Review and Research on Cancer Treatment
Volume 4, Issue 1 (2018) ISSN 2544-2147

Immunohistochemical expression of erythropoietin in invasive breast carcinoma with metastasis to lymph nodes

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Abstract: Introduction: Tumor characteristics, such as size, lymph node status, histological type of the neoplasm and its grade, are well known prognostic factors in breast cancer. The ongoing search for new prognostic factors include Bcl2, Bax, Cox-2 or HIF1-alpha, which plays a key role in phenomenon of tumor hypoxia and might induce transcription of the *EPO* gene. Erythropoietin may influence lymph node metastasis or stimulate tumor progression, thus it seemed interesting to determine its expression in invasive breast cancers with lymph node metastases presenting different basic immunohistochemical profiles (ER, PR, HER2).
Aim: To evaluate the relationship between histological grade, tumor size, lymph node status, expression of ER, PR, HER2 and immunohistochemical expression of erythropoietin in invasive breast cancer with metastasis to lymph nodes.
Materials and methods: The material consisted of histological preparations derived from patients treated for invasive breast cancer with metastasis to lymph nodes. Examined samples were stained using standard methods. Routine tests were additionally performed in order to determine immunohistochemical expression of basic profile of diagnostic markers, such as estrogen receptor (ER), progesterone receptor (PR) and HER2. Expression of erythropoietin (EPO) was assessed through using of an appropriate antibody against its antigen.
Results: Among the studied group of cancers we selected four subgroups with different basic immunohistochemical profiles. Additionally we studied the relationship between histological type of breast cancer and basic immunohistochemical profile and a statistically significant correlation was noted in all cases. Expression of erythropoietin was determined in all histological breast cancer types and it was most frequently identified independently in: invasive ductal carcinoma of no special type (IDC – NST), cancers with the highest histological grade (G3) and cancers evaluated as pT2 and pN1. The largest group expressing EPO consisted of cancers presenting the ER-/PR-/HER2+ basic immunohistochemical profile, while no EPO expression was most often demonstrated in cancers with ER+/PR+/HER2- immunohistochemical profile. Furthermore our study revealed EPO expression in over 40% of cases in the group of TNBC (ER-/PR-/HER2-).
Conclusions: In our study we demonstrated a statistically significant correlation between markers included in the basic immunohistochemical profile (ER, PR, HER2) with histological type of invasive breast cancer. There was no relationship between markers of basic immunohistochemical profile and expression of EPO as well as no statistically significant dependence was found between expression of EPO and basic clinical features. However, conducted studies allow for formulating important conclusions for pathomorphological diagnostics. Moreover, expression of EPO in almost 40% of cancers with ER-/PR-/HER2- immunohistochemical profile (TNBC) suggests that in TNBC erythropoietin might be a prognostic marker.

1. Introduction

Tumor characteristics, such as size, lymph node status and histological type of the neoplasm, are well known prognostic factors in breast cancer. Certain microscopic features of the neoplasm, such as histological grade of malignancy or presence of neoplastic cells in blood and lymphatic vessels, also deserve special attention [1, 2]. Immunohistochemical techniques constitute an excellent tool for determination of new factors characteristic for neoplasms that may be

considered prognostic or predictive factors and therefore, enabling determination of immunohistochemical profiles of various histological types of breast cancer. Standardization of methods of immunohistochemical detection of estrogen receptor (ER), progesterone receptor (PR) and HER2 protein overexpression contributed to inclusion of ER, PR and HER2 status assessment into routine evaluation of prognostic and predictive factors in breast cancer. Assessment of ER, PR and HER2 is currently essential to the therapeutic process. There is a number of factors, such as: Bcl2, Bax, or Cox-2 playing a key role in the process of carcinogenesis and cancer progression, but due to lack of standardized assessment methods, as well as lack of evidence for their clinical value, they are not used in routine pathomorphological evaluation of breast cancer and remain subject of intense research [3]. Since achieving the best antineoplastic effect and providing the patient with optimal quality of life are primary goals of treatment of neoplastic diseases, including breast cancer, there is an ongoing search for prognostic and predictive factors based on morphological features and knowledge of other characteristics of neoplastic cells enabling proper assessment of disease course, malignancy risk and susceptibility to treatment. In cases of neoplasms without lymph node metastases the following factors were proven important for the prognosis: patient age, histological type of neoplasm, histological grade of malignancy, proliferative activity and DNA ploidy. Therefore, the factors currently taken into consideration in the assessment of breast cancers include: markers of proliferation (mitotic index, proportion of cells in the S phase of the cell cycle, expression of nuclear antigen Ki67 and PCNA), DNA ploidy, expression of estrogen and progesterone receptors as well as HER2 protein [4].

Recent years have brought about growing interest in the phenomenon of tumor hypoxia and augmented activity of HIF1-alpha, a marker of hypoxia, which is considered one of the most important factors responsible for activation of tumor angiogenesis. It is believed that HIF1-alpha might induce transcription of the *EPO* gene. Its product, erythropoietin, is a physiological regulator of erythropoiesis sensitive to hypoxia. Biological action of erythropoietin involves induction of erythropoiesis by stimulating proliferation and differentiation of red blood cell precursors into mature erythrocytes. However, the effects of erythropoiesis are not limited to the hematopoietic system. Numerous reports published in the recent years indicate that erythropoietin is a cytokine with autocrine and paracrine properties and affects many non-hematological tissues [5]. Local production of erythropoietin was demonstrated in nervous tissue, genital tract and placenta, while expression of erythropoietin receptor was identified, among other locations, in kidneys, lungs and muscle tissue [5,6]. Erythropoietin receptors were also found in many neoplasms: colon adenocarcinoma, stomach cancer, lung cancer, brain cancer, neoplasms of the head and neck, renal tumors, and in prostate cancer [7]. Ability of erythropoietin to stimulate angiogenesis in both normal and neoplastic cells together with a finding of erythropoietin receptor expression on neoplastic cells and cells of vascular endothelium suggest that erythropoietin might directly influence tumor growth and inhibit apoptosis, stimulating tumor progression or metastasis. It could modify tumor cells' sensitivity to chemo- and radiotherapy [5-7].

The influence of erythropoietin on lymph node metastasis is an interesting, yet scarcely investigated, issue. Therefore, it seemed interesting to determine erythropoietin expression in invasive breast cancers with lymph node metastases presenting different basic immunohistochemical profiles (ER, PR, HER2). From a time perspective, results of this research might become an important parameter in the assessment of the risk of metastasis to lymph nodes and other organs.

