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Expression of Hypoxia-Inducible Factor 1 α in Invasive Breast Cancer with Metastasis to Lymph Nodes: Correlation with Steroid Receptors, HER2 and EPO-R

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

Abstract

Background. Expression of hypoxia-inducible factor 1 α (HIF-1 α) reflects the degree of cell hypoxia and its increased expression was found in most neoplasms, their metastasis as well as in some precancerous lesions.

Objectives. The study aimed to investigate the expression HIF-1 α in invasive breast cancer with metastasis to lymph nodes in correlation with steroid receptors (ER-estrogen receptor, PR-progesterone receptor), HER2 (human epidermal growth factor receptor 2) and EPO-R (erythropoietin receptor).

Material and Methods. A total of 58 cases of invasive breast cancer with metastasis to lymph nodes were assessed for expression of HIF-1 α , EPO-R, ER, PR and HER2.

Results. In our study, among all invasive breast cancers, 36.2% exhibited HIF-1 α expression in the nuclei of neoplastic cells. We also assessed the correlation between histological type of cancer and basic immunohistochemical profile that included HIF-1 α expression and statistical significance was noted only in the case of PR-/ER-/HER2-/HIF-1 α -/ and PR+/ER+/HER2-/HIF-1 α -/ ($p = 0.028$ and $p = 0.008$, respectively). However, only in the case of the PR+/ER+/HER2-/HIF-1 α + immunohistochemical profile and histological grading did we note a statistical significance ($p = 0.006$). Expression of HIF-1 α was most often noted in cancers exhibiting expression of HER2 protein (57.14%). Our study also assessed the relationship between the expression of HIF-1 α in invasive breast cancers and the expression of EPO-R and areas of necrosis, demonstrating a statistically significant dependence ($p = 0.003$).

Conclusions. Expression of HIF-1 α was more often noted in invasive HER2+ cancers characterized by high degree of aggressiveness and poorer prognosis, which might suggest that presence of HIF-1 α protein expression in HER2+ cancers could be an additional prognostic factor, the frequent occurrence of the phenotype of HIF-1 α and EPO-R in cancers invasive HER2+, in the absence of ER and PR, may suggest that HIF-1 α and EPO-R may be an indicator of the aggressiveness of invasive breast cancers, indicating the need for a specific forms of treatment in this group of patients (*Adv Clin Exp Med* 2016, 25, 4, 741–750).

Key words: HIF-1 α , breast cancer, steroid receptors, HER2, EPO-R.

HIF1, one of the main components of cellular response to lack of oxygen, is the main transcription factor involved in cell adaptation to hypoxia. It is a heterodimer built of two subunits, HIF-1 α (120 kDa) and HIF-1 β (91, 93 or 94 kDa) that bind directly to DNA. Interaction between HIF-1 α and HIF-1 β is necessary for binding HIF1 to DNA and for its activity as a transcription factor [1]. HIF-1 β is a protein constitutively located in the cell nucleus, while the activity of HIF-1 α is subject to regulation; hence, its activation is often associated with

the function of HIF1 transcription factor. Under aerobic conditions, HIF-1 α remains inactive [1, 2]. Activation of HIF-1 α first requires its stabilization through the inhibition of proteolysis. Factors other than hypoxia, such as those interfering with iron function (Fe II), essential for proper functioning of dioxygenases, also contribute to the stabilization of HIF-1 α . Effective inhibition of proline 4-hydroxylases facilitates the accumulation of HIF-1 α in the cytoplasm and its subsequent activation [1, 2]. The main way of regulating cellular concentration and

activity of HIF-1 α is oxygen-related mechanism. Heat shock protein (Hsp 90) is an important factor stabilizing HIF-1 α both in normoxemic as well as in hypoxic conditions [1, 2]. There are reports suggesting that HIF-1 α might contribute to the genetic instability of neoplastic cells. The presence of p53 in this process seems necessary, which leads to a suspicion that HIF-1 α induces genome mutations in all stages of tumor development, as most malignancies lose the expression of wild-type p53 [1, 3].

Induction of HIF1 results in the upregulation of a wide variety of target genes, including genes involved in metabolism, angiogenesis, metastasis, as well as apoptosis (GLUT1 – glucose transporter 1, p53, p21, bcl-2 – B-cell lymphoma 2, VEGF – vascular endothelial growth factor and EPO – erythropoietin) [4–6]. A major HIF1 target gene is erythropoietin (EPO). EPO-R (erythropoietin receptor) is also induced by hypoxia but is not regulated by HIF1 [7, 8]. EPO-R belongs to the cytokine receptor superfamily. EPO and its receptor (EPO-R) have recently been found to be expressed on several normal non-hematopoietic cells as well as in cancer tissues. Specifically, high expression of EPO-R has been found on the surface of endothelial cells and various solid tumors [9–11].

