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Hypoxia and breast cancer: prognostic and therapeutic implications

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Abstract. Hypoxia affects many important processes in tumour progression and is a key feature in the tumour microenvironment that needs to be taken into account when evaluating prognostics and therapeutic options for cancer patients. Hypoxia-regulating proteins, i.e. hypoxia inducible factors (HIFs), and associated gene products have been linked to certain tumour behaviours and might be useful as prognostic and predictive markers. Recently, hypoxia-driven gene products have been launched as novel cancer treatment targets with the potential to increase tumour-specific effects. Breast cancer consists of a multitude of different diseases with certain common characteristics, but also clearly disparate behaviours and genetic alterations. In this review we will summarise the role of hypoxia in breast cancer and specifically outline the importance of hypoxia and HIF-1α regarding prognostic and treatment-specific implications. (Part of a Multi-author Review)

Keywords. Breast cancer, HIF-1α, hypoxia, prognosis, treatment.

Introduction

Hypoxia triggers a multitude of cellular processes, including an adaptive response, and is defined as an oxygen level lower than the approximate 7% observed in normal and well-vascularised tissues [1, 2]. When the oxygen supply is diminished, cells need to adapt to the harsh environment as well as initiate the vascularisation process that will increase the local oxygen supply. Central in the hypoxic response is the angiogenic shift, with production of potent angiogenic factors such as vascular endothelial growth factor (VEGF). Hypoxia is present in many solid malignancies, and the mean oxygen level in tumours corresponds to approximately 1.5% [1]. This is partly due to the abnormal vascularisation in tumours, which is insufficient in supplying oxygen to the sometimes rapidly expanding malignant lesion. Besides the generally impaired oxygen supply, there are also areas with an acute lack of oxygen, resulting in necroses and widespread cell death. In breast cancer these types of necrotic processes are often associated with clinically aggressive behaviour. Markers for hypoxia such as HIF-1α have also been linked to highly malignant features and are potentially relevant prognostic markers for identifying subgroups of breast cancer with certain malignant properties [3, 4]. There is an ongoing debate whether hypoxia contributes to more aggressive tumours or if aggressive tumours have more widespread hypoxia, and seemingly one explanation does not necessarily exclude the other. Recent research focusing on hypoxic responses and delineation of important regulators of hypoxia has clearly indicated that 'the hypoxia response' in tumours can be used to define novel treatment strategies [5]. The future arsenal of novel cancer therapies will most certainly include specific targeting of hypoxic processes. In this review we will summarise the general effect of hypoxia in breast cancer, and specifically focus on prognostic and treatment predictive information of hypoxic markers.

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Regulation of Hypoxia Inducible Factor-1

The nuclear transcription factor HIF-1 is accountable for the vast responses during hypoxia by transactivating numerous genes through binding to hypoxia-response elements (HREs) in promoters or enhancers. The protein is a heterodimer consisting of a hypoxia-inducible α subunit and a constitutively expressed β subunit, both members of the basic-Helix-Loop-Helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family of proteins [6]. Three different types of α subunits, HIF-1α, HIF-2α, and HIF-3α have been identified, and although initially thought to have similar function, it is now clear that they have distinctive roles. HIF-1α is by far the most studied and we will only be referring to this type. The activity of HIF-1 is dependent on stabilisation of the α subunit, followed by nuclear translocation and dimerisation with HIF-1β (also referred to as ARNT, aryl hydrocarbon nuclear translocator), and interaction with general transcription activators such as CBP/p300. During normoxia HIF-1α is subjected to protein degradation via the 26S proteasome due to posttranslational modifications, specifically hydroxylation of proline residues [7]. This results in very low levels of HIF-1α during normoxia and basically no activity of HIF-1. HIF-1α does not itself recognise changes in oxygen levels. The actual sensors of oxygen pressure are the prolyl hydroxylase domain (PHD) proteins [8] and asparaginyl hydroxylase [9]. Besides oxygen, hydroxylation by these enzymes requires Fe^{2+} and ascorbate as cofactors. When oxygen is present, the PHDs hydroxylate two specific proline residues (P402 and P564) in the oxygen-dependent degradation domain (ODDD) of the HIF-1α subunit (Fig. 1). This modification is rapidly recognised by the von Hippel Lindau protein (pVHL), that together with the proteins elongin C, elongin B, cullin-2, and Rbx1 forms an E3 ubiquitin ligase complex that targets HIF-1α for proteosomal degradation [10, 11]. In addition, acetylation of a lysine residue (K532) in ODDD by an acetyl-transferase named arrest-defective-1 (ARD1) favours the interaction between HIF-1α and pVHL, and thus destabilises HIF-1α [12]. Three different isoforms of PHDs are known, PHD1, PHD2, and PHD3, and although all of them are able to hydroxylate HIF-1α *in vitro*, PHD2 has been shown to be the key limiting enzyme that controls HIF-1α degradation under normoxic conditions [13]. PHD2 mRNA and protein are also upregulated during hypoxia, a feedback mechanism that ensures rapid degradation of HIF-1α once the oxygen pressure increases.

The second oxygen-sensitive enzyme, asparaginyl hydroxylase, also referred to as Factor Inhibiting HIF-1 (FIH-1), hydroxylates an asparagine residue (N803) in the C-terminal activation domain (C-TAD) of HIF-1α. This abrogates the interaction between HIF-1α and CBP/p300 and hence represses the transactivational activity of HIF-1 [9].

Interestingly, HIF-1α has two transcriptional activation domains [N-TAD (amino) and C-TAD (carboxy)], but only the C-TAD is subjected to hydroxylation by FIH-1. A working model has been proposed on the observation that PHDs have a lower affinity for oxygen than FIH-1, describing induction of two sets of HIF-1-regulated genes depending on the hypoxic gradient. In brief, N-TAD genes are induced during moderately hypoxic conditions when PHDs are inactivated and FIH-1 is still active. Genes dependent on C-TAD activity are transcribed when the oxygen pressure decreases even more and FIH-1 is inactive [14].

