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ORIGINAL ARTICLE

Glutaminase expression is a poor prognostic factor in node-positive triple-negative breast cancer patients with a high level of tumor-infiltrating lymphocytes

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Abstract Glutamine metabolism is emerging as one aspect of dysregulated metabolism of tumors. Triple-negative breast cancer (TNBC) cells are glutamine dependent, whereas luminal-type cells tend to be glutamine independent. Therefore, TNBC patients might benefit from therapies targeting glutamine metabolism. To investigate the clinical significance of glutamine metabolism, we examined expression and prognostic significance of glutaminase in tumor cells and tumor-infiltrating lymphocytes (TILs) in TNBC. We retrieved 658 surgically resected TNBCs and analyzed glutaminase expression in tumor cells and TILs by immunohistochemical staining. Glutaminase expression was observed in 237 cases (36.0%) in tumor cells and 104 cases (15.5%) in TILs. Although glutaminase expression in tumor cells was significantly associated with a low level of TILs ($p = 0.018$), glutaminase expression in TILs was significantly higher in cases with a high level of TILs ($p = 0.031$). Glutaminase expression in tumor cells was significantly associated with poor disease-free survival in patients with lymph node metastasis and high levels of TILs ($p = 0.020$). In addi-

tion, it was an independent poor prognostic factor (hazard ratio = 10.643, 95% confidence interval = 1.999–56.668; $p = 0.006$). Glutaminase expression in tumor cells was observed in a subset of TNBC patients. It was significantly associated with a low level of TILs and poor disease-free survival in TNBCs presenting with lymph node metastasis and high levels of TILs.

Keywords Glutaminase · Tumor-infiltrating lymphocytes · Triple-negative breast cancer · Prognosis

Introduction

Cancer cells depend on energy for survival, proliferation, and invasion [1]. Features of dysregulated tumor metabolism, such as aerobic glycolysis, play a critical role in oncogenesis and represent potential diagnostic markers or therapeutic targets [2–4]. Recent studies indicate that glutamine metabolism is another key feature of dysregulated metabolism in tumors. Glutamine is needed to supply energy to cancer cells by generating cellular ATP and protein synthesis, as a metabolic intermediate for nucleotide synthesis and for c-MYC-mediated oncogenesis [5–10]. However, cancer cell lines show significant variability in response to glutamine deprivation indicating that not all cancer cells need exogenous glutamine for their survival [11]. In breast cancer, basal-type cells are glutamine dependent, whereas luminal-type cells tend to be glutamine independent [1]. Therefore, patients with triple-negative breast cancer (TNBC), which currently have few treatment options, might benefit from therapies that target glutamine metabolism. The conversion of glutamine to glutamate is mediated by

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glutaminolysis, a catabolic process mediated by two distinct glutaminase proteins (GLS1 and GLS2). Glutaminase mediates a signaling cascade associated with the *c-MYC* oncogene and presumably plays a critical role in glutamine metabolism [8, 9, 12, 13].

Tumor-infiltrating lymphocytes (TILs) are associated with good prognosis and predict response to therapy in TNBC [14–16]. TILs also need adequate nutrients and metabolism to function [17]. T cells show different metabolic demands according to their developmental stage. Naïve T cells are metabolically quiescent relative to effector and memory T cells. To promote cell growth and proliferation and ensure effective cell function, effector T cells become metabolically active and increase levels of glycolysis and glutaminolysis [17]. Metabolic competition between cancer cells and cells in the tumor microenvironment, such as TILs, might inhibit the protective role of T cells and subsequently drive cancer progression [18]. Chang et al. demonstrated that glucose consumption by tumors metabolically restricts T cells and promotes tumor progression in a mouse sarcoma model [18]. However, the correlation of glutaminase expression in cancer cells and TILs with respect to metabolic competition and the prognostic significance of glutaminase expression in TNBC has not been investigated. In this study, we examined expression of glutaminase in consecutive breast cancer and TNBC cohorts. We also evaluated the expression of glutaminase in TILs in TNBCs and analyzed its prognostic significance.