Expression of HIF-1 α reflects the degree of cell hypoxia and its increased expression was found in most neoplasms, their metastases as well as in some precancerous lesions. Even a small number of cells exhibiting HIF-1 α overexpression might accelerate tumor growth, indicating that its overexpression is associated with an unfavorable outcome of antineoplastic therapy. In humans the expression of HIF-1 α was identified in breast cancer, lung cancer and brain tumors [1, 5].

Despite numerous studies on the expression of HIF-1 α in breast cancer among women, the mechanism of action of this factor remains unknown and the data regarding its associations with classic established prognostic factors, such as: Expression of Ki-67 nuclear antigen, hormone receptors, indicators of angiogenesis (EPO, EPO-R) or pro- and antiapoptotic proteins, is lacking [1].

It is known from human studies that the expression of HIF-1 α is associated with poor prognosis. A high expression of HIF-1 α is common in cancers; therefore, the authors suggest that it might participate in carcinogenesis as well as in the stimulation of angiogenesis, which promotes tumor growth and invasiveness. Previous studies on breast cancer in women demonstrated the expression of HIF-1 α both in the cytoplasm as well as in the nuclei of cancer cells, although the questions regarding the association of this expression with other prognostic markers remain unanswered [1, 5].

The role of HIF-1 α in tumor progression is not limited to the stimulation of angiogenesis, but also ensures adaptation of tumor cells to functioning under hypoxic conditions, facilitates their survival and spread. HIF-1 α is able to inhibit hypoxia-induced cell death [1, 4, 5]. A correlation between the expression of HIF-1 α and reduced susceptibility to hypoxia-induced apoptosis has been observed in various tumors. HIF-1 α overexpression is also associated with the resistance of cancers to some therapies, e.g. radiotherapy, photodynamic therapy, or chemotherapy. Although the mechanisms regulating HIF-1 α under physiological conditions have been well established, its functioning under pathological conditions (e.g. neoplasms) is still unclear. Despite many controversies and contradictory reports on the role of HIF-1 α in cancer biology, it is an appealing target of antineoplastic treatment and better understanding of its function in physiological and pathological states might help in our combat against cancers [1, 4, 5].

The study aimed to investigate the expression of HIF-1 α in invasive breast cancer with metastasis to lymph nodes in correlation with steroid receptors, HER2 and EPO-R.

Material and Methods

Study material was collected from 58 patients diagnosed with invasive breast cancer and lymph node metastases. Tumor sections were fixed in 10% phosphate buffered formalin. After 24 h, fixation tissues were dehydrated in alcohol of gradually increasing concentration: 50%, 60%, 70%, 80%, 90%, 96%, followed by pure alcohol and xylene, and subsequently embedded in paraffin. Paraffin blocks were cut into slices 4 μ m in thickness. Acquired sections were then stained for diagnostic purposes using various methods. The tumors were classified and graded according to the WHO and the Nottingham modification of the Scarff-Bloom-Richardson systems. In the sections stained with routine H&E method, the following evaluations were carried out: Type of neoplasm (WHO classification), tumor grade including tubule formation, intensity of division as well as the degree of neoplastic cell differentiation and mitotic index expressed as a mean number of mitotic figures in neoplastic cells counted in 10 fields of vision at a $\times 400$ magnification (surface field 0.17 mm²).

Immunohistochemical Methods

Immunohistochemical methods employed paraffin sections attached onto glass slides covered with 2% silane/acetone solution (Sigma) and

dried for 24 h at 42°C. Before commencing the immunohistochemical procedure, sections were dewaxed by inserting them in a series of alcohols of gradually decreasing concentrations, followed by washing in distilled water. The sections were subsequently placed in a buffer solution pH 9 (Dako) in the case of estrogen receptors or pH 6 (Dako) for all other antigens, followed by heat treatment in a 90°C water bath for 30 min in order to uncover the epitope. When antigen epitopes were revealed, sections were cooled for 20 min. After washing twice with distilled water and incubating in 3% hydrogen peroxide for 5 min the sections were washed with TBS buffer (Tris-Buffered Saline Code: S1968, pH 7.6) (Sigma) the first antibody in suitable dilution was applied and incubated in a moisture chamber for 60 min at room temperature. Specimens were subsequently washed in TBS buffer (Tris-Buffered Saline Code: 1968, pH 7.6) (Sigma) for 10 min. Suitable monoclonal or polyclonal sets were used for each antibody in order to visualize the immunohistochemical reaction, then they were washed in TBS buffer (Tris-Buffered Saline, pH 7.6) (SIGMA) and a diaminobenzidine (DAB) (Substrate-Chromogen Solution) solution was applied, prepared according to the procedure provided by the producer for 10 min in order to visualize the color of the reaction. The color intensity of the sections was checked and they were subsequently washed in tap water, stained with Ehrlich's hematoxylin for 5 min, differentiated in 1% acid alcohol solution, and washed in tap water once again. Sections were then dehydrated in graded alcohol series of increasing concentrations, cleared in xylene and embedded in DPX mounting medium by Gurr® Co.

