Expression of glutamine metabolism-related proteins according to molecular subtype of breast cancer

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Abstract

The aim of this study was to investigate the expression of glutamine metabolism-related proteins to determine whether glutamine is metabolized differently according to breast cancer molecular subtype. We generated a tissue microarray of 702 breast cancer patients and performed immunohistochemical staining for glutamine metabolism-related proteins, including glutaminase 1 (GLS1 (GLS)), glutamate dehydrogenase (GDH (H6PD)), and amino acid transporter-2 (ASCT2 (SLC1A5)), which were separately evaluated in tumor and stroma compartments and then analyzed by breast cancer molecular subtypes. Breast cancers were classified as follows: 293 luminal A (41.7%), 166 luminal B (23.6%), 67 HER2 type (9.6%), and 176 TNBC (25.1%). HER2 type showed the highest stromal GLS1 ($P=0.001$), tumoral GDH ($P=0.001$), stromal GDH ($P<0.001$), and tumoral ASCT ($P<0.001$) expression. We identified differential expression of glutamine metabolism-related proteins according to molecular subtype of breast cancer. The highest glutamine metabolic activity was seen in HER2-type breast cancer.

Key Words
- breast cancer
- glutamine
- metabolism

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Introduction

Metabolism in malignant tumors is usually described by the Warburg effect, which is the observation that cancer cells produce energy by glycolysis rather than by oxidative phosphorylation (Warburg 1956). Although aerobic glycolysis is a major metabolic signature of cancer cells, it is not enough to describe cancer cell energy metabolism alone. Metabolic flexibility in cancer cells is a major obstacle in the therapeutic targeting of cancer cell metabolism. In addition to glucose metabolism, glutamine metabolism is an important metabolic pathway for cancer cell survival (DeBerardinis & Cheng 2010). Previous cell culture studies and tumor implantation studies have shown that cancer cell is highly dependent on glutamine, more so than any other amino acid (Collins *et al.* 1997, Friday *et al.* 2011). Therefore, glutamine metabolism has been proposed as an important metabolic phenotype of proliferating cancer cells. Indeed, glutamine plays an important role in cancer cell growth and survival by contributing to ATP synthesis, as well as by providing intermediates for macromolecular synthesis (DeBerardinis & Cheng 2010). The metabolic proteins involved in intracellular glutamine metabolism include amino acid transporter-2 (ASCT2 (SLC1A5); McGivan & Bungard 2007), a transporter-mediating influx of glutamine

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consumed by tumor cells; glutaminase 1 (GLS1 (GLS); Curthoys & Watford 1995), the enzyme involved in deamination of glutamine to glutamate; and glutamate dehydrogenase (GDH (H6PD); Dang 2010), the enzyme converting glutamate to α-ketoglutarate, which is incorporated into the tricarboxylic acid (TCA) cycle.

Breast cancer is a representative heterogeneous tumor with various clinical, histological, and molecular genetic signatures. Through studies performed to categorize breast cancers into subtypes with similar signatures, five molecular subtypes (luminal A, luminal B, HER-2, normal breast-like, and basal-like type) have been identified (Perou *et al*. 2000, Sorlie *et al*. 2001). In addition to its genetic signature, there are differences among the molecular subtypes in terms of histological finding, clinical behavior, therapeutic response, and prognosis. Therefore, metabolic status is also expected to differ according to molecular subtype. Previous studies support this expectation, having identified aerobic glycolysis-related proteins such as GLUT1 (SLC2A1) and CAIX (CA9), which are highly expressed in basal-like type and/or triple-negative breast cancer (TNBC; Hussein *et al*. 2011, Pinheiro *et al*. 2011). However, relatively few studies have considered differences in glutamine metabolism according to the molecular subtypes of breast cancer.

Thus, the aims of this study were to assess the expression of ASCT2, GLS1, and GDH as significant indicators of glutamine metabolism and to evaluate the difference in glutamine metabolism according to breast cancer molecular subtypes.

Materials and methods

Patient selection

A total of 702 patients who were diagnosed with invasive ductal carcinoma, not otherwise specified and underwent surgical excision at Severance Hospital between January 2002 and December 2005, were included in the study group. Patients who received preoperative hormonal therapy or neoadjuvant chemotherapy were excluded. This study was approved by the Institutional Review Board of Yonsei University Severance Hospital. Hematoxylin and eosin (H&E)-stained slides for each case were retrospectively reviewed by a breast pathologist (J S Koo). Histological grade was assessed using the Nottingham grading system (Elston & Ellis 1991). The clinicopathological parameters evaluated in each breast cancer included patient age at initial diagnosis, lymph node metastasis, tumor recurrence, distant metastasis, and patient survival.

