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Yes-associated protein expression in paired primary and local recurrent breast cancer and its clinical significance

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ABSTRACT

Yes-associated protein (YAP) protein acts as tumorigenic factor in many solid tumors, but the situation in breast cancer is under debate. Here, we would analyze its status in breast cancer. YAP expression in the 110 primary breast cancer and their paired local recurrent tumors was investigated. Clinicopathologic data for age, histologic grading, hormone status, lymph nodes and HER2 status were also gathered and analyzed. 46.4% (51/110) primary breast cancer tissues were positive for total YAP expression which was significantly higher than that in the recurrent tissues (10.9%; $P < 0.05$). The expression of total YAP protein in the primary breast cancer tissues was positively associated with the tumor size, especially in triple negative breast cancer (TNBC) subtype ($P < 0.05$). Higher total or nuclear YAP expression in the primary tumor was correlated with poor disease-free survival among patients with TNBC ($P < 0.05$). In the multivariate models, nuclear YAP expression was an independently prognostic factor in TNBC. High total or nuclear YAP expression predicts poor prognosis among patients with TNBC. It might be a therapeutic target for TNBC in the future.

Abbreviations: YAP, yes-associated protein; TNBC, triple-negative breast cancer; IHC, immunohistochemistry.

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Introduction

Yes-associated protein (YAP) is a transcriptional coactivator implicated in organ size control, tissue regeneration, and stem cell self-renewal.[1] As a downstream effector of Hippo pathway, YAP can promote cancer metastasis through interacting with TEAD transcription factors.[2,3] Over expression of YAP or its nuclear localization is correlated with poor prognosis in various cancers, such as lung, colorectal, ovarian, and liver cancer.[4-6] In breast cancer, the role of YAP is still under discussion. Several studies showed YAP as an oncogene in breast cancer, demonstrating that YAP promoted breast cancer cells proliferation and survival, and over expression of YAP-enhanced breast cancer formation and growth in vivo.[7,8] On the contrary, other studies demonstrated YAP as a tumor suppressor, showing decreased expression of YAP in breast cancer tissue compared to the normal breast tissue,[9,10] increased cell migration, invasiveness, and tumor growth of YAP-knockdown breast cancer cells in vitro and in vivo.[11] But, so far, there has been no study investigating the expression of YAP in paired primary and local recurrent breast cancer.

In this study, we compared YAP expression in paired primary and local recurrent breast cancer, and evaluated its possible correlation with clinicopathologic factors.

Materials and methods

Patients and tissues collection

Primary breast cancers and their paired recurrent tumors were collected in Xiangya Hospital of Central South University from January 2005 to January 2017. All the patients were diagnosed with invasive duct carcinoma in the breast and were treated by surgical resection and chemotherapy and/or hormonal therapy and/or radiotherapy. Recurrent tumor tissues were obtained from the same patients. The study was subject to approval by the Institutional Review Board of Xiangya Hospital of Central South University. All the patients were from a Chinese population. Patients and tumor characteristics records were available from hospital medical records, including age at diagnosis, Tumor Node Metastasis (TNM) stage, histologic grade, estrogen receptor (ER) status, progesterone receptor (PR) status, and human epidermal growth factor receptor 2 (HER2) status. The median length of follow-up was 22 months (range: 2 months to 106 months).

Immunohistochemistry staining

Paraffin-embedded sections on polylysine-coated slides were used for staining. Sections were cut at 4 μm. The primary antibody for YAP (CST, 14074, USA) was used. The antibody dilution buffer (ZLI9022, ZSGB-BIO, China), secondary antibody (PV9001, ZSGB-BIO, China), and DAB buffer (ZLI9017, ZSGB-BIO, China) were used. Immunohistochemistry (IHC) was performed as follows: slides were deparaffinized in xylene twice for 5 minutes each time and dehydrated in a graded alcohol series. Antigen retrieval was subjected by microwave in 10 nM sodium citrate buffer at PH = 6.0 for 20 minutes. Peroxidase was blocked with 3% hydrogen peroxide. The primary antibody for YAP was diluted with antibody dilution buffer (1:400) and incubated

overnight in a humidity chamber at 4°C. After the incubation, slides were washed with Phosphate Buffer Saline (PBS) and incubated with secondary antibody and DAB chromogen. The primary antibody incubation step was omitted in the negative control. Positive control tissue was used according to manufacturer's recommendation.

