Recent advances in the biology of tumour hypoxia with relevance to diagnostic practice and tissue-based research

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Abstract

In this review article, we examine the importance of low levels of oxygen (hypoxia) in cancer biology. We provide a brief description of how mammalian cells sense oxygen. The hypoxia-inducible factor (HIF) pathway is currently the best characterised oxygen-sensing system, but recent work has revealed that mammals also use an oxygen-sensing system found in plants to regulate the abundance of some proteins and peptides with an amino-terminal cysteine residue. We discuss how the HIF pathway is affected during the growth of solid tumours, which develop in microenvironments with gradients of oxygen availability. We then introduce the concept of ‘pseudohypoxia’, a state of constitutive, oxygen-independent HIF system activation that occurs due to oncogenic stimulation in a number of specific tumour types that are of immediate relevance to diagnostic histopathologists. We provide an overview of the different methods of quantifying tumour hypoxia, emphasising the importance of pre-analytic factors in interpreting the results of tissue-based studies. Finally, we review recent approaches to targeting hypoxia/HIF system activation for therapeutic benefit, the application of which may require knowledge of which hypoxia signalling components are being utilised by a given tumour.

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Introduction

In this review, we discuss the importance of hypoxia in tumour biology and provide a brief description of how mammalian cells sense oxygen via the hypoxia-inducible factor (HIF) pathway and a newly described mechanism in which hypoxia is sensed by cysteamine (2-aminoethanethiol) dioxygenase. We also introduce the concept of ‘pseudohypoxia’, in which the HIF system is constitutively activated in a number of specific tumour types that are of immediate relevance to diagnostic histopathologists. We provide an overview of the different methods to quantify tumour hypoxia, emphasising the importance of pre-analytic factors in interpreting the results of tissue-based studies. The understanding of these technical issues will become increasingly relevant if knowledge of which hypoxia signalling components are being utilised by a given tumour becomes important in determining treatment choices. Finally, we review recent approaches to targeting hypoxia/HIF system activation for therapeutic benefit.

Oxygen sensing and the hypoxia-inducible factor (HIF) system

About 2.5 billion years ago, the evolution of photosynthesis in cyanobacteria liberated oxygen into Earth’s atmosphere [1]. Under standard temperature and pressure, two atoms of oxygen combine to form molecular oxygen (i.e. O₂ or ‘dioxygen’), a colourless and odourless gas. Increases in atmospheric oxygen coincided with the development of multicellular organisms (Metazoa) [2], which depend upon oxygen for a range of physiological processes, most notably energy production. Although oxygen is essential to generate cellular energy, it is toxic at high concentrations [3]. Thus, oxygen homeostasis must match supply and demand across...
distinct cell types that inhabit environments with varying oxygen levels [4]. In higher animals, the respiratory, cardiovascular, and haematopoietic systems are responsible for oxygen transport. Physiological control occurs through acute regulation of ventilation by peripheral and central chemoreceptors, interwoven with slower adaptive responses, including transcriptional effects mediated through the HIF system [5,6]. Pivotal discoveries pertaining to this pathway have recently been recognised by the award of the Nobel Prize in Physiology or Medicine 2019 [7].

HIF functions as a heterodimer of an oxygen-regulated α subunit (either HIF-1α, HIF-2α, or HIF-3α) and a β subunit (HIF-β), which is widely expressed in an oxygen-independent manner. HIF-α subunits are hydroxylated in an oxygen-dependent manner by the prolyl hydroxylase domain-containing enzymes (PHD1, 2, and 3), facilitating recognition by the von Hippel-Lindau ubiquitin E3 ligase (pVHL) and subsequent proteasomal degradation (Figure 1). The PHDs belong to the 2-oxoglutarate (2-OG)–dependent dioxygenase family, which in humans contains about 70 enzymes that target different substrates. These enzymes require dioxygen, the tricarboxylic acid (TCA) cycle intermediate 2-OG, and ferrous iron (Fe²⁺) as reaction co-factors. Other members of the 2-OG-dependent dioxygenase family include collagen prolyl hydroxylases and epigenetic modifiers such as the Jumonji domain-containing family of histone demethylases and the ten-eleven translocation (TET) DNA 5-methylcytosine hydroxylases. In hypoxia, reduced oxygen availability inhibits the enzymatic activity of PHDs, leading to HIF-α stabilisation, heterodimerisation with HIF-β, co-activator recruitment (itself regulated in an oxygen-dependent manner by the asparaginyl hydroxylase, factor inhibiting HIF [FIH], another member of the dioxygenase family) and transcription of many hundreds of genes. Overall, this entrains a gene-expression profile that facilitates cellular adaptation to hypoxia, for example, by switching cellular metabolism from oxidative phosphorylation to glycolysis (to reduce oxygen demand) and by promoting erythropoiesis and angiogenesis (to restore tissue oxygenation), but also causes responses less intuitively related to oxygenation [8]. Like most physiological systems, activation of the HIF pathway is regulated by a number of feedback loops that shape its overall output [9]. In humans, the HIF-1α-PHD2-VHL axis is ubiquitously expressed and most closely represents ‘archaic’ components of the pathway, whereas HIF-2α, HIF-3α, PHD1, and PHD3 are ‘modern’ genes, derived via gene duplication events, that demonstrate tissue-restricted expression and have evolved to perform specific functions. Reflecting this paradigm, HIF-1α and HIF-2α modulate expression of both overlapping and distinct target genes [10].