Immunohistochemical Staining of Steroid Receptor (ER, PR)

Monoclonal antibodies against receptors for estrogen (Monoclonal Mouse Anti-Human Estrogen Receptor α , 1:50 dilution, Clone: 1D5, Code: IR654, Dako) and progesterone (Monoclonal Mouse Anti-Human Progesteron Receptor, 1:400 dilution, Clone: PR636, Code: IR068, Dako) were used in order to determine the expression of steroid receptors. Immunohistochemistry was performed using the EnVision™ + HRP DakoCytomation (EnVision™ Dual Link System-HRP, DAB+, Code: K4065). The evaluation of immunohistochemical markers was performed by two pathologists as follows: ER and PR were categorized as negative – (0%), low positive – (1 – 10%); nuclear staining in > 10% of tumor cells was considered positive for ER and PR.

Immunohistochemical Staining of HER2

HER2 expression was determined using the HerceptTest™ Dako test (Code: K5204). It enabled the detection of HER2 expression using a polyclonal antibody against this protein (Rb A – Hu HER2 – Rabbit Anti-Human HER2 Protein). HER2 results were determined based on the maximum area of staining intensity according to the instruction in the package insert and the ASCO/ CAP guidelines as follows: strong, circumferential membranous, staining in > 30% of invasive carcinoma cells was graded 3+, moderate, circumferential membranous staining in \geq 10% of invasive tumor cells or strong circumferential membranous staining in \leq 30% of cells was designated as 2+ staining, weak and incomplete membranous staining in invasive tumor cells was scored as 1+ and no staining was marked 0. Tumors with 0 and 1+ staining were considered negative. Results identified as HER2 2+ were verified by fluorescence *in situ* hybridization (FISH). Positive and negative control preparations were previously determined.

Immunohistochemical Staining of HIF-1 α and EPO-R

A total of 58 cases of invasive breast cancer with metastasis to lymph nodes were assessed for the expression of HIF-1 α (Monoclonal Mouse Anti-Human HIF-1 α 1:50 dilution, Clone:28b, Santa Cruz Biotechnology®, Inc.). A visualization system ImmunoCruz™ Mouse ABC Staining System (Santa Cruz Biotechnology®, Inc.) was subsequently applied; tumor-cell immunoreactivity was scored according to both the extent of nuclear staining – relative number of HIF-1 α positive cells, as well as the intensity of the reaction: [–] not detected; [+] < 1% positive cells; [1+] 1–10% weakly to moderately stained cells; [2+] 1–10% intensively stained cells or 10–50% weakly stained cells; [3+] 10–50% positive cells with moderate to marked staining; [4+] > 50% positive cells [12]. Positive controls consisted of HIF-1 α immunoreactive breast cancer tissues. Negative controls were prepared with the omission of primary antibodies. In all examined invasive breast cancers we also assessed the expression of erythropoietin receptor (EPO-R) using an appropriate antibody against EPO-R antigen (Polyclonal Rabbit Anti-Human EPO-R, 1:250 dilution, Clone: C-20, Santa Cruz Biotechnology®, Inc.) and ImmunoCruz™ Rabbit ABC Staining System (Santa Cruz Biotechnology®, Inc.) for visualization. EPO-R staining results were scored according to the percentage of membrane positive cells as follows: (–) < 10%; (+),

10%–20%; (++) > 20%. Moderate expression EPO-R was defined as > 20% tumor cells with positive staining, whereas < 20% was considered low expression [13]. The immunoexpression of EPO-R was located mainly within cell membranes, although in most cases a granular cytoplasmic reaction was also observed. For EPO-R, slides of adult kidney were used as positive controls.

Statistics

All statistical analyses were performed with SPSS software v. 12.0 for Windows. The frequency of HER2 expression according to joint ER/PR status and the distribution of the hormone receptor status (ER, PR, and joint ER/PR) according to HER2 were also calculated. The Fischer test was used to assess the relationship between HIF-1 α expression and expression of steroid receptors (ER, PR), HER2, EPO-R, histological type of tumor, degree of histological malignancy and clinical staging. The results were considered statistically significant if the *p* value was less than 0.05 (*p* < 0.05).

