

Hypoxia-induced chemoresistance in cancer cells: The role of not only HIF-1

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Background. The aim of this review is to provide the information about molecular basis of hypoxia-induced chemoresistance, focusing on the possibility of diagnostic and therapeutic use.

Results. Hypoxia is a common feature of tumors and represents an independent prognostic factor in many cancers. It is the result of imbalances in the intake and consumption of oxygen caused by abnormal vessels in the tumor and the rapid proliferation of cancer cells. Hypoxia-induced resistance to cisplatin, doxorubicin, etoposide, melphalan, 5-fluorouracil, gemcitabine, and docetaxel has been reported in a number of experiments. Adaptation of tumor cells to hypoxia has important biological effects. The most studied factor responsible for these effects is hypoxia-inducible factor-1 (HIF-1) that significantly contributes to the aggressiveness and chemoresistance of different tumors. The HIF-1 complex, induced by hypoxia, binds to target genes, thereby increasing the expression of many genes. In addition, the expression of hundreds of genes can be also decreased in response to hypoxia in HIF-1 dependent manner, but without the detection of HIF-1 in these genes' promoters. HIF-1 independent mechanisms for drug resistance in hypoxia have been described, however, they are still rarely reported. The first clinical studies focusing on diagnosis of hypoxia and on inhibition of hypoxia-induced changes in cancer cells are starting to yield results.

Conclusions. The adaptation to hypoxia requires many genetic and biochemical responses that regulate one another. Hypoxia-induced resistance is a very complex field and we still know very little about it. Different approaches to circumvent hypoxia in tumors are under development.

Key words: HIF-1, hypoxia, chemoresistance, hypoxia-induced chemoresistance

Received: December 22, 2014; Accepted with revision: May 7, 2015; Available online: May 19, 2015
<http://dx.doi.org/10.5507/bp.2015.025>

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INTRODUCTION - HYPOXIA-INDUCED CHEMORESISTANCE

Hypoxia is a common feature of a solid tumor's microenvironment and represents an independent prognostic factor for a variety of human cancers. For the purposes of this paper, references to tumor hypoxia can refer to the tumor as a whole or any part thereof in which hypoxia create a microenvironment. It is the result of imbalances in the intake and consumption of oxygen. This results from vascularization that is structurally and functionally abnormal, coupled with high proliferation rates in tumor cells¹.

The negative impact of hypoxia on cancer cells relative to the efficacy of radio- and chemotherapy has been known for several decades and the survival rate of patients with severely hypoxic tumors is shorter than patients with less hypoxic tumours². In radiotherapy, low oxygen levels prevent the formation of DNA strand breaks induced by radiation, and inhibits repair of DNA damage that induces genetic instability^{3,4}. Hypoxia-induced resistance to cisplatin, doxorubicin, etoposide, melphalan, 5-fluorouracil, gemcitabine, and docetaxel has been previously reported in a number of tumor cell types⁵⁻²³.

Hypoxia regulates approximately 1% of the genes that play a role in the signaling pathways that control various aspects of tumor progression²⁴. Adaptation of tumor cells to hypoxic conditions has important biological effects and

contributes to the aggressive nature of tumors and their dedifferentiation and chemoresistance. In this context, the most studied transcription factor is hypoxia-inducible factor-1 (HIF-1). HIF-1 is an important factor in the adaptation of cells to hypoxic environments, and significantly contributes to the aggressiveness and chemoresistance of number of different tumours^{5,7-9,15,19,21,25-30}. However, these changes can be independent of HIF-1 (ref.^{26,31}). In several cell types, hypoxia-induced drug resistance is unaffected or only partially reversed by HIF-1 inhibition, suggesting the existence of HIF-1 independent mechanisms of hypoxia-induced drug resistance. Other reasons for chemoresistance of cells in hypoxic tumors include acidosis, nutrient starvation that inhibits cell proliferation, increased interstitial fluid pressure, and low drug concentrations. Tumors that are hypoxic because of abnormal vascularization also have decreased drug bioavailability³². Therefore, inadequate pharmacokinetics in these areas may reduce the effectiveness of anticancer drugs even though they are effective under hypoxic conditions or even inhibit HIF-1 α . Furthermore, HIF-1 knockdown cells can normally grow and survive in hypoxic conditions^{7,33}. HIF-1 independent mechanisms of drug resistance in hypoxia are under-investigation, but are still rarely reported.

The aim of this review is to provide general information about the molecular basis of hypoxia-induced chemo-

