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Targeting tumour hypoxia in breast cancer

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ABSTRACT

Breast cancer is the most common malignancy in women. Hypoxia occurs in breast cancer and in other solid tumours due to the tumour outgrowing the existing vasculature. Hypoxia leads to an adaptive response, orchestrated by HIF-1 (hypoxia-inducible factor-1), that is crucial for tumour progression and therapy resistance responsible for poor patient outcome. In several studies, downstream targets of HIF-1α were considered as hypoxia markers. The biological heterogeneity of breast cancer has been investigated through genome profiling technologies. The recent data suggest that treatment outcome depends on individual genetic features and that the hypoxia signature is a significant prognostic factor. The identification of molecular biomarkers with the potential to predict treatment outcome is essential for selecting patients to receive the most beneficial therapy, and in the future may drive stratification in clinical trials.

1. Introduction

Breast cancer is the most commonly occurring malignancy in women, and is responsible for approximately 500,000 deaths per year worldwide. In the recent years, the encouraging trend towards earlier detection and the increasing use of systemic adjuvant treatment have improved the survival rates, but still nearly half of the breast cancer patients treated for localised disease develop metastases.[1]

Hypoxia is the result of an imbalance between oxygen delivery and oxygen consumption resulting in the reduction of oxygen tension below the normal level for a specific tissue.[2] Using Eppendorf histography electrodes, oxygen tensions were measured in several cancer types showing a range of values between 0 and 20 mmHg in the tumour tissues, which were significantly lower than those of the adjacent tissue (24–66 mmHg).[3–5] Oxygen tensions measured in breast cancers of stages T1b-T4 revealed a median p_{O_2} of 28 mmHg compared with 65 mmHg in normal breast tissue.[6]

Hypoxia occurs in many disease processes, and it is widespread in solid tumours due to the tumour outgrowing the existing vasculature. This may result in the death of cancer cells if it is severe and prolonged. *In vivo* two different conditions have been recognised. Chronic or diffusion-limited hypoxia is due to a concentration gradient of diffusion, about 150–200 μM, due to the metabolism of oxygen as it diffuses further away from capillaries and will also be related to the metabolic activity of the tumour. Acute hypoxia is a transient perfusion-limited state, which occurs when an aberrant blood vessel is temporarily shut off, so that the cells adjacent to the capillaries die because of the insufficient blood supply. Intermittent hypoxia occurs when blood vessels are reopened and the hypoxic tissue is reperfused with oxygenated blood, leading to an increase in the levels of reactive oxygen species and resulting in the tissue damage as a result of hypoxia-reoxygenation injury.[7] The recent findings suggest that intermittent hypoxia might protect endothelial cells through a stronger stabilisation of hypoxia-inducible factor-1 (HIF-1) compared with chronic hypoxia.[7]

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In addition to mild hypoxia (0.01–2% O_2), some tumours contain regions of severe hypoxia (<0.01% O_2) called anoxia. This is a functionally different state to hypoxia and leads to coordinated cytoprotective programmes known as the unfolded protein response and integrated stress response, which are critical for tumour survival.[8]

In hypoxic conditions, numerous cellular mechanisms are compromised and an adaptative response occurs which allows cancer cells to adapt to this hostile environment. This renders them more resistant and ability to survive and even proliferate, promoting tumour development.[9]

2. The adaptive response to hypoxia

The cellular response to hypoxia is modulated by the ubiquitous family of transcription factors known as hypoxia-inducible factors consisting of αβ-heterodimers, which include HIF-1α, HIF-2α, HIF-3α and HIF-1α. The HIF-1α subunit is the most ubiquitously expressed and acts as the master regulator of oxygen homeostasis in many types of cells (see Fig. 1). In the presence of oxygen, the von Hippel-Lindau tumour suppressor (pVHL), which is the recognition component of an E3 ubiquitin ligase complex, targets HIF-1α protein which is degraded within minutes by the ubiquitin-proteasome pathway. The interaction of pVHL and HIF-1α requires the hydroxylation of two proline residues, at positions 402 and 564 catalysed by prolyl-hydroxylases. Three prolyl-hydroxylase domain (PHD) enzymes, known as PHD1, PHD2 and PHD3, were identified in mammalian cells and were shown to hydroxylate HIF-1α although at varying levels of activity. In hypoxia, the proline residues are not hydroxylated and thus HIF-1α is stabilised and translocated to the nucleus where, with the recruitment of a number of cofactors including p300, it is dimerised with HIF-1α. The HIF-1 heterodimer targets hypoxia-responsive elements containing genes encoding essential pathways in systemic, local and intracellular homeostasis, providing the essential compensatory mechanism to increase the delivery of oxygen and nutrients while removing the waste products of metabolism.[7,9–12]