To further complicate the story, HIF-1α is regulated by other posttranslational modifications such as phosphorylation, SUMOylation, and nitrosation [7]. A direct phosphorylation of HIF-1α by p42/44 regulated the transcriptional activity of HIF-1α in a reporter assay without affecting the protein stability [15]. Mylonis et al. identified two residues (Ser-641/643) phosphorylated by p42/44, which promoted nuclear accumulation and thereby transcriptional activity by blocking the nuclear export of HIF-1α [16]. Different effects regarding SUMO modification of HIF-1α have been suggested. Bae et al. observed an increased HIF-1α protein stability and transcriptional activity by ectopic expression of SUMO-1 [17], while Brahimi-Horn et al. suggested that SUMOylation decreases the trancriptional activity [7]. Moreover, nitrosation of a cystein residue (Cys-800) in the HIF-1α C-terminal domain by nitric oxide increased HIF-1α transcriptional activity by stimulating the interaction with the co-activator p300 [18]. Under normoxic conditions HIF-1α can be upregulated by various growth factors, such as PDGF, EGF, FGF2, TGF-1β, heregulin, insulin, insulin-like growth factor, and cytokines like TNF-α and interleukin-1β. Receptor-mediated HIF-1α upregulation occurs through two important signalling pathways, the Ras/Mek/MAPK and PI3K/Akt/mTOR cascade. Although most studies on the role of MAPK stimulation show no effect on HIF-1α protein expression, active PI3K signalling leads to increased HIF-1α protein synthesis. Activation of oncogenes, i.e. *ERBB2*, or loss of tumour suppressor genes, i.e. *VHL* or *PTEN*, has also been shown to upregulate HIF-1α through these pathways [19, 20]. There are also other proteins that affect the stability of HIF-1α protein. p53 promotes Mdm2-mediated ubiquitination and degradation of HIF-1α [21], whereas Jab1, a transcriptional co-activator of c-Jun and Jun D, interacts directly with

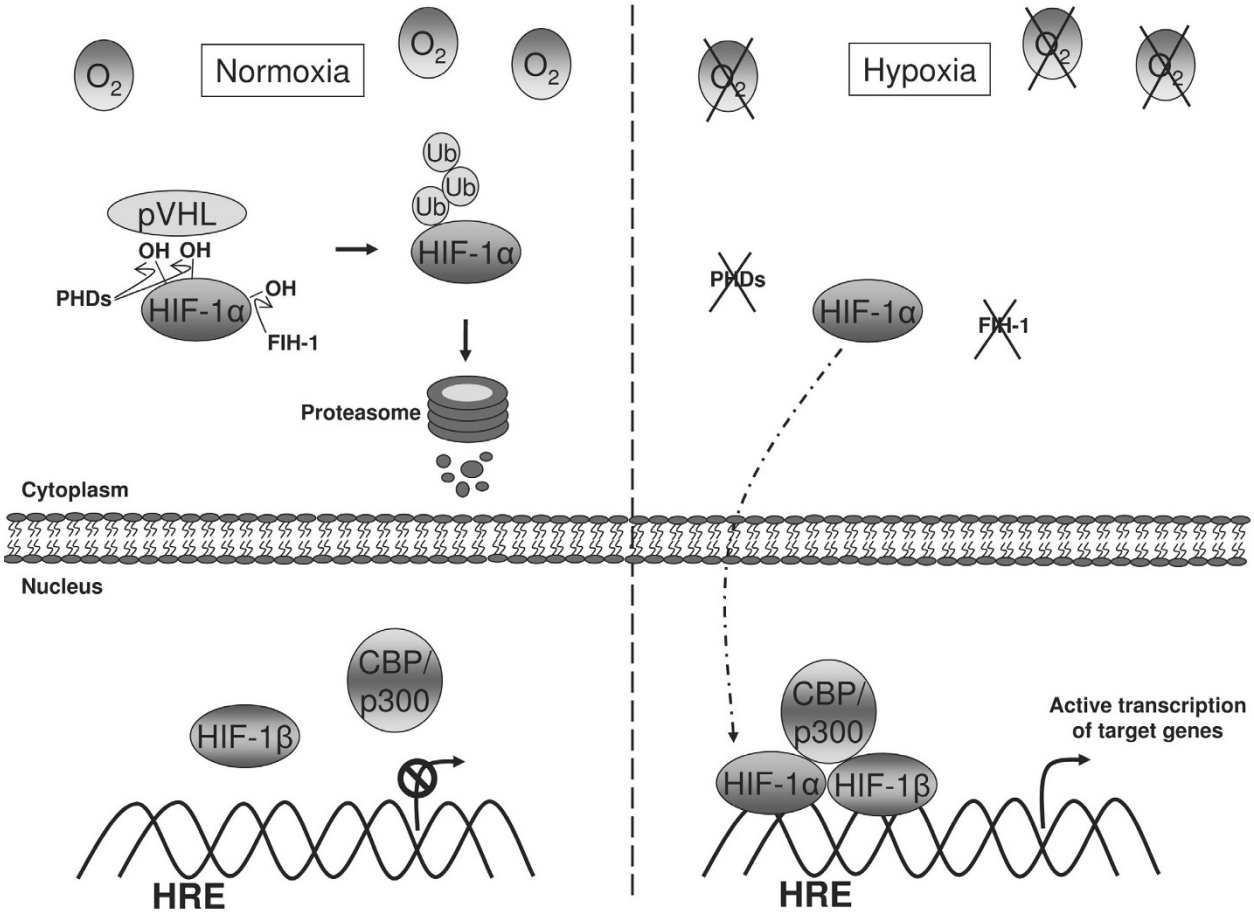

Figure 1. Regulation of HIF-1α during normoxia and hypoxia. Under normoxic conditions, HIF-1α is recognised by the von Hippel Lindau protein (pVHL) through binding to hydroxylated proline residues on HIF-1α, executed by proline hydxolylases (PHDs). This ensures ubiquitination and degradation of HIF-1α by the proteasome. Another hydroxylation of HIF-1α, performed by Factor Inhibiting HIF-1 (FIH-1), abrogates the interaction between HIF-1α and the coactivator CBP/p300. Thus, during normoxia HIF-1α levels and transactivation activity are low, resulting in no transcription of target genes. During hypoxia, inactivation of oxygen-dependent PHDs and FIH-1 stabilises HIF-1α, which translocates to the nucleus and interacts with its partner HIF-1β and the CBP/p300 protein at hypoxia-response elements (HREs), leading to transcription of hypoxia-driven target genes.

HIF-1α and increases its stability, possibly through competition with p53 [22]. Recently, Yoo et al. reported that metastasis-associated protein 1, a component of the nucleosome remodelling histone deacetylation (NuRD) complex, interacts with HIF-1α and enhances its stability by increasing deacetylation of HIF-1α [23].

Hypoxia-associated response

The hypoxia response is manifested differently depending on the cell line or tissue affected, although there is a common regulation of the same biological processes due to the HIF-1 activation, leading to transcription of its target genes (Fig. 2). In order for a cell to survive under hypoxic conditions, a certain adaptation to the limited oxygen supply needs to be provided for. To handle this situation, processes resulting in sparser metabolism, increased angiogenesis, and facilitated motility of cells are favoured.

