Review paper

Hypoxia in breast cancer

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Breast cancer continues to be one of the most common malignancies and is a serious problem particularly in women, although men may also be affected. Such lesions are commonly accompanied by hypoxia, and therefore hypoxia-dependent mechanisms, such as overexpression of hypoxia-inducible factor (HIF), the mechanisms are studied as part of the search for a novel method of cancer treatment. Blocking the activity of HIF and HIF-dependent molecular changes raises hopes for identification of a molecular target to inhibit the tumor growth or even to completely prevent its progression. However, this is difficult due to the crucial role HIF plays in numerous processes occurring not only in cancer cells but mostly in healthy systemic cells in physiological conditions.

Key words: hypoxia in cancer, breast cancer, HIF-1α.

Introduction

Adequate oxygen supply is essential for proper functioning of both individual cells and the human body as a whole. Oxygen is required mainly to maintain appropriate energy reserves in the cells, mostly in the form of ATP, as well as to ensure appropriate oxidoreductive potentials. Some tissues have developed adaptation mechanisms ensuring their function in reduced oxygen supply conditions, while other tissues, such as myocardium or brain cortex, are particularly sensitive to oxygen deficiencies. The term hypoxia is used to describe oxygen deficiency occurring in tissues due to various reasons. Hypoxia can be categorized into the following main types:

- hypoxemic hypoxia associated with lower partial oxygen pressures;
- anoxemic hypoxia associated with reduced oxygen transporting capability in blood;
- stagnant hypoxia resulting from reduced flow of blood in the tissues;
- hypermetabolic hypoxia associated with increased oxygen demand in tissues;
- cytotoxic hypoxia where the quantity of oxygen delivered to tissues is sufficient but toxic damage of tissue has occurred.

The human body has developed a number of mechanisms to adapt to reduced blood oxygen levels. Compensation is possible via numerous mechanisms, the major ones being the increase in cardiac output and lung ventilation, reduced resistance of peripheral vessels, reduced blood viscosity, production of erythropoietin, redistribution of blood flows, increased deposition of myoglobin, switching to anaerobic metabolism or to restore appropriate oxygenation of tissues. Increased ventilation due to hypoxemia is the most efficient of these mechanisms. Thus, hypoxemic hypoxia is the easiest to manage of all forms of oxygen deficiency. Hypoxia due to causes other than hypoxemia appears to be most dangerous for the system, as only the molecular adaptation mechanisms which require more time for full activation compared to the systemic mechanisms are available in such cases [1].

For most cells, hypoxia is defined as molecular oxygen levels reduced to less than 2%, with the level of 1.2% defining moderate hypoxia and the level below

0.2% defining deep hypoxia and anoxia. In order to survive, the cells must either adapt to the new conditions by switching to anaerobic metabolism or restore appropriate oxygenation of tissues. Tumor cells are particularly prone to hypoxia due to their high density and poor vascularization limiting their oxygen supply. Thus, hypoxia is a characteristic feature of most tumors.

Hypoxia is an important factor that mediates the regulation of gene expression; it is capable of both inducing and suppressing the expression of particular genes. The effect of hypoxia on post-transcriptional and post-translational mechanisms determining the efficiency of gene expression was also described. The main transcription factor involved in cellular adaptation to hypoxic conditions is hypoxia-inducible factor 1 (HIF-1).

Characteristics of the hypoxia-inducible factor

Hypoxia-inducible factor belongs to the Per-ARNT-Sim (PAS) family of proteins. It is a transcription factor featuring a helix-loop-helix structure consisting of two subunits. The first subunit is one of three possible a subunits sensitive to the systemic oxygen levels (HIF-1α, HIF-2α or HIF-3α). The other, constitutive subunit β (HIF-β) is common to all HIF variants. Thus formed HIF-1a/β and HIF-2a/β dimers are involved in activation of the transcription of low oxygen level-inducible genes. The role of the third variant of the dimer, HIF-3a/β, has not been precisely determined yet. Expression of a subunits is closely related to the tissue type. HIF-1α is expressed in most tissues, while expression of HIF-2α, known as endothelial protein 1 containing the PAS domain (called EPAS1, endothelial PAS domain-containing protein 1) is limited mainly to hepatocytes, cardiomyocytes, endothelial cells, microglia and type II pneumocytes. HIF-3α is expressed in ocular and cerebellar cells. In contrast to the tissue-specific HIF-2α and HIF-3α, the widespread expression of HIF-1α makes it a universal marker of hypoxia regardless of the location of the tumor. In contrast to subunits sensitive to the oxygen supply, the b subunit is produced in most tissues and is also known as aryl hydrocarbon receptor nuclear translocator (ARNT). Besides being a structural and functional element of HIF, ARNT is also involved in transformation of xenobiotics by inducing the expression of enzymes required for their metabolism [2].