Hydroxylase activity is iron and ascorbate dependent. The recent studies found that physiological concentrations of ascorbate (25 μM) strongly suppress HIF-1α protein levels and HIF transcriptional target. Similar results were observed with iron supplementation.[13]

The factor inhibiting HIF-1 (FIH-1) is another dioxygenase, which hydroxylates a conserved asparagine residue Asn803 within the C-terminal transactivation domain (TAD) under normoxic condition, acting synergistically with the PHD system to block the transcriptional activity of HIF-1α. Recently, it was shown that the cytoplasmic location of FIH-1 in invasive breast cancer is associated with an enhanced hypoxic response and a worse prognosis.[14]

Two different expression patterns of immunohistochemical staining for HIF-1α have been described in primary tumour

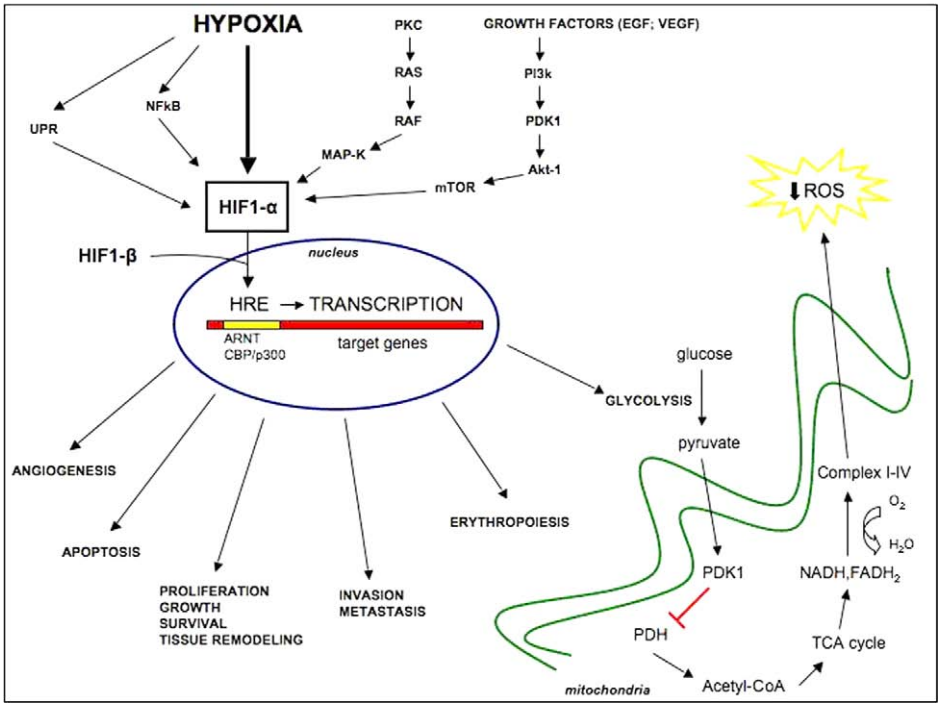

Fig. 1 – Hypoxia upregulates various transcription factors. HIF (hypoxia-inducible factor) plays a key role inducing transcription of genes involved in tumour progression, angiogenesis, erythropoiesis, metabolism, apoptosis and tissue remodelling. By stimulating glycolytic enzymes, HIF promotes glycolysis and prevents the accumulation of ROS (reactive oxygen species) through the induction of PDK1 (pyruvate dehydrogenase kinase 1), which inhibits PDH (pyruvate dehydrogenase) and blocks conversion of pyruvate to Acetyl-CoA, decreasing TCA (tricarboxylic acid) cycle activity. ARNT, aryl hydrocarbon receptor nuclear translocator; HRE, hypoxia-responsive element; PIK3, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; UPR, unfolded protein response.

