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Differential relationship between two hypoxia markers: HIF-1α and GLUT1 and classic prognostic factors in invasive breast carcinoma

Zróżnicowana zależność pomiędzy dwoma markerami hipoksji: HIF-1α i GLUT1 a klasycznymi czynnikami prognostycznymi w inwazyjnym raku piersi

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Abstract

Background: Tumor hypoxia is an adverse prognostic factor which promotes cancer aggressiveness and limits its radio- and chemosensitivity. **The aim of our study** was to explore the relationship between endogenous hypoxia markers and classic prognostic factors, including clinical stage and the expression of ER, PR, and HER2 in primary untreated breast carcinoma. **Methods:** A retrospective immunohistochemical analysis of archived tissue blocks collected from 153 women, who underwent total mastectomy and lymph node dissection, included the expression of two hypoxia-related proteins: HIF-1α and GLUT1. **Results:** GLUT1 labelling index (LI) showed a positive correlation with T stage ($R = 0.18$, $p = 0.026$) and HER2 status ($R = 0.25$, $p = 0.002$), and a negative correlation with the expression of ER ($R = -0.19$, $p = 0.017$) and PR ($R = -0.17$, $p = 0.032$). HIF-1α LI showed a positive correlation with ER expression ($R = 0.16$, $p = 0.045$). In the multivariate regression analysis, a different relationship between classic prognostic factors and the two tested hypoxia proteins was proven. Higher GLUT1 expression correlated with ER and PR negativity ($p = 0.02$ and $p = 0.01$, respectively) as well as with higher expression of HER2 ($p = 0.04$). HIF-1α showed no association with PR and HER2, but a positive correlation with ER ($p = 0.02$). Neither of the hypoxia proteins was associated with a tumor grade. Only one clinical feature, T stage, correlated with both of the hypoxia markers: positively with GLUT1 ($p = 0.049$) and negatively with HIF-1α ($p = 0.01$) expression. **Conclusions:** In breast cancer, GLUT1 expression may be considered an additional prognostic factor which correlates with an adverse status of HER2 and hormonal receptors, and indicates a more hypoxic, radio- and chemotherapy refractory profile of carcinoma.

Key words: hypoxia, HIF-1α, GLUT1, prognostic factors, immunohistochemistry

Streszczenie

Tło: Hipoksja w guzie nowotworowym stanowi niekorzystny czynnik prognostyczny, ogranicza jego promienio- i chemiowrażliwość oraz promuje bardziej agresywny przebieg choroby. Przewidywanie rokowania i odpowiedzi na leczenie wymaga wiedzy o związku hipoksji z uznanymi czynnikami prognostycznymi. **Celem badania** było określenie zależności pomiędzy endogennymi markerami hipoksji w pierwotnym przewodowym raku piersi a klasycznymi czynnikami prognostycznymi, takimi jak stopień zaawansowania klinicznego oraz ekspresja receptorów ER, PR i HER2. **Metody:** Retrospektywna analiza immunohistochemiczna archiwizowanych bloczków tkanek pobranych od 153 kobiet, poddanych mastektomii i limfadenektomii pachowej, objęła ekspresję dwóch związanych z hipoksją białek: HIF-1α i GLUT1. **Wyniki:** Indeks wiązania GLUT1 (GLUT1 LI) wykazał korelację dodatnią z wielkością guza ($R = 0{,}18$, $p = 0{,}026$) i ekspresją HER2 ($R = 0{,}25$, $p = 0{,}002$) oraz ujemną z ekspresją ER ($R = -0{,}19$, $p = 0{,}017$) i PR ($R = -0{,}17$, $p = 0{,}032$). HIF-1α LI korelował wyłącznie z ekspresją ER ($R = 0{,}16$, $p = 0{,}045$). W analizie wieloczynnikowej wykazano zróżnicowaną zależność pomiędzy klasycznymi czynnikami prognostycznymi i testowanymi markerami hipoksji. GLUT1 LI korelował negatywnie z ekspresją ER i PR (odpowiednio $p = 0{,}02$ i $p = 0{,}01$) oraz pozytywnie z ekspresją HER2 ($p = 0{,}04$). Nie udowodniono korelacji pomiędzy HIF-1α LI a ekspresją PR czy HER2, natomiast wykazano jego dodatnią zależność z ekspresją ER ($p = 0{,}02$). Żaden marker hipoksji nie korelował ze stopniem zróżnicowania histologicznego nowotworu. Tylko jeden kliniczny czynnik – wielkość guza (T) – korelował z ekspresją badanych białek: dodatnio z GLUT1 ($p = 0{,}049$), a ujemnie z HIF-1α ($p = 0{,}01$).

