Nuclear Expression of Yes-associated Protein 1 Correlates with Poor Prognosis in Intestinal Type Gastric Cancer

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Abstract. *Background: Yes-associated protein 1 (YAP1) is an effector of the Hippo pathway, which is critical for regulating organ size and cell proliferation in mammals. To investigate the prognostic value of YAP1 in gastric cancer (GC), we assessed its expression in tumors from patients. Materials and Methods: We examined the nuclear expression of the YAP1 protein in 223 cases of GC, particularly of stage II and III disease, using immunohistochemistry. Results: Positive nuclear expression of YAP1 was detected in 27.4% (61/223) of total GCs, 29.1% (34/117) of the intestinal-type GCs (IGC) and 25.5% (27/106) of the diffuse-type GCs (DGC). In the IGC group, we found that the overall survival rate among patients with YAP1 nuclear expression-positive tumors was lower than that in the expression-negative group (p=0.021). Cox multivariate analysis revealed that the nuclear expression of YAP1 was an independent prognosticator of IGC (p=0.018). Conclusion: The nuclear overexpression of YAP1 is an independent biomarker for poor survival, especially for patients with intestinal-type gastric cancer.*

Yes-associated protein 1 (YAP1), a 65-kDa proline-rich phosphoprotein, was initially identified due to its interaction with the SH3 domain of the c-Yes tyrosine kinase (1). Subsequent studies revealed that YAP1 is a transcriptional co-activator interacting with PPXY-motif-containing transcription factors and TEAD-family transcription factors (2-4). YAP1 is considered a nuclear effector of the Hippo pathway (5, 6). The Hippo pathway, a vital regulator of organ size control and tumorigenesis, was initially identified by mosaic screening in *Drosophila melanogaster* (7-11). Components of the Hippo pathway are highly conserved from *Drosophila* to mammals, including *Mst1/2* (*hippo* homolog), *Sav1* (*salvador* homolog), *Lats1/2* (*warts* homolog), *Mob1* (*Mats* homolog), *Yap1* and *Taz* (*Yorkie* homolog) (6, 12, 13). The Hippo pathway kinase cascade phosphorylates YAP1 and induces sequestration of YAP1 in the cytoplasm, with the binding of YAP1 to 14-3-3, thus inhibiting its transcriptional activity (5, 14, 15).

YAP1 has been reported to have several oncogenic properties in human breast epithelium, including anchorage-independent growth, epithelial–mesenchymal transition, and resistance to apoptosis (16). YAP1 is also known to bind to the promoters of many genes which are closely related to stemness and to stimulate the expression of these genes (17). A study using transgenic mice with liver-specific YAP1 overexpression demonstrated a dramatic increase in liver size and the development of liver tumors (5). The *YAP1* gene locus 11q22 is known to be amplified in a wide spectrum of human cancers, including esophageal squamous cell carcinoma, medulloblastoma, and liver cancer (18-20). Consistently, frequent overexpression and nuclear localization of YAP1 has been revealed in many types of human cancer, including those of the liver, lung, ovary, pancreas, colon and prostate (5, 20, 21). YAP1 was reported to be an independent prognostic marker in hepatocellular carcinoma (22). However, with regard to the oncogenic impact of YAP1 in many types of human cancers, some of the reported data are contradictory. Yuan *et al*. found that YAP1 expression was decreased or lost in breast cancer and demonstrated the functional implications of YAP1 as a tumor suppressor (23). Taken together, these results suggest that the biological roles of YAP1 might be variable among different types of human cancers according to the different intrinsic properties of the tumor types.

Gastric carcinoma is one of the leading causes of death worldwide, despite a marked decline in its incidence in the West (24). It is widely accepted that gastric carcinogenesis is a multistep process, especially for intestinal-type gastric cancer (IGC) by Lauren classification, progressing through the stages of chronic gastritis, intestinal metaplasia, dysplasia, and finally gastric carcinoma (25), although *de novo* carcinogenesis also exists (26). There are many molecular

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alterations involved in gastric carcinogenesis, including genetic mutations, amplification of oncogenes, and genetic and epigenetic inactivations of tumor-suppressor genes (26, 27). A few studies have reported overexpression of YAP1 in gastric cancer (28, 29). Recently, a study by Kang *et al.* demonstrated oncogenic roles of YAP1 in gastric carcinoma and showed that nuclear accumulation of YAP1 is a poor prognostic marker (30). In this study, we assessed the value of YAP1 as a prognostic marker in gastric cancer using a large series of advanced stage II and III gastric carcinomas.