Interactions of individual HIF-1α and HIF-1β subunits are required for the formation of the active transcription factor HIF-1 and its effective binding to nuclear DNA with the purpose of expressing the dependent genes. The HIF-1β subunit is constitutively located within the cell nucleus while the availability of HIF-1α is subject to regulation. When the cell is activated by a hypoxic stimulus, HIF-1α is translocated into the nucleus and partakes in formation of the degradation-resistant heterodimer HIF-1 together with associated proteins.

Regulation of the cellular quantity and activity of HIF-1α is mostly related to the function of the aerobic metabolic mechanism, although other proteins have also been found to be involved in its stabilization. Important HIF-1α-stabilizing factors are heat shock protein-90 (Hsp90) and Jun activation domain-binding protein-1 (Jab1) [3]. Although regulation of HIF-1α activity has already been described, the important role of post-translational modifications as a factor responsible for regulation of this activity has been documented only recently. The HIF-1α subunit is encoded by the HIF1A locus located on chromosome 14q21-q24, encoding information for 15 exons. Different functional domains of HIF-1α are encoded by separate exons. Besides the wild type of this subunit (HIF-1αWT) as described in the earliest reports, 8 other splicing variants were identified in human cell lines (HIF-1α827, HIF-1α736, HIF-1α557, HIF-1α516, HIF-1α785, HIF-1α417, HIF-1αTE and an isoform containing an alternative variant of exon 1 (isoform I.2)) [4, 5]. All these forms were capable of dimerizing with the HIF-1β subunit and demonstrated transcriptional activity in human cells; however, only one isoform – HIF-1α736 – had been documented in breast cancer until recently [6].

A study by Dales *et al*. showed for the first time higher expression levels of two HIF-1α splice variants (HIF-1αTAG and HIF-1α736) of estrogen receptor (ER) negative carcinomas compared to normal and benign tissues. Dales *et al*. investigated the prognostic value of HIF-1α transcript expression levels in breast cancer and found a significant relationship between either clinicopathological characteristics or patient metastasis-free survival. The authors found that HIF-1αTAG mRNAs levels were substantially higher in high-grade and steroid hormone receptor-negative tumors. The second and most striking observation was that HIF-1αTAG mRNA levels were indicative of shorter metastasis-free survival, and that this correlated with lymph node status [7, 8]. Microarray tests have shown that there might be as many as 200 proteins with HIF-dependent activation patterns [7]. The number is much higher than previously thought. More than one half of these proteins have not been precisely described yet, and thus their functions remain unexplained. The genes encoding these proteins have one common feature consisting of a single or repeated 5'-CGTG-3' sequence within the promoter region. The sequences have been named hypoxia response elements (HREs). It turned out that not all 5'-CGTG-3' sequences within the genome act as HREs. To date, it has not been explained how HIF is

capable of recognizing functional and non-functional HREs within the promoter region. Some hint may be provided by the HIF-1 ancillary sequence (HAS), reported to be located at a distance of 8 nucleotides from the HRE. However, as evidenced by genomic studies, the sequence is not present in the promoters of all genes containing the HRE [7]. This suggests the need for other sequences to interact with other transcription factors influencing the expression of HIF-dependent genes. The functionality of HRE regions may also depend on the local chromatin structure as determined by methylation or acetylation processes.

Small non-coding RNAs are short RNA molecules whose function is to regulate the expression levels of other genes by several mechanisms. Micro RNA-210 (miR-210) has been strongly linked with the hypoxia pathway, and is upregulated in response to hypoxia-inducible factors. MicroRNA-210, the master hypoxamir, plays pleiotropic roles in certain cancers; however, its role in the development of human cancer remains unclear. Herein, we report that miR-210 is frequently up-regulated in colorectal cancer tissues, with high miR-210 expression significantly correlating with large tumor size, lymph node metastasis, advanced clinical stage and poor prognosis. Functionally, miR-210 overexpression promotes the migration and invasion of colorectal cancer cells. Furthermore, miR-210 can be induced by hypoxia and mediates the hypoxia-induced metastasis of colorectal cancer cells. In addition, vacuole membrane protein 1 (VMP1) is identified as the direct and functional target of miR-210. Thus, miR-210 is a useful biomarker for hypoxic tumor cells and a prognostic factor that plays an essential role in colorectal cancer metastasis [9, 10]. Also mir-210 has been suggested as a useful biomarker to distinguish adrenocortical carcinoma from adrenocortical adenoma [11].

