

Original Article

Yes-associated protein (YAP) is differentially expressed in tumor and stroma according to the molecular subtype of breast cancer

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Abstract: In this study, we aimed to clarify the expression profiles of Yes-associated protein (YAP) and phosphorylated YAP (pYAP) protein and to verify the clinical implication of the expression of YAP protein in human breast cancer. We selected 678 cases of formalin-fixed paraffin-embedded (FFPE) breast cancer tissue to construct tissue microarray (TMA) blocks. We performed immunohistochemical staining of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth receptor-2 (HER-2) and Ki-67 and fluorescent in situ hybridization (FISH) assay for HER-2 on the TMA sections and divided breast cancers into molecular subtypes: luminal A, luminal B, HER-2, triple negative breast cancer (TNBC). Then, we examined YAP and pYAP expression status using immunohistochemical analysis according to the molecular subtypes of breast cancer. We found that HER-2 type breast cancer demonstrated elevated expression level in tumoral cytoplasmic YAP ($P = 0.011$) and pYAP ($P = 0.049$). Expressions of stromal YAP ($P = 0.002$) and pYAP ($P < 0.001$) were higher in luminal B and HER-2 type breast cancer but lower in TNBC. In univariate analysis, nuclear YAP expression of tumor cells was associated with shorter overall survival (OS) ($P = 0.024$). Cytoplasmic YAP expression of HER-2 type breast cancer cells negatively affected disease-free survival (DFS) ($P = 0.034$). In conclusion, we concluded that there was a significant difference in YAP and pYAP expression status according to molecular subtypes and tumoral and cellular components of breast cancers. Finally, we found that nuclear and cytoplasmic YAP expression could be a prognostic marker for breast cancer patients.

Keywords: Breast cancer, molecular subtype, YAP

Introduction

Yes-Associated Protein (YAP) is a recently identified oncogenic transcription coactivator. As a causative oncogene found in the 11q22 amplicon that is frequently observed in human cancer, YAP enhances invasion and proliferation, suppresses apoptosis and is sufficient for transformation [1]. Finally, YAP is the downstream effector molecule of a newly emerging tumor suppressor pathway called the Hippo pathway [2]. Thus, understanding the function of YAP may provide insight to universally conserved control mechanisms of tumorigenesis and tumor progression.

When YAP is phosphorylated on serine 127 by Lats kinase, phosphorylated YAP is sequestered from the nucleus by 14-3-3 and transcription activities of target genes are thus

decreased [3]. In contrast, YAP overexpression, or nuclear localization of YAP, was frequently found in many cancers [4]. Recently, many researchers reported the functions of YAP in breast cancers, but these mechanisms still remain poorly understood. Several studies demonstrated YAP as a tumor suppressor in breast cancer, showing decreased level of YAP expression in human breast cancer tissue relative to normal breast tissue [5], increased cell migration and invasiveness of YAP-downregulated breast cancer cells *in vitro*, and increased tumor growth in the YAP-knockout mouse *in vivo* [6]. On the contrary, other researchers have asserted that YAP acts as an oncogene in breast cancer, demonstrating that YAP overexpression enhanced cellular proliferation [7], and xenograft mice transplanted with a YAP-overexpressing breast cancer cell line promoted tumor formation and growth in compari-

son to the control tumor group [8]. Therefore, it is necessary to clarify the expression status of YAP in human breast cancer tissue and its clinical implications in breast cancer.

In this study, we compared YAP and phosphorylated YAP (pYAP) expression profiles in breast cancers according to their molecular subtypes and cellular compartments, and then evaluated YAP as a prognostic factor in breast cancer patients.

Materials and methods

Patient selection

Patients diagnosed with invasive breast cancer and treated by surgical resection during January 2002 to December 2006 were included in this study. Patients who received preoperative neoadjuvant chemotherapy or hormonal treatment were excluded. This study was approved by the Institutional Review Board of Yonsei University Severance Hospital. A breast pathologist (Koo JS) retrospectively reviewed the histology of all cases using hematoxylin-eosin (H&E) stained slides. The histological grade was assessed using the Nottingham grading system [9]. Clinicopathologic parameters evaluated in each case 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 the corresponding spot was marked on the surface of the paraffin block. Using a biopsy needle, the selected area was punched out and a 3-mm tissue core was placed into a 6 × 5 recipient block. Tissue from the invasive tumor was then extracted. Two tissue cores were extracted to minimize extraction bias. Each tissue core was assigned a unique tissue microarray (TMA) location number that was linked to a database containing other clinicopathologic data.