To date, more than 100 HIF-1 target genes have been reported. Many genes playing roles in glucose metabolism, such as *GAPDH*, enolase 1 (*ENO1*), carbonic anhydrase IX (*CAIX*), lactate dehydrogenase (*LDH-A*), pyruvate kinase M (*PKM*), and *GLUT1* and *GLUT3*, are under the control of the HIF-1 transcription complex. During low oxygen levels, cells switch from the oxygen-dependent tricarboxylic acid (TCA) cycle to oxygen-independent glycolysis [24]. Genes important in amino acid and nucleotide metabolism are also target genes for HIF-1. One of the most important HIF-1 target gene products is VEGF, which together with e.g. TGF-β promotes angiogenesis, in order to reoxygenate the hypoxic cells

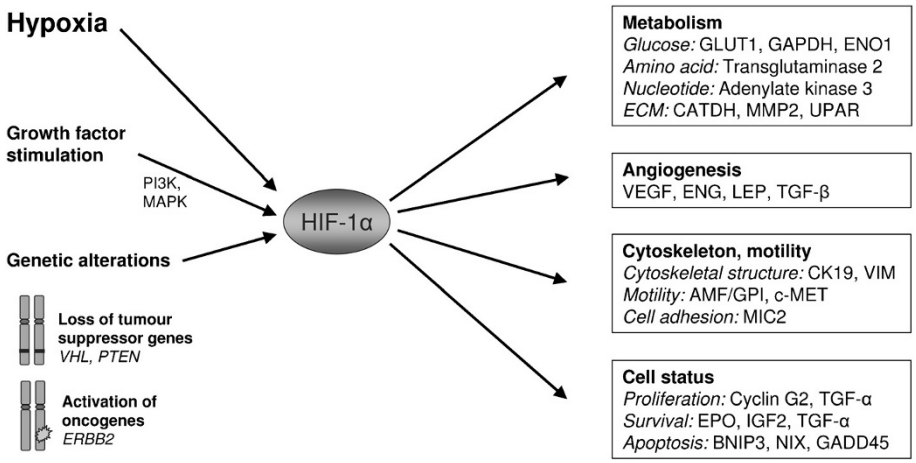

Figure 2. Pathways leading to stabilisation and transcriptional activity of HIF-1α. Hypoxia is the main process regulating HIF-1α activity. PI3K and MAPK signalling via growth factor stimulation and activation of oncogenes or loss of tumour suppressor genes are two other possible mechanisms leading to the stabilisation of HIF-1α. Activation of HIF-1α results in transcription of many different target genes involved in biological processes promoting tumour development. Metabolic shift, induction of angiogenesis, changes in cell adhesion and motility, and promotion of survival or apoptosis are central events resulting from HIF-1 transcription.

on longer terms. Biological processes such as proliferation and apoptosis are also affected by hypoxia. HIF-1 target gene products cyclin G2 and TGF-α are proteins that take part in proliferation, and NIX and GADD45 are important for the apoptosis machinery. Depending on the cellular context, the regulation of these apoptosis-related genes will result in either survival or apoptosis.

Hence many of the biological processes affected by hypoxia are central in carcinogenesis, and this might explain how tumour cells exposed to hypoxia become even more tumorigenic. Other categories of genes transcriptionally activated by HIF-1 are genes involved in erythropoiesis, drug resistance, and epithelial homeostasis. HIF-1α protein is overexpressed in many tumours due to intratumoural hypoxia, but overexpression as a result of genetic alterations in e.g. the *VHL* (tumour suppressor gene) or *ERBB2* (oncogene) has also been reported (Fig. 2) [25].

Cells exposed to hypoxia reconstitute their cytoskeleton and also acquire migratory properties, which are factors important for invasion and metastasising of solid tumours. The acquired resistance to chemotherapy and radiation which denote hypoxic tumours can be explained by mechanisms such as downregulation of pro-apoptotic genes, changes in cell cycle regulation, and reduced uptake and metabolism of nutrients.

Expression of HIF-1α differs between different cell lines exposed to hypoxia and is more or less pronounced depending on the cell line. The breast cancer cell lines T47D and MDA-MB-468 upregulate HIF-1α protein to a higher extent than MDA-MB-231 and MDA-MB-435, but when re-exposed to normoxia, they all reverse their HIF-1α expression completely within an hour [26]. The expression of HIF-1α protein over time is also individual for different cell lines subjected to hypoxia. By immunohistochemical comparison of the two breast cancer cell lines MCF7 and CAMA1, we observed that stabilisation of HIF-1α was more rapid in the MCF7 cells, as illustrated in Figure 3a. The protein expression peaked at approximately 4 h in MCF7 exposed to 0.1 % oxygen, and then declined before a second but weaker peak was observed at 48 h. In CAMA1 the HIF-1α expression was most pronounced at approximately 72 h and then declined. In many cell lines and human tissues HIF-1α mRNA is expressed constitutively and is not further upregulated at hypoxia [26]. As illustrated in Figure 3b, the estrogen receptor α (ERα) is also regulated by hypoxia, as will be further detailed below.

In order to study the cellular response to hypoxia, an expression analysis based on cell lines exposed to hypoxia can be performed. Hedenfalk et al. exposed the three breast cancer cell lines MCF7, T47D, and MDA-MB-468 to 1 % oxygen for up to 48 h and observed both up- and downregulation of many genes, with a clear distinction between the cell lines, as visualised in Figure 4 [27]. Only a small number of genes were altered in two or all three cell lines, but these genes have commonly been reported to be regulated by hypoxia. Genes up or downregulated were further characterised into gene ontology categories, and effects on biological processes from the hypoxia exposure could then be outlined. Different biological processes such as e.g. general metabolism (glucose and alcohol), differentiation, and cytoskeletal structure were affected by hypoxia in all three cell