The effects of HIF pathway activation are dependent upon changes to transcriptional output and, consequently, represent medium- to long-term adaptations to hypoxia occurring over hours, days, and weeks. Other less well-defined, or still to be discovered, oxygen-sensing systems must exist that mediate acute responses over seconds to minutes. Intriguingly, a mammalian homologue of a plant oxygen sensor has recently been identified as cysteamine (2-aminoethanethiol) dioxygenase (ADO) [11]. This enzyme alters target protein stability directly through post-translational modification and, therefore, is likely to transduce more rapid responses to hypoxia than those mediated by the HIF system (Figure 2).

Hypoxia and cancer

Dysregulated oxygen sensing is heavily implicated in the pathophysiology of a wide range of diseases, including cancer [12]. Hypoxia, defined as failure of oxygenation at the tissue level, results when oxygen supply is inadequate to meet demand and is an important pathological feature of solid tumours [13] (Figure 3). The existence of hypoxic regions within solid tumours, manifest histologically as necrosis, has long been recognised [14]. The advent of oxygen-sensitive needle electrodes facilitated direct measurement of tumour oxygen levels, which are reduced when compared to equivalent non-malignant tissues, with some areas being virtually anoxic [15–19]. Inevitably, the development of tumour hypoxia depends upon the interaction of a number of complex factors including the blood’s oxygen-carrying capacity (cancer patients are often anaemic [20]), the integrity and function of tumour blood vessels, and the metabolic demands of tumour and stromal cells. Of particular importance, tumour-associated angiogenesis often results in the formation of vasculature that is both structurally and functionally abnormal [21]. Two subtypes of tumour hypoxia have been proposed. Acute (perfusion-limited) hypoxia develops due to a sudden reduction in blood flow [22], such as upon transient vessel occlusion. In contrast, chronic (diffusion-limited) hypoxia develops due to the abnormally large diffusion distances between centrally located tumour cells and their nearest blood vessel [14]. Nevertheless, the exact distinction between acute and chronic tumour hypoxia remains ill-defined and may be too simplistic [23,24].

The presence of hypoxia within solid human tumours is a poor prognostic factor, irrespective of tumour type [25,26]. Hypoxia may cause a more aggressive malignant phenotype through a number of mechanisms including promoting genetic instability [27], resistance to apoptosis [28], angiogenesis [29], invasion [30], and metastasis [31]. Furthermore, hypoxia is proposed to promote resistance to all major types of cancer therapy by a variety of mechanisms. Surgery is less likely to be curative due to an increased tendency to local invasion and distant metastasis [32,33]. Hypoxia reduces the sensitivity of tumour cells to radiotherapy, which depends upon the generation of reactive oxygen species to cause DNA damage [34]. Chemotherapy may be less effective due to reduced responsiveness of hypoxic tumour cells to cytotoxins [35]. Likewise, the perturbed perfusion of solid tumours affects the delivery of other anti-cancer
drugs, such as small molecule inhibitors, to hypoxic cells [36]. Finally, the hypoxic tumour microenvironment is generally considered to be immunosuppressive and likely reduces the efficacy of immunotherapy [37].

Tumours associated with constitutive HIF system activation (‘pseudohypoxia’)

Although patchy hypoxia is a feature of most solid tumours, a number of specific tumour types are associated with constitutive, oxygen-independent HIF activation, a state termed ‘pseudohypoxia’.

Clear cell renal cell carcinoma (CCRCC)

CCRCC, the most common form of kidney cancer, occurs in both inherited (familial) and the more common sporadic (non-familial) forms. In both forms, this tumour type conforms to Knudson’s ‘two-hit’ hypothesis of cancer development [38]. Familial cases arise in individuals with an inherited germline VHL mutation and subsequent somatic inactivation of the remaining wild-type
cytokine IL-32. However, the
cance of this pathway remains to be con-
stabilized. By acting directly on protein substrates rather than
limited, ADO activity is inhibited and target proteins are rapidly
degraded via the pro-
hydrolysis of Arg-Arg bonds by ADO, the enzyme arginyltransferase 1 (ATE1) and degraded via the pro-

inflammation of the endothelium [39,40]. Loss of
functional pVHL blocks proteosomal degradation of its
targets, leading to stabilisation of HIF-α [41,42]. Subse-
quent activation of HIF-dependent pathways leads to
increased expression of a range of target genes including
VEGF (vascular endothelial growth factor) [43], which
contributes to a distinctive angiogenic phenotype, and
CA9 (carbonic anhydrase nine; CAIX) [44], which is
a useful diagnostic immunohistochemical marker of
CCRCC [45] (Figure 4B). The characteristic clear cell
morphology relates to HIF-mediated cytoplasmic accu-
mulation of glycogen [46–48] and lipid [49,50]
(Figure 4A). In addition, significant overlap is observed
between human RCC-susceptibility polymorphisms and
HIF binding sites [51]. Functional characterisation
of some of these loci has demonstrated roles for HIF in
influencing expression of CCND1 (cyclin D1) [52],
MYC [53], and BHLHE41 (basic helix–loop–helix fam-
ily, member e41) [51]. Of interest, VHL syndrome comprises
several distinct phenotypic subtypes depending
upon the causative mutation. Genotype–phenotype stud-
ies have suggested a correlation between specific VHL
mutations, their quantitative effects on HIF stabilization,
and the spectrum of tumours that develop [54,55]. They
report that mutations that cause complete loss of pVHL
function appear to be required for CCRCC development
but are incompatible with the development of phaeo-
chromocytoma (which is associated with partially inac-
tivating missense mutations that lead to more modest HIF
pathway activation). This work merits further consider-
ation now that the contrasting effects of HIF-1α and
HIF-2α in CCRCC have become apparent (see below).