Immunohistochemistry

The antibodies used for immunohistochemistry in this study are shown in **Table 1**. We performed immunohistochemical staining on formalin-fixed paraffin-embedded (FFPE) tissue

using TMA. Briefly, 5- μ m-thick sections were obtained with a microtome, transferred onto adhesive slides, and dried at 62°C for 30 minutes. After incubation with primary antibodies (**Table 1**), 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 per manufacturer's recommendation. Slides were counterstained with Harris hematoxylin.

Interpretation of immunohistochemical staining

All immunohistochemical markers were assessed by light microscopy. A cut-off value of 1% or more positively stained nuclei was used to define estrogen receptor (ER) and progesterone receptor (PR) positivity [10]. Human epidermal growth receptor-2 (HER-2) 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, less than 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 [11]. HER-2 immunostaining was considered positive when strong (3+) membranous staining was observed whereas cases with 0 to 1+ were regarded as negative. Cases showing 2+ HER-2 expression were evaluated for HER-2 amplification by fluorescent *in situ* hybridization (FISH).

Expression status of YAP and pYAP in tumor cells and stromal cells was assessed according to the cellular compartments such as nucleus and cytoplasm. Immunostaining result was graded 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). We determined positive reactivity when moderate (grade 2) or strong (grade 3) positive cancer cells were identified over 10% of the tumor area.

Ki-67 labeling indices (LI) were scored by counting the number of positively stained nuclei and expressed as a percentage of total tumor cells.

YAP in breast cancer

Table 1. Source, clone, and dilution of antibodies used in this study

antibody	Company	clone	dilution
<i>YAP related</i>			
YAP	Santa Cruz Biotechnology, INC., California, USA	9A1	1:100
Phosphate YAP (ser127)	Abcam, Cambridge, UK	EP1675Y	1:100
<i>Molecular subtype related</i>			
ER	Thermo Scientific, San Diego, CA, USA	SP1	1:100
PR	DAKO, Glostrup, Denmark	PgR	1:50
HER-2	DAKO, Glostrup, Denmark	Polyclonal	1:1500
Ki-67	Abcam, Cambridge, UK	MIB	1:1000

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

Parameters	Total (N = 678) (%)	Luminal A (n = 280) (%)	Luminal B (n = 156) (%)	HER-2 (n = 67) (%)	TNBC (n = 175) (%)	P-value
Age (years)						0.022
≤50	403 (59.4)	160 (57.1)	105 (67.3)	31 (46.3)	107 (61.1)	
>50	275 (40.6)	120 (42.9)	51 (32.7)	36 (53.7)	68 (38.9)	
Histologic grade						<0.001
I/II	453 (66.8)	253 (90.4)	106 (67.9)	35 (52.2)	59 (33.7)	
III	225 (33.2)	27 (9.6)	50 (32.1)	32 (47.8)	116 (66.3)	
Tumor stage						0.014
T1	323 (47.6)	149 (53.2)	77 (49.4)	31 (46.3)	66 (37.7)	
T2/T3	355 (52.4)	131 (46.8)	79 (50.6)	36 (53.7)	109 (62.3)	
Nodal metastasis						0.163
Absent	400 (59.0)	158 (56.4)	86 (55.1)	41 (61.2)	115 (65.7)	
Present	278 (41.0)	122 (43.6)	70 (44.9)	26 (38.8)	60 (34.3)	
Estrogen receptor status						<0.001
Negative	252 (37.2)	5 (1.8)	5 (3.2)	67 (100.0)	175 (100.0)	
Positive	426 (62.8)	275 (98.2)	151 (96.8)	0 (0.0)	0 (0.0)	
Progesterone receptor status						<0.001
Negative	336 (49.6)	46 (16.4)	48 (30.8)	67 (100.0)	175 (100.0)	
Positive	342 (50.4)	234 (83.6)	108 (69.2)	0 (0.0)	0 (0.0)	
HER-2 status						<0.001
Negative	531 (78.3)	280 (100.0)	76 (48.7)	0 (0.0)	175 (100.0)	
Positive	147 (21.7)	0 (0.0)	80 (51.3)	67 (100.0)	0 (0.0)	
Ki-67 LI (%)						<0.001
≤14	380 (56.0)	280 (100.0)	46 (29.5)	27 (40.3)	27 (15.4)	
>14	298 (44.0)	0 (0.0)	110 (70.5)	40 (59.7)	148 (84.6)	
Tumor recurrence	60 (8.8)	15 (5.4)	13 (8.3)	9 (13.4)	23 (13.1)	0.018
Patients' death	58 (8.6)	13 (4.6)	13 (8.3)	9 (13.4)	23 (13.1)	0.007
Duration of clinical follow-up (months, mean ± SD)	70 ± 31	72 ± 29	70 ± 31	66 ± 35	67 ± 33	0.390