Interpretation of immunohistochemical staining

The IHC images used were stained with DAB and hematoxylin. Each batch of staining was accompanied by the same positive and negative control slides. Three representative tumor areas (40× lens) per sample were selected. Before capturing the images, the color density and white balance were standardized for all images. To observe total YAP expression levels, the images were analyzed by 2 blinded investigators or using the software Image J (US National Institutes of Health) with IHC tool box. Using the software Image J, we analyzed 3 representative tumor areas and obtained the mean values of each sample. The mean values of each sample were normalized to the values of positive controls of each batch. According to the total YAP expression results of both manual and software analyses, we classified the samples to the high and low groups. We also analyzed YAP expression levels in cytoplasm and nucleus by 2 blinded investigators and classified to the high and low groups.

Statistical analysis

Statistical analyses were done with SPSS 17.0 (USA). The correlations between staining index and clinicopathologic factors were analyzed using the Pearson Chi-square (χ^2) test or Fisher's exact test for categorical variables. Survival curves were estimated by the Kaplan-Meier method. Multivariate models were done to estimate which factors might have a significant influence on disease-free survival (DFS). $P < 0.05$ was considered to be statistically significant.

Results

Clinical and pathologic data

One hundred ten patients aged from 27 to 65 years at the first diagnosis of invasive duct carcinoma in breast, with a median age of 47 years, were included in the study. According to the clinical measurement, 40% patients had a tumor larger than 5 cm and 20% patients were in stage pN3 (≥10 lymph nodes metastasis). Fifty of 110 patients had ER or PR positive primary tumors (≥1% positive cells). In the paired local recurrent tissues, only 38 patients had ER or PR positive tissues. Fourteen patients had high expression of HER2 protein in primary breast cancer, and 12 cases had high expression of HER2 in the recurrent tumor tissues. The clinical and pathologic data are summarized in Table 1.

Expression of YAP in paired breast cancer tissues and the correlation with clinical data

To compare the expression level of YAP protein in paired primary and recurrent tissue, we investigated its expression in 110 patients with breast invasive duct carcinoma by IHC. Among these patients, 46.4% (51/110) primary breast cancer tissues were high for total YAP expression (Fig 1A), where only 10.9% (12/110) recurrent tissues were high for total YAP expression (Fig 1C). The low expression of YAP was shown in Figure 1B of primary tumor and in Figure 1D of local recurrent tissue. The expression frequency of total YAP protein in primary breast tumor tissues was significantly higher than that in recurrent tissues ($P < 0.05$; Table 1).

Table 1
One hundred ten patients' clinical and pathologic data of primary and recurrent tumors.

Clinical and pathologic data		Number of patients (percentage %) in primary tumors	Number of patients (percentage %) in recurrent tumors
Age	≤35 y	20 (18.2)	–
	>35 y	90 (81.8)	–
Tumor size	≤5 cm	66 (60.0)	–
	>5 cm	44 (40.0)	–
Lymph node metastasis	pN0	28 (25.5)	–
	pN1	28 (25.5)	–
	pN2	32 (29.1)	–
	pN3	22 (20.0)	–
Histologic grading	I and II	70 (63.6)	–
	III	40 (36.4)	–
ER	Negative	62 (56.4)	72 (65.5)
	Positive	48 (43.6)	38 (34.5)
PR	Negative	64 (58.2)	82 (74.5)
	Positive	46 (41.8)	28 (25.5)
HER2	Low expression	96 (87.3)	98 (89.1)
	High expression	14 (12.7)	12 (10.9)
YAP	Low	59 (53.6)	98 (89.1)
	High	51 (46.4)	12 (10.9)

