



Hypoxia and its impact on the tumour microenvironment of gastroesophageal cancers

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Abstract

The malfeasant role of the hypoxic tumour microenvironment (TME) in cancer progression was recognized decades ago but the exact mechanisms that augment the hallmarks of cancer and promote treatment resistance continue to be elucidated. Gastroesophageal cancers (GOCs) represent a major burden of worldwide disease, responsible for the deaths of over 1 million people annually. Disentangling the impact of hypoxia in GOCs enables a better overall understanding of the disease pathogenesis while shining a light on novel therapeutic strategies and facilitating precision treatment approaches with the ultimate goal of improving outcomes for patients with these diseases. This review discusses the underlying principles and processes of the hypoxic response and the effect of hypoxia in promoting the hallmarks of cancer in the context of GOCs. We focus on its bidirectional influence on inflammation and how it drives angiogenesis, innate and adaptive immune evasion, metastasis, and the reprogramming of cellular bioenergetics. The contribution of the hypoxic GOC TME to treatment resistance is examined and a brief overview of the pharmacodynamics of hypoxia-targeted therapeutics is given. The principal methods that are used in measuring hypoxia and how they may enhance prognostication or provide rationale for individually tailored management in the case of tumours with significant hypoxic regions are also discussed.

Key Words: Esophageal cancer; Gastric cancer; Tumor hypoxia; Tumour microenvironment; Gastroesophageal cancer

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onment have allowed for a better understanding of the role of hypoxia and how it contributes to tumour progression and treatment resistance. These methods include non-invasive imaging techniques as well as validated hypoxic molecular signatures. Specific hypoxia-targeted therapies have not matched their expectations but may have potential application in combination with traditional treatment approaches in gastroesophageal cancer.

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INTRODUCTION

One of the major turning points in the study of solid tumours arose with the realization that a critical regulatory influence in the process of angiogenesis was an environmental feature; hypoxia[1,2]. Many studies have since demonstrated the oncogenic transforming power of hypoxia in the microenvironment of different tumour types and the observation that tumour oxygenation status could disrupt the anti-tumour effects of radiation therapy was published over 60 years ago[3-8]. This review will discuss the role of hypoxia in the tumour microenvironment (TME) of gastroesophageal cancers (GOCs) including gastric cancer (GC) and oesophageal cancer (OC), how it augments disease, and additionally its relevance in the setting of prognostication and therapeutic targeting.

GOC is a substantial cause of morbidity and mortality, responsible for 1.2 million deaths per year globally[9-12]. An improved understanding of the risk factors for GC has seen a steady decline in both the incidence and mortality which is in sharp contrast to the rising incidence of OC, particularly oesophageal adenocarcinoma (OAC) globally[13,14]. GOCs develop insidiously and consequently, are commonly diagnosed at an advanced stage where chemotherapy with or without radiation remains the treatment of choice in the neoadjuvant setting[15]. Treatment at this stage is rarely curative and several mechanisms account for this resistance to treatment including tumour cell-intrinsic and extrinsic mechanisms. Hypoxia is a characteristic feature of the TME and a key mediator in conferring and enhancing treatment resistance[16-18]. The TME being the complex reciprocity between both the cellular (resident and infiltrating) and non-cellular components that surround, envelop, and make up the tumour mass, the components of which are summarized in Figure 1[19-21]. The exact mechanisms underlying resistance continue to be elucidated and as such, interest in the role of hypoxia in translational oncology research has garnered increasing interest recently as shown in Figure 2.

Hypoxia mediates aggressive, metastatic, and treatment-resistant disease by augmenting the hallmarks of cancer through various cellular and physiological events including; enhanced tumour cell proliferation, survival, immune evasion, inflammation, induction of angiogenesis, and activation of invasion[16,17,22]. In large part these events are influenced or orchestrated by the relationship between oxygen availability and the genes encoding hypoxia-inducible factors (HIF) and von Hippel Lindau protein (pVHL)[23,24]. HIFs are a family of heterodimeric transcription factors consisting of a labile α subunit and a stable β subunit. There are several HIF isotypes but the most well-studied is HIF1. HIF1- α contains domains amenable to post-translational modifications thereby mediating interactions with the molecular machinery responsible for cellular degradation[25,26]. When induced, HIF1- α associates with the constitutively expressed HIF1- β subunit and together act to bring about the transcription of a multitude of genes involved in complex signalling pathways with a diverse degree of roles. There exists a whole host of HIF target genes that are transcribed in response to hypoxia that have been implicated in driving tumour progression. The roles of these target genes range from receptors to enzymes to further transcription factors and more (Table 1), which are involved in the enhancement of inflammation, angiogenesis, immune evasion, and the other remaining hallmarks of cancer.

Table 1 Hypoxia induces the transcription of a range of genes that mediate diverse roles in promoting the hallmarks of cancer[178-180]

Function	Gene
Enzymes	<i>MMP1, MMP3, LOX, ADAMST1, ACE</i>
Transcription factors	<i>Twist1, Snail, Slug, β-Catenin, c-Myc, Oct4, NF-κB</i>
Receptors	<i>CXCR4, c-Met, TLR4, Notch</i>
Growth factors	<i>VEGF, TGFα</i>
Transporters	<i>Glut-1, MDR1</i>
Intracellular signalling	<i>Cdc42, Rac1, RhoE</i>
Bioenergetics	<i>LDHA, PGK1, PKM2, GAPDH, GPI, ALDOC</i>

In the setting of normoxia, HIF1- α is regulated by two principal mechanisms; oxygen-dependent pVHL-dependent degradation, and oxygen-dependent non-pVHL-dependent inactivation (Figure 3)[25,27,28]. Hydroxylation by oxygen-dependent prolyl hydroxylase domain enzymes trigger recognition by the E3 ubiquitin ligase, pVHL, ensuring proteasomal degradation. In the non-pVHL dependent pathway, induction of factor inhibiting HIF leads to hydroxylation of an asparagine residue preventing HIF1- α from localizing with the co-activators p300 and CBP, hence disabling transcriptional activation[29].

The contribution of hypoxia to disease progression makes it an attractive therapeutic target and potential prognostic aide. However, in the setting of GOC, there are currently no agents specifically targeting hypoxia, nor are there any biomarkers that assess the extent of tumour hypoxia, to guide treatment choice or to indicate the likelihood of treatment response. In this era of precision medicine, a validated biomarker would improve the standard of care for this group of patients.