Although VHL loss is a truncal (early and ubiquitous)
event in CCRCC [56,57], evidence suggests that its inac-
tivation alone, although necessary, is insufficient to
drive CCRCC tumorigenesis. Histological studies of
nephrectomy specimens from VHL syndrome patients
demonstrate that VHL loss, as manifest by positive
immunohistochemical staining for either HIF-α or
CAIX, occurs even in morphologically normal tubular
cells (these lesions were not apparent in sporadic
CCRCC nephrectomy specimens) [58] (Figure 4C–E).
Whilst this event was associated with the induction of
angiogenesis (Figure 4F), cellular proliferation, and
reduced apoptosis, HIF stabilisation was also apparent
in benign cystic lesions that had not progressed to malig-
nancy (Figure 4G–I). Therefore, only a minority of
abnormal single cells appear to progress to dysplastic
multicellular lesions and, presumably, even fewer to
cancer (tumorigenic expansion likely requires additional
[epi]genetic events). This is in keeping with the observa-
tions that acute loss of VHL leads to cellular senescence
(a response predicted to restrict cancer development)
[59] and that mice with conditional Vhl inactivation
do not develop overtly malignant lesions [60]. Furthermore,
tumour genome sequencing has identified other genes
that are recurrently mutated in CCRCC including
PBRM1 (polybromo-1) (41%), BAP1 (BRCA1 associ-
ated protein-1) (15%), SETD2 (SET domain containing
2) (12%), and JARID1C (lysine-specific demethylase
5C [KDM5C]) (14%) [61,62]. Of interest, these genes
are all function as epigenetic regulators and PBRM1,
SETD2, and BAP1 are also located on chromosome 3p
and are frequently co-deleted with VHL upon loss of this
chromosome arm (3p loss is observed in ~90% sporadic
CCRCC [62]). It is possible that, when combined with
VHL loss, disruption to these genes creates an epigenetic
context that favours malignant transformation as
opposed to cellular senescence. There is now evidence
to suggest that mutations in some of these genes
may influence CCRCC behaviour and consequently
patient prognosis. For example, PBRM1 loss has been

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associated with an improved response to immunotherapy [63]. Thus, in the future, it may be necessary to test for such mutations in order to determine optimal patient management.

In addition to chromosome 3p loss, several other chromosomal anomalies have been described in CCRCC including amplification of chromosome 5q (~65–70% cases and associated with a good prognosis [64]) and loss of 14q (~45% cases and associated with a poor prognosis [62,65,66]). It has been demonstrated that chromothripsis (shattering of entire chromosomes or chromosomal regions) can simultaneously generate 3p loss and 5q gain and, in the setting of sporadic CCRCC, this often occurs in only a few hundred cells in childhood or adolescence and thus precedes silencing of the remaining VHL allele (by mutation in 60–70% cases or promoter hypermethylation in 5–10% cases [61,62,67]) and tumour emergence by several decades [68]. Chromosome 5q amplification leads to overexpression of the SQSTM1 oncogene; this has been suggested to increase tumour growth via promoting resistance to redox stress [69], although why this associates with a good prognosis is unclear. It is important to note that HIF1A is located on chromosome 14q and, paradoxically, might act as a tumour-suppressor gene. Indeed, in CCRCC xenograft experiments, HIF-1α overexpression inhibits, whereas HIF-2α overexpression promotes, tumour growth [70]. Supporting this hypothesis, a small

Figure 3. Gradients of oxygen availability exist within solid tumours. Gradients of oxygen availability are common in solid tumours and arise through a complex interaction of factors including the blood’s oxygen carrying capacity, the integrity and function of tumour blood vessels, and the metabolic demands of tumour and stromal cells. In general, perivascular regions are the best oxygenated areas within tumours. Endogenous hypoxia markers are activated via the HIF system at an oxygen partial pressure of approximately 20 mm Hg (reported to arise at about 80 μm from the nearest blood vessel), whereas exogenous hypoxia markers, such as pimonidazole, label more profoundly hypoxic regions (approximately 10 mm Hg/100 μm from the nearest blood vessel). Necrosis (N) occurs within tumour regions that are virtually anoxic (approximately 180 μm from the nearest blood vessel). From left to right, the histological images illustrate immunohistochemical labelling of blood vessel endothelium with CD31 (top left), nuclear accumulation of HIF-1α (arrows), and the hypoxic adducts formed by pimonidazole and an area of necrosis (top right). It is important to note that this schematic representation is rather simplistic and fails to account for acute changes in tumour perfusion, such as occur upon blood vessel blockage or reversal of blood flow.
number of CCRCC harbour HIF1A inactivating mutations [61,71]. The mechanisms underpinning the differential effects of the HIF-α isoforms on tumour growth remain to be fully elucidated but might relate to HIF-2α being more resistant to inhibition by FIH [72,73] or differences in downstream target genes [10,74,75].