Wnioski: Ekspresja GLUT1 w raku piersi może stanowić dodatkowy czynnik prognostyczny, korelujący z niekorzystnym statusem receptora HER2 i receptorów hormonalnych oraz wskazywać na bardziej hipoksyczny, oporny na radio- i chemioterapię, profil raka.

Słowa kluczowe: hipoksja, HIF-1α, GLUT1, czynniki prognostyczne, immunohistochemia

BACKGROUND

Hypoxia is a hallmark of malignant solid tumors, including breast cancer[1]. It has been proven to promote cancer progression and limit treatment efficacy since hypoxic cells are significantly more radio- and chemoresistant[2]. The ability to predict it is an important issue for offering breast cancer patients the most efficient treatment, as is determining so-called classic prognostic factors, including clinical stage, pathology (carcinoma type and grade) and the expression of estrogen (ER), progesterone (PR) and epithelial B2 (ERBB2/HER2) receptors.

A central mediator of the cell's response to hypoxic conditions is a heterodimeric transcription factor, hypoxia-inducible factor 1α (HIF-1α), which promotes the expression of genes involved in glucose metabolism, apoptosis, tumor angiogenesis and invasion. An HIF-1α level increases in many human solid tumors, and although the majority of clinical data confirm the protein's negative prognostic significance[3,4], there are also some contrary results[5,6]. In breast cancer, HIF-1α has been reported to play a role in carcinogenesis[7] and to correlate with poor prognosis[8]. In normoxic conditions, HIF-1α hydroxylation by prolyl hydroxylases (PHDs) promotes its ubiquitin-dependent proteosomal degradation, mainly mediated by von Hippel–Lindau (VHL) E3 ubiquitin-ligase[9]. Under hypoxia, downstream signaling of HIF-1α results from the inhibition of protein degradation (due to impaired VHL recognition by unhydroxylated prolines) and its subsequent overexpression[10].

Glucose transporter 1 (GLUT1) is a downstream target of HIF-1α, which enables aerobic glycolysis in hypoxia conditions by driving glucose transport in cells, thus preventing their death. GLUT1 overexpression has been shown in several carcinomas in which it correlated with a higher tumor grade, greater invasiveness and unfavorable prognosis[11]. In breast cancer, GLUT1 expression has been reported to correlate positively with tumor grade, proliferation rate and the risk of disease progression[12,13].

We decided to explore the link between hypoxia representative proteins: HIF-1α and GLUT1, and classic breast cancer prognostic factors such as clinical stage, tumor grade and ER, PR and HER2 expression.

MATERIAL AND METHODS

Patients

From the Department of Tumor Pathology and Pathomorphology at the Prof. Francis Łukaszczyk Oncology Center, we retrieved tissue samples collected from 153 women, aged 25–74 years (mean age 45.14 years ± 10.76) who underwent total mastectomy and lymph node dissection as first-line therapy for primary invasive ductal breast carcinoma between 2004 and 2005. The study was approved by the Bioethical Board of Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz. Written informed consent for participation in the study was obtained from the participants. A retrospective immunohistochemical analysis of the archived tissue blocks included the expression of HIF-1α and GLUT1 proteins. Prognostic factors, such as clinical stage, tumor grade as well as ER, PR and HER2 expression, were determined at the time of postoperative pathological diagnosis. The histological grade was assessed using the Elston–Ellis system. ER and PR positivity were defined according to the American Society of Clinical Oncology (ASCO) recommendations, and a cut-off value of 10% was used. HER2 status was defined according to the ASCO guidelines, and cases showing 2+ expression were verified for HER2 amplification by fluorescence *in situ* hybridization (FISH).