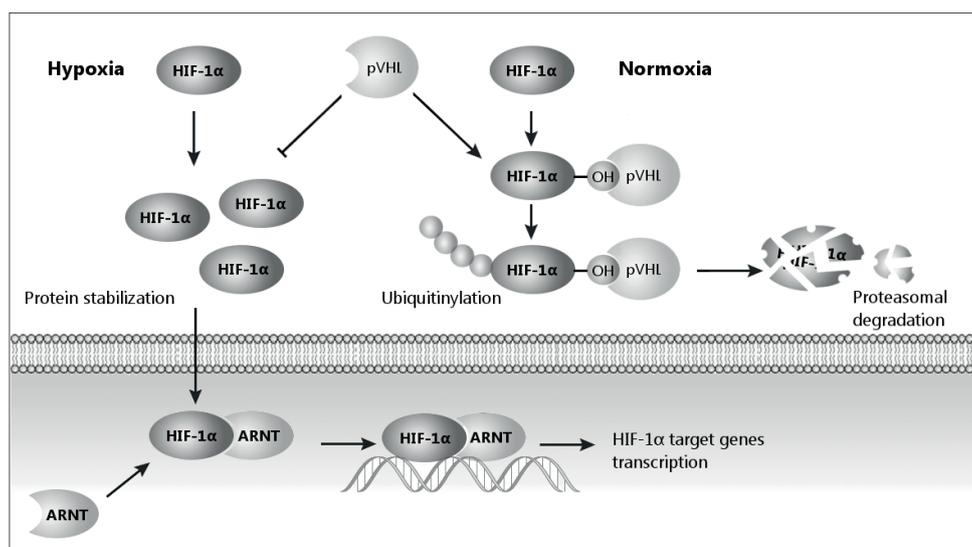


Fig. 1. Regulation of HIF-1 α stability in hypoxia and normoxia (adapted according to ref.⁴¹).

Table 1. Classification of HIF-1 regulated genes (modified according to ref.^{42,43}).

Biological functions	Gene symbol/alias
Transcription factors	TWIST1, Snail, ZEB1, ZEB2, ID2, SMAD7, PPAR γ , GATA1
Histone modifiers	JMJD2B, JMJD2C, MLL1
Enzymes	MMP1, MMP3, LOX, ADAMTS1, ACE, ACE2, XPA, HK1, HK2
Receptors, receptor-associated kinase	CXCR4, CX3CR1, uPAR, PAI-1, 67-kDa laminin receptor, c-Met
Small GTPases, intracellular signaling molecules	VEGF, TGF- α , TGF- β 3, IGF2, Cdc42, Rac1, RhoE, IRS-2
Transporters	glut-1, glut-3, MDR1, VDAC1, transferrin, ceruloplasmin, IGF-BP1 - 3
Membrane proteins	ANGPTL4, L1CAM, α 5 integrin, CD151, CD24, CD147, Galectin-1 and 3, MUC1, Semaphorin 4D, Caveolin-1
Scaffold protein, cytoplasmic protein	HEF1, Lipirin- α 4
Matricellular proteins	CYR61, NOV

resistance, focusing on diagnostic and therapeutic uses in clinical oncology.

THE ROLE OF HIF-1

HIF-1 is a master regulator of oxygen homeostasis with pleiotropic effects. It is a heterodimeric transcription factor composed of 2 subunits – an oxygen-regulated subunit HIF-1 α and a stable constitutively expressed nuclear factor, HIF-1 β (aryl hydrocarbon receptor nuclear translocator, ARNT). Each subunit contains bHLH-PAS domains that mediate heterodimerization and DNA binding. Under normoxic conditions HIF-1 α is hydroxylated by prolyl hydroxylase (PHD) at prolines 402 and 564, and hydroxylated HIF-1 α recruits von Hippel-Lindau protein (pVHL), an E3 ubiquitin protein ligase, and is degraded

by the 26S proteasome after being targeted for ubiquitinylation³⁴⁻³⁶. Recently, an additional mechanism has been recognized (by which HIF-1 α is degraded in lysosomes via chaperone mediated autophagy, including HSC70 and LAMP2A) that is independent of proteasome activity³⁷. Under hypoxic conditions, cytosolic HIF-1 α is stabilized by inhibition of PHD-dependent enzymatic hydroxylation of proline residues and subsequently is translocated to the nucleus, where it binds to HIF-1 β (ref.³⁴⁻³⁶). The HIF-1 complex then binds to the hypoxia-response element (HRE) of the target genes (Fig. 1) (ref.³⁵), which causes increased expression of hundreds of genes (Table 1) (ref.³⁴). In addition, the expression of many other genes is decreased in response to hypoxia (in a HIF-1 dependent manner), but without the detection of HIF-1 in the gene promoters³⁸. Moreover, tumor hypoxia (in a HIF-1 dependent manner) may upregulate receptor tyrosine kinases,

including EGFR, through the repression of rabaptin-5 transcription³⁹. Many effects of HIF-1 are therefore made at the level of gene transcription; however, the direct interactions of HIF-1 with other proteins, e.g. interaction between HIF-1 α and c-myc or p53, remain very important⁴⁰.

During hypoxia, the pVHL-dependent proteasomal degradation of HIF-1 α does not occur. Thus, HIF-1 α is stabilized and transported to the nucleus where it forms a transcriptional complex with the ARNT/HIF-1 β subunit. In the presence of transcriptional coactivators (not shown) HIF-1 complex can cause transactivation of the target genes leading to cell adaptation to hypoxic conditions.