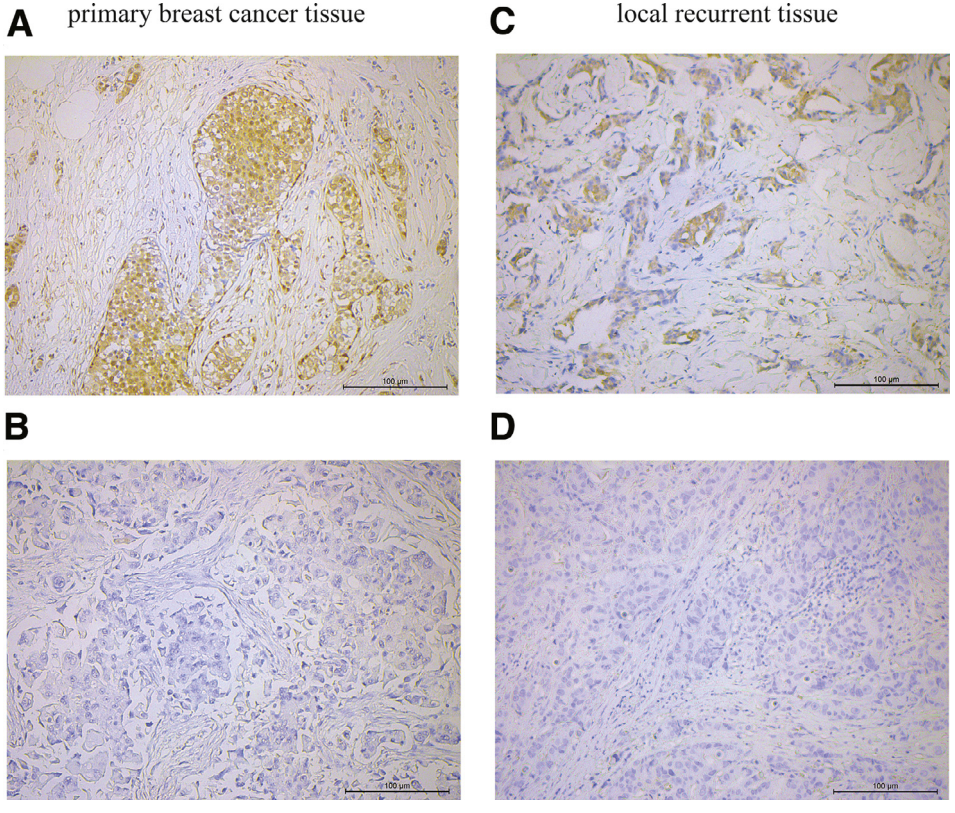

Fig. 1. IHC to detect YAP expression in paired primary and recurrent tissues. (A) High expression of YAP in primary breast cancer tissue in TNBC patient. (B) Low expression of YAP in primary breast cancer tissue in TNBC patient. (C) High expression of YAP in local recurrent breast cancer tissue in luminal-subtype breast cancer patient. (D) Low expression of YAP in local recurrent breast cancer tissue in TNBC patient.

Table 2
Association of YAP expression in 110 primary breast cancer with clinicopathologic factors.