Materials and Methods

Patients and tissue samples. From January 2005 to December 2005, the cases of 223 patients who underwent radical total or subtotal gastrectomy and D2 lymph node dissection were examined. All specimens and surgical slides were obtained from the archives of the Department of Pathology, Yonsei University, Seoul, Korea. Authorization for the use of these tissues for research purposes was obtained from the Institutional Review Board of Yonsei University College of Medicine. Overall survival data were obtained from the Yonsei University Tumor Registry. All 223 cases were reviewed and reclassified according to the World Health Organization classification (31) and categorized as intestinal, diffuse, or mixed according to the Lauren classification (32). The mixed type in the Lauren classification was regarded as intestinal type in the survival analysis.

Immunohistochemistry. Paraffin-embedded tissue blocks were cut into 4-μm sections. Immunohistochemistry was performed using a Ventana XT automated stainer (Ventana Corporation, Tucson, AZ, USA) with antibodies against YAP1 (diluted 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were de-paraffinized using EZ Prep solution (Ventana Corporation). CC1 standard (pH 8.4 buffer containing Tris/borate/EDTA) was used for antigen retrieval and was blocked with inhibitor D (3% H_2O_2) for 4 min at 37˚C. Slides were incubated with primary antibody for 40 min at 37˚C followed by a universal secondary antibody for 20 min, at 37˚C. Slides were incubated in streptavidin-horseradish peroxidase (SA-HRP) D for 16 min at 37˚C and then the substrate, 3,3'-diaminobenzidine tetrahydrochloride (DAB) H_2O_2, was added for 8 min followed by hematoxylin and bluing reagent counterstaining at 37˚C.

The expression of YAP1 was assessed by a three-tiered scoring system which was separately applied to the nucleus and the cytoplasm of the tumor cells. Nuclear YAP1 expression was scored, based on the proportion of nuclear YAP1-expressing cancer cells (negative; focally-positive, ≤50%; diffusely-positive, >50%). Specimens exhibiting diffusely-positive staining were considered the nuclear-positive group, and those with negative- to focally positive- staining were regarded as the nuclear-negative group. Cytoplasmic YAP1 expression was also scored based on the proportion of cytoplasmic YAP1-expressing cancer cells (negative; focally-positive ≤50%; diffusely-positive, >50%). Specimens with diffusely-positive staining were classified as the cytoplasmic-positive group. Scoring was performed independently by two pathologists (Hy Kim and Ho Kim).

Statistical analysis. Statistical calculations were performed with SSPS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). To analyze the association between YAP1 staining and each clinicopathological parameter, the Pearson Chi-square test was performed for comparison of categorical variables between the nuclear YAP1-negative group and the nuclear YAP1-positive group. The student's *t*-test was used to analyze continuous data. Differences were considered significant at $p<0.05$. Overall survival analysis was performed using the Kaplan–Meier method, and the difference between groups was assessed using the log-rank test. Multivariate survival comparisons were carried out using Cox proportional hazard regression models. Estimated relative risks of dying were expressed as adjusted hazard ratios (HR) and corresponding 95% confidence intervals (95% CI).

