Relationships between hypoxia markers and the leptin system, estrogen receptors in human primary and metastatic breast cancer: effects of preoperative chemotherapy.

Mariusz Koda¹, Luiza Kanczuga-Koda¹, Mariola Sułkowska¹, Eva Surmacz², Stanislaw Sułkowski¹.

¹Department of Pathology, Medical University of Bialystok, Bialystok, Poland. ²Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, USA.

E-mail addresses:
MK: kodamar@zeus.amb.edu.pl
LKK: luizakoda@gmail.com
MS: sulek@zeus.amb.edu.pl
ES: surmacz@temple.edu
SS: sulek@zeus.amb.edu.pl

Corresponding Author:
Mariusz Koda, Department of Pathomorphology, Medical University of Bialystok, Waszyngtona13, 15-269 Bialystok, Poland. Tel. +48-85-7485944; Fax. +48-85-7485944; e-mail: kodamar@zeus.amb.edu.pl
Abstract

Introduction. Tumor hypoxia is marked by enhanced expression of hypoxia-inducible factor-α (HIF-1α) and glucose transporter-1 (Glut-1). Hypoxic conditions have also been associated with overexpression of angiogenic factors such as leptin. The aim of our study was to analyze the relationships between hypoxia markers HIF-1α, Glut-1, leptin, and its receptor ObR and other breast cancer biomarkers in primary and metastatic breast cancer in patients treated or untreated with preoperative chemotherapy. Methods. The expression of different biomarkers was examined by immunohistochemistry in 116 primary breast cancers and 65 lymph node metastases. 45 of these samples were obtained from patients who received preoperative chemotherapy and 71 from untreated patients. Results. In primary tumors without preoperative chemotherapy, HIF-1α and Glut-1 were positively correlated (p=0.02, r=0.437). HIF-1α in primary and metastatic tumors without preoperative therapy positively correlated with leptin (p<0.0001, r=0.532; p=0.013, r=0.533, respectively) and ObR (p=0.002, r=0.319; p=0.083, r=0.387, respectively). Hypoxia markers HIF-1α and Glut-1 were negatively associated with estrogen receptor alpha (ERα) and positively with estrogen receptor beta (ERβ). In this group of tumors, we also noted positive correlation between Glut-1 and proliferation marker Ki-67 (p=0.017, r=0.433). In samples with preoperative chemotherapy, associations between HIF-1α and Glut-1, HIF-1α and leptin, HIF-1α and ERα as well as Glut-1 and ERβ were lost. Conclusions. Intratumoral hypoxia in breast cancer is marked by coordinated expression of such markers as HIF-1α, Glut-1, leptin and ObR. The relationships among these proteins can be altered by preoperative chemotherapy.
Introduction

During breast cancer development and progression, rapidly proliferating neoplastic cells are often exposed to hypoxia resulting from insufficient local supply of oxygen and nutrition [1]. Hypoxia can affect developing tumor two-way. On one hand, it might inhibit proliferation and stimulate apoptotic or necrotic cell death, while on the other hand, it can induce progression of neoplasms and cause resistance to anti-cancer treatments. In developing tumors, hypoxic cells are more resistant to ionizing radiation and chemotherapy and display a more invasive and metastatic phenotype, and increased genetic instability. Limited oxygen concentration in tumor tissue is known to modulate (inhibit or stimulate) gene expression as well as affect metabolic processes [2-5].

Hypoxia-inducible factor-1 (HIF-1) and glucose transporter-1 (Glut-1) are two known markers of tissue oxygenation. HIF-1 is heterodimeric transcription factor that consists of a constitutively expressed HIF-1β subunit, and a hypoxia-induced HIF-1α subunit [6, 7]. Under normoxic conditions, HIF-1α is rapidly degraded, but hypoxia or activation of certain intracellular pathways, such as the PI-3K and ERK1/2 pathways, can cause accumulation of this protein and its translocation to the nucleus. Additionally, HIF-1α expression can be induced by loss of tumor suppressor genes PTEN and p53 [7, 8]. HIF-1α binds to Hypoxia Response Elements (HRE, 5’-RCGTG-3’) within regulatory regions of target genes whose protein products are implicated in metabolism, growth, angiogenesis and apoptosis [9-11]. Expression of HIF-1α is characteristic of early stages of carcinogenesis and has been correlated with increased intratumoral angiogenesis, cancer progression, poor patient prognosis and chemo- as well as radio- resistance [12-14].

