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Expression of serine and glycine-related enzymes in phyllodes tumor

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Expression patterns of proteins involved in serine and glycine metabolism, and correlations of these patterns with clinicopathologic factors in phyllodes tumor were investigated. Tissue microarrays were prepared from 203 phyllodes tumors (PT) and stained with antibodies specific for glycine decarboxylase (GLDC), phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH), phosphoglycerate dehydrogenase (PHGDH), and serine hydroxymethyltransferase 1 (SHMT1). These immunohistochemical results and clinicopathologic parameters were analyzed for correlation. Numbers of benign, borderline, and malignant tumors were 155, 32, and 16, respectively. Stromal expression of PHGDH, PSAT1, PSPH, SHMT1, and GLDC increased with increasing tumor grade, and epithelial expression of SHMT1 also increased with increasing tumor grade ($p<0.001$, and $p=0.005$, respectively). On univariate analysis, positive stainings for stromal PHGDH ($p<0.001$), stromal PSAT1 ($p<0.001$), stromal PSPH ($p=0.003$), epithelial SHMT1 ($p=0.001$), stromal SHMT1 ($p=0.022$), and stromal GLDC ($p<0.001$) were each associated with shorter disease-free survival. Stromal GLDC was associated with shorter overall survival ($p<0.001$). In conclusion, expression of proteins related to serine and glycine metabolism increased with increasing histologic grade in stromal components of phyllodes tumor.

Key words: glycine, tumor grade, metabolism, phyllodes tumor, serine

Unlike normal cells, which oxidize glucose through the tricarboxylic acid (TCA) cycle and mitochondrial electron transport, cancer cells may shift into glycolysis despite presence of adequate oxygen. This shift, known as the 'Warburg effect' [1], results in accumulation of glycolytic intermediates and patterns of gene activation that may promote tumor growth. The interconnections of pathways for serine and glycine biosynthesis reveal various levels at which a shift to anerobic metabolism could support cell proliferation [2-5]. In serine biosynthesis, 3-phosphoglycerate (3PG) produced in glycolysis is oxidized by phosphoglycerate dehydrogenase (PHGDH) to 3-phosphohydroxypyruvate (pPYR), which in turn is transaminated by phosphoserine aminotransferase (PSAT) into phosphoserine (pSER), which is dephosphorylated by phosphoserine phosphatase (PSPH) into serine. In glycine metabolism, glycine decarboxylase (GLDC) catalyzes the catabolism of the glycine into methylene-tetrahydrofolate (MTF). The interconversion of serine and glycine by serine hydroxymethyltransferase (SHMT) represents a crossover point from which single carbon units flow from amino acid turnover into the synthesis of purines and pyrimidines (by way of 5, 10-methylenetetrahydrofolate and 5-methyltetrahydrofolate) and into the essential amino acid methionine and the universal methyl-donor S-adenosylmethionine (SAM), by way of 5-MTHF. Increased availability of these substrates for growth may in part explain the advantage to the tumor in the shift to anaerobic metabolism. Recent studies show elevated PHGDH expression in breast cancer and melanoma [3, 4], and GLDC expression in lung cancer, suggesting that changes in serine and glycine metabolism may influence tumorigenesis [5].

Although phyllodes tumors (PTs) contribute only 0.3-1.5% of all breast neoplasms, they may be difficult to distinguish from fibroadenomas, which belong to the same class of fibroepithelial tumors. Both tumors present heterogeneous histologic features, however, some histologic features of the two tumors overlap [6, 7]. In addition, some PTs exhibit malignant behaviors such as occasional recurrence and hematogenous metastasis [8]. Although authors disagree on the histologic classification of PTs, the WHO criteria distinguish PTs as benign, borderline, or malignant tumors [7]. With increasing grade, the frequency of recurrence and/or probability of distant metastasis of PTs also increase. We showed previ-

Table 1. List of enzymes and antibodies

Antibody Target	Source	Clone	Dilution
Serine/glycine-related protein			
Phosphoglycerate dehydrogenase (PHGDH)	Abcam, Cambridge, UK	Polyclonal	1:100
Phosphoserine aminotransferase-1 (PSAT1)	Abcam, Cambridge, UK	Polyclonal	1:100
Phosphoserine phosphatase (PSPH)	Abcam, Cambridge, UK	Polyclonal	1:100
Serine hydroxymethyltransferase (SHMT)	Abcam, Cambridge, UK	Polyclonal	1:100
Glycine decarboxylase (GLDC)	Abcam, Cambridge, UK	Polyclonal	1:100
Proliferation related marker			
Ki-67	Dako Denmark AS, Glostrup, Denmark	MIB-1	1:150

Table 2. Clincopathologic characteristics of patients with phyllodes tumor

Parameters	Total Patients N = 203 (100%)	PT, Benign N = 155 (100%)	PT, Borderline N = 32 (100%)	PT, Malignant N = 16 (100%)	*P*-value
Age (years, mean±SD)	40.2±12.3	38.9±12.2	43.1±11.0	47.6±12.9	0.010
Tumor size (cm, mean±SD)	4.0±2.6	3.6±2.1	4.2±2.5	6.7±4.6	<0.001
Stromal cellularity					<0.001
Mild	122 (60.1)	121 (78.1)	1 (3.1)	0 (0.0)	
Moderate	68 (33.5)	34 (21.9)	27 (84.4)	7 (43.8)	
Marked	13 (6.4)	0 (0.0)	4 (12.5)	9 (56.3)	
Stromal atypia					<0.001
Mild	160 (78.8)	153 (98.7)	7 (21.9)	0 (0.0)	
Moderate	33 (16.3)	2 (1.3)	23 (71.9)	8 (50.0)	
Marked	10 (4.9)	0 (0.0)	2 (6.3)	8 (50.0)	
Stromal mitosis					<0.001
0–4 / 10 HPFs	172 (84.7)	155 (100.0)	17 (53.1)	0 (0.0)	
5–9 / 10 HPFs	20 (9.9)	0 (0.0)	15 (46.9)	5 (31.3)	
≥ 10 / 10 HPFs	11 (5.4)	0 (0.0)	0 (0.0)	11 (68.8)	
Stromal overgrowth					<0.001
Absent	186 (91.6)	155 (100.0)	29 (90.6)	2 (12.5)	
Present	17 (8.4)	0 (0.0)	3 (9.4)	14 (87.5)	
Tumor margin					<0.001
Circumscribed	182 (89.7)	152 (98.1)	24 (75.0)	6 (37.5)	
Infiltrative	21 (10.3)	3 (1.9)	8 (25.0)	10 (62.5)	
Surgical procedure					<0.001
Local excision	150 (73.9)	133 (85.8)	16 (50.0)	1 (6.3)	
Wide excision	39 (19.2)	15 (9.7)	15 (46.9)	9 (56.3)	
Mastectomy	14 (6.9)	7 (4.5)	1 (3.1)	6 (37.5)	
Radiation therapy					0.873
No	177 (87.2)	136 (87.7)	27 (84.4)	14 (87.5)	
Yes	26 (12.8)	19 (12.3)	5 (15.6)	2 (12.5)	
Tumor recurrence	18 (8.9)	5 (3.2)	6 (18.8)	7 (43.8)	<0.001
Distant metastasis	8 (3.9)	0 (0.0)	1 (3.1)	7 (43.8)	<0.001

PT, Phyllodes Tumor; HPFs, high-power fields

ously that expression of the glycolysis-related proteins such as Glut-1, CAIX, and MCT4 in PTs increases with increasing tumor grade [9]. We conducted the present study to investigate the expression of proteins related to serine and glycine metabolism, and to correlate these expressions with clinical and pathologic features of PT.