2. Aim

The aim of this study was to evaluate the relationship between histological grade, tumor size, lymph node status, expression of ER, PR, HER2 and immunohistochemical expression of erythropoietin in invasive breast cancer with metastasis to lymph nodes.

3. Materials and methods

The material consisted of histological preparations derived from patients treated for invasive breast cancer with metastasis to lymph nodes. Histological and immunohistochemical studies were performed at the Department of Pathology, Military Medical Institute in Warsaw. Material

for the study came from biopsies, excisional biopsies and modified radical mastectomies. Samples of tumors were fixed in 10% buffered formalin phosphate. After 24-hour fixation, material was dehydrated using alcohol in gradually increasing concentrations and embedded in paraffin. Paraffin blocks were cut into serial sections 4μm in thickness. They were then stained using standard 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 determinations were carried out: type of neoplasm (WHO classification), tumor grade including tubule formation, and intensity of division as well as the degree of neoplastic cell differentiation and mitotic index defined as a mean number of mitotic figures in neoplastic cells counted in 10 fields of vision at a x400 magnification (surface field 0.17 mm^2).

4. Immunohistochemical Staining Steroid Receptor (ER, PgR) and HER2

Paraffin sections on slides covered with 2% saline solution in acetone at a temperature of 42°C were used for immunohistochemical examination. Routine tests were also performed in order to determine immunohistochemical expression of basic profile of diagnostic markers, such as estrogen receptor (ER), progesterone receptor (PR) and HER2. Immunohistochemistry was performed using the EnVision ™ + HRP DakoCytomation (EnVision ™ Dual Link System-HRP, DAB+, Code: K4065). Monoclonal antibodies against receptors for estrogen (Monoclonal Mouse Anti-Human Estrogen Receptor alpha, 1:50 dilution, Clone: 1D5, Code: IR654, DAKO) and progesterone (Monoclonal Mouse Anti-Human Progesterone Receptor, 1:400 dilution, Clone: PgR636, Code: IR068, DAKO) were used in order to determine the expression of steroid receptors. The study was conducted as follows: sections were incubated at 60°C overnight and subsequently dewaxed. The next step involved revealing the epitope by heating the slides in a buffer for 40 minutes. Subsequently, preparations were left at a room temperature for 20 minutes. Preparations were rinsed in buffer and endogenous peroxidase was blocked by washing in 3% H_2O_2 for 10 minutes. In the next step, preparations were incubated with an appropriate antibody for 30 minutes. After incubation, preparations were rinsed in buffer for 10 minutes, and then incubated with the reagent (Visualization Reagent) for 30 minutes. After incubation with the reagent, preparations were washed in TBS (Tris – Buffered Saline, Code: S1968) with pH 7.6 for 10 minutes, and then incubated with 3,3'– diaminobenidine (DAB) (Substrate Chromogen Solution) for 10 minutes to visualize the color of the reaction. At the end of the procedure, preparations were stained with hematoxylin. Evaluation of the 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. HER2 expression was determined using HerceptTest ™ DAKO test (Code: K5204). It enabled detection of HER2 expression using a polyclonal antibody against this protein (Rb A - Hu HER2 - Rabbit Anti-Human HER2 Protein). Antigen retrieval for HER2 using HerceptTest was performed by immersing and incubating the slides in 10-mmol/L citrate buffer in a calibrated water bath (95-99°C) for 40 minutes (+/- 1 minute). After decanting the epitope- retrieving solution, sections were rinsed in the wash buffer and later, soaked in the buffer for 5 to 20 minutes before staining. The slides were loaded onto the autostainer using the HercepTest program, as described in the manufacturer's insert. In the autostainer, the slides were rinsed, placed in 200μL of peroxidase-blocking reagent for 5 minutes, rinsed, placed in 200μL of primary anti -HER2 protein (or negative control reagent) for 30 minutes, rinsed twice and immersed in 200μL of substrate chromogen solution – DAB for 10 minutes. The slides were counterstained with hematoxylin and finally coverslipped. 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 cell was scored 3+, moderate, circumferential, membranous staining in ≥10% of invasive tumor cells or 3+ staining in ≤30% of cells was designated as 2+ staining, weak and incomplete membranous staining in invasive tumor cells was scored 1+ and no staining was scored 0. Tumors with 0 and 1+ staining were considered negative.

5. Immunohistochemical staining of EPO

Expression of erythropoietin (EPO) was also assessed in all studied invasive breast cancers through use of an appropriate antibody against EPO antigen (Polyclonal Rabbit Anti-Human EPO, 1:100 dilution, Clone: H-162, Santa Cruz Biotechnology ®, Inc.) and subsequent application of the ImmunoCruz ^{TM} Rabbit ABC Staining System for visualization (Santa Cruz Biotechnology ®, Inc.). EPO staining results were scored according to the percentage of cytoplasmic positive cells as follows: (-), <10%; (+), 10%-20%; (++), >20%. Moderate expression EPO was defined as >20% tumor cells with positive staining whereas low expression was <20% [8].

6. Statistical analysis

All statistical analyses were performed with SPSS software v12.0 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 Chi-square (χ^2) was used to assess the relationship between EPO and expression of steroid receptors, HER2, histological type of tumor, degree of histological malignancy and clinical staging. The Fisher exact test was used when the expected cell counts were less than 5. Differences were considered statistically significant at $P < 0.05$.

7. Results

Mean age of studied women was 60.5±11.7 years (median 61 years, range: 43-78 years). Histopathological examination revealed the following percentages of tumors: 68.96% IDC-NST, 18.96% IDC, 3.44% ILC, 6.9% metaplastic carcinoma and 1.72% mixed ductal and lobular carcinoma. Among the studied group of cancers we selected four subgroups with different basic immunohistochemical profiles, such as PR+/ER+/HER2+ (22.4%); PR-/ER-/HER2+ (32.76%); PR-/ER-HER2- (12.07%); PR+/ER+/HER2- (32.76%). In all four subgroups of cancers presenting various basic immunohistochemical profiles IDC-NST was most numerous (68.96%) (Table 1).