Glucose transporter-1 (Glut-1) is another protein whose expression in tumor is hypoxia-dependent [15-17]. Glut-1 exerts cytoprotective effect enabling glucose transport into hypoxic cells, and preventing hypoxia-induced cell death. Glut-1 accumulation was observed in cancer cells in regions near necrotic areas in tumors [18]. Under hypoxic conditions, Glut-1
expression can be enhanced by HIF-1α [19]. Hayashi et al [20] found that this phenomenon involves HIF-1α interaction with a HRE site in the Glut-1 promoter.

In breast cancer, increased expression of Glut-1 was associated with higher invasiveness of breast cancer cell lines and with the poorly differentiated phenotype in human ductal mammary cancers [21]. Glut-1 overexpression in colorectal cancer cells was associated with rapid cancer progression and inversely correlated with prognosis [22]. In breast and lung cancer cell models, blocking of Glut-1 with specific antibodies reduced growth and induced apoptosis [23]. Previously, we found significant co-expression of Glut-1, Bcl-xL, and Bax in colorectal cancer, which could suggest cooperation of these regulatory proteins in different processes, such as cell elimination due to irreversible injury, adaptation to hypoxia, reduction of further damage, and survival of cancer cells [16].

It was also shown that hypoxia upregulates expression of leptin (Ob) – a multifunctional peptide hormone that can be involved in cancer cell growth, transformation, metastasis and resistance to cancer treatments [24-33]. Our recent study indicated that leptin and the leptin receptor (ObR) were significantly overexpressed in primary breast cancer and lymph node metastasis relative to non-cancer mammary epithelium [34]. The promoter of leptin gene contains eight HRE with the minimal core sequence 5′-RCGTG-3′ that can recruit HIF-1α [35]. Recently, we demonstrated in breast cancer cells, leptin and ObR expression can be activated in response to hypoxia or hyperinsulinemia [34, 36]. In case of leptin expression, the process was mediated through HIF-1 and/or Sp-1-dependent transcription [37, 38].

Here we analyzed the relationships among HIF-1α, Glut-1, leptin, ObR and their correlations with other cancer biomarkers in primary breast cancer and lymph node metastases of patients who received or not preoperative chemotherapy.
Materials and methods

Tissue samples

The expression of HIF-1α, Glut-1, leptin, ObR, estrogen receptors ERα, ERβ and Ki-67 was assessed in breast cancer samples obtained from 116 women, aged 30-82 years (mean age 54.4), who underwent partial or total mastectomy and lymph node dissection for primary breast cancer. Forty five patients from this group underwent preoperative chemotherapy (Ansfield or CMF program). Tissue samples were fixed in 10% buffered formaldehyde solution, embedded in paraffin blocks at 56°C and stained with hematoxylin-eosin. Histopathological examination of sections was based on the WHO and pTN classification of breast tumors [39]. The protocol of the present study was reviewed and approved by the local ethical committee. In order to uniform the examined group, the analysis included selected cases of invasive ductal carcinomas in grade G2 and G3 and in stage pT1 and pT2. In the group of tumors without preoperative chemotherapy, the presence of lymph node metastases was revealed in 35 cases (49.3%). 30 patients (66.7%) of patients after preoperative chemotherapy had involved lymph nodes at the time of diagnosis.

Immunohistochemistry

The immunohistochemical analysis of HIF-1α, Glut-1, leptin (Ob), ObR, ERα, ERβ, and Ki-67 expression was carried out using 5μm consecutive tissue sections obtained from tissue samples, as described by us previously in detail [16, 28-30, 34, 40]. The sections were dewaxed in xylene and rehydrated in graded alcohols. After antigen unmasking and endogenous peroxidase removal, nonspecific binding was blocked by incubating the slides for 1 h with 1.5% normal serum in PBS. Next, the sections were incubated with the primary antibodies (Abs). The following Abs were used for marker detection: for HIF-1α rabbit polyclonal Ab (sc-10790; Santa Cruz Biotechnology, Santa Cruz, USA), dilution 1:400; for Glut-1 rabbit polyclonal Ab (A3536; Dako, Denmark), dilution 1:250; for leptin, rabbit
polyclonal Ab (pAb) A-20 (Santa Cruz, USA), dilution 1:100; for ObR, rabbit pAb H-300 (Santa Cruz, USA), dilution 1:75; for ERα, mouse monoclonal Ab (mAb) F-10 (Santa Cruz, USA), dilution 1:200; for ERβ, rabbit pAb H-150 (Santa Cruz, USA), dilution 1:200; and for Ki-67, mouse mAb MIB-1 (Dako, Denmark), dilution 1:100. The studies for leptin, ObR, ERα and ERβ were performed with avidin-biotin-peroxidase complex (ABC Staining System, Santa Cruz, USA), for HIF-1α and Glut-1 with EnVision method (Dako, Denmark), and for Ki-67 with streptavidin-biotin-peroxidase complex (LSAB kit, Dako, Denmark) to reveal Ab-antigen reactions. All slides were counterstained with hematoxylin. Breast specimens previously classified as positive for the expression of the studied markers were used for control and protocol standardization. In negative controls, primary Abs were omitted. The expression of leptin, ObR, ERα, ERβ, and Ki-67 was analyzed by light microscopy in 10 different section fields and the mean percentage of tumor cells displaying positive staining was scored. The expression of leptin and ObR in cancer samples was classified using a four-point scale: 0, <10% positive cells; 1+, 10-50% positive cells with weak staining; 2+, >50% positive cells with weak staining; 3+, >50% positive cells with strong staining. ERα and ERβ were classified as follows: 0, <10% cells with positive staining; 1+, 10-50% cells with positive staining; 2+, 50-80% cells with positive staining; 3+ >80% cells with positive staining. Ki-67 expression was classified as follows: 0, <10% cells with positive staining; 1+, 10-40% cells with positive staining; 2+ >40% cells with positive staining. For HIF-1α and Glut-1, we applied a 3-grade scoring system as follows: 0, <10% immunoreactive cancer cells; 1+, 10-50% immunoreactive cancer cells; 2+, >50% of immunoreactive malignant cells.

