Altered Expression of Yes-associated Protein and β-Catenin in Non-neoplastic and Neoplastic Gastric Surface Epithelia

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Abstract. *Aim: To investigate whether differential expression of Yes-associated protein (YAP) and β-catenin is important in gastric carcinogenesis. Materials and Methods: A total of 284 paraffin-embedded samples collected from 232 patients with gastric adenocarcinoma were used to evaluate YAP and β-catenin expression by immunohistochemistry, and the experimental findings were compared against those for gastric adenocarcinoma cell lines. Results: Nuclear YAP expression gradually increased from non-neoplastic epithelia to tubular or papillary adenocarcinomas (TPADs) and decreased in signet-ring cell carcinoma (SRCC). Cytoplasmic β-catenin expression increased from non-neoplastic epithelia to high-grade dysplasia and was decreased in TPAD and SRCC. YAP-overexpressing cell lines exhibited marked tumor cell invasion, whereas YAP-depleted cells showed reduced invasion. Conclusion: Nuclear YAP and cytoplasmic β-catenin play important roles in carcinogenesis, and the differential patterns YAP and β-catenin expression between TPAD and SRCC imply the existence of different carcinogenic pathways in these conditions.*

Gastric adenocarcinoma is one of the most common types of cancer and a leading cause of cancer-related deaths in both men and women worldwide, especially in Eastern Asia (1). The mechanisms of molecular aberrations of the carcinogenesis of gastric adenocarcinomas have been researched in order to identify novel diagnostic and therapeutic targets.

Yes-associated protein (*YAP*) has been shown to be an interesting oncogene due to its prognostic and therapeutic implications in the progression of various types of cancers (2). YAP is a signal-responsive transcriptional co-activator and a nuclear downstream effector of the Hippo pathway, and is located on human chromosome 11 (3). Elevated YAP expression in cell nuclei has been observed in ovarian carcinoma, colorectal adenocarcinoma, non-small cell lung carcinoma, esophageal squamous cell carcinoma, and hepatocellular carcinoma (4-7). The Hippo pathway is a tumor-suppressor signaling pathway that regulates both pathological tumorigenesis and physiological organ size. The Hippo pathway kinase cascade inactivates YAP by promoting its exportation out of the nucleus, and inactivation of the Hippo pathway results in nuclear overexpression of YAP (8).

β-Catenin is an essential signal transducer in the canonical Wnt signaling pathway. Membranous expression of β-catenin is known to act in the cell–cell adhesion complex linked to E-cadherin and the actin cytoskeleton, and β-catenin expressed in the nucleus acts as a gene transcription factor that is responsible for cell proliferation and differentiation (9, 10). The β-catenin level in the nucleus is elevated by the binding of Wnt to the target cell-surface receptor Frizzled. When the Wnt binds to Frizzled, β-catenin is dissociated from the destruction complex adenomatosis polyposis coli/Axin/glycogen synthase kinase 3b, accumulates in the cytoplasm and is then translocated into the nucleus. β-Catenin has been studied as an oncogene, and its nuclear overexpression has been associated with many types of cancers, including hepatocellular carcinoma, colorectal

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carcinoma, lung cancer, malignant breast tumors, and ovarian and endometrial cancer (11).

The aim of the present study was to determine whether the overexpression of YAP and β-catenin are important factors in the carcinogenesis of human gastric adenocarcinoma. We investigated the expression patterns of YAP and β-catenin in non-neoplastic and neoplastic gastric epithelia. Additionally, the correlation of YAP expression with tumor cell stromal invasion was tested with a three-dimensional organotypic gastric adenocarcinoma cell line model.

Materials and Methods

Patients. We obtained 284 stomach samples collected from 232 patients who underwent curative gastric resection at the Samsung Medical Center, Seoul, South Korea, from 1995 to 2005. The tissues consisted of 74 samples of non-tumor, 19 samples of low-grade dysplasia (LD), 21 samples of high-grade dysplasia (HD), 148 samples of tubular or papillary adenocarcinoma (TPAD), and 22 samples of signet-ring cell carcinoma (SRCC). The patients ranged in age from 27 to 80 years, with a mean of 59.3 years. The male-to-female ratio was 129:70. One hundred and fifty-three out of the 170 (90%) carcinoma samples were from the distal stomach (*i.e.* the distal body, angle, antrum or pylorus). None of the patients had received preoperative chemotherapy or radiotherapy. This study was approved by the Institutional Review Board of Chungnam National University Hospital (CNUH 2014-05-030).