HYPOXIA PROMOTES THE HALLMARKS OF CANCER WITHIN THE TME

Inflammation

Cancer has long been described as a “wound that never heals”, in part due to inflammation, one of the enabling characteristics of cancer originally described by Hanahan and Weinberg[30-31]. Hypoxia and inflammation are intricately intertwined as illustrated through the fact that hypoxia has been shown to directly induce signalling *via* the inflammatory master transcription factor nuclear factor-kappa light chain enhancer of activated B cells (NF- κ B), and likewise NF- κ B induces HIFs[32-37]. In the context of malignancy, there exists a multitude of cancer implicated genes that are regulated by both HIFs and NF- κ B, such as cyclooxygenase 2 and interleukin-6 (IL-6)[38]. This illustrates the complex crosstalk between signalling pathways and the difficulty involved in unravelling the net influence of certain factors in the network. In the setting of GOC, OAC has been described as “a model of inflammatory driven upper gastrointestinal cancer”[39,40]. The paramount importance of inflammation in the aetiology of OC is further validated by the risk reduction conferred by administration of the non-steroidal anti-inflammatory drugs such as aspirin, as demonstrated in a meta-analysis of 9 observational studies by Corley *et al*[41] and Farrow *et al*[42]. In a retrospective study of 53 patients with OAC and the metaplastic precursor lesion, Barrett’s oesophagus (BO), immunohistochemical staining of specimens revealed a significant increase in the expression of HIF1- α in OAC and BO compared to normal tissue but no further elevation between BO and OAC[43]. Furthermore, histological assessment of specimens’ inflammatory status, based on recruitment of neutrophils (reflecting acute inflammation) and monocytes (reflecting chronic inflammation) (known as the Sydney System), demonstrated a significant correlation with HIF1- α expression from normal tissue to metaplastic tissue but no association between other stages or between inflammatory status[43].

Angiogenesis

As previously mentioned, one of the defining discoveries involved in the study of the TME was the effect of hypoxia on angiogenesis[44-46]. This was originally demonstrated in HIF1- β deficient hepatoma cells having markedly reduced vascular endothelial growth factor (VEGF) mRNA levels when cultured under hypoxic

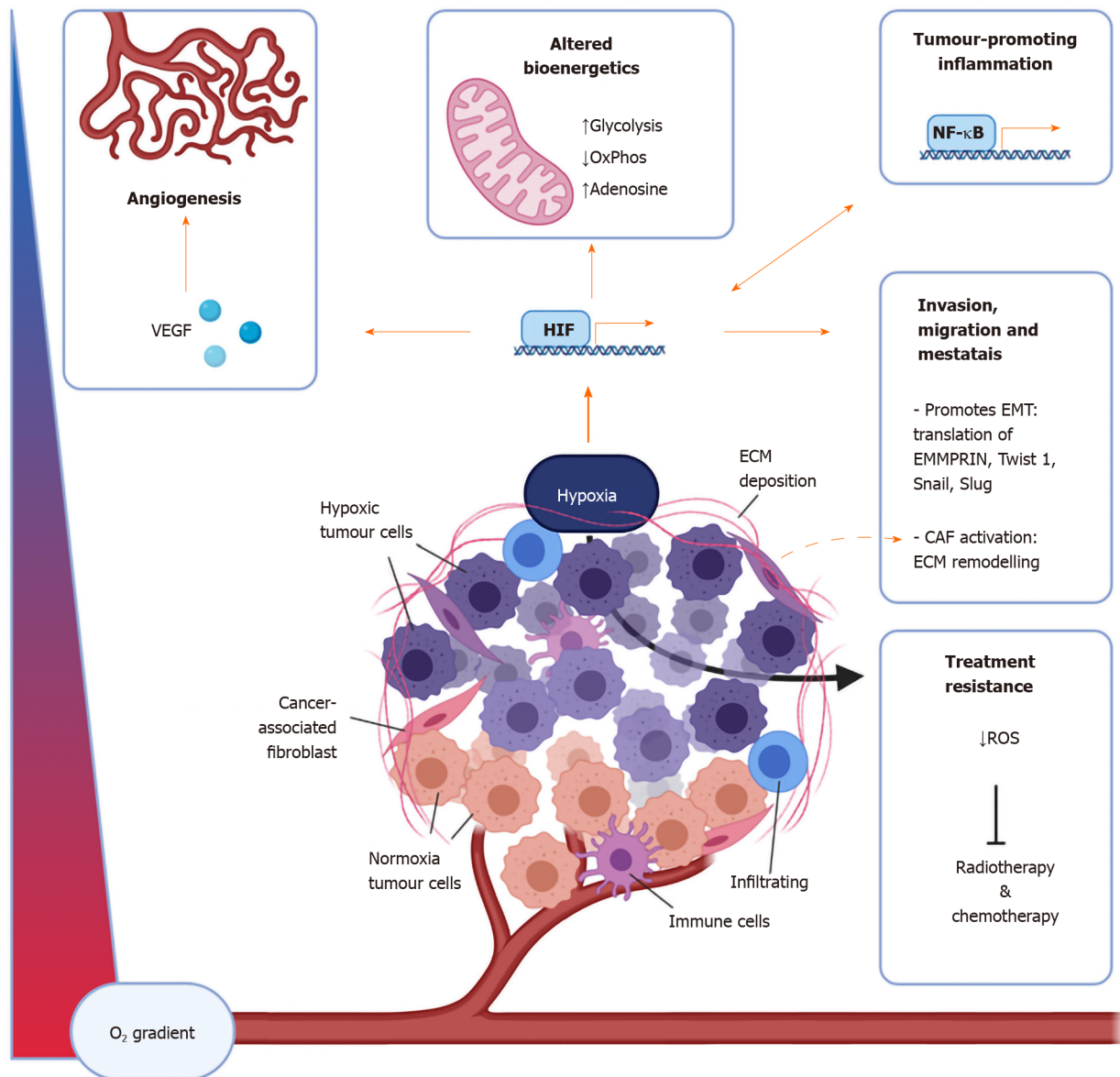


Figure 1 The components of the tumour microenvironment are affected by hypoxia in numerous ways. Important cellular components of the tumour microenvironment include immune cells including macrophages, dendritic cells, myeloid-derived suppressor cells, T cells, natural killer cells, as well as cancer-associated fibroblasts. Non-cellular aspects include the extracellular matrix and signalling molecules such as vascular endothelial growth factor, adenosine, and cytokines and chemokines including interleukin-6, interferon- γ , CXCL1, CXCL3, CCL28[12-14,40]. CAF: Cancer associated fibroblasts; OxPhos: Oxidative phosphorylation; ROS: Reactive oxygen species; VEGF: Vascular endothelial growth factor; NF κ B: Nuclear factor-kappa light chain enhancer of activated B cells; HIF: Hypoxia inducible factor; ECM: Extracellular matrix; EMT: Epithelial-mesenchymal transition.

