Metabolism-Related Proteins Are Differentially Expressed according to the Molecular Subtype of Invasive Breast Cancer Defined by Surrogate Immunohistochemistry

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Key Words

Aerobic glycolysis • Breast cancer • CAIX • Glut-1

Abstract

Objective: The purpose of this study was to investigate the expression of metabolism-related proteins such as Glut-1 and carbonic anhydrase IX (CAIX) according to breast cancer molecular subtype. **Methods:** We generated a tissue microarray of 276 breast cancer patients and performed immunohistochemical staining for known metabolism-related proteins, which were evaluated according to the molecular subtype. **Results:** The expression of IGF-1, MIF, and HIF-1 α was correlated with the HER-2 type ($p < 0.05$). Glut-1 overexpression and CAIX expression were associated with TNBC type, especially with basal-like type, high histologic grade, estrogen receptor negativity, and progesterone receptor negativity ($p < 0.05$). The expression of Glut-1 and CAIX was correlated with statistical significance ($p < 0.001$). **Conclusion:** We identified different patterns of expression of metabolism-related proteins according to the molecular subtypes of breast cancer defined by surrogate immunohistochemistry. Increased expression of HIF-1 α , IGF-1, and MIF was noted in HER-2 type breast cancer and increased expression of Glut-1

and CAIX was noted in TNBC type breast cancer, especially in the basal-like subtype, which exhibited a glycolytic and acid-resistant tumor phenotype.

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Introduction

Differences in the metabolism of neoplastic cells and normal cells originate from the fact that tumor cells gain energy not by aerobic phosphorylation from the tricarboxylic acid cycle but rather by aerobic glycolysis, a phenomenon known as Warburg's effect [1]. Several possible explanations for the selective biochemical processes of neoplastic cells have been suggested. In order to adapt to hypoxic microenvironments caused by oxygen deprivation as a result of the uncontrollable proliferation of neoplastic cells, tumor cells employ the mechanism that consumes the least oxygen, aerobic glycolysis. In addition, the genes that activate aerobic glycolysis may be subjected to different regulating mechanisms in tumors, resulting in altered expression [2, 3]. As a key process in aerobic glycolysis, glucose uptake into the tumor cells is increased and Glut-1 acts as a glucose transporter (fig. 1).

Fig. 1. Overview of aerobic glycolysis-related proteins in cancer cell metabolism. Several factors, including hypoxia, growth factor (IGF-1), and oncogene activation (c-myc), activate HIF-1 α , leading to increases in the expression of Glut-1 in the cell membrane, the uptake of glucose, and the generation of lactate by glycolysis. The generation of lactate results in intracellular acidosis. The expression of CAIX increases to prevent intracellular acidosis, and the expression of PDK1 increases to decrease mitochondrial respiration. In addition, the expression of MIF increases to prevent cell senescence induced by reactive oxygen species.

Increased expression of Glut-1 results in an increase in glucose uptake into cells and in generation of lactate by glycolysis, leading to intracellular acidosis. To prevent acidosis, the expression of carbonic anhydrase IX (CAIX) is increased, as is the expression of pyruvate dehydrogenase kinase 1 (PDK1) in order to suppress mitochondrial respiration. In addition, the expression of macrophage migration inhibitory factor (MIF) is increased to prevent cellular senescence induced by reactive oxygen species [4–11].

Overexpression of Glut-1 during the neoplastic process has been reported in human carcinomas. Overexpression of Glut-1 has been shown to be associated with high-grade tumors, poorly differentiated tumors, tumor invasion, and tumor metastasis [12–15]. Glut-1 overexpression also has been shown to be associated with poor prognosis in oral squamous cell carcinoma [16], non-small cell lung cancer [17], pulmonary adenocarcinoma [18], and thyroid cancer [19], as well as with metastasis in gastric cancer [20] and cervical cancer. [21].

