C	0%, 1–10%, 10–50%	Poor	0.001	0.0009	Abersold *et al.* [28]
Ovarian	A	I–IV	Surgery and ACT	102	69	N	Quartiles	NS	0.6848	N/A	Birner *et al.* [123]
Nasopharyngeal	S	Locally advanced	RT	90	58	N	Quartiles	TP	0.06	N/A	Hui *et al.* [124]
Upper urinary tract	TCC	All	Surgery ± RT ± CT	127	55	N	Positive *vs.* negative	Poor	0.0001	<0.0001	Nakanishi *et al.* [125]

A, adenocarcinoma; ACT, adjuvant chemotherapy; NSCLC, non-small cell lung cancer; N/A, not applicable; ART, adjuvant radiotherapy; O, other tumour type; BCG, intra-vesical bacille Calmette-Guerin; PDT, photodynamic therapy; C, cytoplasmic expression; Poor, poor prognosis; CT, chemotherapy; RT, radiotherapy; CRT, chemo-radiotherapy; S, squamous cell carcinoma; Exp. pat., pattern of expression; TG, trend for good prognosis; Good, good prognosis; TP, trend for poor prognosis; GIST, gastrointestinal stromal tumour; TUR, transurethral resection of bladder tumour; N, nuclear expression; NS, not significant; TCC, transitional cell carcinoma.

[a] Independent prognostic factor when HIF-1α expression was analysed in association with grade.

[b] Overall there was only a borderline significance ($P = 0.059$), however HIF-1α expression was significant in predicting a poor prognosis in 81 patients without lymph node metastases.

[c] Although overall survival was not significant; high HIF-1α expression was associated with a poor outcome for small tumours but good outcome in large tumours.

[d] HIF-2α and the combination of HIF-1α and HIF-2α were significant in multivariate analysis.

[e] HIF-2α expression was a statistically adverse prognostic factor in multivariate analysis.

cell production, and its expression in tumours is thought to contribute to survival of tumour cells in the hypoxic environment by stimulating angiogenesis [34]. iNOS catalyses the formation of nitric oxide (NO), a regulator of vascular permeability and promoter of tumour growth [35]. VEGF is the principal pro-angiogenic growth factor and its stimulation under hypoxia plays a key role in promoting the survival of malignant cells, in local tumour growth and invasion, and in the development of metastases [36]. The expression of HIF-1α has been shown to correlate not only with VEGF but also with the level of angiogenesis in tumours, measured as microvessel density (MVD) [37].

4.3. HIF-1α expression in benign and malignant gastric tissue and its possible role in Helicobacter pylori induced carcinogenesis

4.3.1. HIF-1α expression in benign gastric tissue

HIF-1α is not generally expressed in normal tissue [22,38]. Zhong *et al*. [22] reported no expression in normal gastric mucosa, but only examined 1 specimen. Ito *et al*. [39] studied 71 endoscopic biopsies of normal gastric mucosa from Japanese patients with gastric cancer, peptic ulcer or dyspepsia and found low expression of HIF-1α in benign gastric mucosa. They observed the expression of HIF-1α to be greater in patients receiving acute non-steroidal anti-inflammatory drugs (NSAID) compared with those not taking NSAIDs [39]. The authors hypothesised that this was related to acute ischaemic/hypoxic damage of mucosal cells *in vivo*. A similar situation of acute alcohol injury in a rat model has also been shown to increase HIF-1α stabilisation, especially in the mucosa bordering areas of necrosis [40].

4.3.2. Possible role of HIF-1α in Helicobacter pylori induced gastric carcinogenesis

H. pylori is a Gram-negative bacillus which is thought to be the one of the most important factors in gastric carcinogenesis, with a recent study suggesting it may be a prerequisite for the development of the distal form of the disease [41]. However, the molecular mechanisms of distal intestinal type gastric cancer development remain largely unknown. Several cell signalling pathways have been implicated, including those involved in the control of apoptosis and proliferation, and the activation of tyrosine kinase signalling pathways (EGFR, Her2-Neu and c-Met) [42].