Whilst VHL and chromosome 3p loss are clonal events in CCRCC (i.e. present in all neoplastic cells), intratumoural heterogeneity of mutational burden is well recognised and there is significant spatial variation with regard to other mutations, with up to 75% of all somatic mutations not detectable across every tumour region sampled (i.e. these are sub-clonal events that are present in only a fraction of neoplastic cells) [56,57,76]. Up to 30 driver mutations have been identified per tumour and the extent of sub-clonal diversification correlates with clinical prognosis: ‘low diversity’ tumours with fewer subclones tend to be aggressive whereas highly branched tumours with more than 10 sub-clonal drivers progress less rapidly [77]. This intratumor genetic heterogeneity, a result of Darwinian selection and competition between sub-clones, suggests that a single biopsy may not be representative of the tumour as a whole and presents challenges to the development of biomarkers and targeted therapeutics. A minority of CCRCCs demonstrate wild-type VHL [78] and a subset of these have

Figure 4. Histological features of clear cell renal cell carcinoma (CCRC) and clear cell papillary renal cell carcinoma (CCPRCC). (A) The characteristic clear cell morphology on haematoxylin and eosin (H&E) staining of CCRCC is caused by hypoxia-inducible factor (HIF)-mediated cytoplasmic accumulation of glycogen and lipid. (B) Ubiquitous expression of carbonic anhydrase IX (CAIX) by tumour cells is a useful diagnostic immunohistochemical marker of CCRCC. (C) CAIX is not expressed by non-neoplastic tubular epithelial cells in sporadic cases of CCRCC. (D,E) By contrast, histological studies of nephrectomy specimens from VHL syndrome patients demonstrate that VHL loss, as manifest by positive immunohistochemical staining for CAIX and glucose transporter 1 (GLUT1) in morphologically normal tubular cells. (F) Dual staining for CAIX (red) and the endothelial marker CD31 (brown) demonstrates that angiogenesis develops as an early event after VHL loss/HIF stabilisation. Despite this, the majority of these lesions do not progress to overt malignancy. (G–I) Similar features are observed in benign cystic lesions arising in the kidneys of VHL patients. (J) Histologically, CCPRCC are composed of tumour cells with clear cytoplasm arranged in either a papillary or tubulopapillary architectural pattern. (K) CCPRCC demonstrates a distinctive ‘cup-like’ pattern of CAIX expression, with staining present on the basolateral cell membrane.
mutations in TCEB1 (transcription elongation factor B polypeptide 1), the gene encoding the E3 ubiquitin ligase component elongin C, that also stabilise HIF [67]. As with many of these newly described and rare renal tumours, the long-term prognosis is not fully established, although one case series reports that no patients developed metastases after a median follow-up of 48 months [79]. Further work will be required to characterise the molecular aberrations underpinning CCRCC that harbour neither VHL nor TCEB1 mutations and the clinical significance of these novel tumour subtypes. This is particularly important in the context of entry to clinical trials and eligibility for targeted therapies.

Clear cell papillary renal cell carcinoma (CCPRCC)
CCPRCC is a low-grade renal neoplasm, composed of tumour cells with clear cytoplasm arranged in either a papillary or tubulopapillary architectural pattern [80] (Figure 4J), which is considered to have a favourable prognosis [81]. These tumours express HIF-1α, CAIX, and glucose transporter 1 (GLUT1) but lack VHL loss and chromosome 3p deletion (anomalies associated with CCRCC) as well as trisomies of chromosomes 7 and 17 (anomalies associated with papillary RCC [PRCC]) [82]. On immunohistochemical staining, they are said to show a characteristic ‘cup-like’ membranous pattern of CAIX expression (Figure 4K). Due to its relative rarity (only 4% of all RCC in one study [83]), the exact mechanisms underpinning HIF stabilisation in CCPRCC remain to be elucidated. However, it has been reported that despite harbouring largely wild-type nuclear DNA, mitochondrial DNA in CCPRCC is severely depleted, resulting in oxidative stress that might inhibit PHD activity, potentially explaining the observed upregulation of hypoxic responses [84].

Oncometabolites
Mutations in the TCA cycle enzymes fumarate hydratase (FH), succinate dehydrogenase (SDH) and isocitrate dehydrogenase (IDH) generate ‘oncometabolites’, by-products of mitochondrial metabolism that both act as oncogenic signalling molecules and cause constitutive HIF pathway activation (Figure 5).

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC)
HLRCC, inherited in an autosomal dominant manner, is characterised by the development of cutaneous and uterine benign smooth muscle tumours (leiomyomata), predisposition to RCC (which, in contrast to other inherited RCC, are often solitary but highly aggressive) [85], and adrenocortical adenoma [86]. Germline mutations in FH, which encodes the enzyme that catalyses the hydration of fumarate into malate, are responsible for this syndrome [87]. These inactivating mutations lead to loss of FH protein in tumour cells, which can be detected by immunohistochemistry [88] (Figure 6D). However, immunodetectable FH is maintained in some HLRCC tumours due to missense mutations that produce stable but non-functional protein [89]. Alternatively, in a process known as succination, elevated fumarate modifies cysteine residues in proteins to form S-(2-succino)-cysteine (2SC) [90]. The detection of 2SC can be used as a highly sensitive biomarker for FH mutation [91] (Figure 6E,F) but, at present, this test is not in widespread clinical use. A patient with an FH-deficient tumour should be offered genetic testing and counselling to ascertain whether they have the HLRCC syndrome, and affected individuals should undergo regular imaging-based screening for RCC to facilitate early surgery (the lifetime renal cancer risk for FH mutation carriers is estimated to be 15%) [92]. FH-deficient RCC has a broad spectrum of histological appearances including multiple architectural patterns within a single tumour (papillary, tubular, cystic, and/or solid) (Figure 6A–C), and the tumour cells often have a prominent (‘inclusion-like’) eosinophilic nucleolus and a perinuclear halo [93]. Without a high index of suspicion and appropriate use of FH immunohistochemistry, these tumours may be misclassified as type 2 PRCC. It is notable that not all patients with FH-deficient RCC have HLRCC; in one study only 35% of such tumours were associated with this syndrome [88].