The histopathological features of the gastric carcinomas that were examined were histological differentiation, depth of invasion, and lymph node metastasis. The stages of carcinomas were determined according to the American Joint Committee on Cancer staging system, seventh edition (12). All cases were histologically reviewed by two pathologists (KH. K. and SH. K.), and the two most representative areas of viable tissue were selected and marked on the hematoxylin and eosin-stained slides. To create a tissue microarray, tissue columns (3.0 mm in diameter) were punched from the original paraffin blocks and inserted into new recipient paraffin blocks (each containing 30 holes for tissue columns). The arrays were constructed using two 3-mm diameter cores per tumor.

Cells. Human primary cancer-associated fibroblasts (CAFs) of gastric adenocarcinomas were provided by Seok-Hyung Kim (Samsung Medical Center, Seoul, South Korea) (13). Human gastric adenocarcinoma cell lines AGS (KCLB 21739), MKN-28 (KCLB 80102), MKN-45(KCLB 80103), and MKN-74 (KCLB 80104) (those from Korean Cell Line Bank, Seoul, South Korea) were cultured in RPMI-1640 medium (Gibco®, NY, USA) with 1% fetal bovine serum and 1% penicillin-streptomycin at 37˚C in 5% CO_2.

Tissue immunohistochemistry. Tissue sections on microslides were de-paraffinized with xylene, dehydrated using serial dilutions of alcohol, and immersed in peroxidase-blocking solution (Dako, Glostrup, Denmark) to block the endogenous peroxidase activity. Heat-mediated antigen retrieval was performed with 10 mmol/l sodium citrate (pH 6.0) (Dako) for 15 min using a pressure cooker at full power for 3 min. The sections were incubated overnight at 4˚C with rabbit polyclonal antibody against YAP (1:100; Cell Signaling Tech Inc., Danvers, MA, USA) and rabbit monoclonal antibody against β-catenin (1:200; Abcam Inc., Cambridge, MA, USA). The sections were then incubated in DakoREAL EnVision/HRP rabbit/mouse detection reagent (Dako, Glostrup, Denmark) for 20 min at room temperature. The chromogen was then developed for 2 min, and the slides were counterstained with Meyer's hematoxylin, dehydrated and coverslipped. For the negative controls, isotype-matched irrelevant antibodies or preimmune rabbit/mouse serum was used in place of the primary antibodies.

Evaluation of the immunostained samples. All immunostained slides were digitally scanned using a Scanscope (Aperio ScanScope CS system, Vista, CA, USA). Immunohistochemical staining was scored using digitally scanned files and a light microscope. The nuclear and cytoplasmic expression of YAP, and membranous, cytoplasmic and nuclear expression of β-catenin were observed in the gastric surface epithelial cells of the non-tumor, LD, HD, TPAD and SRCC samples. We used the modified scoring methods of Sinicrope *et al*. (13) and Allred *et al*. (14) to evaluate both the intensity of immunohistochemical staining and the proportion of stained epithelial cells.

The staining intensity was further classified as follows: 1, weak; 2, moderate; and 3, strong. The proportional scores were: 0, 0; 1, >0 to 1/100; 2, >1/100 to 1/10; 3, >1/10 to 1/3; 4, >1/3 to 2/3; 5, >2/3 to 1. To generate the total immunohistochemical score, the intensity score and the proportional score were multiplied for each specimen. Each sample was examined separately and scored by two pathologists (K. H. K. and S. H. K.). Discrepancies in scores were discussed to obtain a consensus.