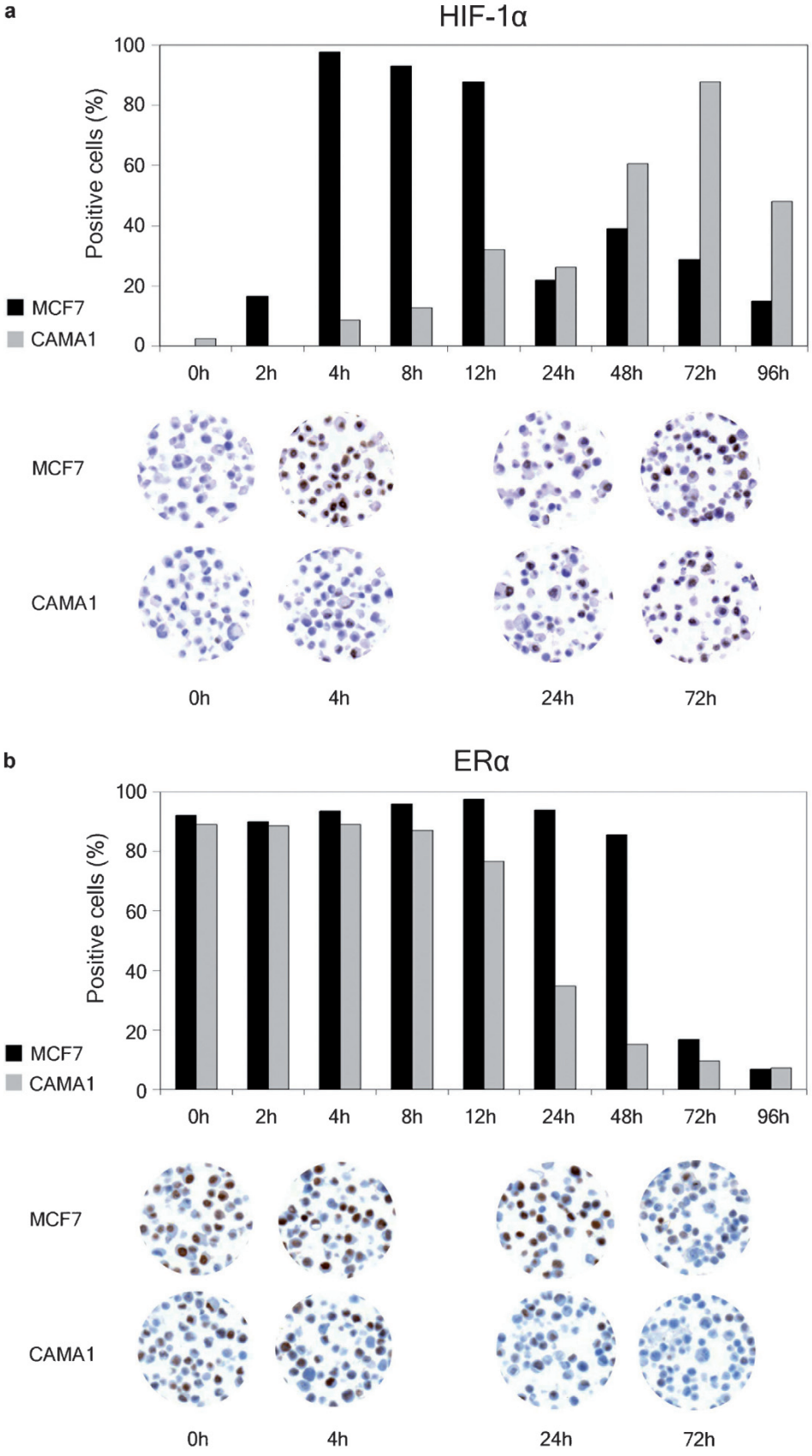

Figure 3. Diagram and immunohistochemical stainings illustrating HIF-1α (*a*) and ERα (*b*) protein expression at different time points for the breast cancer cell lines MCF7 and CAMA1 grown in 0.1 % oxygen. The highest HIF-1α protein content was observed at 4 h in MCF7 and at 72 h in CAMA1. The ERα protein level started to decline at approximately 48 h in MCF7 and at 12 h in CAMA1 and was very low at 96 h in both cell lines.

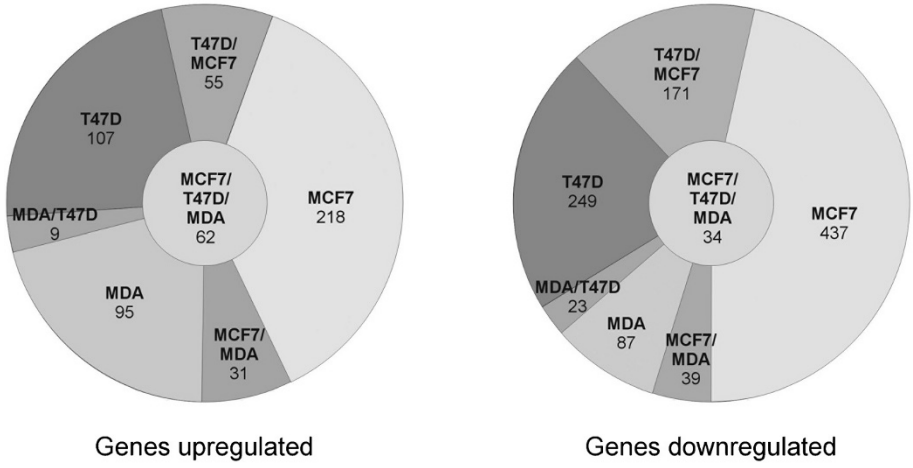

Figure 4. Schematic diagram illustrating the number of genes up- and downregulated, respectively, in response to hypoxia, based on an expression analysis of breast cancer cell lines exposed to 1 % oxygen. The cDNA analysis included ~ 17 500 unique genes, and ~ 10 000 were expressed in each cell line. The number of altered genes is indicated for each cell line individually and for the same alterations in two or all three cell lines [27].

lines. In order to investigate whether the genes affected by hypoxia were regulated via HIF-1 transcriptional activity, an assay to search for HRE motifs was performed. A large number of the regulated genes contained HRE motifs; however, many genes not regulated by hypoxia also contained these HIF-1 binding sites. This study indicates that the overlap in molecular and functional processes regulated by hypoxia is greater than individual gene regulation, when comparing breast cancer cell lines. This suggests that the same biological processes can be affected by hypoxia, although by different genes, depending on the cell line. The extensive change in gene regulation that occurs in cells subjected to hypoxia explains how tumour cells adapt to the new circumstances and escape the obstacles that normally would suppress them.

In vitro model systems for studying different aspects of tumour cell biology are valuable tools, giving us new insights and better understanding of the human carcinogenesis. However, it is important to take into account that cell lines cannot be used as a substitute for the *in vivo* situation. Cell lines can be considered as a reflection of the *in vivo* tumour situation, and they facilitate the experimental approach with large-scale setups for e.g. protein and mRNA analysis, gene transfection, and therapeutic agent analysis. When taken out of their context, tumour cells do not retain their general expression profile, and many features characterising the *in vivo* tumour cell are lost [28, 29]. Nonetheless, the *in vitro* model approach is a powerful alternative, and studies based on cell line experiments are considered as a complement to the *in vivo* studies, when describing biological events taking place in tumorigenesis.

Several *in vivo* models, where the HIF-1α locus has been targeted via homologous recombination creating embryonic stem (ES) cells lacking HIF-1α, have been engineered to better understand the role of HIF-1α during cellular development. Due to different genetic backgrounds of the mice and different analytical methods, the conclusions have not always been concordant. Even so, they offer reasonable directions of the importance of HIF-1 during embryogenesis. Iyer et al. showed that complete deficiency of HIF-1α resulted in developmental arrest and embryonic lethality by E11 [30]. The embryos displayed neural tube defects, cardiovascular malformations, and abnormal vasculature, the latter consistent with a marked reduction in VEGF production. Also, when cultured during hypoxia, HIF-$1\alpha^{-/-}$ cells showed a decreased number of cells but no difference in cell death rate compared to HIF-$1\alpha^{+/+}$ cells. Ryan et al. reported on a similar HIF1-$\alpha^{-/-}$ phenotype but also showed that null cells had a decreased capacity to form teratomas when injected into mice [31]. In contrast to Iyer et al. they observed identical growth rates of wild-type and null cells in culture; however, they could detect an increased apoptosis in the interior neural fold of the null embryo. Interestingly, Carmeliet et al. found that hypoxia and hypoglycaemia reduced proliferation and increased apoptosis in HIF-$1\alpha^{+/+}$ embryonic cells but not in HIF-$1\alpha^{-/-}$ null cells, and that growth of HIF-$1\alpha^{-/-}$ tumours far exceeded that of HIF-$1\alpha^{+/+}$ tumours [32]. This phenomenon could partly be explained be the fact that hypoxia-induced growth arrest and apoptosis through the p53 pathway is HIF-1α-dependent. The results from *in vivo* models clearly show that the HIF-1α network is multifaceted, and the fact that HIF-1α is regulated and post-translationally modified in so many different ways adds even more complexity to the story.

