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Original Article

Expression of Yes-associated protein (YAP) in breast phyllodes tumor

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Abstracts: This study aimed to identify expression profiles of Yes-associated protein (YAP) and its phosphorylated form (pYAP) in phyllodes tumor (PT) of human breast and verify the clinical implications. We selected PTs from the pathologic archive and reviewed the histologic features (141 benign, 27 borderline, and 15 malignant). We made tissue microarray (TMA) block from the formalin-fixed paraffin-embedded (FFPE) tissue corresponding to the representative section. Using TMA block, we performed immunohistochemical staining of YAP and pYAP. In the stromal component, expressions of YAP and pYAP were increased in borderline/malignant PT with comparison of benign PT ($P = 0.002$, and $P < 0.001$, respectively). In the epithelial component, cytoplasmic expression of YAP was highest in borderline PT ($P = 0.001$). Stromal YAP expression ($P < 0.001$) and stromal pYAP expression ($P = 0.042$) were associated with shorter disease-free survival (DFS) and stromal pYAP expression ($P = 0.001$) was associated with shorter overall survival (OS) in univariate Cox analysis. In multivariate Cox analysis, stromal YAP expression was an independent prognostic factor associated with shorter DFS (Hazard ration: 3.206, 95% CI: 1.000-10.27, $P = 0.050$). In conclusion, expression level of YAP in stromal component was increased along with histologic grade of PT and YAP expression in PT was related to tumor progression and poor prognosis.

Keywords: Breast, phyllodes tumor, YAP

Introduction

Phyllodes tumor (PT) is less common consisting 0.3-1.5% of tumors of breast origin. PT, one of fibroepithelial tumors, is composed of stromal component and epithelial component, however, there is no simple criterion to distinguish PT from other fibroepithelial tumors such as fibroadenoma because fibroepithelial tumors share much of histologic features and PT could show a wide spectrum of histologic features resulting in tumor heterogeneity [1, 2]. According to WHO classification, the 3 tiered grading subtypes, such as benign, borderline, and malignant, are recommended for diagnosis of PT and determined by histologic features of stromal component reflecting tumor behavior [2, 3]. Therefore, it is needed to fine adjunctive markers which help to ensure the histologic grades and predict a more precise patient's prognosis of PT.

Yes-associated protein (YAP) is a transcription coactivator and it is believed that YAP could act as an oncogene in the nucleus although phosphorylated YAP (pYAP) is sequestrated in the cytoplasm [4, 5]. In several solid organs including lung, skin, prostate, ovary, liver, and breast, YAP overexpression has been reported in the tumor and solid tumors with YAP overexpression was related to poor prognosis [6-14]. However, expression profiles of YAP in PT and its clinical implications have not been fully studied.

On studying PT in this report, we aimed to verify the expression status of YAP and pYAP in stromal component of tumor according to the histologic grades. To detail, we assessed the expression profiles of YAP and pYAP in epithelial component and stromal component of PT, respectively, and compared differences of YAP and pYAP expression status between these two cellular components. Finally, we identified the relation of YAP expression in each cellular components and survival of PT patients.

Materials and methods

Patient selection

We retrospectively reviewed PTs from the pathologic archive of the department of pathology of