**Statistical analysis.**

Spearman test was used to analyze correlations among leptin, ObR, HIF-1α, Glut-1, ERα, ERβ and Ki-67 expression in primary and metastatic breast cancer in the group with and without preoperative chemotherapy. Values of p<0.05 were taken as statistically significant.
Results

We found a significant correlation between HIF-1α and leptin expression in primary tumors and lymph node metastases of patients who did not receive preoperative chemotherapy (Tab. 1 and 2). This relationship was not observed in breast tumors of patients who received neoadjuvant therapy. On the other hand, the expression of HIF-1α was correlated with the presence of ObR in primary breast cancer regardless of preoperative chemotherapy treatment (Tab. 1 and 2). In lymph node metastases from women without neoadjuvant therapy, a trend toward a positive correlation between HIF-1α and ObR was detected (Tab. 2). However, in lymph node metastases from patients after neoadjuvant therapy, HIF-1α and ObR were not associated (Tab. 2).

Glut-1 did not correlate with leptin and ObR in any studied group of breast tumors (Tab. 1 and 2). On the other hand, positive correlation between HIF-1α and Glut-1 was found in primary tumors from patients without preoperative chemotherapy (Tab. 1). In lymph node metastases and in tumors from patients who received neoadjuvant therapy, no associations between HIF-1α and Glut-1 were observed (Tab. 1 and 2).

Next, we studied relationships between hypoxia markers and estrogen receptor expression. In primary tumors without preoperative chemotherapy we noted a trend toward a negative correlation between HIF-1α expression and ERα (p=0.055, r= -0.194; Tab. 1). These associations were absent in lymph node metastases. HIF-1α and ERα did not correlate in primary tumors and metastases from patients who underwent preoperative chemotherapy (Tab. 1 and 2).

Glut-1 negatively correlated with ERα expression in primary tumors and in lymph node metastases in biopsies from all patients, regardless of preoperative chemotherapy (Tab. 1 and 2). ERβ positively correlated with HIF-1α in primary tumors, both in patients with and without preoperative chemotherapy (Tab. 1), and with Glut-1 in primary tumors without preoperative chemotherapy (Tab. 1).
Finally, we evaluated the link between HIF-1α, Glut-1 and the proliferation marker Ki-67. A positive correlation between Glut-1 and Ki-67 was noted in primary and metastatic cancer biopsies from patients without preoperative chemotherapy (Tab. 1, 2). In the group of primary tumors after neodjuvant therapy, we observed a trend toward a positive correlation in primary tumors only (Tab. 1). On the other hand, HIF-1α did not correlate with Ki-67 in primary tumors or in lymph node metastases in both studied groups of patients (Tab. 1 and 2).