Besides HIF-1 α , there are two other transcription factors in this family of proteins, HIF-2 α and HIF-3 α . The regulation and function of HIF-3 α is not yet entirely clear, it is the least-studied protein of these factors⁴⁴. HIF-2 α , also known as EPAS1 (Endothelial PAS domain-containing protein 1) is highly homologous to HIF-1 α , i.e., they share very similar protein structure and the mechanism of their stabilization and action are also very similar⁴⁵. Despite the similarities, HIF-2 α has several different transcriptional targets than HIF-1 α , which can also be activated during severe hypoxia⁴⁶. HIF-2 α can be detected at near physiological pO₂ and its expression is tissue specific. Neuroblastoma cells in contact with blood vessels, and stromal cells surrounding the tumor have been shown to be HIF-2 α positive⁴⁷. Overexpression of HIF-2 α is also observed in hemangioblastoma cells, which are very rich in blood vessels⁴⁸. One specific HIF-2 α target gene is *Oct-4*, which encodes a transcription factor essential for the pluripotency of stem cells. Its activation is responsible for tumor cell dedifferentiation that has been demonstrated in neuroblastomas, breast cancer, and is probably the same in other tumours⁴⁹⁻⁵¹. Activation of the Notch signaling pathway also participates in the loss of cell differentiation^{52,53}.

Moreover, HIF-1 regulates several miRNAs while other miRNAs target HIF-1. Changes in miRNA expres-

sion, in response to hypoxia, are shown in Table 2. The interaction between miRNAs and HIF-1 may influence many signaling pathways important for angiogenesis, metabolism, apoptosis, cell cycle regulation, proliferation, metastasis, and resistance to anticancer therapy⁵⁴.

HIF-1 AND CHEMORESISTANCE

The association of HIF-1 α overexpression with cell proliferation and poor prognosis has been observed in many human cancers^{35,36,55}. Many of these HIF-1 inducible genes such as VEGF, Glut-1, MDR, and Bcl-2 directly or indirectly mediate chemoresistance¹⁹. Therefore, in a wide range of different tumor types, e.g. hepatocellular carcinoma, neuroblastoma, and lung cancer, inhibition of HIF-1 α resensitizes cells to drug treatment in hypoxia and is then a valid target to reverse hypoxia-induced drug resistance^{8,9,15,19,21,25,27-30,56}. HIF-1 α accumulation has been associated with shorter survival in patients with early stage cervical, breast, ovarian, endometrial, oropharyngeal squamous cell carcinoma, and oligodendroglioma. Significant associations between HIF-2 α overexpression and increased patient mortality have been reported for other diseases, including non-small-cell lung cancer (NSCLC), neuroblastoma, astrocytoma, and head, and neck squamous cell carcinoma⁵⁷.

Hypoxia-induced drug resistance in tumors has been reported for vincristine, doxorubicin, cisplatin, etoposide in neuroblastoma cells^{58,59}; doxorubicin, vincristine, and actinomycin-D in rhabdomyosarcoma and Ewing's sarcoma cells^{15,26}; cisplatin, doxorubicin, and etoposide in osteosarcoma⁶⁰; 5-fluorouracil, paclitaxel, doxorubicin, gemcitabine, and cisplatin in head and neck squamous cell carcinoma^{61,62}; 5-fluorouracil and cisplatin in gastric cancer⁶³; cisplatin and doxorubicin in NSCLC (ref.⁸); and doxorubicin, etoposide, vincristine, and methotrexate in acute lymphoblastic leukemia⁶⁴. The data came mainly from *in vitro* studies. Known HIF-1 transcriptional tar-

Table 2. Examples of changes of miRNAs expression in response to hypoxia (adapted according to ref.⁵⁴). mRNAs can be up-regulated as well as down-regulated, which reflects the complexity of miRNA in hypoxia regulation.

Up-regulated miRNAs	The details of regulation
miR-10b, miR-372/miR-373	regulated by HIF-1 α (via TWIST)
miR-103/miR-107	repress DAPK and KLF4 (role in metastasis)
miR-155	regulated by HIF-1 α , can also negatively influence the stability and activity of HIF-1 α
miR-21	regulated by Akt2 (via NF- κ B and CREB) or RAS/ERK signaling, activate Akt and ERK1/2 pathways leading to the elevation of HIF-1 α and VEGF (via targeting PTEN)
miR-210	stabilize HIF-1 α (via GPD1L repression), inhibit the expression of E2F3 and MNT (control cell cycle progression), down-regulates VMP1
miR-424	suppress CUL2 (can stabilize HIF-1 α)
miR-519c	regulates HIF-1 α (angiogenesis)
Down-regulated miRNAs	
miR-17-92	regulated by wild-type p53, stabilize HIF-1 α
miR-107	reduce the inhibition of HIF-1 β (promotes angiogenesis)
miR-20b, miR-200b	regulated by HIF-1 α
miR-20b, miR-199	stabilize HIF-1 α

Table 3. Overview of HIF-1 mediated drug resistance mechanisms (modified according to ref.³²).

Cancer cell model	Drug/molecule	Resistance phenotype
Breast cancer	etoposide	DNA damage inhibition
	methotrexate	drug efflux
	paclitaxel, docetaxel	apoptosis inhibition
Colon cancer	adriamycin	drug efflux
	etoposide, oxaliplatin	apoptosis inhibition
Fibrosarcoma	cisplatin, etoposide	apoptosis inhibition
Gastric cancer	multiple drugs	drug efflux; apoptosis inhibition
	5-fluoruracil	apoptosis inhibition; senescence inhibition
Glioblastoma	adriamycin	drug efflux
Glioma	etoposide, doxorubicin	drug efflux
HCC	5-fluoruracil	drug efflux
	etoposide	apoptosis inhibition
HeLa cells	4-HPR	apoptosis inhibition
HNSCC	paclitaxel	apoptosis inhibition
Neuroblastoma	etoposide, vincristine	apoptosis inhibition
OSCC	5-fluoruracil, cisplatin	drug efflux; ROS decrease
Prostate cancer	etoposide	DNA damage inhibition
	flutamide	apoptosis inhibition