conditions[24,47]. In the setting of GOC, a study of 92 oesophageal biopsy samples found a significant increase in the expression of HIF1- α in OAC *vs* dysplastic and metaplastic tissues but not between normal and metaplastic tissues[48]. These findings also reflected an increase in VEGF and HIF2- α expression in OAC *vs* dysplastic tissue. Several studies have revealed how hypoxia appears to drive tumour cell plasticity and hence vasculogenic mimicry, a process that allows malignant cells to impersonate endothelial cells and form a network of vessels, and in a sense bypass true angiogenic activity[49-54]. In an *in vitro* analysis of oral squamous cell carcinoma (OSCC) cells, transfection with siRNA targeting HIF1- α was shown to inhibit both vasculogenic mimicry (through three-dimensional culture) and proliferation (as measured by MTT assay)[55]. Validation of these results in a xenograft implant model was then performed; the HIF-1 α knockout mice showed a longer time to tumour formation and had smaller tumours. In an experiment conducted by Chai *et al*[56] of 160 OSCC tumour tissues, both HIF1- α and the degree of vasculogenic mimicry correlated negatively with overall survival (OS). In a separate study, OSCC cell lines cultured under conditions of severe hypoxia (0.5% oxygen) for 5 d secreted exosomes which

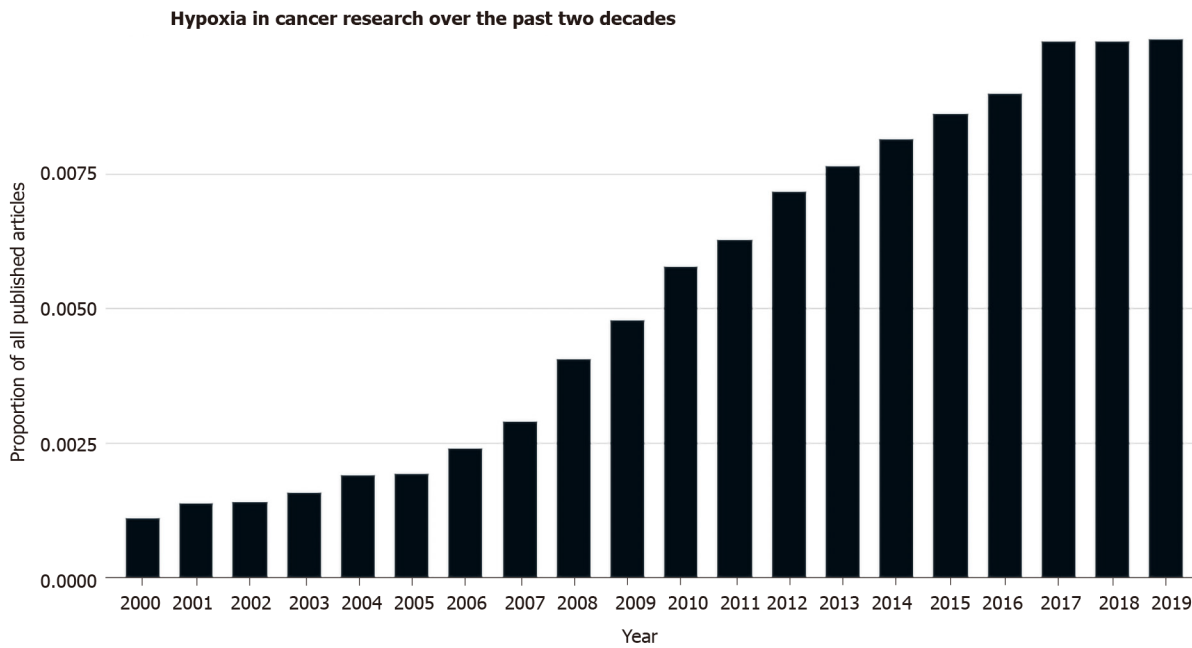


Figure 2 The amount of research investigating the role of hypoxia in cancer has increased over the past 20 yr as seen as a proportion of PubMed listed articles[177].

through tube formation assays, were shown to increase the angiogenic capacity of human umbilical vein endothelial cells when cultured together[57]. Vessel formation was significantly increased compared to umbilical vein endothelial cells cultured with exosomes obtained from OSCC cells exposed to normoxic conditions. When assessed in an *in vivo* implant model, findings reflected those found in the *in vitro* assay. As a consequence of these described phenomena, the blood vessels formed in tumours do not resemble those found in non-malignant tissues. The resulting network is disorganized and highly permeable and this limits the supply of blood and hence oxygen, nutrients, and anti-cancer drugs, further contributing to tumour hypoxia.

Immune evasion

The cancer-immune set point refers to the equilibrium between factors that promote or suppress the anti-cancer immune response[58]. This is of great interest in GOC given the yet unrealized efficacy that was predicted of immune checkpoint inhibitor drugs in treating these cancer types, which are generally characterized as having high tumour mutational burden and evident immune cell infiltration[59]. A hypoxic TME promotes an immunosuppressive phenotype through actions on the diverse array of cellular and non-cellular entities across innate and adaptive immune arms and thus constitutes a vital host factor that may be contributing to a high cancer-immune set point and treatment failure. For example, in the context of cancer, the recruitment of myeloid-derived suppressor cells (MDSCs) is associated with less favourable patient outcomes which are likely mediated by their potent dampening of the anti-tumour immune response[60-62].