TNBC, triple negative breast cancer.

Tumor phenotype classification

In this study, we classified breast cancer phenotypes according to the immunohistochemistry results for ER, PR, HER-2, and Ki-67 LI. FISH results for HER-2 were as follows [12]: *luminal A type*: ER and/or PR positive, HER-2 negative, and Ki-67 LI <14%; *luminal B type*: (HER-2 negative) ER and/or PR positive, HER-2 negative, and Ki-67 LI ≥14% and (HER-2 positive) ER and/or PR positive and HER-2 overexpressed

and/or amplified; *HER-2 type*: ER and PR negative and HER-2 overexpressed and/or amplified; *TNBC type*: ER, PR, and HER-2 negative.

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.

YAP in breast cancer

Table 3. Expression of YAP and pYAP according to breast cancer phenotype

Parameters	Total (N = 678) (%)	Luminal A (n = 280) (%)	Luminal B (n = 156) (%)	HER-2 (n = 67) (%)	TNBC (n = 175) (%)	P-value
YAP (T-Nu)						0.181
Negative	636 (93.8)	261 (93.2)	152 (97.4)	62 (92.5)	161 (92.0)	
Positive	42 (6.2)	19 (6.8)	4 (2.6)	5 (7.5)	14 (8.0)	
YAP (T-Cy)						0.011
Negative	646 (95.3)	265 (94.6)	151 (96.8)	59 (88.1)	171 (97.7)	
Positive	32 (4.7)	15 (5.4)	5 (3.2)	8 (11.9)	4 (2.3)	
YAP (S)						0.002
Negative	574 (84.7)	246 (87.9)	121 (77.6)	51 (76.1)	156 (89.1)	
Positive	104 (15.3)	34 (12.1)	35 (22.4)	16 (23.9)	19 (10.9)	
pYAP (T-Cy)						0.049
Negative	541 (79.8)	224 (80.0)	129 (82.7)	45 (67.2)	143 (81.7)	
Positive	137 (20.2)	56 (20.0)	27 (17.3)	22 (32.8)	32 (18.3)	
pYAP (T-Nu)						0.449
Negative	672 (99.1)	279 (99.6)	155 (99.4)	66 (98.5)	172 (98.3)	
Positive	6 (0.9)	1 (0.4)	1 (0.6)	1 (1.5)	3 (1.7)	
pYAP (S)						<0.001
Negative	615 (90.7)	261 (93.2)	129 (82.7)	57 (85.1)	168 (96.0)	
Positive	63 (9.3)	19 (6.8)	27 (17.3)	10 (14.9)	7 (4.0)	

TNBC, triple negative breast cancer.

Statistical significance was reached 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 the Cox proportional hazards model.

Results

Patients' clinicopathologic characteristics

First, we investigated clinical features of breast cancer patients from the medical records and reviewed pathologic features of patients' breast cancer tissue (**Table 2**). In total, included cases of breast cancers were 280 cases of luminal A type (41.3%), 156 cases of luminal B type (23.0%), 67 cases of HER-2 type (9.9%), and 175 cases of triple negative breast cancer (TNBC) type (25.8%). TNBC-type breast cancers demonstrated higher histologic grade ($P < 0.001$), higher T stage ($P = 0.014$), and higher Ki-67 LI ($P < 0.001$) than other molecular subtypes. HER-2 type breast cancer was associated with older age at diagnosis ($P = 0.022$), more frequent tumor recurrence ($P = 0.018$) and lower survival rate ($P = 0.007$) than other molecular subtypes.