Loss of functional FH protein leads to cellular accumulation of fumarate, a 2-OG analogue that inhibits PHD function and thus stabilises HIF [94,95]. However, studies in knockout mice have demonstrated that renal cyst development in Fh1-deficient animals occurs independently of the HIF/PHD pathway and is instead driven by abnormal antioxidant responses [96,97]. Consequently, survival of FH-deficient cells depends upon induction of stress-response genes including HMOX1 (haem oxygenase 1) [98]. It is important to note that genetic and pharmacological inhibition of HMOX1 is synthetically lethal, providing a potential future treatment option for FH-deficient RCC/HLRCC patients.

Succinate dehydrogenase (SDH)–deficient renal cell carcinoma and hereditary paraganglioma-phaeochromocytoma syndrome (HPGL–PCC)
SDH, a tetrameric enzyme complex composed of subunits encoded by the SDHA, SDHB, SDHC, and SDHD genes, converts succinate to fumarate and contributes to both the TCA cycle and the electron transport chain. Patients with germline mutations in SDHB, SDHC, or SDHD are at increased risk of developing HPGL–PCC, RCC, gastrointestinal stromal tumour (GIST), and pituitary adenoma [99]; germline mutations in SDHA have also recently been implicated in RCC tumorigenesis [100]. In a manner analogous to FH deficiency, loss of functional SDH leads to cellular accumulation of succinate, a 2-OG analogue that can inhibit PHD function and thus stabilises HIF [94,101]. SDH-deficient RCCs are rare (0.05–0.2% of all RCCs), with SDHB mutations being most common [102]. Histologically, they are characterised by tumour cells with voluminous eosinophilic cytoplasm (in keeping with mitochondrial
accumulation) and at least focally evident intracytoplasmic inclusions (representing giant mitochondria) (Figure 6G). Thus, the differential diagnosis of SDH-deficient RCC includes oncocytoma and chromophobe RCC. Lack of immunohistochemical staining for SDHB (Figure 6H) correlates with the presence of mutations in any of the four SDH genes because disrupted SDH complex stability occurs after mutation of any one subunit; the affected gene should then be confirmed by germline mutation testing [103]. Clinical course appears to mirror histological features: low grade tumours behave indolently, whereas the presence of high nuclear grade, necrosis, and/or sarcomatoid change correlates with more aggressive behaviour [102].

Isocitrate dehydrogenase (IDH)
IDH converts isocitrate to 2-OG and exists in three distinct isoforms (IDH1, IDH2, and IDH3). IDH2 and IDH3 are mitochondrial TCA cycle enzymes, whereas IDH1 contributes to cytoplasmic biosynthetic pathways. Somatic mutations in IDH1 and IDH2 have been identified in central nervous system tumours, acute myeloid leukaemia (AML), cartilaginous tumours, and cholangiocarcinoma; mutations in IDH3 have not yet been implicated in tumorigenesis [104]. Heterozygous IDH mutations lead to the production of catalytically inactive heterodimers [105] and consequent accumulation of isocitrate and reduction in 2-OG inhibit dioxygenases, contributing to tumour development. Inhibition of PHDs leads to constitutive HIF activation in an oxygen-independent manner, a state termed ‘pseudohypoxia’. In addition, cancer-associated neomorphic mutations in IDH1 and IDH2 lead to the aberrant production of the D enantiomer of 2-hydoxyglutarate (D-2-HG), which acts as an allosteric inhibitor of some dioxygenases by competing with 2-OG for binding. Under hypoxic conditions, lactate dehydrogenase A (LDHA), cytosolic malate dehydrogenase 1 (MDH1), and mitochondrial malate dehydrogenase 2 (MDH2) convert 2-OG to the L enantiomer of 2-hydoxyglutarate (L-2-HG), which can also inhibit dioxygenases.
imunohistochemical staining with mutation-specific antibodies [115] (Figure 6I,J). On the basis of promising phase I clinical trials, two inhibitors of mutant IDH have been licensed for use in AML and are currently in clinical trials of other tumour types [116].

Phaeochromocytoma (PCC) and paraganglioma (PGL) PCC is a rare catecholamine-producing neuroendocrine tumour of adrenal medulla chromaffin cells, whereas PGL (‘extra-adrenal PCC’) arises in autonomic nervous system ganglia located in the head and neck, thorax, and abdomen. It has long been known that humans exposed to chronic hypoxia through living at altitude demonstrate hyperplasia of the carotid body (the body’s main acute oxygen-sensing organ) [117] and have an increased incidence of carotid body PGL [118]. The HIF system exerts significant influence on the development and function of the sympathoadrenal system [119,120] and its dysregulation, both in the setting of VHL syndrome and SDH mutation, is associated with the development of PCC and PGL. These tumours are highly heritable, with at least 30% harbouring germline mutations [121]; in addition to SDHB (9%), VHL (4%), and SDHD (2%), germline mutations are also frequent in RET (6%, as part of multiple endocrine neoplasia type 2) and NF1 (3%, as part of neurofibromatosis type 1) [122]. Somatic mutations in NF1, RET, VHL, and SDHB are also observed in sporadic tumours, as are mutations in common cancer genes including HRAS, BRAF, and TP53 [122]. In a small number of cases, mutations in other HIF-related genes have been reported including PHD2 [122,123], PHD1 [124], HIF2A [122,125,126], FH [127,128], and IDH1 [122]. In the case of HIF2A, mutations greatly disrupt its ability to bind to pVHL, leading to protein stabilisation [129]. The exact mechanisms linking hypoxic signalling and PCC/PGL remain to be confirmed. However, the HIF-2α-PHD2 couple is critical in controlling the carotid body–mediated ventilatory response to hypoxia in mice [130–133], and selective HIF-2α stabilisation by deletion of Phd2 within oxygen-sensing type I cells leads to the formation of enlarged, PGL-like carotid bodies [134].