Table 1. Correlation between histological type of invasive breast cancer and the basic immunohistochemical profile (ER, PR, HER2)

Immunohistochemistry basal panel for diagnosis of breast cancer	Frequency n=58	Histopathological 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	0.00001132*
PR-/ER-/HER2+	19	10	7	0	1	1	0.00005*
PR-/ER-/HER2-	7	6	0	0	1	0	0.012051*
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

In our material we studied the relationship between histological type of breast cancer and basic immunohistochemical profile and a statistically significant correlation was noted in all cases ($p<0.05$) (Table 1). In our study basic immunohistochemical profile was extended to include a new marker – erythropoietin (EPO) – its expression was determined in all histological breast cancer types (Figure 1). Depending on expression of EPO in invasive breast cancers we differentiated eight subgroups of cancers presenting different immunohistochemical profiles taking into consideration this novel marker – EPO: PR+/ER+/HER2+/EPO+ (10.35%); PR-/ER-/HER2+/EPO+ (12.06%); PR-/ER-/HER2-/EPO+ (5.17%); PR+/ER+/HER2-/EPO+ (8.62%); PR+/ER+/HER2+/EPO- (12.07%); PR-/ER-/HER2+/EPO- (20.69%); PR-/ER-/HER2-/EPO- (6.90%); PR+/ER+/HER2-/EPO- (24.14%) (Table 2). In our study, among all breast cancers 36.2% exhibited EPO expression in neoplastic cells, while in 63.8% EPO expression was not found. IDC-NST was the most numerous group among all histological types of cancers positive for EPO expression (24.1%) (Table 2). No expression of EPO was found in 43.1% of IDC-NST. Among the remaining cancers EPO expression was found in 3.5% of IDC and in 8.6% of metaplastic carcinomas, while no expression of EPO was demonstrated in 15.5% of IDC, 2.5% of ILC and 1.7% of mixed ductal and lobular carcinomas (Table 2). A correlation between histological type of cancer and immunohistochemical profile that included EPO expression was studied and no statistically significant relationships were found (Table 2).

Table 2. Correlation between the histological type of invasive breast cancer and the basic profile of immunohistochemical, including EPO expression

Immunohistochemistry basal panel for diagnosis of breast cancer and expression of EPO	Frequency n=58	Histopathological type of invasive breast cancer					P-value*
		IDC-NST	IDC	ILC	Metaplastic carcinoma	Mixed ductal and lobular	
PR+/ER+/HER2+/EPO+	6	5	0	0	1	0	0.948854
PR-/ER-/HER2+/EPO+	7	5	1	0	1	0	0.95319379
PR-/ER-/HER2-/EPO+	3	1	0	0	2	0	0.5119721
PR+/ER+/HER2-/EPO+	5	3	1	0	1	0	0.9427068
PR+/ER+/HER2+/EPO-	7	4	3	0	0	0	0.764538
PR-/ER-/HER2+/EPO-	12	5	6	0	0	1	0.1861714
PR-/ER-/HER2-/EPO-	4	4	0	0	0	0	0.4695367
PR+/ER+/HER2-/EPO-	14	12	0	2	0	0	0.153851

*Statistically significant results ($P < 0.05$)

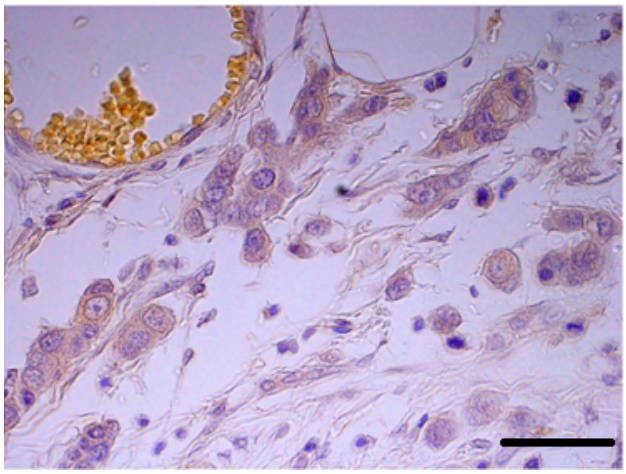

Fig.1. Immunohistochemical image of invasive breast cancer – positive immunohistochemical staining for EPO (original magnification, x200; scale bar, 50µm)

In our study we also assessed the dependence between the degree of histological malignancy (G1-G3), tumor size (pT) or presence of lymph node metastases (pN), and basic immunohistochemical profile that included EPO expression, but no statistically significant correlations were found (Tables 3-5). Among G3 cancers 47% exhibited expression of EPO, while no EPO expression was found in 53% of cases. In a group of G2 cancers 24% were positive for EPO, while 76% of them did not exhibit EPO expression. Among all cancers, the largest group expressing EPO were the G3 cancers (67%) (Table 3).

Table 3. Correlation between the basic profile of immunohistochemical, including EPO expression, and histological grading of invasive breast cancer

Immunohistochemistry basal panel for diagnosis of breast cancer and expression of EPO	Frequency n=58	Histological grade			
		G1	G2	G3	P-value*
PR+/ER+/HER2+/EPO+	6	0	2	4	0.83946
PR-/ER-/HER2+/EPO+	7	0	1	6	0.76498
PR-/ER-/HER2-/EPO+	3	0	0	3	0.50667
PR+/ER+/HER2-/EPO+	5	1	3	1	0.25208
PR+/ER+/HER2+/EPO-	7	0	2	5	0.45285
PR-/ER-/HER2+/EPO-	12	1	7	4	0.97190
PR-/ER-/HER2-/EPO-	4	0	1	3	0.89404
PR+/ER+/HER2-/EPO-	14	1	9	4	0.55822

*Statistically significant results ($P < 0.05$)

We also evaluated the relationship between tumor size (pT) and basic immunohistochemical profile that included expression of EPO and it was demonstrated that among cancers exhibiting EPO expression the pT2 cancers comprised the largest group (52.3%) (Table 4). As much as 36.2% of cancers with metastases to lymph nodes expressed EPO, while no EPO expression was found in 63.8% of cancers with lymph node metastases (Table 5).

Table 4. Correlation between the basic profile of immunohistochemical, including EPO expression, and tumor stage of invasive breast cancer

Immunohistochemistry basal panel for diagnosis of breast cancer and expression of EPO	Frequency n=58	Tumor stage				
		pT1	pT2	pT3	pT4	P-value*
PR+/ER+/HER2+/EPO+	6	1	3	0	2	0.80270
PR-/ER-/HER2+/EPO+	7	0	5	1	1	0.48037
PR-/ER-/HER2-/EPO+	3	1	2	0	0	0.74510
PR+/ER+/HER2-/EPO+	5	4	1	0	0	0.16947
PR+/ER+/HER2+/EPO-	7	5	1	0	1	0.45243
PR-/ER-/HER2+/EPO-	12	7	4	0	1	0.81794
PR-/ER-/HER2-/EPO-	4	1	2	1	0	0.55984
PR+/ER+/HER2-/EPO-	14	3	10	0	1	0.23777

*Statistically significant results *(P < 0.05)*

Table 5. Correlation between the basic profile of immunohistochemical, including EPO expression, and nodal stage of invasive breast cancer

