Yes-associated Protein Is Not an Independent Prognostic Marker in Breast Cancer

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Abstract. *Background: The development of tissue microarray (TMA) technology has provided the opportunity to perform analyses of tissue samples on a large scale in an uniform fashion. This study was designed with the use of TMA to explore the Yes-associated protein (YAP) status in breast cancer. Patients and Methods: YAP expression in tumor and tumor-free samples from 94 patients with primary breast cancer was analyzed by TMA. The clinicopathological data for age, estrogen receptor status, histological grading and TNM staging were also collected. Results: There were 29 patients (30.8%) with 1^+ expression, in YAP, 59 patients (62.8%) with 2^+ expression and 6 (6.4%) with 3^+ expression. There was no significant relationship between YAP expression and the other clinicopathological variables. By multivariate analysis, YAP expression failed to produce any significant relationship with the overall survival rate. Conclusion: YAP expression is not an independent prognostic factor in patients with breast cancer.*

The development of carcinogenesis is complicated, and multiple mechanisms are believed to contribute to the development of malignant tumors, following the disruption of the balance between apoptosis and cell proliferation (1, 2). Molecular pathways with a role in the maintenance of tissue hemostasis have been claimed to play a critical role in the proper control of cell proliferation and apoptosis (3, 4).

The Hippo pathway, a vital growth regulator of cell proliferation and apoptosis, was initially identified by mosaic screening in *Drosophila melanogaster* (5, 6). Yes-associated protein (YAP) is the mammalian ortholog of Drosophila Yorkie (Yki), which is a negatively-regulated downstream target of the Hippo signaling pathway, and acts as a transcriptional coactivator involved in the regulation of cell growth, proliferation and apoptosis (7, 9). The potent effects of YAP upon cell growth, division, and apoptosis support the concept that YAP normally functions to maintain tissue hemostasis, however, once dysregulated, it may contribute to a malignant cellular phenotype (10).

Breast cancer is not a single disease with variable morphological features and biomarkers, but rather a combination of molecularly and clinically distinct neoplastic diseases (11-13). Identification of the molecular characteristics of breast cancer is nowadays possible and the gene expression information found using several approaches may contribute to the designing of an optimal treatment plan, as well as for deliverance of prognostic information (14, 15).

The recent development of the tissue microarray (TMA) technology has provided the opportunity to perform analyses of tissue samples on a large scale in an uniform fashion with minimal damage to the original tissue blocks (16, 17).
This study was designed with the application of TMA to analyze the YAP status in breast cancer and with the hope of elucidating the possible relationship between YAP expression and breast cancer.

Patients and Methods

Patients and clinical samples. A retrospective study of 94 patients with primary invasive breast cancer selected from the pathology files of Kaohsiung Chang Gung Memorial Hospital between January 1994 and December 1998 was performed. The hematoxylin-eosin-stained slides of the paraffin-embedded tumor specimens were

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reviewed by a pathologist to confirm the accuracy of the histological diagnoses and lymph node status. Patients, except those with stage VI disease, underwent modified radical mastectomy due to invasive breast cancer, defined as carcinoma with invasion to or beyond the basement membrane, regardless of histological classification (ductal or lobular) (18). The data regarding primary tumor staging, age, estrogen receptor status (19-22), lymph node status, histological grading and TNM staging were also collected.

Construction of the tissue microarray. Representative areas of both tumor and non-tumor parts for each case were selected and circled to match the blocks for the tissue microarray. Then the blocks matching the circled slides were retrieved to prepare the recipient block for the microarray. To assure the representativity of the selected cores, three areas, each for both tumor and non-tumor parts per case were determined for assembling the recipient blocks. Each target area on the selected blocks was punched to form a 0.6 mm-diameter tissue core and was placed consecutively on the recipient blocks, of approximately 3 cm × 2 cm, with a precision instrument (Beecher Instruments, Silver Spring, MD, USA), as described elsewhere (23).