In the previous gene expression profile study, breast carcinomas were classified into the following five molecular subtypes: luminal A, luminal B, HER-2, normal breast-like, and basal-like [22]. Apart from these five molecular subtypes, estrogen receptor (ER)-, progesterone receptor (PR)-, and HER-2-negative carcinomas were defined clinically as triple-negative breast cancer (TNBC) [23]. Previous studies regarding the role of metabolism-related proteins in breast cancer have shown that overexpression of Glut-1 is correlated with high grade [24, 25], proliferative activity [26, 27], poor differentiation [27],

and poor prognosis [28], and overexpression of CAIX is correlated with high grade [29], metastatic breast cancer cell lines [30], and poor prognosis [31–33]. Accordingly, since the HER-2 type and basal-like type of breast cancer exhibit high-grade histology, increased proliferative activity, and areas of geographic tumor necrosis, increased expression of metabolism-related proteins in these specific types of breast cancer is to be expected [34–39]. However, a limited number of studies regarding the expression of metabolism-related proteins according to the molecular subtypes of breast cancer have been conducted [40]. The purpose of this study was to investigate the expression of metabolism-related proteins such as Glut-1 and CAIX according to breast carcinoma subtypes and to determine the implications of these expression patterns.

Materials and Methods

Patient Selection

From the Department of Pathology at Severance Hospital, we retrieved tissue samples collected from patients with invasive ductal breast carcinoma that were archived between January 2000 and December 2001. This study was approved by the Institutional Review Board of Severance Hospital. Formalin-fixed and paraffin-embedded tissue specimens from 276 cases of primary breast cancer were included. All archival hematoxylin and eosin (H&E)-stained slides for each case were reviewed by two pathologists (J.S. Koo and J. Choi). Histological grade was assessed using the Nottingham grading system [41]. The clinical parameters that were evaluated for each tumor included patient age at initial diagnosis, lymph node status, local recurrence, systemic recurrence, and patient survival.

Table 1. Source, clone, and dilution of antibodies

Antibody	Clone	Dilution	Company
Aerobic glycolysis			
IGF-1	Polyclonal	1:100	Abcam, Cambridge, UK
c-myc (phospho Ser62)	Polyclonal	1:100	Abcam
HIF-1 α	EP1215Y	1:100	Biocare, Yorba Linda, Calif., USA
Glut-1	SPM498	1:200	Abcam
MIF	Polyclonal	1:100	Abcam
CAIX	Polyclonal	1:100	Abcam
PDK1	Polyclonal	1:100	Abcam
Molecular subtype			
ER	SP1	1:100	Thermo Scientific, USA
PR	PgR	1:50	DAKO, Glostrup, Denmark
HER-2	Polyclonal	1:1500	DAKO
CK5/6	D5/16B4	1:50	DAKO
EGFR	EGFR.25	1:50	Novocastra, Newcastle, UK

Tissue Microarray

A representative area was selected on each H&E-stained slide and a corresponding spot was marked on the surface of the paraffin block. Using a biopsy needle, the selected area was punched out and a 3-mm tissue core was placed into a 6 × 5 recipient block. The tissue of the invasive tumor was extracted. Two tissue cores were extracted to minimize extraction bias. Each tissue core was assigned a unique tissue microarray location number that was linked to a database containing other clinicopathologic data.

Immunohistochemistry

The antibodies used for immunohistochemistry in this study are shown in table 1. All immunostaining was performed using formalin-fixed, paraffin-embedded tissue sections. Briefly, 5- μ m-thick sections were obtained with a microtome, transferred onto adhesive slides, and dried at 62°C for 30 min. After incubation with primary antibodies, immunodetection was performed with biotinylated anti-mouse immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3'-diaminobenzidine chromogen as the substrate. The primary antibody incubation step was omitted in the negative control. Slides were counterstained with Harris hematoxylin.

Interpretation of Immunohistochemical Staining

All immunohistochemical markers were assessed by light microscopy. A cut-off value of 1% or more of positively stained nuclei was used to define ER and PR positivity [42]. HER-2 staining was analyzed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines using the following categories: 0 = no immunostaining; 1+ = weak incomplete membranous staining in less than 10% of tumor cells; 2+ = complete membranous staining, either uniform or weak in at least 10% of tumor cells, and 3+ = uniformly intense membranous staining in at least 30% of tumor cells. HER-2 immunostaining was considered positive when strong (3+) membranous staining was observed, whereas cases with 0–1+ staining were regarded as negative [43]. Cases with 2+ HER-2 expression were