Results

The mean age of studied women was 59.9 \pm 12.3 years (median: 58.5 years, range: 30–79 years). In histopathological examination we found 67.26% of IDC-NST (invasive ductal carcinoma of no special type) (Fig. 1), 18.96% of IDC (invasive ductal carcinoma), 3.44% of ILC (invasive lobular carcinoma), 8.62% of metaplastic carcinoma and 1.72% of mixed ductal and lobular carcinomas. Among the group of studied cancers we differentiated four subgroups characterized by different basic immunohistochemical profiles, such as: PR+/ER+/

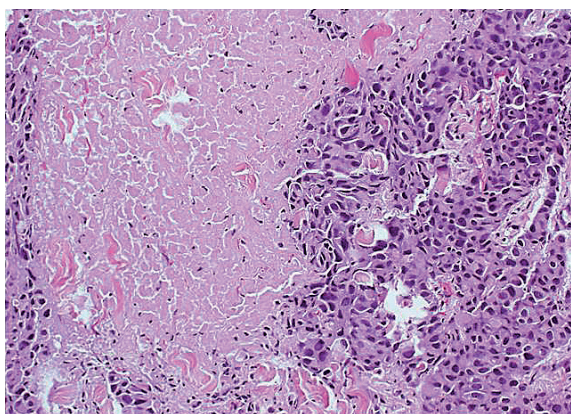


Fig.1. Histopathological image of invasive ductal carcinoma of no special type of the breast with necrosis, Grade 3 ($\times 400$ magnification)

/HER2+ (22.4%); PR-/ER-/HER2+ (32.76%); PR-/ER-/HER2- (12.07%); PR+/ER+/HER2- (32.76%). In all four subgroups of cancers with different basic immunohistochemical profiles, the IDC-NST group was most numerous (68.96%). In our studies we assessed the relationship between histological type of breast cancer and basic immunohistochemical profile – statistical significance was noted in all cases (*p* < 0.05) (Table 1).

Basic immunohistochemical profile (ER, PR, HER2) assessment was broadened by a new marker, HIF-1 α , in our study (Fig. 2 A, B, C, D). In the assessment of HIF-1 α expression in invasive breast cancers we distinguished eight subgroups of cancers with various immunohistochemical profiles taking the new marker into consideration (Table 2):

PR+/ER+/HER2+/HIF-1 α + (5.17%);
PR-/ER-/HER2+/HIF-1 α + (15.51%);
PR-/ER-/HER2-/HIF-1 α + (5.17%);
PR+/ER+/HER2-/HIF-1 α + (10.34%);
PR+/ER+/HER2+/HIF-1 α - (17.24%);
PR-/ER-/HER2+/HIF-1 α - (17.24%);
PR-/ER-/HER2-/HIF-1 α - (6.89%);
PR+/ER+/HER2-/HIF-1 α - (22.41%).

In our study among all invasive breast cancers 36.2% exhibited HIF-1 α expression in the nuclei of neoplastic cells, while no HIF-1 α expression was noted in 63.8% of cases (Fig. 2 A). IDC-NST comprised the most numerous group (61.9%) among all histological types of cancers positive for HIF-1 α expression (Table 2) (Fig. 1 B). We also assessed the correlation between the histological type of cancer and the basic immunohistochemical profile that included HIF-1 α expression, and statistical significance was noted only in the case of PR-/ER-/HER2-/HIF-1 α - and PR+/ER+/HER2-/HIF-1 α - (*p* = 0.028 and *p* = 0.008, respectively). As much as 57.1% of “triple negative cancers” (TNBC) did not demonstrate the expression of HIF-1 α (Table 2).

In our study, we assessed the relationship between the histological grade of malignancy (G1-G3), tumor size (pT) and the presence of lymph node metastases (pN) and a basic immunohistochemical profile, including HIF-1 α expression. However, only in the case of the PR+/ER+/HER2-/HIF-1 α + /immunohistochemical profile and histological grading (G1-G3) did we note a statistical significance (*p* = 0.006) (Table 3). Among the G3 cancers, 57.14% of them expressed HIF-1 α , while in the group of G2 cancers, 33.3% of them expressed HIF-1 α . Taking all cancers into consideration the largest group exhibiting HIF-1 α consisted of G3 cancers (Table 3). We assessed the correlation between tumor size (pT) and basic immunohistochemical profile including HIF-1 α expression and demonstrated that among all cancers exhibiting HIF-1 α expres-