Hypoxia and cancer

In vivo, two conditions of hypoxia have been described: the acute and the chronic hypoxia [3]. Acute hypoxia is defined as perfusion-limited and is denoted by cells adjacent to capillaries that die due to the insufficient blood supply [3, 33]. This state of hypoxia occurs when an aberrant blood vessel is shut down. Chronic or diffusion-limited hypoxia involves tumour cells remotely from the capillaries that adapt to the hypoxia and undergo a selection process where they gradually become clinically aggressive. The harsh microenvironment created by hypoxia results in tumour cells showing genetic instability and hence supports the hypothesis claiming that hypoxia promotes malignant progression [3, 33].

Regional tumour hypoxia develops in early stages of carcinogenesis, before the tumour metastasises, and is often observed in non-invasive tumours [3]. Whether hypoxia generates an aggressive tumour phenotype or an aggressive tumour phenotype generates hypoxia is continuously discussed, yet no definite answer exists. As mentioned earlier, hypoxia affects many important properties that render a cell more resistant to a harsh environment. Many of these properties are most likely of selective advantage in cancer cells that altogether makes them more robust and resistant to the hostile environment, constituting the inflammatory response and stromal reaction. Intuitively, tumours expressing hypoxic markers would therefore possess more aggressive properties than tumours lacking these markers. On the other hand, it could be that aggressive tumours express hypoxic markers as a consequence of a deranged vascularisation and rapid growth and that hypoxia is only a passive process secondary to other, more important selective properties [34]. The hypoxic response might further not always be a selective advantage for tumours, and a diminished response could speculatively be a selective advantage for certain tumour types. Further studies are needed to uncover this complicated network of important selective properties in tumour cells, but it is likely that a combination of the different scenarios described above exists and that various subgroups of cancer might handle the hypoxic response and selective properties in different ways.

Besides the fact that hypoxia affects general processes such as glycolysis, apoptosis, and proliferation, recent data have linked hypoxia to a dedifferentiated phenotype. Several studies in neuroblastoma have indicated that hypoxia is associated with a dedifferentiated phenotype with loss of the expression of neuronal markers [34–36]. Recent studies have further suggested cross-talk between hypoxia and the notch-signalling pathway, a cell-cell communication mechanism closely associated with cell differentiation [37, 38]. This highlights additional levels of complexity within cell differentiation pathways but also highly relevant links to hypoxia. The association between hypoxia and differentiation is interesting with regard to tumour biology, and as will be detailed below, poorly differentiated tumours are in general more malignant than highly differentiated ones. If hypoxia could force the tumour cells into a poorly differentiated state, this could partially explain some of the more aggressive features observed in hypoxic tumours as also will be detailed further. For breast cancer, 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 [39]. In parallel with HIF-1α expression, there was a decline in the ERα protein content as well as an increase in cytokeratin 19 expression. Similar findings were also observed in breast cancer cell lines grown in hypoxia, further supporting these data. Even though the presence of ERα and cytokeratin 19 not is exclusively linked to differentiation, this could suggest that hypoxia will affect processes intimately linked to a general differentiation state of the tumour cells. This further motivates the use of hypoxia-targeting treatment protocols that potentially also have an effect on the differentiation machinery.

Breast cancer

Breast cancer affects approximately one out of every nine women, making it the most common female malignancy. The expected survival rate after 10 years is ~75%, and the majority of patients die from other causes than the diagnosed breast cancer. It has been increasingly clear that breast cancer constitutes a mixture of different cancers with varying behaviours. Some breast cancer types are highly malignant, with rapid progression and spreading of the disease, whereas other types are of low malignant grade. Breast cancer is traditionally divided according to microscopic appearance and growth behaviours into a few well-established tumour types, where ductal breast cancer is the most common type [40]. Recently, a more modern subdivision of breast cancer was proposed based on the presence of common genetic alterations and expression profiles dividing breast cancer in one basal-like, one *ERBB2*-overexpressing, two luminal-like, and one normal breast tissue-like subgroup [41]. Future research and clinical protocols must address how these subgroups should be implemented in the clinic, and more important how these subgroups are

linked to clinical behaviours and response to treatment strategies.

During progression into invasive breast cancer there are several defined precancerous stages, such as *in situ* cancer, that are of importance when detailing hypoxia. Especially DCIS has been linked to the presence of large central necrotic processes, and it is obvious that these often poorly differentiated high-grade in situ lesions have widespread hypoxic areas and probably a general hypoxic phenotype [42, 43]. The corresponding invasive lesion also exhibits similar hypoxic areas that are associated with high HIF-1α expression and aggressive disease [43].

Due to the obvious difference in malignant behaviours between different subtypes of breast cancer, it is important to define aggressive properties in tumours. This can be done by using prognostic markers that predict the probability of disseminated disease and death for a specific breast cancer patient [44]. Well-established prognostic markers that are used today are tumour size, the presence of lymph-node metastases, and differentiation grade [45]. The tumour differentiation grade is determined by evaluating the presence of tubular formations, nuclear atypia, and mitoses, altogether creating a standardised differentiation spectrum that clearly reflects clinical behaviours of the tumour [44]. The presence of lymph-node metastases is a prognostic criterion linked to worse outcome and often aggressive disease, although, it should be noted that lymph-node metastases are not equal to distant metastases and that this local metastatic ability is probably the net result of extensive lymph vessel growth, proliferation, as well as increased but uncertain ability to establish distant metastases. Patients with large tumours also have poorer prognosis, and this together with increased proliferation is a classical prognostic parameter in many cancer forms.

Breast cancer therapy is today based on surgical removal of the primary tumour, postoperative radiation followed by adjuvant endocrine and/or chemotherapy. Specific treatments targeting ERBB2-overexpressing cancer with trastuzumab are also an important additive treatment modality. The selective estrogen receptor modulator (SERM) tamoxifen, together with aromatase inhibitors, represents main endocrine treatment schedule for breast cancers that express hormone receptors ERα and/or the progesterone receptor (PR). Unfortunately, not all ERα-positive breast cancer patients respond to endocrine therapy, and several potential mechanisms inducing resistance to various endocrine treatments have been outlined [46, 47].

To predict the efficiency of a certain treatment, there is a limited set of treatment-predictive markers that are analysed in breast cancer samples. A good example of a treatment-predictive marker is the ERα. If the breast cancer cells do not express ERα, the patient will not respond to endocrine treatment in most cases. There are a few reports of patients with ERα-negative tumours that do respond to endocrine treatment, but the reason behind this is unknown. The combination of ERα and PR positivity has in some studies been suggested to be indicative of a pronounced tamoxifen response, whereas contradictory results have been observed in other studies [48]. The overexpression of ERBB2 is a predictive marker for trastuzumab response [49]. In general, the few predictive markers used today have limitations, and there is a great need for better treatment-predictive markers both for conventional therapy strategies as well as for novel tumour-targeting treatments. As discussed below, markers for hypoxia might have the potential to add predictive treatment information, even though we are far from implementing these novel markers into the clinic.

