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Expression of lipid metabolism-related proteins in breast phyllodes tumors

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The aim of this study was to investigate the expression of lipid metabolism-related proteins and the implications thereof in phyllodes tumor (PT) of the breast. A tissue microarray (TMA) was constructed using paraffin blocks from 194 PT patient tissue samples. Immunohistochemical staining for lipid metabolism-related proteins, namely hormone-sensitive lipase (HSL), perilipin 2, fatty-acid-binding proteins 4 (FABP4), carnitine palmitoyltransferase-1 (CPT-1), acyl-CoA oxidase 1 (ACOX-1), and fatty acid synthase (FASN) was performed, and the immunohistochemical staining results were analyzed with respect to clinicopathologic parameters. The numbers of benign, borderline, and malignant PTs were 151, 27, and 16, respectively. The expression of HSL, perilipin 2, FABP4, CPT-1, and FASN in stromal components was higher in higher grade tumors. On univariate analysis, shorter disease-free survival (DFS) was associated with stromal perilipin 2 positivity ($p<0.001$) and stromal CPT-1 positivity ($p=0.004$). Shorter overall survival (OS) was associated with stromal perilipin 2 positivity ($p<0.001$), stromal FABP4 positivity ($p<0.001$), stromal CPT-1 positivity ($p=0.004$), and stromal FASN positivity ($p<0.001$). Multivariate Cox analysis revealed that stromal perilipin 2 positivity (hazard ratio=31.693, 95% CI: 1.341-748.8, $p=0.032$) was an independent factor for shorter DFS. In conclusion, higher expressions of HSL, perilipin 2, FABP4, CPT-1 and FASN in the stromal component were observed in higher grade PT.

Key words: lipid, metabolism, phyllodes tumor

Phyllodes tumor (PT) is a relatively rare tumor that accounts for 0.3-1.5% of breast neoplasms. Because it shares morphologic characteristics with another fibroepithelial tumor, fibroadenoma, and because PT can have histologically heterogeneous features within a given tumor, it is sometimes difficult for definitive diagnosis [1, 2]. In addition, phyllodes tumor may manifest as a clinically malignant tumor, showing recurrence or hematogenous metastasis [3]. In histologic classification, although there are discrepancies among classification schemes, it is classified into 3 categories; benign, borderline, or malignant, using World Health Organization (WHO) classification standards. High grade PTs display tumor aggressive features, such as tumor recurrence and distant metastasis.

Tumor cells produce energy by aerobic glycolysis, while normal cells do so by aerobic phosphorylation through the tricarboxylic acid (TCA) cycle, a phenomenon called the Warburg effect [4]. Glycolysis is an important element of cancer metabolism, but cancer cell metabolism is not the result of a single metabolic system. Previous studies have reported that the dominant form of metabolism can be glycolysis or oxidative phosphorylation [5], depending on the tumor type. This flexibility of cancer cell metabolic system is a major obstacle to therapies targeting cancer cell metabolism. In addition to glycolysis and mitochondrial metabolism, lipid metabolism is also an element of cancer metabolism. Important components of lipid metabolism include lipolysis, lipid transfer, and β-oxidation.

The molecules that play important roles in lipolysis include hormone sensitive lipase (HSL), which disassembles

Abbreviations: PT – phyllodes tumor; WHO – World Health Organization; TCA – tricarboxylic acid; HSL – hormone-sensitive lipase; FABP – fatty-acid-binding proteins; CPT – Carnitine palmitoyltransferase; ACOX1 – acyl-CoA oxidase 1; CAIX – Carbonic anhydrase IX; MCT4 – Monocarboxylate transporter 4; H&E – hematoxylin and eosin; FASN – fatty acid synthase; EMT – epithelial-mesenchymal transition; PKM2 – pyruvate kinase muscle isozyme 2; TGIF2, TGFB-Induced Factor Homeobox 2, ALDH – aldehyde dehydrogenase; GD2 – disialoganglioside