There is emerging evidence that HIF-1α may be involved in the aetiology of gastric cancer (Fig. 2). Infection with *H. pylori* is the major initiating and driving factor, with the proposal that the formation of reactive oxygen species (ROS) due to neutrophil infiltration in response to *H. pylori* infection causes epithelial cell injury and progressive DNA damage [43]. There is evidence that gastric epithelial ROS, both endogenous and *H. pylori*-induced, may lead to HIF-1α expression under normal oxygen conditions [44]. A recent cell line study showed that ROS from the more virulent cytotoxin-associated gene-A (*cagA*) bearing strains of *H. pylori* cause HIF-1α stabilisation and accumulation in gastric cancer cells under normoxic conditions [44]. In cells under the influence of ROS produced by *H. pylori*, HIF-1α is continuously present regardless of cellular oxygen status.

In addition to ROS, another important mediator in the chronic inflammatory process is nitric oxide (NO) which, in response to *H. pylori* infection, is produced by gastric epithelial and non-epithelial cells from L-arginine via iNOS. Increased iNOS expression is seen in *H. pylori* infected gastric mucosa [45,46]. NO has been shown to interfere with HIF-1α prolyl hydroxylases under normoxia, preventing degradation and resulting in HIF-1α accumulation and activation [47].

4.3.3. Possible role of HIF-1α in non-steroidal anti-inflammatory drug (NSAID) induced protection of gastric carcinogenesis

In contrast to Ito *et al*. [39] study showing the expression of HIF-1α in NSAID-related gastritis, there is some evidence that NSAIDs can also decrease HIF-1α expression. NSAID use is known to be associated with a decreased risk of gastric cancer [48,49]. The anti-cancer effects of NSAIDs relate to their ability to inhibit the expression of cyclooxygenase-2 (COX-2), which is upregulated in malignancy. Although the exact mechanism of action has yet to be completely defined, there is some evidence for a role for HIF-1α.

COX-2 functions to convert arachidonic acid to prostaglandins in inflamed and neoplastic tissue. Expression of COX-2 is induced by pro-inflammatory cytokines, oncogenes, growth factors and hypoxia. Overexpression is common in human cancers, including gastric, and is associated with advancing tumour progression and metastatic potential [50]. COX-2 expression induces the synthesis of prostaglandins, especially PGE2 [51]. PGE2 causes the degradation of VHL protein, thereby increasing HIF-1α expression (Fig. 2) [52].

A cell line study by Jones *et al*. [53], showed that NSAIDs (both non-selective and COX-2 specific types) inhibited angiogenesis in a model of rat gastric cancer. Decreased HIF-1α levels were seen in the cells exposed to NSAIDs. A mechanism was proposed where the NSAIDs increased the expression of the VHL tumour suppressor protein (Fig. 2), resulting in HIF-1α ubiquitination and an inhibition of hypoxia-induced angiogenesis. NSAIDs have also been shown to reduce both HIF-1α and HIF-2α levels in prostate cancer cells [54]. In the latter study, the HIF inhibition was independent of the COX expression. A recent study has confirmed the COX-2/PGE2/HIF-1α/VEGF pathway to play an important role in angiogenesis in gastric cancer [55].

Table 2
Immunohistochemical expression of HIF-1 inducible genes which are known to be important in gastric adenocarcinoma