Tissue microarray

On H&E-stained slides of tumors, a representative area was selected and a corresponding spot was marked on the surface of the paraffin block. Using a punch machine, the selected area was punched out and a 3 mm tissue core was placed into a 6×5 recipient block. The invasive tumor tissue was then extracted, and more than two tissue cores were extracted to minimize extraction bias. Each tissue core was assigned a unique tissue microarray location number linked to a database containing other clinicopathological data.

Immunohistochemistry

The antibodies used for immunohistochemistry in this study are shown in Table 1. All immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections. Briefly, 5 μm-thick sections were obtained with a microtome, transferred to adhesive slides, and dried at 62 °C for 30 min. After incubation with primary antibodies, immunodetection was performed with biotinylated anti-mouse immunoglobulin, followed

Table 1 Source, clone, and dilution of used antibodies.

Antibody	Clone	Dilution	Company	Specific reference
Molecular subtype related				
ER	SP1	1:100	Thermo Scientific, CA, USA	
PR	PgR	1:50	Dako, Glostrup, Denmark	
HER2	Polyclonal	1:1500	Dako	
Ki-67	MIB-1	1:150	Dako	
Glutamine metabolism related				
GLS1	Polyclonal (ab93434)	1:50	Abcam, Cambridge, UK	Colombo *et al*. (2011)
GDH	Polyclonal (ab153973)	1:100	Abcam	
ASCT2	Polyclonal (ab78371)	1:100	Abcam	

ER, estrogen receptor; PR, progesterone receptor, GLS1, glutaminase 1, GDH, glutamate dehydrogenase, ASCTS, amino acid transporter-2.

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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. Immunostaining for positive control tissues (GLS1 and GDH, liver tissue; ASCT2, testis tissue) was performed to confirm the specificity of the antibodies.

Interpretation of immunohistochemical staining

All immunohistochemical markers were accessed by light microscopy. A cutoff value of 1% or more positively stained nuclei was used to define estrogen receptor (ER) and progesterone receptor (PR) positivity (Hammond *et al.* 2010). HER2 (ERBB2) 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, <10% of tumor cells; 2+, complete membranous staining, either uniform or weak in at least 10% of tumor cells; and 3+, uniform intense membranous staining in at least 30% of tumor cells (Wolff *et al.* 2007). HER2 immunostaining was considered positive when strong (3+) membranous staining was observed, whereas cases with 0 to 1+ were considered negative. Cases with 2+ HER2 immunoexpression were evaluated for HER2 amplification by fluorescent *in situ* hybridization (FISH). Immunohistochemical stain results for Ki-67 were scored by counting the number of positively stained nuclei and expressed as a percentage of total tumor cells (Ki-67 labeling index (LI)). Immunohistochemical staining results for ASCT2, GLS1, and GDH were evaluated separately for the tumor and stroma compartments, which were scored based on the intensity of expression (0, negative; 1, weak; 2, moderate; and 3, strong). Moderate or strong staining was considered positive.

FISH analysis

Before conducting FISH analysis, invasive tumors were examined on H&E-stained slides. FISH was subsequently performed on the tested tumor tissue using a PathVysion *HER2* DNA Probe Kit (Vysis, Downers Grove, IL, USA) according to the manufacturer's instructions. *HER2* gene copy number was evaluated using an epifluorescence microscope (Olympus, Tokyo, Japan). At least 60 tumor cell nuclei in three separate regions were investigated for *HER2* and chromosome 17 signals. *HER2* gene amplification was determined according to the ASCO/CAP guidelines (Wolff *et al.* 2007). An absolute *HER2* gene copy number lower than 4 or a *HER2* gene/chromosome 17 copy number ratio (HER2:Chr17 ratio) <1.8 was considered *HER2* negative. An absolute *HER2* copy number between 4 and 6 or a HER2:Chr17 ratio between 1.8 and 2.2 was considered *HER2* equivocal. An absolute *HER2* copy number >6 or a HER2:Chr17 ratio higher than 2.2 was considered *HER2* positive.

Tumor phenotype classification

Breast cancer molecular subtypes were classified according to the immunohistochemistry results for ER, PR, HER2, and Ki-67 and the FISH results for HER2 as follows (Goldhirsch *et al.* 2011): luminal A type: ER or/and PR positive and HER2 negative and Ki-67 LI <14%; luminal B type: (HER2 negative) ER or/and PR positive and HER2 negative and Ki-67 LI ≥14% (HER2 positive), ER or/and PR positive and HER2 overexpressed or/and amplified; HER2 type: ER and PR negative and HER2 overexpressed or/and amplified; and TNBC type: ER, PR, and HER2 negative.