Hypoxia in breast cancer

As mentioned earlier, hypoxic areas can often be observed in breast cancer samples. The old definition 'comedo cancer' is a highly malignant breast cancer with low differentiation and the presence of hypoxic areas [50, 51]. Besides these tumours with an obvious hypoxic and necrotic manifestation, some type of hypoxic response in a large fraction of invasive breast cancer samples is commonly detected. By using HIF-1α as a marker for hypoxia, approximately 25–40% of all invasive breast cancer samples are hypoxic [52–57]. In general, HIF-1α positivity is linked to large tumour size, high grade, high proliferation, lack of lymph-node metastases and hormone-receptor negativity. It should be noted that it is not obligate with necrotic areas in large tumours and there could accordingly be marked HIF-1α positivity even in small tumours despite the link between hypoxia and tumour size. The large variation of HIF-1α-positive tumours in the different studies probably depends on the cutoff used for scoring positive tumours. Although hypoxia is the major factor regulating HIF-1α, it can also be triggered by growth factor stimulation and/or genetic alterations in oncogenes and suppressor genes (Fig. 2), and a fraction of the breast cancer samples expressing HIF-1α are probably not hypoxic. Vleugel et al. have shown that out of 44% HIF-1α-positive samples, 13.5% expressed HIF-1α in a perinecrotic fashion, whereas 30.5% had a more diffuse HIF-1α staining indicative of an alternative activation of HIF-1α [53]. The HIF-1α downstream target CAIX was

further coexpressed with HIF-1α only in the perinecrotic and scattered HIF-1α-expressing tumours. The lack of association between HIF-1α and downstream targets in certain HIF-1α high expressing breast cancers also supports differing cellular response depending on the reason HIF-1α is expressed and in what context the hypoxia marker appears. When analysing CAIX in large breast cancer cohorts, there was on the other hand a strong link to HIF-1α expression as well as to large tumour size, grade, proliferation, and hormone receptor negativity [58]. Van den Eynden et al. have also shown that the presence of hypoxia in a primary breast cancer corresponds to hypoxia markers in lymph-node metastasis, strengthening the hypothesis that besides effects from the microenvironment, the intrinsic angiogenic hypoxia potential of a tumour is kept during tumour progression [59].

Chen et al. observed an association between HIF-1α and MET overexpression in breast cancer, suggesting that HIF-1α promotes aggressive breast cancer disease by induction of MET [60]. Another report has shown a strong correlation between HIF-1α and VEGF-C as well as lymphatic microvessel density in human breast cancer. This suggests that HIF-1α could affect tumour-associated lymphangiogenesis, which is a critical parameter in the spreading of breast cancer cells [61]. Hypoxia can further stimulate breast carcinoma cell invasion through general effects on a multitude of invasion/migration-associated gene products, exemplified by MT1-MMP and MMP2 activation as well as stabilisation of microtubules, and promoting the Rab11 trafficking of the α6β4 integrin [27, 62, 63].

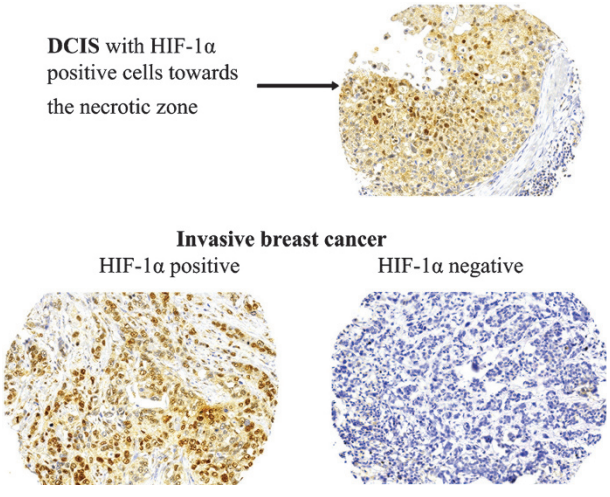

Figure 5. HIF-1α immunohistochemical stainings of a ductal cancer *in situ* (DCIS) of the breast and invasive breast cancer samples.

HIF-1α and ERα in breast cancer

The presence of ERα is an important marker for endocrine treatment response in breast cancer and constitutes a key molecule in targeted treatment of the disease. ERα is also implied in the development and progression of breast cancer and is altogether intimately linked to several important breast cancer features. Interestingly, hypoxia has been shown to decrease the ERα protein content in breast cancer cell lines, and several groups have reported specific downregulation of ERα and PR under various hypoxic conditions (1 % and 0.1 % oxygen) [39, 64–67]. We observed that in the breast cancer cell lines MCF7 and CAMA1, the expression of ERα was decreased over time during hypoxia, with a slightly different pattern depending on the cell line. At 0.1 % oxygen, downregulation of the receptor started at approximately 12 h in CAMA1 cells and somewhat later in the MCF7 cells (Fig. 3b). At the time point of 96 h only a very low amount of ERα protein was detectable. The hormone receptors are further downregulated when HIF-1α is transiently overexpressed or stabilized with iron chelators. Importantly, the response to tamoxifen is also diminished in hypoxic breast cancer cells compared to cells grown under normoxic conditions [64, 68]. The mechanisms of ERα downregulation during hypoxia are probably dependent on both increased proteasomal degradation of ERα and decreased transcriptional ERα-activation [66, 68]. The transcriptional inhibition of ERα during hypoxia seems to be mediated by the ERK pathway, since downregulation could be inhibited by MEK and ERK inhibitors [68]. Regulation of ERα and the ERα response during hypoxia is nevertheless complex, and intermittent hypoxia seems to be able to alter the proteasomal machinery, causing prolonged ERα downregulation not attributed to direct acute hypoxic effects [69]. Recent findings also suggest that hypoxia can trigger ligand-independent ERα activation, possibly by direct HIF-1α and ERα interaction [70]. Despite the somewhat contradictory findings, it is important to take hypoxia into account when analysing ERα in breast cancer samples as well as when designing novel treatment strategies. The presence of ERα in breast cancer is definitely a dynamic phenomenon influenced by the microenvironment and hypoxia. Strategies to increase the effect of endocrine treatments could be directed to the hypoxia driven ERα downregulation, potentially better targeting tumour cells in hypoxic compartments by restoring ERα protein content [68].