samples. One depends on the distance from blood vessels associated with a decreased oxygen concentration. The other expression pattern is diffuse throughout the entire tumour, indicating that HIF-1α can be triggered by factors other than hypoxia.[15] Growth factors (e.g. IGF2, TGFα, IGF1R and EGFR), cytokines and other signalling molecules stimulate HIF-1α synthesis via activation of the phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathways in a cell-type-specific manner. PI3K mediates its effects through its target AKT and the downstream kinase mTOR (mammalian target of rapamycin which is inhibited by rapamycin, a macrolid antibiotic), which have a regulating role in protein synthesis. Stimulation of the human breast cancer cell line MCF-7 with heregulin activates the human epidermal growth factor receptor 2 (HER)/Neu receptor tyrosine kinase, and results in an increased HIF-1α protein synthesis, dependent upon activity of PI3K, AKT and mTOR. Oncogenes (e.g. v-Scr and H-Ras) induce constitutive expression of HIF-1α. The signalling pathway mediated by wingless-type (Wnt) proteins is implicated at several stages of mammary gland growth and differentiation, and the recent evidences suggest a role in breast carcinogenesis.[16] Wnt/βcatenin pathway is involved in the epithelial-mesenchymal transition (EMT), a crucial process in tumour development, increasing tumour cells proliferation, migration and invasion.[17,18] Although the process has not been well elucidated, the possibility that HIF-1 induces tumour cells to undergo EMT has been demonstrated in colon cancer[19] and prostate cancer,[20] and the recent data indicate that the Wnt/βcatenin signalling pathway may be critical in the signal of HIF-1α for inducing prostate cancer cell to undergo EMT.[21] Genetic abnormalities observed frequently in human cancers, including loss-of-function mutations (e.g. VHL, p53 and PTEN), are also associated with increased expression of HIF-1α and HIF-1 inducible genes.[22–24]

In microenvironments, where oxygen is scarce and glucose consumption is high, a metabolic shift from oxidative to glycolytic metabolism occurs. The important role of the family of glucose transporters (GLUT-1 and GLUT-3 being hypoxia-inducible) has been extensively investigated in breast cancer cell lines and surgical specimens.[25] However, while HIF-1 stimulates glycolysis, it also actively downregulates mitochondrial function and oxygen consumption by inducing pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates pyruvate dehydrogenase (PDH), the mitochondrial enzyme that converts pyruvate into acetyl-CoA. HIF-1 also induces the expression of genes encoding lactate dehydrogenase A (LDHA), which converts pyruvate into lactate, and cytochrome c oxidase subunit COX4-2, which replaces COX4-1 and increases the efficiency of mitochondrial respiration under hypoxia. These events result in a drop in mitochondrial oxygen consumption and reduced free radical generation, thereby decreasing cell death in response to hypoxia.[26–28]

A well-defined link between the upregulation of HIF-1 in hypoxia and the maintenance of pH balance is a group of genes that encode for transmembrane carbonic anhydrases (CAs). CAs have been described in a variety of tumour types, including breast cancer, where its expression increases with increasing distance from blood vessels and decreasing oxygen concentration, and is extreme in perinecrotic areas (see Table 1).[29–31]

Hypoxia also plays a crucial role in modulation of tumour angiogenesis that is required for tumour growth and metastasis.[32,33] The most characterised HIF-regulated gene is vascular endothelial growth factor (VEGF), which is involved in regulating endothelial cell proliferation and blood vessel formation in both normal and cancer cells.[34] Other than VEGF (or VEGF-A), the predominant factor that influences angiogenesis, its family includes VEGF-C, D, E and placental growth factor (PLGF). Alternative splicing of VEGF-A forms four isoforms including $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$.[35] However, the recent studies suggested a HIF-1-independent mechanism that regulates pro-angiogenic activity of VEGF by showing induction of tumour angiogenesis before the activation of HIF-1.[36]

Activation of nuclear factor-kB (NF-KB) under hypoxia was identified, which may enhance its role in oncogenic signalling pathways, apoptosis and cell adhesion. A role of NF-kB in TNFα-mediated HIF-1 accumulation by hypoxia-independent mechanisms was described.[37] The recent studies have further suggested an important link between hypoxia and the notch-signalling pathway, a cell–cell communication mechanism closely associated with cell differentiation.[38]