Statistical analyses

Data were processed using SPSS for Windows, version 12.0 (SPSS, Inc.). Student's *t* and Fisher's exact tests were used to examine any differences in continuous and categorical variables respectively. In the case of analyzing data with multiple comparisons, a corrected *P* value with the application of the Bonferroni's multiple comparison procedure was used. Significance was assumed when $P<0.05$. Kaplan–Meier survival curves and log-rank statistics were employed to evaluate the time to tumor metastasis and survival time. Multivariate regression analysis was performed using Cox proportional hazards model.

Results

Patient clinicopathological characteristics

Table 2 shows the clinicopathological characteristic of the study group. The 702 breast cancers were classified into subtypes, including 293 luminal A (41.7%), 166 luminal B (23.6%), 67 HER2 type (9.6%), and 176 TNBC (25.1%).

Expression of glutamine metabolism-related proteins according to breast cancer phenotype

The expressions of glutamine metabolism-related proteins according to breast cancer molecular phenotype are

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

Parameters	Total (*n*=702 (%))	Luminal A (*n*=293 (%))	Luminal B (*n*=166 (%))	HER2 type (*n*=67 (%))	TNBC (*n*=176 (%))	*P* value
Age (years, mean±S.D.)	49.8±11.0	50.7±10.5	48.5±10.1	52.8±9.9	48.3±12.4	0.006
Histological grade						<0.001
I	114 (16.2)	88 (30.0)	18 (10.8)	1 (1.5)	7 (4.0)	
II	354 (50.4)	177 (60.4)	90 (54.2)	34 (50.7)	53 (30.1)	
III	234 (33.3)	28 (9.6)	58 (34.9)	32 (47.8)	116 (65.9)	
Tumor stage						0.008
T1	342 (48.7)	162 (55.3)	86 (51.8)	29 (43.3)	65 (36.9)	
T2	346 (49.3)	124 (42.3)	78 (47.0)	37 (55.2)	107 (60.8)	
T3	14 (2.0)	7 (2.4)	2 (1.2)	1 (1.5)	4 (2.3)	
N stage						0.051
N0	414 (59.0)	166 (56.7)	91 (54.8)	41 (61.2)	116 (65.9)	
N1	190 (27.1)	88 (30.0)	43 (25.9)	13 (19.4)	46 (26.1)	
N2	62 (8.8)	26 (8.9)	17 (10.2)	9 (13.4)	10 (5.7)	
N3	36 (5.1)	13 (4.4)	15 (9.0)	4 (6.0)	4 (2.3)	
Ki-67 LI (%, mean±S.D.)	17.4±18.5	4.7±3.7	19.7±12.7	19.6±12.7	35.4±23.0	<0.001
Tumor recurrence	61 (8.7)	15 (5.1)	12 (7.2)	10 (14.9)	24 (13.6)	0.003
Patient death	58 (8.3)	13 (4.4)	11 (6.6)	11 (16.4)	23 (13.1)	0.001
Duration of clinical follow-up (months, mean±S.D.)	69.9±31.4	72.1±29.7	70.3±30.3	65.7±34.6	67.7±33.7	0.285

TNBC, triple-negative breast cancer.

summarized in Table 3, Figs 1 and 2. Stromal GLS1 (P=0.001), stromal GDH (P<0.001), and tumoral ASCT2 expressions (P<0.001) were the highest in HER2 type and lowest in luminal A type. Tumoral GDH expression was the highest in HER2 type and the lowest in TNBC (P=0.001).

Correlation between the expression of glutamine-related proteins and clinicopathological factors

Correlation of the clinicopathological factors (age (≤35 vs >35), histological grade (I/II vs III), T stage (T1 vs T2/3),

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

		Tumor phenotype				
Antibodies	Total (*n*=702 (%))	Luminal A (*n*=293 (%))	Luminal B (*n*=166 (%))	HER2 type (*n*=67 (%))	TNBC (*n*=176 (%))	*P* value
Tumoral GLS1						0.171
Negative	208 (29.6)	83 (28.3)	60 (36.1)	20 (29.9)	45 (25.6)	
Positive	494 (70.4)	210 (71.7)	106 (63.9)	47 (70.1)	131 (74.4)	
Stromal GLS1						**0.001**
Negative	466 (66.4)	218 (74.4)	105 (63.3)	38 (56.7)	105 (59.7)	
Positive	236 (33.6)	75 (25.6)	61 (36.7)	29 (43.3)	71 (40.3)	
Tumoral GDH						**0.001**
Negative	113 (16.1)	44 (15.0)	25 (15.1)	2 (3.0)	42 (23.9)	
Positive	589 (83.9)	249 (85.0)	141 (84.9)	65 (97.0)	134 (76.1)	
Stromal GDH						**<0.001**
Negative	325 (46.3)	177 (60.4)	59 (35.5)	20 (29.9)	69 (39.2)	
Positive	377 (53.7)	116 (39.6)	107 (64.5)	47 (70.1)	107 (60.8)	
Tumoral ASCT2						**<0.001**
Negative	539 (76.8)	246 (84.0)	127 (76.5)	42 (62.7)	124 (70.5)	
Positive	163 (23.2)	47 (16.0)	39 (23.5)	25 (37.3)	52 (29.5)	
Stromal ASCT2						0.079
Negative	606 (86.3)	260 (88.7)	147 (88.6)	53 (79.1)	146 (83.0)	
Positive	96 (13.7)	33 (11.3)	19 (11.4)	14 (20.9)	30 (17.0)	

TNBC, triple-negative breast cancer. *P* values <0.05 are shown in bold.