evaluated for HER-2 amplification by fluorescent in situ hybridization (FISH). The immunostained slides were scored according to the percentage or intensity of tumor cells exhibiting nuclear (c-myc and HIF- α), cytoplasmic [insulin-like growth factor (IGF)-1, c-myc, Glut-1, MIF, CAIX, PDK1, HIF- α , and cytokeratin (CK) 5/6], and membranous epidermal growth factor receptor (EGFR) staining. Immunohistochemical stain results for EGFR, PDK1, c-myc, CAIX, and IGF-1 were evaluated based on the intensity of expression (0 = negative, 1 = weak, 2 = moderate, and 3 = strong). Moderate or intense staining was considered positive. Results for HIF- α , MIF, and CK5/6 were considered positive when more than 10% of the tumor cells were stained. The results for Glut-1 were scored according to the percentage of tumor cells with Glut-1 positivity as follows: low (less than 50%) and high (more than 50%).

FISH Analysis

Invasive tumors were examined on H&E-stained slides and FISH was subsequently performed on the tested tumor using a PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, Ill., USA) according to the manufacturer's instructions. The HER-2 gene copy number on the slides was evaluated using an epifluorescence microscope (Olympus, Tokyo, Japan). At least 60 tumor cell nuclei in three separate regions were investigated for HER-2 and chromosome 17 signals. HER-2 gene amplification was determined according to ASCO/CAP guidelines [43]. An absolute HER-2 gene copy number lower than 4 or an HER-2 gene/chromosome 17 copy number ratio (HER-2 /Chr17 ratio) of less than 1.8 was considered HER-2 negative. An absolute HER-2 copy number between 4 and 6 or an HER-2/Chr17 ratio between 1.8 and 2.2 was considered HER-2 equivocal. An absolute HER-2 copy number greater than 6 or an HER-2/Chr17 ratio higher than 2.2 was considered HER-2 positive.

Tumor Phenotype Classification Using Surrogate Markers

In this study, we classified breast cancer phenotypes according to immunohistochemistry results for ER, PR, HER-2, and FISH

Table 2. Clinicopathologic characteristics of patients according to breast cancer phenotype

Parameter	Total (n = 276)	TNBC (n = 97)	HER-2 type (n = 25)	Luminal B (n = 33)	Luminal A (n = 121)	p value
Mean age ($\pm$ SD), years	49.0 $\pm$ 10.6	47.5 $\pm$ 12.4	50.9 $\pm$ 10.2	47.3 $\pm$ 8.9	50.2 $\pm$ 9.4	0.175
Histologic grade						<0.001
I	33 (12.0)	4 (4.1)	0	1 (3.0)	28 (23.1)	
II	147 (53.3)	35 (36.1)	14 (56.0)	19 (57.6)	79 (65.3)	
III	96 (34.8)	58 (59.8)	11 (44.0)	13 (39.4)	14 (11.6)	
Tumor stage						0.002
T1	123 (44.6)	30 (30.9)	10 (40.0)	22 (66.7)	61 (50.4)	
T2	149 (54.0)	63 (64.9)	15 (60.0)	11 (33.3)	60 (49.6)	
T3	4 (1.4)	4 (4.1)	0	0	0	
Nodal stage						0.792
N0	153 (55.4)	56 (20.3)	15 (60.0)	21 (63.6)	61 (50.4)	
N1	78 (28.3)	27 (27.8)	5 (20.0)	9 (27.3)	37 (30.6)	
N2	28 (10.1)	9 (9.3)	4 (16.0)	1 (3.0)	14 (11.6)	
N3	17 (6.2)	5 (5.2)	1 (4.0)	2 (6.1)	9 (7.4)	
Tumor recurrence	27 (9.8)	17 (17.5)	5 (20.0)	1 (3.0)	4 (3.3)	<0.001
Patient deaths	23 (8.3)	15 (15.5)	4 (16.0)	1 (3.0)	3 (2.5)	0.002
Mean duration of clinical follow-up ($\pm$ SD), months	67.0 $\pm$ 17.6	67.5 $\pm$ 24.8	61.5 $\pm$ 16.8	68.5 $\pm$ 11.5	67.4 $\pm$ 11.2	0.430

Values in parentheses are percents.

as follows: luminal A type, ER or/and PR positive and HER-2 negative; luminal B type, ER or/and PR positive and HER-2 overexpressed and/or amplified; HER-2 overexpression type, ER and PR negative and HER-2 overexpressed and/or amplified, and TNBC type, ER, PR, and HER-2 negative. The TNBC type was sub-classified into basal-like (CK5/6 positive and/or EGFR positive) or non-basal-like types (CK5/6 and EGFR negative).

