Evaluation of the association between HIF-1α and HER-2 expression, hormone receptor status, Ki-67 expression, histology and tumor FDG uptake in breast cancer

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Abstract. The present study aimed to examine hypoxia-inducible factor (HIF)-1α expression and its association with glucose uptake in invasive breast cancer. In addition, connections between glucose uptake and several other prognostic parameters of breast cancer were studied. Between August 2013 and April 2015, 92 patients with biopsy-diagnosed breast cancer were subjected to ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography. The primary tumor and nodal maximum standardized uptake values (SUVmax) were recorded, and HIF-1α expression and clinical parameters, including tumor mass, estrogen receptor (ER) and progesterone receptor (PgR) levels, human epidermal growth factor receptor-2 (HER-2), Ki-67 index, grade and histology, were analyzed. SUVmax was compared with clinicopathological parameters and HIF-1α expression. The median SUVmax values of the ER-negative and PgR-negative tumors were significantly increased compared with ER and PgR-positive tumors, respectively (P=0.004 and P=0.008). SUVmax differed significantly between the T2 and T3 tumors and the T1 tumors. The median SUVmax levels were higher in the Ki-67 expression >10% group than the Ki-67 index <10% group (P=0.001). Although the median SUVmax values in HER-2-positive and -negative tumors were similar, triple-negative tumors demonstrated significantly higher values (P=0.04). With regard to tumor grade, the median SUVmax was greater in the high-grade tumors compared with the low-grade tumors. SUVmax did not exhibit a significant correlation with HIF-1α expression; however, HIF-1α expression was associated with tumor size and PgR expression. HIF-1α expression increased with a larger tumor size (r=0.27; P=0.008) and decreased PgR expression (r=-0.26; P=0.0002). The axillary nodal SUVmax of the N1 tumors was significantly lower than the N2 and N3 tumors (P<0.0001). In the multivariate analysis, tumor size, Ki-67 expression and ER Allred score were independent factors that impacted SUVmax. The results of the present study indicated strong associations between tumor size, tumor grade, Ki-67 expression, triple-negativity, downregulated hormone receptor expression and SUVmax values. Conversely, there was no association observed between glucose uptake and levels of HIF-1α. Based on these results, it is suggested that the lack of assiocation between hypoxia and glucose uptake indicates phenotypic independence.

Introduction

Breast cancer is the most prevalent malignancy in women, accounting for ~29% of all female cancer cases (1). Despite progress in early diagnosis and treatment, breast cancer remains the second most frequent cause of cancer-associated mortality in women in United States (1). Traditionally, histologic tumor grade, tumor size, estrogen receptor (ER) and progesterone receptor (PgR) expression, axillary lymph node involvement, Ki-67 expression and human epidermal growth factor receptor-2 (HER-2) status have been employed as prognostic factors for breast cancer patients.

The association between tumorigenesis, hypoxia and tumor development is well-documented (2). Tumor hypoxia is facilitated by various factors, including tumor localization, tumor size and blood flow, all of which affect oxygen accessibility (3). The most recognized hypoxia signaling factor is hypoxia-inducible factor (HIF)-1 (3). HIF-1 is made up of two subunits: HIF-1α and HIF-1β (4,5). While HIF-1β is expressed constitutively, HIF-1α is only expressed during hypoxic conditions and is a measurement of overall activity (4-7). Increased

levels of HIF-1α have been documented in multiple tumors, in particular breast cancer (8). Notably, cells under low oxygen stress often switch to aerobic glycolysis (lactate production), a low energy-generating state, from oxidative phosphorylation, which is termed the Warburg effect (9). Previous studies have reported that aerobic glycolysis increases ~2-fold under acute hypoxia, which results in augmented cytoplasmic hexokinase and membrane-localized glucose transporters (GLUT), including GLUT-1 and GLUT-3 (10). Although hypoxia has been generally recognized for its impact on glucose metabolism, certain hypoxic tumors demonstrate marginal increases in glucose uptake, glycolytic shift and lactate production (9).

Augmented and differential glucose metabolism was established in 1931 by Otto Warburg (11). A measure of glucose uptake is an important feature of the Warburg effect phenomenon and may be measured in tumors with ^{18}F-fluorodeoxyglucose (FDG) tracers and positron emission tomography (PET). FDG-PET provides semiquantitative measurement of glucose standardized uptake value (SUV), which may aid in predicting tumor activity, treatment monitoring, staging and detection of disease recurrence (12,13). The level of FDG uptake in breast tumors has been documented for its heterogeneity compared with other forms of cancer (14). Notably, breast cancer FDG uptake is reported to be associated with several tumorigenic factors, including histologic grade, tumor size and hormone receptor expression levels, among others (15-17). Based on this, breast cancer with augmented glucose uptake is more aggressive compared to breast cancer with low glucose uptake (15-17).