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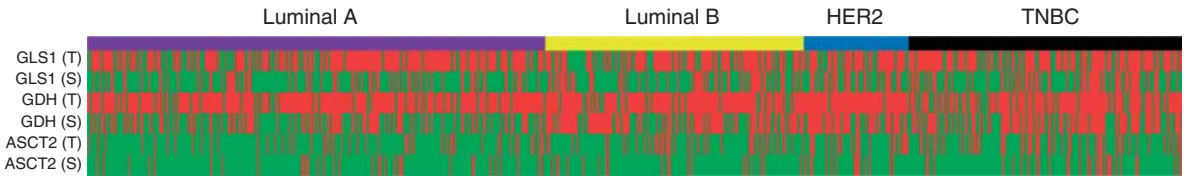

Figure 1
Heat map of immunohistochemical results for glutamine metabolism-related proteins. Red, positive; green, negative.

and N stage (N0 vs N1–3)) and expression of glutamine metabolism-related proteins were analyzed. Stromal GDH expression was correlated with high histological grade ($P=0.012$), and tumoral GDH expression was correlated with lymph node metastasis ($P=0.036$). ER, PR, and HER2 status was significantly different according to the expression of glutamine metabolism-related proteins shown in Fig. 3. ER negativity was associated with stromal GLS1 positivity ($P=0.024$), stromal GDH positivity ($P=0.006$), and tumoral ASCT2 positivity ($P<0.001$). PR negativity was associated with stromal GDH positivity ($P=0.012$) and tumoral ASCT2 positivity ($P=0.012$). HER2 positivity was associated with tumoral GDH positivity ($P=0.006$), stromal GDH positivity ($P<0.001$), and tumoral ASCT2 positivity ($P=0.042$).

Further analysis confirmed several positive correlations among the glutamine metabolism-related proteins (shown in Table 4): tumoral GLS1–stromal GLS1 ($r=0.191$, $P<0.001$), tumoral GLS1–tumoral GDH ($r=0.302$, $P<0.001$), tumoral GLS1–tumoral ASCT2 ($r=0.091$, $P=0.016$), tumoral