Statistical Analysis

Data was analyzed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, Ill., USA). For the determination of statistical significance, Student's t test and Fisher's exact test were used for continuous and categorical variables, respectively. $p < 0.05$ was considered statistically significant. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate the time to tumor recurrence. Multivariate regression analysis was performed using the Cox proportional hazards model.

Results

Patient Characteristics

The clinicopathologic characteristics of 276 patients, which comprised 97 (35.1%) cases of TNBC, 25 (9.1%) cases of the HER-2 type, 33 (12.0%) cases of luminal B, and 121 (43.8%) cases of the luminal A type, are summarized in table 2. The TNBC and HER-2 types had higher histo-

logic grades ($p < 0.001$), higher tumor stage ($p = 0.002$), higher tumor recurrence rates ($p < 0.001$), and a higher number of patient deaths ($p = 0.002$).

Metabolic Signature according to Molecular Subtype of Breast Cancer

Metabolism-related protein expression according to the molecular subtype of breast cancer is summarized in table 3 and figure 2. The HER-2 type showed higher rates of positivity for IGF-1 ($p = 0.020$), HIF-1 α ($p = 0.005$), and MIF ($p < 0.001$). The TNBC type showed higher rates of Glut-1 overexpression and positive expression of CAIX ($p < 0.001$ and $p = 0.012$, respectively), whereas the rate of CAIX expression in the tumor stroma was the lowest ($p = 0.012$). The luminal A type had the lowest rates of expression of IGF-1, HIF-1 α , and Glut-1. The patterns of metabolism-related protein expression in the basal-like type and non-basal-like type TNBCs are summarized in table 4. A total of 33 cases of the basal type (34.0%) and 64 cases of the non-basal-like type (66.6%) were studied. The rates of high Glut-1 expression and CAIX positivity were higher in the basal-like type compared with the non-basal-like type ($p = 0.010$ and $p < 0.001$, respectively).

Table 3. Immunohistochemical characteristics of cancer cell metabolism-related proteins according to breast cancer phenotype

Antibody	Total (n = 276)	Tumor phenotype (mean $\pm$ SD)				p value
		TNBC (n = 97)	HER-2 (n = 25)	Luminal B (n = 33)	Luminal A (n = 121)	
c-myc						0.207
Negative	160 (58.0)	62 (63.9)	17 (68.0)	16 (48.5)	65 (53.7)	
Positive	116 (62.0)	35 (36.1)	8 (32.0)	17 (51.5)	56 (46.3)	
IGF-1						0.020
Negative	263 (95.3)	92 (94.8)	21 (84.0)	31 (93.9)	119 (98.3)	
Positive	13 (4.7)	5 (5.2)	4 (16.0)	2 (6.1)	2 (1.7)	
HIF-1 α						0.005
Negative	263 (95.3)	90 (92.8)	21 (84.0)	32 (97.0)	120 (99.2)	
Positive	13 (4.7)	7 (7.2)	4 (16.0)	1 (3.0)	1 (0.8)	
Glut-1						< 0.001
Low	244 (88.4)	69 (71.1)	23 (92.0)	32 (97.0)	120 (99.2)	
High	32 (11.6)	28 (28.9)	2 (8.0)	1 (3.0)	1 (0.8)	
MIF						< 0.001
Negative	240 (87.0)	89 (32.2)	17 (68.0)	24 (72.7)	110 (90.9)	
Positive	36 (13.0)	8 (8.2)	8 (32.0)	9 (27.3)	11 (9.1)	
CAIX						0.012
Negative	186 (67.4)	56 (57.7)	16 (64.0)	29 (87.9)	85 (70.2)	
Positive	90 (32.6)	41 (42.3)	9 (36.0)	4 (12.1)	36 (29.8)	
CAIX in the tumor stroma						0.012
Negative	242 (87.7)	92 (94.8)	18 (72.0)	28 (84.8)	104 (85.9)	
Positive	34 (12.3)	5 (5.2)	7 (28.0)	5 (15.2)	17 (14.1)	
PDK1						0.522
Negative	123 (44.6)	48 (49.5)	12 (48.0)	15 (45.5)	48 (39.7)	
Positive	153 (55.4)	49 (50.5)	13 (52.0)	18 (54.5)	73 (60.3)	

Values in parentheses are percents.