Immunohistochemistry basal panel for diagnosis of breast cancer and expression of EPO	Frequency n=58	Nodal stage			
		pN1	pN2	pN3	P-value*
PR+/ER+/HER2+/EPO+	6	2	2	2	0.91074
PR-/ER-/HER2+/EPO+	7	2	3	2	0.91759
PR-/ER-/HER2-/EPO+	3	2	1	0	0.91308
PR+/ER+/HER2-/EPO+	5	3	2	0	0.78999
PR+/ER+/HER2+/EPO-	7	4	3	0	0.62719
PR-/ER-/HER2+/EPO-	12	6	3	3	0.81628
PR-/ER-/HER2-/EPO-	4	2	0	2	0.46813
PR+/ER+/HER2-/EPO-	14	10	3	1	0.70751

*Statistically significant results *(P < 0.05)*

A relationship between expression of EPO and basic immunohistochemical profile (ER, PR, HER2) was also examined in the studied group of cancers and it was shown that the largest group expressing EPO consisted of cancers presenting the ER-/PR-/HER2+ basic immunohistochemical profile (33.3%), while no EPO expression was most often demonstrated in cancers with ER+/PR+/HER2-immunohistochemical profile (37.8%) (Table 6). Our study also showed that in the group of "triple-negative cancers" (TNBC) (ER-/PR-/HER2-) EPO expression was found in over 40% of cases.

Table 6. The relationship between the basic profile immunohistochemistry and expression of EPO

Immunohistochemistry basal panel for diagnosis of breast cancer	Frequency n=58	Expression of EPO		P-value*
		Positive[1]	Negative[2]	
PR+/ER+/HER2+	13	6	7	0.3968007
PR-/ER-/HER2+	19	7	12	0.943628
PR-/ER-/HER2-	7	3	4	0.97477
PR+/ER+/HER2-	19	5	14	0.421906

*Statistically significant results (P < 0.05)
[1] (+) 10%-20%; (++) >20%
[2] (-) <10%

8. Discussion

It is presently known that erythropoietin stimulates the process of angiogenesis, both in normal and neoplastic cells, results of some studies suggesting that erythropoietin might influence tumor size and inhibit apoptotic mechanisms [9], which can induce metastasis

[10, 11]. Therefore, such an effect may change neoplastic cells' susceptibility to chemo- and radiotherapy, although this suggestion requires numerous further studies. Data regarding expression of erythropoietin in invasive breast cancers with lymph node metastasis in the context of various histological types and basic immunohistochemical profiles (ER, PR, HER2) is scarce. There is also little data on the influence of erythropoietin on lymph node metastasis thus, it seemed interesting to determine expression of erythropoietin in invasive breast cancers depending on the histological type of cancer, presence of lymph node metastasis, expression of estrogen and progesterone receptors, as well as expression of HER2 – markers determined routinely in immunohistochemical diagnostics. Our study was conducted on a group of 58 patients with various histological types of invasive breast cancer. Expression of basic markers used in routine immunohistochemical diagnostics, such as ER, PR and HER2, was assessed and a statistically significant relationship was demonstrated between all histological types of invasive breast cancers and expression of estrogen and progesterone receptors, as well as HER2 expression ($p<0.05$) (Table 1). Since the main goal of this research was to assess the expression of EPO in all studied histological types of invasive breast cancers, it seemed important to distinct four subgroups of cancers exhibiting different immunohistochemical profiles: PR+/ER+/HER2+ (22.4%); PR-/ER-/HER2+ (32.76%); PR-/ER-HER2- (12.07%); PR+/ER+/HER2- (32.76%). In all four subgroups of invasive cancers presenting various basic immunohistochemical profiles expression of erythropoietin was examined using immunohistochemical techniques. In invasive breast cancer, we found weak-to-moderate, granular, cytoplasmic staining for EPO in 36.2% of specimens. In their studies Acs, Zhang et al. (2002) found the presence of weak-to-moderate, granular, cytoplasmic EPO immunostaining in benign mammary epithelial cells in 91.8% and 95.6% of the specimens, respectively [12]. Taking into consideration histological type of invasive breast cancer the largest EPO-positive group in our material comprised the IDC-NST (24.1%). Among the remaining cancers EPO expression was demonstrated in 3.5% of IDC and in 8.6% of metaplastic carcinomas, while in 15.5% of IDC, 3.5% of ILC and in 1.7% of mixed ductal and lobular carcinomas expression of EPO was not found. No statistically significant relationship between histological type of invasive cancers and expression of EPO was found ($p>0.05$). In studies Acs, Zhang et al. (2002) EPO immunostaining was similar in invasive ductal and lobular carcinomas and in carcinomas with mixed ductal and lobular features. Acs, Zhang et al. (2002) found no differences in EPO immunostaining between DCIS and LCIS. Also, the authors found no statistically significant relationship between the expression of EPO and histological type of breast cancer [12]. In our study we assessed the dependence between histological grade of malignancy (G1-G3) and basic immunohistochemical profile, including EPO expression, but no statistically significant correlations were found ($p>0.05$) (Table 3). Among all cancers the most numerous group exhibiting EPO expression were the G3 cancers (67%) (Table 3). We also assessed the relationship between tumor size (pT), presence of lymph node metastasis and expression of EPO, finding that among all cancers expressing EPO, cancers assessed as pT2 comprised the largest group (52.3%) (Table 4). As much as 36.2% of cancers with lymph node metastasis exhibited EPO expression., while in 63.8% of cancers, expression of EPO was not demonstrated (Table 5). We also assessed the relationship between expression of EPO and basic immunohistochemical profile (ER, PR, HER2) in studied cancers and found that the most numerous group exhibiting EPO expression were the cancers presenting ER-/PR-/HER2+ basic immunohistochemical profile (33.3%), while no EPO expression was most often demonstrated in cancers of ER+/PR+/HER2- basic immunohistochemical profile (37.8%) (Table 6). No statistically significant correlation was demonstrated between EPO expression and tumor size (pT), lymph node metastasis (pN) or expression of estrogen and progesterone receptors as well as HER2 ($p>0.05$). Similarly, Acs, Zhang et al. (2002) found in their studies no correlation between EPO immunostaining and tumor size, tumor grade, lymph node status, hormone receptor status, or HER2/neu overexpression [12]. In a study by Acs, Zhang et al. (2002), when the analysis was performed using the differential tumor score for EPO, we found a significant correlation between increased EPO immunostaining in the tumors and the presence of lymph node metastases [12]. From a clinical point of view it is important to determine whether adverse effects of rhEPO therapy correlate with the expression of EpoR in neoplastic cells, since

