High Nuclear Expression of Yes-Associated Protein 1 Correlates With Metastasis in Patients With Breast Cancer

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Background: Yes-associated protein 1 (YAP1) is a transcription factor regulated by the Hippo pathway and functions as an oncogene in various solid tumors under dysregulated Hippo pathway. However, the role of YAP1 in breast cancer remains controversial. Here, we investigated the impact of different levels of nuclear YAP1 expression on the clinical characteristics and survival outcome in patients with breast cancer.

Patients and Methods: Retrospectively obtained 455 breast tumor samples at Gangnam Severance Hospital were examined for YAP1 expression by immunohistochemistry, and the clinical data were analyzed. External validation was performed using a retrospective cohort and tissues in 482 patients from Severance Hospital.

Results: High nuclear YAP1 expression was associated with hormone receptor negativity and aggressive tumor behavior, including lymph node metastasis, high Ki67 labeling index and inferior distant metastasis-free survival (DMFS, hazard ratio [HR] 2.271, 95% confidence intervals [CIs] 1.109–4.650, P = 0.0249), and also confirmed inferior disease free survival (HR 3.208, 95% CIs 1.313–7.833, P = 0.0105) in external validation cohort. In patients with triple-negative breast cancer (TNBC), high nuclear YAP1 expression was an independent significant determinant of poor DMFS (HR 2.384, 95% CIs 1.055–5.386, P = 0.0367).

Conclusion: Our findings suggest that nuclear YAP1 expression is a biomarker of adverse prognosis and a potential therapeutic target in patients with breast cancer, especially in TNBC.

Keywords: Yes-associated protein 1, breast cancer, prognosis, metastasis, triple-negative breast cancer

BACKGROUND

Breast cancer is the most common cancer among women worldwide, and approximately 15 million people are diagnosed with this disease each year. In recent years, owing to advanced treatment modalities and the identification of new drug targets, breast cancer mortality has decreased by approximately 2.3% per year (1). So far, well known biomarkers such as including ER and HER2, as well as others, including pSTAT3 expression, LDH, and tumor-infiltrating lymphocytes, etc. – have been studied to elucidate breast cancer biology and survival outcome (2–4), further endeavor is still required to understand tumor nature. Despite these improvements, metastatic breast cancer still results in poor survival. In order to further enhance treatment efficacy, new drug targets with specific roles in metastatic cascades must be unveiled.

Lymph node metastasis is the most important prognostic factor in breast cancer, and is associated with high relapse and mortality (5–8). The mechanisms underlying lymph node metastasis are still poorly understood. It has been reported that the transcriptional co-activator, Yes-associated protein 1 (YAP1), plays an important role in lymph node metastasis (9). Enhanced YAP1 activity increases fatty acid oxidation, ultimately leading to lymph node metastasis. The Hippo signaling regulates organ size and development (10), and restricts transcription co-factor YAP1 and transcriptional coactivator with PDZ-binding motif (TAZ) by cytoplasmic retention followed by protein degradation (11). However, dysregulated Hippo signaling results in the nuclear accumulation of non-phosphorylated YAP1 and TAZ. These interact with transcriptional enhanced associated domain (TEAD)-containing transcription factors in nucleus, promoting the expression of genes related to cell proliferation and epithelial-mesenchymal transition (EMT) (12, 13). Moreover, YAP1 was reported to induce TEAD-dependent focal adhesion kinase phosphorylation, ultimately promoting tumor invasiveness (14). Activated YAP1 contributes to cancer development by promoting a malignant tumor phenotype. In particular, YAP1 stimulates cancer stem cell proliferation and epithelial-mesenchymal transition, induces drug resistance, inhibits apoptosis, and promotes tumor overgrowth (10, 15–18). Several studies have reported a correlation of YAP1 expression with aggressive clinical characteristics and low survival (19–29). This evidence suggests that YAP1 is a potential therapeutic target, and that its pharmacologic or genetic inactivation may suppress tumor progression and improve drug sensitivity. However, the association between clinical data and YAP1 expression in patients with breast cancer has been poorly explored. In addition, the possible clinical relevance of YAP1 subcellular localization is not clearly defined.

The aim of this study was to correlation between the level of nuclear YAP1 expression and the clinical characteristics and survival rates of patients with breast cancer. The impact of YAP1 expression on survival was also evaluated in patients with triple negative breast cancer (TNBC).