Western blot analysis. Nuclear and cytoplasmic proteins were extracted from AGS, MKN-28, MKN-45, and MKN-74 cells separately using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo science 78833; Pierce, Rockford, IL, USA). Protein concentrations were determined using Bio-Rad protein assay kits (Bio-Rad Laboratories Inc., Hercules, CA, USA). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (50 μg protein in each well), the samples were transferred to Polyvinylidene fluoride membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). Western blotting was performed with YAP (1:2000; Cell Signaling Tech Inc.) and β-actin (1:2,000; Cell Signaling Tech Inc.) antibodies. The membranes were incubated overnight at 4˚C with a rabbit polyclonal anti-YAP antibody (1:1000; Cell Signaling Tech Inc.). The protein bands were detected with enhanced chemiluminescence plus (GE Healthcare Life science, Armersham, NJ, USA), and the images were digitalized using an UVITEC Cambridge alliance mini 4M system (UVItec Limited, Cambridge, UK).

Cell invasion assay. The cell invasion assay was performed using Lab-Tek II 8-Chamber Slide™ (Thermo Fisher Science Inc., Rochester, NY, USA). Each chamber was filled with matrix [seven volumes Matrigel, one volume culture medium containing the CAFs (1×10^5 cells/ml), one volume culture medium, one volume FBS; Corning® Matrigel® Basement membrane matrix, 10 ml vial] and overlaid with RPMI-1640 medium with AGS, MKN-28, MKN-45, or MKN-74 cells (2×10^4 cell/each chamber) (14). After 4 days, the cells in the invasion assays were fixed in 4% formaldehyde and 0.25% glutaraldehyde, paraffin-embedded, and sectioned for staining with H&E to assess the tumor cell invasion into the extracellular matrix.

Statistics. The associations of the immunohistochemical expressions of YAP and β-catenin with the different types of gastric surface

Table I. *Analyses of the Yes-associated protein (YAP) and β-catenin expression in the gastric surface epithelia of normal tissue (non-tumor), dysplasia, tubular or papillary adenocarcinoma (TPAD) and signet ring cell carcinoma (SRCC). Data are median values (range).*

Clinicopathological feature	Nuclear YAP expression			Cytoplasmic β-catenin expression		
	Value	No.	*p*-Value	Value	No.	*p*-Value
Age (years)			0.686*			0.389*
<65	8 (0-12)	89		3 (0-10)	112	
≥65	8 (0-12)	47		3 (0-10)	56	
Sex			0.869*			0.666*
Male	8 (2-12)	47		3 (0-10)	62	
Female	8 (0-12)	89		3 (0-10)	106	
Gastric surface epithelia			<0.001†			<0.001†
Non-tumor	0 (0-8)	63		0 (0-5)	78	
Dysplasia (LD+HD)	8 (1-9)	38		5 (0-10)	40	
TPAD	8 (0-12)	121		3 (0-10)	150	
SRCC	4 (1-8)	19		0 (0-10)	20	
Differentiation‡			0.053*			0.299*
WDAC+MDAC	8 (2-12)	74		3 (0-10)	93	
PDAC	8 (2-12)	46		3 (0-8)	55	
Tumor stage			0.628*			0.238*
T1	8 (2-12)	35		3 (0-10)	38	
T2-T4	8 (0-12)	100		3 (0-10)	126	
Nodal stage			0.474*			0.318*
N0	8 (2-12)	51		3 (0-10)	59	
N1-N3	8 (0-12)	85		3 (0-10)	106	
pTNM stage			0.737*			0.980*
I	8 (2-12)	46		3 (0-10)	50	
II-IV	8 (0-12)	91		3 (0-10)	116	

*Mann-Whitney U-test; †Kruskal–Wallis test; LD, low-grade dysplasia; HD, high-grade dysplasia; TPAD, tubular or papillary adenocarcinoma; ‡differentiation of tubular or papillary adenocarcinoma; WDAC, well-differentiated adenocarcinoma; MDAC, moderately differentiated adenocarcinoma; PDAC, poorly differentiated adenocarcinoma.

epithelial lesions were examined with the non-parametric Kruskal-Wallis analyses combined with Scheffé post hoc comparisons. The clinicopathological variables were analyzed for statistical significance using Mann-Whitney *U*-tests. Statistical significance was set at $p<0.05$ (SPSS 21; SPSS Inc., Chicago, IL, USA).