HIF system activation in cancer—causative or consequential?

A critical question is ‘does HIF system activation primarily drive the malignant phenotype or is it a secondary phenomenon occurring within, and shaping the subsequent behaviour of, aggressive tumours that have already outgrown their blood supply?’ Early experiments assessing in vivo growth of murine tumours showed that HIF-deficient tumours grew more slowly and were less well vascularised than wild-type counterparts [135,136], demonstrating that an intact HIF response contributes to tumour angiogenesis and growth. Furthermore, the finding that RCC-susceptibility loci preferentially affect HIF-binding
regions [51], and that such overlap is not observed among other cancers in which HIF is instead upregulated by microenvironmental hypoxia [137], strongly suggests that precise modulation of particular outputs of the HIF pathway is specifically crucial in the development of RCC and not other common tumours. However, care must be exercised when interpreting the role of HIF in pseudohypoxic human tumours. In addition to HIF-α, pVHL has been suggested to target a range of other cancer-related substrates for proteasomal degradation [138–140] (by comparison, rigorous testing suggests PHDs act only on HIF-α [141]). Thus, tumorigenesis in CCRC is likely multi-factorial and not solely dependent on HIF stabilisation. A similar caveat applies to FH, SDH, and IDH mutated cancers because their associated oncometabolites inhibit many 2-OHG-dependent dioygenases above and beyond PHDs. Epigenetic aberrations due to inhibition of TETs [111,142,143] and histone demethylases [112,143,144] and abnormalities of collagen maturation due to inhibition of collagen prolyl and lysyl hydroxylases [145] almost certainly contribute to malignant transformation. In other words, HIF stabilisation in pseudohypoxic tumours does not necessarily imply causality, as evidenced by the development of renal cysts in mice defective for both Fhl alone and Fh1 and Hif1α in combination [97]. It is notable that activating mutations of HIF-α and inactivating mutations of PHDs have not been commonly observed in large scale cancer genome sequencing programmes (with the exception of a minority of cases of PCC/PGL). Thus, genetic evidence does not support a simple, or general, causal effect of HIF system activation leading to tumour development. Instead, activation of this physiological pathway in cancer, by either microenvironmental or oncogenic factors, stimulates numerous downstream effectors, some of which are pro-tumorigenic, others anti-tumorigenic, and yet others neutral (the ‘co-selection’ hypothesis proposed by Ratcliffe [146]). The net output of these signals is likely to be highly context specific and to depend upon the exact cellular and microenvironmental circumstances under which HIF activation occurs [137,147], which may explain, at least in part, the exquisite tissue specificity of tumorigenesis in VHL syndrome and other inherited tumour syndromes characterised by HIF stabilisation.

Quantification of tumour hypoxia

The establishment of robust approaches for tumour hypoxia quantification, which is not routinely evaluated in clinical practice, would facilitate improved patient stratification for treatment with hypoxia-modifying therapies [148]. Histological analysis of tumour samples provides unparalleled ability to profile the highly heterogeneous spatial distribution of hypoxia within the regions of tissue sampled [149–152]. Unfortunately, it is not possible to predict the extent of tumour hypoxia purely on the basis of routine histological features such as necrosis and vascularity [149]. This limitation can be overcome through immunohistochemical detection of hypoxia markers, which are either endogenous (requiring the administration of a drug) or exogenous (proteins involved in the cellular response to hypoxia), although great care is required to avoid pre-analytic artefacts.

Exogenous hypoxia markers

Immunohistochemical staining for pimonidazole or EF5 is widely considered the ‘gold standard’ approach to the histological identification of tumour hypoxia. These compounds are 2-nitroimidazole derivatives that selectively undergo reduction when oxygen levels are profoundly low (below 10 mm Hg [153,154]), producing a highly reactive intermediate species that binds irreversibly to intracellular thiol-containing molecules [155]. These adducts can be identified subsequently by immunohistochemistry [156,157] (Figure 7D–F) and are commonly expressed at a distance of more than 100 μm from the nearest blood vessel [158]. An advantage of this approach is that if these drugs are administered long enough before specimen retrieval (16–24 h before in human studies and 1–2 h before in murine studies) the free drug will have been cleared by the time of surgery/biopsy, preventing any artefactual adduct formation as a result of the procedure. However, the requirement to administer these drugs, either intravenously or orally, prior to tissue collection means that although they are in widespread use in pre-clinical studies, only limited cohorts of labelled human tumours exist. Therefore, a more commonly used approach is to detect endogenous hypoxia markers such as HIF-α, CAIX, and GLUT1.

Endogenous hypoxia markers

HIF-α

As the master transcription factor coordinating the cellular response to hypoxia, HIF-α proteins are themselves endogenous hypoxia markers [159]. Both HIF-1α (Figure 7A) and HIF-2α can be detected in human tumours by immunohistochemistry [160] but HIF-α is extraordinarily labile upon re-oxygenation and immunostaining is technically demanding, necessitating careful validation of protocols [161,162]. In addition, HIF-α can be stabilised in an oxygen-independent manner in cancer [163], whereas its expression is frequently lost in highly hypoxic peri-necrotic tumour cells due to nutrient depletion [164]; thus, care must be taken when interpreting patterns of HIF-α expression. Finally, HIF-α antigenicity is recognised to deteriorate with increasing age of the paraffin block [165], which has direct relevance for studies conducted on archival human material. Given these complexities, more stable endogenous hypoxia markers may be easier to detect.