MATERIALS AND METHODS

Patients

We retrospectively collected the tumor tissues from the patients undergoing primary curative surgery for breast cancer at the Gangnam Severance Hospital in Seoul, Korea from February 1992 to April 2017 and at the Severance Hospital in Seoul, Korea from January 2000 to December 2010. A validation cohort consisted of 482 patients from the Severance Hospital. Inclusion and exclusion criteria were as follows:

1) Inclusion criteria:
    - Patients age ≥ 20 years
    - Breast cancer confirmed by pathologic diagnosis (stage I–III)
    - Available YAP1 immunohistochemical staining with tissue microarray
2) Exclusion criteria:
    - Any other carcinoma *in situ*
    - Other cancer history (except thyroid cancer and carcinoma in situ)
    - Not assessable electronic medical record

All subjects were diagnosed with stage I–III primary breast cancer. All patient treatments were performed according to standard protocols. The following data were collected: age at surgery, tumor size, lymph node status, histological grade (HG), status of estrogen receptor (ER), status of progesterone receptor (PR), status of human epidermal growth factor receptor-2 (HER2), lymphovascular invasion (LVI), Ki67 leveling index, tumor-infiltrating lymphocytes (TILs), treatment modalities, recurrence, and death. Tumor HG was determined by applying the modified Scarff-Bloom-Richardson grading system. The study protocol was approved by the institutional review board (IRB) of the Gangnam Severance Hospital (local IRB No. 3-2019-0188). The need for informed consent was waived under the approval of the IRB due to the retrospective design.

IMMUNOHISTOCHEMISTRY (IHC) AND MOLECULAR SUBTYPING

As previously described (30), 3-μm thick tissue sections were cut from formalin-fixed paraffin-embedded (FFPE) tissue microarray (TMA) blocks. After deparaffinization and

rehydration with xylene and alcohol graded solutions, respectively, IHC was performed by using a Ventana Discovery XT Automated Slide Stainer (Ventana Medical System, Tucson, AZ, USA). Cell Conditioning 1 (CC1) buffer (citrate buffer, pH 6.0; Ventana Medical System) was used for antigen retrieval. The appropriate positive and negative controls were included.

IHC staining was evaluated with light microscopy (BX53 upright microscope, Olympus, Tokyo, Japan). Nuclear staining values of 1% or higher were considered indicative of ER (clone SP1; dilution 1:100; Thermo Scientific, San Diego, CA, USA) and PR (clone PgR; dilution 1:50; DAKO, Glostrup, Denmark) positivity (31). HER2 (polyclonal; dilution 1:1500; DAKO) staining was interpreted based on the 2018 American Society of Clinical Oncology/College of American Pathologists guidelines (32). Only samples with strong and circumferential membranous HER2 immunoreactivity (3+) were considered positive, while those with 0 and 1+ HER2 staining were considered negative. Cases with equivocal HER2 expression (2+) were further evaluated for HER-2 gene amplification by silver in situ hybridization (SISH). Positive nuclear Ki-67 (clone MIB; dilution 1:1,000; Abcam, Cambridge, UK) staining was assessed based on the percentage of positive tumor cells, defined as Ki-67 labeling index.

The specimens were categorized as follows: i) Luminal/HER2-negative (ER- and/or PR-positive and HER2-negative); ii) HER2-positive (HER2-positive regardless of ER and PR status); iii) TNBC (ER-, PR-, and HER2-negative).

EVALUATION OF NUCLEAR YAP1 EXPRESSION BY TISSUE MICROARRAY AND IHC STAINING

Hematoxylin and eosin-stained slides from the resected breast cancer specimens were examined, and the representative areas were marked. The matched tissue cores (2 mm) were extracted from FFPE tumor blocks and placed into 5 × 10 recipient TMA blocks.