Patients and methods

Patient selection. Tissue samples were obtained from phyllodes tumors (PTs) removed during surgeries performed in the years 2000 to 2010, and prepared and archived at the Department of Pathology at Severance Hospital. All tis-

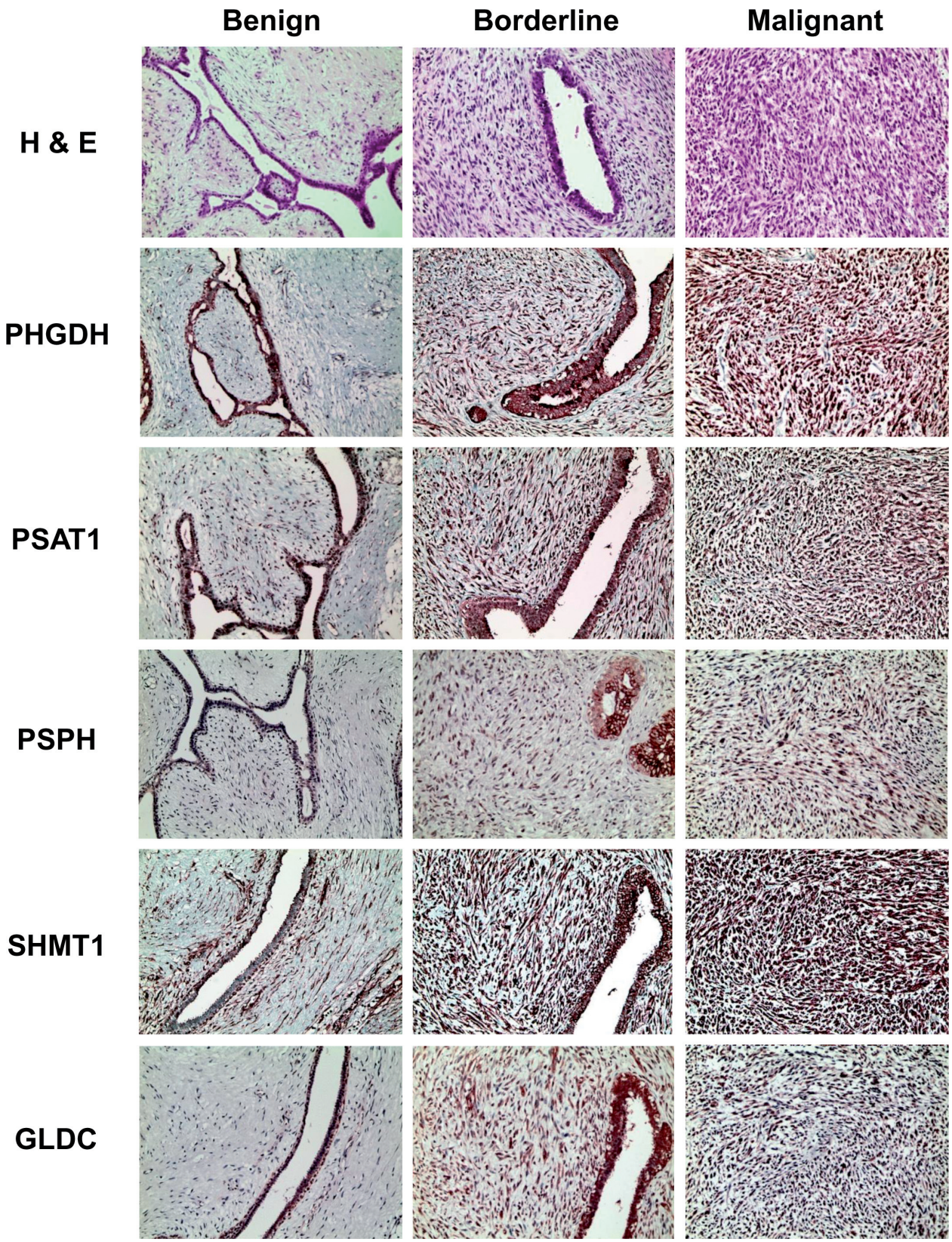

Figure 1. Expression of proteins related to serine and glycine metabolism in phyllodes tumor. As histologic grade of the tumor increased, the expression of PHGDH, PSAT1, PSPH, SHMT1, and GLDC in the stromal component also increased.

sues were fixed in 10% buffered formalin and embedded in paraffin prior to staining with hematoxylin and eosin (H&E). Two pathologists (JS Koo and W Jung) reviewed all of the archived H&E slides. The PTs were graded according to criteria in the World Health Organization Classification of Tumors 2003 [7]. Clinical factors assessed were patient age, tumor recurrence, distant metastasis, and survival. The Institutional Review Board of Yonsei University Severance Hospital approved the study.

Tissue microarray (TMA). A representative area was selected fom 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 the 5-mm tissue core was placed in a 5 x 6 recipient block. Two tissue cores were extracted to minimize extraction bias. Each separate tissue core was assigned a unique tissue microarray location number that was linked to a database including other clinical-pathologic data.

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

Table 3. Expression of proteins related to serine and glycine metabolism according to phyllodes tumor grade