Results

Determination of YAP1 expression by immunohistochemical analysis. YAP1 expression was evaluated in 223 gastric cancer cases by immunohistochemistry. Among the 223 cases, 93 (41.7%) were stage II and 130 (58.3%) were stage III disease. In the normal gastric mucosa, most non-cancerous epithelial cells displayed absent or weak expression of YAP1 (Figure 1A); only some cells in the proliferative area had moderate YAP1 expression. In gastric cancer cells, YAP1 expression was detected not only in the nucleus, but also in the cytoplasm (Figure 1B-D). Because nuclear localization is essential for YAP1 to function as a transcriptional effector, in this study, we restricted the evaluation of YAP1 to nuclear expression. The specimens were classified based on immunohistochemical results as belonging either to the nuclear-positive group or the nuclear-negative group. Positive nuclear expression of YAP1 was found in 61 out of 223 total gastric cancer specimens (27.4%). Nuclear positivity was detected in 29.1% (34/117) of intestinal-type gastric cancer (IGC) specimens and in 25.5% (27/106) of diffuse-type gastric cancer (DGC) specimens.

Correlations between nuclear expression of YAP1 and clinicopathological characteristics. The clinicopathological characteristics of the 223 primary gastric cancer biopsies are summarized in Table I. The results revealed a positive association of nuclear expression of YAP1 with younger age. Nuclear positivity for YAP1 was significantly more frequent in patients <65 years than in patients ≥65 years ($p=0.031$). However, we did not find any significant correlation between nuclear YAP1 expression and other clinicopathological variables, including gender, tumor location, Lauren classification, histological differentiation, invasion depth, lymph node metastasis, or TNM stage.

Nuclear expression of YAP1 correlates with poor survival in IGC. To evaluate whether nuclear expression of YAP1 in gastric cancer is associated with prognosis, Kaplan–Meier survival curves were constructed using overall cumulative survival (follow-up time of 5-75 months) to compare the