There were 53 (34.64%) pT1, 90 (58.82%) pT2, 5 (3.27%) pT3 and 5 (3.27%) pT4 patients as well as 89 (58.17%) pN0, 23 (15.03%) pN1, 21 (13.72%) pN2 and 20 (13.07%) pN3 patients. The histological grade was determined in 128 of 153 patients. Grade I represented 3 (2.34%) tumors, grade II – 98 (76.56%) and grade III – 27 (21.09%) tumors. There were 100 (65.36%) ER-positive, 95 (62.09%) PR-positive and 17 (11.11%) HER2-positive carcinomas (Tab. 1).

Parameter category	T (*n* = 153)	N (*n* = 153)	G (*n* = 128)	ER (*n* = 153)	PR (*n* = 153)	HER2 (*n* = 153)
Parameter-value (percentage)	**1** – 53 (34.64%)	**0** – 89 (58.17%)	**I** – 3 (2.34%)	**(+)** 100 (65.36%)	**(+)** 95 (62.09%)	**(+)** 17 (11.11%)
	2 – 90 (58.82%)	**1** – 23 (15.03%)	**II** – 98 (76.56%)	**(–)** 53 (34.64%)	**(–)** 58 (37.91%)	**(–)** 136 (88.89%)
	3 – 5 (3.27%)	**2** – 21 (13.72%)	**III** – 27 (21.09%)			
	4 – 5 (3.27%)	**3** – 20 (13.07%)				

Tab. 1. The clinical-pathological data of the studied material

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Immunohistochemistry

The analysis was conducted with the EnVision method using the EnVision+System HRP (horseradish peroxidase, K 4001 and K 4002, DAKO, USA) kit and adequate monoclonal antibodies (monoclonal anti-HIF-1α, Chemicon International, USA; polyclonal anti-GLUT1, Cell Marque, USA). Sections (5-μm-thick) derived from 10% formalin-fixed and paraffin-embedded tumors were placed onto basic adhesive slides and incubated for 2 hours at 60°C in a chamber thermostat. The entire pretreatment process of deparaffinization, rehydration and epitope retrieval was conducted with PTLink (a pretreatment module for tissue specimens) using the EnVision Flex Target Retrieval Solution, High pH (DM828). The endogenous enzymatic activity of peroxidase was inhibited with 3% hydrogen peroxide solution. The sections were incubated with primary antibodies (1:100 for HIF-1α, 1:100 for GLUT1), and afterwards with an EnVision+System HRP reagent. Chromogen DAB (3,3-diaminobenzidine, K3468, DAKO, USA) was used to demonstrate the examined cellular structures. Cell nuclei were stained with hematoxylin (S 2020, DAKO, USA). As the last step, the sections were hydrated in increasing ethanol dilutions, cleared in xylene and mounted in medium (Consul Mount, Thermo Shandon, USA).

Biomarker assessment

All slides were reviewed by two pathologists (HA, JL) using a light microscope with a micrometric insertion (Olympus Poland). The samples chosen for evaluation came from hypoxic tumor regions. Viewing fields were evaluated at 40-fold magnification under an objective lens. At least 500 (max. 1000) breast cancer cells were counted in several randomly selected viewing fields. A spatial correlation between the presence of positive staining of both markers was visible. The results of nuclear staining of HIF-1α and cytoplasmic staining of GLUT1 (Fig. 1) in cancer cells were shown in the form of labelling indices (LI) interpreted as the percentage of positively stained cells in the total number of examined breast cancer cells.