Marker	Patients/specimens	Expression (%)	Cut-offs	Expression correlated with:	Other comment	Author, Ref.
Glut-1	617 patients	30	0–1%, 2–30% and >30%	Increasing tumour stage, lymphatic and vascular invasion, peritoneal and hepatic metastases Shorter survival ($P = 0.0001$)	Peri-necrotic expression	Kawamura *et al.* [68]
Glut-1	70 patients	19	< or >25%	Tumour invasion, lymphatic and vascular invasion, and lymph node metastases	Significant association with overall survival in univariate ($P = 0.0009$) and multivariate ($P = 0.03$) analysis	Noguchi *et al.* [65]
CA IX	74 tumour specimens, 67 benign tissue, 22 adenomas, 33 intestinal metaplasia	N/A	N/A	CA IX expression was high in normal and hyperplastic mucosa and lower in dysplasia and gastric malignancy. There was no correlation with tumour stage	CA IX has a physiological role producing gastric acid and high expression occurs in benign tissue	Leppilampli *et al.* [61]
CA IX	59 patients	N/A	IRS < or >3	Associated with reduced post-operative survival ($P = 0.03$) (assessed in 23 patients)	Staining observed in the invasive tumour edge	Chen *et al.* [64]
Epo-R	40 patients	N/A	N/A	Grade of tumour and extent of angiogenesis		Ribatti *et al.* [69]
VEGF	206 patients	74	Positive *vs.* negative	Associated with shorter disease free survival ($P < 0.02$) and overall survival ($P < 0.01$)	Statistically significant in multivariate analysis	Fondevila *et al.* [78]
VEGF	76 patients	39	< or >10%	Lymph node metastasis ($P = 0.009$) Shorter overall survival ($P < 0.05$)		Ichikura *et al.* [126]
VEGF	50 patients	50	N/A	Not assessed	Staining observed more often in the invasive margin of the tumour rather than the centre	Liu *et al.* [75]
VEGF	129 patients	43	< or >5%	Lymphatic invasion ($P < 0.05$), venous invasion ($P < 0.05$), lymph nodes metastases ($P < 0.01$) and liver metastases ($P < 0.01$)	Poor overall prognosis in multivariate analysis	Maeda *et al.* [76]
VEGF	195 patients	31	Positive or negative	VEGF expression was observed more frequently in the patients who developed recurrence	Assessed on pre-operative biopsies in patients with early gastric cancer. Poor overall prognosis in multivariate analysis	Maeda *et al.* [77]
VEGF	80 patients	67	IRS 0–2 *vs.* 3–6	Degree of differentiation ($P < 0.01$), lymph node metastases ($P < 0.01$) and micro-vessel density (MVD) ($P < 0.05$)		Du *et al.* [79]
iNOS	55 patients	44	< or >10%	Lymph node metastases ($P = 0.014$) p53 expression ($P = 0.005$)	Increased expression of iNOS, VEGF and p53 also found in intestinal metaplastic tissue	Feng *et al.* [70]
iNOS	55 patients	53	Median score	Advanced tumours ($P = 0.015$), size >5 cm ($P = 0.025$) and metastases ($P = 0.002$)	p53 expression was correlated ($P = 0.018$)	Rajnakova *et al.* [71]
iNOS	46 patients	59	Positive or negative	Advanced tumour stage ($P = 0.019$) and lymph node metastases ($P < 0.05$), but not associated with MVD	Reduced 5-year survival ($P < 0.05$)	Song *et al.* [72]
iNOS	85 patients	59	IRS 0–1, 2, 3–4, 5	Associated with advanced stage and lymph node metastasis	VEGF expression and iNOS expression correlated ($P = 0.018$)	Song *et al.* [73]

CA IX, carbonic anhydrase IX; Epo-R, erythropoietin receptor; Glut-1, glucose transporter-1; IRS, immunoreactive score; iNOS, inducible nitric oxide synthase; VEGF, vascular endothelial growth factor.

Both high levels of HIF-1α and VEGF were observed in cells that over-expressed COX-2.

4.4. Prognostic significance of HIF-1α and hypoxia-inducible product expression in gastric cancer

There is a paucity of data on HIF-1α and gastric cancer. In the large immunohistochemical study of HIF-1α expression in human cancers by Zhong *et al.* [22], expression was found in gastric cancer. However, only two specimens with gastric adenocarcinoma were stained for HIF-1α in this study, both of which were positive (one with 1% staining and the other with over 50% staining). Unfortunately, unlike other cancers (Table 1), few published studies have described HIF-1α expression in gastric adenocarcinomas.

Two studies have assessed HIF-1α as a prognostic marker in gastrointestinal stromal tumours (GIST) of the stomach. Takahashi and colleagues [56] examined the expression of HIF-1α, VEGF, anti-CD31 (to score MVD) and Ki-67 using immunohistochemistry in 53 patients with GIST affecting the stomach. HIF-1α expression was shown in 32% of the specimens and correlated significantly with tumour size, liver metastasis and overall prognosis. HIF-1α expression correlated well with VEGF expression and the level of angiogenesis measured histologically as MVD. A further study, which assessed 62 patients with GIST, confirmed these findings and also found that high HIF-1α was associated with a high incidence of tumour recurrence and distant metastasis [57].