Results

Differential expression of YAP and β-catenin in non-tumor, dysplastic epithelia, TPAD, and SRCC. The nuclear expression of YAP and cytoplasmic expression of β-catenin in the non-tumors, LDs, HDs, TPADs and SRCCs were measured by immunohistochemical staining. The Kruskal–Wallis test revealed a significant main effect of tissue type ($p<0.001$) (Table I and Figure 1). All non-neoplastic gastric surface epithelia expressed lower levels of YAP than did the neoplastic epithelia of dysplasia and TPAD ($p<0.001$ for non-tumor *vs*. dysplasia; $p=0.047$ for dysplasia *vs*. TPAD; and $p<0.001$ for TPAD *vs*. SRCC) (Figure 2A). The malignant cells (TPAD and SRCC) exhibited lower membranous expression of β-catenin than non-neoplastic epithelia ($p<0.001$). In dysplastic epithelia, the immunoreactivity for cytoplasmic β-catenin was higher than that observed in non-neoplastic surface epithelia, TPAD, and SRCC ($p<0.001$ for non-tumor *vs*. dysplasia; $p=0.002$ for dysplasia *vs*. TPAD; and $p=0.003$ for TPAD *vs*. SRCC) (Figure 2B). Nuclear expression of β-catenin was only observed in 1 out of the 21 HD cases (4%), 5 out of the 148 TPAD cases (3%), 1 out of the 22 SRCC of cases (4%), and none of the non-neoplastic gastric mucosa or LD (0%). No significant correlations of nuclear YAP or cytoplasmic β-catenin expression with the other clinicopathologic parameters were observed (Table I).

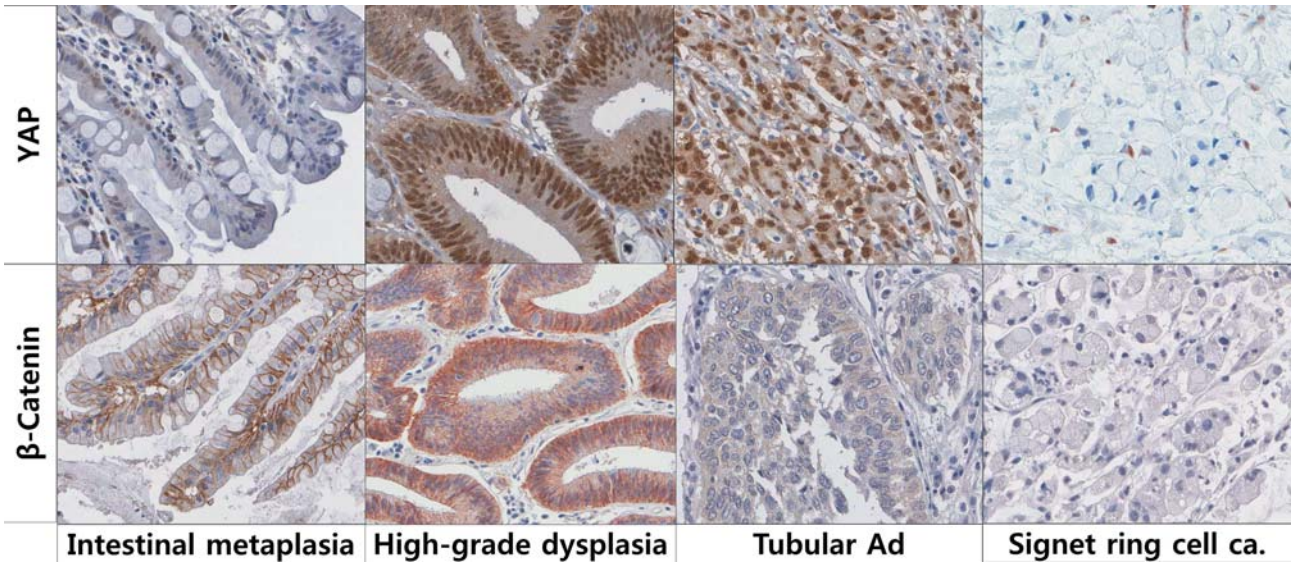

Figure 1. *Representative Yes-associated protein (YAP) and β-catenin expression in intestinal metaplasia, high-grade dysplasia, tubular adenocarcinoma (Ad) and signet ring cell carcinoma (ca.) of the stomach, as revealed by immunohistochemical staining (magnification, ×400). The strong nuclear expressions of YAP in the high-grade dysplasia and tubular adenocarcinoma contrasted with the staining in the signet ring cell carcinoma. The strong membranous expression of β-catenin in the intestinal metaplasia contrasted with the cytoplasmic expression in the high-grade dysplasia.*