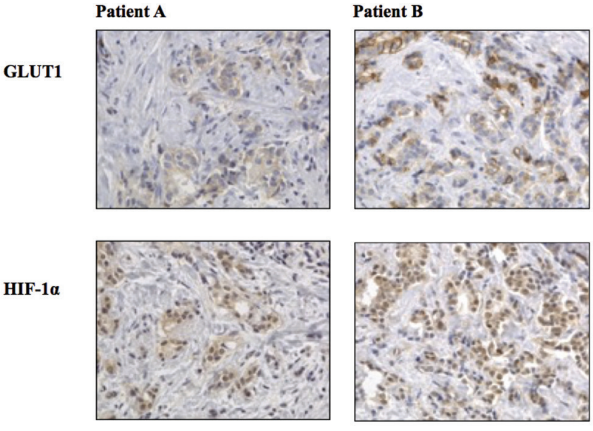

Fig. 1. HIF-1α nuclear staining and GLUT1 cytoplasmic staining in breast cancer cells of two different patients. Original magn. 400×

Statistics

The statistical analysis of GLUT1 and HIF-1α expression in relation to T and N stage, histological grade as well as ER, PR, and HER2 status was performed using Statistica (StatSoft, Inc., 2011, STATISTICA, version 10.0, www.statsoft.com) with the generalized regression model (GRM). In the systems that exhibited a statistical significance of the main effect, post-hoc tests (Tukey's and LSD) were carried out to identify homogeneous groups. In all analyses, $p < 0.05$ was considered statistically significant. The results are shown as graphs in the form of average values, with 95% confidence intervals.

RESULTS

The mean labelling index of HIF-1α ($LI_{HIF\text{-}1}α$) was 2.25% ± 4.71 (range 0–35%). Based on the median $LI_{HIF\text{-}1}α$ value, we distinguished the "HIF-1α = 0%" (87/153 = 56.86%) and "HIF-1α > 0%" (66/153 = 43.14%) subgroups of tumors. GLUT1 expression was observed in 141/153 (92.16%) tumors. The mean labelling index of GLUT1 (LI_{GLUT1}) was 48.49% ± 32.71 (range 0–100%). Based on the median LI_{GLUT1} value, we divided GLUT1-positive tumors into GLUT1 < 50% (75/153 = 49.02%) and GLUT ≥ 50% (78/153 = 50.98%) subgroups.

Associations between the expression of the hypoxia-related proteins and classic prognostic factors, utilizing the Spearman test, are summarized in Tab. 2. In this analysis, the GLUT1 labelling index showed a positive correlation with T stage ($R = 0.18$, $p = 0.026$) as well as HER2 status ($R = 0.25$, $p = 0.002$), and a negative correlation with the expression of both hormonal receptors: ER ($R = -0.19$, $p = 0.017$) and PR ($R = -0.17$, $p = 0.032$). HIF-1α labelling index showed only one significant relationship which was a positive correlation with ER expression ($R = 0.16$, $p = 0.045$).

In the multivariate regression analysis, a different relationship between clinical-pathological prognostic factors and the two tested hypoxia proteins, HIF-1α and GLUT1, was also proven. The results are summarized in Fig. 2 and Fig. 3. A higher GLUT1 expression correlated with ER and PR negativity ($p = 0.02$ and $p = 0.01$, respectively) and with a higher expression of HER2 ($p = 0.04$) (Fig. 2). HIF-1α showed no correlation with PR and HER2 and, in contrast to GLUT1, a higher expression in ER-positive tumors ($p = 0.02$) (Fig. 2). None of the hypoxia proteins was associated with a tumor grade (Fig. 3). Only one clinical feature, T stage, correlated significantly, but mutually inversely, with both of the hypoxia markers (Fig. 3). We observed a positive correlation with GLUT1 expression ($p = 0.049$), which was highest for T4 tumors, and a negative correlation with HIF-1α expression ($p = 0.01$), which was highest for T1 tumors.