Table 1. Relationship between histological type of invasive breast cancer with metastasis to lymph nodes and the basic immunohistochemical profile (ER, PR, HER2)

Immunohistochemistry – basal panel for diagnosis of breast cancer	Frequency n = 58	Histological type of invasive breast cancer					p-value
		IDC-NST	IDC	ILC	metaplastic carcinoma	mixed ductal and lobular	
PR+/ER+/HER2+	13	9	3	0	1	0	< 0.001*
PR-/ER-/HER2+	19	10	7	0	1	1	< 0.001*
PR-/ER-/HER2-	7	5	0	0	2	0	0.012*
PR+/ER+/HER2-	19	15	1	2	1	0	< 0.001*

* statistically significant results ($p < 0.05$); IDC-NST – invasive ductal carcinoma of no special type; IDC – invasive ductal carcinoma; ILC – invasive lobular carcinoma.

Table 2. Relationship between histological type of invasive breast cancer with metastasis to lymph nodes and basic immunohistochemical profile, including HIF-1 α expression

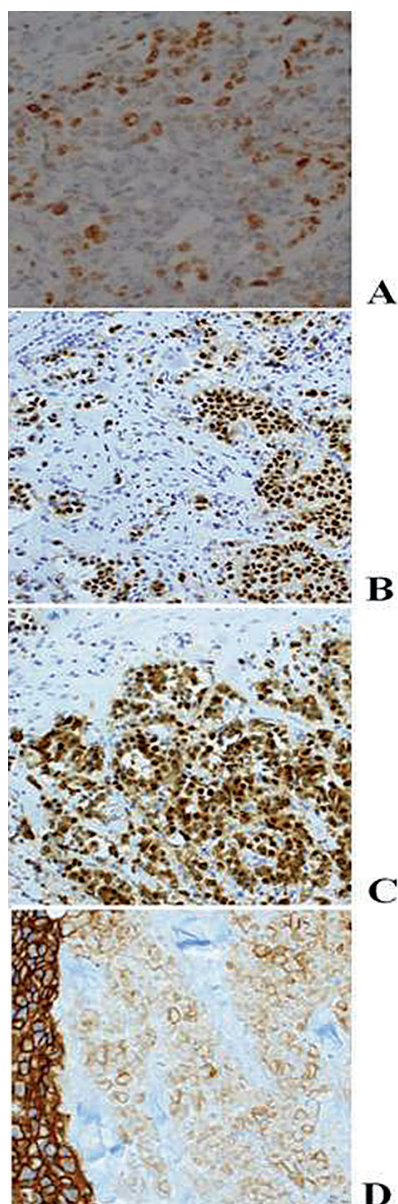
Immunohistochemistry – basal panel for diagnosis of breast cancer and expression of HIF-1 α	Frequency n = 58	Histological type of invasive breast cancer					p-value
		IDC-NST	IDC	ILC	metaplastic carcinoma	mixed ductal and lobular carcinoma	
PR+/ER+/HER2+/HIF1- α +	3	1	2	0	0	0	0.212
PR-/ER-/HER2+/HIF1- α +	9	5	2	0	1	1	0.888
PR-/ER-/HER2-/HIF1- α +	3	2	0	0	1	0	0.060
PR+/ER+/HER2-/HIF1- α +	6	5	0	0	1	0	0.578
PR+/ER+/HER2+/HIF1- α -	10	8	1	0	1	0	0.902
PR-/ER-/HER2+/HIF1- α -	10	5	5	0	0	0	0.153
PR-/ER-/HER2-/HIF1- α -	4	3	0	0	1	0	0.028*
PR+/ER+/HER2-/HIF1- α -	13	10	1	1	1	0	0.008*

* statistically significant results ($p < 0.05$).

sion the most numerous group comprised of cancers with pT2 staging (47.61%) (Table 3). As much as 36.2% of cancers with lymph node metastases exhibited HIF-1 α expression, although no statistically significant dependence was demonstrated between HIF-1 α expression and the presence of lymph node metastases (Table 3). We also examined the relationship between HIF-1 α expression and the basic immunohistochemical profile (ER, PR, HER2) in the examined cancers and noted that tumors with ER-/PR-/HER2+ profile comprise the largest group expressing HIF-1 α (42.86%), while the lack of HIF-1 α expression was most often noted in cancers with ER+/PR+/HER2- basic immunohistochemical profile (35.1%). The expression of HIF-1 α was most often noted in cancers exhibiting the expression of HER2 protein (57.14%). However, there were no statistically significant correlations between HIF-1 α expression and basic immunohistochemical profiles of studied cancers ($p > 0.05$).