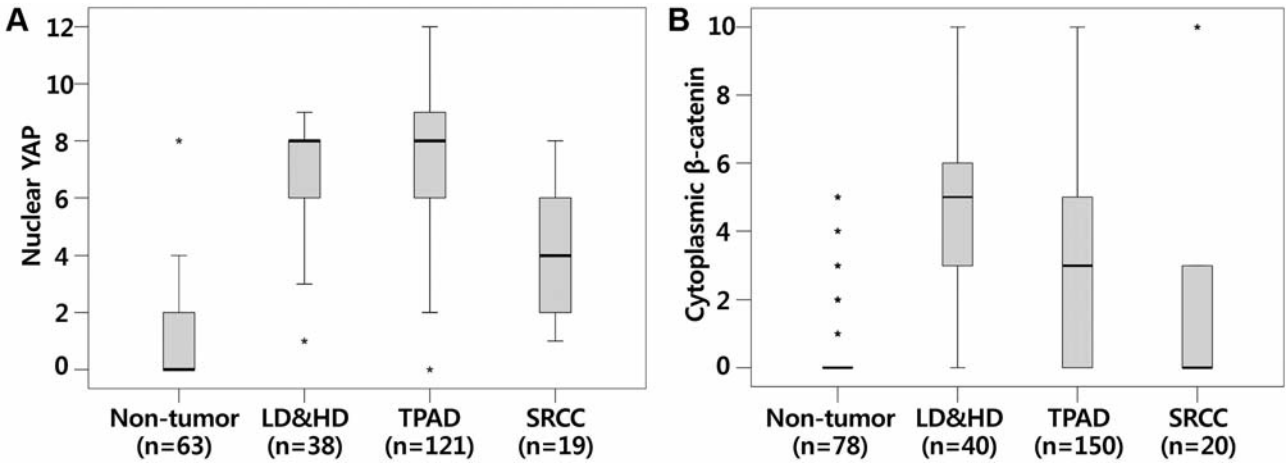

Figure 2. *Comparison of the expression of Yes-associated protein (YAP) and β-catenin between non-tumor, dysplasia, tubular or papillary adenocarcinoma (TPAD) and signet-ring cell carcinoma (SRCC) groups. A: Nuclear expression of YAP (Scheffé post hoc analyses: p<0.001 for non-tumor vs. dysplasia, p=0.047 for dysplasia vs. TPAD, and p<0.001 for TPAD vs. SRCC). B: Cytoplasmic expression of β-catenin (Scheffé post hoc analyses: p<0.001 for non-tumor vs. dysplasia, p=0.002 for dysplasia vs. TPAD, and p=0.003 for TPAD vs. SRCC). The dark line in the middle of the boxes is the median. The box length indicates the interquartile range. The ends of the whiskers represent maximum and minimum values excluding outliers. The asterisks are outlers greater than 3 times the inter quartile range.*

YAP expression in the gastric adenocarcinoma cell lines. By western blot, it was seen that nuclear and cytoplasmic YAP expressions were more reduced in the MKN45 cells than the AGS, MKN28 and MKN74 cells (Figure 3A). The invasion of the MKN45 cells in the three-dimensional organotypic cell culture model was less extensive than that of the AGS, MKN28 and MKN74 cells (Figure 3B).