Immunohistochemistry results	Number of Patients N = 203 (100%)	PT, Benign n = 155 (100%)	PT, Borderline n = 32 (100%)	PT, Malignant n = 16 (100%)	*P*-value
PHGDH (E)*					0.096
Negative	58 (31.4)	51 (33.6)	7 (25.0)	0 (0.0)	
Positive	127 (68.6)	101 (66.4)	21 (75.0)	5 (100.0)	
PHGDH (S)					<0.001
Negative	165 (81.3)	139 (89.7)	22 (68.8)	4 (25.0)	
Positive	38 (18.7)	16 (10.3)	10 (31.3)	12 (75.0)	
PSAT1 (E)*					0.073
Negative	91 (49.2)	80 (52.6)	9 (32.1)	2 (40.0)	
Positive	94 (50.8)	72 (47.4)	19 (67.9)	3 (60.0)	
PSAT1 (S)					<0.001
Negative	155 (76.4)	133 (85.8)	18 (56.3)	4 (25.0)	
Positive	48 (23.6)	22 (14.2)	14 (43.8)	12 (75.0)	
PSPH (E)*					0.125
Negative	82 (44.3)	70 (46.1)	12 (42.9)	0 (0.0)	
Positive	103 (55.7)	82 (53.9)	16 (57.1)	5 (100.0)	
PSPH (S)					<0.001
Negative	184 (90.6)	150 (96.8)	25 (78.1)	9 (56.3)	
Positive	19 (9.4)	5 (3.2)	7 (21.9)	7 (43.8)	
SHMT1 (E)*					0.005
Negative	132 (71.4)	115 (75.7)	15 (53.6)	2 (40.0)	
Positive	53 (28.6)	37 (24.3)	13 (46.4)	3 (60.0)	
SHMT1 (S)					<0.001
Negative	86 (42.4)	79 (51.0)	6 (18.8)	1 (6.3)	
Positive	117 (57.6)	76 (49.0)	26 (81.3)	15 (93.8)	
GLDC (E)*					0.575
Negative	20 (10.8)	18 (11.8)	1 (3.6)	1 (20.0)	
Positive	165 (89.2)	134 (88.2)	27 (96.4)	4 (80.0)	
GLDC (S)					<0.001
Negative	178 (87.7)	146 (94.2)	24 (75.0)	8 (50.0)	
Positive	25 (12.3)	9 (5.8)	8 (25.0)	8 (50.0)	

*Eighteen tumors without an epithelial component were excluded.
E, epithelial; S, stromal; Enzyme abbreviations as in Table 1

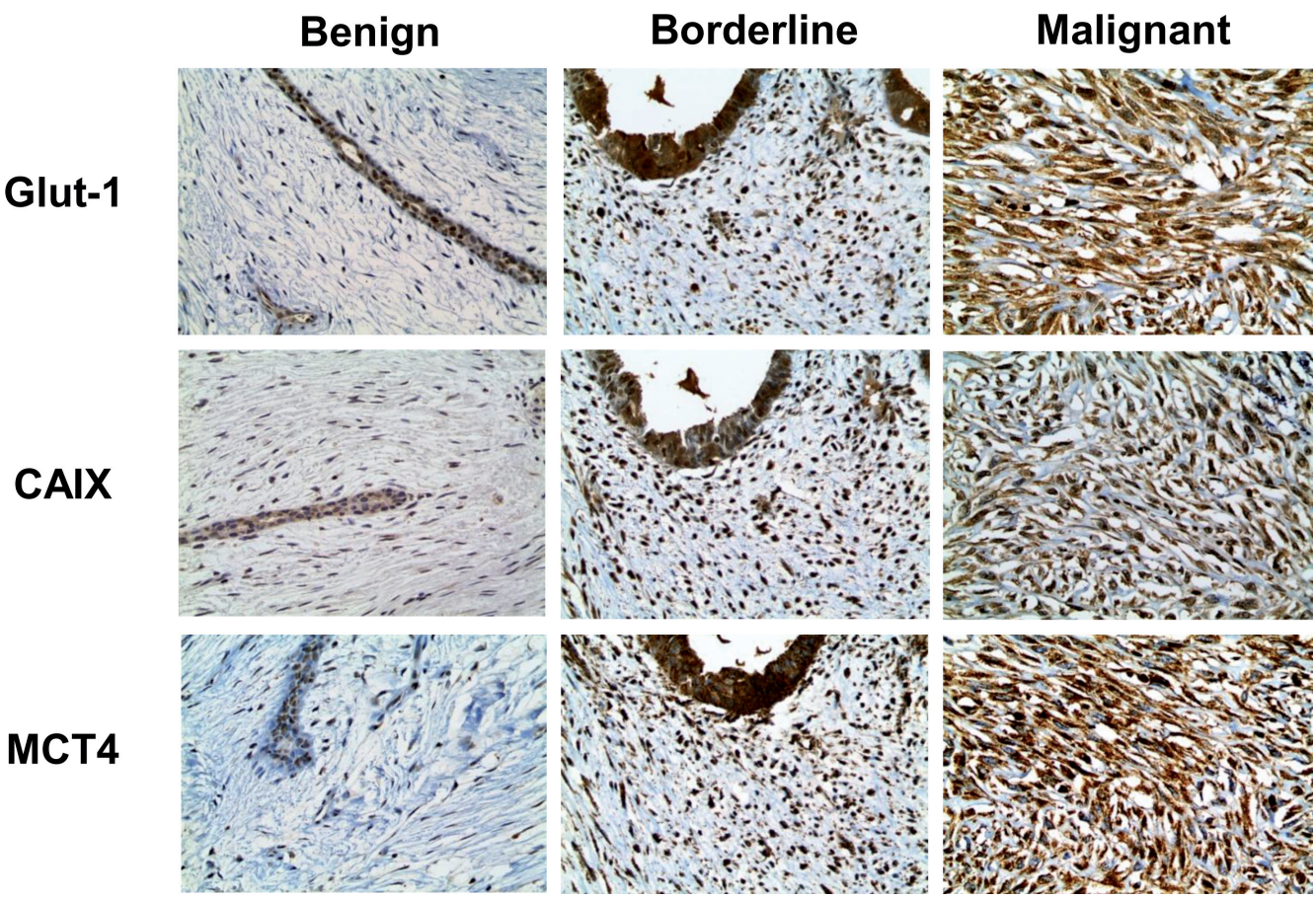

Figure 2. Representative images for glycolysis-related proteins in phyllodes tumor.

Table 4. Correlation between expression status of serine/glycine metabolism related proteins and Ki-67 LI

Parameters	Ki-67 LI of Epithelial component* (%, mean±SD)	p-value	Parameters	Ki-67 LI of Stromal component (%, mean±SD)	p-value
PHGDH (E)*		**0.019**	PHGDH (S)		**<0.001**
Negative	1.0±1.7		Negative	1.0±4.8	
Positive	2.1±3.6		Positive	7.0±12.8	
PSAT1 (E)*		0.639	PSAT1 (S)		**<0.001**
Negative	1.7±3.5		Negative	1.1±5.1	
Positive	1.9±2.9		Positive	5.4±11.5	
PSPH (E)*		**0.005**	PSPH (S)		0.051
Negative	1.0±1.6		Negative	1.8±7.3	
Positive	2.4±3.9		Positive	5.3±6.8	
SHMT1 (E)*		0.370	SHMT1 (S)		**0.002**
Negative	1.6±3.4		Negative	0.3±1.1	
Positive	2.1±2.5		Positive	3.5±9.4	
GLDC (E)*		0.260	GLDC (S)		**<0.001**
Negative	1.0±1.8		Negative	1.1±4.7	
Positive	1.9±3.3		Positive	9.3±15.1	

* Eighteen tumors without an epithelial component were excluded.
E, epithelial; S, stromal; Enzyme abbreviations as in Table 1