In our studies we also assessed the relationship between clinical features, such as histological grade of malignancy, tumor size or presence of lymph node metastases and the expression of HIF-1 α , EPO receptor, but no statistically significant correlations were shown ($p > 0.05$). The expression of HIF-1 α and EPO-R was most often noted in G3 cancers. In our material, cancers exhibiting HIF-1 α expression were also characterized by EPO-R expression. EPO-R staining was uniform throughout the tumor and an increased expression was seen in tumor cells adjacent to necrotic areas. EPO-R expression was found in the tumor vasculature.

We also evaluated the relationship between the expression of HIF-1 α , EPO-R and the basic immunohistochemical profile (ER/PR/HER2), but no statistically significant correlations were found ($p > 0.05$). The expression of HIF-1 α and EPO-R were most often found in cancers with PR-/ER-/HER2+ immunohistochemical profile (42.85%).



Figs. 2 A, B, C, D. Immunohistochemical image of invasive ductal carcinoma of no special type of the breast (IDC-NST)

A – showing positive nuclear staining of HIF-1 α ; B – showing positive nuclear staining of ER; C – showing positive nuclear staining of PR; D – showing positive membrane staining of HER2 with necrosis, ($\times 400$ magnification)

Our study also assessed the relationship between the expression of HIF-1 α in invasive breast cancers and the expression of EPO-R and areas of necrosis, demonstrating a statistically significant dependence ($p = 0.003$) (Table 4).

Discussion

In most malignancies we may observe a chronic hypoxia phenomenon caused by the lack of vasculature or structural and functional underdevel-

opment of the existing vessels that are unable to supply oxygen to the intensely proliferating neoplastic tissue [9]. In solid tumors hypoxia is a result of growing metabolic activity and oxygen consumption by rapidly proliferating tumor cells and a drop in pH of surrounding environment [10]. Cells located closest to the vessels are supplied with relatively high oxygen concentrations, which becomes reduced as the distance from the vessel increases. Neoplasms are also accompanied by areas of necrosis, where cells die due to inadequate oxygen supply [11]. In malignancies hypoxia also selects cells of malignant phenotype capable of functioning in hostile environment. Hypoxia leads to the activation of a number of adaptive processes associated with the activation of anaerobic metabolism, intense neovascularization, changes in regulation of cell cycle and increased proliferation. These processes are aimed at adapting cells to survival under difficult conditions of reduced oxygen supply [14]. Moreover, hypoxia influences intercellular interactions, activates invasion and migration [15], increasing invasive potential of cells, enabling them to initiate metastasis. Cells that activate this response and generate a more aggressive phenotype are subject to selection among the population of tumor cells through emergence of hypoxia-induced genetic instability [16]. In order to survive and grow under difficult hypoxic conditions cells activate a number of adaptive mechanisms, such as: Switching to anaerobic metabolism, resistance to apoptosis, uncontrolled replication, instability of genomic DNA, escape from immunological reaction, induction of angiogenesis, migration to the regions of the body where hypoxia is less pronounced.

Correlation between hypoxia and poor prognosis was observed in many malignancies, such as breast, lung, colon and cervical cancer [17–20]. It was demonstrated in a number of publications on breast cancers [21], brain tumors [22], cervical cancers [23] as well as head and neck tumors [24] that overexpression of HIF-1 α is an unfavorable predictive and prognostic factor, particularly with regard to radiation treatment. However, in the studies by Filles et al. [25] and Volm et al. [26] on squamous cell carcinoma of the oral cavity and non-small cell lung cancer, authors found that overexpression of HIF-1 α protein is associated with higher probability of survival after radiotherapy [25, 26]. However, it should be noted that such conclusions might result from a small number of patients included in the studies as well as the heterogeneity of study groups in terms of tumor advancement and treatment. Overexpression of HIF-1 α is an unfavorable predictive factor since this protein is involved in stimulation of angiogenesis. Górski et al. [27] cor-

Table 3. Relationship between basic immunohistochemical profile, including HIF-1 α expression, and clinicopathological parameters of invasive breast cancer with metastasis to lymph nodes