gets may induce drug resistance by affecting drug transporters, e.g. increased p-glycoprotein (MDR1, multidrug resistance 1) (ref.⁶⁵); drug targets, e.g. decreased topoisomerase II (ref.²⁵); or by changing the response to drugs, for instance by modifying drug-induced apoptosis⁵⁸, reducing drug induced senescence⁹, or inducing autophagy in response to drugs (Table 3) (ref.²⁷). It has been shown that HIF-1 inhibition reverses multidrug resistance in colon cancer⁶⁶ and HIF-1 α knockout cells are more sensitive to cytostatics and irradiation than wild-types³².

HIF-1 AS A PRO-OR ANTIAPOPTOTIC FACTOR

Besides the protective responses, HIF-1 and/or hypoxia have also been shown to be either anti-apoptotic or proapoptotic, based on cell type and severity of hypoxia. More severe or prolonged hypoxia induces apoptosis that is, at least in part, initiated by the direct association of HIF-1 α and p53 (ref.^{67,68}). Among proapoptotic genes regulated by HIF-1 are p53 (whose relation to HIF-1 is, however, very complicated and often seems to be controversial) and genes belonging to the Bcl-2 family of proteins – BNIP3, NIX and NOXA (ref.^{69,70}).

BNIP3 (Bcl-2/adenovirus E1B 19 kDa interacting protein 3) is expressed in the brain and skeletal muscles under normal conditions⁷¹, but its function is unknown. In most other tissues, BNIP3 expression is induced by HIF-1 under hypoxic conditions. BNIP3 has also been shown to induce cell type specific necrosis, apoptosis, and autophagy^{72,67}. Proapoptotic functions of BNIP3 have been well documented in cardiomyocytes. On the other

hand, while BNIP3 is expressed in hypoxic regions of tumors it fails to induce cell death. In ovarian cancer, the overexpression of BNIP3 truncated forms with mutations in bcl2-binding domain fail to induce cell death. Furthermore, the BNIP3 proapoptotic effect can be circumvented by EGFR signaling in epithelial cancer cells. Therefore, whether hypoxia-induced BNIP3 has a pro- or anti-apoptotic effect depends on the context and upon what genetic changes the cancer cells experience. From this point of view, hypoxia creates a strong selective pressure and selects cells with survival advantages and cell death resistance. *Pro-apoptotic transcription factor p53* is considered to be the most common inhibitor of cellular proliferation as well as inducer of apoptosis. When cells sense a decrease in oxygen, HIF-1 can enhance the stability of p53 and these two transcription factors cooperate with each other to induce apoptosis that is p53-HIF-1-dependent⁶⁸. The direct interaction between p53 and HIF-1 α results in not only p53 stabilization but also inhibition of HIF-1-dependent transcription. This effect may explain the competition between p53 and HIF-1 for the p300 coactivator as well as MDM2 dependent degradation of HIF-1 α via formation of the p53/HIF-1/MDM2 complex (ref.^{73,74}). However, the precise relationship of HIF-1 and p53 is still very complicated and unclear. The interaction between HIF-1 and p53 has been previously and comprehensively reviewed⁷⁵. Based on the above-mentioned information, apoptosis or cell survival in hypoxic regions of tumors probably depends on the type of tumor and the presence or absence of genetic alterations that affect the balance between pro-apoptotic and anti-apoptotic roles of hypoxia-induced factors⁷⁶.

OTHER FACTORS RESPONSIBLE FOR HYPOXIA-INDUCED CHEMORESISTANCE

Other factors besides HIF-1 must be responsible for the cellular response to hypoxia and hypoxia-induced chemoresistance. A number of other cellular pathways are differentially regulated in hypoxia and may also contribute to hypoxia-induced drug resistance⁷⁷⁻⁸¹. It has been shown that the suppression of HIF-1 α through gene knockdown or the use of small molecule inhibitors reduces resistance to cytostatics, such as cisplatin, etoposide, doxorubicin, and ellipticine, but does not suppress it completely^{20,33,59,60}. The fact that in some cell lines (e.g. neuroblastoma, osteosarcoma, and lung adenocarcinoma) chemoresistance persists or is only partially reversed, despite HIF-1 being eliminated, points to alternative hypoxia-induced survival pathways³³.

Hypoxia selects more resistant cells having aggressive and metastatic phenotypes, which are often associated with poor prognoses, e.g., prostate cancer cells. This resistance to hypoxia-induced apoptosis may be due to the increased expression of anti-apoptotic factors including the IAP3 and Bcl-2 family of proteins and is independent on HIF-1 (ref.⁸²). Inhibition of the phosphoinositol-3-kinase (PI3K) pathway, nuclear factor kappa-B (NF- κ B), cyclooxygenase-2 (COX-2), activator protein-1 (AP-1), c-jun, Pim-1, and STAT-3 can reverse resistance to cytostatics in hypoxia, implying a role for these pathways in hypoxia-induced drug resistance^{10,18,22,23,33,77,83,84}. However, the degree in which they are dependent on functional HIF-1 is uncertain.