For IHC, each TMA slide was stained with anti-YAP1 antibody (clone 63.7; dilution 1:200; Santa Cruz Biotechnology, Dallas, TX, USA). After staining, nuclear YAP1 expression was assessed by a pathologist (400× magnification). YAP1 expression was evaluated in both the cytoplasm and nuclei of the tumor cells. Cytoplasmic staining was evaluated by the H-score, which was obtained by multiplying staining intensity (0, 1, 2, or 3) by percentage of stained area (%). As nuclear expression was rare and mostly focal, only the intensity of nuclear staining was examined (0, 1+, 2+, 3+), regardless of the corresponding cytoplasmic staining. The intensity of nuclear staining of the myoepithelial cells was assigned a value of 2+ and used as an internal control. Weaker and stronger signals were assigned a value of 1+ and 3+, respectively. Negative and weak (1+) nuclear staining were considered indicative of low expression, while moderate (2+) and strong (3+) nuclear expression were indicative of high expression (**Figure 1**). The IHC results were interpreted blindly, without any information regarding clinical parameters or outcomes.

STATISTICAL ANALYSIS

Distant metastasis-free survival (DMFS) was defined as the time from the primary curative surgery to the first breast cancer-derived distant metastasis, or death due to any cause, or end of follow-up. Overall survival (OS) was defined as the time from the primary curative surgery to the end of follow-up, or death due to any cause. Disease-free survival (DFS) was defined as the time from the primary curative surgery to cancer recurrence, second cancer, or death. The data of patients who did not exhibit relevant events were censored at the end of follow-up.

The continuous variables between the two groups were compared using the Student's *t*-test or the Mann-Whitney test. The categorical variables were compared by using the *Chi*-square test or the Fisher's exact test. Survival curves were obtained by the Kaplan-Meier method and two-group comparisons were made using log-rank test. Univariate and multivariate Cox proportional hazard models were used to identify the factors associated with survival outcome (DMFS and OS). The variables showing statistically significant differences in the univariate analysis were used in the multivariate Cox proportional hazard models.

Statistical analysis was performed by using SPSS version 24 (SPSS: Chicago, IL, USA) software. The threshold for statistical significance was set at $P < 0.05$, with a 95% confidence interval not including 1.

RESULTS

Impact of Nuclear YAP1 Expression on the Baseline Characteristics of Patients With Breast Cancer

A total of 455 breast cancer patients at Gangnam Severance Hospital were included in this study. The median age was 50 (25–86) years. The median DFS and OS were 59 (10–325) and 60 (12–325) months. Low and high nuclear YAP1 expression were found in the tumors of 344 (75.6%) and 111 (24.4%) patients, respectively. The clinical characteristics were examined in relation to nuclear YAP1 expression (**Table 1**). High nuclear YAP1 expression was associated with aggressive tumor features, including hormone receptor negativity, high HG, lymph node metastasis, and high Ki67 expression. Patients were classified into three subtypes based on IHC analysis: luminal/HER2-negative (243 patients), HER2-positive (62 patients), and TNBC (146 patients). High nuclear YAP1 expression was associated with the TNBC subtype.

Validation cohort included 482 patients at Severance Hospital. Median DFS and OS were 65 (5–139) and 65 (12–241) months. Of the 482 patients, 428 (88.8%) exhibited low nuclear YAP1 expression, and 54 (11.2%) exhibited high nuclear YAP1 expression. Clinical characteristics were compared to nuclear YAP1 expression in **Supplementary Table 1**. Also, high nuclear YAP1 expression was related to TNBC subtype.