Materials and methods

Patient and tissue specimens

We evaluated two groups of patients with primary breast cancer. One group comprised 157 consecutively enrolled patients with breast cancer, who underwent surgical resection at the Asan Medical Center in Seoul, Korea in 1998 and from whom formalin-fixed, paraffin-embedded tissue samples were available for analysis. In this cohort, 97 of the 157 patients received six cycles of an adjuvant methotrexate-based regimen (cyclophosphamide, 500 mg/m^2; methotrexate, 40 mg/m^2; and 5-fluorouracil, 500 mg/m^2) or an anthracycline-based regimen (cyclophosphamide, 500 mg/m^2; adriamycin, 50 mg/m^2; and 5-fluorouracil, 500 mg/m^2). Five patients received adjuvant chemotherapy with an unknown regimen. In addition, 16.5 and 75.8% patients received radiotherapy and hormone therapy, respectively. The mean follow-up period was 13.4 years. The second group comprised 682 TNBC patients who underwent surgical resection between 2004 and 2010 at the Asan Medical Center, from whom tissue samples were available. In this group, 473 of the 682 patients did not present with lymph node metastasis. They received four cycles of adjuvant anthracycline and cyclophosphamide (AC, adriamycin, 60 mg/m^2, and cyclophosphamide, 600 mg/m^2). The remaining 209 patients presented with lymph node metastasis and were treated with four cycles of AC followed by either four
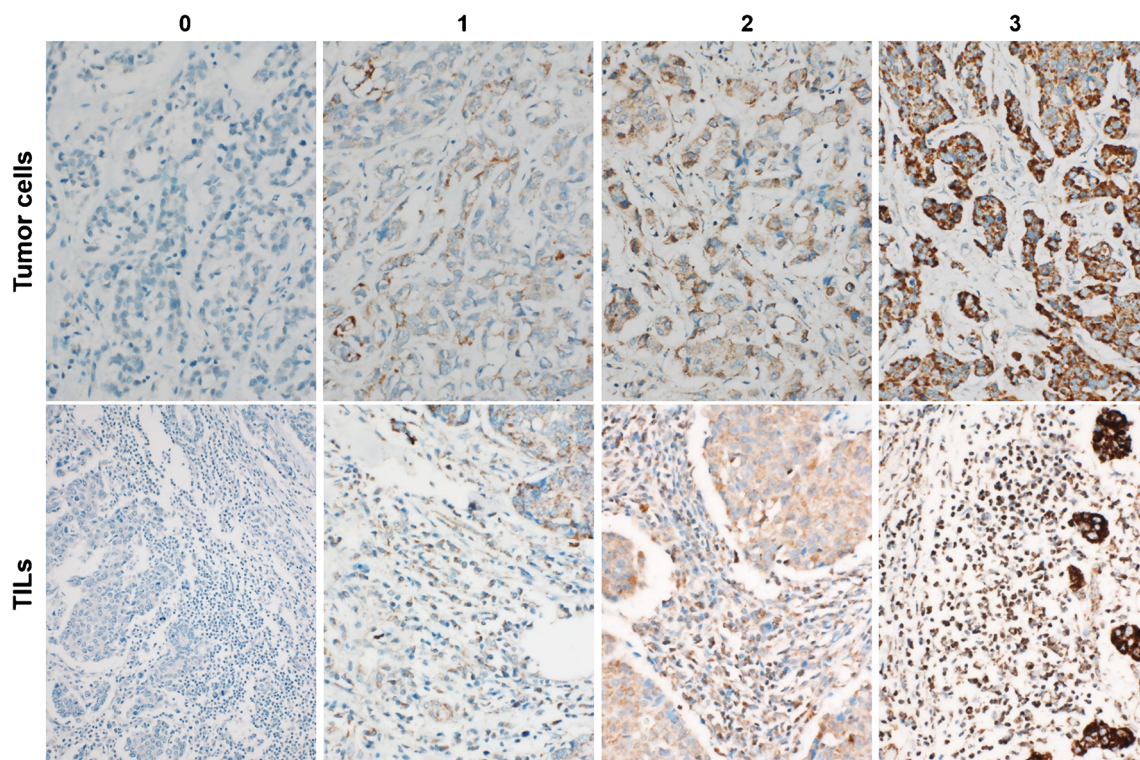