Nuclear factor- κ B (NF- κ B) is a ubiquitous transcription factor that is composed mainly of homo- or heterodimers of p50 and p65. NF- κ B has been found to be overexpressed in tumors and to be activated by hypoxia^{85,86}. However, whether hypoxia-induced NF- κ B activation contributes to tumor cell resistance or sensitivity to chemotherapy is still unknown. The contribution of NF- κ B to chemoresistance has been reported in many cancers. Under resting conditions, NF- κ B is held inactive in the cytoplasm, bound to NF- κ B inhibitors (I κ B α , I κ B β , I κ B γ , and I κ B ϵ) (ref.⁸⁷). Upon stimulation with different substances, such as tumor necrosis factor alpha (TNF- α), I κ Bs are ubiquitinated and degraded and NF- κ B is released and translocated to the nucleus^{88,89}. NF- κ B then activates the transcription of target genes involved in angiogenesis, cell proliferation, and survival⁹⁰. NF- κ B up-regulates the expression of anti-apoptotic genes such as Bcl-2 (ref.⁹¹), cellular inhibitors of apoptosis (c-IAPs), and Bcl-xL (ref.^{92,93}). However, other reports have suggested that NF- κ B could also function as a tumor suppressor and the inhibition of its activity could counteract the effects of chemotherapy^{94,95}. Moreover, NF- κ B subunits p50 and p65 have been shown to directly interact with HIF-1, during hypoxia, at a distinct element in the proximal promoter of the HIF-1 α gene^{96,97}, and these two transcription factors appear to share common regulatory pathways. Hypoxia may stop suppression of NF- κ B activity through decreased PHD-dependent hydroxylation of IKK- β (I κ B kinase subunit beta) (ref.⁹⁸). All the observations, taken together,

leave no doubt that the cross-talk between NF- κ B and HIF-1 is of an importance in the treatment of cancer.

Cyclooxygenase-2 (COX-2) and sphingosine kinase 2 (SphK2). It is thought that COX-2 inhibitors prevent the development of resistance to different cytostatics and that COX-2 overexpression induces increased multidrug resistance protein 1 (MRP1) expression in different cancer cells⁹⁹⁻¹⁰². Schnitzer et al. showed that COX-2 is up-regulated under hypoxia in a HIF-1-independent fashion and sphingosine-1-phosphate (S1P) is responsible for COX-2 induction under hypoxia in A549 lung cancer cells¹⁰³. Simultaneous inhibition of HIF-1 and COX-2 strongly suppresses chemoresistance and the inhibition of only COX-2 decreases chemoresistance of cancer cells in hypoxia³³. S1P formation and release are promoted by SphK2 expression, which is also responsible for enhancing its activity. Most likely, hypoxia causes an increase in SphK2 by achieving post-translational protein modification. However, the enhancing of mRNA transcription and translation or affecting mRNA stability is also possible¹⁰⁴⁻¹⁰⁶. The extracellular S1P activity leads to chemoresistance mediated via S1P1/S1P3 receptors and p42/44 MAPK-dependent signaling pathways as well¹⁰⁶.

Protein survival kinases PI3K and Akt. Phosphatidylinositol-3 kinases (PI3K) phosphorylate inositol phospholipids, generate the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) on the inner surface of the plasma membrane. Phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) interacts with these phospholipids, causing its translocation to the inner membrane where it is phosphorylated and activated by pyruvate dehydrogenase kinases 1 and 2 (PDK1 and PDK2). Activated Akt modulates the function of numerous substrates involved in the regulation of cell survival, cell cycles, and cell growth¹⁰⁷. The PI3K/Akt signaling pathway is frequently affected in human cancers, which can induce angiogenesis and oncogenic transition, both in a HIF-1-dependent and -independent manner. Activation of PI3K and Akt protect cells against apoptosis and induces drug resistance during both normoxia and hypoxia. PI3K/Akt inhibition is able to reverse this resistance^{10,80}. In pancreatic cancer, hypoxia-induced resistance to gemcitabine was shown to be also partially dependent on the MAPK (Erk) signaling pathway and NF- κ B (see above). Therefore, a combination of tyrosine kinase inhibitor with gemcitabine should be an effective therapy for pancreatic cancer. It has been shown that hypoxia increases phosphorylation of Akt (ref.¹⁰), but still, similar to other signals, the mechanism of hypoxia-mediated PI3K/Akt-dependent chemoresistance has not been laid out in detail.

Signal transducer and activator of transcription 3 (STAT3). It has been shown that enhanced glycolysis in hypoxic tumor cells is dependent on HIF-1 α up-regulation, while reduced mitochondrial activity is HIF-1 α -independent and likely caused by STAT3-mediated down-regulation of mitochondrial proteins. Constitutively active pro-oncogenic transcription factor STAT3 acts as a regulator of cell metabolism, inducing aerobic glycolysis and down-regulating mitochondrial activity, both in primary fibroblasts and in STAT3-dependent tumor cell