Also, miR-210 is a useful diagnostic marker for breast cancer in women. Hong *et al*. concluded that high expression of miR-210 might predict poor survival in patients with breast cancer [12]. Camps *et al*. found that higher expression levels of miR-210 were correlated with shorter disease-free and overall survival in breast cancer [13]. Foekens *et al*. found that a higher expression level of miR-210 was correlated with lymph node-negative estrogen receptor positive breast cancers [14]. Toyama *et al*. concluded that the degree of miR-210 expression might be a clinically useful prognostic factor for decision-making regarding treatment in the adjuvant setting, especially in node-negative triple-negative breast cancer patients [15].

Upon sufficient oxygen supply, HIF-1α and HIF-2α are hydroxylated and form a complex with VHL protein comprising a part of the E3 ubiquitin ligase complex. This binding leads to degradation of HIF subunits in the proteasome pathway and prevents the expression of hypoxia-induced genes. Ubiquitination and proteasomal proteolysis of the subunit is a very rapid process that is immediately inhibited in the case of oxygen shortage. Hydroxylation that initiates the a subunit degradation process occurs at three locations of each of these subunits at proline and asparagine moieties, i.e. Pro402, Pro564 and Asn803 within HIF-1α, and Pro405, Pro531 and Asn851 within HIF-2α [16].

Hydroxylation of these amino acid moieties is irreversible as it forms very stable, covalent bonds. The process requires the substrate protein as well as molecular oxygen, iron ions, 2-oxoglutarate and ascorbate ions. The cellular levels of HIF-1 in the cell depend on many factors, such as distribution of hydroxylases that take part in degradation and tissue vascularization responsible for the availability of oxygen depending on cell location. The oxygen level gradient formed as a certain distance from the vessel affects the activity of hydroxylases. At sites of low partial oxygen pressure, i.e. at increasing distances from the vessel supplying the particular region, the activity of these enzymes is inhibited. This is also associated with stabilization of the a subunit of the HIF protein, its translocation into the nucleus, dimerization with the β subunit and the binding of the transcription factors.

The role of hypoxia in cancer

Carcinoma develops from epithelial tissue and mimics the epithelium of the affected organ. Tumor cells are capable of differentiation, maturation and metaplasia. Cancer is characterized by unrestricted replication, growth and other processes independent of the remaining cells as well as by its ability to infiltrate surrounding tissues and form distant metastases. Hypoxia is an important element of the microenvironment of cancer development. It is due to insufficient angiogenesis in the region of the developing lesion. The angiogenesis process is always a step behind the growing tumor, contributing to insufficient supply of oxygen and other nutrients to cancer cells. Oxygen is capable of diffusing through the tissues to the depth of 100-180 mm, which is largely insufficient for the supply in the malignant growth region. As a consequence, the tumor tissue oxygen levels are usually not higher than 7%, and often lower than 1%, while the average value for normal tissue is about 7% (53 mmHg) [2, 17]. Scarce quantities of oxygen in the tumor region lead to stabilization of HIF-α protein, which in turn stimulates endothelial cells to migrate into the region affected by the tumor growth and to initiate the angiogenesis process. However, blood vessels are formed in this process in

a disorderly manner. This hampers appropriate oxygen supply to the tissues, leading to continuously worsening hypoxia.

The presence of hypoxic regions within solid tumors is a reliable indicator of poor prognosis. Hypoxia contributes to the increased aggressiveness of the malignant process and protects tumors from conventional treatment methods. Hypoxia within solid tumors is usually associated with increased resistance to chemotherapy, immunotherapy and radiation therapy [18].