Fig. 1 Representative images of glutaminase immunohistochemical staining of tumor cells and TILs

cycles of paclitaxel (175 mg/m^2) or four cycles of docetaxel (75 mg/m^2). Five hundred fifty-one patients (80.8%) received radiotherapy, and one patient received hormone therapy. The median follow-up period was 78.3 months. All of the patients were chemotherapy naïve and radiotherapy naïve preoperatively. Clinical information was obtained from medical records and pathology reports from the surgery. Exemption from informed consent after de-identification of information was approved by the Institutional Review Board of the Asan Medical Center.

Histological evaluation

Hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists (H.J.L. and G.G.). Slides were histopathologically analyzed for the presence of TILs (defined as the percentage of the stroma of invasive carcinoma infiltrated by lymphocytes in 10% increments) [14], histological grade, tumor size, pT stage, pN stage, and lymphovascular invasion. TIL presence was dichotomized into high or low using 60% as cutoff, in agreement with the definition of lymphocyte-predominant breast cancer [19, 20]. Histological grade was assessed using the modified Bloom–Richardson classification [21].

Tissue microarray construction and immunohistochemistry

From formalin-fixed, paraffin-embedded tissue samples, a tissue microarray was constructed using an arraying instrument, as previously described [22]. Of each sample, three 1-mm-diameter cores were included in the array, to minimize tissue loss and mitigate the effects of tumor heterogeneity. Tissue microarray sections were stained using an automatic immunohistochemical staining device (Benchmark XT; Ventana Medical Systems, Tucson, AZ). Antibodies to target the estrogen receptor (ER, 1:100, Leica Biosystems, Newcastle, UK), progesterone receptor (PR, 1:200, Leica Biosystems), HER2 (1:8, Ventana Medical Systems), and glutaminase (1:100, Abcam, Cambridge, UK) were used. ER and PR levels were scored as positive if at least 1% tumor nuclei stained positive [23]. The hormone receptor (HR)-positive group was defined as ER- or PR-positive tumors. HER2-overexpressing tumors were defined as those with scores of 3+ by immunohistochemistry or gene amplification by fluorescence in situ hybridization (FISH) or silver in situ hybridization (SISH) [24].

The intensity of glutaminase expression in the tumors was scored on a scale of 0–3 (Fig. 1). Cytoplasmic expression of glutaminase was also evaluated using an immunoreactivity score, which was generated by multiplying intensity and percentage of positive cells (0–300); scores >10 were classified as positive. The intensity of glutaminase staining in TILs was classified as none, weak, moderate, or strong (Fig. 1). The

cases with moderate or strong intensity were considered positive [25].

Statistical analysis

All statistical analyses were conducted using SPSS statistical software version 18 (SPSS, Chicago, IL). The Chi-square test, log-rank test, and forward conditional Cox proportional

Table 1 Comparison of pathologic factors in consecutive invasive carcinoma cohort according to tumoral glutaminase expression

Variables	Negative	Positive	*p* value
Tumor subtype			<0.001
HR+/HER2−	90 (63.4)	1 (6.7)	
HR+/HER2+	12 (8.5)	0 (0.0)	
HR−/HER2+	14 (9.9)	0 (0.0)	
TNBC	26 (18.3)	14 (93.3)	
Histologic grade			0.011
1	27 (19.0)	0 (0.0)	
2	72 (50.7)	5 (33.3)	
3	43 (30.3)	10 (66.7)	
pT			0.710
1	48 (33.8)	5 (33.3)	
2	86 (60.6)	8 (53.3)	
3	4 (2.8)	1 (6.7)	
4	4 (2.8)	1 (6.7)	
Lymphovascular invasion			0.947
Negative	103 (72.5)	11 (73.3)	
Positive	39 (27.5)	4 (26.7)	
Lymph node metastasis			0.078
Negative	74 (53.2)	4 (28.6)	
Positive	62 (46.8)	10 (71.4)	
pTNM stage			0.021
I	36 (25.4)	3 (20.0)	
II	72 (50.7)	7 (46.7)	
III	34 (23.9)	4 (26.7)	
IV	0 (0.0)	1 (6.7)	
Radiation therapy			0.066
Negative	121 (85.2)	10 (66.7)	
Positive	21 (14.8)	5 (33.3)	
Adjuvant systemic therapy			0.421
Negative	51 (35.9)	4 (26.7)	
Unknown	4 (2.8)	1 (6.7)	
Anthracycline-based	26 (18.3)	5 (33.3)	
Methotrexate-based	61 (43.0)	5 (33.3)	
Tumor-infiltrating lymphocyte			0.602
≤60%	85 (59.9)	7 (46.7)	
>60%	57 (40.1)	8 (53.3)	