lines. As a result, cells are protected from apoptosis and senescence while becoming highly sensitive to glucose deprivation¹⁰⁸. STAT3 activation is significantly increased by the tumor hypoxia and is accompanied by increased ROS generation²³. Moreover, STAT3 directly regulates both cell-proliferation and pro-survival genes, both of which provide growth advantages to tumor cells by blocking pro-apoptotic genes^{23,109}. Pharmacological inhibitors of STAT3 have been shown to significantly reduce cell proliferation capacity under hypoxic conditions and induce apoptosis in a variety of human cancer cells, e.g. breast, prostate, colon, and ovarian cancers¹¹⁰⁻¹¹². Also, STAT3 inhibition partially overcame hypoxia-induced chemoresistance to cisplatin and taxol treatment²³. Therefore, it seems that STAT3 activation might contribute to cellular resistance to anticancer drug-induced apoptosis. However, how STAT3 is activated in hypoxic cancer cells remains an intriguing question and it is obvious that STAT3 can act both HIF-1 α dependently or independently. It is reported that the VEGF promoter contains various transcription factor binding sites including STAT3 (ref.¹¹³) and HIF-1 (ref.¹¹⁴). In hypoxia, STAT3 can interact with HIF-1 α , increase its levels¹⁰⁹ while both transcription factors control VEGF transcriptional activation leading to maximum activity and angiogenesis. Therefore, targeting STAT3/HIF-1 α /VEGF signaling can be a potent anti-angiogenic strategy in tumors^{115,116}.

Pim kinases. The Pim serine/threonine kinases Pim-1, Pim-2, and Pim-3 regulate signaling pathways that contribute to tumor cell proliferation, expression of survival proteins and drug efflux transporters, and inhibit apoptosis. They affect tumor cell proliferation and survival through multiple mechanisms that include inhibition of major signaling pathways, interference with the activity of specific cell cycle molecules, and changes in gene expression¹¹⁷. Overexpression of Pim kinases has been observed in patients with non-Hodgkin's lymphoma, leukemia, and prostate cancer¹¹⁸⁻¹²¹. The up-regulation of Pim kinases by hypoxia appears to be limited to Pim-1 (ref.¹¹⁷) and is HIF-1 independent^{18,19}. Hypoxia-induced expression of Pim-1 is associated with metastasis and worse treatment response, while inhibition of Pim-1 suppresses cell proliferation and migration, induces apoptosis, and increases sensitivity to cytotoxic drugs, e.g. to cisplatin, doxorubicin, and gemcitabine in pancreatic carcinoma¹⁸ or to docetaxel in prostate cancer cells^{122,123}; this was found to be true under hypoxic conditions both *in vitro* and *in vivo*^{18,117}. The Pim-1 expression level also correlates with tumor prognosis¹²⁴.

Pim-1 induces resistance to anticancer drugs through stabilization of the mitochondrial transmembrane potential and regulates apoptosis, the cell cycle, and gene transcription by phosphorylating target proteins such as Bad, p21/waf1, c-Myb, and c-Myc (ref.^{18,125-130}). Pim-1 down-regulates p27(Kip1) both at the transcriptional and post-translational level. p27(Kip1) is a cyclin-dependent kinase inhibitor 1B (CDKN1B) and its mutation or down-regulation can lead to the loss of control over the cell cycle and to the uncontrolled cell proliferation¹³¹. Pim-1 may also affect the NF- κ B pathway by phosphorylation and stabilization of RelA/p65, an important component

of the NF- κ B signaling pathway¹³², and facilitates signaling through the NF- κ B pathway by down-regulating the expression of TNFAIP3/A20, a negative inhibitor of NF- κ B signaling¹³³. Pim-1 can also phosphorylate P-glycoprotein (MDR-1 gene product), which results in its protection from ubiquitination and proteasomal degradation. Its inhibition reverses resistance to doxorubicin in ovarian cancer cells that overexpress P-glycoprotein¹³⁴. Experiments that directly link the effects of Pim-1 on NF- κ B signaling and increased resistance to cytostatic drugs constitute a valuable and interesting field for further study¹¹⁷.

Carbonic anhydrases (CAs) are metalloenzymes that catalyze the interconversion of carbon dioxide and water to bicarbonate and protons to maintain the acid-base balance in blood and tissues, and to help transport carbon dioxide out of tissues. Hypoxia induces expression of carbonic anhydrase isoform IX (CA IX) via the HIF-1 pathway in many tumors. The role of CA IX in hypoxic tumor acidification processes has been demonstrated¹³⁵. CA IX decreases extracellular pH, disrupts cellular adhesion by degrading the catenin-cadherin complex or the integrin-mediated cell adhesion system, and induces up-regulation of the genes involved in invasion and migration, and causes resistance to weakly basic cytostatics^{136,137}. An explanation of the changes to the cell adhesion system appears to be linked to acidosis caused by carbonic acid produced by the CA IX-mediated catalytic transformation of carbon dioxide. It is assumed that CA IX inhibition, which normalizes tissue pH, might cause tumor regression.

METABOLISM, MITOCHONDRIA AND ATP

The adaptation to hypoxia requires not only the above mentioned changes of expression, but also biochemical responses that counter-balance the decrease in energy supply from mitochondrial respiration. The metabolites influence expression of genes which regulate biochemical pathways that contribute to the metabolic signature of hypoxic cells¹³⁸. Altered energy metabolism in cancer cells, such as decreased mitochondrial functions and enhanced aerobic glycolysis, is not only a fundamental phenotype of malignant tumors, but also play important roles during tumor progression, metastasis, and relapse^{139,140} and is suspected to contribute to chemoresistance. The precise mechanisms, though, are still unclear¹⁴¹.