Correlations between the Expression of Metabolism-Related Proteins and Clinicopathologic Factors

The correlations between the expression of metabolism-related proteins and clinicopathologic factors are summarized in table 5. The expression of c-myc was correlated with ER positivity ($p = 0.037$) and PR positivity ($p = 0.032$), and the expression of IGF-1 was correlated with high histologic grade ($p = 0.038$), ER negativity ($p = 0.019$), and HER-2 positivity ($p = 0.023$). The expression of HIF-1 α was correlated with high histologic grade ($p = 0.038$), high T stage ($p = 0.006$), lymph node metastasis ($p = 0.003$), ER negativity ($p = 0.004$), and PR negativity ($p = 0.032$), and high Glut-1 expression was correlated with high histologic grade ($p < 0.001$), absence of lymph node metastasis ($p = 0.006$), ER negativity ($p < 0.001$), and PR negativity ($p < 0.001$). The expression of MIF was cor-

related with HER-2 positivity ($p < 0.001$), and the expression of CAIX was correlated with high histologic grade ($p = 0.019$) and ER negativity ($p = 0.008$).

The Impact of Metabolism-Related Proteins on Disease-Free Survival and Overall Survival

The relationships between the expression of metabolism-related proteins and disease-free survival (DFS) and overall survival (OS) are summarized in table 6. HIF-1 α positivity was correlated with a shorter DFS and OS ($p < 0.001$), and high Glut-1 expression was correlated with a shorter DFS ($p = 0.028$). Multivariate Cox regression analysis was used to identify clinicopathologic and immunohistochemical parameters affecting patient survival. Parameters associated with a shorter DFS included younger age (≤ 35 vs. > 35 , $p = 0.014$, OR = 3.285, 95% CI 1.268–8.501), high T stage (T1 vs. T2–3, $p = 0.018$, OR =

Fig. 2. Expression of metabolism-related proteins according to molecular subtype. The HER-2 type expresses IGF-1, HIF-1 α , and MIF. The TNBC type expresses high levels of CAIX and Glut-1, particularly in the basal-like subtype.

Table 4. Clinicopathologic and immunohistochemical characteristics of cancer cell metabolism-related proteins in TNBC

	TNBC		p value
	basal-like (n = 33)	non-basal-like (n = 64)	
<i>Clinicopathologic parameters</i>			
Mean age ($\pm$ SD), years	47.3 $\pm$ 11.9	47.6 $\pm$ 12.8	0.918
Histologic grade			0.085
I	2 (6.1)	2 (3.1)	
II	7 (21.2)	28 (43.8)	
III	24 (72.7)	34 (53.1)	
Tumor stage			0.789
T1	10 (30.3)	20 (31.3)	
T2	21 (63.6)	42 (65.6)	
T3	2 (6.1)	2 (3.1)	
Nodal stage			0.436
N0	19 (57.6)	37 (57.8)	
N1	7 (21.2)	20 (31.2)	
N2	5 (15.2)	4 (6.3)	
N3	2 (6.1)	3 (4.7)	
Tumor recurrence	7 (21.2)	10 (15.6)	0.471
Patient deaths	7 (21.2)	8 (12.5)	0.246
Mean duration of clinical follow-up ($\pm$ SD), months	67.3 $\pm$ 25.0	67.5 $\pm$ 24.9	0.967
<i>Immunohistochemical parameters</i>			
c-myc			0.626
Negative	20 (60.6)	42 (65.6)	
Positive	13 (39.4)	22 (34.4)	
IGF-1			0.772
Negative	31 (93.9)	61 (95.3)	
Positive	2 (6.1)	3 (4.7)	
HIF-1 α			0.180
Negative	29 (87.9)	61 (95.3)	
Positive	4 (12.1)	3 (4.7)	
Glut-1			0.010
Low	18 (54.5)	51 (79.7)	
High	15 (45.5)	13 (20.3)	
MIF			0.574
Negative	31 (93.9)	58 (90.6)	
Positive	2 (6.1)	6 (9.3)	
CAIX			0.000
Negative	11 (33.3)	45 (70.3)	
Positive	22 (66.7)	19 (29.7)	
CAIX in tumor the stroma			0.772
Negative	31 (93.9)	61 (95.3)	
Positive	2 (6.1)	3 (4.7)	
PDK1			0.474
Negative	18 (54.5)	30 (46.9)	
Positive	15 (45.5)	34 (53.1)	
Values in parentheses are percents.			