triglycerides into free fatty acids [6], and perilipin 2, a lipid droplet gate-keeper in lipolysis [7]. The key molecule in the lipid transfer process is fatty-acid-binding protein (FABP), which serves as a free fatty acid transporter [8]. Carnitine palmitoyltransferase-1 (CPT-1) [9] and acyl-CoA oxidase 1 (ACOX1) [10] are known to play key roles in β-oxidation. In previous studies, higher expression of glycolysis-related proteins, such as Glut-1, carbonic anhydrase IX (CAIX), and MCT4, was found in PTs of higher grade [11], and the expression of serine/glycine metabolism-related proteins was also higher in higher grade PTs [12]. These results suggest that the expression of lipid metabolism-related proteins may also differ according to PT grade, but there has been no study published to date on this issue. Thus, the aim of this study was to investigate the expression of lipid metabolism-related proteins in different grades of PTs, and the clinical implications thereof.

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

antibody	clone	dilution	company
HSL	Polyclonal	1:100	Abcam, Cambridge, UK
Perillipin 2	Polyclonal	1:100	Abcam, Cambridge, UK
FABP4	Polyclonal	1:100	Abcam, Cambridge, UK
CPT-1	8F6AE9	1:200	Abcam, Cambridge, UK
Acyl-CoA oxidase 1	Polyclonal	1:50	Abcam, Cambridge, UK
FASN	Polyclonal	1:200	Abcam, Cambridge, UK

Patients and methods

Patient selection. Tissue samples from patients who underwent surgical resection at Severance Hospital after being diagnosed with PT from 2000 to 2010 were enrolled in this study. The study was approved by the Institutional Review

Table 2. Clinicopathologic characteristics of patients with phyllodes tumor

Parameters	Total N = 194 (100%)	PT, Benign N = 151 (100%)	PT, Borderline N = 27 (100%)	PT, Malignant N = 16 (100%)	*P*-value
Age (years, mean±SD)	40.1±12.4	38.9±12.2	42.3±11.5	47.6±12.9	0.017
Tumor size (cm, mean±SD)	4.0±2.6	3.6±2.2	4.3±2.5	6.7±4.6	0.001
Stromal cellularity					<0.001
Mild	119 (61.3)	118 (78.1)	1 (3.7)	0 (0.0)	
Moderate	63 (32.5)	33 (21.9)	23 (85.2)	7 (43.8)	
Marked	12 (6.2)	0 (0.0)	3 (11.1)	9 (56.2)	
Stromal atypia					<0.001
Mild	154 (79.4)	149 (98.7)	5 (18.5)	0 (0.0)	
Moderate	30 (15.5)	2 (1.3)	20 (74.1)	8 (50.0)	
Marked	10 (5.2)	0 (0.0)	2 (7.4)	8 (50.0)	
Stromal mitosis					<0.001
0–4 / 10 HPFs	152 (78.4)	151 (100.0)	1 (3.7)	0 (0.0)	
5–9 / 10 HPFs	31 (16.0)	0 (0.0)	26 (96.3)	5 (31.2)	
≥ 10 / 10 HPFs	11 (5.7)	0 (0.0)	0 (0.0)	11 (68.8)	
Stromal overgrowth					<0.001
Absent	177 (91.2)	151 (100.0)	24 (88.9)	2 (12.5)	
Present	17 (8.8)	0 (0.0)	3 (11.1)	14 (87.5)	
Tumor margin					<0.001
Circumscribed	174 (89.7)	148 (98.0)	20 (74.1)	6 (89.7)	
Infiltrative	20 (10.3)	3 (2.0)	7 (25.9)	10 (62.5)	
Surgical procedure					<0.001
Local excision	146 (75.3)	131 (86.8)	14 (51.9)	1 (6.2)	
Wide excision	34 (17.5)	13 (8.6)	12 (44.4)	9 (56.2)	
Mastectomy	14 (7.2)	7 (4.6)	1 (3.7)	6 (37.5)	
Radiation therapy					0.989
No	171 (88.1)	133 (88.1)	24 (88.9)	14 (87.5)	
Yes	23 (11.9)	18 (11.9)	3 (11.1)	2 (12.5)	
Tumor recurrence	18 (9.3)	5 (3.3)	6 (22.2)	7 (43.8)	<0.001
Distant metastasis	8 (4.1)	0 (0.0)	1 (3.7)	7 (43.8)	<0.001

PT, Phyllodes Tumor; HPFs, high-power fields

Board of Yonsei University Severance Hospital. All tissues were fixed in 10% buffered formalin and embedded in paraffin. All archival hematoxylin and eosin (H&E)–stained slides for each case were reviewed by 2 pathologists (JS Koo and W Jung). The histologic grade of PT was determined using the H&E-stained slides according to the WHO classification system [2]. Clinical factors including patients' ages, tumor recurrence, distant metastasis, and survival were collected via review of electronic medical records.