Prognostic Significance of Nuclear YAP1 Expression

There were 41 patients with developing distant metastasis. Among them, 14 had bone metastasis, 11 lung metastasis, five
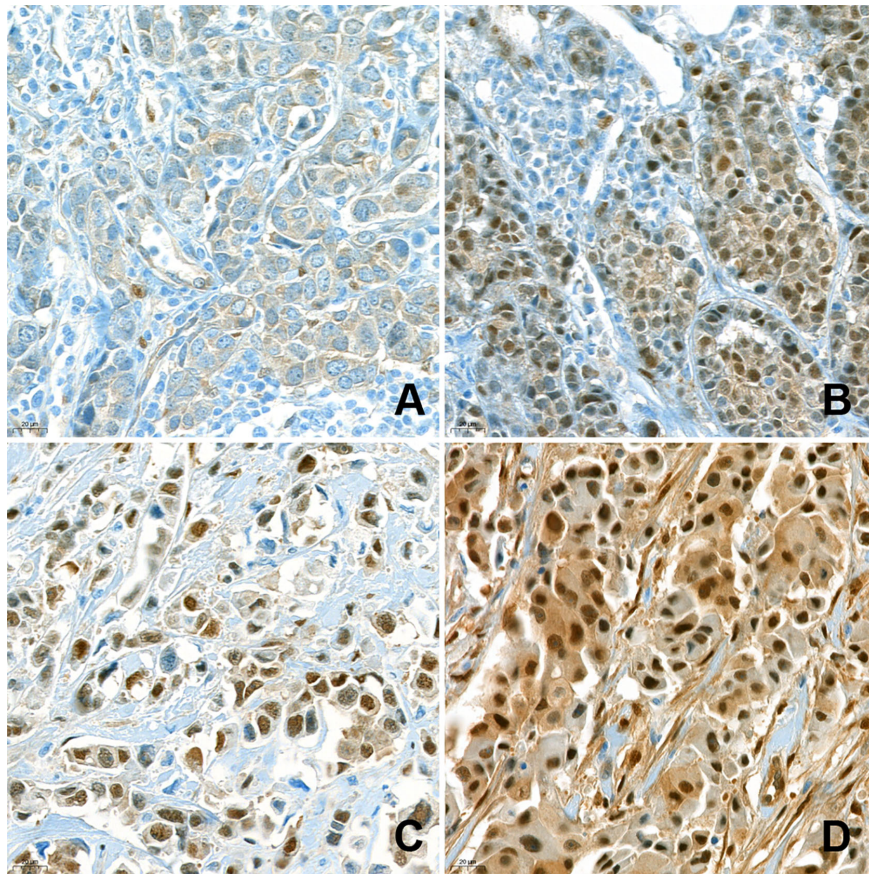
FIGURE 1 | Immunohistochemical analysis of nuclear YAP1 expression. Nuclear YAP1 expression was evaluated in high-power fields (400× magnification) by an experienced pathologist. The samples were classified as negative **(A)**, 1+ **(B)**, 2+ **(C)**, and 3+ **(D)**, based on the intensity of YAP1 nuclear staining.

liver metastasis, three brain metastasis, while 17 had developed metastases to other sites (including duplication). There were 18 mortality events. High nuclear YAP1 expression was significantly associated with decreased distant metastasis-free survival (DMSF) [**Figure 2A**; hazard ratio (HR), 2.271, 95% confidence intervals (CIs) 1.109–4.650, P = 0.0249, log rank test], and was a significant predictor of poor overall survival (**Figure 2B**; HR 3.856, 95% CIs 1.321–11.26, P = 0.0135, log rank test).

Negative hormone receptor status, tumor size >2 cm, and high nuclear YAP1 expression were significantly associated with decreased DMFS, as assessed by univariate analysis (**Table 2**). High nuclear YAP1 expression was still a significant determinant of decreased DMFS after adjustment for hormone receptor status, tumor size, and nuclear YAP1 expression by the Cox proportional hazards model (HR 1.893, 95% CIs 1.009–3.552, P = 0.047).

In the univariate Cox proportional hazard model, negative hormone receptor status, high histologic grade, tumor size > 2 cm, and high nuclear YAP1 expression were found to be significant prognostic factors for OS (**Supplementary Table 2**). However, in the multivariate analysis, nuclear YAP1 expression

was not a significant predictor of OS (**Supplementary Table 2**, HR 1.576, 95% CIs 0.616–4.034, P = 0.343).

In validation cohort, high nuclear YAP1 expression was significantly predictive of decreased DFS (**Supplementary Figure 1**; HR, 3.208, 95% CIs 1.313–7.833, P = 0.0105, log rank test). ER negativity, PR negativity, tumor size >2 cm, lymph node metastasis, and high nuclear YAP1 expression were significant factors in the multivariate analysis of DFS (**Supplementary Table 3**). When adjusted for other factors, high nuclear YAP1 expression was a significant factor in reduced DFS (HR 2.112, 95% CIs 1.083–4.119, P = 0.028).