Cells adapt to chronic, gradually worsening hypoxia better than to rapid, acute hypoxia episodes. Adaptation to chronic oxygen deficiency includes mainly intensification of the expression of genes encoding glycolytic enzymes that facilitate the provision of energy from ATP along anaerobic pathways. Increased expression of genes encoding for lactate dehydrogenase, pyruvate kinase within skeletal muscles and astrocytes as well as phosphoglycerate kinase, i.e. enzymes responsible for anaerobic metabolism of glucose, were observed in hypoxic conditions. Anaerobic glycolysis is much less efficient than oxidative phosphorylation. One molecule of glucose leads to formation of two ATP molecules, while oxidative phosphorylation leads to formation of (insert source) molecules. The lower efficacy of the process leads to a higher cellular demand for glucose, and thus the increase in sugar uptake is required by means of increased expression of glucose transporters (GLUT). Increased expression of GLUT 1 was demonstrated as a response to hypoxia in stem cells, adipocytes, neurons, astrocytes and chondrocytes [19]. Increased expression of GLUT 4 was observed in skeletal muscle cells and cardiomyocytes [20, 21].

The metabolic shift from oxygen respiration to anaerobic glycolysis is reflected by morphological and biochemical changes occurring within mitochondria. Mitochondrial volume reduction, matrix condensation, crista thickening and a significant drop (ca. 30%) in the activity of the respiratory chain enzymes were observed in chronic hypoxia. Elevated expression of HIF as a result of hypoxia is observed in about 53% of all cancers, including in 56-76% of breast cancers [22, 23].

Hypoxia-inducible factor 1 is responsible mainly for cellular adaptation to hypoxic conditions; therefore, genes triggered by this factor are responsible mainly for the improvement in oxygen supply (by increasing angiogenesis, broadening the lumen of existing vessels, increasing erythropoiesis or increasing iron consumption), adaptation of cells to anaerobic metabolism conditions as well as for other changes facilitating cell survival in insufficient oxygen availability and modifying the main metabolic pattern. The most important genes and effects of proteins encoded by them are listed in Table I.

Tumor cells are particularly prone to hypoxia. This is due to the sparse network of blood vessels, morphological and functional vessel disorders, large distances between cells and vessels, dense packing of tumor-transformed cells as well as ischemia due to progression of the pathology or to the adverse effects of the initiated treatment. These conditions promote selection of cells that became most resistant to these adverse conditions by developing the most effective adaptation mechanisms. The growth of thus selected cells leads to worsened hypoxia, thus closing and escalating the vicious circle of the disease.

Stimulation of angiogenesis promotes the increased risk of distant metastases. Better accessibility of blood vessels increases the chance of tumor cells finding their way into the bloodstream and being transported to niches allowing settlement and formation of a metastatic lesion.

Breast cancer

Breast cancer is a widespread disease with 1.3 million new cases being diagnosed each year and an annual mortality rate of nearly 450,000 individuals. Factors predisposing to breast cancer include familial susceptibility (genetic factor), female gender, age, age at menarche and menopause, exposure to ionizing radiation and other diseases of the breast [24]. The breast consists of mammary gland lobules, lactiferous ducts connecting the lobules with the papilla and stroma consisting of adipose tissue, connective tissue and a network of blood and lymph vessels. Most cancers occur within the lactiferous duct tissues; however, lesions may develop in all breast structures. Cancers developing within lactiferous ducts without infiltration of surrounding tissues is referred to as non-invasive ductal carcinoma (ductal carcinoma *in situ* – DCIS) and amounts to about 20% of all breast cancers. The most common type of invasive breast cancer (observed in ca. 70-80% of cases) is invasive ductal carcinoma of no special type. Much less common types of cancer lesions include lobular carcinoma, medullary carcinoma, mucinous carcinoma, apocrine carcinoma and papillary carcinoma. Molecular characteristics of the cancer lesions play the key role in the selection of an optimum treatment in breast cancer patients. Determination of the expression of estrogen and progesterone receptors and of the expression of human epidermal growth factor receptor 2 (HER2/neu) seem to be the most important issues. Activation of growth factor-dependent pathways is also a very important mechanism of breast cancer progression. About 20% of these cancers are characterized by overexpression of HER2 (*EGFR2* or ErbB2), a transmembrane receptor of tyrosine kinase activity. Estrogens play an important role in breast cancers. They exert numerous effects by interacting