The present study aimed to investigate the association between FDG uptake and HIF-1α expression in breast cancer. The link between known breast cancer parameters (tumor size, axillary lymph node involvement, ER and PgR expression, HER-2, Ki-67 expression, grade and histology) and FDG uptake was also evaluated.

Materials and methods

Patient characteristics and tissue samples. A total of 92 patients, who were diagnosed with no particular type of breast carcinoma between August 2013 and April 2015 in the Department of Surgical Oncology, Süleyman Demirel University (Isparta, Turkey), were included in the study. Diagnosis of primary breast cancer was defined by coreneedle biopsy at least 15 days prior to PET/computed tomography (CT). Initial diagnosis and re-analysis of biopsies were performed by a pathologist (Department of Pathology, Süleyman Demirel University) for diagnostic confirmation. The tumors were subjected to the modified Scarff-Bloom-Richardson grading system (18) and categorized based on the World Health Organization classification system (19). FDG-PET analysis was performed on all 92 patients between August 2013 and April 2015 to determine the stage of the disease prior to therapy. Patients were eligible for surgery if no indication of distant metastatic spread was determined. Patients diagnosed with excisional biopsy and/or lesions <1 cm (based on CT images) were not included in the study. Each individual was subjected to surgery three weeks after FDG-PET examination. No patients received neoadjuvant therapy. The study was approved by the Ethics Committee of

the Süleyman Demirel Universty and informed consent was obtained from all patients prior to examination.

FDG-PET image acquisition. Whole-body FDG-PET scans were perfomed as described using the Philips Gemini TF PET/CT scanner (Philips Medical Systems B.V., Eindhoven, The Netherlands). Patients were prepared by a 6-h fast, as serum glucose levels had to be <150 mg/dl prior to glucose tracer administration. At 60 min after the intravenous injection of 3.7 MBq/kg (0.1 mCi/kg) ^{18}F-FDG (Monrol, Eczacıbaşı, İstanbul, Turkey), PET/CT was performed. Subsequently, an emission scan was recorded in three-dimensional mode following CT for 2 min per position. PET and CT images were examined in the cross-sectional planes view and in the rotating maximum-intensity projection. FDG uptake in the tumor and lymph nodes were semiquantified using maximum SUV (SUVmax).

Immunohistochemistry. HIF-1α expression was evaluated by immunohistochemistry using monoclonal rabbit anti-human HIF-1α antibodies (clone, EP1215Y; dilution, 1:100; Abcam, Cambridge, MA, USA). A biotynilated goat anti-polyvalent secondary antibody (TP-125-BN; Thermo Fisher Scientific, Inc., Waltham, MA, USA) experiment was run in parallel as a negative control and human ovarian carcinoma was used as positive control. The avidin-biotin-peroxidase complex method was used while performing immunostaining. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded resection specimens. Tissues were kept in 10% formalin at room temperature for overnight fixation and processed in a fully automated tissue processor. Sections from paraffin-embedded tissues (4 μm thick) were obtained for immunohistochemical staining. The level of staining was determined using a light microscope. In the evaluation of immunostaining, nuclear immunoreactivity in neoplastic cells was considered as positive. A value ≥10% was set as the cut-off to distinguish positive and negative immunoreactivity (20).

Statistical analysis. Median and interquartile ranges (IQR) for the SUVmax for each prognostic indicator, including hormone receptor expression, Allred score above or below 4, grade 1, 2 vs. grade 3, HIF-1α, HER-2, Ki67 index, histology, and tumor (T1, T2 and T3) and nodal (N0, N1, N2 and N3) status, were calculated (21). SUVmax and prognostic indicators were analyzed by the Mann-Whitney U test or Kruskal Wallis test. Categorical data were studied using the χ^2 test. Mean IQR (IQR_M) was calculated and used instead of mean ± standard deviation. Multiple regression analysis was employed using the forward enter method by recruiting predictors with P<0.05 and excluding if P>0.10. Outliers were identified and excluded from analysis. Multiple regression analysis yielded the best predictors of SUVmax. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using by MedCalc v12.5 (MedCalc Software bvba, Ostend, Belgium).