Several tumour suppressor genes (*VHL*, *PTEN*), which are relevant to the HIF pathway, are frequently inactivated in gastric cancer [58,59]. The inactivation of these genes are known to cause the upregulation of HIF-1α in normoxic conditions [60]. It will be of interest in future studies to correlate HIF-1α expression with these proteins. Several studies have used immunohistochemistry to evaluate hypoxia-inducible proteins in gastric cancer (Table 2).

5. Carbonic anhydrase (CA) IX and gastric cancer

Overexpression of CA IX has been reported in various cancer types. However, expression is low or even lost in most gastric cancers [61,62], indicating that the biological function of CA IX in gastric cancer might be more complex than in other types of cancer. The gastric mucosa is one of the most predominant sites of physiological CA IX expression as biologically it is involved in the production of gastric acid. Leppilampi *et al.* [61] performed an immunohistochemical study of CA IX expression in normal gastric mucosa, gastric adenomas and different grades of gastric carcinomas. CA IX expression was high in normal and hyperplastic mucosa and lower in dysplasia and gastric malignancy. This loss of expression of CA IX may be related to neoplastic alteration, including dedifferentiation during gastric carcinogenesis. CA IX may also be involved in early gastric carcinogenesis as CA IX deficient mice show increased cellular proliferation and develop gastric hyperplasia [63].

CA IX was expressed in the invasive edge of gastric cancer resection specimens [64]. Although high CA IX expression was associated with shorter post-operative survival ($P = 0.028$), only 23 patients were included in the survival analysis. In the same paper, an *in vitro* study revealed that cells expressing CA IX had increased proliferation rates and were much more invasive than cells that lacked CA IX [64].

6. Glut-1 and gastric cancer

Glut-1 is expressed in gastric cancer [65–68]. Noguchi *et al.* [65] found high (>25%) Glut-1 expression was associated with a poor post-operative survival in 70 gastric cancer patients. A more detailed study by Kawamura *et al.* [68] investigated 617 gastric carcinomas and found 182 (30%) were positive for Glut-1. Staining was mainly localised in the central part of tumour nests, with a preferential association with central necrosis. Staining varied between histological sub-type: papillary (44%), tubular (32%), poorly-differentiated (28%), signet-ring (1%) and mucinous (1%). The expression of Glut-1 increased progressively with increasing tumour stage. There were also significant associations between Glut-1 positivity and adverse tumour features and survival (Table 2).

7. Erythropoietin receptor (Epo-R) and gastric cancer

Ribatti *et al.* [69] studied Epo-R expression immunohistochemically in 40 patients with gastric adenocarcinoma. They found that Epo-R expression correlated with angiogenesis, measured as MVD, and progression of disease. Moreover, Epo-R expression increased with advancing tumour grade.

8. Inducible nitric oxide synthase (iNOS) and gastric cancer

Several papers have examined iNOS expression in gastric cancer specimens and found associations with adverse pathological features and reduced survival [70–73]. iNOS expression is found in both diffuse and intestinal types of the disease [74]. Increased expression correlates with increasing tumour stage and number of lymph node metastases [70–72]. In a recent paper by

Song *et al.* [73], the rate of expression of iNOS was 59% and correlated with VEGF expression.

9. Vascular endothelial growth factor (VEGF) and microvessel density (MVD) in gastric cancer

VEGF expression is associated with an increased risk of metastatic disease and reduced survival in patients with gastric cancer [72,75]. In three studies, VEGF expression was also found to be an independent adverse prognostic factor for survival [76–78]. Du *et al.* [79] studied VEGF expression and MVD in 80 patients with gastric cancer. VEGF expression was found in 68% (54/80) and was closely related to the degree of tumour differentiation, lymph node metastases and MVD. In addition, positive staining for VEGF tended to be located at the centre of the tumour or at the edge of areas of necrosis, consistent with hypoxia-induced expression.

Koukarakis *et al.* [80] studied MVD in a range of human tumours including 98 patients with locally-advanced inoperable gastric cancers. They all received chemotherapy with a median follow-up of 9 months (range 2–60 months). When survival was plotted against MVD the results revealed a 'U-shaped' distribution. Patients with low and high tumour MVD had a poor prognosis, whilst patients with an intermediate MVD survived longer. In the low MVD group, poor oxygenation and reduced drug delivery were believed to be the underlying reason for poor outcome. The highly vascularised tumours or the high MVD group were thought to have a poor prognosis due to their highly aggressive, angiogenic phenotype and early metastatic capabilities.