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Parameter		LI GLUT1 < 50% *N* = 75 (49.02%)	LI GLUT1 > 50% *N* = 78 (50.98%)	*p/R*	LI HIF-1α = 0 *N* = 87 (56.86%)	LI HIF-1α < 0 *N* = 66 (43.14%)	*p/R*
T stage	T1 T2 T3 T4	32 (20.92) 40 (26.14) 2 (1.31) 1(0.65)	21 (13.73) 50 (32.68) 3 (1.96) 4 (2.61)	0.026/0.18	27 (17.65) 54 (35.29) 3 (1.96) 3 (1.96)	26 (16.99) 36 (23.53) 2 (1.31) 2 (1.31)	NS
N stage	N0 N1 N2 N3	45 (29.41) 14 (9.15) 6 (3.92) 10 (6.54)	44 (28.76) 9 (5.88) 15 (9.80) 10 (6.54)	NS	49 (32.03) 15 (9.80) 15 (9.80) 8 (5.23)	40 (26.14) 8 (5.23) 6 (3.92) 12 (7.84)	NS
ER	Positive Negative	56 (36.60) 19 (12.42)	44 (28.76) 34 (22.22)	0.017/–0.19	51 (33.33) 36 (23.53)	49 (32.03) 17 (11.11)	0.045/0.16
PR	Positive Negative	53 (34.64) 22 (14.38)	42 (27.45) 36 (23.53)	0.032/–0.17	49 (32.03) 38 (24.84)	46 (30.07) 20 (13.07)	NS
HER2	(–) (+) (++) (+++)	30 (19.61) 35 (22.88) 6 (3.92) 4 (2.61)	20 (13.07) 29 (18.95) 16 (10.46) 13 (8.50)	0.002/0.25	32 (20.92) 29 (18.95) 14 (9.15) 12 (7.84)	18 (11.76) 35 (22.88) 8 (5.23) 5 (3.27)	NS
Grade	I II III	*N* = 64 (50%) 2 (1.56) 50 (39.06) 12 (9.38)	*N* = 64 (50%) 1 (0.78) 48 (37.50) 15 (11.72)	NS	*N* = 74 (57.81%) 2 (1.56) 57 (44.53) 15 (11.72)	*N* = 54 (42.19%) 1 (0.78) 41 (32.03) 12 (9.38)	NS

Tab. 2. Associations between the expression of hypoxia-related proteins and classic prognostic factors. The correlation between the pairs of parameters is expressed with the Spearman's correlation coefficient (R)

DISCUSSION

Based on the literature and our previous results on the relationship of hypoxia with classic prognostic factors in prostate cancer[14] and its meaning in carcinogenesis[15], we also expected to find a correlation between the approved clinical-pathological prognostic factors and hypoxia-related proteins in breast carcinoma.

We found an association between GLUT1 expression and other tumor biological markers, including a negative correlation with the recognized favorable prognostic factors, such as ER and PR expression, and a positive correlation with an unfavorable prognostic factor, i.e. HER2 overexpression. In contrast to GLUT1, HIF-1α did not correlate with either PR or HER2 expression but correlated positively with ER expression. Since studies conducted on tissues and cell lines have indicated a close dependence of GLUT expression on HIF-1α activation[10,11], we rather expected consistent results for both hypoxia markers. When discussing a differential relationship of GLUT1 and HIF-1α with standard receptor prognostic factors, we took into consideration relatively low labelling indices of HIF-1α obtained in our study with a high proportion of $LI_{HIF\text{-}1}\alpha = 0\%$ (about 57%) and low mean $LI_{HIF\text{-}1}\alpha$ reaching 2.25%, a factor which might have influenced the results of the statistical analysis. One should also allow for the fact that transient stabilization and short half-life of endogenous HIF proteins may limit their usefulness in detection of tumor hypoxic response[16]. Finally, in cancer cells, the expression of both proteins can be up-regulated not only by hypoxia. GLUT1 expression also depends on growth factors[17], suppressor genes[18], oncogenes[19,20] and the PI3K/AKT/ mTOR molecular pathway[21], and may not be even connected with HIF-1α activity[20]. In contrast, HIF-1α expression can be up-regulated by activation of the PI3K and ERK1/2 intracellular pathways as well as due to the loss of tumor suppressor genes such as *PTEN* and *p53*[22,23]. Additionally, HIF-1α proteosomal degradation may be the result of the expression of either von Hippel–Lindau E3 ubiquitin-ligase[9] or Sharp-1, a basic helix–loop–helix transcription factor[24]. Molecular studies by Surazynski *et al.* might be to some extent a confirmation of our findings on the relationship between HIF-1α and ER[25]. The authors suggested that α-estrogen receptor up-regulates the activity of prolidase, which in turn contributes to an increase in HIF-1α nuclear localization.