Expression of YAP and pYAP according to molecular subtypes of breast cancer

Next, we performed immunohistochemical staining for identification of YAP and pYAP expression status according to the molecular subtype of breast cancer (**Table 3** and **Figure 1**). We further evaluated expression profiles of YAP and pYAP dividing cellular components into the nucleus and cytoplasm. Our results showed increased tumoral cytoplasmic YAP expression ($P = 0.011$) and tumoral cytoplasmic pYAP expression ($P = 0.049$) in HER-2 type breast cancer tissue compared to other molecular subtypes. In stromal component, YAP ($P = 0.002$) and pYAP ($P < 0.001$) were increased in luminal B and HER-2 type breast cancer tissues but relatively decreased in TNBC type breast cancer tissue. However, there was no significant difference in nuclear YAP expression according to the molecular subtype of breast cancer.

Correlation between expression of YAP and pYAP proteins and clinicopathologic factors

We investigated various clinicopathologic parameters known to affect patients' prognosis according to expression status of YAP and pYAP:

YAP in breast cancer

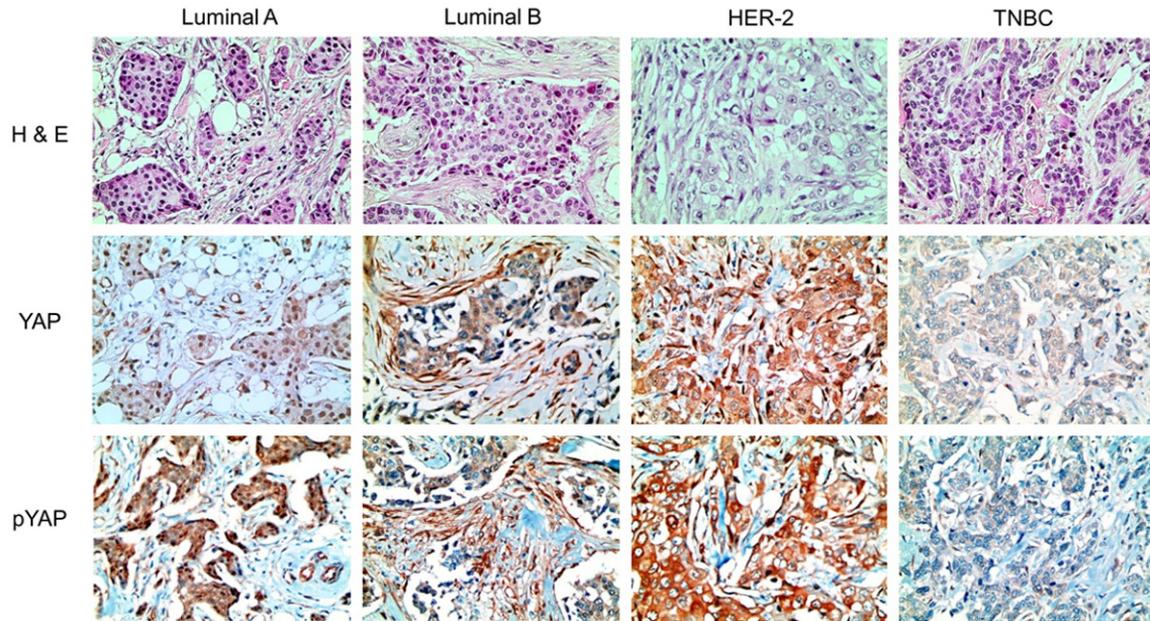


Figure 1. Expression of YAP and pYAP according to the molecular subtype of breast cancer. HER-2 type breast cancer demonstrated cytoplasmic YAP and pYAP in cancer cells. Luminal B and HER-2 type breast cancer revealed a higher expression rate of stromal YAP and pYAP, but TNBC did not. Microscopic magnification, 200X.

age at diagnosis, tumor stage, nodal metastasis, histologic grade, ER status, PR status, HER-2 status, and Ki-67 L.I. (**Tables 4 and 5**). Both YAP expression ($P = 0.001$) and pYAP expression ($P = 0.001$) were increased in the stromal cells of HER-2 type breast cancer. Cytoplasmic pYAP expression was associated lower tumor stage ($P = 0.003$). However, nuclear YAP expression in tumor cells was not associated with any significant differences in various clinicopathologic parameters.