Immunohistochemical analysis. A rabbit polyclonal antibody against YAP (ab52771) was purchased from Abcam PLC (Cambridge, UK) and was diluted 1:100 in phosphate-buffered saline (PBS). Five-micrometer sections were cut from the recipient blocks of the TMA, incubated overnight in and over at 37˚C, de-waxed in xylene, and dehydrated in a series of graded alcohols. The sections were then treated with 3% hydrogen peroxide for 10 min to neutralize the endogenous peroxidase activity and microwaved in 10 mM citrate buffer at pH 6.0 to unmask the epitopes. After antigen retrieval, the sections were incubated with diluted YAP antibody for 1 h followed by PBS wash. Horseradish peroxidase/Fab polymer conjugate (PicTure™ -Plus kit) (Zymed, South San Francisco, CA, USA) was then applied to the sections for 30 min. After washing, the sections were incubated with the peroxidase substrate diaminobenzidine for 5 min and counterstained with hematoxylin.

Grading for YAP immunoreactivity. The immunoreactivity for YAP was scored by a four-tier grading system: 0, no staining in tumor cells; 1^+, weak cytoplasmic and/or nuclear staining in tumor cells; 2^+, an intermediate staining intensity between 1^+ and 3^+ in tumor cells; and 3^+, diffuse (more than 90% cells) and strong nuclear and cytoplasmic staining in tumor cells (Figure 1).

Patients and follow-up. All of the patients were women of 29 to 73 years of age, with a mean age of 49.5±10.2 years. The mean follow-up was 68.7±27.2 months (range=5 to 98 months). Follow-up was usually performed every three months for the first two years and then every six months for the next three years. After five years, follow-up became annual. Chest radiography, serum alkaline phosphatase level, and detailed physical examination were usually performed at follow-up. Annual mammography or breast sonography (for younger patients) were performed. Radionuclide bone scan, abdominal sonography or other image studies were performed if specific symptoms, signs or elevated serum alkaline phosphatase levels were noted. Data regarding patient survival, clinical status, and clinicopathological factors were obtained from medical records, contact with the patients at the outpatient clinics or by telephone, or both.

Table I. *Yes-associated protein (YAP) expression in relation to clinicopathological variables*.

Variable	YAP expression						
	1+		2+		3+		
	No.	%	No.	%	No.	%	*p*-Value
Age (years)							0.469
<50	17	58.6	28	47.5	4	66.7	
≥50	12	41.4	31	52.5	2	33.3	
ER status							0.815
Negative	16	55.2	36	61.0	4	66.7	
Positive	13	44.8	23	39.0	2	33.3	
Grading							0.460
1	3	10.4	10	16.9	0	0.0	
2	13	44.8	39	66.2	3	50.0	
3	13	44.8	10	16.9	3	50.0	
T-stage							0.598
T1	7	24.2	12	20.3	0	0.0	
T2	15	51.7	29	49.2	4	66.7	
T3	3	10.3	12	20.3	2	33.3	
T4	4	13.8	6	10.2	0	0.0	
N-stage							0.811
N0	13	44.8	29	49.2	3	50.0	
N1	5	17.2	9	15.3	2	33.3	
N2	7	24.2	10	16.9	1	16.7	
N3	4	13.8	11	18.6	0	0.0	
TNM-stage							0.567
I	3	10.4	9	15.3	0	0.0	
II	13	44.8	26	44.1	4	66.7	
III	13	44.8	20	33.9	2	33.3	
IV	0	0.0	4	6.7	0	0.0	

ER: Estrogen receptor.

Statistics. All analyses were carried out using the Statistical Package for the Social Sciences, release 17.0 (SPSS, Inc. Chicago, IL USA). Differences of clinicopathological features among groups by immunostaining were assessed with the χ^2 method and Fisher's exact test, where appropriate. Overall survival was calculated using univariate analysis by the Kaplan-Meier method. Differences in survival were tested using the log-rank test. To control for confounding factors, the Cox proportional hazard model was used. Survival plots were constructed using the Kaplan-Meier method. All tests were two-sided. Statistical significance was set at $p<0.05$.