Table 5. Correlations between serine- and glycine-related proteins expressed in the epithelial component of phyllodes tumor and pathologic parameters*

Parameters	PHGDH			PSAT1			PSPH			SHMT1			GLDC		
	–	+	*P*-value†	–	+	*P*-value†	–	+	*P*-value†	–	+	*P*-value†	–	+	*P*-value†
Stromal cellularity			0.420			1.810			0.280			0.230			0.576
Mild	44 (75.9)	75 (59.1)		63 (69.2)	56 (59.6)		60 (73.2)	59 (57.3)		92 (69.7)	27 (50.9)		17 (85.0)	102 (61.8)	
Moderate	13 (22.4)	47 (37.0)		25 (27.5)	35 (37.2)		21 (25.6)	39 (37.9)		37 (28.0)	23 (43.4)		3 (15.0)	57 (34.5)	
Marked	1 (1.7)	5 (3.9)		3 (3.3)	3 (3.2)		1 (1.2)	5 (4.9)		3 (2.3)	3 (5.7)		0 (0.0)	6 (3.6)	
Stromal atypia			2.105			1.505			1.850			**0.005**			3.635
Mild	52 (89.7)	105 (82.7)		81 (89.0)	76 (80.9)		73 (89.0)	84 (81.6)		120 (90.9)	37 (69.8)		18 (90.0)	139 (84.2)	
Moderate	5 (8.6)	20 (15.7)		9 (9.9)	16 (17.0)		8 (9.8)	17 (16.5)		10 (7.6)	15 (28.3)		2 (10.0)	23 (13.9)	
Marked	1 (1.7)	2 (1.6)		1 (1.1)	2 (2.1)		1 (1.2)	2 (1.9)		2 (1.5)	1 (1.9)		0 (0.0)	3 (1.8)	
Stromal mitosis			2.865			0.955			2.070			**0.010**			3.790
0–4/10 HPFs	54 (93.1)	114 (89.8)		86 (94.5)	82 (87.2)		76 (92.7)	92 (89.3)		126 (95.5)	42 (79.2)		19 (95.0)	149 (90.3)	
5–9/10 HPFs	4 (6.9)	11 (8.7)		4 (4.4)	11 (11.7)		6 (7.3)	9 (8.7)		5 (3.8)	10 (18.9)		1 (5.0)	14 (8.5)	
≥10/10 HPFs	0 (0.0)	2 (1.6)		1 (1.1)	1 (1.1)		0 (0.0)	2 (1.9)		1 (0.8)	1 (1.9)		0 (0.0)	2 (1.2)	
Stromal overgrowth			1.190			2.900			0.595			0.710			2.715
Absent	58 (100.0)	124 (97.6)		90 (98.9)	92 (97.9)		82 (100.0)	100 (97.1)		131 (99.2)	51 (96.2)		20 (100.0)	162 (98.2)	
Present	0 (0.0)	3 (2.4)		1 (1.1)	2 (2.1)		0 (0.0)	3 (2.9)		1 (0.8)	2 (3.8)		0 (0.0)	3 (1.8)	
Tumor margin			**0.045**			3.110			0.095			0.330			3.230
Circumscribed	58 (100.0)	113 (89.0)		85 (93.4)	86 (91.5)		80 (97.6)	91 (88.3)		125 (94.7)	46 (86.8)		19 (95.0)	152 (92.1)	
Infiltrative	0 (0.0)	14 (11.0)		6 (6.6)	8 (8.5)		2 (2.4)	12 (11.7)		7 (5.3)	7 (13.2)		1 (5.0)	13 (7.9)	
Tumor recurrence			1.285			1.280			0.815			**0.015**			2.495
Absent	56 (96.6)	117 (92.1)		87 (95.6)	86 (91.5)		79 (96.3)	94 (91.3)		128 (97.0)	45 (84.9)		18 (90.0)	155 (93.9)	
Present	2 (3.4)	10 (7.9)		4 (4.4)	8 (8.5)		3 (3.7)	9 (8.7)		4 (3.0)	8 (15.1)		2 (10.0)	10 (6.1)	
Distant metastasis			1.685			0.810			1.025			0.125			0.365
Absent	58 (100.0)	125 (98.4)		91 (100.0)	92 (97.9)		82 (100.0)	101 (98.1)		132 (100.0)	51 (96.2)		19 (95.0)	164 (99.4)	
Present	0 (0.0)	2 (1.6)		0 (0.0)	2 (2.1)		0 (0.0)	2 (1.9)		0 (0.0)	2 (3.8)		1 (5.0)	1 (0.6)	

*Eighteen cases without an epithelial component were excluded.
† *P*-values are corrected for multiple testing using the Bonferroni correction.
HPF, high-powered fields; Enzyme abbreviations as in Table 1

hematoxylin. Immunohistochemical markers for GLDC, PSAT, PSPH, PHGDH, and SHMT1 were assessed by light microscopy. The staining results were semi-quantitatively scored by multiplying the proportion of stained cells by immunostaining intensity. The proportion of cells stained was categorized as 0 (negative), 1 (< 30% of tumor cells), or 2 (> 30% of tumor cells), and the intensity was categorized as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The total score was determined as negative (0 to 1) or positive (2 to 6) [10]. Ki-67 labeling indices (LI) were scored by