10. Targeting HIF-1α as a therapeutic approach in gastric cancer

There is interest in HIF-1α as a cancer therapeutic target [81]. This stems in part from studies implicating HIF-1α in tumour resistance to chemotherapy and radiation [28,82]. Blocking HIF-1α activity has potential to inhibit cancer progression by depriving cancer cells of the means to adapt to hypoxia and a nutrient depleted environment. Several compounds are currently undergoing assessment in murine gastric tumour models.

YC-1 [3-(5′-hydroxymethyl-2′-furyl1)-1-benzylindazole] is a soluble guanylyl cyclase stimulator developed for its ability to inhibit platelet aggregation and vascular contraction, and for the treatment of circulation disorders. Yeo *et al.* [83] studied its therapeutic use *in vivo* in gastric tumours induced subcutaneously in nude mice. They found that YC-1 blocked angiogenesis and inhibited tumour growth, resulting in fewer blood vessels, lower expression of HIF-1α and HIF-1 regulated genes. They concluded that YC-1 is a potent inhibitor of HIF-1 with the potential to become the first anti-angiogenic anticancer agent to target HIF-1α.

A recent study by Stoeltzing *et al.* [84] assessed a gene therapy approach for inhibiting HIF-1α in nude mice transfected with human gastric cancer cell lines both subcutaneously and orthotopically. Inhibition was carried out by overexpressing a dominant-negative construct of HIF-1α that dimerised with endogenous HIF-1β to produce non-transcriptionally active HIF-1. Inhibition of HIF-1α reduced VEGF expression, angiogenesis and tumour size. They concluded that HIF-1α was a valid target for the treatment of gastric cancer.

11. Future research and conclusion

11.1. HIF-1α and gastric carcinogenesis

The role of ROS, both *H. pylori*-induced and endogenous, in stabilising HIF-1α is an area that requires further study. Experiments investigating the role of a ROS-directed post-translational modification of the HIF-1α protein in the regulation of HIF-1α stability will be of interest [44]. COX-2 expression induces the synthesis of prostaglandins (especially PGE2), which degrade VHL and increase the stability of HIF-1α. Investigation of the possible HIF-1α related mechanism of gastric cancer chemo-prevention with long-term NSAID use, especially COX-2 specific agents, is warranted. More detailed exploration of the relationship between HIF-1α and COX-2 would also be of interest.

11.2. HIF-1α as a prognostic factor for treatment response

There is a need for large studies exploring the relationship between HIF-1α expression and treatment outcome in gastric adenocarcinoma. There is currently considerable interest in microarray technology for the investigation of global gene expression patterns. Microarrays are being used to study *H. pylori* gene diversity and changes in gene expression associated with *H. pylori* infection and gastric carcinogenesis [85]. These studies are starting to provide information on not only the molecular basis of gastric cancer, but also new targets for the development of diagnostic markers for gastric cancer and therapeutic drug discovery [86]. They are also being used to define genomic profiles that predict lymph node status and survival, and a recent study revealed a profile that discriminated a group of patients with a high probability of survival [87]. It is important to be aware, however, that HIF-1α is unlikely to emerge from oligonucleotide microarray experiments as a factor involved in gastric cancer development and prognosis. This is because changes in HIF-1α associated with cancer occur post-translation (*i.e.* they occur at the protein

rather than the RNA level) and previous cDNA microarray studies have found constant transcript levels [88]. Nevertheless, cDNA microarrays will still be a valuable technique for surveying the expression of genes related to hypoxia and/or HIF-1α levels derived from other techniques.

Tissue microarrays offer an efficient way of examining protein expression on a large number of tumour cases on a single glass slide. They can be used in retrospective studies to examine the expression of a number of proteins in a large number of gastric cancers in a uniform way. Preliminary work has highlighted the feasibility and validity of carrying out tissue microarrays using gastric cancer biopsy tissue [89]. Tissue array studies, therefore, will be of interest to examine molecular marker profiles at the protein level (that include HIF-1α) in relation to treatment response.