HR hormone receptor

hazards regression model were used to evaluate the data. All tests were two-sided, and $p > 0.05$ was considered statistically significant.

Results

Glutaminase expression in consecutive breast cancer cases

We analyzed glutaminase expression in the tumor cells of each subtype in a consecutive series of breast cancer cases. Glutaminase was expressed in 15 consecutive breast cancer cases (9.6%), 14 of which were TNBC (14 of 40 TNBC cases, 35%, $p < 0.001$, Table 1). There was one glutaminase-positive case in the HR-positive (weak ER expression, ER Allred score 4) and HER2-negative group. All HER2-positive cases were negative for glutaminase expression.

Glutaminase expression in the TNBC cohort and the correlation of glutaminase expression with clinical factors

As glutaminase expression was more prevalent in TNBC samples, we analyzed glutaminase expression in a large cohort of TNBC cases. Evaluation of immunohistochemical staining for glutaminase was possible on 658 cases regarding tumor cells and 673 cases regarding TILs. Clinicopathological characteristics of TNBC patients are summarized in Table 2. Glutaminase expression was positive in 237 cases (36.0%) in tumor cells and 104 cases (15.5%) in TILs. While positive glutaminase expression in tumor cells was significantly associated with a low level of TILs ($p = 0.018$), glutaminase expression in TILs was significantly higher in cases with a high level of TILs ($p = 0.031$, Table 2). There were no significant differences in histological grade, pT stage, lymphovascular

Table 2 Comparison of pathologic factors in another triple-negative invasive carcinoma patient cohort according to glutaminase expression

Variables	Tumor		p value	TILs		p value
	Negative	Positive		Negative	Positive	
Age			0.951			0.506
<50	253 (60.1)	143 (60.3)		348 (61.2)	60 (57.7)	
≥50	168 (39.9)	94 (39.7)		221 (38.8)	44 (42.3)	
Histologic grade			0.322			0.351
2	105 (24.9)	51 (21.5)		139 (24.4)	21 (20.2)	
3	316 (75.1)	186 (78.5)		430 (75.6)	83 (79.8)	
pT stage			0.637			0.746
1	180 (42.8)	103 (43.5)		245 (43.1)	48 (46.2)	
2	227 (53.9)	123 (51.9)		304 (53.4)	51 (49.0)	
3	13 (3.1)	11 (4.6)		19 (3.3)	5 (4.8)	
4	1 (0.2)	0 (0.0)		1 (0.2)	0 (0.0)	
Lymphovascular invasion			0.191			0.249
Negative	315 (74.8)	188 (79.3)		440 (77.3)	75 (72.1)	
Positive	106 (25.2)	49 (20.7)		129 (22.7)	29 (27.9)	
Lymph node metastasis			0.402			0.616
Negative	287 (68.2)	169 (71.3)		397 (69.8)	70 (67.3)	
Positive	134 (31.8)	68 (28.7)		172 (30.2)	34 (32.7)	
pTNM stage			0.893			0.027
I	149 (35.4)	83 (35.0)		197 (34.6)	43 (41.3)	
II	212 (50.4)	123 (51.9)		301 (52.9)	41 (39.4)	
III	60 (14.3)	31 (13.1)		71 (12.5)	20 (19.2)	
Radiation therapy			0.642			0.546
Negative	79 (18.8)	48 (20.3)		113 (19.9)	18 (17.3)	
Positive	342 (81.2)	189 (79.7)		456 (80.1)	86 (82.7)	
TILs			0.018			0.031
≤60%	275 (65.3)	176 (74.3)		400 (70.3)	62 (59.6)	
>60%	146 (34.7)	61 (25.7)		169 (29.7)	42 (40.4)	