Impact of expression of YAP and pYAP proteins on patient prognosis

We analyzed overall survival (OS) rate and disease-free survival (DFS) rate of breast cancer patients according to YAP and pYAP expression using Kaplan-Meier survival curve (**Figure 2**). Distinctively, patients with breast cancer who showed nuclear YAP expression in tumor cells had inferior OS compared to patients with nuclear YAP-negative breast cancer (**Figure 2A**, $P = 0.024$). When we analyzed OS and DFS according to the molecular subtype of breast cancer, cytoplasmic YAP expression of tumor cells in HER-2 type negatively affected DFS of breast cancer patients (**Figure 2B**, $P = 0.034$). However, nuclear YAP expression of tumor cells did not affect either OS or DFS in each molecular subtype of breast cancer.

Next, we investigated predictable variables associated with patients' survival according to YAP expression status using Cox regression analysis (**Table 6**). In univariate analysis, only nuclear YAP expression of tumor cells was correlated with shorter OS ($P = 0.024$) and demonstrated a tendency of negative correlation with DFS ($P = 0.079$).

When the predictable parameters were adjusted for by multivariate analysis (**Table 7**), a tendency for nuclear YAP expression of tumor cells to independently affect inferior OS was revealed (Hazard ratio: 2.073, 95% CI: 0.973-4.416, $P = 0.059$). As expected, lymph node metastasis was associated with shorter DFS (Hazard ratio: 2.210, 95% CI: 1.295-3.771, $P = 0.004$) and shorter OS (Hazard ratio: 1.879, 95% CI: 1.098-3.216, $P = 0.021$). Higher tumor stage was also associated with shorter DFS (Hazard ratio: 2.024, 95% CI: 1.109-3.693, $P = 0.022$).

Discussion

We investigated the expression profiles of YAP and pYAP in various molecular subtypes of breast cancers. Previous studies have reported the expression frequency of YAP in breast cancer to range from 45-75%, which is much higher than our results showing 6.2% of tumors having nuclear expression and 4.7% of tumors having

YAP in breast cancer

Table 4. Correlations between the expression of YAP and clinicopathologic parameters

Parameters	YAP (T-Nu)			YAP (T-Cy)			YAP (S)		
	Negative n = 636 (%)	Positive n = 42 (%)	P-value*	Negative n = 646 (%)	Positive n = 32 (%)	P-value*	Negative n = 574 (%)	Positive n = 104 (%)	P-value*
Age (years)			0.737			0.138			0.046
≤50	377 (59.3)	26 (61.9)		388 (60.1)	15 (46.9)		332 (57.8)	71 (68.3)	
>50	259 (40.7)	16 (38.1)		258 (39.9)	17 (53.1)		242 (42.2)	33 (31.7)	
Histologic grade			0.512			0.812			0.908
I/II	423 (66.5)	30 (71.4)		431 (66.7)	22 (68.8)		383 (66.7)	70 (67.3)	
III	213 (33.5)	12 (28.6)		215 (33.3)	10 (31.2)		191 (33.3)	34 (32.7)	
Tumor stage			0.522			0.784			0.756
T1	305 (48.0)	18 (42.9)		307 (47.5)	16 (50.0)		272 (47.4)	51 (49.0)	
T2/T3	331 (52.0)	24 (57.1)		339 (52.5)	16 (50.0)		302 (52.6)	53 (51.0)	
Nodal metastasis			0.564			0.435			0.938
Absent	377 (59.3)	23 (54.8)		379 (58.7)	21 (65.6)		339 (59.1)	61 (58.7)	
Present	259 (40.7)	19 (45.2)		267 (41.3)	11 (34.4)		235 (40.9)	43 (41.3)	
Estrogen receptor status			0.264			0.678			0.715
Negative	233 (36.6)	19 (45.2)		239 (37.0)	13 (40.6)		215 (37.5)	37 (35.6)	
Positive	403 (63.4)	23 (54.8)		407 (63.0)	19 (59.4)		359 (62.5)	67 (64.4)	
Progesterone receptor status			0.706			0.959			0.743
Negative	314 (49.4)	22 (52.4)		320 (49.5)	16 (50.0)		286 (49.8)	50 (48.1)	
Positive	322 (50.6)	20 (47.6)		326 (50.5)	16 (50.0)		288 (50.2)	54 (51.9)	
HER-2 status			0.230			0.074			0.001
Negative	495 (77.8)	36 (85.7)		510 (78.9)	21 (65.6)		462 (80.5)	69 (66.3)	
Positive	141 (22.2)	6 (14.3)		136 (21.1)	11 (34.4)		112 (19.5)	35 (33.7)	
Ki-67 LI (%)			0.639			0.138			0.177
≤14	355 (55.8)	25 (59.5)		358 (55.4)	22 (68.8)		328 (57.1)	52 (50.0)	
>14	281 (44.2)	17 (40.5)		288 (44.6)	10 (31.2)		246 (42.9)	52 (50.0)	