Tissue microarray. A representative area of each H&E-stained tumor slide was selected and the corresponding spot was marked on the surface of the corresponding paraffin block. Using a biopsy needle, the selected area was punched out and a 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, 5-μm-thick sections were created 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 secondary immunoglobulin, followed by incubation with 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. All immunohistochemical markers were accessed via light microscopy. The proportion of positively stained cells on each slide was graded as 0, 1, and 2, corresponding to negative, positive in fewer than 30% of tumor cells, and positive in 30% or more of tumor cells, respectively. The immunostaining intensity was graded as 0, 1, 2, or 3, corresponding to negative, weak, moderate, or strong staining, respectively. The results were ultimately scored by

Table 3. Expression of proteins related to lipid metabolism related proteins according to phyllodes tumor grade

Immunohistochemistry results	Total N = 194 (100%)	PT, Benign N = 151 (100%)	PT, Borderline N = 27 (100%)	PT, Malignant N = 16 (100%)	*P*-value
HSL (E)*					0.951
Negative	124 (67.4)	102 (67.5)	17 (65.4)	5 (71.4)	
Positive	60 (32.6)	49 (32.5)	9 (34.6)	2 (28.6)	
HSL (S)					**0.001**
Negative	147 (75.8)	123 (81.5)	17 (63.0)	7 (43.8)	
Positive	47 (24.2)	28 (18.5)	10 (37.0)	9 (56.2)	
Perilipin 2 (E)*					0.289
Negative	136 (73.9)	115 (76.2)	16 (61.5)	5 (71.4)	
Positive	48 (26.1)	36 (23.8)	10 (38.5)	2 (28.6)	
Perilipin 2 (S)					**<0.001**
Negative	189 (97.4)	151 (100.0)	26 (96.3)	12 (75.0)	
Positive	5 (2.6)	0 (0.0)	1 (3.7)	4 (25.0)	
FABP4 (S)					**<0.001**
Negative	155 (79.9)	133 (88.1)	18 (66.7)	4 (25.0)	
Positive	39 (20.1)	18 (11.9)	9 (33.3)	12 (75.0)	
CPT-1 (E)*					0.485
Negative	158 (85.9)	128 (84.8)	23 (88.5)	7 (100.0)	
Positive	26 (14.1)	23 (15.2)	3 (11.5)	0 (0.0)	
CPT-1 (S)					**0.034**
Negative	169 (87.1)	136 (90.1)	22 (81.5)	11 (68.8)	
Positive	25 (12.9)	15 (9.9)	5 (18.5)	5 (31.2)	
FASN (E)*					0.625
Negative	163 (88.6)	133 (88.1)	23 (88.5)	7 (100.0)	
Positive	21 (11.4)	18 (11.9)	3 (11.5)	0 (0.0)	
FASN (S)					**<0.001**
Negative	190 (97.9)	151 (100.0)	27 (100.0)	12 (75.0)	
Positive	4 (2.1)	0 (0.0)	0 (0.0)	4 (25.0)	

*Ten tumors without an epithelial component were excluded. E, epithelial component; S, stromal component
Bold numbers represent statistically significant results ($p<0.05$).

multiplying the score for proportion of stained cells by the score for staining intensity. Tumors with score of 0-1 were counted as negative, and tumors with a score of 2-6 were counted as positive [13].

Statistical analysis. Data were analyzed using SPSS for Windows, Version 12.0 (SPSS Inc., Chicago, IL, USA). To determine statistical significance, Student's *t*- and Fisher's exact tests were used for continuous and categorical variables, respectively. Statistical significance was assigned if $p < 0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor recurrence. Multivariate regression analysis was performed using a Cox proportional hazards model.