MDSCs are defined as “a heterogenous population of cells of myeloid origin that consist of myeloid progenitors, immature macrophages, immature granulocytes, and immature dendritic cells” (DCs)[63,64]. In a murine model of OSCC, intratumoural MDSC percentages were shown to correlate with the tumour progression sequence[65]. The role of IL-6 was then explored in the context of MDSCs and tumour progression. In patients with OSCC compared to healthy controls, serum IL-6 was significantly increased. Also, the percentage of intratumoural MDSCs correlated with general serum IL-6 levels. Delving further into this, the murine model of OSCC was utilized with 3 cohorts; IL-6 knockout, IL-6 stimulation (*via* 100 ng intraperitoneal injection twice weekly for 6 wk), and normal wild type. The cohort receiving IL-6 had a significant 3-fold increase in the percentage of MDSCs compared to the IL-6 deficient cohort (15% to 5% respectively). These findings were analogous when examining tumour invasiveness. As mentioned previously, HIF has been shown to upregulate the transcription of inflammatory factors including IL-6, and overall, the results demonstrate the importance of hypoxia in driving the pro-tumour immunosup-

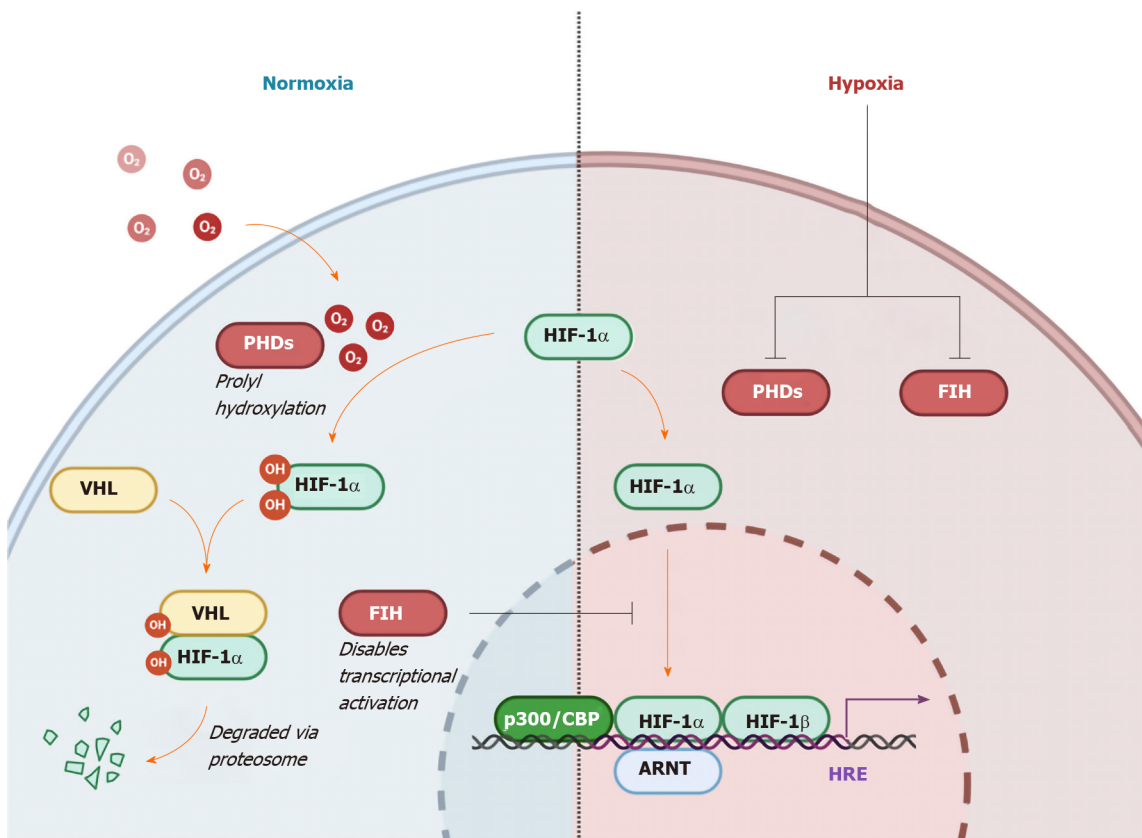


Figure 3 Regulation of hypoxia-inducible factor 1- α by oxygen levels and von Hippel Lindau protein. Hydroxylation by oxygen-dependent prolyl hydroxylase domain enzymes triggers recognition by the E3 ubiquitin ligase von Hippel Lindau, ensuring proteasomal degradation. In the non-von Hippel Lindau protein dependent pathway, induction of Factor Inhibiting hypoxia-inducible factor (FIH) leads to hydroxylation of an asparagine residue preventing HIF-1 α from localizing with the co-activators p300 and CBP, hence disabling transcriptional activation[30]. The HIF pathway functions to conduct and orchestrate the cellular response to low oxygen availability[24,25]. HRE: Hypoxia response element; ARNT: Aryl hydrocarbon receptor nuclear translocator; PHD: Prolyl hydroxylase domain enzymes; VHL: Von Hippel Lindau; HIF1- α : Hypoxia-inducible factor 1- α ; FIH: Factor inhibiting hypoxia-inducible factor.

pressive functions of MDSCs[38,66]. Others have shown the hypoxic TME to drive MDSC differentiation to tumour associated macrophages (TAMs), again in a manner that is orchestrated by HIF1- α [67].

TAMs comprise a large part of the cellular TME and as such are gaining further infamy for their role in driving tumour progression[68,69]. Studies have demonstrated how TAM recruitment and infiltration into the TME is in part mediated by the hypoxic response and HIF-driven regulation of chemoattractant including CCL2, CCL5, and receptors such as CXCR4[70-73] (Figure 4). There is strong evidence that macrophage infiltration and density are associated with worse patient outcomes in the setting of malignancy[74-76]. A meta-analysis of 16 OC cancer studies ($n = 2292$), found that M2-polarised pro-tumour macrophage density to be predictive of worse OS and disease stage[77,78]. In addition, *in vitro* evidence suggests that TAM density is significantly associated with an increase in programmed death-ligand 1 expression on OSCC cells[78]. Once infiltrated into the TME, low oxygen tension enhances the oncogenic role of TAMs *via* the promotion of proliferative and angiogenic growth signalling pathways[79,80]. Notably, while two studies have characterized the correlation between HIF1- α expression, TAM infiltration, and patient survival in the setting of gastric malignancy, the impact of hypoxia on the biology of TAMs could be further expanded in the context of GOC[81,82].