The significance of hypoxia proteins in breast cancer has been also explored in several clinical studies. Koda *et al.* showed, similarly to our results, a negative correlation between ER expression and GLUT1 in primary breast cancers and lymph node metastasis, which they did not prove for HIF-1α[26]. Choi *et al.* reported, in turn, that the expression of both GLUT1 and HIF-1α correlated with ER and PR negativity. HIF-1α was associated with HER2 overexpression, while high GLUT1 expression was correlated with a triple (ER, PR, HER2) negative subtype of breast cancer[27].

Hypoxia increases with tumor growth, the consequence of which should be a link between hypoxia markers

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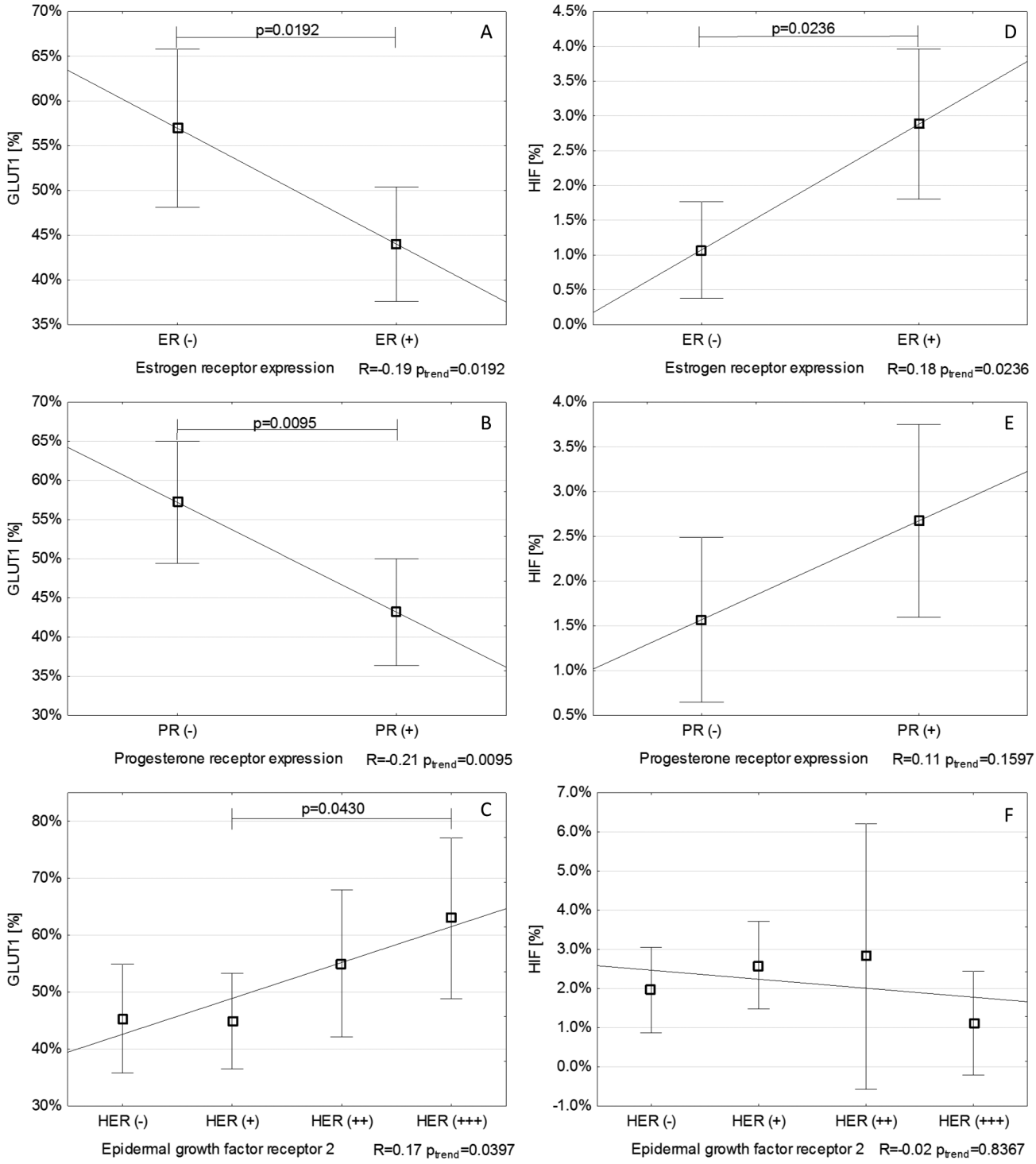