Hypoxia and HIF-1α as prognostic markers in breast cancer

In general there is a rather strong link between the expression of hypoxia markers in breast cancer and aggressive disease, and consequently impaired patient prognosis. Several large studies have observed that HIF-1α protein expression is linked to a worse prognosis, though with some variations in the specific subgroup of breast cancer where HIF-1α was an independent prognostic marker [52–57]. The majority of the studies analysing HIF-1α in breast cancer in relation to prognosis have been conducted using immunohistochemistry and formalin-fixed paraffin-embedded material or frozen material. It should be noted that many of the antibodies used to detect HIF-1α by immunohistochemistry are difficult to handle and occasionally react with other proteins besides HIF-1α, making the evaluation of HIF-1α troublesome. It is therefore important to include tissues with defined hypoxic areas when analysing HIF-1α in clinical materials, as e.g. high-grade DCIS with central necrotic areas [39]. This ensures that the antibodies detect nuclear HIF-1α staining under the current staining conditions, thereby increasing the validity of the results obtained. The use of CAIX as well as HIF-1α antibodies can serve as positive controls in tissue-based analyses, whereas a suitable negative control not directly affected by hypoxia could be a proliferation marker such as Ki-67.

Dales et al. studied HIF-1α in an unselected material of 745 invasive breast cancer samples and observed that high HIF-1α expression significantly correlated to poor overall survival and high metastatic risk [54]. Multivariate statistical analysis further showed that the prognostic value of HIF-1α was independent of other current prognostic markers. The prognostic features of HIF-1α were also significant for the subgroup of lymph node-negative patients. Other studies have shown that HIF-1α is linked to poor survival also in lymph node-positive disease, and both Schindl et al. and Kronblad et al. have observed significantly independent prognostic information of HIF-1α in this subgroup of breast cancer with a somewhat worse outcome [52, 56]. Kronblad et al. also studied the true prognostic information of HIF-1α by analysing a control group of patients who did not receive any adjuvant treatment after surgery [52]. This ensures that the specific link between HIF-1α and tumour aggressiveness was studied without interference of potential associations between HIF-1α and treatment effects. Also, in this study there was a correlation between HIF-1α and prognosis, but specifically in the subgroups of lymph node-positive disease or in grade 1–2 tumours. Vleugel et al. further studied the relation between prognosis and the specific expression pattern of HIF-1α in breast cancer and observed rather striking and contrasting results between diffuse and scattered HIF-1α staining. Tumours with scattered HIF-1α staining, indicative of hypoxia-driven HIF-1α expression, were in general more aggressive compared to tumours with diffuse HIF-1α staining [53]. This difference in the HIF-1α staining pattern is difficult to analyse in tissue micro-

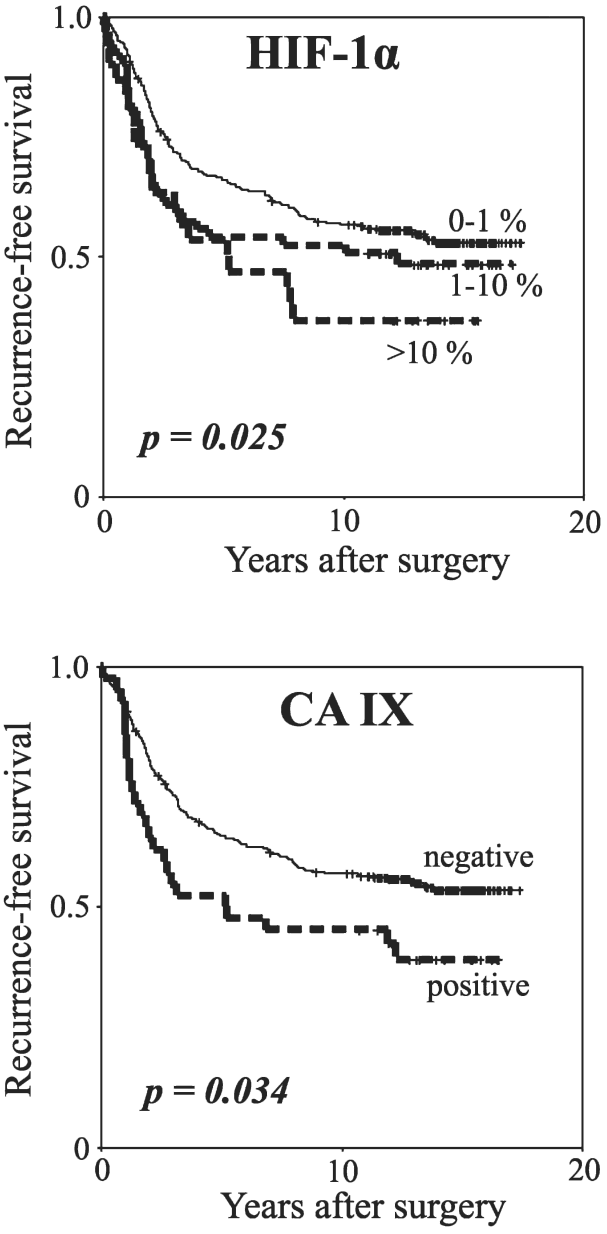

Figure 6. Kaplan-Meier curves illustrating the recurrence-free survival for premenopausal breast cancer patients in relation to HIF-1α-positive cells (n = 376) and CAIX (n = 399) protein expression. All patients were included in a randomised treatment trial and received either 2 years of tamoxifen or no further treatment after surgery [52, 58].

array biopsies of formalin-fixed material, which is the most common way of analysing prognostic parameters in large collections of cancer samples. Larger studies of whole sections of breast cancer samples are probably needed in order to fully clarify the different roles for scattered and diffuse HIF-1α in breast cancer. The different studies presented earlier also used slightly different end-points. This should be considered when comparing results from larger studies, and a future aim would be to use the same criteria for tumour aggressiveness.

Studies of downstream targets of HIF-1 in breast cancer, indicative of a "true" hypoxic response, produce results rather similar to those with HIF-1α. Brennan et al. studied CAIX in premenopausal breast cancer patients and reported that CAIX was an independent prognostic parameter in lymph node-positive patients, similar to what was observed for HIF-1α in the same cohort of patients [52, 58].

HIF-1α as a predictive marker for treatment response in breast cancer

Only a few studies have outlined any potential use of HIF-1α as a marker for treatment-predictive information in breast cancer. Generali et al. studied the relationship between HIF-1α/CAIX and the response to epirubicin, and they observed a significant link between expression of HIF-1α and poor treatment response to epirubicin [71]. In the same study, there was no significant difference in treatment response in relation to HIF-1α for patients receiving epirubicin and tamoxifen. Kronblad et al. have also studied the difference in tamoxifen response in relation to HIF-1α expression in a randomised treatment trial including premenopausal breast cancer patients receiving either 2 years of tamoxifen or no adjuvant treatment after surgery [52]. However, there was no difference in tamoxifen treatment response in the different HIF-1α subgroups. In summary, more studies are needed before the role of HIF-1α as predictive marker for treatment responses can be clarified, but studies like the one by Generali et al. are encouraging, and indeed support the idea that the hypoxia pathway is enrolled in major events directly affecting the efficiency of many treatment protocols.