Table I. Genes and effects of the encoded proteins

Adaptation process	Activated gene/ gene product	Produced protein	References
Stimulation of angiogenesis	VEGF	vascular endothelial growth factor	[2, 3, 8]
	CYR61	cysteine-rich angiogenic inducer 61	
Voltage drop across the vascular wall leading to extension of the increased blood flow	iNOS	inducible nitric oxide synthase	[7, 17, 19]
	ADM	adrenomedullin	
Increased red blood cell production	EPO	erythropoietin	[42, 48]
Iron turnover growth, increasing its supply of dividing cells	TF	transferrin	[36, 37, 39]
	CP	ceruloplasmin	
Decoupling of ATP production by oxygen availability in the environment	LDHA LDHB OKLR PGK1 PGK2	all enzymes of the glycolytic cycle (e.g., lactate dehydrogenase, pyruvate kinase, phosphoglycerate kinase)	[10, 38]
Increasing the transport of glucose into cells	GLUT1	glucose transporter type 1	[19]
	GLUT4	glucose transporter type 4	
Adjusting the pH of the medium	CAIX/CA9	carbonic anhydrase 9	[36]
Control of proliferation and differentiation of most cell types	IGF-2	insulin-like growth factor 2	[27, 30]
	TGF-β3	transforming growth factor β3	
Inhibition of apoptosis through activation of oncogenes	Ras	proteins RAS	[22, 23, 35]
	HER2/neu	protein HER2/neu	
	SRC	cytoplasmic tyrosine kinase Src	
Inhibition of cell differentiation	ID2	inhibitor of DNA binding 2	[43]
Resistance to chemotherapeutic agents participating in multi-drug resistance	ABCB1 (ATP-binding cassette subfamily B member 1)	P-glycoprotein	[41, 46]
ECM metabolism, reconstruction of the surrounding tissue	MMP2	metallopeptidase 2	[44, 45, 47]
	CTSD	cathepsin D	
Role in induction and maintenance of multidrug resistance	ABCG2	ABC transporter family	[42]
Increased production of ATP	ALDOA	aldolase A	[41, 42, 43]
	GAPDH	glyceraldehyde 3 phosphate dehydrogenase	
	ENO1	enolase 1	
Role in adhesion, cell proliferation, cell migration and angiogenesis	CTGF	connective tissue growth factor	[25, 26]
Allowing an unlimited number of cell divisions	TERT	telomerase	[29]
Stabilization of the genome and control of the division of centromeres during mitosis	NPM1	nucleophosmin 1	[35]
Stabilization of proteins involved in the process of correct folding, oligomerization, translocation and degradation	HSP90	heat shock protein 90	[41, 43]
Participation in the remodeling of tissues	MMP14	metalloproteinase 14	[42]

with estrogen receptors (ER) α and β. Binding between these hormones and receptors leads to both genomic and non-genomic changes in epithelial cells within the mammary glands. Interactions with the cellular genome involve transcriptional activation and repression of genes. Genes encoding progesterone receptor, cathepsin D, c-myc, cyclin D1 and numerous other proteins are dependent on these interactions. Extragenomic effects are much less studied. They include mainly modulation of intracellular signaling pathways and the activity of membrane ion channels. Overexpression of ER is observed in up to 70% of lesions diagnosed as invasive breast cancer. Ligand binding causes heterodimerization of the receptor, followed by phosphorylation and activation of subsequent signaling pathways, including the MAPK pathway. Activation of the MAPK pathway leads to increased growth, proliferation and migration of cells as well as to increased angiogenesis [25, 26].

Regulation of protein expression of hypoxia-inducible factor as a new strategy in the diagnosis of breast cancer

The mean oxygen level in breast cancer tissues is about 10 mmHg compared to 63 mmHg in healthy tissue. Thus reduced partial pressure of oxygen results in increased expression of HIF. It was also noted that HIF-1α levels were increased in DCIS – the preinvasive stage of breast cancer – and were associated with increased microvascular density, indicating that HIF-1α expression might have an important role early in breast cancer progression [24].

Adaptation to hypoxia is mediated by HIF-1, which stimulates angiogenesis and high anaerobic metabolism. It has been shown that high microvessel density, VEGF over-expression, and increased lactate production are associated with a poor prognosis in human malignancies. Bos *et al.* found that high levels of HIF-1 were correlated with overexpression of VEGF, one of its main downstream effectors, confirming angiogenesis as one of the proposed mechanisms by which HIF-1 activation stimulates tumor growth and metastasis. Analysis of other clinicopathological variables in this study confirmed previous data revealing a positive association between increased proliferation, poor histologic grade, and high levels of HIF-1α [24, 25, 26].