In future, these types of studies should lead a greater individualisation of treatment in patients with gastric cancer and the selection of therapy to those most likely to respond whilst avoiding morbidity in those unlikely to benefit. Although adjuvant chemoradiotherapy improves the survival of patients with gastric cancer, the controversy regarding sub-optimal surgery in patients included in the Intergroup 0116 trial has prevented its adoption into routine clinical practice in Europe. There is already some evidence that HIF-1α expression might predict those patients unlikely to respond to radiotherapy in several tumour sites. Investigation of the value of HIF-1α and its associated proteins in predicting chemotherapy and radiation response will, therefore, also be of interest in gastric cancer patients.

11.3. HIF-1α as a therapeutic target

Future development of HIF-1α blocking agents of is an exciting prospect. Several strategies to inactivate or exploit HIF-1α are being explored in preclinical models. These include gene therapy approaches [90], small molecule inhibitors of HIF-1α transcription [91] and promoters of HIF-1α protein degradation [92]. The preliminary demonstration of the efficacy of targeting HIF-1α in gastric cancer [84] should provide a stimulus for further work in this area. As for other molecularly targeted therapy, future clinical studies will need to examine the efficacy of HIF-1α directed therapy in combination with standard therapeutic approaches. Moreover, carrying out clinical trials in unselected groups of patients may not be the most appropriate way forward. Selection of patients based on either HIF-1α status or measurements of tumour hypoxia may be required.

11.4. Oxygenation status of gastric cancers

There are currently no published studies of measurements of the oxygenation status of gastric cancers. Therefore, in addition to work examining the prognostic significance of HIF-1α expression, studies examining the oxygenation status of gastric cancer would be useful. Oxygen electrodes have proved useful in a number of tumour types [93–95], but have limited use in gastric cancer because of poor accessibility. Other approaches such as the immunohistochemical expression of hypoxia-specific markers such as pimonidazole [93] and non-invasive imaging [96] are being developed and could be carried out in patients with gastric cancer. It would also be important to further correlate oxygenation results with the potential hypoxia-related markers (HIF-1α, Glut-1, CA IX, Epo, VEGF) to gain further insight into their functional roles in gastric cancer.

11.5. HIF subunits and variants

A number of proteins have been identified recently that are closely related to HIF-1α and regulate the transcription of hypoxia-regulated genes in a similar way to HIF-1α (HIF-2α and HIF-3α). A study in non-small cell lung cancer showed that HIF-2α expression was related to poor outcome, whereas HIF-1α was not [97]. Another study showed a predominant role of HIF-2α over HIF-1α in the regulation of the transcriptional response of hypoxia in renal cell carcinoma [98]. These findings raise the possibility of tissue specific differences in the relative importance of HIF proteins in determining tumour progression and prognosis. Different isoforms of the various HIF proteins have also been identified [99–102]. There is some evidence that HIF polymorphisms have a role in generating differences in the potential for tumour progression between individuals [100]. The clinical relevance of different HIF proteins and variants, therefore, will also be of interest for future research in gastric cancer.

11.6. Conclusion

Over the past 5 years, HIF-1α has emerged as a key regulator of the growth of solid tumours. Although studies in gastric cancer are lacking, there is some evidence suggesting that HIF-1α may be an involved in gastric tumourigenesis. HIF-1α can be expressed under normal oxygen conditions in gastric cancer cells due to endogenously generated ROS. *H. pylori* infection, associated with the development of gastric cancer, induces ROS and results in the constant expression of HIF-1α in normoxia. NSAIDs, shown to reduce the risk of gastric cancer, can decrease the expression of HIF-1α. With the exception of two studies showing that the expression of HIF-1α in GISTs is an adverse prognostic feature [56,57], there are no published studies correlating HIF-1α expression with treatment outcome in gastric adenocarcinoma. However, the immunohistochemical expression of several HIF-1α target genes (*Glut-1*, *iNOS*, *VEGF*, *CA IX*) are associated with a poor prognosis.

Future clinical studies are required to investigate the expression of HIF-1α in pre-malignant and malignant gastric lesions and to determine its value in predicting treatment outcome. The targeted inhibition of HIF-1α has also been shown to inhibit the growth of gastric tumours in animals. Increased understanding of the importance of HIF-1α and hypoxia, therefore, may hold the key to a greater individualisation of therapy and new treatments for patients with gastric cancer.

Conflict of interest statement

None declared.

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