Signifying the potential of innate immune research in cancer, Gilead recently invested \$4.8 billion for ownership of magrolimab[83], a monoclonal antibody that works through the disruption of CD47 which is expressed on cancer cells and acts to downregulate the anti-tumour phagocytic capability of macrophages. Targeting hypoxia-mediated CD47 function may also extend to cancers of the alimentary tract. Immunohistochemical staining and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) of OSSC specimens taken from 14 patients demonstrated a significant increase in expression of CD47 while another preclinical study revealed an augmented response to immune checkpoint inhibition in

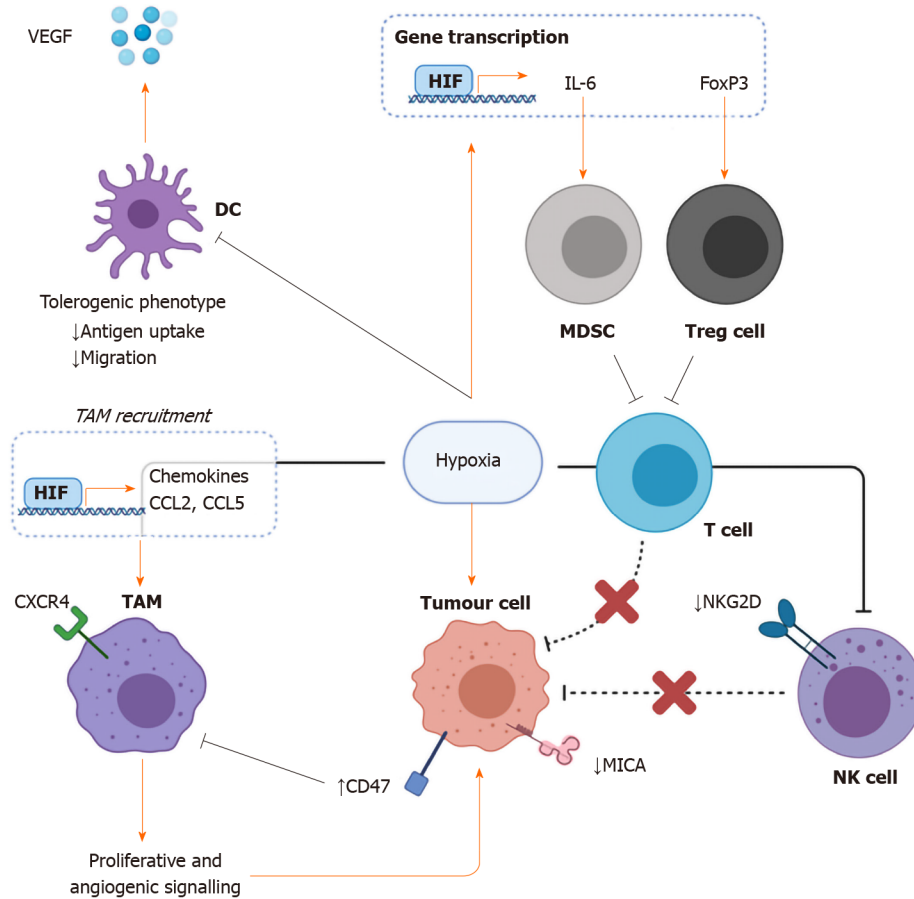


Figure 4 The effects of hypoxia on immune evasion. Hypoxia has been shown to impair antigen uptake and migration in dendritic cells while at the same time increasing vascular endothelial growth factor production thus impairing the bridge between the innate anticancer immune response and the adaptive response while also enhancing angiogenic signalling. Hypoxia-inducible factor-mediated transcription of the cytokine interleukin-6 and FoxP3 results in the subsequent recruitment of immunosuppressive myeloid derived suppressor cells and in increased proportion of protumorigenic Tregs respectively. Low oxygen status is also linked with decreased tumour expression of the natural killer (NK) cell receptor ligand MHC class I chain-related molecule A, as well as its receptor NKG2D on NK cells. Hypoxia-dependent transcription of chemokines such as CCL2 and CCL5 enhance the recruitment of tumour associated macrophages through receptors such as CXCR4. DC: Dendritic cell; MDSC: Myeloid derived suppressor cell; NK cell: Natural killer cell; TAM: Tumour associated macrophage; Treg cell: T regulatory cell; VEGF: Vascular endothelial growth factor; HIF: Hypoxia inducible factor; IL: Interleukin; MICA: MHC class I chain-related molecule A.

combination with CD47 antagonism[84,85]. CD47 expression has also been shown to predict prognosis in OSCC[86].

Natural killer (NK) cells are a type of innate lymphoid cell that are capable of recognizing tumour cells through two principal mechanisms; altered expression of self or missing-self[87,88]. For example, in the absence of cellular stress, MHC class I chain-related molecules (MICA and MICB) are not normally expressed on cells. In one study of prostate cancer cells, culture under hypoxic conditions is shown to result in the shedding of MICA hence characterizing an immune evasive phenotype[89]. Hypoxia also affects both resting and activated NK cells directly by curtailing the expression of costimulatory NKG2D and other NK cell receptors (NKP46, NKP30) which enable NK cell function[90]. Furthermore, a low oxygen environment has revealed impaired NK cell differentiation in one *in vitro* study[91]. The density of infiltrating NK cells has been shown to be prognostic in OSCC[92]. In a study of OSCC xenografts implanted in nude mice, NK cell depletion was shown to restore tumour growth following treatment with an anti-PD-1 (programmed death-1) agent illustrating the important anti-tumour role of NK cells which is tightly regulated by the PD-1 pathway[93]. In human OC, NK cells that demonstrate high expression of a novel inhibitory regulator protein, T cell immunoglobulin domain and mucin domain 3 (Tim-3) are predisposed to apoptosis and hence fail to combat tumour progression[94]. Increased expression of Tim-3 in this context occurs through NF- κ B signalling thus linking hypoxia to NK cell-mediated anti-tumour dysfunction. NK cells are also an important entity in GC. Tumour infiltrating NK cells expressing high levels of Tim-3 have been correlated with adverse prognosis in a study of 62 patients with the disease[95].