In their studies on invasive breast cancer, Bos *et al*. demonstrated the relationship between the expression of HIF-1α and the levels of cyclin A, Ki-67 and p53 protein. These associations were most evident in lymph node-negative cases and might therefore contribute to the poor prognosis of HIF-1α-positive cancers described previously [27]. In addition, diverse expression of these biomarkers in cancers with ER expression was observed, suggesting possible interactions between HIF-1α and ER. The expression of cyclin A or Ki-67 suggests that the cells are in mitotic division phase S or G2. The expression of cyclin A is stimulated by tyrosine phosphatase cdc25A and associated with non-differentiated and ER-negative breast cancers characterized by poor prognoses. Ki-67 is also commonly used as a cellular proliferation marker, although the role of this protein is not fully understood. It is present in higher quantities in phase S cells, but also in phase G1-G2 cells. The increased expression of both these proteins is indicative of high proliferation potential of the studied tissue, and thus of poor prognosis, and is associated with increased cellular concentration of HIF-1α. The relationship between HIF-1 and proliferation rate was reported before, but the relationship has not been fully explained yet. The role of estrogens in breast cancer development is well known and confirmed by epidemiological data as well as by numerous reports on the efficient treatment of this cancer by means of anti-estrogen therapy. Estrogens are responsible for the increase in the rate of cellular divisions; on the other hand, cancers with increased ER expression were shown to be less prone to metastases and more sensitive to antiestrogens. This may suggest that the hormones have an impact on the rate of tumor progression. Estrogens regulate the metabolism of collagen [28, 29]. Their effect on the shift of the equilibrium of collagen synthesis and degradation facilitates the growth and migration of cells. Although degradation is largely dependent on the activity of extracellular metalloproteinases, the final degradation reaction is mediated by cytoplasmic prolidase. Prolidase is an enzyme that catalyzes the last stage of degradation of extracellular matrix components. It is responsible for the cleavage of proline and hydroxyproline from the carboxylic termini of the degraded peptides. Studies conducted by Surazynski *et al*. showed that the products of prolidase activity, i.e. proline and hydroxyproline, regulate degradation of HIF-1α. Overexpression of prolidase leads to an increased concentration of HIF-1α and therefore increased cellular levels of VEGF and GLUT-1. The mechanism of HIF-1α accumulation is associated with blocking the von Hippel-Lindau protein-dependent HIF degradation pathway [30]. Surazynski *et al*. also decided to examine the relationship between the impact of estrogens on breast cancer and the concentration of HIF-1α to finally confirm the impact of estrogens on the disease [31]. To this end, they conducted studies on estrogen-dependent MCF-7 breast cancer cells expressing ER α and ER β as well as on estrogen-independent cells MDA-MB-231 expressing only β receptors. The study showed that the prolidase activity is correlated with the nuclear location of HIF-1α in breast cancer cells. In addition, the phenomenon was found only in cells expressing the ER α. Studies showed that the increase in prolidase activity occurred in MCF-7 cells

only, suggesting that the process is dependent only on the presence of the a receptor. MDA-MB-231 cells lacking this receptor showed no effect manifesting in a change of prolidase activity. The increased prolidase activity leads to secretion of larger quantities of proline and hydroxyproline which inhibit HIF-1α degradation, thus contributing to increased concentrations of the transcription factor within the nucleus [31]. The correlation of hypoxia with the extracellular matrix proves the existence of special pathways in which the detection of intracellular matrix degradation is interpreted as a tissue stress signal and leads to induction of angiogenesis.

For many years, the B-cell lymphoma gene (BCL-6) has been ascribed only the role of immortalization of murine fetal fibroblasts and B lymphocyte precursor cells by increasing the activity of cyclin D1. Increased expression of cyclin D1 in various cancers is nothing unusual; however, it turned out that overexpression of BCL-6 is not limited to lymphomas. In their studies on invasive breast cancer, Bos *et al*. used immunohistochemical assays to assess the expression of BCL-6 in material collected from breast cancers and normal breast tissues. The results showed overexpression of BCL-6 in 16% of tested cancer lesions while unchanged tissue was characterized by overexpression in less than 1% of cases. The analysis showed that the increase in BCL-6 activity was correlated with increased expression of cyclin D1, p53 protein and HIF-1α [32]. The study was the first to demonstrate that BCL-6 plays an important role also in cancers other than lymphomas. Overexpression of this protein in breast cancer, although possible, seems to be so uncommon that its role as a potential biomarker is very unlikely. This, however, requires further studies.