CAIX

CAIX is a hypoxia-inducible transmembrane protein that converts carbon dioxide to carbonic acid, contributing to...
an acidic extracellular pH [166]. Expression of CAIX increases below oxygen tensions of approximately 20 mmHg [167], with the median distance between areas of CAIX expression and the nearest blood vessel being 80 μm [168]. CAIX is expressed in the hypoxic regions of many (Figure 7B), but not all (Figure 7G–I), human carcinomas but not in most non-neoplastic tissues [169], providing good ‘signal-to-noise’ upon immunostaining. However, expression may be modulated by factors other than hypoxia, such as extracellular pH, glucose availability, and oncogenic signalling pathways [170], so this cannot be relied on in isolation.

GLUT1

GLUT1 facilitates transportation of glucose into cells across their plasma membranes; it is upregulated via HIF-1α due to the increased reliance of hypoxic cells upon anaerobic glycolysis [171]. However, because GLUT1 is basally expressed even in normoxia (including by red blood cells), its histological expression is diffuse and less well-demarcated than that of CAIX [172] (Figure 7C).

Other proteins that have been assessed as potential endogenous hypoxia markers include VEGF [173,174], erythropoietin (EPO) [175,176], and osteopontin (OPN) [177,178]. However, these secreted proteins are not particularly suited for localisation of hypoxia, although their mRNAs may be (e.g. through utilisation of chromogenic RNA in-hybridisation techniques [179]).

Of note, exogenous and endogenous hypoxia markers frequently display regions of both overlapping and non-overlapping expression within the same specimen [180,181]. The extent to which this observation is attributable to methodological variations (such as specimen fixation, staining technique, and so on) remains unclear. However, when selecting for a marker of hypoxia, it is important to consider biological differences between markers including hypoxia severity (exogenous markers label radiobiological hypoxia [<10 mm Hg], whereas...
endogenous markers depend upon HIF system activation ([<20 mmHg]) and duration (HIF-α is stabilised rapidly after the onset of hypoxia and has a short half-life, whereas downstream target genes are induced more slowly and have longer half-lives), as well as potential regulation by non-hypoxic factors. Thus, it is good practice to confirm the presence of tissue hypoxia by staining for at least two, and ideally more, different markers. Another limitation of histological markers of hypoxia is a tendency for researchers to dichotomise tumour regions as being either ‘hypoxic’ or ‘non-hypoxic’. It is more appropriate to consider staining patterns in the context of the oxygen gradients that exist from the best oxygenated perivascular tumour regions (~30 mmHg [182]) to virtually anoxic necrotic areas (Figure 3). Of interest, the analysis of multiple endogenous hypoxia markers (with differing half-lives) in the same specimen, which will be facilitated by the recent development of robust multiplex platforms [183], can generate oxygen supply maps to represent these gradients [181]. With choice of appropriate markers, this approach may also facilitate distinction between pre-existing tissue oxygenation and effects occurring during specimen handling and obviate the need for exogenous markers in future studies. Li and colleagues have outlined protocols for pimonidazole and HIF-α immunohistochemistry [184].

Non-histological approaches

A range of non-histological approaches to detect tumour hypoxia exist including direct measurement with oxygen-sensitive electrodes [185], quantification of circulating hypoxia-inducible proteins such as VEGF [186] and OPN [187], cross-sectional imaging with positron electron tomography (PET) or magnetic resonance imaging (MRI) [188], and analysis of tumour gene expression data to identify molecular signatures of hypoxia [189].

Therapeutic targeting of tumour hypoxia and the HIF system

The importance of oxygen in influencing response to treatment has fostered interest in developing therapies that modify tumour hypoxia. The predominant approaches to modulating the hypoxic tumour microenvironment have been to either increase oxygen supply and/or decrease oxygen consumption [190], to sensitise hypoxic tumour cells to radiotherapy effects [191], or to selectively eliminate hypoxic tumour cells by the administration of hypoxia-activated cytotoxic prodrugs [192]. Several studies have reported small but consistent improvements in outcome with such approaches, particularly in the setting of squamous cell carcinoma of the head and neck [193]. It is exciting that data from pre-clinical studies suggest that improved tumour oxygenation might also promote anti-tumour immunity [194]. Despite this evidence, no such treatment has become firmly established in routine clinical practice.

Given the critical role of the HIF system in disease pathophysiology, it is an attractive pharmacological target across a range of medical conditions if effects can be appropriately targeted. Stabilisation of HIF-α, leading to erythropoiesis and improved iron metabolism, is an emerging therapeutic strategy for the treatment of anaemia of chronic kidney disease. Recent phase III randomised clinical trials of roxadustat, an orally administered 2-OG analogue that reversibly inhibits PHDs, have demonstrated superiority to placebo in increasing haemoglobin among patients who are not undergoing dialysis [195] and non-inferiority to recombinant EPO in patients receiving dialysis [196]. Due to their pleiotropic effects, PHD inhibitors may also have benefits in cardiovascular and metabolic disease [197]. Theoretically, long-term widespread PHD inhibition might promote the development of tumours, particularly PGL. Although mature clinical studies are lacking, one pre-clinical study has reported effective erythropoiesis without promotion of tumour progression in a VEGF-sensitive murine breast cancer model [198].