Clinical data	YAP expression (total)		P	YAP expression (cyto)		P	YAP expression (nucl)		P
	low cases (percentage)	high cases (percentage)		low cases (percentage)	high cases (percentage)		low cases (percentage)	high cases (percentage)	
Age									
≤35 y	10 (45.5)	12 (54.5)		17 (77.3)	5 (22.7)		11 (50.0)	11 (50.0)	
>35 y	49 (55.7)	39 (44.3)	0.390	60 (68.2)	28 (31.8)	0.405	50 (56.8)	38 (43.2)	0.565
Tumor size									
≤5 cm	43 (65.2)	23 (34.8)		43 (65.2)	23 (34.8)		43 (65.2)	23 (34.8)	
>5 cm	16 (36.4)	28 (63.6)	0.003*	34 (77.3)	10 (22.7)	0.143	18 (40.9)	26 (59.1)	0.053
Lymph node									
pN0	14 (50.0)	14 (50.0)		20 (71.4)	8 (28.6)		15 (53.6)	13 (46.4)	
pN1	12 (42.9)	16 (57.1)		15 (53.6)	13 (46.4)		13 (46.4)	15 (53.6)	
pN2	22 (68.8)	10 (31.3)		27 (84.4)	5 (15.6)		22 (68.8)	10 (31.3)	
pN3	11 (50.0)	11 (50.0)	0.211	15 (68.2)	7 (31.8)	0.078	11 (50.0)	11 (50.0)	0.318
Histologic grading									
I and II	32 (45.7)	38 (54.3)		45 (64.3)	25 (35.7)		34 (48.6)	36 (51.4)	
III	27 (67.5)	13 (32.5)	0.028*	32 (80)	8 (20)	0.084	27 (67.5)	13 (32.5)	0.054
ER									
Negative	34 (54.8)	28 (45.2)		43 (69.4)	19 (30.6)		35 (56.5)	27 (43.5)	
Positive	25 (52.1)	23 (47.9)	0.774	34 (70.8)	14 (29.2)	0.867	26 (54.2)	22 (45.8)	0.811
PR									
Negative	35 (54.7)	29 (45.3)		44 (68.8)	20 (31.3)		36 (56.3)	28 (43.8)	
Positive	24 (52.2)	22 (47.8)	0.794	33 (71.7)	13 (28.3)	0.736	25 (54.3)	21 (45.7)	0.843
HER2									
Low	51 (53.1)	45 (46.9)		68 (70.8)	28 (29.2)		52 (54.2)	44 (45.8)	
High	8 (57.1)	6 (42.9)	07778	9 (64.3)	5 (35.7)	0.617	9 (64.3)	5 (35.7)	0.477
Molecular type									
HR + HER2-	26 (54.2)	22 (45.8)		34 (70.8)	14 (29.2)		27 (56.3)	21 (43.8)	
HR + HER2+	1 (50)	1 (50)		2 (100)	0 (0)		1 (50)	1 (50)	
HR-HER2+	7 (58.3)	5 (41.7)		7 (58.3)	5 (41.7)		8 (66.6)	4 (33.4)	
HR-HER2-	25 (52.1)	23 (47.9)	0.982	34 (70.8)	14 (29.2)	0.644	25 (52.1)	23 (47.9)	0.833

Fisher's exact test was used, if the theoretical frequency in the cell was less than 5.
* $P < 0.05$.

The correlation between YAP expression in primary breast cancer and the clinicopathologic factors was statistically analyzed. The higher expression of total YAP in primary tumor was significantly associated with the larger tumor size and histologic grading ($P < 0.05$; Table 2). However, there were not any correlations between total YAP and other clinical factors, for example, age, histologic grading, ER, PR, HER2, or molecular subtype. YAP expression in cytoplasm and nucleus of primary tumor were analyzed separately, but no significant difference was found (Table 2).

Expression of YAP in primary breast cancer was not correlated with DFS

Univariate and multivariate analysis were used to determine the prognostic significance of clinical parameters. As shown in Supplemental Table 1 and Table 2, higher histologic grading (III), negative ER, negative PR, or high expression of HER2 were independently associated with poor DFS of patients with breast cancer ($P < 0.05$). The expression of total YAP in primary tumor was not associated with DFS of breast cancer patients (log-rank test, $\chi^2 = 1.057$, $P = 0.304$, Fig 2A).