Values in parentheses are percents.

3.285, 95% CI 1.250–10.951), HIF-1 α positivity (p = 0.002, OR = 5.208, 95% CI 1.835–14.778), and high Glut-1 expression (p = 0.049, OR = 2.574, 95% CI 1.004–6.601). Parameters associated with shorter OS were younger age (≤ 35 vs. >35 , p = 0.022, OR = 3.338, 95% CI 1.188–9.376), high T stage (T1 vs. T2–3, p = 0.016, OR = 4.565, 95% CI 1.333–15.638), and HIF-1 α positivity (p = 0.009, OR = 4.538, 95% CI 1.459–14.114).

Discussion

We investigated the expression of cancer cell metabolism-related proteins according to the molecular subtypes of breast cancer defined by surrogate immunohistochemistry. The expression of HIF-1 α was associated with the HER-2 subtype (p = 0.005), a high histologic grade (p = 0.038), a high T stage (p = 0.006), lymph node metastasis (p = 0.003), ER negativity (p = 0.004), and PR negativity (p = 0.032), which was consistent with previous results that showed that HIF-1 α is correlated with a high histologic grade, ER negativity, and PR negativity [29, 44].

The association between the expression of HIF-1 α and ER and PR negativity may be explained by several mechanisms. Since it has been reported that hypoxia decreases the expression of hormone receptors [45], it is possible that the expression of HIF-1 α induced by hypoxia results in the loss of hormone receptors. Another explanation is related to the fact that the loss of hormone receptors is a known characteristic of aggressive breast carcinomas exhibiting high proliferative activity, which tends to generate hypoxic areas where the expression of HIF-1 α is increased [29]. However, HIF-1 α seems to have a stronger association with the HER-2 subtype than the TNBC subtype with a high histologic grade, ER negativity, and PR negativity, and this result is supported by the fact that HIF-1 α protein expression is increased via the HER-2 signaling pathway in breast carcinoma [46].

In this study, the expression of HIF-1 α was not strongly associated with HER-2 positivity per se, but it was associated with the HER-2 subtype. This finding suggests that HIF-1 α expression is not associated with the luminal B subtype (ER+, PR+, and HER-2+), although it was associated with the HER-2 subtype (ER-, PR-, and HER-2+). We are unsure of the underlying mechanism for this difference, but it may be because factors other than HER-2 expression status could also affect HIF-1 expression. Previous studies have suggested that the expression patterns of HIF-1 α reflect different mechanisms. It has been reported that perinecrotic or focal patterns of expression

Table 5. Correlations between the expressions of metabolism-related proteins and clinicopathologic factors

Parameter	c-myc			IGF-1			HIF-1 α			Glut-1			MIF			CAIX			PDK1		
	-	+	p	-	+	p	-	+	p	low	high	p	-	+	p	-	+	p	-	+	p
Age, years			0.077			0.381			0.254			0.417			0.934			0.937			0.322
≤35	18	6		22	2		24	0		20	4		21	3		16	8		13	11	
>35	142	110		241	11		239	13		224	28		219	33		170	82		110	142	
Histologic grade			0.730			0.038			0.038			<0.001			0.858			0.019			0.479
I/II	103	77		175	5		175	5		173	7		157	23		130	50		83	97	
III	57	39		88	8		88	8		71	25		83	13		56	40		40	56	
T stage			0.073			0.490			0.006			0.217			0.482			0.187			0.493
T1	64	59		116	7		122	1		112	11		105	18		88	35		52	71	
T2-3	96	57		147	6		141	12		132	21		135	18		98	55		71	82	
N stage			0.749			0.906			0.003			0.006			0.731			0.978			0.595
N0	90	63		146	7		151	2		128	25		134	19		103	50		66	87	
N1-3	70	53		117	6		112	11		116	7		106	17		83	40		57	66	
ER			0.037			0.019			0.004			<0.001			0.803			0.008			0.198
Negative	81	44		115	10		114	11		95	30		108	17		74	51		61	64	
Positive	79	72		148	3		149	2		149	2		132	19		112	39		62	89	
PR			0.032			0.116			0.032			<0.001			0.491			0.328			0.051
Negative	98	56		144	10		143	11		123	31		132	22		100	54		77	77	
Positive	62	60		119	3		120	2		121	1		108	14		86	36		46	76	
HER-2			0.852			0.023			0.114			0.086			<0.001			0.062			0.732
Negative	127	91		211	7		210	8		189	29		199	19		141	77		96	122	
Positive	33	25		52	6		53	5		55	3		41	17		45	13		27	31	