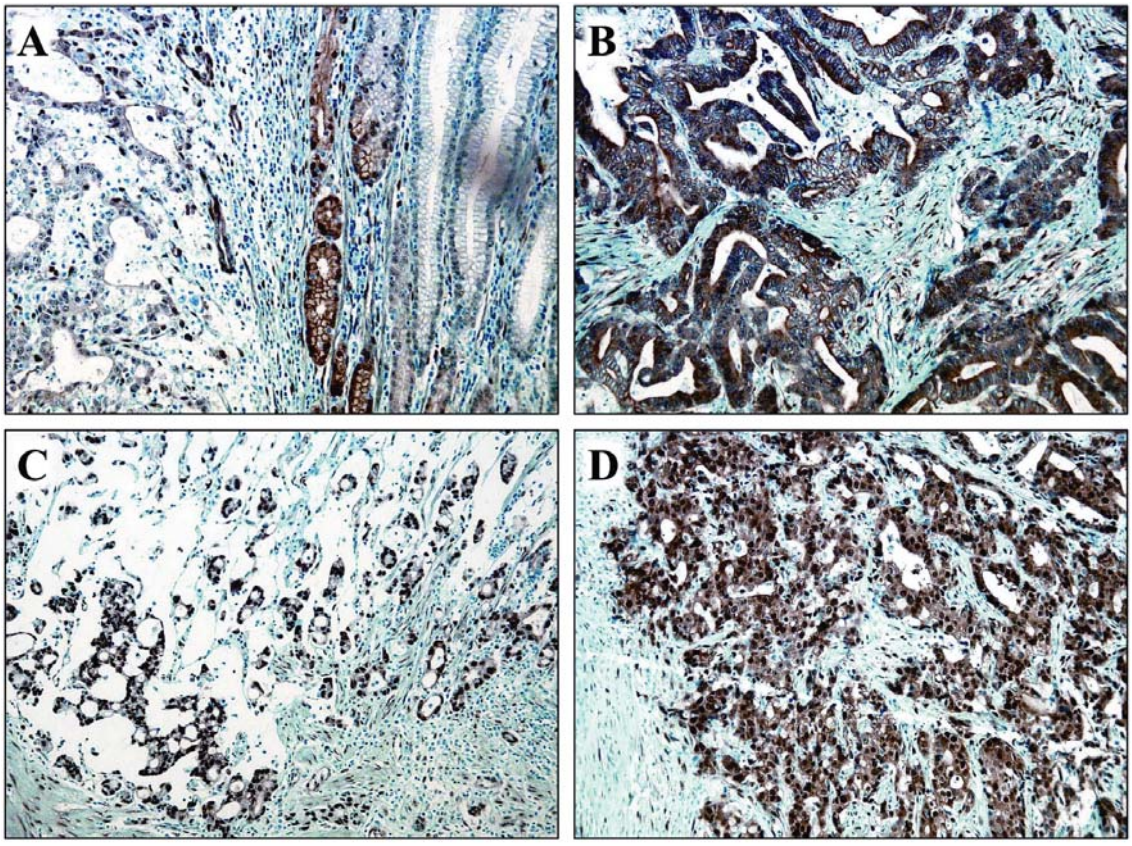

Figure 1. *Representative results of Yes-associated protein 1 (YAP1) expression in gastric cancer. Non-cancerous gastric epithelium (right) with negative staining and a cancerous area (left) with weakly positive cytoplasmic staining (A). Representative cases of cytoplasmic expression (B), positive nuclear expression (C) and both nuclear and cytoplasmic expression (D) of YAP1 in gastric cancer.*

nuclear YAP1-positive group to the nuclear YAP1-negative group. When all gastric cancer patients were analyzed together, nuclear YAP1 expression did not correlate with overall survival (p=0.356) (Figure 2A). However, when we divided the 223 gastric cancer patients into IGC (n=117, 52.5%) and DGC (n=106, 47.5%) groups for survival analysis, the five-year survival rate in patients with IGC with positive nuclear expression of YAP1 was significantly lower than that in corresponding patients with negative expression (p=0.021) (Figure 2B). We also analyzed the prognostic value of YAP1 in the IGC group according to cancer stage. In stage II IGC, those whose tumors were nuclear YAP1-positive had much worse survival rates than those with nuclear-negative expression (p=0.026) (Figure 2C). Similarly, there was a trend indicating that those in the nuclear YAP1-positive stage III group also had a worse survival rate compared to their nuclear-negative counterparts (Figure 2D). In the DGC group, the nuclear expression of YAP1 did not correlate with the five-year survival rate (p=0.303) (data not shown).

Nuclear expression of YAP1 in IGC cancer is an independent prognostic factor of poor survival outcome. We explored factors related to patient prognosis. On univariate analysis, depth of tumor invasion (p=0.030), lymph node metastasis (p=0.012), and TNM stage (p<0.001) were significantly associated with post-operative survival in all 223 cases, while nuclear YAP1 expression, age, gender, tumor location, Lauren classification, and histological differentiation had no prognostic value. In contrast, the nuclear expression status of YAP1 in the IGC group was significantly correlated with overall survival (p=0.024), along with depth of tumor invasion (p=0.018), lymph node metastasis (p=0.030) and TNM stage (p=0.001) (Table II). Moreover, multivariate Cox regression analysis of the overall patient group revealed that only tumor stage was an independent prognostic factor (p<0.001). However, analysis of the IGC group revealed that nuclear YAP1 expression (p=0.018) and TNM stage (p<0.001) were independent prognostic factors; the nuclear expression of YAP1 had an HR of 2.082 (95% CI=1.134-3.820) (Table III).

Table I. *Relationship between clinicopathological characteristics and nuclear Yes-associated protein 1 (YAP1) expression in patients with gastric cancer.*