Besides the fact that hypoxia affects general processes such as glycolysis, apoptosis and proliferation, the recent data linked hypoxia to a dedifferentiated phenotype. Helczynska et al. have used a model system of ductal carcinoma *in situ* (DCIS) to investigate the presence of various markers in relation to the hypoxic region surrounding the central necrotic areas. They found that, in parallel with HIF-1α expression, there was a decline in the oestrogen receptor α (ERα) protein content as well as an increase in cytokeratin 19 expression, suggesting that hypoxia affects processes intimately involved in cellular differentiation. These data are supported in studies conducted with breast cancer cell lines grown in hypoxia.[39,40]

From a clinical point of view, hypoxia is a potential therapeutic problem as the adaptative changes in response to hypoxia lead towards treatment resistance to both radio- and chemotherapy. An additional physical effect of hypoxia, which was recognised 50 years before HIF was discovered, relates to oxygen free radicals. It has been recognised for many years that the oxygenation status of a tumour is an important factor affecting the cytotoxicity of radiation, and it has become well established that cells in oxygen-deficient areas may cause solid tumours to become radioresistant. This phenomenon is known as 'hypoxic radioresistance', and is the result of a lack of oxygen in the radiochemical process by which ionising radiation is known to interact with cells. The phenomenon is most clearly seen after large single doses of radiation, but also exists in normal fractionated radiotherapy.[41] Hypoxia also directly induces resistance of solid tumours to chemotherapy by reducing the generation of free radicals by agents such as bleomycin and doxorubicin, and by the inhibition of cell cycle progression and proliferation, since a number of drugs specifically target highly proliferating cells.[42,43] The oxygen level is an important factor in the action of many antineoplastic agents, several of which have been classified *in vitro* and *in vivo* by their selective cytotoxicity towards

Table 1 – Genes regulated by HIF-1.

Iron metabolism: ceruloplasmin, transferrin, transferrin receptor
Erythropoietin: erythropoietin
pH regulation: carbonic anhydrase-9 and -12
Apoptosis: BNIP3, BNIP3L, RTP801
Angiogenesis: adrenomedullin, angiopoietin-2, plasminogen activator inhibitor-1, transforming growth factor-α, transforming growth factor-β3, vascular endothelial growth factor
Cell proliferation and survival: cyclin G2, insulin-like growth factor-2, insulin-like growth factor-binding protein-1, -2, -3, nitric oxide synthase-2, P21, WAF1
Vascular tone: α_{1B}-adrenergic receptor, endothelin-1, haeme oxygenase-1, nitric oxide synthase-2
Collagen metabolism: aldolase-A and C, hexokinase-1 and 2, glucose transporter-1 and 3, glyceraldehyde-3-Pdehydrogenase, lactate dehydrogenase-A, phosphofructokinase-L, phosphoglycerate kinase-1, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3, pyruvate kinase-M, triosephosphate isomerase
Regulation of HIF-1 activity: p35srj

Abbreviations: BNIP3, Bcl2 and adenovirus E19 19 KDa interacting protein 3; HIF, hypoxia-inducible factor.

oxygenated and hypoxic tumour cells in animal models (see Table 2).

3. Hypoxia: prognostic and predictive marker in breast cancer

Various methods have been developed to measure tumour hypoxia directly or indirectly, including imaging by blood oxygen level-dependent magnetic resonance (BOLD MRI), hypoxia-activated scanning agents (e.g. nitroimidazoles, fluoromisonidazole) and immunohistochemical analysis for hypoxia-induced genes. Currently, the Eppendorf polarographic oxygen electrode is the rarely used method considered the 'gold standard', but it correlates poorly with other markers.[4,44] However, all these techniques have limitations due to their invasiveness or necessity for pre-injection of a non-approved agent (e.g. pimonidazole), or lack of approved imaging agents.[7,45]