High nuclear or total YAP expression in the TNBC primary tumor is associated with poor DFS

We found no correlation between YAP expression and molecular subtype (Table 2); however, we noticed that none of the recurrent TNBC tumors had high YAP expression (Supplemental

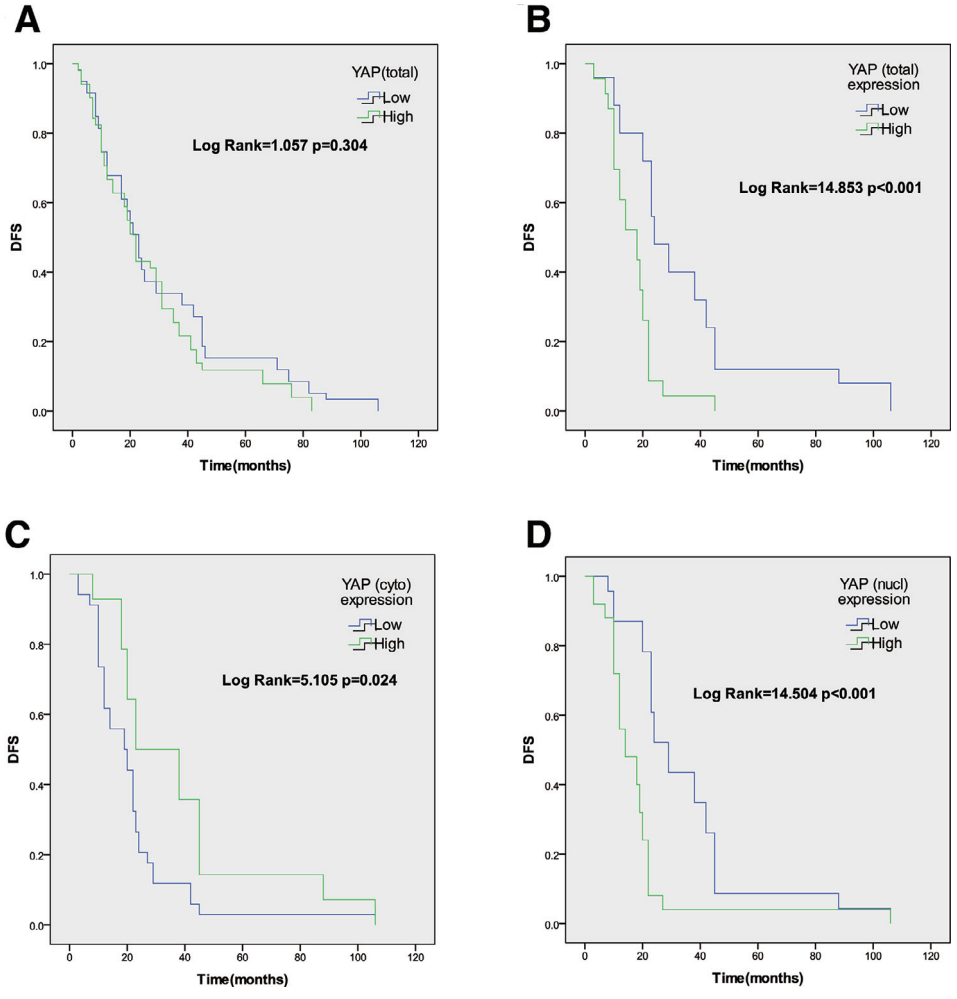

Fig. 2. High total or nuclear YAP expression in primary TNBC was associated with poor DFS. (A) The expression of total YAP was not associated with DFS of the whole cohort of breast cancer patients. (B) Patients with high total YAP expression had a significantly worse DFS than that of patients with low YAP expression in TNBC patients. (C) Patients with high cytoplasmic YAP expression had a significantly better DFS than that of patients with low cytoplasmic YAP expression in patients with TNBC. (D) Patients with high nuclear YAP expression had a significantly worse DFS than that of patients with low YAP expression in TNBC patients.

Table 3). Then the clinical and pathologic data of 48 TNBC patients were shown in Supplemental Table 3. As shown in the Table 3, high expression of total YAP in primary tumor was associated with larger tumor size ($P = 0.036$). Interestingly, the YAP expression in cytoplasm of primary tumor was negatively correlated with tumor size, and its expression in nucleus was positively correlated with tumor size ($P < 0.05$).