TILs tumor-infiltrating lymphocytes

Table 3 Univariate and multivariate disease-free survival analyses of clinicopathological variables in triple-negative invasive carcinoma patient cohort

Variables	Univariate analyses			Multivariate analyses		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Age: ≥50 vs. <50 years	0.895	0.609–1.316	0.572			
Histologic grade: 3 vs. 1–2	0.821	0.540–1.248	0.356			
pT: 2–4 vs. 1	1.884	1.258–2.821	0.002			
Lymphovascular invasion: positive vs. negative	3.317	2.285–4.816	<0.001			
Lymph node metastasis: positive vs. negative	2.715	1.871–3.941	<0.001	2.979	2.032–4.366	<0.001
TIL: > 60 vs. ≤ 60%	0.978	0.966–0.990	<0.001	0.371	0.221–0.623	<0.001
Tumoral glutaminase expression: positive vs. negative	1.138	0.770–1.683	0.517			
TIL glutaminase expression: positive vs. negative	0.997	0.749–1.275	0.865			

CI confidence interval, *TILs* tumor-infiltrating lymphocytes

invasion, lymph node metastasis, and radiotherapy between glutaminase-negative and glutaminase-positive cases.

Prognostic significance of glutaminase expression in TNBC

In the survival analysis, a higher pT stage (*p* = 0.002), the presence of lymphovascular invasion, metastasis, and low TIL levels (all, *p* < 0.001) were significantly associated with poor disease-free survival (Table 3). Lymph node metastasis and low level of TILs (both, *p* < 0.001) were independent poor prognostic factors. Subgroup analysis was done according to lymph node metastasis and levels of TILs. The tumor cell glutaminase expression was significantly associated with poor disease-free survival in patients with lymph node metastasis and a high level of TILs (*p* = 0.020, Table 4, Fig. 2a). In multivariate analysis, glutaminase expression in tumor cells was an independent poor prognostic factor (hazard ratio = 10.643, 95% confidence interval = 1.999–56.668; *p* = 0.006) along with the presence of lymphovascular invasion (hazard ratio = 16.372, 95% confidence interval = 1.920–139.629; *p* = 0.011). In other subgroups, glutaminase

expression was not associated with disease-free survival (Tables 4 and 5, Fig. 2b, c, and d).

Discussion

Glutamine metabolism is important for cancer cell survival [5, 26]. Glutamine plays a role in cancer cell proliferation by participating in ATP synthesis and generating intermediates for the synthesis of macromolecules [5]. Using immunohistochemical staining with an antibody against glutaminase, we demonstrate that glutaminase is almost exclusively expressed in TNBCs compared with that in other types of breast cancer. Kim et al. also noted that tumor glutaminase expression is higher in TNBC than in other subtypes of breast cancer, and in HR-positive tumors, glutaminase tends to be negative [25], which was in accordance with our results. A previous study reported that in luminal-type breast cancer, glutamine is synthesized by glutamine synthetase, but not in basal-like type breast cancer [1]. Thus, basal-like breast cancer or TNBC show a high level of glutaminase expression and therefore appear more glutamine dependent than other subtypes of

Table 4 Univariate subgroup disease-free survival analyses of triple-negative invasive carcinoma patient with lymph node metastasis according to glutaminase expression

Variables	Low levels of TILs			High levels of TILs		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Age: ≤50 vs. >50 years	1.326	0.765–2.298	0.315	0.770	0.149–3.968	0.754
Histologic grade: 3 vs. 1–2	1.327	0.665–2.650	0.423	0.916	0.110–7.612	0.935
pT: 2–4 vs. 1	1.280	0.641–2.556	0.484	3.466	0.417–28.822	0.250
Lymphovascular invasion: positive vs. negative	1.878	1.039–3.393	0.037	10.838	1.304–90.094	0.027
Tumoral glutaminase expression: positive vs. negative	1.077	0.608–1.906	0.800	6.962	1.349–35.938	0.020
TIL glutaminase expression: positive vs. negative	1.024	0.702–1.495	0.902	0.842	0.292–2.427	0.750