Results

Basal characteristics of phyllodes tumors. The basal characteristics of 194 total patients with PT are summarized in Table 2. Overall, 151, 27, and 16 were diagnosed with benign, borderline, or malignant PT, respectively. Patient age and tumor size increased with greater tumor grade (p=0.017, and p=0.001, respectively), and the rate of tumor recurrence and distance metastasis also increased with greater tumor grade (p<0.001). The site of metastasis was the lung in every instance of metastatic disease.

Expression of lipid metabolism-related proteins according to phyllodes tumor grade. The expression of lipid metabolism-related proteins was analyzed according to PT grade (Table 3). The expression of HSL, perilipin 2, FABP4, CPT-1, and fatty acid synthase (FASN) in the stromal component was higher in higher grade PTs (p<0.05, Figure 1). FABP4 was not expressed in the epithelial component, and ACOX-1 expression was undetectable in both the epithelial and stromal components.

Correlations between the expression of lipid metabolism-related proteins in phyllodes tumor and pathologic parameters. Correlations between the expression of lipid metabolism-related proteins in phyllodes tumor and patho-

Table 4. Univariate analysis of expression of lipid metabolism related proteins 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
HSL (E)*			0.384		0.865
Negative	124/12/2	160 (152-169)		174 (170-178)	
Positive	60/3/1	173 (163-184)		180 (174-185)	
HSL (S)			0.233		0.836
Negative	147/12/6	168 (160-176)		175 (170-181)	
Positive	47/6/2	147 (125-169)		167 (158-177)	
Perilipin 2 (E)*			0.445		0.088
Negative	136/10/1	166 (158-173)		177 (175-180)	
Positive	48/5/2	163 (147-179)		173 (161-186)	
Perilipin 2 (S)			**<0.001**		**<0.001**
Negative	189/15/5	168 (161-175)		178 (174-182)	
Positive	5/3/3	50 (0-106)		62 (10-114)	
FABP4 (S)			0.096		**<0.001**
Negative	178/15/4	167 (160-175)		179 (175-182)	
Positive	16/3/4	110 (86-135)		92 (56-128)	
CPT-1 (E)*			0.940		N/A
Negative	158/13/3	168 (160-175)		N/A	
Positive	26/2/0	101 (90-111)		N/A	
CPT-1 (S)			**0.004**		**0.028**
Negative	169/12/5	170 (163-177)		177 (173-182)	
Positive	25/6/3	85 (68-101)		97 (85-109)	
FASN (E)*			N/A		N/A
Negative	163/15/3	N/A		N/A	
Positive	21/0/0	N/A		N/A	
FASN (S)			0.140		**<0.001**
Negative	190/17/6	166 (159-174)		177 (172-181)	
Positive	4/1/2	28 (15-41)		23 (10-35)	

*Ten tumors without an epithelial component were excluded. E, epithelial component; S, stromal component
Bold numbers represent statistically significant results (p<0.05).