Discussion

In this study we analyzed the relationships among hypoxia-inducible proteins (i.e., HIF-1α, Glut-1, leptin, and ObR) and other biomarkers (i.e., estrogen receptors and Ki-67) in primary and metastatic breast cancer. Our second goal was to evaluate possible influence of preoperative chemotherapy on associations among studied proteins. The major findings of our study can be summarized as follows: 1) HIF-1α and Glut-1 are positively correlated in primary tumors without preoperative chemotherapy; 2) HIF-1α positively correlate with the leptin system in primary and metastatic breast cancer without preoperative chemotherapy; 3) the leptin system is not associated with Glut-1 expression in all studied groups; 4) Glut-1 correlates negatively with ERα in primary and metastatic tumors, regardless of preoperative chemotherapy; 5) hypoxia markers correlate positively with ERβ expression in primary tumors, especially in the group without preoperative therapy; 6) Glut-1 expression is positively associated with Ki-67 in primary tumors, while in lymph node metastases, a trend toward positive correlation between these proteins is found in the group without therapy; 7) preoperative chemotherapy influences the associations between HIF-1α and leptin in primary and metastatic tumors, HIF-1α and ObR in metastatic tumors, HIF-1α and Glut-1 in primary tumors; Glut-1 and ERβ in primary tumors; Glut-1 and Ki-67 in primary and metastatic tumors.
Our results suggest that hypoxic conditions resulted in coordinated upregulation of HIF-1α and Glut-1 in primary tumors. However, the association between the two markers was lost in lymph node metastases as well as in tumors after preoperative chemotherapy. This could be related to changes in proliferative activity in metastases and to therapeutic influences on cancer cells as indicated our previous studies [41, 42]. We showed that mean percentage of Ki-67 cancer cells was lower in primary and metastatic tumors after chemotherapy compared to tumors without chemotherapy [42].

Hypoxia in solid tumors is associated with the accumulation of HIF-1α and activation of HIF-1-dependent transcription of genes regulating cell motility, invasion, and angiogenesis [14, 43]. Bos et al [12, 44] reported that HIF-1α overexpression was associated with more aggressive breast cancer. We speculate that HIF-1 could also improve glucose consumption in hypoxic cancer cells by the stimulation of glucose transporter Glut-1 expression.

The associations between HIF-1α and the leptin system identified by us in this study confirm the results obtained by Cascio et al [37], Bartella et al [36] and Garofalo et al [34] who demonstrated that physiologic hypoxia and/or accumulation of HIF-1α due to hypoxia-mimicking conditions or stabilization of HIF-1a by growth factors can stimulate leptin and/or ObR expression in breast cancer cells.

According to our knowledge, our analysis for the first time revealed positive correlation between HIF-1α and leptin, as well as between HIF-1α and ObR in human breast cancer biopsies from primary and metastatic tumors. We reported analogous associations in human colorectal cancer [30]. Cumulatively, our data strongly suggest the involvement of tissue hypoxia in the stimulation of leptin and ObR expression in human cancers [28, 30, 34]. The overexpression of the leptin system could lead to leptin-enhanced tumor growth and progression under hypoxic conditions.

One of the features of breast cancer progression is the development of resistance to hormonal therapy. The possible mechanism of this phenomenon could be related to
differential ER expression in metastatic and primary sites, as described by us before [40]. Our present study suggests that tumor malignancy might correlate with loss of ERα expression. Indeed, hypoxia could induce ERα downregulation via proteasome-dependent pathway [45, 46]. Cho J et al [47] suggested that ERα down-regulation under hypoxic conditions in human breast cancer involves protein interactions between ERα and HIF-1α.

Negative correlation between Glut-1 and ERα found in this study suggests that loss of ERα in breast cancer is associated with overexpression of Glut-1, which could facilitate proliferation, survival and possibly progression and dedifferentiation of cancer cells [48, 49].

In contrast to ERα, we noted a positive correlation between ERβ and hypoxia markers HIF-1α, Glut-1. Our observations are consistent with results of Cordadini et al [50], who reported that hypoxic conditions upregulated ERβ protein levels in breast cancer cells. Moreover, sequence analysis of the ERβ promoter region contains specific sequence for HRE, which might explain coexpression of ERβ and HIF-1α.

Preoperative chemotherapy is an integral part of management of patients with advanced breast cancer. However, its influence on biological factors and relationships between them in primary and metastatic tumors is poorly recognized. Our present study clearly suggest that preoperative chemotherapy alters the expression of and relationships among several biomarkers, specifically, HIF-1α and leptin in primary and metastatic tumors, HIF-1α and ObR in metastatic tumors, HIF-1α and Glut-1 in primary tumors; Glut-1 and ERβ in primary tumors; Glut-1 and Ki-67 in primary and metastatic tumors. The results suggest that the expression of above markers in breast cancer is not a stable phenotype and can be modified by preoperative chemotherapy. These data are consistent with our previous reports on differential expression of ERα, ERβ and Ki-67 expression in metastatic breast cancer versus primary cancer, as well as significant influence of preoperative chemotherapy on ERα, ERβ, and Ki-67 expression [40-42]. Also Jain et al [51] observed substantial effects of preoperative chemotherapy on ER expression in breast cancer.
Conclusions

In conclusion, our results suggest that breast tumor cells experience hypoxic conditions, as indicated by the expression of such markers as HIF-1α, Glut-1, leptin and ObR. Interestingly, the relationships among these proteins differ in primary and metastatic tumors and might be influenced by preoperative chemotherapy. Thus, analysis of biomarkers in both primary and metastatic tumors before and after chemotherapy could help in understanding the mechanisms of breast cancer progression and select the optimal individualized treatment options.