Results

There were 29 patients (30.8%) with YAP expression of 1^+, 59 patients (62.8%) with 2^+ expression and 6 (6.4%) with 3^+ expression. There was no significant relationship between YAP expression and the other clinicopathological variables. By χ^2 comparisons between groups were made. There was no significant relationship between YAP expression and age (p=0.469), histological grading (p=0.460), primary tumor staging (p=0.598), lymph node status (p=0.811), estrogen receptor positivity (p=0.815) or TNM stage (p=0.567, Table I).

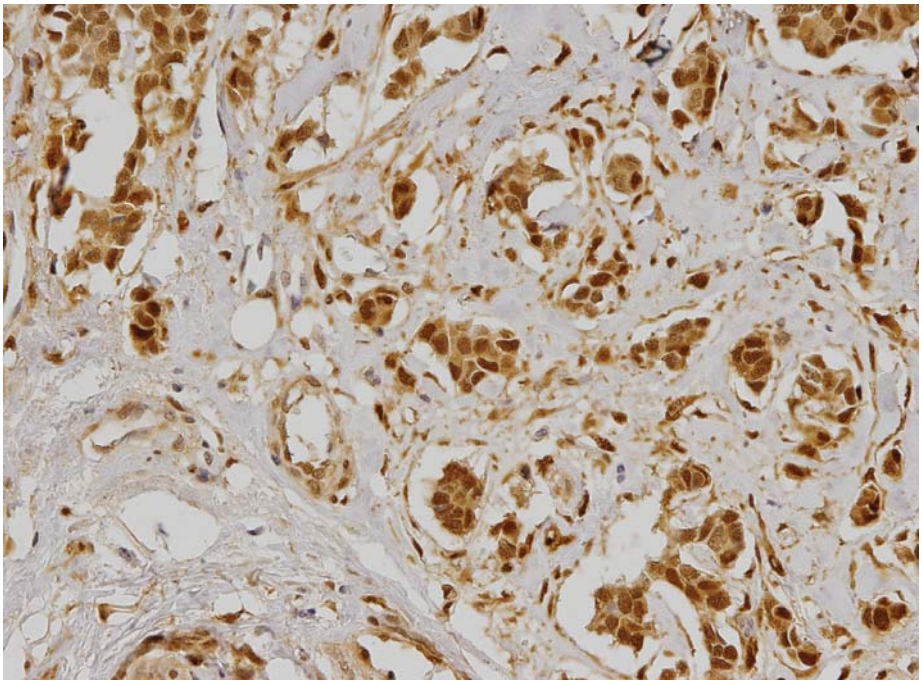

Figure 1. *A representative case of breast cancer scored as 3+ for (YAP) immunostaining. Diffuse and strong nuclear and cytoplasmic staining for YAP is noted in the tumor cells. Original magnification, ×200.*

For survival analyses, the end point was overall survival. The overall 5-year survival rates for different categories are listed in Table II. By multivariate analyses, YAP expression failed to have any significant relation to the overall 5-year survival rate (p=0.367, Table III).

Table II. *Overall 5-year survival rate according to clinicopathological category.*

Variable	Category	5-Year survival rate (%)	p-Value
Age (years)	<50	77.3	0.24
	≥50	64.4	
ER status	Negative	62.5	0.037
	Positive	81.2	
Grading	1	61.5	0.395
	2	76.3	
	3	65.4	
TNM stage	I	100.0	1.03×10^{-22}
	II	95.3	
	III	40.0	
	IV	0.0	
YAP expression	1^+	72.4	0.656
	2^+	72.8	
	3^+	50.0	

ER: Estrogen receptor. YAP: Yes-associated protein.