Hypoxia and HIF-1α as targets for novel treatment strategies

Hypoxic tumours are difficult to treat, as they are often resistant to current therapies. Regarding radiotherapy, the dose of ionising radiation needs to be more than the double required for oxygenated tumours [72]. The fact that hypoxic cells reside in regions with inadequate blood supply makes the delivery of therapeutic drugs difficult. Hypoxic tumour cells are often low-proliferative, and this reduces the effectiveness of cytostatic drugs [33]. The higher dose required against these resistant cells further increases the potential damage to the surrounding tissue. Therapeutics directed against hypoxic tumour cells hence need to have high delivery efficiency, high specificity and selective cytotoxicity to be good candidates.

One way to approach tumour hypoxia therapy is to increase the oxygenation of the hypoxic cells before administration of chemotherapy or radiotherapy. This has been routine in the treatment of hypoxic tumours for several years, and includes blood transfusion to raise the haemoglobin concentration prior to conventional therapy [3, 33]. The administration of EPO (erythropoietin) has also been reported to improve the efficiency of chemotherapy or radiotherapy [3].

Another approach to effectively target hypoxic tumour cells to use bioreactive drugs. These are compounds that are converted to active metabolites in the absence of oxygen and hereby target the hypoxic cells with high specificity. Tirapazamine (TPZ) is a bioreactive drug with great potential and has been reported to be a promising agent in combination with cisplatin and radiotherapy in several clinical trials [72]. In hypoxic conditions TPZ is reduced to a radical that induces single- and double-strand breaks together with base damage [33].

Solid tumours are often infiltrated by macrophages which accumulate in areas of hypoxia, and this phenomenon underlies another relatively new treatment strategy. The idea is to equip activated macrophages with the ability to express therapeutic genes. These macrophages will seek the tumour, and expression of the gene or genes will result in killing of the hypoxic tumour cells. To date this cancer treatment strategy is not in use, but clinical protocols involving transfer of activated patient-derived macrophages have been described [73].

Given the overexpression of HIF-1α and the fact that hypoxia drives tumour cells to become more aggressive through induction of genes that favour blood vessel formation, cell survival, migration, and invasion has led to the idea that HIF-1 might be an important therapeutic target in solid tumors. It has been shown in several cancer types, such as breast [56, 57], cervical [74], endometrial [75], and oligodendroglioma [76], that high expression of HIF-1α correlates to poor prognosis and increased mortality, supporting the idea that HIF-1 would make a suitable drug target. However, a few reports have in contrast indicated a

link between HIF-1α and apoptosis and favourable prognosis [77, 78]. Several known anticancer compounds have an effect on HIF-1, even though they were not developed as direct inhibitors of HIF-1. Also, inhibitors of angiogenesis, one of the main effects of active HIF-1 signalling, are currently used in clinical trials. The search for small-molecule inhibitors of HIF-1 is in progress by screening chemical and natural product libraries. So far, only a few have been identified, and the specific HIF-1α inhibitor PX-478 [79] is currently being evaluated in clinical trials.

The anticancer compounds known to inhibit HIF-1 exert their function by inhibiting HIF-1α interaction with its modulating protein partners or by inhibiting different signalling pathways involving HIF-1. Inhibitors against the chaperone protein Hsp-90, which is important for the proper folding of HIF-1α, disrupt the stabilisation of HIF-1α. Examples of Hsp-90 inhibitors are geldanamycin and radicicol, where geldanamycin is the less toxic agent and hence a potentially clinically useful inhibitor [80]. Geldanamycin promotes the degradation of HIF-1α, which results in decreased protein levels [81]. HIF-1 transcriptional activity is under regulation of the PI3K/AKT pathway, and inhibiting PI3K pharmacologically results in decreased HIF-1α activation. By inhibiting mTOR with rapamycin, the stabilization and transactivation of HIF-1α have been reported to decline. It is believed that mTOR is an intermediate between AKT and HIF-1 which are not thought to be directly interacting. Since mTOR has important functions in translation of many proteins, inhibiting this protein will not only influence HIF-1α regulation. Inhibitors of MEK1 (PD098059) and PI3K (LY294002) also lower the levels of HIF-1α protein as well as the protein levels of the downstream target gene product VEGF [81].

Another group of compounds with effects on HIF-1 transcriptional activity and the stabilisation of HIF-1α are topoisomerase I inhibitors such as topotecan [80]. The mechanism of action of this topoisomerase I inhibitor on HIF-1α is not yet fully determined. Also, inhibitors of topoisomerase II, such as GL331, decrease HIF-1α levels presumably via transcriptional repression. Two other cytostatic agents, taxol (taxane) and vincristine (vinca alkaloid), have been reported to decrease HIF-1α protein levels and also affect transactivating activity [81]. The mechanism of action is mediated through disruption of the microtubule cytoskeleton and reveals the importance of the cytoskeleton for HIF-1α functionality.

In general, solid tumours develop hypoxic areas, and in order to survive the harsh environment the tumour cells adapt by changing their genetic expression profile, which ultimately leads to a more aggressive cancer phenotype, as detailed earlier. By inhibiting the central mediator of this shift, HIF-1 tumours might become more responsive to additional treatment, such as radiation and chemotherapy. However, a problem with this HIF-1 targeting remains: the dose of the anticancer compounds administrated often needs to be considerably higher than the therapeutically relevant concentration to also have an effect on HIF-1 regulation [81]. Nevertheless, HIF-1 seems to be a pertinent target for drug development directed at anticancer therapy, especially since hypoxia plays such a central role in solid tumour development. It is clear that the search for and development of HIF-1 inhibitors has been intensified, and it is now important to rapidly evaluate these promising novel cancer compounds in clinical trials. The best therapeutic approach in the treatment of hypoxic tumors is most likely a combination of different types of anti-cancer drugs, since the hypoxia response is such a complex process.

Conclusions and perspectives

Hypoxia is a common phenomenon in malignancies that affects many important tumour biological properties. For breast cancer there are numerous reports studying large numbers of breast cancer samples with various methods, indicating the presence of hypoxia-driven HIF-1α activation as well as alternative activation of HIF-1α in different subsets of the disease. In general, there is a link between expression of hypoxic markers and poor prognosis as well as ERα negativity. HIF-1α might also provide treatment-predictive information by identifying subsets of breast cancer patients who do not respond to conventional treatment even though further studies are needed to better clarify this issue. Hypoxia and many hypoxia-regulated gene products can also serve as novel treatment targets, and in summary the specific targeting of hypoxic processes might be an important additive treatment for breast cancer in the future. The exact role of hypoxia in the selection of tumour properties is complicated, and the question whether hypoxia contributes to a tumour's aggressiveness or whether an aggressive tumour has more widespread hypoxia still stands, even though we favour a combination of both these explanations. An aggressive tumour is prone to consume more oxygen, which in turn results in hypoxia. Alternatively, hypoxic tumours are known to upregulate genes important for tumour growth, invasiveness, angiogenesis, and evading apoptosis, which in turn will lead to a more aggressive phenotype [3,72]. These two hypotheses are contrary to each other in the sense that in the first model reoxygenation

and HIF-1 inhibition would enhance tumour growth and in the second this approach would reduce tumour growth. It is clear that we need to learn more about this important and common process, and indeed consider hypoxia and hypoxia-related mechanisms as important key players in malignancies.

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