Prognostic Significance of Nuclear YAP1 Expression in TNBC Patients

The impact of nuclear YAP1 expression on survival and clinical characteristics was evaluated in patients with TNBC (**Supplementary Table 4**). Of the 146 TNBC patients, 80 (54.8%) had tumors with low nuclear YAP1 expression, while 66 (45.2%) had tumors with high nuclear YAP1 expression. In TNBC patients, the clinical characteristics were not significantly affected by the level of nuclear YAP1 expression. However, high nuclear YAP1 expression was

TABLE 1 | Clinical characteristics in relation to nuclear YAP1 expression.

|	YAP1-low, n = 344 (%)	YAP1-high, n = 111 (%)	P value
Age (year, mean ± SD)	50.78 ± 10.47	49.51 ± 9.50	0.256
HR			<0.001
Positive	238 (69.2)	35 (31.5)	
Negative	106 (30.8)	76 (68.5)	
HER2[a]			0.301
Positive	50 (14.5)	12 (10.8)	
Negative	290 (84.3)	99 (89.2)	
Missing	4 (1.2)	0	
HG[a]			0.005
I, II	223 (64.8)	54 (48.6)	
III	115 (33.4)	52 (46.8)	
Missing	6 (1.7)	5 (4.5)	
Subtype[a]			<0.001
Luminal/HER2(−)	210 (61.0)	33 (29.7)	
HER2 (+)	50 (14.5)	12 (10.8)	
TNBC	80 (23.3)	66 (59.9)	
Missing	4 (1.2)	0	
Tumor size[a]			0.298
≤2 cm	174 (50.6)	50 (45.0)	
>2 cm	169 (49.1)	61 (55.0)	
Missing	1 (0.3)	0	
Lymph node metastasis[a]			0.602
Negative	231 (67.2)	72 (64.9)	
Positive	111 (32.3)	39 (35.1)	
Missing	2 (0.6)	0	
Ki67 (%)[a]			0.001
≤20%	258 (75.0)	62 (55.9)	
>20%	74 (21.5)	40 (36.0)	
Missing	12 (3.5)	9 (8.1)	
Lymphovascular invasion[a]			0.413
Negative	268 (77.9)	81 (73.0)	
Positive	55 (16.0)	21 (18.9)	
Missing	21 (6.1)81	9 (8.1)	
TILs (%, mean ± SD)[a]	33.84 ± 26.15 (n = 164)	33.54 ± 30.77 (n = 72)	0.943

SD, standard deviation; HR, hormone receptor; HER-2, human epidermal growth factor receptor-2; HG, histological grade; TNBC, triple negative breast cancer; TILs, tumor-infiltrating lymphocytes.
[a]*Percentages calculated without missing values.*

FIGURE 2 | Kaplan-Meier survival curves of distant metastasis-free survival (DMFS) and overall survival (OS) in relation to nuclear YAP1 expression. Patients with high nuclear YAP1 expression exhibited poor DMFS and overall survival (OS) (**A**, HR 2.271, 95% CI 1.109–4.650, P = 0.0249; **B**, HR 3.856, 95%CI 1.321–11.26; P = 0.0135, log-rank test, respectively).

associated with poor DMFS (**Figure 3**; HR 2.384, 95% CIs 1.055–5.386, P = 0.0367, log rank test). Lymph node metastasis and high nuclear YAP1 expression were significant determinants of poor DMFS in the univariate analysis (**Supplementary Table 5**). Adjustment for significant factors in the univariate analysis confirmed that high nuclear YAP1 expression was significantly associated with DMFS (HR 2.329, 95% CIs 1.016–5.339, P = 0.046).