Table 1. Clincopathologic characteristics of patients with phyllodes tumor

Parameters	Number of Patients n = 183 (%)	PT, Benign n = 141 (%)	PT, Borderline n = 27 (%)	PT, Malignant n = 15 (%)	*P*-value
Age (y, mean ± SD)	40.3 ± 12.2	39.1 ± 11.9	42.7 ± 11.9	47.6 ± 13.4	0.019
Tumor size (cm, mean ± SD)	4.0 ± 2.6	3.7 ± 2.2	4.3 ± 2.5	6.2 ± 4.3	0.001
Stromal cellularity					< 0.001
Mild	111 (60.7)	110 (78.0)	1 (3.7)	0 (0.0)	
Moderate	61 (33.3)	31 (22.0)	23 (85.2)	7 (46.7)	
Marked	11 (6.0)	0 (0.0)	3 (11.1)	8 (53.3)	
Stromal atypia					< 0.001
Mild	145 (79.2)	139 (98.6)	6 (22.2)	0 (0.0)	
Moderate	29 (15.8)	2 (1.4)	19 (70.4)	8 (53.3)	
Marked	9 (4.9)	0 (0.0)	2 (7.4)	7 (46.7)	
Stromal mitosis					< 0.001
0-4/10 HPFs	143 (78.1)	141 (100.0)	2 (7.4)	0 (0.0)	
5-9/10 HPFs	30 (16.4)	0 (0.0)	25 (92.6)	5 (33.3)	
≥ 10/10 HPFs	10 (5.5)	0 (0.0)	0 (0.0)	10 (66.7)	
Stromal overgrowth					< 0.001
Absent	168 (91.8)	141 (100.0)	25 (92.6)	2 (13.3)	
Present	15 (8.2)	0 (0.0)	2 (7.4)	13 (86.7)	
Tumor margin					< 0.001
Circumscribed	165 (90.2)	139 (98.6)	20 (74.1)	6 (40.0)	
Infiltrative	18 (9.8)	2 (1.4)	7 (25.9)	9 (60.0)	
Tumor local recurrence	17 (9.3)	5 (3.5)	5 (18.5)	7 (46.7)	< 0.001
Distance metastasis	7 (3.8)	0 (0.0)	0 (0.0)	7 (46.7)	< 0.001
Surgical treatment					< 0.001
Lumpectomy	139 (76.0)	124 (87.9)	14 (51.9)	1 (6.7)	
Partial mastectomy	33 (18.0)	12 (8.5)	12 (44.4)	9 (60.0)	
Total mastectomy	11 (6.0)	5 (3.5)	1 (3.7)	5 (33.3)	
Radiation therapy					< 0.001
No	176 (96.2)	140 (99.3)	25 (92.6)	11 (73.3)	
Yes	7 (3.8)	1 (0.7)	2 (7.4)	4 (26.7)	

Severance hospital. They had been surgically excised and diagnosed from 2000 to 2010. The study was approved by The Institutional Review 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). We assessed histologic grade of PT according to the recommended criteria by WHO classification [2]. Histologic features of PT were reviewed by three breast pathologists. Histologic parameters such as stromal cellularity, stromal atypia, stromal mitosis, stromal overgrowth, and tumor margin were evaluated on H&E-stained slides. Included clinical parameters were patient age at initial diagnosis, tumor size, local recurrence, distant metastasis, methods of surgical resection, and postoperative radiation therapy.

Tissue microarray

On H&E stained slides of tumors, a representative area was selected and the 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. Two tissue cores were extracted to minimize extraction bias. Each tissue core was assigned a unique tissue microarray location number that was linked to a database containing other clinicopathologic data.

Immunohistochemistry

We used formalin-fixed, paraffin-embedded (FFPE) tissue sections for immunohistochemical staining. Briefly, 5-*μ*m-thick sections were obtained with a microtome, transferred into

Table 2. Expression of YAP and pYAP according to phyllodes tumor grade

Parameters	Number of Patients n = 183 (%)	PT, Benign n = 141 (%)	PT, Borderline n = 27 (%)	PT, Malignant n = 15 (%)	*P*-value
YAP (E-Nu)*					0.784
Negative	173 (98.9)	139 (98.6)	26 (100.0)	8 (100.0)	
Positive	2 (1.1)	2 (1.4)	0 (0.0)	0 (0.0)	
YAP (E-Cy)*					0.001
Negative	164 (93.7)	136 (96.5)	20 (76.9)	8 (100.0)	
Positive	11 (6.3)	5 (3.5)	6 (23.1)	0 (0.0)	
YAP (S)					0.002
Negative	165 (90.2)	133 (94.3)	20 (74.1)	12 (80.0)	
Positive	18 (9.8)	8 (5.7)	7 (25.9)	3 (20.0)	
pYAP (E)*					0.194
Negative	82 (46.9)	66 (46.8)	10 (38.5)	6 (75.0)	
Positive	93 (53.1)	75 (53.2)	16 (61.5)	2 (25.0)	
pYAP (S)					< 0.001
Negative	158 (86.3)	132 (93.6)	19 (70.4)	7 (46.7)	
Positive	25 (13.7)	9 (6.4)	8 (29.6)	8 (53.3)	

Abbreviations: PT, Phyllodes Tumor; E, epithelial component; S, stromal component; Nu, nuclear; Cy, cytoplasmic. *8 cases without an epithelial component were excluded.

adhesive slides, and dried at 62°C for 30 minutes. After incubation with primary antibodies for YAP (1: 100, Santa cruz biotechnology), phosphorylated YAP (ser127) (1: 100, Cell signaling), 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. Positive control tissue was used as manufacturer's recommendation. Slides were counterstained with Harris hematoxylin.