rhEPO remains an important element of treatment scheme for anemia of malignancy. EpoR protein has been proposed to be nonfunctional in tumor cells due to a non-cell surface location, and therefore, presumably, is not available for activation by rhEPO [13]. Todaro, Turdo et al. (2013) found that breast cancer stem-like cells (BCSCs) isolated from patient tumors express the EpoR. Among all types of breast cancer basal-like subtype demonstrated the greatest expression of EpoR. This trial also showed that BCSCs respond to EPO treatment with increased cell proliferation and self-renewal rate. Importantly, EPO stimulation increased BCSCs resistance to chemotherapeutic agents and activated cellular pathways responsible for survival and drug resistance [14]. Specifically, the Akt and ERK pathways were activated in BCSCs at early time points following EPO treatment, whereas Bcl-xL levels increased at later times. In vivo, EPO administration counteracted the effects of chemotherapeutic agents on BCSCs-derived orthotopic tumor xenografts and promoted metastatic progression both in the presence and in the absence of chemotherapy treatment [14]. All this data shows that EPO acts directly on the breast cancer stem-like cells through activation of specific signal transduction pathways responsible for tumor protection from chemotherapy and accelerates disease progression. According to the latest results of research, the population of neoplastic stem cells seems to be responsible for progression of neoplastic disease, its recurrence and metastasis. In light of those discoveries it is necessary to develop treatment that would target this population of cells. It has been known for several years now that use of rhEPO negatively influences survival among breast cancer patients. Studies by Todaro, Turdo et al. (2013) were the first to show the effect of EPO and EpoR on breast cancer stem cells. These results confirmed that EPO contributes to development of chemotherapy resistance and acceleration of tumor growth [14]. Several years before, Phillips, Kim et al. (2007) investigated the effect of erythropoietin on cancer stem cells in breast cancer cell lines. They found that pharmacological concentrations of rhEPO increased the number of putative breast cancer initiating cells (BCICs) in established breast cancer cell lines. This increase was mediated by the activation of the Notch signaling pathway. Primarily, the Notch pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes. The increase in the number of BCICs observed after rhEPO treatment was significant and the cells were not only viable but, what is more important, exhibited an increased self-renewal capacity as demonstrated by primary in vitro sphere formation [15]. Phillips, McBride et al. (2006) have previously proved that activation of the Notch signaling pathway is a part of the cellular stress response to clinical doses of ionizing radiation [16]. This effect was mediated by increased expression of the Notch receptor ligand Jagged-1 in the non-BCIC population that activated Notch signaling in BCICs. As for radiation, Phillips, Kim et al. (2007) have demonstrated that rhEPO treatment activated Notch signaling pathway in BCICs [15]. Research conducted for the past several years enabled determining function of EPO and EpoR in various types of cells, including demonstration of high activity within neoplastic cells. Over a decade ago Acs, Chen et al. (2004) described autocrine regulation of apoptosis by these proteins in breast cancer. MCF-7 breast cancer cell lines were incubated in increasing concentrations of O_2 and it was shown that increased expression of EPO mRNA correlates tightly with increasing degree of hypoxia in cell culture [17]. Similarly, escalation of acute hypoxic conditions induced transcription of EpoR mRNA. Verification with Western blot corroborated the differences in EPO and EpoR protein expression in a manner corresponding to mRNA transcripts. The study also confirmed that autocrine EPO signaling induced by moderate levels of hypoxia inhibits hypoxia-induced apoptosis and promotes survival in MCF-7 human breast cancer cells. Anti-apoptotic effect of EPO correlated with upregulation of Bcl-2 and Bcl-X_L, thus its mechanisms appear to be similar to those described in hematopoietic cells. The above-described mechanism, colloquially known as "apoptosis escape of tumors", plays a key role in development and progression of neoplastic process. Evasion of apoptosis is also one of the most important features enabling determination of the degree of tumor malignancy. Acs, Zhang et al. (2003) previously shown that EPO can inhibit chemotherapeutic drug-induced apoptosis and cytotoxicity [18]. In the next study their results suggested that the increased EPO signaling induced by tumor hypoxia can play a significant role in resistance to therapy of hypoxic tumors [19]. Hypoxia is a common phenomenon accompanying solid tumors, such as breast cancer. Inadequate oxygen distribution, which most often

affects central regions of rapidly growing lesions, leads to rearrangement of gene expression in tumor tissues subjected to hypoxia. In effect, there is increased synthesis of proteins directed at protecting the cell from adverse conditions. It is achieved by, among other things, blocking cell's ability to initiate apoptosis. This phenomenon is advantageous in most cells, but very dangerous from a point of view of neoplastic process. Characteristic proteins that exhibit increased expression under hypoxic conditions include EPO, EpoR and HIF-1α. Increased intracellular concentration of STAT3 (signal transducer and activator of transcription 3) may be also one of the markers of hypoxia. This transcription factor is activated through phosphorylation of tyrosine 705. Despite the fact that this protein becomes overexpressed in neoplasms and was even considered a protooncogene, accumulation of STAT3 can be detected under various non-neoplastic conditions of increased cell turnover and enhanced biosynthesis of various proteins [20, 21]. Links between expressions of the proteins suggests functional dependences between STAT, HIF-1α, EPO and EpoR in cell-to-cell signaling in breast cancer [22]. Breast cancer also involves upregulation of transcriptional agents like STAT3, STAT3 activator – EpoR (erythropoietin receptor), and a HIF-1 downstream protein – EPO [23]. STAT3 contributes to increase of EPO expression, which is also HIF-1α dependent. Tyrosine phosphorylation of STAT3 is triggered by EPO. HIF-1α overexpression is an indicator of poor prognosis and significantly decreased survival among patients with breast cancer [24]. HIF-1 upregulates transcription of angiogenic genes like EPO and vascular endothelial growth factor (VEGF), which induce sprouting of new vessels and result in increased risk of metastasis as they increase contact surface between tumor cells and vasculature. HIF-1 is responsible mainly for cellular adaptation to hypoxic conditions. Genes triggered by this factor are responsible mainly for the improvement in oxygen supply, adaptation of cells to anaerobic metabolism conditions as well as for other changes facilitating cell survival in insufficient oxygen availability. HIF-1 induces transcription of cyto-protective proteins in malignant cells in hypoxic conditions [25,26]. HIF-1 and STAT3 actions have been associated indirectly with each other. This relationship was highlighted mainly by interference of STAT3 transcription with small-molecule inhibitor and resultant downregulation of HIF-1 and VEGF that delayed tumor growth and angiogenesis [27].