Characteristic	Total no. of patients (n=223)		Nuclear YAP1 expression				*p*-Value
			Negative (%) 162 (72.6)		Positive (%) 61 (27.4)		
Age (years)							0.031
<65	155	(69.5)	106	(65.4)	49	(80.3)	
≥65	68	(30.5)	56	(34.6)	12	(19.7)	
Gender							0.076
Male	140	(62.8)	96	(59.3)	44	(72.1)	
Female	83	(37.2)	66	(40.7)	17	(27.9)	
Location							0.526
Upper	31	(13.9)	23	(14.2)	8	(13.1)	
Middle	54	(24.2)	36	(22.2)	18	(29.5)	
Lower	138	(61.9)	103	(63.6)	35	(57.4)	
Lauren classification							0.548
Intestinal	117	(52.5)	83	(51.2)	34	(55.7)	
Diffuse	106	(47.5)	79	(48.8)	27	(44.3)	
Histological differentiation							0.173
Well-	12	(5.4)	7	(4.3)	5	(8.2)	
Moderately-	62	(27.8)	46	(28.4)	16	(26.2)	
Poorly-	93	(41.7)	63	(38.9)	30	(49.2)	
Other	56	(25.1)	46	(28.4)	10	(16.4)	
Invasion depth							0.472
T1	5	(2.2)	4	(2.5)	1	(1.6)	
T2	31	(13.9)	22	(13.6)	9	(14.8)	
T3	83	(37.2)	64	(39.5)	19	(31.1)	
T4	104	(46.6)	72	(44.4)	32	(52.5)	
Lymph node metastasis							0.850
N0	43	(19.3)	29	(17.9)	14	(23.0)	
N1	52	(23.3)	43	(26.5)	9	(14.8)	
N2	58	(26.0)	37	(22.8)	21	(34.4)	
N3	70	(31.4)	53	(32.7)	17	(27.8)	
TNM stage							
II	93	(41.7)	68	(42.0)	25	(41.0)	0.893
III	130	(58.3)	94	(58.0)	36	(59.0)	

Discussion

The Hippo pathway is an important signaling pathway controlling organ size and regulating cell proliferation and apoptosis, and dysfunction of this pathway often contributes to tumorigenesis (12, 33). YAP1 is a downstream target of the Hippo pathway and plays a role as a transcription co-activator (6). Previous studies have also reported elevated YAP1 protein levels in various types of cancer, including gastric cancer (21, 28-30). Kang *et al*. found that YAP1 exhibited oncogenic properties in gastric cancer, and its nuclear accumulation was associated with a poor prognosis (30). According to Lauren classification, the intestinal and diffuse subtypes of gastric cancer have distinct pathogenesis, and specific biomarkers related to the IGC and DGC types remain to be identified (34, 35). In the current study, we investigated YAP1 expression in 223 gastric cancer specimens in order to assess whether YAP1 has different clinical presentations or oncogenic roles between the two major gastric cancer histological subtypes. In order to diminish the influence of tumor stage, we selected only patients with stage II and III gastric cancer.

By immunohistochemical staining, we observed that the YAP1 expression level in gastric cancer was significantly higher than in normal mucosa. This result was consistent with previous reports (28, 30). Frequent nuclear accumulation of YAP1 has been reported in many types of human cancers, including colon, ovary, liver, prostate and lung cancer (21, 22, 36, 37). YAP1 is inactivated by phosphorylation and sequestered in the cytoplasm *via* interaction with 14-3-3, whereas loss of Hippo signaling induces nuclear accumulation of YAP1 and increases its transcriptional activity (5, 14, 15). It is expected that a subcellular localization is important for