Gort *et al*. defined the pathophysiological importance of HIF-1α activity regulation by phosphatidylinositol 3 kinase (PI-3 kinase)/Akt. In the study, hypoxia within the tumor lesion was modeled by culturing cancer cells in low partial oxygen pressure conditions. It was observed that hypoxia-induced expression of HIF-1α was reduced by serum deprivation. Overexpression of dominant-active Akt1 restored the expression of HIF-1α expression, whereas inhibition of PI-3 kinase activity reduced hypoxic HIF-1α protein levels to a similar extent as serum deprivation. Immunohistochemical analysis of human breast cancers revealed that the lack of Akt1 phosphorylation is correlated with low HIF-1α levels. These results allow us to conclude that the activity of Akt is physiologically important for the expression of HIF-1α in breast cancer. This may potentially mean that HIF-1α function could be regulated by inhibitors of the PI-3 kinase/Akt pathway [33]. Recent studies suggest an important role of Wnt and Notch pathways in breast cancer. Ectopic expression of Wnt-1 was shown to be responsible for transformation of human breast epithelial cells via a Notch pathway-activating mechanisms. Chen *et al*. demonstrated that the activity of the Notch pathway in breast cancer is enhanced by chronic hypoxia, or more specifically by accumulation of HIF-1α and HIF-2α. Expression of the Notch pathway target genes (HES1 and HEY1) was induced in cells incubated in low oxygen conditions, confirming the researchers' assumptions. In addition, an increase in ligand concentration and stabilization of Notch receptors also contributed to Notch pathway activation in hypoxic conditions. The expression of HES1 and HEY1 was observed in most cancer cells studied in hypoxic conditions. However, the increase in HEY1 expression in hypoxia is more dramatic than the increase in HES1 expression, suggesting that HEY1 might be a better marker of Notch pathway activation in breast cancer. One of the direct, most important consequences of Notch pathway activation is reduced expression of E-cadherin in breast cancer, being an important cause of increased tumor invasiveness and a tendency to metastatic spread [34].

The relationship between inflammation and tumor progression is widely accepted. It is also the case for breast cancer. Besides playing a central role in the induction of inflammatory processes, interleukin 1β (IL-1β) was also identified as a factor important for progression of the tumor and stimulation of angiogenesis as well as being responsible for the increase in the invasiveness of cancer lesions. Recently, there has been considerable interest in understanding the non-hypoxic upregulation of the hypoxia-inducible factor HIF-1α by IL-1 in neoplastic cells since aberrant expression of HIF-1α correlates with tumor progression. Naldini *et al*. studied the effect of IL-1β on cell migration and HIF-1α accumulation in the human invasive breast cancer cell line MDA-MB-231. It was found that hypoxia-independent induction of HIF-1α by IL-1β in MDA-MB-231 cells was associated with an increase in cell migration and a simultaneous increase in the activity of phosphorylated p38 MAPK and CXCR1 expression. Inhibition of HIF-1α by siRNA led to a significant reduction in CXCR1 expression and cell migration, confirming the role of HIF-1α in hypoxia-independent, IL-1β-induced migration of the MDA-MB-231 line cells. The results of the studies present a new role of IL-1 in breast cancer cells. The therapeutic approach focused on inhibition of IL-1β activity appears to be a new target for the research aimed at the development of novel methods to treat invasive breast cancer [35].

Central foci of fibrotic tissue are quite commonly reported in female breast tumors classified as malignant lesions. Observations have revealed that the presence of such central scars was closely correlated with higher scores in malignancy scales as well as with the presence of metastases and shorter patient survival. The key el-

ement associated with fibrosis and aggressive cancer phenotype is hypoxia within the pathological lesion.

Jemal *et al*. studied these correlations, reporting them for the first time in male breast cancer. Male breast cancer is very rare. It accounts for less than 1% of all breast cancer cases [36]. Much of the information on male breast cancer is generalized and gathered from data on female cases. However, a small number of conducted studies suggest numerous phenotypic and genomic differences between the two types of lesions. Fibrotic foci present in breast cancers are scar lesions consisting mainly of collagen, featuring numerous fibroblasts. Most commonly, they are located in central parts of the tumor. Fibrotic foci have been described in detail in female breast cancers as well as in other tumors, such as lung cancer, pancreatic cancer and colon cancer. Hypoxia is believed to be the element connecting the presence of a fibrotic focus with the cancer phenotype and progression rate. Fibroblasts and cancer cells present in such regions clearly express HIF-1α and carbonate anhydrase IX (CAIX) – two factors closely related to hypoxia [37]. Necrotic regions are easy to assess by histological means and provide important prognostic information. Studies conducted by Anderson *et al*. demonstrated a positive correlation of the presence of necrosis with a high histological grade of the tumor, high mitotic activity, numerous lymph node metastases and poor prognosis. No relationship with patients' age and ER expression was observed. Similar relationships were reported previously in female breast cancer [38].