Results

Patient characteristics. A total of 92 patients were enrolled in the present study. The mean age was 58.9±11.5 years (IQR_M, 58.3 years). Tumors were determined in the right breast of 35

(38.0%) patients and in the left breast of 57 (62.0%) patients (P=0.02). The IQR_M tumor size, number of metastatic lymph nodes, tumor SUVmax and axillary tumor SUVmax were 2.5 cm, 2.2, 7 and 2.6, respectively.

Tumor characteristics. The characteristics of all tumors are presented in Table I. The tumors were grade 1, 2 and 3 in 16.3, 53.3 and 30.4% of patients, respectively. In addition, 32.6, 30.4, 21.7 and 15.2 of patients were N0, N1, N2 and N3, respectively, and tumor size was T1, T2 and T3 in 20.7, 69.6 and 9.8% of patients, respectively. ER was positive in 82.6% of patients and negative in 17.4%, while PgR was positive in 81.5% of patients and negative in 18.5%. The IQR_M estrogen and progesterone Allred scores were 6.2 and 5, respectively. HER-2 expression was classified as 0, 1, 2 and 3 in 44.6, 16.3, 14.1 and 25.0% of patients, respectively. Silver *in situ* hybridization (SISH) was applied to 13 patients and 7 (53.8%) were positive. A total of 84 (91.3%) patients were triple-negative. Ki-67 expression was ≤10% in 34 (37.0%) patients and >10% in 58 (63.0%) patients. HIF-1α was positive in 83.7% of patients (Table I; Fig. 1A and B).

Clinicopathological parameters, SUVmax and HIF-1α. Comparisons between SUVmax, HIF-1α and clinicopathological parameters of the primary tumors are presented in Table II. The median SUVmax values of ER- and PgR-negative tumors were significantly increased (P=0.004 and P=0.008). This difference in SUVmax was also evident in the Allred score of ER and PgR. The SUVmax values of the T2 and T3 tumors were significantly different from those of the T1 tumors (P=0.02), and the SUVmax values between the Ki-67 >10% group and the Ki-67 <10% group were also significantly different (P=0.01). Although median SUVmax values were not different in HER-2-positive and -negative tumors, it was higher in triple-negative tumors (P=0.04). With regard to tumor grade, median SUVmax was significantly higher in high-grade tumors (Fig. 2A and B). SUVmax did not exhibit a significant correlation with HIF-1α expression (P=0.28); however, HIF-1α was associated with tumor size and PgR levels. HIF-1α expression increased with a larger tumor size (r=0.27; P=0.008) and decreased PgR expression (r=-0.26; P=0.0002).

Axillary nodal status and SUVmax. The IQR_M of axillary nodal SUVmax was 2.7. SUVmax according to nodal status is presented in Table III. Axillary SUVmax of N1 was statistically lower than N2 and N3 (P<0.0001) (Fig. 3).

Multiple regression analysis. Multiple regression analysis was performed to determine the association between SUVmax and independent factors affecting SUVmax (Table IV). The SUVmax of the axillary lymph nodes was only predicted by nodal status in multiple regression analysis (coefficient, 2.7; $r_{partial}$=0.70; t=9.1; r^2=0.49; P<0.0001).

Discussion

Various studies have demonstrated an association between FDG uptake in tumors and multiple prognostic indicators (22-24). Additional studies have evaluated the association among hypoxia, namely HIF-1α, and FDG uptake in breast

Table I. Breast cancer tumor characteristics.

Characteristic	Number	%
Grade		
1	5	16.3
2	49	53.3
3	28	30.4
Nodal status		
N0	30	32.6
N1	28	30.4
N2	20	21.7
N3	14	15.2
Tumor size		
T1	19	20.7
T2	64	69.6
T3	9	9.8
ER		
Negative	16	17.4
Positive	76	82.6
PgR		
Negative	17	18.5
Positive	75	81.5
ER Allred score		
<4	20	21.7
≥4	72	78.3
PgR Allred score		
<4	29	31.5
≥4	63	68.5
HER-2		
0	41	44.6
1	15	16.3
2	13	14.1
3	23	25
Ki-67		
≤10%	34	37
>10%	58	63
HIF-1α		
Negative	15	16.3
Positive	77	83.7

ER, estrogen receptor; PgR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; HIF-1α, hypoxia inducible factor-1α.

cancer (25). To the best of our knowledge, no study has yet investigated the combined association between hypoxia, FDG uptake and other clinicopathological prognostic factors.