DISCUSSION

In this study, the nuclear expression of YAP1 was evaluated in a large number of breast cancer specimens, was found to be significantly associated with the occurrence of distant metastasis (HR 2.271, 95%CIs 1.109–4.650, P = 0.0249). Furthermore, nuclear YAP1 expression was a strong determinant of metastasis in TNBC (HR 2.384, 95%CIs 1.055–5.386, P = 0.0367), an aggressive subtype of breast cancer. These
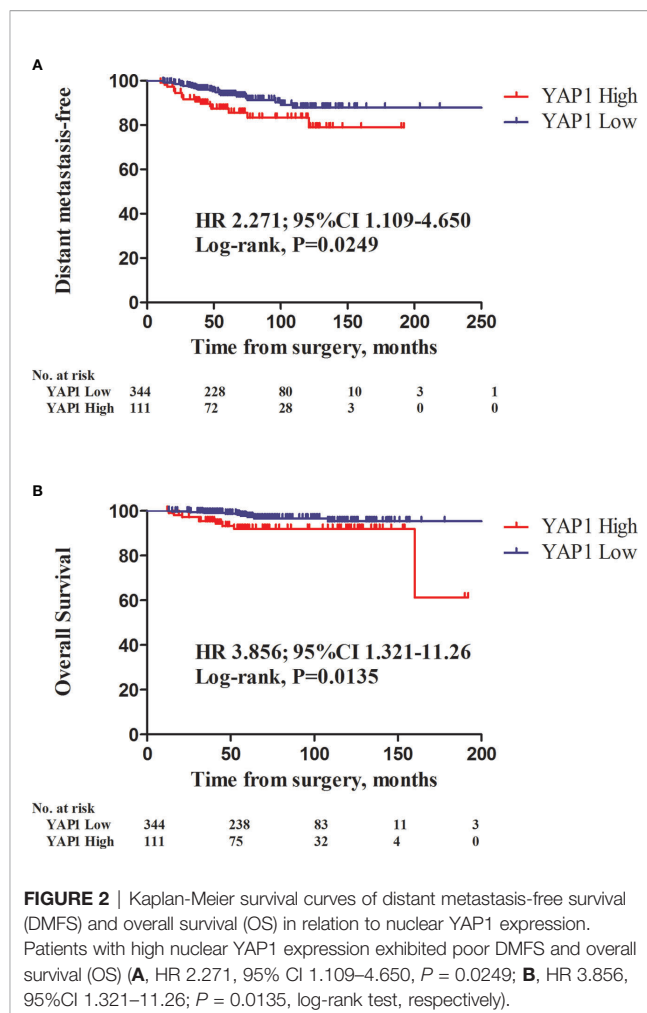
findings indicate that targeted therapy for YAP1 may potentially improve the survival outcomes, particularly metastasis, of the patients with breast cancer.

The activation of YAP1, along with that of another transcriptional co-activator TAZ, is associated with dysregulated Hippo signaling (33, 34). YAP1 overexpression promotes EMT, inhibits apoptosis, and induces growth factor-independent cell proliferation. Based on these findings, it was speculated that YAP1 may play a role as a proto-oncogene (23). In addition, several studies have reported a negative impact of YAP1 activation on the survival of patients with gastric, colorectal, ovarian, bladder, and non-small cell lung cancer (22, 29, 35–37).

Currently, the role of YAP1 in breast cancer remains controversial. For instance, Lehn and colleagues reported that YAP1 expression is inversely correlated with HG and tumor cell proliferation, and that low YAP1 mRNA levels are associated with decreased recurrence-free survival and tamoxifen-resistance in luminal A subtype breast cancer (38). Also, some have reported that YAP1 promotes cell proliferation, tumorigenesis, EMT, and drug resistance, and is associated with TP53 mutation, ER negativity, and poor survival (18, 39). However, several other

TABLE 2 | Hazard ratios (HRs) and 95% confidence intervals (CIs) for distant metastasis-free survival (DMFS).

	Univariate analysis		Multivariate analysis	
	HRs (95% CIs)	P value	HRs (95% CIs)	P value
Age	0.983 (0.953–1.014)	0.284		
HR		0.015		0.158
Negative	Reference		Reference	
Positive	0.449 (0.236–0.854)		0.607 (0.303–1.215)	
HER2		0.295		
Negative	Reference			
Positive	0.575 (0.204–1.619)			
HG		0.101		
I, II	Reference			
III	1.670 (0.904–3.083)			
Tumor size		0.009		0.012
≤2 cm	Reference		Reference	
>2 cm	2.525 (1.263–5.049)		2.426 (1.211–4.861)	
Lymph node metastasis		0.051		
Negative	Reference			
Positive	1.840 (0.996–3.397)			
YAP1 expression		0.028		0.047
Low	Reference		Reference	
High	2.020 (1.078–3.784)		1.893 (1.009–3.552)	
Ki67 (%)		0.237		
≤20%	Reference			
>20%	1.530 (0.756–3.095)			
Lymphovascular invasion		0.391		
Negative	Reference			
Positive	1.411 (0.643–3.099)			
TILs	0.988 (0.971–1.006)	0.200		

HR, hormone receptor; HER-2, human epidermal growth factor receptor-2; HG, histologic grade; TILs, tumor-infiltrating lymphocytes.

studies have suggested that survival is not significantly affected by YAP1 expression in breast cancer (39, 40), or YAP1 may in fact act as a tumor suppressor (26, 41). Among the most of previous studies mentioned above, the specific argument on subcellular localization of YAP1 in previous studies were unclear. Although Vlug et al. showed upregulated YAP1 expression in invasive lobular carcinoma, but its prognostic impact was not evaluated (39). Kim et al. evaluated the differential expression of YAP and phosphorylated YAP in the each of molecular subtypes of breast cancer and found that nuclear YAP expression was associated with shorter survival (20). However, in previous study, nuclear YAP expression in multivariate analysis did not have statistical significance. Given that nuclear YAP1 expression is a surrogate marker of an activated form of YAP1 (9), we focused on the nuclear YAP1 expression in this study, and found that nuclear YAP1 expression is an independent prognostic predictor for distant metastasis, particularly in the TNBC patients.