*P-value was corrected by the Bonferroni method.

YAP in breast cancer

Table 5. Correlations between the expression of pYAP and clinicopathologic parameters

Parameters	pYAP (T-Cy)			pYAP (T-Nu)			pYAP (S)		
	Negative n = 541 (%)	Positive n = 137 (%)	P-value*	Negative n = 672 (%)	Positive n = 6 (%)	P-value*	Negative n = 615 (%)	Positive n = 63 (%)	P-value*
Age (years)			0.066			0.691			0.882
≤50	331 (61.2)	72 (52.6)		400 (59.5)	3 (50.0)		365 (59.3)	38 (60.3)	
>50	210 (38.8)	65 (47.4)		272 (40.5)	3 (50.0)		250 (40.7)	25 (39.7)	
Histologic grade			0.755			0.994			0.087
I/II	363 (67.1)	90 (65.7)		449 (66.8)	4 (66.7)		417 (67.8)	36 (57.1)	
III	178 (32.9)	47 (34.3)		223 (33.2)	2 (33.3)		198 (32.2)	27 (42.9)	
Tumor stage			0.003			0.688			0.113
T1	242 (44.7)	81 (59.1)		321 (47.8)	2 (33.3)		287 (46.7)	36 (57.1)	
T2/T3	299 (55.3)	56 (40.9)		351 (52.2)	4 (66.7)		328 (53.3)	27 (42.9)	
Nodal metastasis			0.723			1.000			0.753
Absent	321 (59.3)	79 (57.7)		396 (58.9)	4 (66.7)		364 (59.2)	36 (57.1)	
Present	220 (40.7)	58 (42.3)		276 (41.1)	2 (33.3)		251 (40.8)	27 (42.9)	
Estrogen receptor status			0.161			0.202			0.227
Negative	194 (35.9)	58 (42.3)		248 (36.9)	4 (66.7)		233 (37.9)	19 (30.2)	
Positive	347 (64.1)	79 (57.7)		424 (63.1)	2 (33.3)		382 (62.1)	44 (69.8)	
Progesterone receptor status			0.864			0.447			0.264
Negative	269 (49.7)	67 (48.9)		332 (49.4)	4 (66.7)		309 (50.2)	27 (42.9)	
Positive	272 (50.3)	70 (51.1)		340 (50.6)	2 (33.3)		306 (49.8)	36 (57.1)	
HER-2 status			0.090			1.000			0.001
Negative	431 (79.7)	100 (73.0)		526 (78.3)	5 (83.3)		492 (80.0)	39 (61.9)	
Positive	110 (20.3)	37 (27.0)		146 (21.7)	1 (16.7)		123 (20.0)	24 (38.1)	
Ki-67 LI (%)			0.815			0.413			0.251
≤14	302 (55.8)	78 (56.9)		378 (56.2)	2 (33.3)		349 (56.7)	31 (49.2)	
>14	239 (44.2)	59 (43.1)		294 (43.8)	4 (66.7)		266 (43.3)	32 (50.8)	

*P-value was corrected by the Bonferroni method.