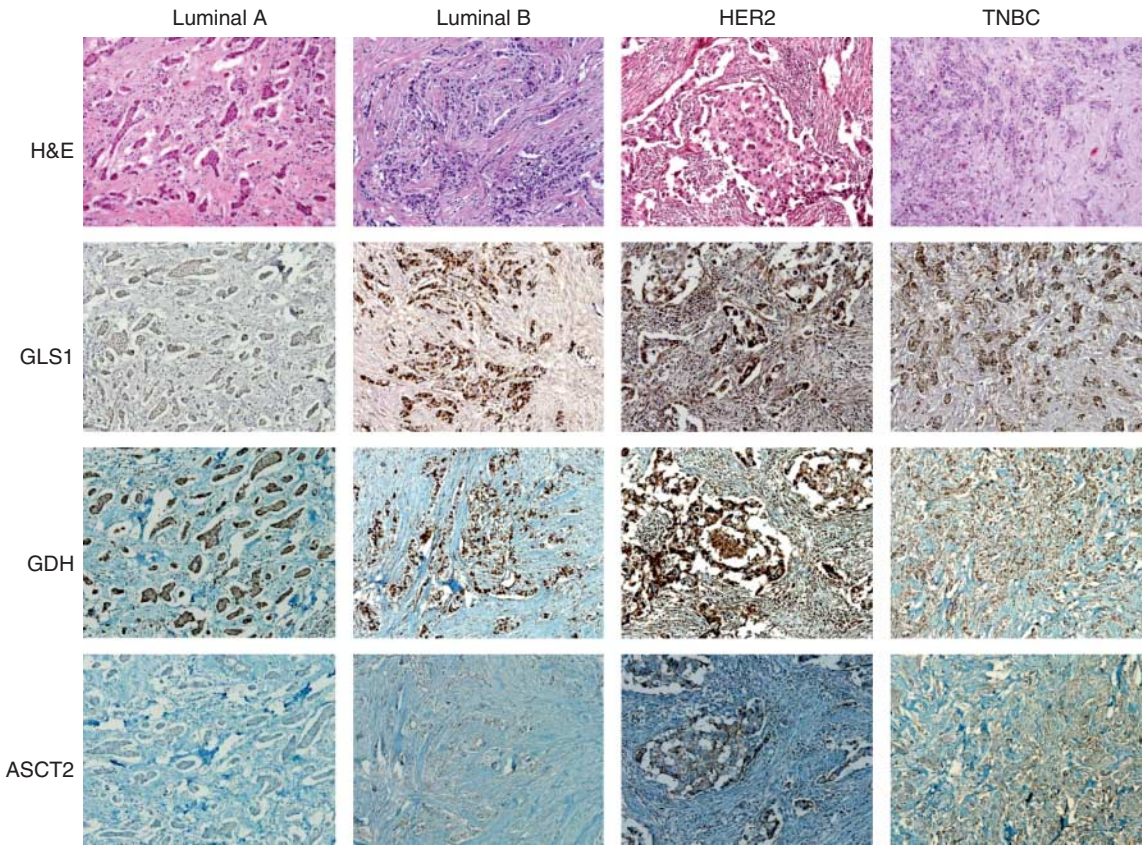

Figure 2
Expression of glutamine metabolism-related proteins according to the molecular subtypes of breast cancer. HER2-type tumors exhibited the highest immunoexpression of stromal GLS1, tumoral GDH, tumoral ASCT2, and stromal ASCT2. Triple-negative breast cancer (TNBC) exhibited the lowest tumoral GDH expression of all molecular subtypes.

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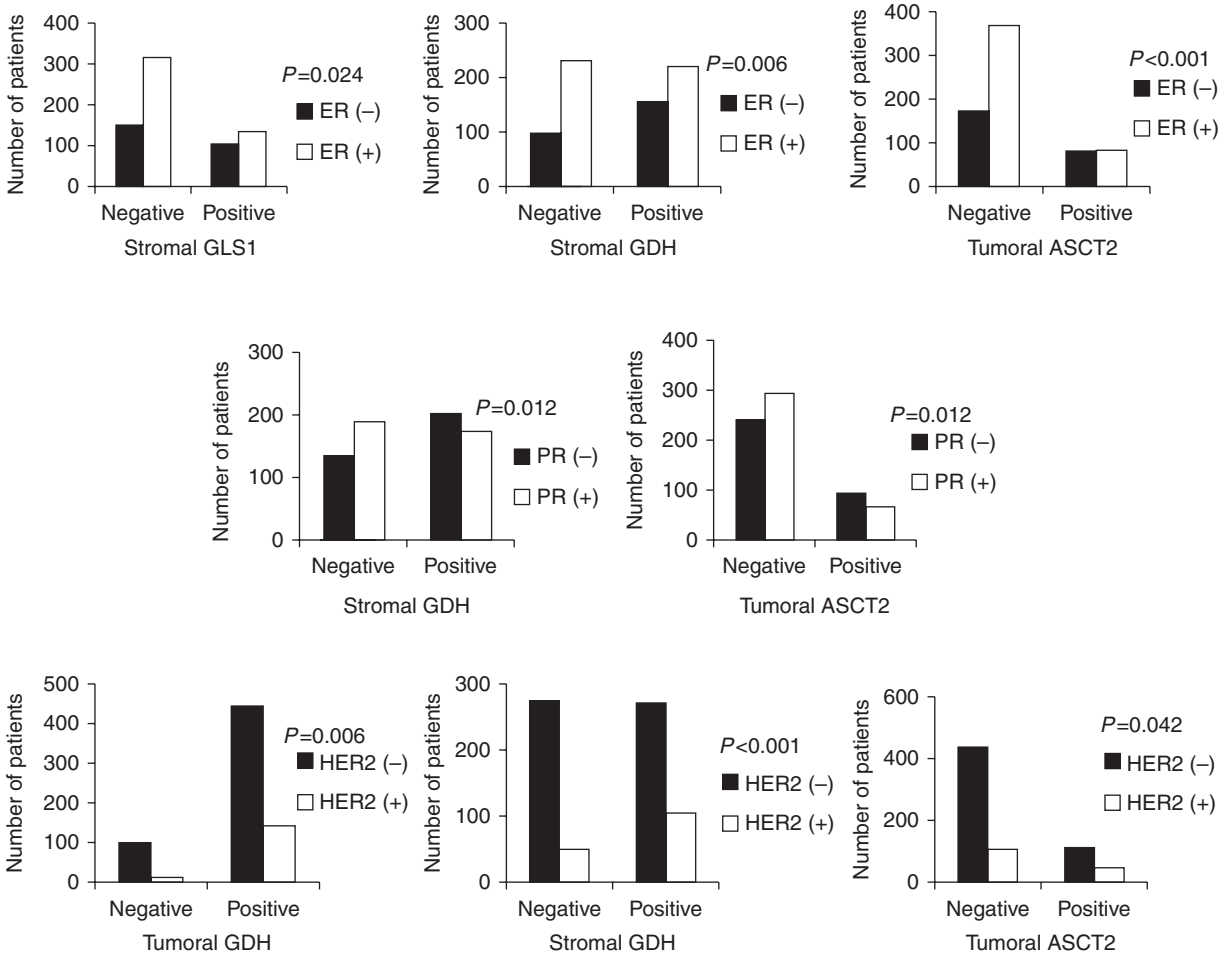