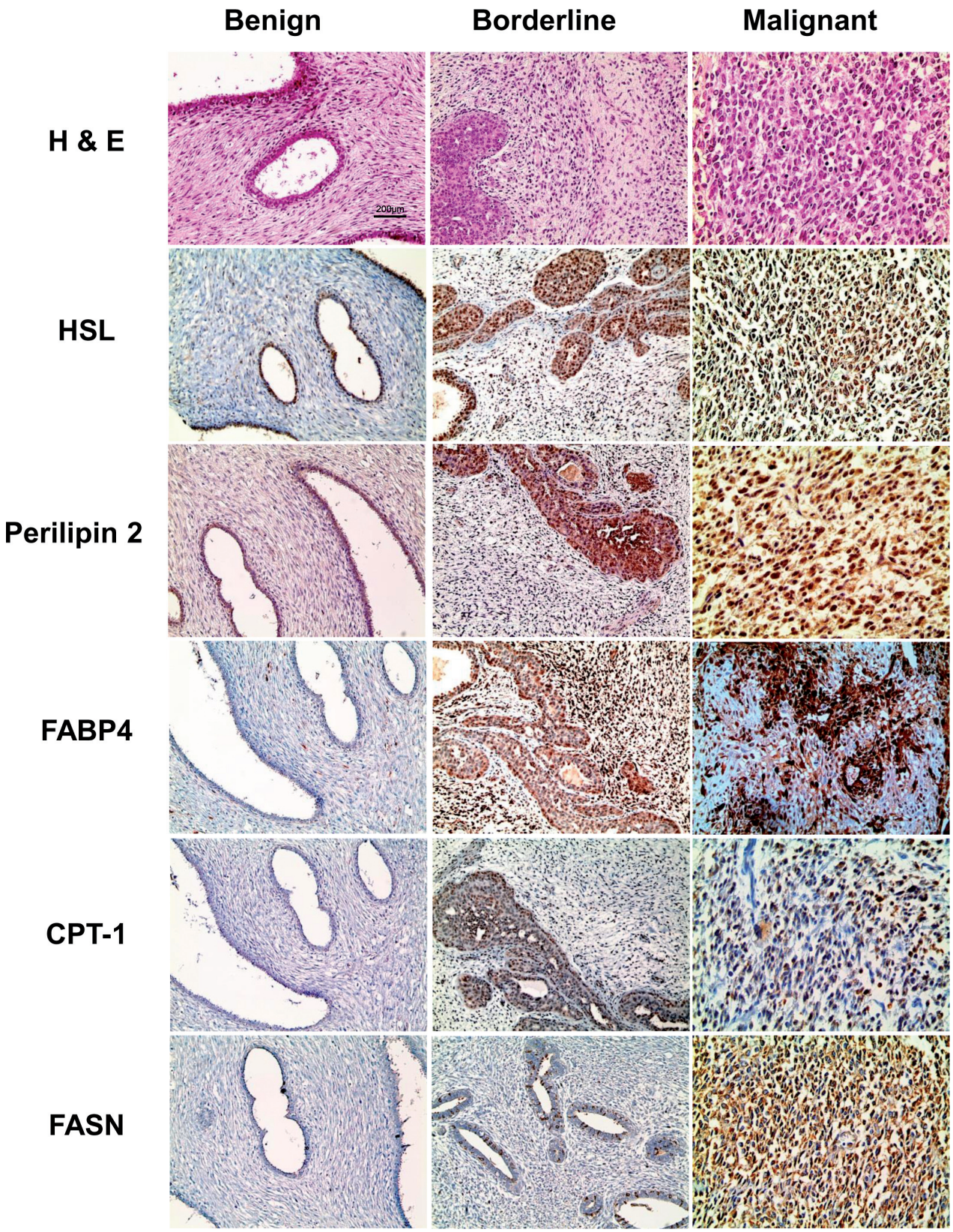

Figure 1. Expression of lipid metabolism-related proteins according to histologic grade of phyllodes tumor (x 200). Higher expression of HSL, perilipin 2, FABP4, CPT-1, and FASN in the stromal component was noted in higher grade PTs.

logic parameters were evaluated (Figure 2). The factors that were associated with increased stromal cellularity, increased stromal mitosis, and infiltrative tumor margin were stromal perilipin A expression and stromal FABP4 expression ($p<0.05$); in addition, increased stromal atypia correlated with stromal perilipin A expression ($p=0.001$). The factors associated with stromal overgrowth included stromal perilipin A expression ($p<0.001$), stromal FABP4 expression ($p<0.001$), and stromal FASN expression ($p<0.001$).

Impact of the expression of lipid metabolism-related proteins on patients' prognoses. A univariate analysis of the impact of expression of lipid metabolism-related proteins on patient prognosis was performed. Shorter disease-free survival (DFS) was associated with stromal perilipin 2 positivity ($p<0.001$), and stromal CPT-1 positivity ($p=0.004$), whereas shorter overall survival (OS) was associated with stromal perilipin 2 positivity ($p<0.001$), stromal FABP4 positivity ($p<0.001$), stromal CPT-1 positivity ($p=0.004$), and stromal FASN positivity ($p<0.001$) (Table 4 and Figure 3). On a multivariate Cox analysis, the independent predictive factors of shorter DFS included higher histologic grade (hazard ratio=8.037, 95% CI: 2.024-31.90, $p<0.001$), stromal overgrowth (hazard ratio=18.501, 95% CI: 2.299-148.8, $p=0.006$) and stromal perilipin 2 positivity (hazard ratio=31.693, 95% CI: 1.341-748.8, $p=0.032$). Stromal overgrowth was an independent factor of shorter OS (hazard ratio=93.583, 95% CI: 3.209-2728, $p=0.008$) (Table 5).