In other types of cancers, this technique has generated many correlations between hypoxia and cancer treatment and outcome.[46] For this reason, efforts have been encouraged to non-invasively detect and localise regions of poor oxygenation in tumours. The recent studies suggested that hypoxia-regulated genes could be used alternatively as endogenous hypoxia markers, which are strongly related to aggressive disease and poor prognosis.[47] Although HIF-1α expression may also be influenced by other pathways, a significant correlation between oxygen tension and HIF-1α has been reported in cervical cancer, suggesting that HIF-1α might be used as a surrogate for tumour hypoxia.[47] By using HIF-1α as a marker for hypoxia, approximately 25–40% of all invasive breast cancer samples are hypoxic; the frequency of HIF-1α-positive cells increases in parallel with increasing pathologic stage and is associated with a poor prognosis. HIF-1α expression is associated with reduced survival in a variety of human cancers, and may also influence resistance to therapy in several cancer types. In a recent work, Generali et al. showed that in the human breast cancer HIF-1α expression is also a predictive marker of chemotherapy failure, with a significant inverse correlation between pre-treatment levels of HIF-1α and disease response.[48] In addition, they found that HIF-1α is upregulated in patients with higher risk of relapse, identifying ER

Table 2 – Anticancer agents that target HIF-1 activity.

HSP90 inhibitor: geldanamycin, 17-AAG (geldanamycin analogue), radiciol, KF58333 (radicicol analogue)
Topoisomerase inhibitor: topotecan, GL331, anthracycline
Microtubule modifier: taxane (paclitaxel, docetaxel), vinca alkaloid (vincristine, vinoblastine), 2-methoxyoestradiol (2ME2), epothilone B, colchicine
sGC stimulator: YC-1
Trx-1 inhibitor: pleurotin, PX-12/1-methylpropyl 2-imidazolyl-disulphide
Histone deacetylase inhibitor: FK228
P300 CH1 inhibitor: chetomin
Proteasome inhibitor: bortezomib
PIK3 inhibitor: wortmannin, LY294002
mTOR inhibitor: rapamycin, CCI-779, rad-001
MEK inhibitor: PD98059, BAY43-9006 (sorafenib)
ErbB2 receptor tyrosine kinase inhibitor: trastuzumab (herceptin)
Tyrosine kinase inhibitor: imatinib (Glivec)
EGFR tyrosine kinase inhibitor: ZD-1839 (Iressa), erlotinib (Tarceva)
COX2 inhibitor: celecoxib
Tyrosine kinase inhibitor: genistein

Abbreviations: HSP90, heat-shock protein 90; HIF, hypoxia-inducible factor; sGC, soluble guanylate cyclase; Trx, thioredoxin-1; cGMP, cycline guanosine monophosphate; PIK3, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; MEK, MAP/ERK Kinase; ErbB2, epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor; COX2, cyclooxigenase-2.

positive patients with a poor outcome, similar to that of ER negative patients. Dales et al. investigated HIF-1α in 745 breast cancer samples using immunohistochemical assays on frozen sections and observed that high HIF-1α expression was associated with poor overall survival and high metastasis risk. This was in node-negative and node-positive patients.[49] HIF-1α was found to be an indicator of poor prognosis in both node-negative and node-positive breast cancer.[50,51]

Gene amplification of the c-erb gene is associated with a poor prognosis and subsequent resistance to chemotherapy, radiotherapy and anti-oestrogen therapy. Upregulation of HIF-1α is observed in both Her2/erb2 overexpressing and Her2/erb2 negative tumours, but a recent analysis revealed that the poor survival was mainly correlated with tumours exhibiting c-erbB2 and HIF-1α reactivity simultaneously. These findings indicate that c-erbB2-mediated tumour aggressiveness in breast cancer could be partly due to HIF-1α activation, through the coactivation of angiogenesis and migration pathways in the HIF-1α-positive/c-erbB2-positive group of patients.[52]

In several studies, downstream targets of HIF-1α were considered as hypoxia markers. Expression of CAIX is localised to the perinecrotic area of tumours and has been observed to start at a median distance of 80 μM from a blood vessel, where the oxygen tension drops to 1% or less.[53] Previous studies showed that CAIX is a marker in tumour samples and that its expression was associated with poor prognosis, independently of the other commonly recognised prognostic parameters. However, using a primary chemo-endocrine setting of therapy, Generali et al. showed that CAIX expression was significantly associated with poor DFS and OS but failed to be an independent predictor of DFS in multivariate analysis, although they suggested a contribution of CAIX expression to tamoxifen resistance.[30] Other authors found that CAIX was rarely expressed in normal epithelium and benign lesions, but present in a significant percentage of DCIS and invasive breast carcinoma. Loss of CAXII and/or gain of CAIX expression may be associated with a high risk of progression, and thus may be of prognostic significance.[54] Recently, Brennan et al. studied CAIX in premenopausal breast cancer patients and reported that CAIX was an independent prognostic parameter in lymph node-positive patients.[55]