Interpretation of immunohistochemical staining

We assessed YAP and pYAP expression in tumor cells of each epithelial and stromal component. In detail, YAP and pYAP expression in the nucleus and the cytoplasm of each tumor cells were also evaluated. Immunostaining result was graded 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). Because normal ductal cells also occasionally demonstrated weak positivity of YAP and pYAP, we estimated positive criteria as over grade 2+ tumor cells were identified in more than 10% of tumor area.

Statistical analysis

Data were analyzed using SPSS for Windows, Ver-sion 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. Sta-tistical significance was when $P < 0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor recurrence and overall survival. Multivariate regression analysis was performed using Cox proportional hazards model.

Results

Patients' basic characteristics and pathologic features of PT

183 cases of PT were included in this study. We summarized clinical characteristics of PT patients and reviewed histologic features (**Table 1**). The worse tumor grade, the more age at diagnosis ($P = 0.019$) and the larger tumor size ($P = 0.001$). Local recurrence and distant metastasis were more frequently identified in malignant PT ($P < 0.001$). 33.3% and 26.7% of malignant PT patients received total mastectomy and radiation therapy, respectively, which were much higher incidence rather than patients of benign or borderline PTs ($P < 0.001$). As defined, stromal cellularity, stromal atypia, stromal mitosis, and stromal over growth was marked as the histologic grade of PT increased ($P < 0.001$). Infiltrative tumor margin rather than circumscribed margin was also most frequently identified in malignant PT ($P < 0.001$).