The role of the central fibrotic region in cancer progression and phenotype determination may be explained by the induction of HIF-1α and CAIX in these regions. As mentioned before, low oxygen levels activate HIF-1α to induce processes allowing the cells to avoid death in hypoxic conditions; these include angiogenesis, adaptation to hypoxic conditions and development of an aggressive cancer phenotype. Several studies have demonstrated the presence of a rich and dense network of microcirculation vessels in tumors featuring central fibrotic foci, particularly large fibrotic foci with necrotic regions. In addition, fibroblasts present in these regions were characterized by particularly rapid proliferation and high expression of proteinase crucial for metastatic spread [39].

Hypoxia and tumor-associated macrophages (TAMs) as potential diagnostic and prognostic biomarkers in breast cancer

Tumor-associated macrophages (TAM) have been linked with the progression of cancer by favoring tumor angiogenesis, growth, and metastasis. The precise mechanisms that maintain the protumor phenotype of TAM are poorly understood, but recent research has highlighted a number of signaling pathways that are important in TAM phenotype and function. Nuclear factor-κB (NF-κB) is considered the master regulator of inflammatory and immune responses. Recently several genetic studies have indicated that NF-κB is an important pathway in TAM for the integration of signals from the tumor microenvironment that promote carcinogenesis [40].

Clinical evidence compellingly indicates the association between a high TAM influx and poor prognosis in patients with breast cancers. The pan-macrophage marker CD68 is now generally used to identify TAMs in diagnostic biopsy samples, and some other TAM-related biomarkers are also used in prognosis prediction, including CD163, vascular endothelial growth factor (VEGF), hypoxia-inducible factors (HIFs), proliferation cellular nuclear antigen (PCNA), ferritin light chain (FTL) and C-C motif chemokine ligand 18 (CCL18) [41].

Aggressive tumors grow very rapidly. In these conditions, it is impossible to develop a sufficient network of supplying blood vessels. This contributes to formation of chronically ischemic and hypoxic regions. These are the locations of accumulation of macrophages involved in angiogenesis. Hypoxia leads to HIF-a stability in TAMs, contributing to their transcriptional activity and expression of HIF-dependent genes encoding e.g. VEGF, FGF, IL-1β, IL-8, cyclooxygenase-2, metalloproteinases 7, 9, 12, and angiopoietin 1, and thus to initiation of new vessel formation. The increase in the number of HIF-2α-positive macrophages in the natural history of invasive breast cancer is closely correlated with increased angiogenesis and poor prognosis. The increase in HIF-2α expression was also correlated with increased grade of tumor malignancy. What is interesting, the study revealed a negative correlation between the expression of HIF-2α and the expression of thymidine phosphorylase (TP) – an enzyme present in tumor tissues and involved in angiogenesis – in TAMs. Previous studies showed that tumors with TP overexpression in TAMs were associated with much worse prognoses regardless of the status of lymph node involvement, density of blood vessel network or the size of the tumor. The results are somewhat contradictory, as the TP gene lacks the hypoxia response element (HRE). This partially explains why the expression of TP is lower at sites with high HIF-2α activity [42]. It is suspected that breast cancer is associated with two alternative pathways for the induction of angiogenesis:

- activation of HIF-2α in TAMs of hypoxic cancer cells;
- TP-mediated induction of oxidative stress in the presence of oxygen, involving formation of reactive oxygen species that induce the expression of strong angiogenic factors VEGF, MMP-1 and IL-8 [43].

The increasing oxygen demand of the growing tumor leads to chronic hypoxia that contributes to accumulation of TAMs and increased expression of HIF-2α, leading to formation of a sort of angiogenic phenotype macrophages. On the other hand, in regions less exposed to hypoxia, with the supply of oxygen and other nutrients still depending on the formation of new blood vessels, an alternative angiogenesis activation pathway, involving the TP-dependent mechanism, is predominant. These observations show that the expression of VEGF, HIF and TP and the presence of hypoxic macrophages are important indicators of angiogenesis associated with high risk of metastatic spread, high malignancy grade and poor prognosis [44].

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