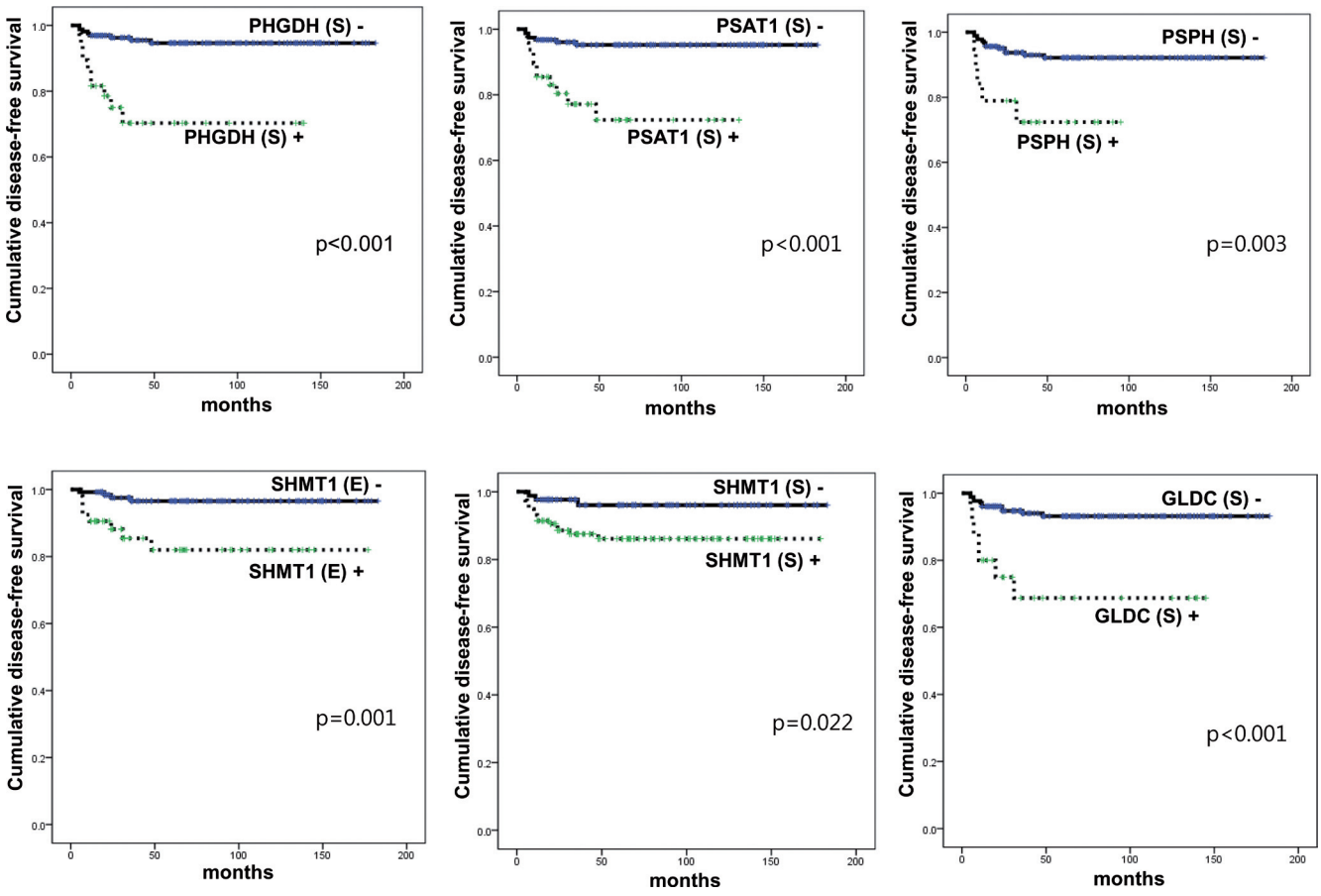

Figure 3. Disease-free survival according to expression of proteins related to serine and glycine metabolism.

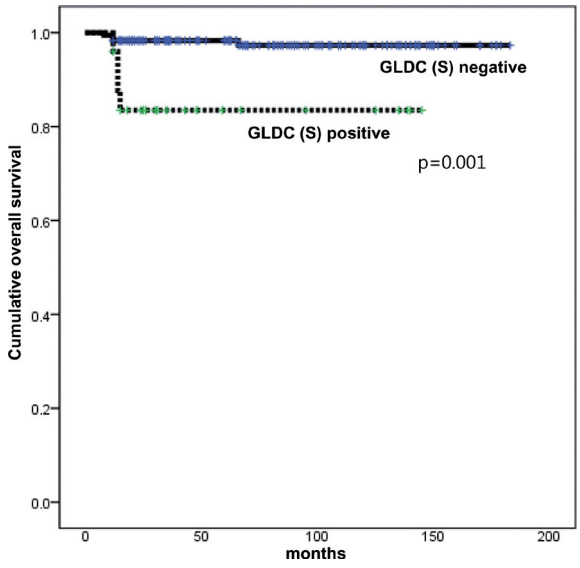

Figure 4. Overall survival according to status of GLDC in stromal component of phyllodes tumor.

counting the number of positively stained nuclei and is expressed as a percentage of total tumor cells.

Statistical analysis. Data were analyzed using SPSS for Windows, Version 12.0 (SPSS Inc., Chicago, IL, USA). For determination of statistical significance, Student's *t* and Fisher's exact tests were used for continuous and categorical variables, respectively. A p-value less than 0.05 was considered significant. Time to tumor recurrence was evaluated using Kaplan-Meier survival curves and the log-rank test. Multivariate regression analysis was performed using Cox proportional hazards model.

Results

Basal characteristics of phyllodes tumor. Table 2 shows basal characteristics of 203 patients with PTs that included 155 (76.4%) benign, 32 (15.8%) borderline, and 16 (7.9%) malignant tumors. Higher tumor grade was associated with patient age (p=0.010), tumor size (p=<0.001), tumor recurrence (p<0.001), and distant metastasis (p<0.001). Eight patients developed distant metastasis, exclusively to the lung.

Table 6. Correlations between serine- and glycine-related proteins expressed in the stromal component of phyllodes tumor and pathologic parameters