Conversely, a number of different approaches to HIF inhibition in cancer have been tested in pre-clinical models and early phase clinical trials, including those that block HIF-α synthesis, stabilisation, DNA binding, and transcriptional effects [199]. However, such approaches may be too simplistic due to the different effects of HIF-1α and HIF-2α in individual tumour types and risk entraining unacceptable off-target effects due to the complexities of manipulating a ‘hard-wired’ physiological system. Given the importance of elevated HIF-2α signalling in CCRCC, specifically abrogating the effects of this isoform is a particularly attractive therapeutic strategy. It has been shown that use of short hairpin RNAs targeting this isoform is effective at suppressing VHL-defective tumour growth in mice [200]. Traditionally, transcription factors have been considered to be ‘undruggable’ [201]. Thus, current therapeutic strategies in CCRCC focus on blocking downstream effectors of HIF signalling, such as with VEGF-neutralising monoclonal antibodies [202] or VEGF receptor antagonists [203–205]. The discovery that HIF-2α contains a large (290 Å) internal cavity in its PAS-B domain that accommodates ligand binding [206], in a manner that antagonises its ability to heterodimerise with HIF-1α [207], has suggested that small molecules could specifically inhibit this isoform (HIF-1α does not possess this cavity) (Figure 8). Indeed, proof of efficacy of two selective HIF-2α inhibitors (PT2385 and PT2399) in pre-clinical in vitro and in vivo models soon followed [208–210]. A recent phase I trial of PT2385 in humans with previously treated, advanced CCRCC reported evidence of anti-tumour activity in 14% patients with a favourable safety profile, although the predictable side-effect of anaemia occurred in 45% patients [211]. Of interest, dyspnoea and hypoaemia are also recognised side-effects and presumably relate to inhibition of HIF-2α signalling in the carotid body [212]. These drugs may also be effective in treating other tumours in which HIF-2α is implicated in tumorigenesis, including RCCs caused by mutations in FH, SDH, and TCEB1 and PGL/PCC [213,214]. Furthermore, HIF-2α is implicated...
in maintenance of glioblastoma multiforme (GBM) stem cells [215] and its inhibition has been proposed to be a credible therapeutic approach in this tumour type [216]. However, disappointingly, a planned interim analysis of a phase II clinical trial in patients with recurrent GBM reported acceptable safety but minimal anti-tumour activity [217]. These findings suggest that targeting this pathway is only likely to be of clinical benefit in tumours in which HIF activation is a bona fide driver, rather than a co-selected, event. It is notable that the development of protocols for more robust HIF-1/2α immunostaining may facilitate patient selection for this new form of therapy.

Conclusions and future perspectives

Although the HIF system has evolved for physiological purposes, its regulation is perturbed across a broad range of diseases including cancer. Evaluation of a large body of experimental and clinical data suggests that, although not likely to be directly causative of cancer by themselves, hypoxia and HIF activation do contribute to a more aggressive tumour phenotype. Nevertheless, the exact effects of HIF activation in cancer are complex and both context and HIF-α isoform specific. Of immediate relevance to diagnostic pathology, oncogenic activation of HIF occurs in a group of ‘pseudohypoxic’ tumours with distinct morphological, immunohistochemical, and/or genetic characteristics. A number of different techniques are available in the research setting for quantification of tumour hypoxia and activation of the HIF pathway, but the most clinically meaningful strategies remain unclear. Rapid changes in oxygen levels and the short half-life of HIF proteins when oxygen is available mean that pre-analytic factors may influence results; caution is therefore required in the interpretation of results from tissues obtained through ‘routine’ diagnostic pathways. New pharmacological...
approaches to the manipulation of tumour hypoxia and HIF activity have emerged recently and are displaying efficacy in early phase clinical trials in CCRCC. It will be of much clinical interest to discover if these results are reproduced in larger studies, whether the same drugs are effective in the treatment of other tumour types or even other diseases and, given the plasticity of both tumours and oxygen-sensing pathways, for how long their effects are maintained. The targeted application of these drugs will require knowledge of which hypoxia signalling components are being utilised by a given tumour; accurate determination of this will require special precautions to be taken in the handling of diagnostic tissues.

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Author contributions statement

PSM drafted the manuscript and figures. AY drafted figures and revised the manuscript. MH provided histological images for figures and revised the manuscript. MBP provided histological images for figures and revised the manuscript. JA provided histological images for figures and revised the manuscript. CWP revised the manuscript and figures. All authors approved the final version for publication.

Abbreviations

2-HG, 2-hydroxyglutarate; 2-OG, 2-oxoglutarate (also referred to as a-ketoglutarate); 2SC, S-(2-succino)-cysteine; ADO, adenosine; ADH, alcohol dehydrogenase; ATR, adenosine triphosphate; CAIX, carbonic anhydrase; CCPRCC, clear cell papillary renal cell carcinoma; CCRCC, clear cell renal cell carcinoma; EPO, erythropoietin; FH, fumarate hydratase; HIF, hypoxia-inducible factor; HLRCC, hereditary leiomyomatosis and renal cell carcinoma; HPGL-PCC, hereditary paraganglioma-phaeochromocytoma syndrome; HRE, hypoxia response element; IDH, isocitrate dehydrogenase; MRE, magnetic resonance imaging; OPN, osteopontin; PCC, pheochromocytoma; PET, positron electron tomography; PGL, paraganglioma; PHD, prolyl hydroxylase domain-containing enzyme; PRCC, papillary RCC; RCC, renal cell carcinoma; SDH, succinate dehydrogenase; TCA, tricarboxylic acid; TET, ten-eleven translocation enzyme; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau

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Recent advances in tumour hypoxia

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