DCs present antigens to T cells including CD4⁺ T helper cells, resulting in the initiation of the adaptive anti-tumour immune response[96]. In cancer, impaired DC function is associated with defective anti-tumour immune responses and hence cancer progression[97-99]. While there are contrasting studies, the net effect of the hypoxic TME may be skewed towards a tolerogenic DC phenotype[100,101]. An *in vitro* study of peripheral blood mononuclear cells isolated from a healthy human cohort and cultured under hypoxic conditions (1% oxygen) showed that hypoxia impairs DC uptake of antigens and causes modulation of their cytokine expression patterns in both resting and activated states[100]. Hypoxia increased VEGF production and CXCR4 expression and lead to a reduction in DC production of tumor necrosis factor- α thereby revealing the pro-angiogenic and immunosuppressive effect of reduced oxygen tension on DCs. Lysosomal-associated membrane protein (LAMP3) is a marker of mature DCs and it has been shown to be induced by hypoxia in breast cancer both *in vitro* and *in vivo*[102]. It is thought to be implicated in metastasis[103]. RT-qPCR analysis of 157 OSCC tissues as well as immunohistochemical staining of 46 specimens reveal its expression to be correlated with poor patient outcomes, further emphasizing the tolerogenic capacity of DCs[104]. Again, in the context of OAC, co-culture with DCs has been shown to induce Treg (T regulatory) differentiation supporting the tolerogenic DC phenotype in these malignancies[105]. Given that successful activation of adaptive T cell responses is dependent on DC migration to peripheral lymphoid organs, further research and investigation of the effect of hypoxia in the TME on DCs is required to fully dissect the potential clinical impact regarding patient outcomes and treatment resistance[106].

Hypoxia-induced HIF1- α expression is also associated with the upregulation of the transcription factor Forkhead Box Protein P3 (FoxP3), highlighting the role of hypoxia in regulating the abundance and function of Treg cells, further illustrating the potential immunosuppressive effect of a hypoxic TME on anti-tumour immunity[107,108]. In a study of GC, the frequency of Treg cells was significantly higher in the tumour compared with peripheral circulation wherein, intratumoural levels of FoxP3 correlated with TNM stage[109]. In a complementary study, elevated Treg/CD8⁺ cell ratio was shown to be an independent predictor for worse OS in a study of 133 patients with GC. Tregs are also crucially important in OC; one study found an increased percentage of peripheral Treg cells in OC patients *vs* healthy controls and they further demonstrated that a higher proportion of Tregs was inversely correlated to survival[110]. The administration of an agent that disrupts Treg recruitment to a hypoxic TME may represent a potential therapeutic target capable of improving outcomes[111].

Invasion, migration, and metastasis

The activation of cancer-associated fibroblasts (CAFs) in hypoxic TMEs has been implicated in the altered deposition, remodelling and degradation of the extracellular matrix (ECM) and hence invasion, migration, and metastasis[112-114]. In a study of 183 patients with OAC, characteristic expression of CAF marker α -SMA was found to be correlated with worse OS[115]. It was initially hypothesized that increased collagen production and fibrosis would present an obstacle to tumour cell invasion and metastasis, but evidence suggests that this is a lot more complex. In one study of pancreatic carcinoma cells, collagen has been shown to increase expression of the key epithelial to mesenchymal transition (EMT) transcription factor Snail in a transforming growth factor- β -mediated manner[116]. Thus, this series of events is thought to be involved in the activation of CAFs thereby, ensuring enhanced migratory capacity, invasiveness, survival, and ECM deposition in a positive feedback loop[117,118]. In the area of GOC, an *in vitro* assay revealed extracellular matrix metalloproteinase inducer (EMMPRIN) promoted EMT and hence invasion and migration of an OC cell line[119]. The authors followed up this study by showing, through HIF1- α interference and culture under hypoxic (1% oxygen) conditions, that EMMPRIN was regulated by HIF1- α . Further research probing the relationship between traditionally neglected components of the TME like CAFs and hypoxia in upper gastrointestinal cancers is required.

Altered energetics

Cells deprived of oxygen promote tumour proliferation and survival through reprogramming of energy metabolism[30]. The observation that neoplastic cells shift their metabolism from aerobic to anaerobic respiration was first observed nearly 100 years ago by Otto Warburg[120,121]. This shift is orchestrated by the hypoxia master regulator HIF which upregulates enzymes involved in glycolysis such as pyruvate dehydrogenase kinase 1, and ultimately the production of lactate from pyruv-

ate[122,123]. Immunoblot analysis of both gastric and OSCC specimens has demonstrated reductions in the expression of the β catalytic subunit of a key protein involved in oxidative phosphorylation, ATP synthase, further implicating the role of metabolic reprogramming in upper gastrointestinal malignancies[124]. It is also probable that an altered bioenergetic phenotype contributes to treatment resistance in a hypoxia-driven manner. In one study, the expression of 4 proteins involved in metabolic respiration in the setting of OAC ($n = 23$), were assessed prior to chemoradiation[125]. Increased levels of the oxidative phosphorylation protein ATP5B were significantly increased in those with poor response to chemoradiation as defined per tumour regression grade. This suggests that tumours that retain some sense of metabolic plasticity may predict treatment-refractory disease.

Lactate dehydrogenase is responsible for converting pyruvate to lactate under hypoxic conditions[126]. In a study of 152 patients with GC, immunohistochemical staining for lactate dehydrogenase (LDH) isoenzyme 5 demonstrated significant associations between immunoreactivity and a number of different tumour features such as tumour size, venous and lymphatic invasion, and tumour stage[127]. Inoculation of mice with LDH knock-out pancreatic cancer cells has been shown to result in reduced tumour size[128]. Furthermore, the quantity of MDSCs isolated from the LDH knock-out cancer mice both in tumour and spleen was significantly less in controls, and they demonstrated lower suppressive activity.