The association between clinical parameters and DFS was analyzed among patients with TNBC tumors. Data of univariate analysis among patients with TNBC tumors were present in Supplemental Table 4. As shown in Figure 2B and D, patients with high total or nuclear YAP expression in primary tumor had a significantly worse DFS than that of patients with low YAP expression (log-rank test, $P < 0.001$, respectively). As shown in Figure 2C, patients with low cytoplasmic YAP expression in primary tumor had a significant shorter DFS than that of patients

Table 3
Association of YAP expression in 48 TNBC primary breast cancer with clinicopathologic factors.

Clinical data	YAP expression (total)		P	YAP expression (cyto)		P	YAP expression (nucl)		P
	Low cases (percentage)	High cases (percentage)		Low cases (percentage)	High cases (percentage)		Low cases (percentage)	High cases (percentage)	
Age									
≤35 years	4 (40.0)	6 (60.0)		8 (80)	2 (20)		3 (30.0)	7 (70.0)	
>35 years	21 (55.3)	17 (44.7)	0.390	26 (68.4)	12 (31.6)	0.474	23 (68.8)	15 (31.3)	0.085
Tumor size									
≤5 cm	21 (61.8)	13 (38.2)		21 (61.8)	13 (38.2)		22 (64.7)	12 (35.3)	
>5 cm	4 (28.6)	10 (71.4)	0.036*	13 (92.9)	1 (7.1)	0.031*	4 (28.6)	10 (71.4)	0.022*
Lymph node									
pN0	10 (55.6)	8 (44.4)		12 (66.7)	6 (33.3)		10 (55.6)	8 (44.4)	
pN1	6 (75.0)	2 (25.0)		6 (75)	2 (25)		6 (75.0)	2 (25.0)	
pN2	4 (33.3)	8 (66.7)		11 (91.7)	1 (8.3)		4 (33.3)	8 (66.7)	
pN3	5 (50.0)	5 (50.0)	0.324	5 (50)	5 (50)	0.184	6 (60.0)	4 (40.0)	0.302
Histologic grading									
I and II	13 (50.0)	13 (50.0)		18 (69.2)	8 (30.8)		13 (50.0)	13 (50.0)	
III	12 (54.5)	10 (45.5)	0.753	16 (72.7)	6 (27.3)	0.791	13 (59.1)	9 (40.9)	0.529

Fisher's exact test was used, if the theoretical frequency in the cell was less than 5.
* $P < 0.05$.

Table 4
Multivariate analysis 48 TNBC patients' clinicopathologic factor and DFS.

Variate	HR	95%CI	P
Tumor size (>5 cm vs ≤5 cm)	1.573	0.749-3.303	0.231
Histologic grading (III vs I/II)	2.161	1.140-4.097	0.018*
YAP(cyto) (high vs low)	0.923	0.425-2.008	0.840
YAP(nucl) (high vs low)	3.199	1.489-6.874	<0.003*

* $P < 0.05$.

with high YAP expression ($P = 0.024$). Data of multivariate analysis were shown in Table 4. Histologic grading and nuclear YAP expression were independently correlated with DFS of TNBC patients ($P < 0.05$). Tumors with high grading and high nuclear YAP expression in primary tumor predicted poor DFS of the TNBC patients.

Discussion

In this study, we investigated the YAP protein expression in paired primary and local recurrent breast cancer tissues. The expression frequency of YAP was 46.4% in the primary invasive duct breast cancer tissues, and this was in line with previous data showing the YAP expression frequency was 45.1%.[9] We also analyzed the expression frequency of YAP in paired primary and recurrent tissues, and we found the expression of YAP was significantly lower in the recurrent tissues than that in the paired primary breast cancer tissues. One study has explored the YAP expression in the metastasis breast cancer, including metastatic tissues from bone, brain, liver, and lung. The authors concluded that the level of YAP expression varied according to metastatic site, and high YAP expression was correlated with poor survival.[12] But there was no study comparing the YAP expression in the paired primary and local recurrent tissues. In our study, we also analyzed the correlation between YAP expression and DFS in the cohort of patients, but there was no significant correlation.