In the present study, univariate analysis identified that augmented SUVmax values were associated with a higher nuclear grade, larger tumor size, negative hormone receptor status, triple-negativity and high Ki-67 expression. The median SUVmax value was higher in HER-2-positive breast cancer, but this was not significant statistically (P=0.07). Conversely,
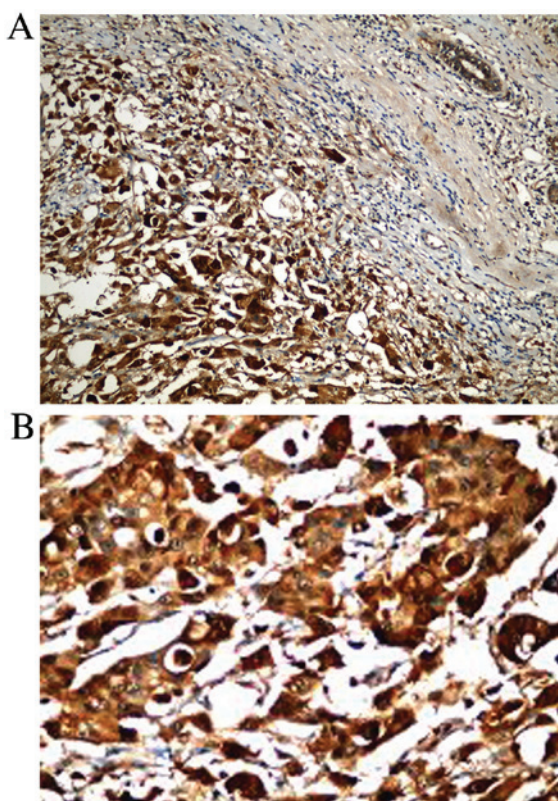
Figure 1. Immunohistochemical staining of breast cancer tissue. (A) Strong nuclear and cytoplasmic HIF-1α positivity in the neoplastic breast carcinoma cells (left), and negative nuclear staining in the adjacent normal breast duct (upper right) (magnification, x200). (B) High power view of strong nuclear and cytoplasmic HIF-1α expression in invasive breast carcinoma tissue (magnification, x400). HIF-1α, hypoxia induciblefactor-1α.

Table II. Univariate analysis of median SUVmax for different tumor characteristics.

Characteristic	Median SUVmax (IQR)	P-value
ER		
Negative	9.6 (4.0)	0.004[a,c]
Positive	6 (6.2)	
PgR		
Negative	9.8 (3.3)	0.008[a,c]
Positive	6 (6.1)	
ER Allred score		
<4	9.2 (3.6)	0.01[a,c]
≥4	6 (6.3)	
PgR Allred score		
<4	9.1 (4.2)	0.03[a,c]
≥4	5.9 (6.1)	
HER-2		
Negative	5.7 (6.6)	0.07[a]
Positive	7.9 (3.8)	
Triple-negative		
Non-TN	6.8 (5.9)	0.04[a,c]
TN	10.1(3.6)	
Ki-67		
≤10%	4.7 (6.8)	0.01[a,c]
>10%	7.8 (5.5)	
HIF-1α		
Negative	6.0 (6.0)	0.28[a]
Positive	7.8 (5.5)	
Grade		
1-2	5.7 (5.8)	0.001[a,c]
3	9.6 (4.1)	
Tumor size		
T1	4.8 (3.7)	0.02[b,c,d]
T2	7.8 (6.2)	
T3	7.9 (3.8)	

[a]Mann-Whitney U test; [b]Kruskal Wallis test; [c]P<0.05; [d]SUVmax value of T1 is different from SUVmax value of T2 and T3. SUVmax, maximum standardized uptake value; IQR, interquartile range; ER, estrogen receptor; PgR, progesterone receptor; HER-2, human epidermal growth factor receptor-2; HIF-1α, hypoxia induciblefactor-1α.

there was no association observed between HIF-1α expression and FDG uptake. Factors for tumor proliferation and metastasis were significantly associated with augmented SUVmax. Oshida *et al* (26) reported that high FDG uptake in breast tumors is associated with poor prognosis. Conversely, results regarding the link between tumor proliferation and FDG uptake in multiple tumors remains controversial (27,28). On the other hand, in a number of previous studies positive associations were observed between proliferation rates and FDG uptake in breast cancer (15,16,22,25,29,30). The present study is consistent with this, as Ki-67 expression and tumor size were considered independent factors for SUVmax in breast cancer according to multivariate regression analysis. Several studies have produced contradictory results, with some demonstrating a correlation between FDG uptake and tumor size (16,17,30,31), while others have not had consistent results (14,15,22,23,32).