Parameters	PHGDH			PSAT1			PSPH			SHMT1			GLDC		
	–	+	*P*-value	–	+	*P*-value	–	+	*P*-value	–	+	*P*-value	–	+	*P*-value
Stromal cellularity			**<0.001**			**<0.001**			**<0.001**			**<0.001**			**<0.001**
Mild	117 (70.9)	5 (13.2)		112 (72.3)	10 (20.8)		118 (64.1)	4 (21.1)		71 (82.6)	51 (43.6)		117 (65.7)	5 (20.0)	
Moderate	44 (26.7)	24 (63.2)		37 (23.9)	31 (64.6)		58 (31.5)	10 (52.6)		14 (16.3)	54 (46.2)		55 (30.9)	13 (52.0)	
Marked	4 (2.4)	9 (23.7)		6 (3.9)	7 (14.6)		8 (4.3)	5 (26.3)		1 (1.2)	12 (10.3)		6 (3.4)	7 (28.0)	
Stromal atypia			**<0.001**			**<0.001**			**<0.001**			**<0.001**			**<0.001**
Mild	144 (87.3)	16 (42.1)		137 (88.4)	23 (47.9)		154 (83.7)	6 (31.6)		81 (94.2)	79 (67.5)		150 (84.3)	10 (40.0)	
Moderate	18 (10.9)	15 (39.5)		16 (10.3)	17 (35.4)		24 (13.0)	9 (47.4)		3 (3.5)	30 (25.6)		23 (12.9)	10 (40.0)	
Marked	3 (1.8)	7 (18.4)		2 (1.3)	8 (16.7)		6 (3.3)	4 (21.1)		2 (2.3)	8 (6.8)		5 (2.8)	5 (20.0)	
Stromal mitosis			**<0.001**			**<0.001**			**<0.001**			**<0.001**			**<0.001**
0–4/10 HPFs	152 (92.1)	20 (52.6)		143 (92.3)	29 (60.4)		165 (89.7)	7 (36.8)		85 (98.8)	87 (74.4)		159 (89.3)	13 (52.0)	
5–9/10 HPFs	10 (6.1)	10 (26.3)		9 (5.8)	11 (22.9)		12 (6.5)	8 (42.1)		0 (0.0)	20 (17.1)		14 (7.9)	6 (24.0)	
≥10/10 HPFs	3 (1.8)	8 (21.1)		3 (1.9)	8 (16.7)		7 (3.8)	4 (21.1)		1 (1.2)	10 (8.5)		5 (2.8)	6 (24.0)	
Stromal overgrowth			**<0.001**			**<0.001**			**<0.001**			**0.001**			**<0.001**
Absent	160 (97.0)	26 (68.4)		150 (96.8)	36 (75.0)		174 (94.6)	12 (63.2)		85 (98.8)	101 (86.3)		169 (94.9)	17 (68.0)	
Present	5 (3.0)	12 (31.6)		5 (3.2)	12 (25.0)		10 (5.4)	7 (36.8)		1 (1.2)	16 (13.7)		9 (5.1)	8 (32.0)	
Tumor margin			**<0.001**			**0.001**			**<0.001**			**0.030**			**<0.001**
Circumscribed	154 (93.3)	28 (73.7)		145 (93.5)	37 (77.1)		170 (92.4)	12 (63.2)		83 (96.5)	99 (84.6)		166 (93.3)	16 (64.0)	
Infiltrative	11 (6.7)	10 (26.3)		10 (6.5)	11 (22.9)		14 (7.6)	7 (36.8)		3 (3.5)	18 (15.4)		12 (6.7)	9 (36.0)	
Tumor recurrence			**<0.001**			**<0.001**			**0.025**			0.105			**<0.001**
Absent	157 (95.2)	28 (73.7)		148 (95.5)	37 (77.1)		171 (92.9)	14 (73.7)		83 (96.5)	102 (87.2)		167 (93.8)	18 (72.0)	
Present	8 (4.8)	10 (26.3)		7 (4.5)	11 (22.9)		13 (7.1)	5 (26.3)		3 (3.5)	15 (12.8)		11 (6.2)	7 (28.0)	
Distant metastasis			**0.005**			**<0.001**			**0.015**			0.065			**0.005**
Absent	163 (98.8)	32 (84.2)		154 (99.4)	41 (85.4)		180 (97.8)	15 (78.9)		86 (100.0)	109 (93.2)		175 (98.3)	20 (80.0)	
Present	2 (1.2)	6 (15.8)		1 (0.6)	7 (14.6)		4 (2.2)	4 (21.1)		0 (0.0)	8 (6.8)		3 (1.7)	5 (20.0)	

* *P*-values are corrected for multiple testing using the Bonferroni correction.
HPF, high-powered fields; Abbreviations as in Table 1

Expression of proteins related to serine and glycine metabolism according to phyllodes tumor grade. The immunohistochemical analysis of TMA revealed that stromal expression of PHGDH, PSAT1, PSPH, SHMT1, and GLDC ($p<0.001$, Figure 1) and epithelial expression of SHMT1 ($p=0.005$) were associated with increasing histologic grade (Table 3). Additionally, we evaluated the association between expression of serine/glycine metabolism-related proteins and Ki-67 LI. Epithelial expression of PHGDH and PSPH was positively correlated with Ki-67 LI of epithelial component

Table 7. Correlations between serine/glycine-related and glycolysis-related proteins expressed in the epithelial component of PT*

Parameters	Glut-1			CAIX			MCT4		
	Negative n = 180 (%)	Positive n = 5 (%)	*P*-value†	Negative n = 107 (%)	Positive n = 78 (%)	*P*-value†	Negative n = 169 (%)	Positive n = 16 (%)	*P*-value†
PHGDH (E)			0.981			0.072			3.000
Negative	58 (32.2)	0 (0.0)		41 (38.3)	17 (21.8)		53 (31.4)	5 (31.3)	
Positive	122 (67.8)	5 (100.0)		66 (61.7)	61 (78.2)		116 (68.6)	11 (68.8)	
PHGDH (S)			3.000			1.608			2.142
Negative	152 (84.4)	5 (100.0)		89 (83.2)	68 (87.2)		144 (85.2)	13 (81.3)	
Positive	28 (15.6)	0 (0.0)		18 (16.8)	10 (12.8)		25 (14.8)	3 (18.8)	
PSAT1 (E)			0.177			1.377			1.305
Negative	91 (50.6)	0 (0.0)		50 (46.7)	41 (52.6)		85 (50.3)	6 (37.5)	
Positive	89 (49.4)	5 (100.0)		57 (53.3)	37 (47.4)		84 (49.7)	10 (62.5)	
PSAT1 (S)			3.000			1.743			3.000
Negative	143 (79.4)	4 (80.0)		83 (77.6)	64 (82.1)		134 (79.3)	13 (81.3)	
Positive	37 (20.6)	1 (20.0)		24 (22.4)	14 (17.9)		35 (20.7)	3 (18.8)	
PSPH (E)			3.000			0.306			2.379
Negative	80 (44.4)	2 (40.0)		53 (49.5)	29 (37.2)		74 (43.8)	8 (50.0)	
Positive	100 (55.6)	3 (60.0)		54 (50.5)	49 (62.8)		95 (56.2)	8 (50.0)	
PSPH (S)			3.000			3.000			0.834
Negative	168 (93.3)	5 (100.0)		100 (93.5)	73 (93.6)		159 (94.1)	14 (87.5)	
Positive	12 (6.7)	0 (0.0)		7 (6.5)	5 (6.4)		10 (5.9)	2 (12.5)	
SHMT1 (E)			0.072			0.099			0.234
Negative	131 (72.8)	1 (20.0)		83 (77.6)	49 (62.8)		124 (73.4)	8 (50.0)	
Positive	49 (27.2)	4 (80.0)		24 (22.4)	29 (37.2)		45 (26.6)	8 (50.0)	
SHMT1 (S)			1.971			0.159			0.363
Negative	79 (43.9)	3 (60.0)		54 (50.5)	28 (35.9)		78 (46.2)	4 (25.0)	
Positive	101 (56.1)	2 (40.0)		53 (49.5)	50 (64.1)		91 (53.8)	12 (75.0)	
GLDC (E)			3.000			**0.027**			2.055
Negative	20 (11.1)	0 (0.0)		17 (15.9)	3 (3.8)		18 (10.7)	2 (12.5)	
Positive	160 (88.9)	5 (100.0)		90 (84.1)	75 (96.2)		151 (89.3)	14 (87.5)	
GLDC (S)			3.000			3.165			0.195
Negative	161 (89.4)	5 (100.0)		97 (90.7)	69 (88.5)		154 (91.1)	12 (75.0)	
Positive	19 (10.6)	0 (0.0)		10 (9.3)	9 (11.5)		15 (8.9)	4 (25.0)	

*Eighteen cases without an epithelial component were excluded.
† *P*-values are corrected for multiple testing using the Bonferroni correction.