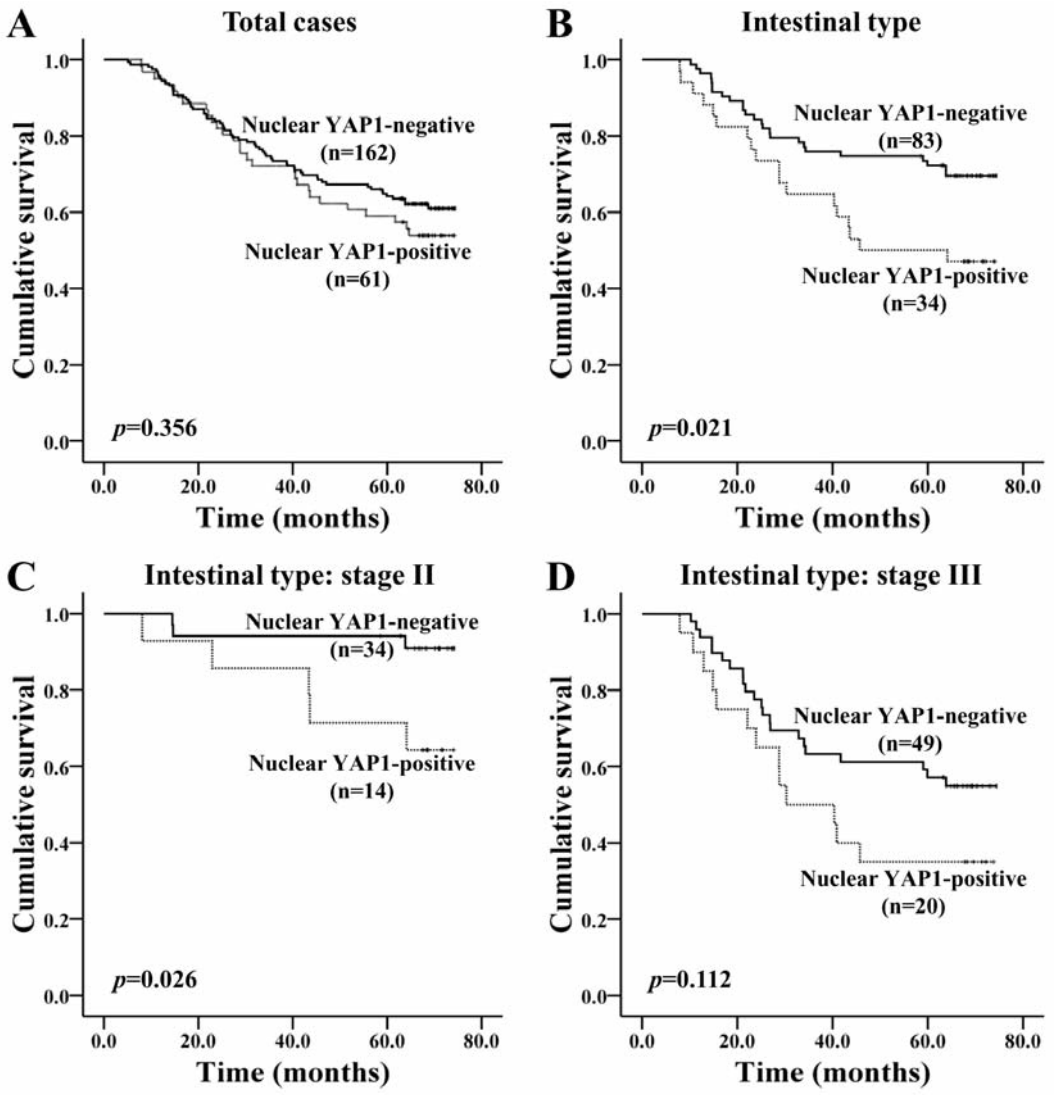

Figure 2. *Kaplan–Meier survival curves of patients with stage II or III gastric cancer according to nuclear Yes-associated protein 1 (YAP1) expression status. The five-year overall survival rate was analyzed for all patients with gastric cancer (n=223, p=0.357) (A), patients with intestinal-type gastric cancer (n=117, p=0.021) (B), and patients with stage II (n=48, P=0.026) (C) and stage III (n=69, p=0.112) (D) intestinal-type gastric cancer.*

YAP1; YAP1 functions oncogenically when it is accumulated in the nucleus of tumor cells. Consistent with a previous report (30), we did not identify any statistical correlation between the cytoplasmic expression of YAP1 and survival, or any of the clinicopathological factors (data not shown). For this reason, our current study was focused on the nuclear expression of YAP1 in gastric cancer.

Functionally, there is strong evidence suggesting YAP1 as a potential oncogene. Overexpression of YAP1 in non-transformed mammary MCF10A epithelial cells causes epithelial–mesenchymal transition, a phenomenon critical for cancer metastasis (16). YAP1 also promotes tumor growth in nude mice by cooperating with the *Myc* oncogene (20). Additionally, liver size is significantly increased when YAP1 is specifically up-regulated in the livers of transgenic mice (5). It was shown that ectopic expression of YAP1 in gastric cancer cells promotes anchorage-independent colony formation, inducing a more invasive phenotype, and accelerates cell growth both *in vitro* and *in vivo* (30). Several studies have also demonstrated the interplay between the YAP1 protein and some important signaling pathways: YAP1 interacts with SMAD family member 7 (SMAD7) and enhances the inhibitory activity of SMAD7 towards transforming growth factor, beta (TGF-β) signaling (38); inhibition of Notch signaling largely overcomes YAP-induced dysplasia and differentiation in mammals (39); and YAP1 was found