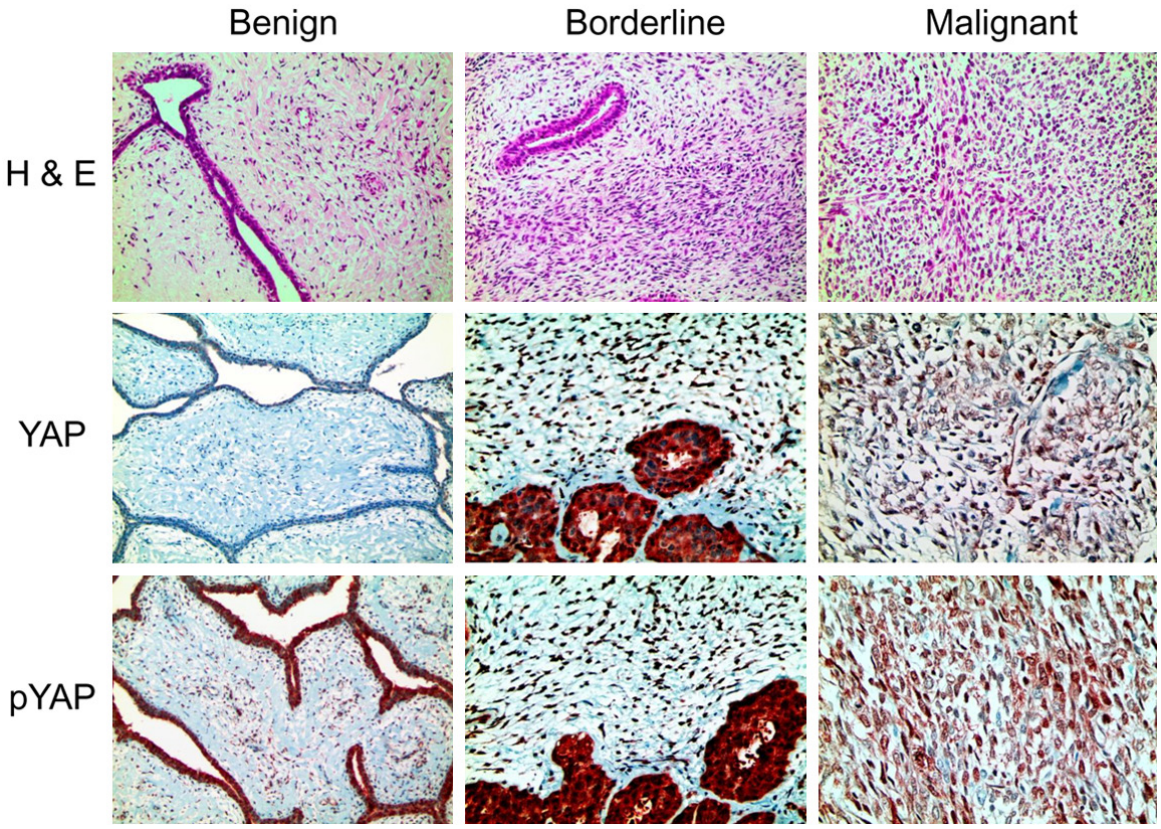

Figure 1. Expression for YAP and pYAP according to the histologic grade of phyllodes tumor. YAP and pYAP expressions in stromal component were identified more frequently in borderline and malignant PTs.

Expression of YAP and pYAP according to the histologic grade of phyllodes tumor

We performed immunohistochemical staining of YAP and pYAP on PTs with various histologic grades and then assessed their expression profiles by epithelial and stromal components (**Table 2** and **Figure 1**). Eight out of 183 PTs were excluded because we could not detect epithelial component in the TMA cores made from these eight cases. Although nuclear expression of YAP in epithelial component were rarely identified, cytoplasmic YAP expression was more frequently identified in benign and borderline tumors rather than in malignant PT ($P = 0.001$). pYAP expression of epithelial component was also increased in benign and borderline PTs rather than in malignant PT, but it was not statistically significant ($P = 0.194$). Therefore, we suggested that YAP might not play an important role as a transcription coactivator in the nucleus of epithelial component of PT because YAP protein was predominantly located in the cytoplasm. Next, we analyzed the expression profiles of YAP and pYAP in stromal component. Interestingly, nuclear YAP expression was more frequently identified in borderline and malignant PTs ($P = 0.002$). pYAP expression was also increased according to histologic grade: the worse histologic grade of PT, the more frequently expressed pYAP ($P < 0.001$). These result indicated that YAP expression was generally increased in stromal cells of high grade PTs.

Correlation between YAP and pYAP with clinicopathologic parameters

We investigated the associations of YAP and pYAP expressions of PTs and their clinicopathologic parameters (**Figure 2**). As a result, increased levels of YAP and pYAP expressions were associated with marked stromal cellularity, stromal atypia, increased number of stromal mitosis, and stromal overgrowth ($P < 0.05$). pYAP expression in stromal component was more frequently associated with infiltrative tumor margin rather than circumbstrcibed tumor margin ($P = 0.005$). On the other hand,