EPO and EpoR are induced by hypoxia in breast cancer and could contribute to increased survival rate of tumor cells via counteraction to hypoxic injury [28]. EPO counteracts outflow of cytochrome c from mitochondrion by upregulation of Bcl-xL. EPO prevents apaf-1 complex dependent activation of caspase 9 and 3 by inhibition of binding cytochrome c to apaf-1 and cyt-c in cytoplasm [23]. Wincewicz, Sulkowska et al. (2007) described the above correlations in ductal breast carcinoma. STAT3 was detected in 50% of cancers, HIF-1α in 72% of cases, EPO in 89% of all the cancers and EpoR in 72%. There were significant associations between expression of STAT3 and HIF-1α. STAT3 was significantly associated with expressions of EPO and EpoR in cancers of all patients. HIF-1α was correlated with EPO and EpoR in most of analyzed groups. This data indicates tight interrelationships between all those proteins in breast cancer. They all become overexpressed under hypoxic conditions and they all significantly influence the biology of the tumor. Occurrence of HIF-1α was significantly increased in chemotherapy spared tumors compared to chemotherapy treated cancers because chemotherapy could destructively affect cancer cells via inhibition of protein expression [23]. Correlations between STAT3 and EPO suggest their action in accord to support survival of breast cancer cells in human tumors in the same fashion as in cell lines [29].

Results of studies by Reinbothe, Larsson et al. (2014) somewhat revolutionized the perception of the role of EPO and EpoR in breast cancer. According to these results, rhEPO stimulation of cultured EpoR-expressing breast cancer cells did not result in increased proliferation, overt activation of EpoR (receptor phosphorylation) or a consistent activation of canonical EpoR signaling pathway mediators such as JAK2, STAT3, STAT5, or AKT. However, EpoR knockdown experiments suggested functional EPO receptors in estrogen receptor positive ($ER\alpha^+$) breast cancer cell lines, as reduced EpoR expression resulted in decreased proliferation, but not cell death. This effect on proliferation was not seen in estrogen receptor negative ($ER\alpha^-$) cells. These observations suggest that decreased expression of EpoR reduced the ERα-dependent proliferation in breast cancer [30]. EpoR expression seems to play a role in proliferation control of $ER\alpha^+$ breast cancers while survival seems to be unaffected by reduction of EpoR expression. Studies by

Reinbothe, Larsson et al. (2014) show that in neoplasms of the breast exhibiting EpoR expression proliferation is induced in conjunction with this receptor, but by an EPO-independent mechanism in $ER\alpha^{+}$ breast cancers. Molecular mechanisms affecting the interactions between EpoR and ERα as well as their effect on the biology of tumor cells require further studies [30]. It is currently unknown how these proteins cooperate to regulate cell proliferation in breast cancer. Questions need to be addressed in order to find out how EpoR should be targeted to modulate the potent ERα signaling pathways. Answers to this query may reveal the new potent therapeutic methods in breast cancer. Conclusions from the studies by Reinbothe, Larsson et al. (2014) that use of rhEPO does not influence proliferative activity or survival in the five tested breast cancer cell lines are consistent with the results of studies by LaMontagne, Butler et al. (2006) [30,31]. On the other hand, contradictory findings were reported in a study by Acs, Acs et al. (2001) [28]. There are also discrepancies in the published data that demonstrate that rhEPO stimulation of breast cancer cells results in changes in cell signaling mediators, such as AKT, ERK1/2 and STATs [32, 33].

Despite the fact that initially EPO was only attributed a role in regulation of erythropoiesis, this protein turned out to be an important link connecting numerous signal transduction pathways, both in many normal as well as cancerous non-hematopoietic tissues. Moreover, in the recent years there have been reports describing functional autocrine/paracrine EPO/EpoR systems in human neoplastic cells derived from breast cancer, cervical cancer, melanoma and prostate cancer. This data suggests that EPO/EpoR axis may affect tumor growth, disease progression and metastasis [17, 34].

Liang, Qiu et al. (2014) described the autocrine/paracrine functions of EPO produced by breast cancer cells under both normoxic and hypoxic conditions. They found that the level of EPO produced by these cells was higher in hypoxia than in normoxia. This observation is consistent with the knowledge that EPO is a product of hypoxia-inducible gene expression. Unfortunately, this study did not allow for determining whether the effects of EPO are produced as a result of auto- or paracrine signaling. However, it was determined with high certainty that both types of intercellular communication influenced the observed effects in cultured cell lines. Results presented in the publication demonstrated also that EPO/EpoR autocrine/paracrine signaling could mediate migration and influence invasion potential of breast cancer cells. It suggests that autocrine/paracrine EPO signaling could be one of the effector mechanisms, through which HIF-1 factor influences the invasiveness of breast cancer [35]. Targeting of HIF-1, which is being actively investigated as a potential strategy for cancer therapy, could inhibit the effects of autocrine/paracrine EPO signaling on cell migration and invasiveness. Inhibition of EPO autocrine/paracrine signaling pathways in cancer cells could be one of the mechanisms explaining the anticancer effects of several anti-HIF-1 agents reported in the literature [36]. Furthermore, we found that autocrine/paracrine production of EPO also played a role in stimulating tumorsphere growth of breast cancer cells.

In recent studies Zhou, Damrauer et al. (2015) corroborated the hypothesis that exogenous EPO does not significantly influence cell proliferation and does not protect them from chemotherapy-induced apoptosis in vitro. It is somewhat different under in vivo conditions, where EPO distinctly promotes progression of breast cancer. According to these results, researches hypothesized that EPO's tumor promoting effects are seen in vivo but not with in vitro assays because it affects a limited fraction of cells, such as breast tumor initiating cells. Thus, its effects might only be seen with a longer period of EPO administration, such as those achieved in vivo [37].

Moreover, it was observed that treatment of breast tumor-initiating cells (TICs) with EPO activated JAK/STAT signaling as well as promoted their self-renewal. Studies also confirmed the protumorigenic role of endogenous EPO in breast tumorigenesis. It turned out that both breast cancer cells, as well as tumor-associated endothelial cells can produce and release EPO into the microenvironment of the tumor. Endogenous EPO expression was hypoxia-inducible in breast cancer cell lines, but not in human mammary epithelial cells. EPO overexpression in breast cancer cells correlates negatively with progression-free survival [37].