Discussion

The present study investigated the expression of lipid metabolism-related proteins in breast PTs. In higher grade PTs, the expression of HSL, perilipin 2, FABP4, CPT-1, and FASN was higher in the stromal component. There is no previous study regarding lipid metabolism-related proteins in PTs to which a direct comparison can be made. However, previous studies have reported that perilipin 2 is expressed in a proportion of sebaceous carcinomas [14], that FABP4 is associated with cancer progression [15, 16], and that CPT-1 is an important factor in cancer cell survival [17, 18]. In addition, FASN is reported to be involved in tumor progression in various types of neoplasms [19, 20], and these facts suggest that lipid metabolism-related proteins play important roles in tumor growth and progression. The metabolism of PT has not been well investigated so far, but in previous studies, the expression of glycolysis-related proteins and serine/glycine metabolism-related proteins were higher in higher grade PTs [11, 12], suggesting increased metabolic activity and increased lipid metabolism occur in higher grade PTs. One possible explanation of the differences in metabolic activity according to PT grade is the association between stem cells and metabolism. In previous studies, metabolic enzymes have been reported to affect cancer stemness [21], of which the epithelial-mesenchymal transition (EMT) process has been reported to be an important feature. In this process, the expression of pyruvate kinase muscle isozyme 2 (PKM2) increases, and it

Table 5. 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			**<0.001**			N/A
Benign vs. Borderline or Malignant	8.037	2.024-31.90		N/A	N/A	
Stromal cellularity			0.243			0.977
Mild vs. moderate or marked	0.151	0.006-3.597		1.062	0.019-60.74	
Stromal atypia			0.274			0.987
Mild vs. moderate or marked	0.215	0.014-3.389		1.024	0.058-18.04	
Stromal mitosis			0.933			0.212
0-4/10 HPFs vs. >4/10 HPFs	1.153	0.042-31.95		0.064	0.001-4.763	
Stromal overgrowth			**0.006**			**0.008**
Absent vs. Present	18.501	2.299-148.8		93.583	3.209-2728	
Tumor margin			0.087			0.118
Circumscribed vs. Infiltrative	0.215	0.037-1.250		0.138	0.011-1.658	
Perilipin 2 (S)			**0.032**			0.080
Negative vs. Positive	31.693	1.341-748.8		35.677	0.651-1956	
FABP4 (S)			0.483			0.817
Negative vs. Positive	0.536	0.094-3.062		1.332	0.118-15.07	
CPT-1 (S)			0.476			0.338
Negative vs. Positive	1.671	0.407-6.858		3.450	0.274-43.37	
FASN (S)			0.881			0.318
Negative vs. Positive	0.804	0.046-14.08		4.050	0.260-63.05	

S, stromal component

Bold numbers represent statistically significant results (p<0.05).