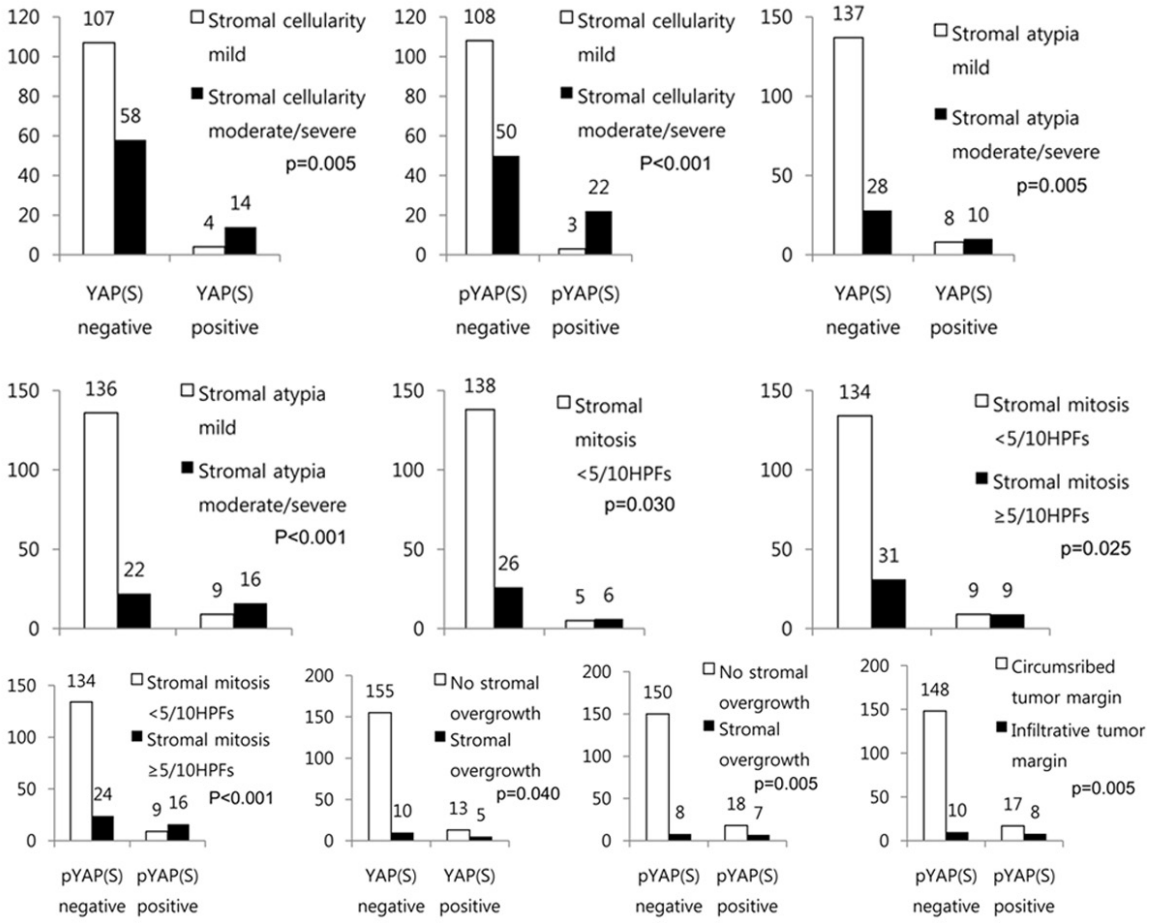

Figure 2. Correlation between clinicopathologic factors and YAP/pYAP expression in phyllodes tumor.

Table 3. The impact of YAP and pYAP expression on prognosis by univariate analysis using the log-rank test

Parameters	Total number/ recurrence/death	Disease-free survival		Overall survival	
		Median survival (95% CI) months	*p*-value	Median survival (95% CI) months	*p*-value
YAP(E-Nu)*			n/a		n/a
Negative	173/13/2	n/a		n/a	
Positive	2/0/0	n/a		n/a	
YAP (E-Cy)*			0.169		n/a
Negative	164/11/2	170 (163-177)		n/a	
Positive	11/2/0	99 (75-122)		n/a	
YAP (S)			< 0.001		0.051
Negative	165/11/5	170 (163-177)		177 (172-182)	
Positive	18/6/2	80 (56-105)		106 (90-121)	
pYAP (E)*			0.532		0.933
Negative	82/5/1	168 (159-177)		176 (172-180)	
Positive	93/8/1	166 (156-177)		180 (175-185)	
pYAP (S)			0.042		0.001
Negative	158/12/3	169 (161-176)		179 (176-183)	
Positive	25/5/4	120 (97-143)		126 (105-147)	

Abbreviations: E, epithelial component; S, stromal component; Nu, nuclear; Cy, cytoplasmic. *8 Cases without an epithelial component were excluded.