Tumor and axillary lymph node grade are major predictive factors for breast cancer. In the present study, the most significant correlation was observed between SUVmax and tumor grade (P=0.001), which is consistent with previous studies (16,17,26,30,33,34). Additionally, multiple regression analysis performed in current study demonstrated that the SUVmax of axillary lymph nodes was only predicted by nodal status (coefficient, 2.7; $r_{partial}$=0.70; t=9.1; r^2=0.49; P<0.0001). Song *et al* (12) reported that axillary nodal SUVmax on pretreatment FDG-PET may be an independent factor for disease relapse and prognosis in patients with invasive ductal carcinoma and axillary lymph node involvement.

Several studies have observed FDG uptake in invasive lobular cancer, which was reduced compared with other types of aggressive tumors (14,15,23,30). This finding is explained by diffuse infiltration, lower tumor cell density, low levels of GLUT-1 and reduced proliferation (23,35,36).

Although studies regarding hormone receptor expression and FDG uptake are inconsistent, the present study detected a significantly higher FDG uptake in ER- and PgR-negative tumors than positive ones (P=0.004 and P=0.008, respectively). Meanwhile, ER Allred score was identified to be an

Table III. Axillary SUVmax according to nodal status.

Nodal status	Median SUVmax (IQR)	Statistical significance
N0	0 (0)	Statistically different from N1, N2 and N3
N1	2.2 (2.4)	Statistically different from N0, N2 and N3
N2	5.7 (5.4)	Statistically different from N0 and N1
N3	5.8 (6.3)	Statistically different from N0 and N1

The Kruskal Wallis test was used to determine statistical significance. SUVmax, maximum standardized uptake value; IQR, interquartile range.

Table IV. Multivariate model demonstrating the effects of different factors on SUVmax.

	Coefficient	$r_{partial}$	T	P-value
ER Allred score	-0.3322	-0.2436	-2.302	0.0238
Ki-67	0.04052	0.2306	2.172	0.0327
Size	0.6996	0.2557	2.424	0.0175

SUVmax, maximum standardized uptake value; ER, estrogen receptor.

Figure 3. Axillary nodal SUVmax in the N1 stage were significantly lower than those in the N2 and N3 stages (*P<0.0001). SUVmax, maximum standardized uptake values.
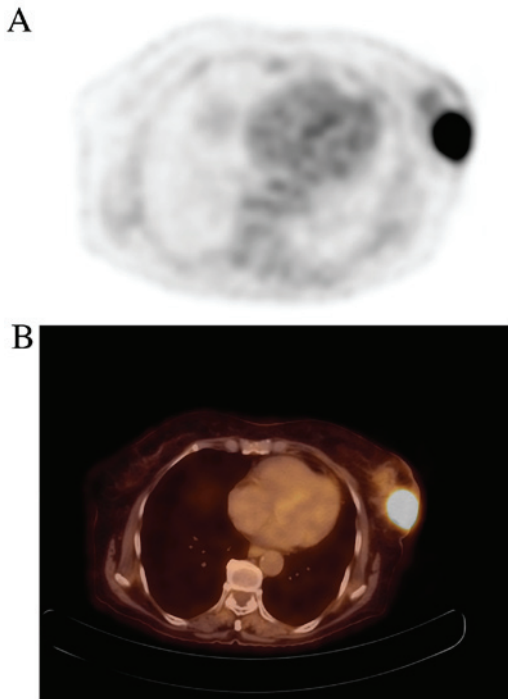

Figure 2. (A) FDG PET and (B) hybrid PET/computed tomography axial images demonstrating a left breast cancer lesion with notably increased FDG uptake (maximum standardized uptake value, 11.9) in a 76-year-old woman with invasive breast carcinoma (no special type). Histopathological and immunohistochemical features of the tumor were as follows: pT2 (45 mm); N0; histologic grade 3; ER-; PgR-; HER-2 3+++; and Ki-67, 70%. FDG, ^{18}F-fluorodeoxyglucose uptake; PET, positron emission tomography.