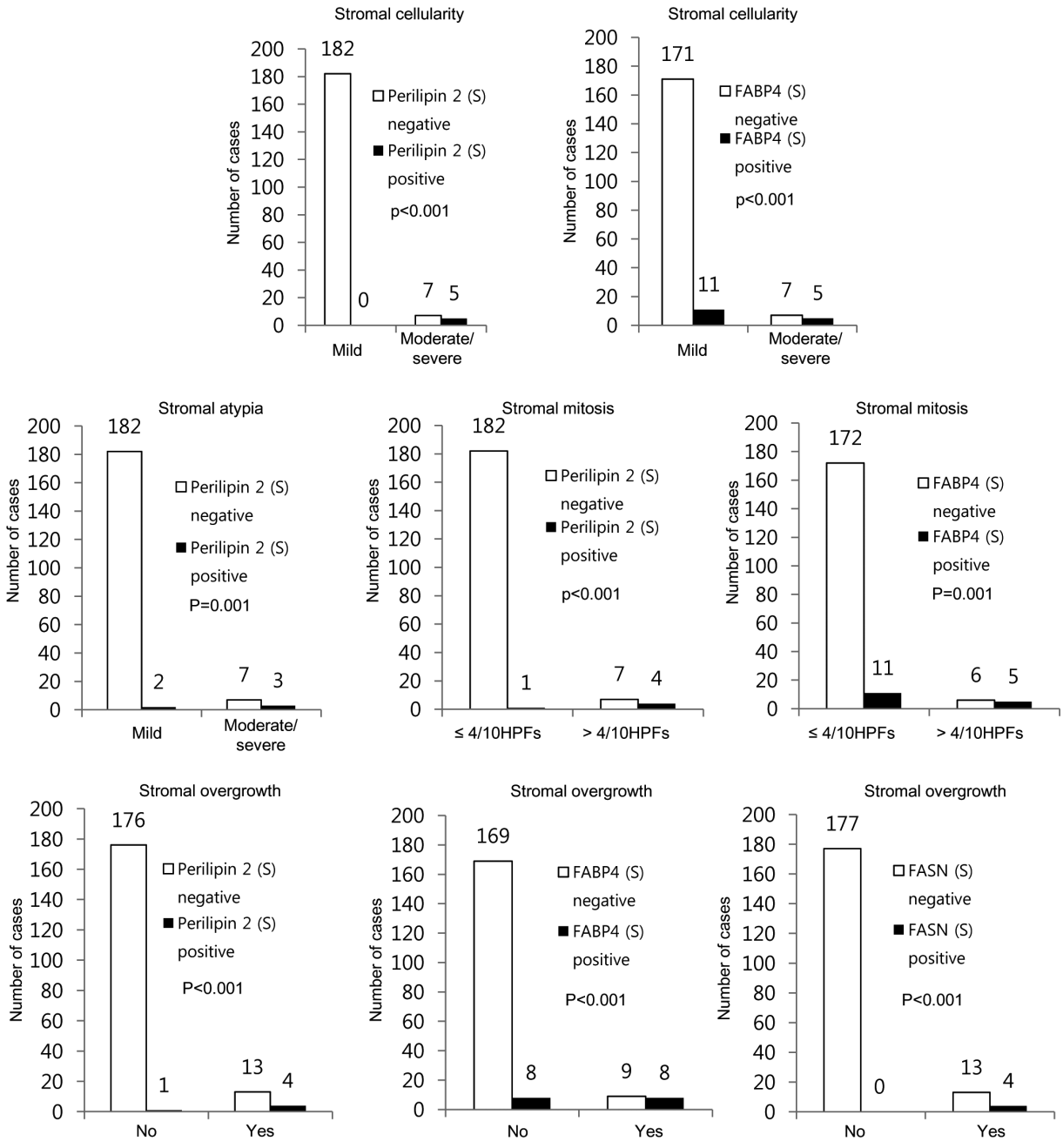

Figure 2. Correlation between the expression of lipid metabolism-related proteins in phyllodes tumor and pathologic parameters. S, stromal component

translocates to the nucleus and interacts with TGFB-Induced Factor Homeobox 2 (TGIF2) and controls the EMT. PKM2 is a key protein in glycolysis in cancer cells, which suggests an association between metabolic enzymes and cancer stemness. Malignant PTs can contain heterologous tumor cell types of stromal origin, such as rhabdomyosarcoma, liposarcoma, and osteosarcoma, suggesting a role for mesenchymal stem cells in PT development. It has also been reported that malignant PTs express elevated levels of aldehyde dehydrogenase (ALDH) and disialoganglioside (GD2), which are characteristic of mesenchymal stem cells [22]. Further study is required to elucidate connections between mesenchymal stem cells and lipid metabolism.

In the present study, perilipin 2 was an independent prognostic factor, and in previous studies, it has been reported to be a prognostic factor in clear cell renal cell carcinoma [23, 24], suggesting its role as a tumor prognostic marker. The clinical implication of the differences observed in lipid metabolism-re-