Figure 3
Correlation between the expression of glutamine metabolism-related proteins with ER, PR, and HER2 status. *P* values are corrected for multiple testing using the Bonferroni's correction method.

GLS1–stromal ASCT2 ($r=0.086$, $P=0.023$), stromal GLS1–tumoral GDH ($r=0.131$, $P<0.001$), stromal GLS1–stromal GDH ($r=0.467$, $P<0.001$), stromal GLS1–stromal ASCT2 ($r=0.191$, $P<0.001$), tumoral GDH–stromal GDH ($r=0.106$, $P=0.005$), tumoral GDH–tumoral ASCT2 ($r=0.140$, $P<0.001$), tumoral GDH–stromal ASCT2 ($r=0.118$, $P=0.002$), stromal GDH–tumoral ASCT2 ($r=0.091$, $P=0.016$), stromal GDH–stromal ASCT2 ($r=0.195$, $P<0.001$), and tumoral ASCT2–stromal ASCT2 ($r=0.302$, $P<0.001$).

Impact of the expressions of glutamine-related proteins on prognosis

Univariate analysis failed to reveal a relationship between glutamine-related protein expression and prognostic factors (Table 5). Multivariate Cox analysis (Table 6) demonstrated that younger age (≤35 years, hazard ratio: 2.425, 95% CI: 1.240–4.740, $P=0.010$), high T stage (hazard ratio: 2.322, 95% CI: 1.258–4.287, $P=0.007$), and lymph node metastasis (hazard ratio: 2.457, 95% CI: 1.439–4.195, $P=0.001$) were independent prognostic factors for shorter disease-free survival (DFS). Lymph node metastasis (hazard ratio: 1.907, 95% CI: 1.111–3.271, $P=0.019$) and stromal GDH negativity (hazard ratio: 2.024, 95% CI: 1.176–3.485, $P=0.011$) were independent prognostic factors for shorter overall survivial (OS).

Discussion

In this study, we examined the expression of glutamine metabolism-related proteins according to breast cancer molecular subtype. Although little is known about the differences in glutamine metabolism among the different molecular subtypes of breast cancer, a previous study

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Table 4 Correlation of the expression of glutamine metabolism-related molecules in breast cancer.

Parameters	Tumoral GLS1	Stromal GLS1	Tumoral GDH	Stromal GDH	Tumoral ASCT2	Stromal ASCT2
Tumoral GLS1						
Correlation coefficient		0.191	0.302	0.048	0.091	0.086
P value		<0.001	<0.001	0.202	0.016	0.023
Stromal GLS1						
Correlation coefficient	0.191		0.131	0.467	0.059	0.191
P value	<0.001		<0.001	<0.001	0.121	<0.001
Tumoral GDH						
Correlation coefficient	0.302	0.131		0.106	0.140	0.118
P value	<0.001	<0.001		0.005	<0.001	0.002
Stromal GDH						
Correlation coefficient	0.048	0.467	0.106		0.091	0.195
P value	0.202	<0.001	0.005		0.016	<0.001
Tumoral ASCT2						
Correlation coefficient	0.091	0.059	0.140	0.091		0.302
P value	0.016	0.121	<0.001	0.016		<0.001
Stromal ASCT2						
Correlation coefficient	0.086	0.191	0.118	0.195	0.302	
P value	0.023	<0.001	0.002	<0.001	<0.001	

showed that basal-like breast cancer has a higher level of GLS1 expression and is more glutamine dependent than luminal type because glutamine is sufficiently synthesized by glutamine synthetase in luminal type but not in basal-like type (Kung *et al*. 2011). In this study, even though it was not statistically significant, tumoral GLS1 expression was higher in TNBC than in luminal type or HER2 type, and GLS1 negativity in tumor was correlated with ER and PR positivity. Tumoral ASCT2 expression was correlated with ER and PR negativity ($P<0.001$ and $P=0.002$ respectively), which reflects the high level of glutamine uptake in basal-like type, which was in accordance with the results of the previous study (Kung *et al*. 2011).