The effects of these processes are not restricted to neoplastic cells, as the evidence implicates hypoxia-driven metabolic shifts in other cellular components of the TME, particularly immune cells. Tissue hypoxia in cancerous or non-cancerous cells results in the build-up of the purine adenosine, extracellularly which augments a plethora of the hallmarks of cancer[129-134]. Evidence suggests this is conferred predominantly through the release and metabolism of ATP by the surface membrane nucleotidases CD39 and CD73[133,135,136]. In one *in vitro* experiment, an epithelial cell line demonstrated increased CD73 expression when exposed to hypoxic conditions, and examination of the CD73 gene has identified a binding site for HIF1[137]. Subsequent binding to purinergic receptors and adenosinergic signalling is known to mediate an anti-tumour immunosuppressive phenotype through effects on Tregs, MDSCs, TAMs, and B lymphocytes across various solid tumours types including OC[135,138-141]. In the context of GOC, a gene expression study of several radiotherapy resistant OC cell lines, CD73 expression was shown to be increased in TE-2, TE-13, and KYSE170 when compared to parent cell lines[142]. Once again, given the hypoxia-driven mechanism, this highlights the pro-inflammatory, tumour-promoting effect of the adenosine axis, thereby signifying another potential method of clinically targeting hypoxia pathways in the treatment of GOC.

Also, hypoxia (oxygen of 1.5%) driven reprogramming of energetic metabolism is linked to PD-1 immune checkpoint blockade resistance[143]. *In vivo* treatment with metformin, decreases OCR in tumour cells, while increasing consumption in T cells resulting in reduced hypoxia. The authors further examined the effect of anti-PD-1 agents in concert with metformin administration *in vivo* in a melanoma tumour type that traditionally fails to respond to immune checkpoint blockade. The synergistic effect demonstrated substantially increased tumour elimination[143]. These intricately woven hypoxia-mediated effects exist in concert with one another to contribute to an aggressive phenotype characterized by treatment resistance and poor prognosis.

MEASURING HYPOXIA

Measuring tissue and tumour hypoxia is challenging. There are four principal methods for measuring oxygen levels *in vivo*; the Eppendorf oxygen electrode, exogenous markers, endogenous markers, and imaging techniques. The Eppendorf electrode quickly became the gold standard for measuring oxygen tension when it was introduced at the beginning of the millennium after studies confirmed that low tumour oxygenation status was associated with worse outcomes in cervical as well as head and neck cancer[144,145]. However, it fell out of favour just as quickly for a variety of reasons. It was notably limited to tumours that were accessible and it was steadfastly invasive. It was additionally prone to sampling error[146]. Although hypoxia can be arbitrarily classified as acute/perfusion limited or chronic/diffusion-limited, there remains significant spatiotemporal variation in tumour oxygen tension and hence multiple sampling observations must be taken[147]. The literature on the use of endogenous hypoxia markers in GOC is extensive and is discussed in the context of prognosis and treatment and resistance[148-152]. Exogenous markers such

as pimonidazole are administered to a patient and undergo chemical modification in hypoxic cells and are then amenable to visualization in specimens. A summary of the major methods used to measure tumour hypoxia and their associated advantages and disadvantages can be found in [Table 2](#).

In the last decade, several studies have characterized gene expression signatures corresponding to oxygenation status[153-155]. Using a 15 gene expression panel derived from these studies, Ye *et al*[156] classified 24 cancer types from The Cancer Genome Atlas into a hypoxia score of high, low, and intermediate after adjusting for confounding factors such as sex and ethnicity. They were further able to validate this categorization with independent proteomic data where hypoxic status was known. 135/193 (70%) of GC samples had high hypoxic status while only 34/124 (27%) of OC samples fell into this category. There may be differences between OSCC and OAC but they were grouped together in this study. They further built on these findings by comparing molecular characteristics such as miRNA expression, highly mutated genes, and significant copy number alterations between the hypoxia score high and low tumours. In both OC and GC samples that had molecular signatures of high hypoxic status, a number of miRNAs that target the tumour suppressor gene tumour protein p53 inducible nuclear protein 1 (TP53INP1), were significantly downregulated[156].

TREATMENT RESISTANCE AND PROGNOSIS

Ionizing radiation generates free radicals from molecules of oxygen which then induce double-stranded DNA breaks resulting in mitotic catastrophe. This is one of the key mechanisms for radiation-induced tumour cell death and it is reliant on the presence of oxygen within the TME[144,157]. GC and OC cells cultured *in vitro* under hypoxic conditions (1% oxygen) were more resistant to radiation-induced cell death compared to GC and OC cells cultured under normoxic conditions, as assessed by colony formation assay[158]. The contribution of hypoxia to radiotherapy treatment resistance is relatively well established but its role in conventional chemotherapy and molecularly targeted therapy is less clear cut, particularly in GOC. Functional inactivation of HIF1- α in GC cell lines demonstrated increased susceptibility to 5-fluorouracil and cisplatin as determined by proliferation and apoptosis assays which lends support to the use of HIF1- α in predicting response to therapy[159]. Analysis of cell cycle distribution patterns following treatment with 5-fluorouracil revealed a greater proportion of senescent HIF1- α deficient cells compared with controls. Likewise, the apoptotic cell fraction as determined by caspase 3 cleavage of HIF1- α deficient cells was greatly increased. The mechanism for this is thought to be mediated by HIF1- α dependent suppression of P53 induction in response to 5-fluorouracil[159,160]. Another potential mechanism is suggested by a different study, using RT-PCR and Western blot to demonstrate the HIF1- α dependent upregulation of p-glycoprotein in GC cells incubated at 1% oxygen levels[161]. P-glycoprotein is a transporter protein that augments the efflux of drugs from cells and hence is associated with chemoresistance in GOC[162,163].

There are a large number of studies that have investigated the prognostic value of hypoxia in OC. A systematic review carried out by Peerlings *et al*[152] evaluated 22 studies assessing various hypoxia-related markers and established that increased expression of HIF1- α in early-stage OSCC was associated with increased resistance to chemoradiotherapy treatment. They also conclude that radiologically, the positron emission tomography (PET) marker 18F-FETNIM was significantly predictive for response to combined chemoradiation in the setting of OSCC[164]. In brief, these tracers work by diffusing into cells non-specifically. In the absence of oxygen, they undergo a chemical reaction and their resultant physicochemical properties do not allow diffusion out of the cell[165]. PET with 18F-FAZA (18F-fluoroazomycin arabinoside) has been shown to predict radiotherapy response in OAC murine xenografts[166]. Validation of the tracer 18F-HX4 has been performed in OC but is yet to be studied as a potential prognostic factor[167]. Overall, imaging of hypoxia continues to be an attractive approach for studying the TME and subsequent patient outcomes.