Next, we analyzed total YAP expression in distinct molecular subtypes of breast cancer. In line with previous study, the expression of YAP in HER2 positive-subtype and triple negative-

subtype breast cancer was around 35% and 50%, respectively.[9,13] But total YAP expression was much lower in luminal-subtype breast cancer tissues in this study (46% vs 59.3%) than that from a study which showed significant higher YAP expression in luminal-A subtypes breast cancer with a larger cohort of samples.[13] This may be ascribed to selection bias. In this study, we have collected patients whose paired tissues of primary and local recurrent tumors had been preserved. We have excluded patients with metastatic samples of liver, lung, and bone due to easy access of local recurrent tissue samples.

Focused on the TNBC subtype, we observed that high total YAP expression was associated with larger tumor size, so do the nuclear YAP expression. We also observed that high cytoplasmic YAP expression was negatively correlated with tumor size. More interestingly, we observed that high total or nuclear YAP expression was correlated with poor DFS. But high cytoplasmic YAP expression was correlated with better DFS. This was verified in the multivariate model, which showed histologic grading and nuclear YAP expression were independently prognostic factors in patients with TNBC. Some previous studies have observed that higher YAP expression was associated with poor DFS and/or OS,[12] but there were also disputes showing no significant correlation or even negative correlation.[13,14] But these studies have not paid close attention to survival in distinct molecular subtype. Our results showed in TNBC that the YAP expression was a prognostic factor of patients' survival. There was some analysis of public databases, and it showed that YAP mRNA and protein expression were positively associated with TNBC subtype in patients and in cell lines.[15] Another study retrospectively evaluated YAP expression in TNBC patients with neoadjuvant chemotherapy, and it showed that YAP expression in tumor cells conferred poor survival outcomes.[16] In our study, there was another interesting phenomenon showing low YAP expression in all of those recurrent tissues of TNBC patients. We found patients without changes of YAP expression had better DFS than that of patients with a decrease expression of YAP. This needs further studies for the verification with more breast cancer samples.

Previous studies[10,13] have shown that YAP expression is not correlated with tumor size of breast cancer. This was distinct from our results, which demonstrated that the high expression of total YAP was correlated with large tumor size in the cohort of breast cancer patients. In the TNBC subtype, the tumor size was positively correlated with total and nuclear YAP expression, and negatively correlated with cytoplasmic YAP expression. We considered that the difference might be explained by the classification according to the tumor size large than 5 cm or not in this study, whereas the cut-off value is 2 cm in other studies. As for the correlation between total YAP expression and histologic grading, our results supported this positive correlation between them, although there was controversy about it.[17] In this study, YAP expression was not related to the lymph nodes metastasis ER, PR, or HER2, which have been reported in previous study.[14] These gave us a clue that YAP might play a major role in TNBC cells, but not if cancer cells had positive ER, PR, and HER2 molecular. As for the TNBC, chemotherapy is the only treatment, and the finding of new therapeutic target is of great importance.[18,19] More investigations are needed for the regulation mechanism in TNBC.

Conclusion

In this study, we evaluated the YAP expression in both nucleus and cytoplasm. This study compared YAP expression in primary breast cancer tissues with that in paired recurrent tissues, and showed that YAP expression in both nuclei and cytoplasm was significantly higher in primary breast cancers than that in the recurrent tissues. The expression of total YAP protein in the primary breast cancer tissues was positively associated with the tumor size, especially in TNBC patients. Higher total or nuclear YAP expression in primary tumor was correlated with poor DFS among patients with TNBC. The observation of no YAP expression in the local recurrent tissues of TNBC needs further investigation in future.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.currproblcancer.2018.12.005.

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