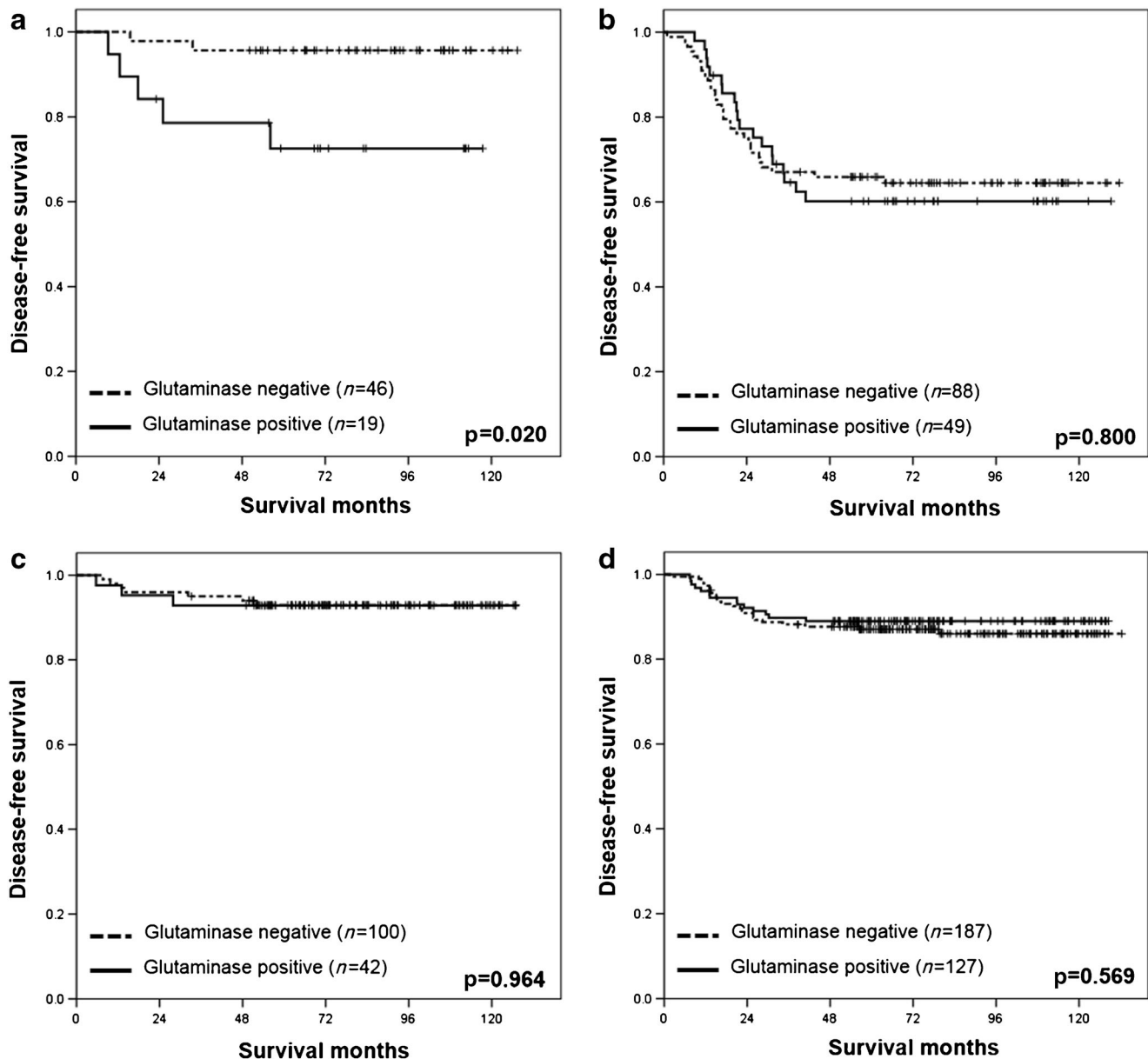*CI* confidence interval, *TILs* tumor-infiltrating lymphocytes