TNBC is histologically characterized by high histological grade, poor differentiation, increased mitosis, and tumor necrosis, features that are shared among tumors with high metabolic activity (Reis-Filho & Tutt 2008, Rakha & Ellis 2009, Foulkes *et al*. 2010, Venkitaraman 2010), and thus, TNBC is assumed to exhibit the highest level of glutamine metabolic activity. However, in this

Table 5 Univariate analysis of glutamine-related proteins in breast cancer, time to disease-free survival, and overall survival according to log-rank testing.

Parameters	No. of patients ($n=702$)		Disease-free survival		Overall survival	
	Tumor recurrence ($n=61$ (%))	Patient death ($n=58$ (%))	Mean survival (95% CI) months	*P* value	Mean survival (95% CI) months	*P* value
Tumoral GLS1				0.404		0.288
Negative	21 (34.4)	21 (36.2)	124 (118–129)		127 (122–132)	
Positive	40 (65.6)	37 (63.8)	127 (122–131)		130 (127–133)	
Stromal GLS1				0.607		0.701
Negative	43 (70.5)	41 (70.7)	124 (119–128)		129 (126–132)	
Positive	18 (29.5)	17 (29.3)	128 (122–133)		129 (125–134)	
Tumoral GDH				0.273		0.832
Negative	7 (11.5)	9 (15.5)	129 (123–135)		130 (124–136)	
Positive	54 (88.5)	49 (84.5)	125 (121–129)		129 (126–132)	
Stromal GDH				0.382		0.097
Negative	32 (52.5)	34 (58.6)	122 (115–128)		127 (123–131)	
Positive	29 (47.5)	24 (41.4)	128 (125–132)		131 (128–134)	
Tumoral ASCT2				0.466		0.060
Negative	45 (73.8)	39 (67.2)	125 (121–129)		131 (128–134)	
Positive	16 (26.2)	19 (32.8)	125 (119–132)		125 (119–131)	
Stromal ASCT2				0.892		0.761
Negative	53 (86.9)	51 (87.9)	125 (121–130)		129 (126–132)	
Positive	8 (13.1)	7 (12.1)	121 (114–127)		127 (121–133)	

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Table 6 Multivariate analysis of prognosis in breast cancer.

	Disease-free survival			Overall survival		
Parameters	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
Age (years)			**0.010**			0.154
≤35 vs >35	2.425	1.240–4.740		1.716	0.817–3.606	
Histological grade			0.551			0.829
I/II vs III	1.191	0.670–2.119		0.829	0.459–1.495	
T stage			**0.007**			0.072
T1 vs T2–4	2.322	1.258–4.287		1.704	0.953–3.047	
N stage			**0.001**			**0.019**
N0 vs N1–3	2.457	1.439–4.195		1.907	1.111–3.271	
ER status			0.640			0.521
Negative vs positive	1.629	0.211–12.582		1.965	0.249–15.481	
PR status			0.522			0.129
Negative vs positive	1.321	0.564–3.092		1.956	0.829–4.616	
HER2 status			0.564			0.589
Negative vs positive	1.406	0.443–4.462		0.719	0.217–2.378	
Tumor phenotypes			0.986			0.657
Luminal A						
Luminal B	1.185	0.416–3.380		1.857	0.683–5.054	
HER2	1.191	0.090–15.813		2.082	0.155–27.995	
TNBC	1.194	0.121–11.766		1.122	0.113–11.142	
Stromal GDH			0.131			**0.011**
Negative vs positive	1.496	0.887–2.521		2.024	1.176–3.485	
Tumoral ASCT2			0.898			0.127
Negative vs positive	1.039	0.576–1.875		1.560	0.881–2.761	

TNBC, triple-negative breast cancer. *P* values <0.05 are shown in bold.

study, we found that HER2-type breast cancer exhibited the most frequent expression of glutamine metabolism-related proteins both in tumor and in stroma, with the exception of tumoral GLS1 expression and stromal ASCT2 expression. In addition, luminal B type showed higher glutamine metabolic activity than luminal A type, and HER2 positivity was correlated with tumoral GDH, stromal GDH, and tumoral ASCT2 positivity ($P=0.001$, $P<0.001$, and $P=0.007$ respectively). As for the impact of HER2 amplification/overexpression on the higher glutamine metabolism, we considered the role of *c-myc*. It is well known that c-myc regulates glutamine metabolism by regulating the expression of glutamine transporter (ASCT2) and glutaminase (GLS1) (Wise *et al*. 2008, Gao *et al*. 2009). In addition, c-myc amplification was more common in breast cancers with HER2 amplification than in breast cancers without HER2 amplification (Park *et al*. 2005). Taken together, we postulated that higher glutamine metabolism of molecular subtypes with HER2 amplification could be described by an association with c-myc amplification.