(p=0.019, and 0.005, respectively), and stromal expression of PHGDH (p<0.001), PSAT1 (p<0.001), SHMT1 (p=0.002) and GLDC (p<0.01) was positively correlated with Ki-67 LI of stromal component (Table 4).

Correlations between serine and glycine-related proteins expressed in phyllodes tumor and pathologic parameters. In this set of patients with PT, PHGDH expression in epithelial component was correlated with infiltrative tumor margin (p=0.045), and SHMT1 expression in epithelial component was correlated with increased stromal atypia (p=0.005), increased stromal mitosis (p=0.010), and tumor recurrence (p=0.015) (Table 5).

In stromal regions of these PTs, expression of PHGDH, PSAT1, PSPH, SHMT1, and GLDC were all correlated with increased stromal cellularity, increased stromal atypia, increased mitosis, stromal overgrowth, and infiltrative tumor margin (p<0.05). In addition, PHGDH, PSAT1, PSPH, and GLDC expressions were correlated with tumor recurrence and distant metastasis (p<0.05, Table 6).

Correlations between serine and glycine-related and glycolysis-related proteins expressed in PT. Previously we reported on expression of glycolysis-related proteins Glut-1, CAIX, and MCT4 [9]. In this study, we investigated associations between expression of Glut-1, CAIX, and MCT4 and expression of proteins involved in serine and glycine metabolism. In epithelial portions of the tumors we found no correlations (Table 7). Within stromal regions, MCT4 expression was correlated with stromal PHGDH (p<0.001),

Table 8. Correlations between serine/glycine-related and glycolysis-related proteins expressed in the stromal component of PT

Parameters	Glut-1			CAIX			MCT4		
	Negative n = 198 (%)	Positive n = 5 (%)	*P*-value*	Negative n = 142 (%)	Positive n = 61 (%)	*P*-value*	Negative n = 173 (%)	Positive n = 30 (%)	*P*-value*
PHGDH (E)*			n/a			0.195			3.000
Negative	58 (31.4)	0 (0.0)		46 (34.6)	12 (23.1)		53 (31.7)	5 (27.8)	
Positive	127 (68.6)	0 (0.0)		87 (65.4)	40 (76.9)		114 (68.3)	13 (72.2)	
PHGDH (S)			0.138			0.051			**<0.001**
Negative	163 (82.3)	2 (40.0)		122 (85.9)	43 (70.5)		150 (86.7)	15 (50.0)	
Positive	35 (17.7)	3 (60.0)		20 (14.1)	18 (29.5)		23 (13.3)	15 (50.0)	
PSAT1 (E)*			n/a			0.744			3.000
Negative	91 (49.2)	0 (0.0)		64 (48.1)	27 (51.9)		82 (49.1)	9 (50.0)	
Positive	94 (50.8)	0 (0.0)		69 (51.9)	25 (48.1)		85 (50.9)	9 (50.0)	
PSAT1 (S)			0.261			0.147			**<0.001**
Negative	153 (77.3)	2 (40.0)		114 (80.3)	41 (67.2)		141 (81.5)	14 (46.7)	
Positive	45 (22.7)	3 (60.0)		28 (19.7)	20 (32.8)		32 (18.5)	16 (53.3)	
PSPH (E)*			n/a			0.150			1.365
Negative	82 (44.3)	0 (0.0)		65 (48.9)	17 (32.7)		76 (45.5)	6 (33.3)	
Positive	103 (55.7)	0 (0.0)		68 (51.1)	35 (67.3)		91 (54.5)	12 (66.7)	
PSPH (S)			0.210			0.102			**<0.001**
Negative	181 (91.4)	3 (60.0)		133 (93.7)	51 (83.6)		163 (64.2)	21 (70.0)	
Positive	17 (8.6)	2 (40.0)		9 (6.3)	10 (16.4)		10 (5.8)	9 (30.0)	
SHMT1 (E)*			n/a			1.416			0.156
Negative	132 (71.4)	0 (0.0)		97 (72.9)	35 (67.3)		123 (73.7)	9 (50.0)	
Positive	53 (28.6)	0 (0.0)		36 (27.1)	17 (32.7)		44 (26.3)	9 (50.0)	
SHMT1 (S)			1.194			**<0.001**			**<0.001**
Negative	85 (42.9)	1 (20.0)		77 (54.2)	9 (14.8)		82 (47.4)	4 (13.3)	
Positive	113 (57.1)	4 (80.0)		65 (45.8)	52 (85.2)		91 (52.6)	26 (86.7)	
GLDC (E)*			n/a			0.594			1.257
Negative	20 (10.8)	0 (0.0)		17 (12.8)	3 (5.8)		17 (10.2)	3 (16.7)	
Positive	165 (89.2)	0 (0.0)		116 (87.2)	49 (94.2)		150 (89.8)	15 (83.3)	
GLDC (S)			0.042			**<0.001**			**0.003**
Negative	176 (88.9)	2 (40.0)		134 (94.4)	44 (72.1)		158 (91.3)	20 (66.7)	
Positive	22 (11.1)	3 (60.0)		8 (5.6)	17 (27.9)		15 (8.7)	10 (33.3)	

* *P*-values are corrected for multiple testing using the Bonferroni correction.
E, epithelial; S, stromal, Glut1, glucose transporter 1; CAIX, carbonic anhydrase; MTC4, monocarboxylate transporter 4; other enzymes as in Table 1

stromal PSAT1 (p<0.001), stromal PSPH (p<0.001), stromal SHMT1 (p<0.001), and stromal GLDC (p=0.006) expression. In addition, stromal CAIX expression correlated with stromal SHMT1 and stromal GLDC expression (p<0.001, Table 8).

Associations of serine and glycine-related protein expression with patient outcome in PT. In univariate analysis, positive staining for stromal PHGDH (p<0.001), stromal PSAT1 (p<0.001), stromal PSPH (p=0.003), epithelial SHMT1 (p=0.001), stromal SHMT1 (p=0.022), and stromal GLDC (p<0.001) in the PT were associated with shorter disease-free survival (DFS) (Table 9). Positive staining for stromal GLDC in the PT was associated with shorter overall survival (OS) (p<0.001, Figure 4). Multivariate Cox analysis revealed no factor independently associated with shorter DFS or shorter OS (Table 10).