reflect HIF-1 α overexpression induced by severe hypoxia [47] and that diffuse patterns of expression reflect HIF-1 α overexpression by HER-2 [46], HIF-1 α gene amplification [48], or mutation [49] rather than hypoxia. In this study, we were unable to compare results directly with previous studies since a diffuse pattern of HIF-1 α expression was not noted in more than 50% for any tumor.

MIF expression was strongly associated with the HER-2 subtype and HER-2 positivity ($p < 0.001$). A previous study demonstrated a positive correlation between MIF expression and HER-2 expression [50], which was consistent with the results of our study, whereas MIF expression was correlated with ER and PR positivity in another study [51]. Thus, additional research is required to elucidate the role of MIF in breast cancer.

Glut-1 high expression was associated with TNBC type ($p < 0.001$), high histologic grade ($p < 0.001$), absence of lymph node metastasis ($p = 0.006$), ER negativity ($p < 0.001$), and PR negativity ($p < 0.001$) in this study. Previous studies have shown that in breast cancer Glut-1 overexpression is associated with high histologic grade, high proliferative activity, poor differentiation, BRCA1-related breast cancer, ER negativity, PR negativity, and the basal-like subtype [24–27, 33, 47, 52], and the results are consistent with those of this study. The expression of

CAIX was associated with TNBC type ($p = 0.012$), high histologic grade ($p = 0.019$), and ER negativity ($p = 0.008$), and these results are consistent with previous studies showing that the expression of CAIX in breast cancer is correlated with a high histologic grade, BRCA1-related breast cancer, ER negativity, PR negativity, and the basal-like subtype [31–33, 47]. The expression of Glut-1 and that of CAIX were associated with similar parameters, consistent with a previous study that showed a highly positive correlation between Glut1 expression and CAIX expression [30]. The expression of Glut-1 and that of CAIX were closely related in this study ($p < 0.001$, data not shown).

The expression of HIF-1 α was not related with that of either Glut-1 or CAIX in this study. A previous study using invasive breast cancer tissue was also unable to identify a relationship between the expression of HIF-1 α and Glut-1 or CAIX [30, 40, 53, 54]. Although both Glut-1 and CAIX are known target genes of HIF-1 α as part of the metabolic process, there was no association between HIF-1 α and their expression levels. One possible explanation for this lack of association is the difference in the half-lives of HIF-1 α and Glut-1/CAIX. HIF-1 α is undetectable a few minutes after re-oxygenation, whereas Glut-1 and CAIX persist due to their relative stabilities [54, 55]. Another possible explanation is that the expression of Glut-1

Table 6. Univariate analysis of metabolism-related proteins in breast cancer and time to DFS and OS by log-rank test