The markers assessed in the systematic review by Peerlings *et al*[152] included HIF1- α , VEGF, carbonic anhydrase IX, GLUT1, Beclin-2, HIF2- α , as well as PET. The most common method used to assess these markers was immunohistochemical staining of surgical or biopsied specimens i.e. an invasive technique. The authors indicate that HIF1- α overexpression was associated with worse outcomes for OS and disease-free

Table 2 Techniques used in the measurement of tissue oxygenation and their associated advantages and disadvantages[10,181-183]

Technique	Advantages	Disadvantages
Needle Electrodes	Instrumental in establishing the link between hypoxia and treatment failure	Prone to sampling error due to poor spatial resolution
	Real time direct measurement	Invasive and requires direct access to tumours
Exogenous Markers	More sensitive than electrodes at lower oxygen levels	Requires biopsy and immunohistochemistry
	Reproducible	
	Precise spatial resolution	
Endogenous Markers	Precise spatial resolution	Requires biopsy and immunohistochemistry
	Can be serological such as Osteopontin	
	Can be tissue based such as HIFs or carbonic anhydrase IX	
Radiological	Non-invasive	Expensive
	Reproducible	Radiation exposure
	Precise spatial resolution	Relatively less well established

HIF: Hypoxia inducible factor.

survival in OSCC but the evidence for its association in OAC was inconclusive, mainly due to the absence of data. VEGF expression correlated with patient outcomes in OSCC but not OAC[152]. In contrast, carbonic anhydrase IX appears to be an independent predictor of survival in OAC. Carbonic anhydrase IX is a glycoprotein expressed on the cell surface and its primary function is the catalytic conversion of carbon dioxide to bicarbonate and protons[150,168]. Under the transcriptional control of HIF1- α , the metalloenzyme is thought to contribute to tumour growth and proliferation through the regulation of pH, ECM degradation, and EMT[168,169]. In the majority of studies assessing endogenous markers, the determination of what constituted "hypoxic" was based on relatively arbitrary thresholds of immunohistochemical expression, with very little in the way of standardized protocols across studies. For example, Munipalle *et al*[151] defined "high" HIF-1 α expression as greater than 10% of OSCC cells showing positive staining. Birner *et al*[170] devised a score based on intensity and percentage of cells showing positive expression in a cohort of 333 OCs. Anything above the median was then considered a "high" expression while those below were considered a "low" expression.

In a more recent systematic review and meta-analysis, Luo *et al*[148] examined the clinical predictive value of HIF2- α . It included 40 studies with 4345 cancer cases but only 2 of these studies assessed upper gastrointestinal cancers. Of these 2 studies, 1 was solely GC ($n = 127$), while the other was both GC and OC ($n = 177$)[149,171]. Based on the Newcastle Ottawa score, the authors determined that both of these papers were of high quality. Both of these studies demonstrated a statistically significant association between HIF2- α and OS on univariate analysis but not multivariate. In the pooled analysis, the authors conclude that high HIF2- α expression was associated with a lower OS.

While there is a non-insignificant aggregate of clinical evidence denoting a statistically significant association between endogenous markers of tumour oxygenation and clinical outcomes, the heterogeneity in study methods and contrasting results ultimately indicates a need for more prospective research with greater adherence to the standardization of reporting. The REMARK recommendations for tumour marker prognostic studies published by the Equator Network lay out a checklist for researchers to improve both quality and transparency in research[172]. The wealth of data as discussed above, demonstrating the correlation between outcomes or treatment resistance and tumour hypoxia further illustrates the importance of the development and clinical implementation of new techniques in measuring tumour hypoxia such as non-invasive imaging[148,149,151,170,171].

HYPOXIA-TARGETED THERAPIES

Hypoxic areas of the TME inherently suffer from poor perfusion and disorganized vasculature and this has been one of the primary limitations to systemically administered therapeutics[173]. Nevertheless, a number of agents have been tested in clinical studies. Hypoxia-targeted therapies mainly consist of bioreductive prodrugs (hypoxia-activated prodrugs) but molecularly targeted agents that inhibit effectors in hypoxia-responsive pathways such as HIF1- α target genes or receptor tyrosine kinases like the VEGF receptor could be grouped here as well[173].

Bioreductive agents such as tirapazamine work in a similar manner to exogenous markers of hypoxia; they undergo chemical modification in hypoxic cells resulting in hypoxia-selective cytotoxicity. The bioreductive alkylating agent apaziquone demonstrated efficacy as a first-line agent in early clinical studies of bladder cancer but in a phase II study in 20 patients with GC, there was no clinical benefit[174,175]. In a preclinical murine model of OSCC and OAC, administration of the bioreductive prodrug evofosfamide was shown to delay tumour growth in combination with radiotherapy *vs* radiotherapy alone[176]. This came with the added benefit of no additional toxicity. As of the time of writing, there have been no clinical trials investigating the potential use of evofosfamide or other bioreductive prodrugs in OC and although the efficacy of these agents has largely been disappointing as first-line treatment in other cancer types, they may potentially improve sensitivity when used in combination with conventional chemoradiation.

CONCLUSION

The myriad of components that comprise the TME and the effects imposed on them by oxygen deprivation ensures that researchers have yet to scratch the surface in disentangling the key processes amenable to overcoming treatment-refractory disease and prognostication. Hypoxia plays a role in promoting immunosuppressive cells and subverting anti-tumour immune responses within the TME. Hypoxia also promotes the additional hallmarks of cancer including inflammation, angiogenesis, and reprogramming of metabolism. The intricate nature of these hypoxia-mediated effects is very complex and further research is required to elucidate the mechanisms as they pertain to GOC. Standardization of methodology in hypoxia focused basic research and clinical reporting would be conducive to driving this area forward. This deeper understanding will hopefully reveal novel therapeutic targets to control disease progression in GOC but currently, this remains out of reach. However, hypoxia as a clinical marker to stratify patients into certain treatment pathways or aid prognosis is something that is firmly within our grasp.

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