Immunohisto- chemistry – basal panel for diagnosis of breast cancer and expression of HIF-1 α	Frequency n = 58	Prognostic parameters												
		histological grade				tumor stage					nodal stage			
		G1	G2	G3	p-value	pT1	pT2	pT3	pT4	p-value	pN1	pN2	pN3	p-value
PR+/ER+/HER2+/ HIF1- α +	3	0	1	2	0.831	3	0	0	0	0.159	1	2	0	0.268
PR-/ER-/HER2+/ HIF1- α +	9	1	2	6	0.322	2	5	1	1	0.452	3	4	2	0.391
PR-/ER-/HER2-/ HIF1- α +	3	0	0	3	0.228	1	2	0	0	0.731	1	1	1	0.744
PR+/ER+/HER2-/ HIF1- α +	6	1	4	1	0.006*	3	3	0	0	0.835	3	2	1	0.849
PR+/ER+/HER2+/ HIF1- α -	10	0	3	7	0.401	3	4	0	3	0.188	5	3	2	0.900
PR-/ER-/HER2+/ HIF1- α -	10	0	6	4	0.632	5	4	0	1	0.851	5	2	3	0.507
PR-/ER-/HER2-/ HIF1- α -	4	0	1	3	0.405	1	2	1	0	0.730	3	0	1	0.485
PR+/ER+/HER2-/ HIF1- α -	13	1	8	4	0.229	4	8	0	1	0.840	10	3	0	0.095

* statistically significant results (p < 0.05).

Table 4. Relationship between expression of HIF-1 α , and EPO-R and necrosis invasive breast cancer with metastasis to lymph nodes

Parameter	Frequency n = 58	Expression of HIF-1 α		p-value
		positive	negative	
EPO-R positive	21	21	0	0.003*
EPO-R negative	37	0	37	0.003*
Necrosis positive	21	21	0	0.003*
Necrosis negative	37	0	37	0.003*

* statistically significant results (p < 0.05).

roborated this hypothesis, demonstrating a correlation between increased level of VEGF cytokine after radiation and higher rate of survival of vascular endothelial cells as well as faster tumor growth in a murine model [27].

In studies by van der Groep et al. [28] HIF-1 α overexpression was observed in ductal breast carcinoma *in situ* – DCIS (respectively: 63% of BRCA1, 62% of BRCA2 and 34% of non-BRCA mutation) [28].

In studies by Gruber et al. [12] HIF-1 α overexpression was observed in breast cancer, respective-

ly: 44% patients for the negative HIF-1 α expression group, 33% patients for the moderate HIF-1 α expression group and 23% for the intense HIF-1 α expression group [12], while in our studies among all invasive breast cancers 36.2% exhibited HIF-1 α expression in the nuclei of neoplastic cells and no expression of HIF-1 α was demonstrated in 63.8% of cases. In studies by Vleugel et al. HIF-1 α expression was detectable in 44% patients with breast cancer. In these studies, high HIF-1 α expression was significantly correlated with a poor histological grade and the presence of necrosis [29].

In our study, we also evaluated the relationship between the degree of histological malignancy (G1–G3) and the basic immunohistochemical profile, including HIF-1 α expression. However, a statistically significant correlation was noted only in the case of PR+/ER+/HER2-/HIF-1 α + profile and histological grade of malignancy (G1–G3) ($p = 0.006$) (Table 3). Authors of other studies have not investigated the relationship between HIF-1 α and the degree of histological grade, taking into account the basic immunohistochemical profile.

In our material, among G3 cancers, 57.14% exhibited HIF-1 α expression, while 33.3% of G2 cancers expressed HIF-1 α . Taking all cancers into consideration the most numerous group exhibiting HIF-1 α comprised of G3 cancers (Table 3).

Van der Groep et al. found no correlation between the expression of HIF-1 α and histological grade, and found no statistical relationship between the expression of HIF-1 α and the expression of ER, PR and HER2 [28]. We obtained similar results in our own research – no statistically significant correlation was found between the expression of HIF-1 α and the basic immunohistochemical profile (ER/PR/HER2) in the examined tumors ($p > 0.05$). We also examined the relationship between HIF-1 α expression and basic immunohistochemical profile (ER/PR/HER2) in the examined cancers and noted that tumors with ER-/PR-/HER2+ profile comprise the largest group expressing HIF-1 α (42.86%), while the lack of HIF-1 α expression was most often noted in cancers with ER+/PR+/HER2- basic immunohistochemical profile (35.1%). In our study, expression of HIF-1 α was most often noted in cancers exhibiting expression of HER2 protein (57.14%). Similar results were obtained by Yehia et al. (2015) finding in the whole group of breast cancer 39% of cancers that express HIF-1 α (own research – 36.2%), and lack of expression of HIF-1 α found in 61% (respondents own – 63.8%). Also showed the expression of HIF-1 α at 50% TNBC (own research 42.9% TNBC), and showed the expression of HIF-1 α at 50% of cancers that express HER2(+) (researches own 57.14%). Yehia et al. in their study found that half of TNBC is linked with a marker of hypoxia (HIF-1 α), but pointed out that this relationship is not only unique to TNBC, as similar findings were observed in the present study. Also Yehia et al. demonstrated that half of cancers HER2(+) showed the expression of HIF-1 α , and similar results were observed in the present study [30].