It has been shown that the increase in glucose uptake during hypoxia was not reversed by HIF-1 α silencing, indicating that bio-energetic needs can also be met by direct biochemical alterations without long-term genetic adaptation¹³⁸. ATP deprivation occurs in all types of cell death, during late-stage apoptosis, in autophagy, and during necrosis. Therefore, it is suggested that energy metabolism plays a critical role in the survival of cancer cells under stress^{142,143}. Moreover, drug-resistant cells were characterized by defective mitochondrial ATP production, elevated aerobic glycolysis, higher levels of intracellular ATP, and enhanced HIF-1-mediated signaling. Chemoresistant cells need faster ATP generating mechanisms to survive. They consume higher amounts of ATP via the endoplasmic

reticulum enzyme UDPase ENTPD5, which in turn enhances aerobic glycolysis/fermentation¹⁴⁴. Under these conditions, HIF-1 acts as a key glycolysis regulator and is up-regulated independently of oxygen levels¹⁴¹. HIF-1 also regulates mitochondrial functions. It has been shown that HIF-1 stabilization under hypoxia leads to the expression of PDK1 (ref.¹⁴⁵) protein that phosphorylates and inactivates pyruvate dehydrogenase (PDH), and limits the conversion of pyruvate to acetyl-CoA in the mitochondria. Consequently, PDK1 induction decreases the citric acid cycle (TCA cycle) activity and reduces oxygen consumption¹³⁸.

Intracellular ATP levels are a core determinant in the development of acquired drug resistance of human colon cancer cells that harbor different genetic backgrounds. ATP induced resistance to oxaliplatin and to 5-fluorouracil can be seen in colorectal cancer cells. Conversely, depleting ATP by cell treatment with a glycolysis inhibitor, sensitizes cells resistant to oxaliplatin and also increased sensitivity to 5-fluorouracil¹⁴¹.

It seems that under hypoxia, autophagy is required to support ATP production in addition to glycolysis and its inhibition might be used to selectively target hypoxic regions of tumors. Based on the above mentioned, an understanding of metabolic adaptations under hypoxia could reveal novel pathways that, if targeted, might lead to specific death of hypoxic regions while sparing normal cells that are rarely exposed to hypoxia. Mitochondria are the major oxygen consumers in the cell and are in the heart of several biochemical pathways involved in both energy production and generation of anabolic intermediates that are required for proliferation. Therefore, changes in mitochondrial function under hypoxia are likely to contribute to the altered metabolic profile of cells, making mitochondria important oxygen sensors. They can respire at very low oxygen levels and the rate of respiration of cells adapted to chronic hypoxia demonstrates that they retain active respiration, though at significantly reduced rates. Still, while genetic reprogramming during hypoxia has been characterized and many players in this process have been identified, the contribution of mitochondria to the hypoxic response is remains uncertain¹³⁸.

SIDE POPULATION AND CD133

Cancer stem cell (CSC) models propose that tumors consist of a variety of cells with different proliferative and tumorigenic capacities. According to this theory, small populations of CSC have been suggested as drivers of tumor growth and are responsible for resistance to therapy, recurrence, and metastasis. In hypoxic tumors, CSCs are thought to be regulated by HIF-1 α and HIF-2 α for survival and protection of tumor growth¹⁴⁶. CD133 is one of the most important markers for CSC in a variety of solid tumors. It has been reported in glioma cells that the number of CD133+ cells (CSC) (ref.¹⁴⁷) or expression of CD133 in both SCLC and NSCLC cells¹⁴⁸ was correlated with hypoxia-induced increase of HIF-1 α . In pancreatic cancer cells, effects of hypoxia on up-regulation of CD133 was

diminished after knocking down HIF-1 α (ref.¹⁴⁹). Another study suggested that laryngeal cancer cells, subjected to hypoxia followed by normoxia, exhibited enhanced capacities for proliferation, invasion, sphere, and colony formation in comparison with cells which were maintained consistently under normoxia. These events were associated with an increasing CD133+ fraction denoting regulation of cancer stem cells by hypoxic microenvironments¹⁵⁰. Moreover, Zhu et al. has demonstrated the role of HIF-1 in intermittent hypoxia on promoting the metastatic ability and invasiveness of CD133+ pancreatic cancer stem-like cells¹⁵¹.

Even though, most of the literature data shows the up-regulation of CD133 during hypoxia, controversy of the regulatory mechanisms of CD133 expression via mTOR signaling and HIF-1 α in cancer cells might lead insights into the role of mTOR and oxygen-sensitive intracellular pathways in the maintenance of stemness in CSC (ref.¹⁵²). It is possible to speculate that in some tumors, one of the mechanisms for hypoxia-induced resistance, may be induction of CSC.

FUTURE PERSPECTIVES

Targeting hypoxic cells within solid tumors seems to be a promising treatment modality to overcome resistance and prevent recurrence¹⁵³. Many different approaches to overcome hypoxia-induced chemo- and/or radioresistance, including inhibitors of HIF-1, or hyperoxia, have been developed.