List of abbreviations
HIF-1α, Hypoxia-inducible factor-α; Glut-1, glucose transporter-1; Ob, leptin; ObR, leptin receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; HRE, Hypoxia Response Elements.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MK designed the study, analyzed data and drafted the manuscript; LKK analyzed statistical analysis and edited the manuscript; MS collected the samples, performed pathological evaluation and edited the manuscript; ES analyzed results and edited the manuscript; SS designed the study, collected the samples, performed pathological evaluation and edited the manuscript.
References


Table 1. Correlation between HIF-1α, GLUT-1 and other studied biomarkers in primary breast cancer in patients without and after preoperative chemotherapy.

<table>
<thead>
<tr>
<th>Compared biomarkers</th>
<th>Primary tumors without preoperative chemotherapy</th>
<th>Primary tumors after preoperative chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>HIF-1α – Ob</td>
<td>&lt;0.0001</td>
<td>0.532</td>
</tr>
<tr>
<td>HIF-1α – ObR</td>
<td>0.002</td>
<td>0.319</td>
</tr>
<tr>
<td>HIF-1α – ERα</td>
<td>0.055</td>
<td>-0.194</td>
</tr>
<tr>
<td>HIF-1α – ERβ</td>
<td>0.002</td>
<td>0.408</td>
</tr>
<tr>
<td>HIF-1α – Ki-67</td>
<td>0.822</td>
<td>-0.031</td>
</tr>
<tr>
<td>HIF-1α – GLUT-1</td>
<td>0.02</td>
<td>0.437</td>
</tr>
<tr>
<td>GLUT-1 – Ob</td>
<td>0.930</td>
<td>0.018</td>
</tr>
<tr>
<td>GLUT-1 – ObR</td>
<td>0.472</td>
<td>0.139</td>
</tr>
<tr>
<td>GLUT-1 – ERα</td>
<td>0.022</td>
<td>-0.410</td>
</tr>
<tr>
<td>GLUT-1 – ERβ</td>
<td>0.008</td>
<td>0.467</td>
</tr>
<tr>
<td>GLUT-1 – Ki-67</td>
<td>0.017</td>
<td>0.433</td>
</tr>
</tbody>
</table>
Table 2. Correlation between HIF-1α, GLUT-1 and other studied biomarkers in lymph node metastases in patients without and after preoperative chemotherapy.

<table>
<thead>
<tr>
<th>Compared biomarkers</th>
<th>Lymph node metastases without preoperative chemotherapy</th>
<th>Lymph node metastases after preoperative chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>HIF-1α – Ob</td>
<td><strong>0.013</strong></td>
<td>0.533</td>
</tr>
<tr>
<td>HIF-1α – ObR</td>
<td><strong>0.083</strong></td>
<td>0.387</td>
</tr>
<tr>
<td>HIF-1α – ERα</td>
<td>0.133</td>
<td>-0.327</td>
</tr>
<tr>
<td>HIF-1α – ERβ</td>
<td>0.119</td>
<td>0.474</td>
</tr>
<tr>
<td>HIF-1α – Ki-67</td>
<td>0.604</td>
<td>-0.167</td>
</tr>
<tr>
<td>HIF-1α – GLUT-1</td>
<td>0.776</td>
<td>-0.177</td>
</tr>
<tr>
<td>GLUT-1 – Ob</td>
<td>0.405</td>
<td>0.242</td>
</tr>
<tr>
<td>GLUT-1 – ObR</td>
<td>0.261</td>
<td>-0.309</td>
</tr>
<tr>
<td>GLUT-1 – ERα</td>
<td><strong>0.017</strong></td>
<td>-0.606</td>
</tr>
<tr>
<td>GLUT-1 – ERβ</td>
<td>0.677</td>
<td>-0.117</td>
</tr>
<tr>
<td>GLUT-1 – Ki-67</td>
<td><strong>0.078</strong></td>
<td>0.468</td>
</tr>
</tbody>
</table>