Many studies have confirmed the clinical relevance of VEGF expression as a significant and independent prognostic variable for relapse-free and overall survival.[56–65] The recent studies observed that HER-2/neu receptors play an important role in heregulin-induced angiogenesis.[66,67]

In addition, many studies have suggested that microvessel density (MVD), a surrogate marker of tumoural angiogenesis, is correlated with poor prognosis invasive breast cancer.[33] However, measurements of MVD are poorly reproducible[68] and standardised methods will be needed for MVD assessment.[69,70]

The association of macrophages with angiogenesis and poor prognosis in invasive breast cancer have been described.[71,72] Hypoxia stimulates transendothelial migration of monocytoid cells from the peripheral circulation into tumour tissue, where they exhibit a tumourigenic phenotype and show pro-angiogenic activity under VEGF stimuli.[73–76] An emerging area of angiogenesis regulated by hypoxia is the recruitment of circulating endothelial progenitor cells by cytokines induced by hypoxia, e.g. VEGF and then localisation to the hypoxic tumour by CXCR4 and other pathways.[77,78]

4. Profiling breast cancer for hypoxia: towards personalised therapy

4.1. *Gene profiles*

Understanding the association between biological factors and treatment response is important in order to identify patients, who will derive benefit from certain therapeutic regimens. This would enable the design of management plans optimised for the individual patient. The recognition of prognostic and predictive markers is also crucial to identify novel targets for specific therapeutics.

As microarray techniques allow the analysis of thousands of expressed genes, this should be a promising approach for identifying multiple factors acting in concert to influence outcome and response to therapy.

Although hypoxia has been recognised as an important determinant of clinical outcomes in human cancers, it has been difficult to define tumour phenotypes based on hypoxia responses. Recently, Winter et al. assessed the mRNA profile of head and neck cancer (HNSCC) samples defining an *in vivo* hypoxia metagene by clustering around the RNA expression of a set of well-known hypoxia-regulated genes (e.g. CAIX, GLUT1 and VEGF). The metagene contained many previously described *in vitro*-derived hypoxia response genes, and was prognostic for treatment outcome in independent data sets including breast cancer.[79]

Chi et al., using DNA microarrays, found that in breast cancer samples the expression of most of the genes in the hypoxia response signature varied, and were separated into two groups by hierarchical clustering based on the level of hypoxia response. All the normal breast samples and fibroadenomas were clustered in a group characterised by low expression of the hypoxia signature, while ductal adenocarcinoma samples were split between low and high hypoxia response groups. In this way, the authors were able to stratify human cancers according to the presence and amplitude of a hypoxia response and showed that breast cancer tumours with a strong gene expression signature of the hypoxia response had a significantly worse prognosis and correlated with cancer progression and metastasis.[80]

Seigneuric et al. focused their attention on the time dependency of hypoxia-regulated genes expression, and described how the early and the late hypoxia responses are very different at the transcriptional level. Using published data from the microarray data of Chi et al., they showed that survival differences are correlated with early hypoxia signatures, but not late hypoxia responses.[81]

This evidence suggests that treatment response and outcomes come to depend on individual genetic features. The identification of molecular biomarkers with the potential to predict treatment response outcome is essential for selecting patients to receive the most beneficial therapy, and it might drive stratification in clinical trials. Hypoxia is a key physiological difference interacting independently with many key pathways, and will need to be incorporated into the

algorithms used. Examples of drugs already developed particularly relate to VEGF blockade, but many signal transduction blockers targeting HER2 and EGFr will also inhibit hypoxia signalling. Many enzymes and signalling pathways described above are targets for drugs in phase I trials and for cost effectiveness we need to understand the biology to select appropriate patients.

Conflict of interest statement

We can confirm that there are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within that could inappropriately influence (bias) our work.

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