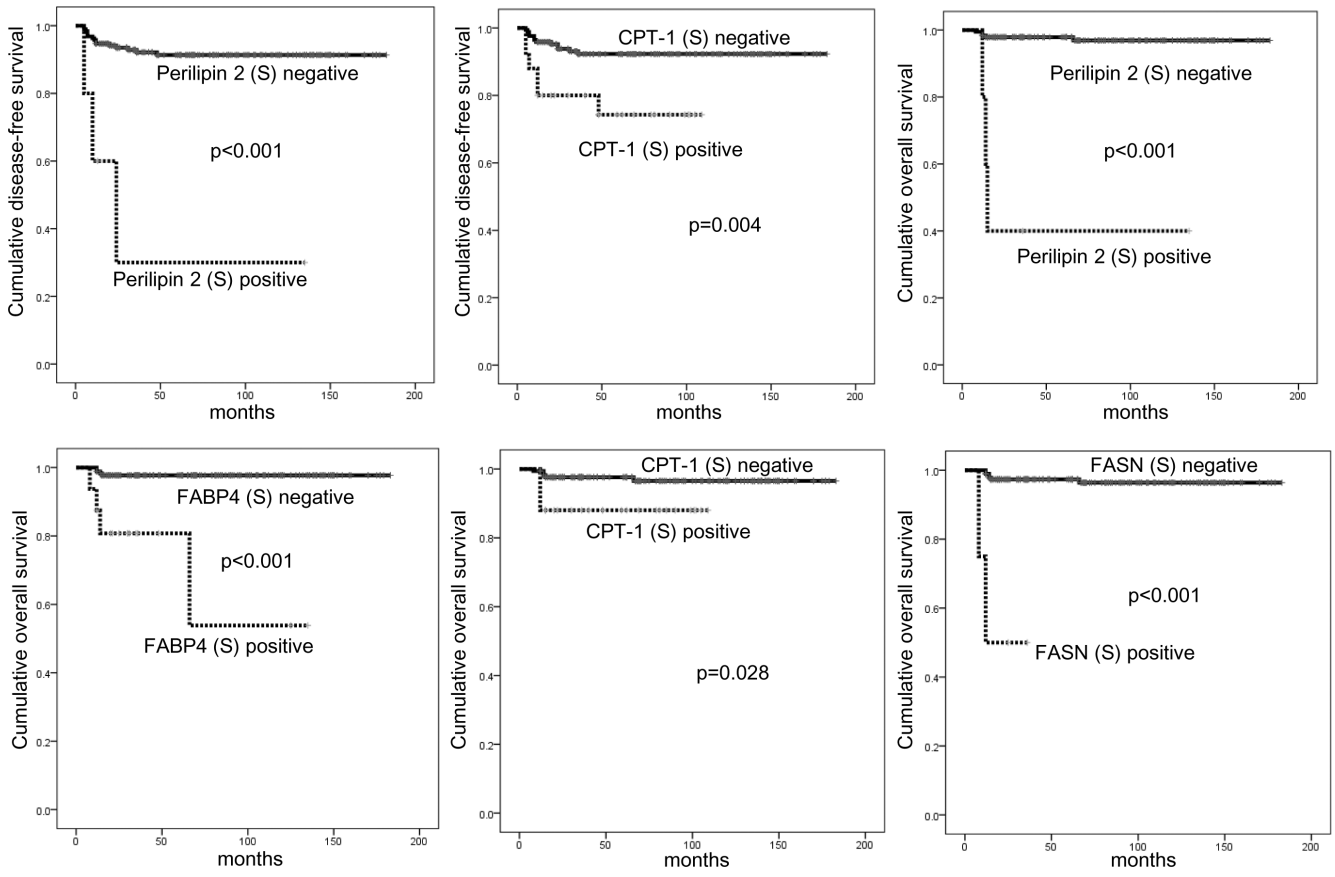

Figure 3. Disease-free survival (a, b) and overall survival (c, d, e, f) according to the status of lipid metabolism-related proteins in phyllodes tumors. S, stromal component

lated protein expression according to PT grade is the potential for lipid metabolism-related proteins to serve as therapeutic targets. Supporting this idea, inhibitors of glycolysis-related molecules such as HIF-1α [25, 26], Glut1 [27, 28], CAIX [29], and MCT4 [30] have been reported to inhibit tumor growth in preclinical studies of various neoplasms, suggesting that metabolic inhibitors are candidate therapeutic agents. Among lipid metabolism-related proteins, the inhibition of CPT-1 [31] and of FASN [32-34] have been reported to inhibit tumor growth. The results of this study support the validity of this therapeutic strategy, which should be pursued further.

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