independent factor affecting SUVmax. A number of previous studies reported that there was correlation between hormone receptor status and SUVmax (14,15,32,34,35,37). However,
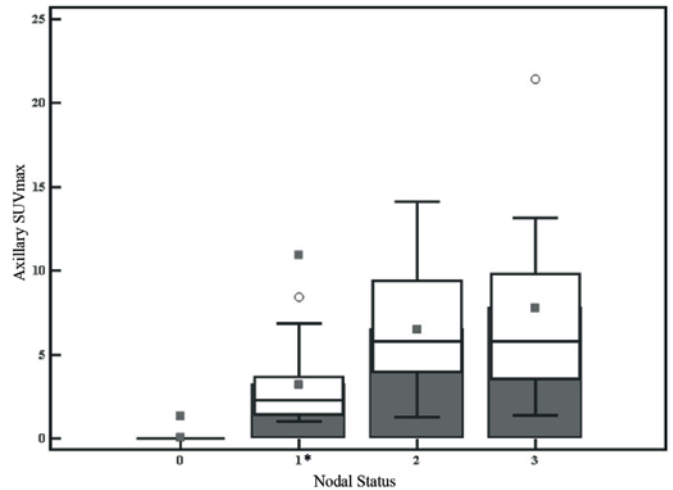
recent studies have demonstrated a positive association between SUVmax values and ER negativity in tumors (30,32,37,38). Triple-negative tumors have been documented as more invasive and are known for their poor prognosis compared with non-triple negative tumors (37). The present study also observed that the triple-negative tumors had augmented SUVmax compared with the non-triple-negative disease (P=0.04), which is consistent with previous reports (23,37).

Previously, several studies reported that there was a marked improvement in the survival of patients with HER-2 receptor-positive breast cancer due to the use of antibody treatment with trastuzumab (39-41). Although a few previous studies have observed an association between SUVmax and c-Erb-B2 positivity (16,17), the current study did not identify a significant correlation, which is supported by a number of other studies (15,32,37,42). This may suggest that HER-2 does not have any major effect on glycolytic pathways (23).

Intratumoral hypoxia has been demonstrated to lead to poor prognosis (43-45). Hypoxic tumors have been documented for their association with aggressiveness, augmented metastasis, and resistance to chemo and radiation therapy (43-45). Increased expression of HIF-1α has been reported in a various forms of common cancer. Bos et al (8) observed that HIF-1α was upregulated during breast pathogenesis following the identification of enhanced HIF-1α levels in ductal carcinoma in situ and invasive cancer specimens. However, augmented levels were not observed in normal breast and ductal hyperplasia (8).

Subsequently, Bos *et al* (25) investigated the association between FDG uptake and HIF-1α, GLUT-1, hexokinase I-II-III, vascular endothelial growth factor (VEGF), intratumoral microvessel density and mitotic index. Despite reporting a significant positive association with FDG uptake and GLUT-1 and hexokinase I, no correlation was observed between FDG uptake and HIF-1α or other biomarkers (25). This was consistent with the results of the present study. Following investigation of Ki-67 expression through immunohistochemistry, Evans *et al* (46) suggested that hypoxic localized cell proliferation of tumors was decreased (46). In the current study, HIF-1α levels were associated with tumor size and PgR negativity.

Clavo *et al* (47) examined FDG uptake in multiple types of carcinoma cells after varying the oxygen levels *in vitro*. An enhancement in FDG level was observed following moderate hypoxic treatment. Based on these results, it was suggested that hypoxia regulates FDG uptake (28,47). Toba *et al* (48) investigated the association between HIF-1α, GLUT-1, VEGF and FDG uptake in thymic epithelial tumors. The results demonstrated a moderate association between FDG uptake and the expression of HIF-1α (48). Furthermore, Rajendran *et al* (9) examined the association between hypoxia and glycolysis with ^{18}F-fluoromisonidazole (FMISO) and FDG uptake on PET images in four diverse tumors, including breast cancer. A moderate association was identified between FDG and FMISO uptake in head and neck cancer, but no association was observed in other cancer forms, including breast cancer and glioblastoma (9).

In conclusion, the results of the current study suggested that increased FDG uptake was associated with various poor prognostic indicators, such as large tumor size, high tumor grade and Ki-67 expression, reduction in hormonal receptors, and triple-negative primary breast tumors. However, no assoication was identified between FDG uptake and HIF-1α level. Although hypoxia impacts glycolysis, tumor hypoxia is a heterogeneous phenomenon. Therefore, there may be inconsistent patterns observed between glucose utilization and hypoxia. A major limitation of the present study may be that that only patients with invasive breast carcinoma were included. Thus, future studies may benefit from including a range of histological types, subsequently allowing a comparison between different types of breast cancer.

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