Fig. 2. Relationship between pathological prognostic factors: ER, PR as well as HER2 and hypoxia-related proteins: HIF-1α and GLUT1 (average values with 95% confidence intervals)

and a tumor stage. In our study, such a correlation was observed only for GLUT1, whose expression (understood as hypoxia level) was proportional to the tumor size. We did not notice the same effect for HIF-1α, for which the highest expression was present in the smallest tumors. Having analyzed this, we allowed for a heterogeneous distribution of T stage in the studied group which was dominated by T1 and T2 groups (94%). The systemic review of studies on hypoxia marker expression in breast cancer by Adams *et al.* revealed no association between GLUT1 and tumor size[28]. However, such a correlation has been reported for other carcinomas[29,30].

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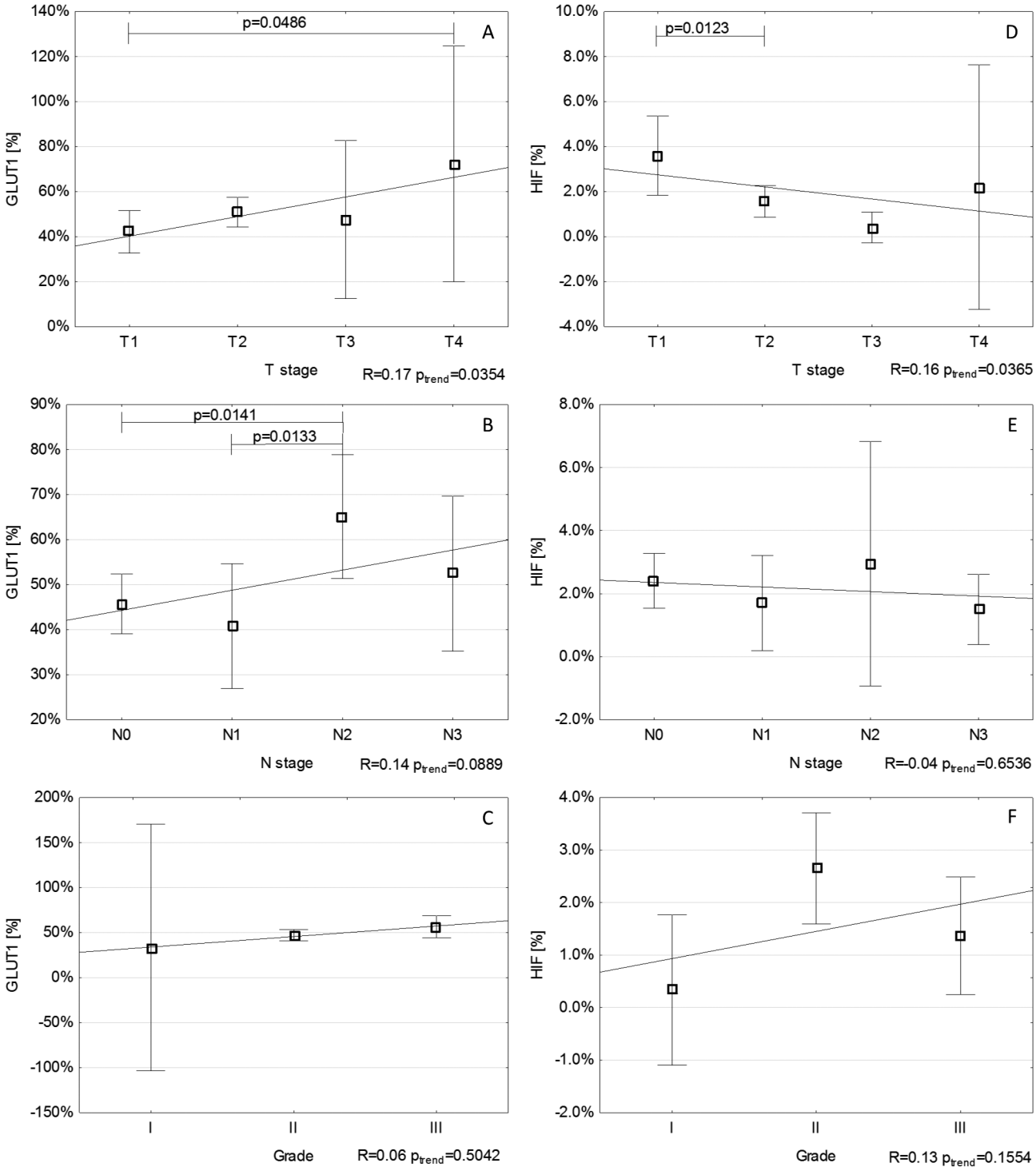

Fig. 3. Relationship between clinical prognostic factors: T stage, N stage as well as tumor grade and hypoxia-related proteins: HIF-1α and GLUT1 (average values with 95% confidence intervals)

We did not confirm any relationship with a histological grade either for GLUT1 or for HIF-1α, which is contrary to some other observations[7,28] and undoubtedly influenced by the dominance of grade II tumors that accounted for about 80% of the whole studied group.

CONCLUSIONS

The results of our study suggest that GLUT1 might be a valuable prognostic factor in breast cancer patients. As an indicator of a tumor hypoxic profile, it correlates with adverse hormonal and HER2 receptor status as well

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as tumor stage. A differential link of GLUT1 and HIF-1α with the classic prognostic factors might to some extent reflect a methodological advantage of glucose transporter testing, but may also provoke further studies on a very complicated network of molecular pathways in which both hypoxia markers are engaged. Since one rather looks for straightforward tools to be used for classifying patients into prognostic groups in clinical practice, GLUT1 seems to be a promising answer.

List of abbreviations

ER – estrogen receptor; **PR** – progesterone receptor; *ERBB2/HER2* – epithelial B2 receptor; **HIF-1α** – hypoxia-inducible factor 1α; **PHDs** – prolyl hydroxylases; **VHL** – von Hippel–Lindau; **GLUT1** – glucose transporter 1; **FISH** – fluorescence *in situ* hybridisation; **GRM** – generalized regression model; **LI** – labelling index.

Conflict of interest

The authors declare that they have no competing interests.

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