Research conducted in the recent years ascribes EPO another, clinically important role. Observations allow saying that EPO antagonizes treatment with the anti-HER2 antibody, trastuzumab, by activating EpoR/JAK2 downstream effectors, effectively bypassing HER2 signaling [38]. Moreover, the antagonizing effect should be only apparent in patients treated with anti-HER2

antibodies. The fact that adverse effects of rhEPO were observed among various subtypes of breast cancer as well as patients with anemia of malignancy who had not been treated with trastuzumab strongly suggests that there are other pathways not related to HER2, through which EPO promotes tumor progression. The above observations demonstrate an important role of both exogenous and endogenous EPO in the course of breast cancer. Results of studies by Liang, Esteva et al. (2010) show that JAK2 inhibition in combination with chemotherapy is a promising therapeutic strategy rather than EPO/EpoR inhibition in the treatment of breast cancer patients [33].

9. Conclusions

Literature data suggest that EPO might influence tumor growth, disease progression and metastasis. However, numerous studies are conducted on cell lines, but there is scarce data from studies conducted on tissues using immunohistochemical methods routinely performed in pathomorphological diagnostics. There is an ongoing search for prognostic markers in the diagnostics of breast cancer in women and researchers strive to expand the basic immunohistochemical profile in the diagnostics of breast cancer. In our study we demonstrated a statistically significant correlation between markers included in the basic immunohistochemical profile (ER, PR, HER2) with histological type of invasive breast cancer. There was no relationship between markers of basic immunohistochemical profile and expression of EPO as well as no statistically significant dependence was found between expression of EPO and basic clinical features, such as tumor size, grade of histological malignancy or lymph node status.

However, conducted studies allow for formulating important conclusions for pathomorphological diagnostics. Based on the analysis, it may be concluded that:

- Expression of erythropoietin (EPO) in the cytoplasm of cancer cells, was most frequently identified in invasive ductal carcinoma of no special type (IDC – NST);
- Expression of EPO was most frequently identified in invasive breast cancers evaluated as pT2 (52.3%);
- Expression of EPO was most frequently identified in invasive breast cancers evaluated as pN1;
- Expression of erythropoietin (EPO) in the cytoplasm of cancer cells was most often identified among cancers with the highest histological grade (G3). Our research results suggest that increased EPO signaling may represent a novel mechanism modulating differentiation of cancer cells and is connected with low degree of differentiation of tumor cells;
- Cancers with ER-/PR-/HER2- immunohistochemical profile, the so-called "triple-negative" (TNBC) are characterized by, among other things: greater clinical advancement of the disease at the moment of diagnosis, poor histological differentiation (acc. to Bloom-Richardson classification; G3). In our study the G3 cancers most often expressed EPO; also, among the TNBC expression of EPO was confirmed in over 40% of cases, suggesting that in TNBC erythropoietin might be a prognostic marker.

References

1. Badowska-Kozakiewicz A. M., Patera J., Sobol M., Przybylski J., *The role of estrogen and progesterone receptors in breast cancer - immunohistochemical evaluation of estrogen and progesterone receptor expression in invasive breast cancer in women*, Contemp Oncol, 3 (2015), s. 1-6
2. Badowska-Kozakiewicz A. M., Sobol M., Patera J., Kozłowski W., *Immunohistochemical evaluation of human epidermal growth factor receptor 2 and estrogen and progesterone receptors in invasive breast cancer in women*, Arch Med Sci, 9 (2013), s. 466-471
3. Trost N., Stepisnik T., Berne S., Pucer A., Petan T., Komel R., Debeljak N., *Recombinant human erythropoietin alters gene expression and stimulates proliferation of MCF-7 breast cancer cell*, Radiol Oncol, 47 (2013), s. 382-389
4. Lopez T. V., Lappin T. R., Maxwell P., Shi Z., Lopez-Marure R., Aguilar C., Rocha-Zavaleta L., *Autocrine/paracrine erythropoietin signalling promotes JAK/STAT-dependent proliferation of human cervical cancer cells*, Int J Cancer, 129 (2011), s. 2566-2576
5. Arcasoy M. O., Amin K., Vollmer R. T., Jiang X., Demark-Wahnefried W., Haroon Z. A., *Erythropoietin and erythropoietin receptor expression in human prostate cancer*, Mod Pathol, 18 (2005), s. 421-430