Discussion

The balance between cell proliferation and apoptosis is necessary to optimal tissue growth, development, and function. Destruction of the equilibrium can lead to excessive tissue loss, with resultant loss of function as in the case of excessive apoptosis (2), or it may cause tumorigenesis if there is inadequate apoptosis linked to uncontrolled cell proliferation. The Hippo pathway, which has been identified with the aid of Drosophila genetic screening, is a potent regulator of tissue homeostasis through controlling cell growth, division, and apoptosis (5, 6). It also has been claimed to be a potent developmental pathway as mice, deficient in YAP have an embryonic lethal phenotype (24). Given that this pathway controls the fundamental processes of cell division and death, it may be a primary target for dysregulation in cancer (25).

It has been claimed that the development of malignancy requires the following processes: self-sufficiency in growth signals, insensitivity to antigrowth signals, ability to evade apoptosis, unlimited replicative potential of the tumor cells, ability to sustain angiogenesis, and an ability to invade tissues and metastasize (1, 25). Genomic instability due to inactivation of tumor suppressors can lead to the above-mentioned processes of malignancy, resulting in the

Table III. *Multivariate analysis for overall survival rate.*

Variable	Category	Odds ratio	95% CI	*p*-Value
Age	≥50 *vs.* <50 years	1.21	0.51-2.84	0.998
ER status	Positive *vs.* Negative	0.38	0.4-0.98	0.047
Histological grading	1, 2, 3	2.11	1.11-4.03	0.023
TNM stage	I, II, III, IV	17.3	6.88-43.53	1.37×10^{-9}
YAP expression	1, 2, 3	1.36	0.70-2.67	0.367

95% CI: 95% confidence interval; ER: estrogen receptor; YAP: yes-associated protein.

appearance of pre-malignant cells (1). It is probable that YAP is not responsible for each of these processes, but it may be involved in providing an environment suitable for inhibition of apoptosis and promoting cellular proliferation by increasing the genomic instability of pre-malignant cells and enabling the critical processes in the development of malignancy (25, 26).

The application of TMA technology for immunohistochemical staining is an advantageous development, since this method guarantees the homogenous and synchronous analysis of different tissue samples and their gene expression under uniform test conditions (17, 27, 28).

As an array-based high-throughput technique, TMA allows for analysis of very large numbers of tumors at once, either at the DNA, RNA, or protein level in a parallel fashion, with minimal damage to the original tissue blocks (29). In contrast to conventional immunohistochemical analyses on large sections, TMA allows for a high level of standardization for immunohistochemical staining because all tumor samples are pre-treated and stained under exactly the same conditions. In contrast to the reading of large sections, which always is an attempt to integrate the observations in multiple, different regions of a tissue section, the morphological classification and interpretation of immunoreactivity are based on the findings within one small, highly-defined tissue area in TMA. The criteria for diagnostic decisions are therefore much easier to establish between the individual samples on the array and to compare among different observers (29-31).

Nevertheless, critique about TMA arises as to whether these small specimens (diameter 0.6 mm) are really representative of their donor tumors. It has been reported that some alterations are not detected if the analysis of heterogenous tumors is restricted to samples measuring 0.6 mm (28). However, Moch *et al.* (30) pointed out that the TMA approach has been designed to examine tumor populations and not to survey individual tumors. This group analyzed the impact of tissue heterogeneity on TMA data comparing results obtained from TMA with results from large sections in multiple different studies, and found that the results did show heterogeneity within tumors but suggested that this heterogeneity did not influence the identification of prognostic parameters. The reliability of TMAs in detecting protein expression and gene amplification in breast cancer has been confirmed (16, 32). Our study analyzed the YAP expression in breast cancer by immunohistochemical staining with TMA and the results were smoothly obtained).

In summary, YAP expression by immunohistochemical staining with TMA was not shown to be an independent prognostic factor in patients with breast cancer.

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