In our study we also evaluated a relationship between tumor size (pT) and basic immunohistochemical profile, which included HIF-1 α expression, and demonstrated that among cancers ex-

pressing HIF-1 α pT2 tumors comprised the most numerous group (47.61%) (Table 3). However, no statistically significant correlations were found between HIF-1 α expression and tumor size (pT), which is in agreement with studies by Vleugel et al. who failed to demonstrate a statistically significant relationship between tumor size and HIF-1 α expression ($p = 0.08$) [29].

Also in studies by Gruber et al. found no correlation with well-known prognostic parameters, such as the T stage, the grade or the number of positive lymph nodes ($p > 0.05$). They showed only a statistical significance between the expression of HIF-1 α and the expression of progesterone receptor ($p = 0.006$).

Studies by Gruber et al. demonstrated the expression of HIF-1 α in the majority of patients with lymph node metastasis. Gruber et al. (2004) in their study showed that the presence of HIF-1 α is predictive of a poor outcome, although its impact is less evident than an advanced T stage or the number of positive lymph nodes [12].

In our study 36.2% of cancers with lymph node metastasis exhibited HIF-1 α expression, although no statistically significant correlations were demonstrated between HIF-1 α expression and presence of lymph node metastasis (Table 3). Similarly, Vleugel et al. did not demonstrate in their studies a statistically significant relationship between the expression of HIF-1 α and lymph node metastasis [29].

In our study, we found EPO-R expression in 36.2% of cases, and Pelekanou et al. showed that in 50 cases (82%) the tumor mass was positive for EPO-R, with staining being more intense at the growing edge of the tumor [31]. In our material we also evaluated the relationship between clinical features, such as histological grade of malignancy, tumor size or presence of lymph node metastases and expression of HIF-1 α , and receptor for EPO, but no statistically significant correlations were demonstrated. Acs et al. also revealed in their study no correlation between the expression of EPO-R and the size of the tumor [32]. Gruber et al. also revealed in their study no correlations HIF-1 α with well-known prognostic parameters, such as the T stage, the grade or the number of positive lymph nodes [12].

However, the expression of HIF-1 α and EPO-R was most often found in G3 cancers. In our study, the cancers exhibiting HIF-1 α expression also expressed EPO-R. In our material, we evaluated the relationship between the HIF-1 α expression in invasive breast cancers and the expression of EPO-R, demonstrating a statistically significant correlation ($p = 0.003$) (Table 4). Similar results were obtained by Wincewicz et al. [4].

Furthermore, in our study, no statistically significant dependence was found between the expression of HIF-1 α and ER or PR ($p > 0.05$). Gruber et al. published different results, showing a statistically significant association that exists only between HIF-1 α expression and the progesterone receptor expression, and also showed no statistically significant relationship in the case of ER expression [12].

The majority of “triple negative” invasive breast cancers (ER-/PR-/HER2-) did not exhibit the expression of HIF-1 α protein or EPO-R, which might indicate better prognosis in this group of patients, since the lack of HIF-1 α and the EPO-R expression limits the process of angiogenesis and reflects better oxygenation of neoplastic cells, which might increase tumor susceptibility to radiotherapy, but it can also suggest that targeted therapies consisting of agents that reduce HIF-1 α expression in cancer cells might be less effective in TNBC. However, such a suggestion requires further studies on a larger group of patients diagnosed with TNBC. The expression of HIF-1 α

was more often noted in invasive HER2+ cancers characterized by a high degree of aggressiveness and poorer prognosis, which might suggest that the presence of HIF-1 α protein expression in HER2+ cancers could be an additional prognostic factor. The frequent occurrence of the phenotype of HIF-1 α and EPO-R in cancers invasive HER2+, in the absence of ER and PR, may suggest that HIF-1 α and EPO-R may be an indicator of the aggressiveness of invasive breast cancers, indicating the need for a specific forms of treatment in this group of patients. The expression of EPO-R was found most frequently in poorly differentiated carcinomas, but the lack of correlation between EPO-R and the histological grade of malignancy, histological type of cancer, tumor size or presence of lymph node metastases does not substantiate establishing EPO-R as a prognostic factor in the diagnostics of breast cancer. A correlation was demonstrated between the expression of HIF-1 α protein and EPO-R in invasive breast cancers, suggesting that HIF-1 α stimulates vessel formation by the tumor cells.

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Conflict of interest: None declared

Received: 14.01.2016

Revised: 4.05.2016

Accepted: 12.05.2016