	Number of patients			DFS		OS	
		tumor recurrence (n = 27)	patient death (n = 23)	mean survival (95% CI), months	p	mean survival (95% CI), months	p
c-myc					0.786		0.368
Positive	158	17 (10.8)	16 (10.1)	102.6 (97.6–107.5)		103.9 (99.4–108.4)	
Negative	116	10 (8.6)	7 (6.0)	96.7 (92.6–100.8)		99.4 (96.4–102.4)	
IGF-1					0.066		0.027
Negative	261	24 (9.2)	20 (7.7)	103.8 (100.0–107.7)		106.0 (102.8–109.3)	
Positive	13	3 (23.1)	3 (23.1)	67.8 (55.7–80.0)		68.5 (57.2–79.9)	
HIF-1 α					<0.001		<0.001
Negative	261	22 (8.4)	19 (7.3)	104.3 (100.4–108.2)		106.2 (102.9–109.4)	
Positive	13	5 (38.5)	4 (30.8)	65.6 (47.9–83.3)		72.8 (58.1–87.5)	
Glut-1					0.028		0.053
Low	243	21 (8.6)	18 (7.4)	104.6 (100.8–108.4)		106.1 (102.8–109.4)	
High	31	6 (19.4)	5 (16.1)	74.6 (64.9–84.3)		86.8 (77.9–95.6)	
MIF					0.382		0.186
Negative	238	22 (9.2)	18 (7.6)	103.5 (99.4–107.7)		105.9 (102.5–109.3)	
Positive	36	5 (13.9)	5 (13.9)	87.7 (80.5–95.0)		88.8 (82.5–95.0)	
CAIX					0.271		0.195
Negative	186	16 (8.6)	13 (7.0)	102.4 (97.9–106.9)		104.6 (100.9–108.3)	
Positive	88	11 (12.5)	10 (11.4)	101.8 (95.5–108.1)		104.0 (98.6–109.4)	
CAIX in the tumor stroma					0.731		0.985
Negative	240	23 (9.6)	20 (8.3)	103.1 (99.0–107.3)		105.1 (101.5–108.7)	
Positive	34	4 (11.8)	3 (8.8)	102.0 (93.3–110.7)		105.4 (99.1–111.7)	
PDK1					0.616		0.626
Negative	122	14 (11.5)	12 (9.8)	100.1 (94.4–105.7)		102.6 (97.8–107.4)	
Positive	152	13 (8.6)	11 (7.2)	104.9 (100.5–109.3)		106.3 (102.3–108.6)	

Values in parentheses are percents unless otherwise stated.

and CAIX may be regulated by mechanisms other than HIF-1 α . The expression of Glut-1 may be increased by inhibitors of oxidative phosphorylation such as mitochondrial inhibitors [56]. In addition, CAIX is known to be activated by the phosphatidylinositol-3-kinase (PI3K) pathway [57] and the mitogen-activated protein kinase (MAPK) pathway [58].

Glut-1 and CAIX expression was highest in the TNBC type, which seems to most closely resemble the glycolytic and acid-resistant phenotype. The glycolytic and acid-resistant phenotype exhibits the most powerful adaptive advantage since it uses glycolysis to supply energy to tumors with a high proliferative activity, thereby enabling continuous tumor growth. Active glycolysis results in intracellular acidosis, which usually leads to acid-mediated cell death. However, overexpression of CAIX, such as in the TNBC type, neutralizes the acidic environment and allows the tumor to become acid resistant. CAIX expression was found in the tumor stroma, as previously re-

ported [29, 54, 59]. There was a discrepancy between the results of previous studies regarding the expression of CAIX in the tumor stroma and its implication in the prognosis of breast cancer, thus it was not possible to clarify the role of CAIX expression in the stroma of breast cancer in this study.

In this study, the expression of CAIX in the stroma was the lowest in the TNBC subtype, suggesting that the tumor stroma is acid sensitive in spite of the high CAIX expression, enabling tumor cells of the TNBC subtypes to be acid resistant. It has been reported that increased acidity in the tumor stroma enables tumor invasion through degradation of the extracellular matrix [60] and generation of genetic mutations in the cells [61], which may be related to the aggressiveness of the TNBC type. The basal-like subtype of TNBC had higher rates of Glut-1 and CAIX expression than the non-basal-like subtype ($p = 0.010$ and $p < 0.001$, respectively) in this study, which is consistent with the results of previous studies that

showed that the expression of Glut-1 and CAIX is strongly associated with the basal-like subtype [33, 40].

Although there is some overlap in their characteristics, TNBC and basal-like breast cancers are not coincident entities. TNBC is a heterogeneous tumor group comprised of the basal-like subtype, the molecular apocrine subtype (HER-2 enriched type), and the claudin-low subtype [62]. The basal-like subtype of TNBC showed a more glycolytic and acid-resistant phenotype than the non-basal-like subtype in this study, and this result may be linked to the more aggressive behavior of tumors showing the basal markers of TNBC.

In conclusion, we identified different expression patterns of metabolism-related proteins according to the

molecular subtypes of breast cancer defined by surrogate markers. Increased expression of HIF-1 α , IGF-1, and MIF was noted in the HER-2 type, and increased expression of Glut-1 and CAIX was noted in the TNBC type, especially in the basal-like subtype, which had a glycolytic and acid-resistant phenotype.

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