Based on the positive correlations between tumoral GLS1–stromal GLS1 ($r=0.191$, $P<0.001$), tumoral GDH–stromal GDH ($r=0.106$, $P=0.005$), and tumoral ASCT2–stromal ASCT2 expression ($r=0.302$, $P<0.001$), we identified a relationship between tumoral and stromal glutamine metabolism. Previous studies have reported that the byproducts of glutaminolysis in cancer cells, such as ammonia, diffuse into the stroma and stimulate autophagy and glutamine synthesis in cancer-associated fibroblasts, which subsequently feeds cancer cells in a vicious cycle (Eng & Abraham 2010, Marino & Kroemer 2010, Pavlides *et al*. 2010, Ko *et al*. 2011, Martinez-Outschoorn *et al*. 2011). One previous study utilized co-culturing of MCF-7 breast cancer cells (luminal A type) with fibroblasts and found increased expression of GLS, GDH, and SLC6A14 (glutamine importer) and reduced glutamine neosynthesis when compared with breast cancer single-cell culture without fibroblast, thus demonstrating stroma–tumor glutamine transportation (Ko *et al*. 2011). Moreover, glutamine uptake as well as glutaminase expression were mainly observed in cancer cells, while glutamine synthetase expression was restricted to stromal cells (Ko *et al*. 2011). Even though glutamine synthetase was not examined in this study, expression of each of the three glutamine metabolism-related proteins (GLS1, GDH, and ASCT2) exhibited more common and stronger immunoexpression in cancer cells than in stromal cells,

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which was in accordance with the results of previous studies. Because neither autophagy-related molecules nor glutamine synthetase was investigated in this study, we could not demonstrate whether stromal cells provided the cancer cells with glutamine. However, we did confirm that higher glutamine metabolic activity exhibited by tumors was associated with increased glutamine metabolic activity in the stroma.

In this study, the frequency of stromal GLS1, stromal GDH, tumoral ASCT2, and stromal ASCT2 positivity was HER2 type > TNBC > luminal A type, even though stromal ASCT2 was not statistically significant. However, TNBC exhibited the lowest tumoral GDH expression rate of all molecular subtypes. A previous cell line study of glioblastoma reported that GLS metabolized about $58 \pm 1\%$ of the total glutamine consumed by cells, while GDH only metabolized $\sim 10\%$ of the GLS flux, with the contributions of other glutamate-consuming enzymes such as alanine aminotransferase or aspartate aminotransferase (Yang *et al*. 2009). The relative activity of GDH and other glutamate-consuming enzymes is determined by intracellular glucose metabolic activity. Briefly, GDH activity is stimulated under glucose deprivation conditions, while its activity is repressed during robust glycolysis. Likewise, as GDH activity is influenced by various factors, it is presumed that GDH activity is not directly proportional to glutamine uptake by ASCT2 or GLS activity. Furthermore, basal-like type and/or TNBC has been shown to exhibit the highest glycolysis-related protein activity, including Glut1 (SLC2A1) and CAIX (CA9) (Hussein *et al*. 2011, Pinheiro *et al*. 2011). Taken together, the low level of GDH activity observed in TNBC in this study can be explained by its high level of glycolytic activity.

This study investigated the expression of the glutamine metabolism-related proteins using immunohistochemistry; therefore, the specificity of each antibody is very important for the reliability of the study results. During immunohistochemical staining procedures, we first immunostained positive control tissues (GLS1 and GDH, liver tissue; ASCT2, testis tissue) that were recommended by the antibody manufacturers. After verifying the staining quality, immunostaining of the experimental tissues was performed with positive control tissues.

It is ideal to perform immunohistochemical staining with a negative control. Nevertheless, most of the immunohistochemistry antibodies are presented without negative control. However, this study performed immunohistochemical staining on the tissue microarrary containing 29 tissue cores in one slide; thus, expressional differences are distinctly recognized among the cores on same slide; some cores showed distinct positivity. However, the other cores have distinct negativity under the same condition for staining. In addition, significant zonal variation of immunoexpression was observed within one core. For example, tumor cells are positive, while surrounding stromal cells are negative. Namely, immunohistochemistry on tissue microarray has advantages similar to staining with internal positive and negative controls.

In this study, we investigated the expression of glutamine metabolism-related proteins according to breast cancer molecular subtypes. In conclusion, HER2-type breast cancer had the highest expression of stromal GLS1, tumoral GDH, stromal GDH, and tumoral ASCT, while TNBC had the lowest tumoral GDH expression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S Kim participated in the design of the study and performed the statistical analysis and drafted the manuscript. D H Kim carried out the immunoassays. W-H Jung participated in its design. J S Koo conceived the study and participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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