TNBC is simply defined as a breast cancer that satisfied the ER, PR, and HER2 negativities, however it is not a uniform subtype rather a complex heterogeneous collection of molecularly different subtypes (42). Unlike hormone receptor-positive type or HER2-type breast cancer, there is currently limited therapeutic targets for TNBC, Although immune
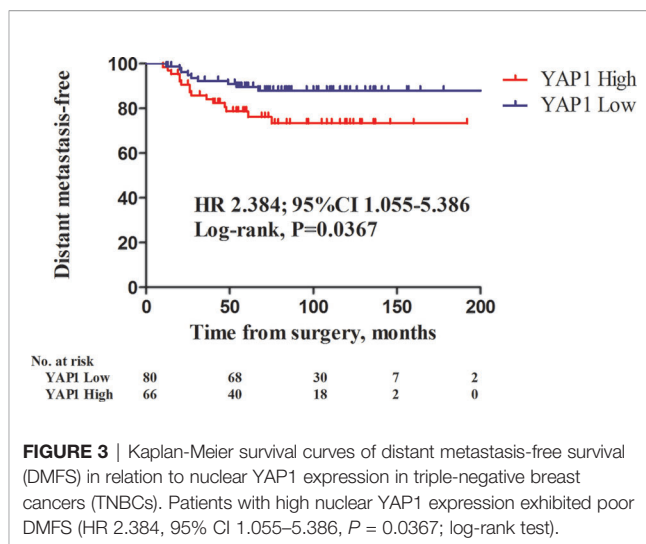
FIGURE 3 | Kaplan-Meier survival curves of distant metastasis-free survival (DMFS) in relation to nuclear YAP1 expression in triple-negative breast cancers (TNBCs). Patients with high nuclear YAP1 expression exhibited poor DMFS (HR 2.384, 95% CI 1.055–5.386, *P* = 0.0367; log-rank test).

checkpoint inhibitors are now applied in TNBC in combination with chemotherapy (43, 44), demanding on targeted therapy is still existed, and YAP1 could be the one of candidate of potential therapeutic target.

Our study has several limitations. First, it was a retrospective study. Second, the TNBC subtype was overrepresented due to selection bias during sample collection. Third, TMA slide staining may underestimate the rate of YAP1 positivity compared to whole-slide examination. Although our study is difficult to compare with previous IHC studies (20, 26, 38, 40, 41, 45), as these did not clarify whether the employed antibodies were specific for phosphorylated YAP1, nor was the intracellular localization of the antigen established, we specifically examined the nuclear YAP1 expression and confirmed its prognostic effect. In our study, no correlations were found between cytosolic YAP1 staining and survival (data not shown). Despite these limitations, the results demonstrated that nuclear YAP1 expression was a clinical prognostic factor in breast cancer, especially TNBC.

In conclusion, we suggested that nuclear YAP1 expression is a clinical prognostic factor for breast cancer. In addition, YAP1 is a potentially valuable therapeutic target for patients with breast cancer, especially in TNBC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki

Declaration and its later amendments or comparable ethical standards. The protocol was approved by the institutional review board (Local IRB number: 3-2019-0188) of Gangnam Severance Hospital. The need for informed consent was waived under the approval of the IRB due to the retrospective design.

AUTHOR CONTRIBUTIONS

YC and CY had full access to all of data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. Conceptualization, YC and CY. Data curation, YC, SB, DK, JK, T-KY, W-CP, AL, and CY. Funding acquisition, YC. Investigation, YC, SB, DK, SA, JJ, and CY. Methodology, YC and CY. Resources, JJ and JK. Formal analysis, YC and CY. Supervision, JJ. Writing—original draft, YC and CY. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.609743/full#supplementary-material

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