YAP in breast cancer

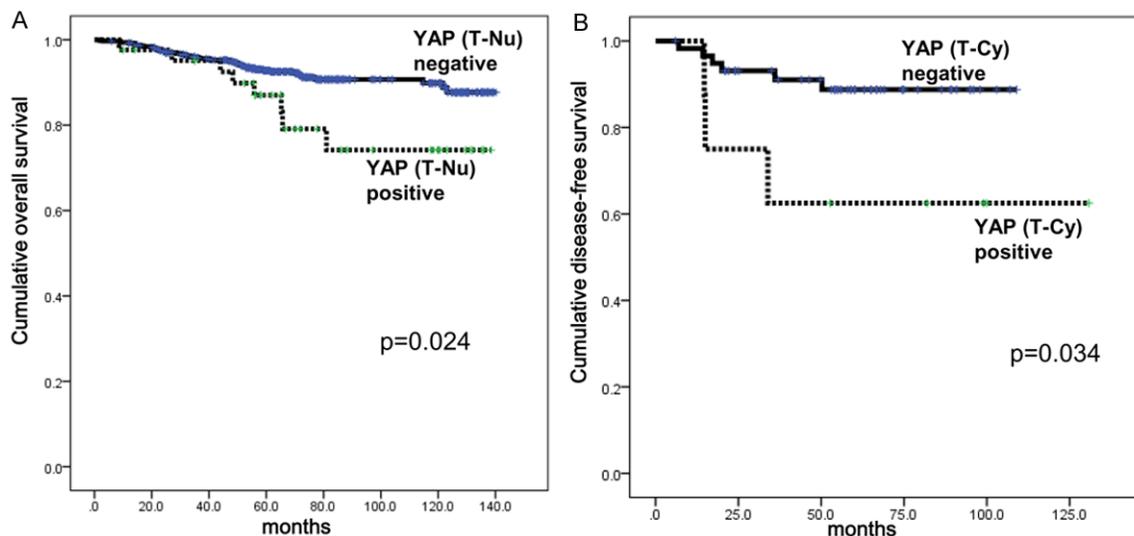


Figure 2. Survival analysis of breast cancer patients according to the expression status of YAP protein. A. Overall survival curve according to the nuclear expression status of YAP in breast cancer cells. B. Disease-free survival curve according to the cytoplasmic expression status of YAP in HER-2 type breast cancer cells. T-Nu, nuclear expression of tumor cells. T-Cy, cytoplasmic expression of tumor cells.

cytoplasmic expression [5, 8]. We believe that this difference may be explained by the cut-off value of positive reactivity. In this study, we determined positive reactivity of YAP protein when moderately (grade 2) or strongly (grade 3) positive cancer cells were identified over 10% of tumor area, because normal luminal cells or myoepithelial cells also had weak positivity of YAP protein. Based on the same criteria, a previous report indicated that 62% of breast cancers had moderate positivity and only 6% had strong positivity [13]; and in another study, 29% of breast cancers demonstrated YAP [8].

As YAP protein functions as a transcription coactivator in the nucleus and phosphorylated YAP protein is sequestered in the cytoplasm, it is needed to localize YAP and pYAP protein to identify the oncogenic function of YAP in breast cancer cells. We divided cellular components such as the nucleus and cytoplasm and sorted through the expression status of YAP and pYAP in each component using immunohistochemical assay. Our results showed that the expression of YAP protein differed between each molecular subtype of breast cancer. We discovered that HER-2 type breast cancer demonstrated increased expression level of cytoplasmic YAP and pYAP among subtypes, although previous studies reported that YAP expression of breast cancer cells was associated with inva-

sive lobular carcinoma [14] and ER/PR positivity [5]. As we mentioned above, YAP protein is phosphorylated in the cytoplasm by a kinase cascade associated with the Hippo signaling pathway. Because HER-2 is a transmembrane receptor which has an intrinsic tyrosine kinase [15], we suggest that cytoplasmic overexpression of YAP and pYAP, but not nuclear overexpression, in HER-2 type breast cancer could be due to the possibility that HER-2 amplification/overexpression increases intrinsic kinase activity and, thus, a significant amount of YAP protein can be phosphorylated in the cytoplasm. However, the precise mechanism of increased phosphorylation of YAP in HER-2 type breast cancer should be studied further. Unexpectedly, the nuclear pYAP expression in a few cases of breast cancer ranged from 0.4 to 1.7% according to molecular subtype. This result may be due to non-specific or over staining and nuclear pYAP expression may not, in fact, be associated with any clinicopathologic features of breast cancer.