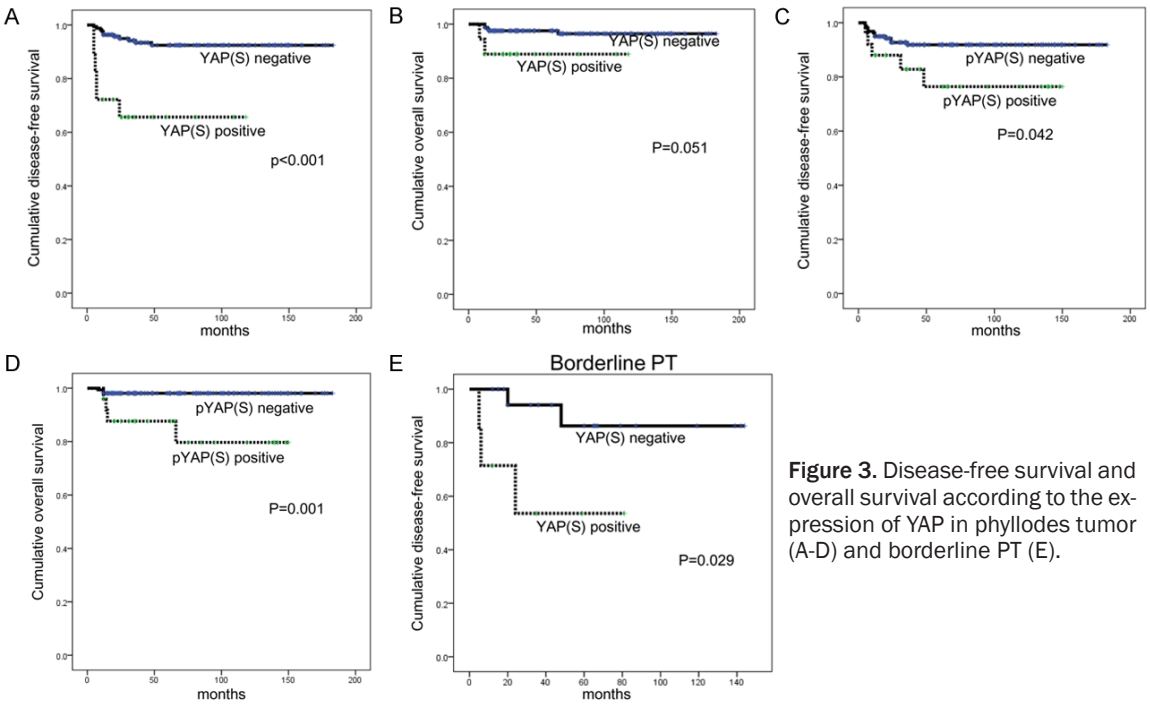

Figure 3. Disease-free survival and overall survival according to the expression of YAP in phyllodes tumor (A-D) and borderline PT (E).

Table 4. Multivariate analysis in patients with phyllodes tumors

Factor	*Disease-free survival*			*Overall survival*		
	Hazard ratio	*95% CI*	*P-value*	*Hazard ratio*	*95% CI*	*P-value*
Stromal cellularity			0.931			n/a
Mild *vs.* moderate/marked	0.914	0.119-7.011		n/a	n/a	
Stromal atypia			0.680			n/a
Mild *vs.* moderate/marked	0.639	0.076-5.372		n/a	n/a	
Stromal mitosis			0.108			n/a
0-4/10 HPFs *vs.* ≥ 5/10 HPFs	8.063	0.631-103.0		n/a	n/a	
Stromal overgrowth			0.041			n/a
Absent *vs.* present	4.008	1.059-15.16		n/a	n/a	
Tumor margin			0.360			n/a
Circumscribed *vs.* infiltrative	0.549	0.152-1.985		n/a	n/a	
YAP (S)			0.050			0.799
Negative *vs.* Positive	3.206	1.000-10.27		1.314	0.161-10.733	
pYAP (S)			0.661			0.110
Negative *vs.* Positive	1.309	0.393-4.364		4.708	0.704-31.48	

HPFs, high-power fields.

cytoplasmic YAP expression of epithelial component was associated with increased stromal cellularity ($P = 0.030$).