6. Kaushansky K., *Lineage-specific hematopoietic growth factors*, N Engl J Med, 354 (2006), s. 2034-2045
7. Lai S. Y., Grandis J. R., *Understanding the presence and function of erythropoietin receptors on cancer cells*, J Clin Oncol, 24 (2006), s. 4675-4676
8. Wang L., Li H. G., Xia Z. S., *Prognostic significance of erythropoietin and erythropoietin receptor in gastric adenocarcinoma*, World J Gastroenterol, 17 (2011), s. 3933-3940
9. Aapro M., Leonard R. C., Barnadas A., Marangolo M., Untch M., Malamos N., Mayordomo J., Reichert T., Pedrini J. L., Ukarma L., Scherhag A., Burger H., *Effects of once-weekly epoetin beta on survival in patients with metastatic breast cancer receiving anthracycline- and/or taxane-based chemotherapy. Results of the Breast Cancer-Anemia and the Value of Erythropoietin (BRAVE) Study*, J Clin Oncol, 26 (2008), s. 592-598
10. Volgger B., Kurz K., Zoschg K., Theurl I., Ciresa-Konig A., Marth C., Weiss G., *Importance of erythropoietin receptor expression in tumour*, Anticancer Research, 30 (2010), s. 3721-3726
11. Hershman D. L., Buono D. L., Malin J., McBride R., Tsai W. Y., Neugut A. I., *Patterns of use and risks associated with erythropoiesis-stimulating agents among Medicare patients with cancer*, J Natl Cancer Inst, 101 (2009), s. 1633-1641
12. Acs G., Zhang P. J., Rebbeck T. R., Acs P., Verma A., *Immunohistochemical expression of erythropoietin receptor in breast carcinoma*, Cancer, 95 (2002), s. 969-981
13. Kokhaei P., Abdalla A. O., Hansson L., Mikaelsson E., Kubbies M., Haselbeck A., Jernberg-Wiklund H., Mellstedt H., Osterborg A., *Expression of erythropoietin receptor and in vitro functional effects of epoetins in B-cell malignancies*, Clin Cancer Res, 13 (2007), s. 3536–3544
14. Todaro M., Turdo A., Bartucci M., Iovino F., Dattilo R., Biffoni M., Stassi G., Federici G., De Maria R., Zeuner A., *Erythropoietin activates cell survival pathways in breast cancer stem–like cells to protect them from chemotherapy*, Cancer Res, 73 (2013), s. 6393-6400
15. Phillips T. M., Kim K., Vlashi E., McBride W. H., Pajonk F. *Effects of recombinant erythropoietin on breast cancer–initiating cells*, Neoplasia, 9 (2007), s. 1122-1129
16. Phillips T. M., McBride W. H., and Pajonk F., *The response of CD24(-/low)/CD44+ breast cancer – initiating cells to radiation*, J Natl Cancer Inst, 98 (2006), s. 1777-1785
17. Acs G., Chen M., Xu X., Acs P., Verma A., Koch C. J., *Autocrine erythropoietin signaling inhibits hypoxia-induced apoptosis in human breast carcinoma cells*, Cancer Lett, 214 (2004), s. 243-251
18. Acs G., Zhang P. J., McGrath C. M., Acs P., McBroom J., Mohyeldin A., Liu S., Lu H., Verma A., *Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression*, Am J Pathol, 162 (2003), s. 1789-1806
19. Fu P., Jiang X., Arcasoy M. O., *Constitutively active erythropoietin receptor expression in breast cancer cells promotes cellular proliferation and migration through a MAP-kinase dependent pathway*, Biochem Biophys Res Commun, 379 (2009), s. 696-701
20. Sawa S., Kamimura D., Jin G. H., Morikawa H., Kamon H., Nishihara M., Ishihara K., Murakami M., Hirano T., *Autoimmune arthritis associated with mutated interleukin (IL)-6 receptor gp130 is driven by STAT3/IL-7-dependent homeostatic proliferation of CD4+ T cells*, J Exp Med, 203 (2006), s. 1459-1470
21. Murray P. J., *STAT3-mediated anti-inflammatory signaling*, Biochem Soc Trans, 34 (2006), s. 1028-1031
22. Wincewicz A., Koda M., Sulkowska M., Kanczuga-Koda L., Wincewicz D., Sulkowski S., *STAT3 and hypoxia induced proteins – HIF-1alpha, EPO and EPOR in relation with Bax and Bcl-xL in nodal metastases of ductal breast cancers*, Folia Histochem Cytobiol, 47 (2009), s. 425-430
23. Wincewicz A., Sulkowska M., Koda M., Leśniewicz T., Kanczuga-Koda L., Sulkowski S., *STAT3, HIF-1α, EPO and EPOR - signaling proteins in human primary ductal breast cancers*, Folia Histochem Cytobiol, 45 (2007), s. 81-86
24. Schindl M., Schoppmann S. F., Samonigg H., Hausmaninger H., Kwasny W., Gnant M., Jakesz R., Kubista E., Birner P., Oberhuber G., *Austrian Breast and Colorectal Cancer Study Group, Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer*, Clin Cancer Res, 8 (2002), s. 1831-1837
25. Dales J. P., Garcia S., Meunier-Carpentier S., Andrac-Meyer L., Haddad O., Lavaut M. N., Allasia C., Bonnier P., Charpin C., *Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients*, Int J Cancer, 116 (2005), s. 734-739
26. Kronblad A., Jirstrom K., Ryden L., Nordenskjold B., Landberg G., *Hypoxia inducible factor-1alpha is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response*, Int J Cancer, 118 (2006), s. 2609-2616
27. Xu Q., Briggs J., Park S., Niu G., Kortylewski M., Zhang S., Gritsko T., Turkson J., Kay H., Semenza G. L., Cheng J. Q., Jove R., Yu H., *Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways*, Oncogene, 24 (2005), s. 5552-5560
28. Acs G., Acs P., Beckwith S. M., Pitts R. L., Clements E., Wong K., Verma A., *Erythropoietin and erythropoietin receptor expression in human cancer*, Cancer Res, 61 (2001), s. 3561-3565

29. Gritsko T., Williams A., Turkson J., Kaneko S., Bowman T., Huang M., Nam S., Eweis I., Diaz N., Sullivan D., Yoder S., Enkemann S., Eschrich S., Lee J. H., Beam C. A., Cheng J., Minton S., Muro-Cacho C. A., Jove R., *Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells*, Clin Cancer Res, 12 (2006), s. 11-19
30. Reinbothe S., Larsson A. M., Vaapil M., Wigerup C., Sun J., Jögi A., Neumann D., Rönnstrand L., Pĺhlman S., *EPO-independent functional EPO receptor in breast cancer enhances estrogen receptor activity and promotes cell proliferation,* Biochem Biophys Res Commun, 445 (2014), s 163-169
31. LaMontagne K. R., Butler J., Marshall D. J., Tullai J., Gechtman Z., Hall C., Meshaw A., Farrell F. X., *Recombinant epoetins do not stimulate tumor growth in erythropoietin receptor-positive breast carcinoma models*, Mol Cancer Ther, 5 (2006), s. 347–355
32. Jin W., Lin Z., Zhang X., Kong L., Yang L., *Effects and mechanism of recombinant human erythropoietin on the growth of human breast cancer MDA-MB-231 cells in nude mice*, Pathol Res Pract, 211 (2015), s. 570-576
33. Liang K., Esteva F. J., Albarracin C., Stemke-Hale K., Lu Y., Bianchini G., Yang C. Y., Li Y., Li X., Chen C. T., Mills G. B., Hortobagyi G. N., Mendelsohn J., Hung M. C., Fan Z., *Recombinant human erythropoietin antagonizes trastuzumab treatment of breast cancer cells via Jak2-mediated Src activation and PTEN inactivation*, Cancer Cell, 18 (2010), s. 423-435
34. Pelekanou V., Kampa M., Kafousi M., Dambaki K., Darivianaki K., Vrekoussis T., Sanidas E., Tsiftsis D. D., Stathopoulos E. N., Castanas E., *Erythropoietin and its receptor in breast cancer: correlation with steroid receptors and outcome*, Cancer Epidemiol Biomarkers Prev, 16 (2007), s. 2016-2023
35. Liang K., Qiu S., Lu Y., Fan Z., *Autocrine/paracrine erythropoietin regulates migration and invasion potential and the stemness of human breast cancer cells*, Cancer Biol Ther, 15 (2014), s. 89-98
36. Garber K., *New drugs target hypoxia response in tumors*, J Natl Cancer Inst, 97 (2005), s. 1112-1114
37. Zhou B., Damrauer J. S., Bailey S. T., Hadzic T., Jeong Y., Clark K., Fan C., Murphy L., Lee C. Y., Troester M. A., Miller R., Jin J., Darr D., Perou C. M., Levine R. L., Diehn M., Kim W. Y., *Erythropoietin promotes breast tumorigenesis through tumor-initiating cell self-renewal*, J Clin Invest, 124 (2014), s. 553-563
38. Fillmore C. M., Kuperwasser C., *Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy*, Breast Cancer Res, 10 (2008), R25