The first clinical studies focused on diagnosis of hypoxia and on the inhibition of hypoxia-induced changes in tumor cells have begun to yield results. Hu et al. have tested (18)F-fluoroerythronitroimidazole PET/CT that uses an assessment of tumor hypoxia to evaluate the prognosis on a group of thirty-two NSCLC patients¹⁵⁴. A clinical study examining antisense oligodeoxynucleotide binding HIF-1 α showed a decrease in HIF-1 protein levels and mRNA levels in some of its target genes in tumor cells¹⁵⁵. Kummar et al. proved that topotecan can decrease expression of HIF-1 α and some HIF-1 target genes like VEGF and GLUT-1 in solid tumors¹⁵⁶. Other clinical studies have shown that celecoxib, a selective COX-2 inhibitor, decreased tumor cell proliferation, microvessel density, angiogenesis, HIF-1 levels, and enhanced apoptosis in prostate cancer¹⁵⁷. The newly developed prodrug TH-303 (which consist of 2-nitroimidazole covalently linked to brominated isophosphoramidate mustard i.e. the prodrug) and which, under hypoxic conditions, is converted into brominated isophosphoramidate mustard (i.e. the active cytotoxic drug) seems to be promising. Phase I clinical trials with this drug have already been completed¹⁵⁸.

Studies using hyperbaric oxygen therapy (HBOT) with chemotherapy have shown synergistic effects both *in vitro* and *in vivo* (for review see ref.¹⁵⁹). Moreover, clinical studies in patients with gliomas¹⁶⁰ and NSCLC (ref.¹⁶¹) have shown promising results, and several studies with the combination of HBOT and radiotherapy have been also performed¹⁶². However, this approach is still in its

infancy and a number of questions, e.g. a sequence of individual modalities, duration, and intensity of HBOT need to be resolved.

CONCLUSIONS

In this review we have summarized the most important factors and pathways that contribute to hypoxia-induced chemoresistance in cancer cells. The adaptation to hypoxia requires many genetic and biochemical responses that regulate one another, revealing that hypoxia-induced resistance is a very complex and intricate field about which we still know very little. Since hypoxia is closely associated with chemo- and radioresistance, different approaches are continuously being developed in an effort to circumvent hypoxia-induced resistance.

ABBREVIATIONS

AP-1, Activator protein-1; ARNT, Aryl hydrocarbon receptor nuclear translocator; Bad, Bcl-2-associated death promoter; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra-large; bHLH-PAS, basic helix-loop-helix - period circadian protein Ah receptor nuclear translocator protein and single-minded protein; BNIP3, Bcl-2/adeno-virus E1B 19 kDa interacting protein 3; CA IX, Carbonic anhydrase isoform IX; c-Myb, myeloblastosis family of transcription factors; c-myc, v-myc avian myelocytomatosis viral oncogene homolog c; COX-2, Cyclooxygenase-2; CDKN1B, Cyclin-dependent kinase inhibitor 1B; EGFR, Epidermal growth factor receptor; EPAS1, Endothelial PAS domain-containing protein 1; Glut-1, Glucose transporter 1; HBOT, Hyperbaric oxygen therapy; HIF-1, Hypoxia-inducible factor-1; HRE, Hypoxia-response element; HSC70, Heat shock cognate protein 70; IAP31, inhibitor of apoptosis 31; c-IAPs, Inhibitors of apoptosis; I κ B α , I κ B β , I κ B γ , and I κ B ϵ , NF- κ B inhibitors α , β , γ , and ϵ ; LAMP2A, Lysosome-associated membrane protein 2; MDM2, Mouse double minute 2 homolog; MDR, Multiple drug resistance gene; MRP1, Multidrug resistance protein 1; mTOR, Mammalian target of rapamycin; NF- κ B, Nuclear factor- κ B; NIX, Nip3-like protein X; NSCLC, Non-small-cell lung cancer; Oct-4, Octamer-binding transcription factor 4; p21/waf1, Cyclin-dependent kinase inhibitor 1; PDK1 and PDK2, Pyruvate dehydrogenase kinases 1 and 2; PDH, Pyruvate dehydrogenase; PET/CT, Positron emission tomography-computed tomography; PHD, Prolyl hydroxylase; PI3K, Phosphoinositol-3-kinase; Pim-1, Pim-2, and Pim-3, Pim serine/threonine kinases 1, 2 and 3; PIP₃, Phosphatidylinositol (3,4,5)-trisphosphate; RelA/p65, Nuclear factor NF-kappa-B p65 subunit; S1P, Sphingosine-1-phosphate; SCLC, Small-cell lung cancer; SphK2, Sphingosine kinase 2; STAT3, Signal transducer and activator of transcription 3; TCA cycle, Citric acid cycle; TNFAIP3/A20, Tumor necrosis factor, alpha-induced protein 3; TNF- α , Tumor necrosis factor α ; VEGF, Vascular endothelial growth factor; VHL - von Hippel-Lindau protein.

ACKNOWLEDGMENT

Supported by Charles University in Prague, project GA UK No 620612 and by the Ministry of Health of the Czech Republic for conceptual development of research organization 00064203 (University Hospital Motol, Prague, Czech Republic).

Author contributions: JH: wrote the chapters "Hif-1 as a pro- and anti-apoptotic factor" and "Other factors responsible for hypoxia induced chemoresistance"; MAK: wrote the chapter "Side population and CD133"; TE: wrote the chapters "Future perspectives" and "Conclusions" and final revision; HD: wrote all other chapters. All co-authors participated in the conception of the manuscript and revised the whole manuscript.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

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