Impact of the expression of YAP and pYAP on patient prognosis

According to the above results, we could find that YAP and pYAP expressions of stromal component were related to higher histologic grade and histologic features which were suggested with malignancy of PT. Therefore, we determined to clarify the association of YAP and pYAP expression and patient's prognosis of PT (**Table 3**). First, we produced Kaplan-Meier survival curves according to YAP and pYAP expression status by tumor components (**Figure 3**). As expected, patient with PTs which demonstrated increased level of YAP expression in the stromal component had shorter disease-free survival

(DFS, $P < 0.001$, **Figure 3A**) and overall survival (OS, $P = 0.051$, **Figure 3B**). In like manner, patient with PTs which demonstrated increased level of pYAP expression in the stromal component had shorter DFS ($P = 0.042$, **Figure 3C**) and OS ($P = 0.001$, **Figure 3D**). When we analyzed patients' survival according to the histologic grade of PT, YAP expression of the stromal component was related to decreased DFS in borderline PT ($P = 0.029$, **Figure 3E**). Next, we analyzed statistical correlation of YAP with several predictable variables and patients' survival of PTs using multivariate Cox regression (**Table 4**). As a result, only stromal overgrowth demonstrated about fourfold increase of hazard ratio in patient's DFS (hazard ratio: 4.008, 95% CI: 1.059-15.16, $P = 0.041$). Interestingly, stromal YAP expression was independently associated with increased DFS (Hazard ration: 3.206, 95% CI: 1.000-10.27, $P = 0.050$).

Discussion

YAP is a recently identified oncogenic transcription coactivator [15]. As a causative oncogene found in 11q22 amplicon frequently observed in human cancer, YAP enhances invasion and proliferation, suppresses apoptosis and is sufficient for transformation [16]. Hippo pathway consists of a kinase cascade including Lats kinase, the mammalian homolog of Warts [17], and YAP is phosphorylated by Lats kinase to be sequestered from the nucleus by 14-3-3 [5]. However, the functions of YAP had remained very poorly understood until the discovery of the Hippo tumor suppressor pathway.

Although PT which belongs to fibroepithelial tumor of breast has a malignant potential, there is no standard immunohistochemical panel helping to predict of biologic behavior of PT. Previous reports demonstrated increased level of YAP expression in lobular carcinoma [13] and the relation of YAP expression and ER/PR status in breast cancer [14]. In mesenchymal origins, YAP expression of tumor cells in osteosarcoma [18], Ewing sarcoma [19], and undifferentiated pleomorphic sarcoma [20] have been studied. In this study, we assessed expression profiles of YAP and pYAP in benign, borderline, and malignant PTs according to their epithelial and stromal components.

Interestingly, we could find that stromal expressions of YAP and pYAP were increased in borderline and malignant PTs with comparison of benign PT along with other malignant mesenchymal tumors. As well, YAP expression in the stromal component was associated with increased stromal cellularity, increased stromal atypia, increased stromal mitosis, stromal overgrowth, and infiltrative tumor margin, which have been known as histologic features of malignant PT. This result is compatible with the previous reports about malignant mesenchymal tumors of which YAP expression were associated with tumor progression, tumor growth, and tumor staging [18-20].

We thought that the above results suggested the stromal overexpression of YAP and pYAP might be associated with patient's prognosis because tumor aggressiveness is determined by the histologic features of stromal component in PT. As expected, we could determine that YAP expression in stromal component was related to the poor prognosis. Other researchers also found the correlation of YAP expression and poor prognosis in various epithelial tumors: ovarian cancer [6], urinary bladder cancer [7], colorectal cancer [8], esophageal cancer [9], stomach cancer [10], and lung cancer [11]. The excitement of this study was that stromal YAP expression in borderline PTs was related to shorter DFS and this suggested the possibility of YAP protein as a prognosis predictor for borderline PT.

Conventional treatment of choice for PTs is a surgical resection. In consideration of target therapy, it is needed to develop methods of inactivation of YAP protein which might be more frequently expression in borderline and malignant PTs than in benign PT. Experimental studies proposed the suggestions by presenting the decrease of cancer cell migration and proliferation *in vitro* [21, 22] and the inhibition of tumor growth *in vivo* [21] by YAP inactivation.

We believe that this study is the first to reveal the expression profiles of YAP and pYAP in PTs according to their histologic grades and cellular components. Furthermore, we could find the clinical implication of YAP and pYAP expressions in PT as an indicator of tumor progression and a poor prognostic marker.

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Disclosure of conflict of interest

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