Discussion

A principal finding in this study was that stromal expression of proteins related to serine and glycine metabolism increased in phyllodes tumors with increasing histologic tumor grade. Although we found no previous study appropriate for comparison, reported associations of PHGDH with breast carcinoma [4] and melanoma [3], and of GLDC with tumor aggressiveness in pulmonary non-small cell carcinoma [5], are compatible with our results. In a previous study we found that stromal expression of the glycolysis-related proteins

Table 9. Univariate analysis of serine- and glycine-related proteins expressed with respect to patient prognosis using the log-rank test

Parameters	No. of patients Total/recurrence/metastasis	Disease-free survival		Overall survival	
		Median survival (95% CI) months	*P* –value	Median survival (95% CI) months	*P* -value
PHGDH (E)			0.222		n/a
Negative	58/2/0	172 (164-181)		n/a	
Positive	127/10/2	168 (159-177)		n/a	
PHGDH (S)			**<0.001**		0.527
Negative	165/8/6	174 (168-180)		176 (171-181)	
Positive	38/10/2	102 (83-122)		133 (123-142)	
PSAT1 (E)			0.165		0.846
Negative	91/4/1	169 (162-176)		175 (171-178)	
Positive	94/8/1	166 (154-177)		179 (173-186)	
PSAT1 (S)			**<0.001**		0.268
Negative	155/7/5	174 (169-180)		177 (172-182)	
Positive	48/11/3	103 (86-119)		127 (118-135)	
PSPH (E)			0.129		0.947
Negative	82/3/1	176 (168-183)		180 (176-185)	
Positive	103/9/1	161 (151-171)		175 (172-178)	
PSPH (S)			**0.003**		0.094
Negative	184/13/6	170 (163-176)		177 (172-181)	
Positive	19/5/2	72 (55-89)		86 (74-97)	
SHMT1 (E)			**0.001**		n/a
Negative	132/4/5	177 (172-182)		n/a	
Positive	53/8/0	148 (130-166)		n/a	
SHMT1 (S)			**0.022**		0.077
Negative	86/3/1	176 (169-183)		181 (177-184)	
Positive	117/15/7	156 (145-167)		168 (160-176)	
GLDC (E)			0.526		n/a
Negative	20/2/0	153 (132-174)		n/a	
Positive	165/10/2	171 (165-178)		n/a	
GLDC (S)			**<0.001**		**0.001**
Negative	178/11/4	171 (165-178)		178 (174-182)	
Positive	25/7/4	104 (78-129)		123 (103-142)	

Abbreviations: S, stromal component; E, epithelial component; enzymes as in Table 1

[a] 14 cases without an epithelial component were excluded.

glucose transporter-1 (Glut-1), carbonic anhydrase (CAIX), and monocarboxylate transporter 4 (MCT4) increased with increasing tumor grade in PT [9], consistent with an increase in demand for energy and anabolic substrates. Results in the present study confirmed a similar increase in serine and glycine metabolism with tumor grade. In addition, we observed an association between glycolysis-related proteins and proteins related to serine and glycine metabolism, consistent with the interdependence of these pathways. Specifically, expression of the glycolysis-related proteins CAIX and MCT4 in stromal regions of PT were significantly correlated with stromal expression of proteins related to serine and glycine metabolism.

A previous study suggested that increased biosynthesis of serine influences breast cancer behavior through production of α-ketoglutarate, a TCA cycle intermediate, rather than by other conversions of serine [4]. This may result in suggestion that the increased glycolysis and increased serine metabolism that we observed in phyllodes tumor, as products of these pathways feed into mitochondrial metabolism. Studies on the expression of ATP synthase, glutaminase 1 (GLS1) and succinate dehydrogenase (SDH) may resolve this question.

In this study, increased stromal expression of SHMT1 in PT with increasing histologic grade is consistent with a role for glycine in rapid cancer cell proliferation as previously reported [2]. In the epithelial component of PT, only SHMT1 among serine/glycine metabolism-related proteins showed increased expression with increasing tumor grade. Although malignant PTs may occasionally display vigorous stromal proliferation resulting in no identifiable epithelial component,

Table 10. Multivariate analysis of disease-free survival in patients with phyllodes tumors

Included factor	Disease-free survival			Overall-survival		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Histologic grade			0.219			1.000
Benign vs. Borderline or Malignant	7.923	0.293-214.2		1.000	0.018-57.02	
Stromal cellularity			0.897			1.000
Mild vs. moderate or marked	0.852	0.076-9.554		1.000	0.141-7.094	
Stromal atypia			0.239			1.000
Mild vs. moderate or marked	0.195	0.013-2.962		1.000	0.024-43.71	
Stromal mitosis			0.200			1.000
0-4/10 HPFs vs. >4/10 HPFs	5.867	0.392-87.93		1.000	0.023-43.71	
Stromal overgrowth			0.077			1.000
Absent vs. Present	10.872	0.774-152.6		1.000	0.016-61.74	
Tumor margin			0.361			1.000
Circumscribed vs. Infiltrative	0.298	0.022-3.991		1.000	0.050-19.94	
PHGDH (S)			0.472		Not included	
Negative vs. Positive	2.061	0.288-14.76				
PSAT1 (S)			0.942		Not included	
Negative vs. Positive	0.936	0.159-5.511				
PSPH (S)			0.573			1.000
Negative vs. Positive	0.552	0.070-4.349		1.000	0.060-16.73	
SHMT1 (E)			0.060		Not included	
Negative vs. Positive	4.783	0.939-24.37				
SHMT1 (S)			0.330			1.000
Negative vs. Positive	0.343	0.040-2.953		1.000	0.205-4.885	
GLDC (S)			0.088			1.000
Negative vs. Positive	5.145	0.782-33.84		1.000	0.084-11.83	

Abbreviations: HPFs, high-power fields; S, stromal component; E, epithelial component; enzymes as in Table 1.

epithelial proliferation, when present, is more often observed in borderline PTs than in benign ones, suggesting that single-carbon metabolism may influence progression in the epithelial component of PT.

The GLDC expression also increased with increasing histologic grade in this set of phyllodes tumors. The exact mechanism for this is unknown; however, it may possibly be explained as a secondary effect of oncogene overexpression. The expression of GLDC is shown to increase in cultured breast epithelial cells following transformation by KRAS, PIK3CA and MYC oncogenes (MCF10A) [5], and stromal c-myc [11] and EGFR [12] expression may increase with increasing histologic grade in PTs.

Our results demonstrating the significant association between stromal expression of serine/glycine metabolism-related proteins of PT and poor prognosis are consistent with the results of previous studies; PHGDH expression and GLDC expression were reported to be correlated with poor prognosis in cervical cancer [13] and lung cancer [5], respectively. Expression of glycolysis-related proteins such as Glut-1[14, 15], CAIX[16, 17], MCT-4[18-21] was reported to be associated with poor prognosis in various type of cancer, indicating that increased glycolysis-related metabolism has a correlation with poor prognosis.

Based on the roles of serine and glycine in single-carbon metabolism, increased stromal expression of enzymes that metabolize these amino acids with increasing histologic grade in PTs may have therapeutic significance. Ongoing preclinical studies of drugs targeting multiple sites of one-carbon metabolism, including GLDC, PSAT, PSPH, PHGDH, may yield new possibilities for the therapy of PTs [14, 22].

In conclusion, expression of proteins related to serine and glycine metabolism increased in the stromal component of phyllodes tumor with increasing tumor histologic grade, and expression of these proteins correlated with expression of the glycolysis-related proteins CAIX, and MCT4.

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