This is the first study to focus on the stromal expression of YAP and pYAP proteins in breast cancer. YAP and pYAP expression of stromal cells were increased in luminal B type and HER-2 type breast cancers but decreased in TNBC. We struggled to understand this phenomenon and considered the possibility of

YAP in breast cancer

Table 6. Univariate analysis of the impact of the expression of YAP and pYAP in breast cancers on disease-free survival or overall survival by log-rank test

Parameters	Number of patients/ recurrence/death	Disease-free survival		Overall survival	
		Mean survival (95% CI) months	P-value	Mean survival (95% CI) months	P-value
YAP (T-Nu)			0.079		0.024
Negative	636/53/50	126 (123-130)		130 (127-133)	
Positive	42/7/8	111 (99-123)		116 (103-130)	
YAP (T-Cy)			0.579		0.924
Negative	646/56/55	127 (124-130)		129 (126-132)	
Positive	32/4/3	117 (105-129)		124 (115-134)	
YAP (S)			0.163		0.110
Negative	574/55/54	124 (120-128)		128 (125-131)	
Positive	104/5/4	132 (126-138)		134 (130-139)	
pYAP (T-Cy)			0.920		0.894
Negative	541/49/47	127 (123-130)		129 (126-132)	
Positive	137/11/11	117 (110-124)		127 (120-134)	
pYAP (T-Nu)			n/a		n/a
Negative	672/60/58	n/a		n/a	
Positive	6/0/0	n/a		n/a	
pYAP (S)			0.981		0.894
Negative	615/55/53	126 (122-130)		129 (126-132)	
Positive	63/5/5	98 (92-103)		121 (114-128)	

Table 7. Multivariate analysis of breast-cancer survival

Included parameters	Disease-free survival			Overall survival		
	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
T stage			0.022			0.100
T1 versus T2-3	2.024	1.109-3.693		1.629	0.910-2.914	
N stage			0.004			0.021
N0 versus N1-3	2.210	1.295-3.771		1.879	1.098-3.216	
Histologic grade			0.507			0.726
I/II versus III	1.213	0.686-2.144		0.900	0.500-1.621	
ER status			0.291			0.223
Negative versus Positive	1.496	0.709-3.158		1.575	0.758-3.272	
PR status			0.274			0.086
Negative versus Positive	1.534	0.713-3.300		1.976	0.909-4.296	
HER-2 status			0.531			0.618
Negative versus Positive	1.208	0.669-2.183		1.164	0.640-2.115	
YAP (T-Nu)			0.125			0.059
Negative versus Positive	1.867	0.841-4.147		2.073	0.973-4.416	

mechanotransduction of tumor cells in surrounding stromal cells regulated by the Hippo signaling pathway. It is known that the actin cytoskeleton could decrease the phosphorylation of YAP as a key molecule of the Hippo signaling pathway and de-phosphorylated YAP is translocated into the nucleus, increasing tran-

scripts of downstream growth factors such as CCN1/CYR61 and CCN2/CTGF [16, 17]. In breast cancer, the concept of cancer associated fibroblasts consisting of tumor stroma is explained by several types of cells with a well-known component being the myofibroblast in which α -smooth muscle actin is expressed

[18]. To sum up these findings, we believe that YAP and pYAP expression of tumor stromal cells composed of cancer-associated fibroblasts may be affected by cancer cells.

In this study, we investigated the prognosis of patients with nuclear YAP-expressing breast cancer. When the survival analysis included all subtypes of breast cancer, nuclear YAP expression of the cancer cells was associated with inferior OS. A detailed analysis of molecular subtypes showed cytoplasmic YAP expression of tumor cells in HER-2 type breast cancer was related to shorter DFS. In the same manner, previous studies have suggested that YAP expression is associated with poor prognosis in various solid tumors including ovarian cancer [19], urinary bladder cancer [20], colorectal cancer [21], esophageal cancer [22], stomach cancer [23], and lung cancer [24]. In breast cancer, it has been thought that YAP was not an independent prognostic marker [13]. Accordingly, we also did not consider YAP expression to be an independent predictable parameter in Cox multivariate analysis. Although only one report has demonstrated that decreased levels of YAP expression in luminal A type breast cancer was associated with decreased DFS [25], it is necessary to verify the effect of YAP expression of each molecular subtype on patients' prognosis.

To conclude, YAP and pYAP proteins were expressed differently according to the molecular subtypes of breast cancer and its cellular components. In this study, we show that increased nuclear YAP expression correlated with decreased OS in breast cancer and overexpression of cytoplasmic YAP in HER-2 type breast cancer was associated with shorter DFS.

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Disclosure of conflict of interest

None.

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YAP in breast cancer

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