Table II. *Univariate Cox proportional regression analysis of data for gastric cancer overall and for intestinal-type gastric cancer.*

Characteristic	Total cases			Intestinal-type		
	No.	HR (95% CI)	*p*-Value	No.	HR (95% CI)	*p*-Value
Invasion depth						
T1 and T2	36	1.00 (–)		21	1.00 (–)	
T3 and T4	187	2.230 (1.079-4.610)	0.030	96	5.548 (1.341-22.948)	0.018
Lymph node metastasis						
N0/N1	95	1.00 (–)		51	1.00 (–)	
N2/N3	128	1.766 (1.134-2.748)	0.012	66	2.057 (1.072-3.947)	0.030
TNM stage						
II	93	1.00 (–)		48	1.00 (–)	
III	130	2.361 (1.478-3.772)	<0.001	69	3.919 (1.814-8.465)	0.001
YAP1						
Nuclear-negative	162	1.00 (–)		83	1.00 (–)	
Nuclear-positive	61	1.233 (0.789-1.927)	0.357	34	2.010 (1.096-3.687)	0.024

HR: Hazard ratio; CI: confidence interval; YAP1: Yes-associated protein 1.

Table III. *Multivariate Cox regression analysis of data for gastric cancer overall and for intestinal-type gastric cancer.*

Characteristic	Total cases			Intestinal-type		
	No.	HR (95% CI)	*p*-Value	No.	HR (95% CI)	*p*-Value
TNM stage						
II	93	1.00 (–)		48	1.00 (–)	
III	130	2.363 (1.479-3.774)	<0.001	69	3.998 (1.850-8.641)	<0.001
YAP1						
Nuclear-negative				83	1.00 (–)	
Nuclear-positive				34	2.082 (1.134-3.820)	0.018

HR: Hazard ratio; CI: confidence interval; YAP1: yes-associated protein 1.

overexpressed in subsets of medulloblastomas, which was driven by activated Sonic hedgehog (SHH) or wingless-type MMTV integration site family (WNT) signaling, indicating a crosstalk between YAP1 and SHH signaling as well as between YAP1 and the WNT signaling pathway (19, 39).

To explore the clinical impact of nuclear YAP1 expression in gastric cancer, we first analyzed the relationship between nuclear YAP1 expression and clinicopathological parameters. Our data revealed that except for age (<65 years *vs*. ≥65 years), there was no significant association between the nuclear expression of YAP1 and any other clinicopathological factor, which is in keeping with previous results (30). We also analyzed the influence of nuclear YAP1 expression on the survival of patients with gastric cancer. Interestingly, although we did not observe distinct expression patterns for IGC and DGC, expression in the two subtypes had different clinical significance. Our results indicated that in the IGC group, patients with nuclear YAP1 expression in their tumors had significantly poorer survival compared to those with negative expression. When we further analyzed the prognostic value of YAP1 in stage II or III IGC, we found that YAP1 expression was statistically associated with poor prognosis in stage II, but not in stage III cancer. In addition, nuclear expression of YAP1 was not significantly associated with the five-year survival rate for patients overall, nor in the DGC group. Furthermore, multivariate analysis showed that YAP1 expression was an independent prognostic factor for patients in the IGC group. Taken together, our findings provide evidence that elevated nuclear expression of YAP1 in IGC might contribute to the malignant potential and worse prognostic outcome.

In conclusion, we revealed that the nuclear accumulation of YAP1 protein might serve as an independent biomarker for poor prognosis, particularly in patients with IGC. Together with the previous report from Kang *et al*. (30), our findings strongly suggest that YAP1 has an important oncogenic role in gastric cancer, especially in IGC. To understand the precise function of YAP1, its underlying mechanism, and its impact on cancer prognosis, further investigations are still required.

Conflict of Interest

No conflicts of interest are declared.

Acknowledgements

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