Fig. 2 Disease-free survival analysis of TNBC subgroups according to tumoral glutaminase expression. **a** In TNBC patients with lymph node metastasis and high levels of TILs, glutaminase expression is associated with poor disease-free survival ($p = 0.020$). **b–d** In TNBC patients not presenting with lymph node metastasis or high levels of TILs, glutaminase expression is not significantly associated with disease-free survival. (**b** TNBC patients with lymph node metastasis and low TIL levels, $p = 0.800$; **c** without lymph node metastasis and high levels of TILs, $p = 0.964$; **d** without lymph node metastasis and low levels of TILs, $p = 0.569$)

breast cancer [1]. TNBC might show a high rate of glutamine metabolism, because TNBC usually have features correlated with high metabolic activity, such as high histologic grade, increased mitosis, and tumor necrosis [25].

Glutaminase expression in tumor cells was significantly correlated with a low level of TILs, suggesting that active consumption of glutamine in tumor cells precludes effective utilization of glutamine by TILs and hinders proliferation of TILs in TNBCs with a high expression of glutaminase. We evaluated the prognostic significance of glutaminase expression in TNBCs and found that tumor glutaminase expression is significantly associated with poor disease-free survival in TNBCs with lymph node metastasis and a high level of TILs. Although a high level of TILs was associated with better clinical outcome in patients with TNBC, not all patients with a high level of TILs remained free of recurrence or alive [27]. Few prognostic indicators are associated with TNBCs with a high level of TILs. Our findings suggest that glutaminase expression in tumor cells might be a valuable prognostic marker of TNBCs presenting with a high level of TILs. Our findings are also consistent with the concept of metabolic competition in the tumor microenvironment, as a high level of glutaminase

Table 5 Univariate subgroup disease-free survival analyses of triple-negative invasive carcinoma patient without lymph node metastasis according to glutaminase expression

Variables	Low level of TILs			High level of TILs		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Age: ≤50 vs. >50 years	0.588	0.306–1.131	0.112	0.439	0.095–2.030	0.292
Histologic grade: 3 vs. 1–2	0.622	0.337–1.146	0.127	0.968	0.124–7.560	0.975
pT: 2–4 vs. 1	1.386	0.757–2.540	0.290	2.161	0.573–8.145	0.255
Lymphovascular invasion: positive vs. negative	1.772	0.848–3.705	0.128	4.996	1.323–18.858	0.018
Tumoral glutaminase expression: positive vs. negative	0.827	0.430–1.591	0.569	1.031	0.267–3.988	0.964
TIL glutaminase expression: positive vs. negative	1.003	0.393–2.560	0.995	0.957	0.203–4.508	0.956

CI confidence interval, *TILs* tumor-infiltrating lymphocytes

expression in tumor cells might result in glutamine deprivation in TILs, thereby hindering the protective role of T cells in cancer progression [18].

Poor prognosis of TNBC with lymph node metastasis and a high level of TILs might be associated with hypoxia-inducible factor 1 (HIF1) promoting invasion, growth, and metastasis of TNBC cells [28–30]. High glutaminase activity induces a high level of extracellular glutamate, which inhibits intracellular cysteine uptake via the xCT glutamate-cystine antiporter [28]. Depletion of intracellular cysteine directly inhibits degradation of HIF1α resulting in intracellular HIF1α accumulation [28].

In developing new modalities of cancer treatment, observations on cancer metabolism beyond the Warburg effect, such as discovery of targetable metabolomic pathway and identification of somatic mutations in metabolic enzymes, might be relevant [31–36]. However, it would be important to selectively target metabolism of cancer cells because TILs also need increased glycolysis and glutaminolysis for cell survival and function and compete with cancer cells for metabolite consumption [17, 18, 37].

In summary, glutaminase expression in tumor cells was almost exclusively present in TNBCs and significantly correlated with a low level of TILs. High glutaminase in TNBCs presenting with lymph node metastasis and high levels of TILs was significantly associated with poor disease-free survival.

Compliance with ethical standards This study was approved by the Institutional Review Board of Asan Medical Center and confirmed to the provisions of the Declaration of Helsinki.

Conflict of interest The authors declare that they have no conflict of interest.

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