Discussion

The present study showed the de-regulation of YAP and β-catenin during human gastric carcinogenesis. Our data provide evidence that nuclear expression of YAP gradually increased from non-tumor, LD and HD to TPAD but in SRCC. The cytoplasmic accumulation of YAP and β-catenin increased

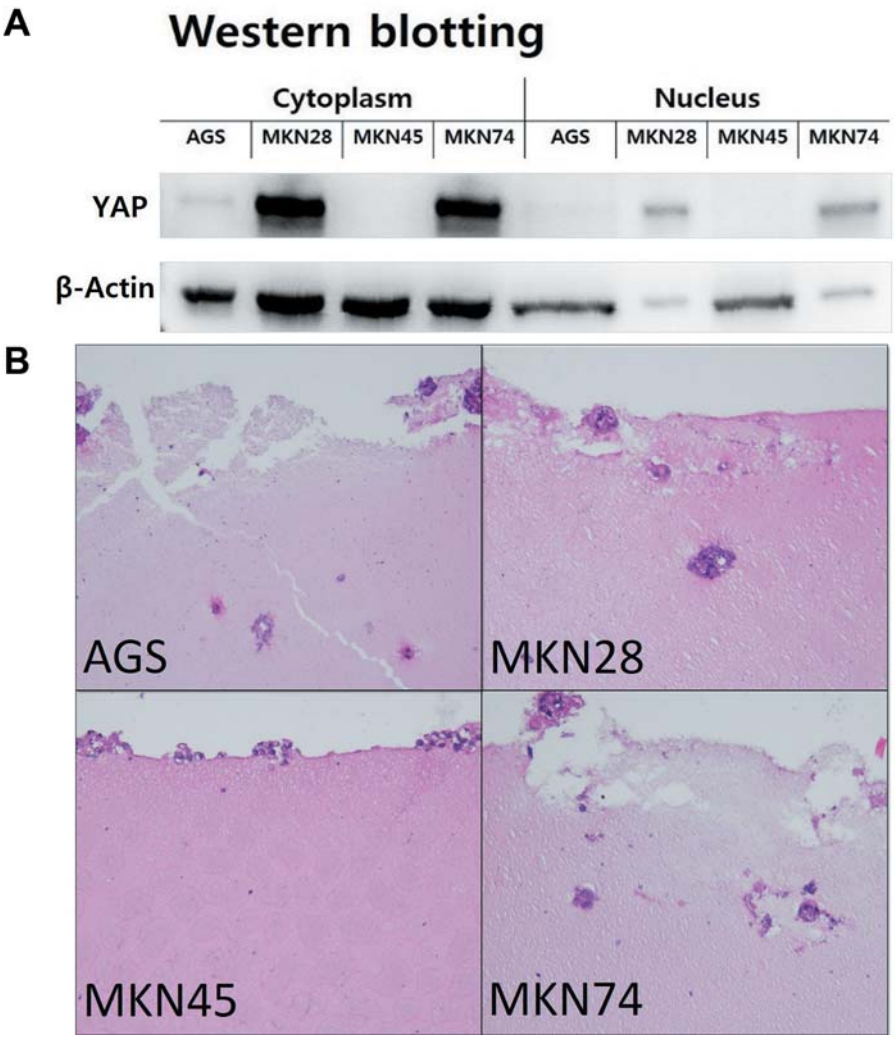

Figure 3. *Yes-associated protein (YAP) production in gastric cancer cell lines and tumor invasion assay with the three-dimensional organotypic cell culture model using a basement membrane matrix (BD Matrigel™). A: YAP was detected by western blot in the nuclear and cytoplasmic fractions. B: The three-dimensional organotypic cell culture model showed invasion of the gastric cancer cells. The MKN45 cancer cells in which YAP was down-regulated exhibited markedly suppressed invasion of the extracellular matrix.*

from non-tumor and LD to HD but decreased in TPAD and dropped in SRCC (Figure 1A). Nuclear expression of β-catenin was present in only one out of 21 HDs (4%), five out of 148 TPADs (3%), one out of 22 SRCCs (4%), and none of the non-tumors or LDs (0%). Our results show that YAP and β-catenin are involved in gastric carcinogenesis from LD to HD and that nuclear localization of YAP is involved in gastric TPAD but not in SRCC. The carcinogenesis of SRCCs might, therefore, occur along a different pathway from that of TPADs. Our results imply that nuclear localization of YAP might be a common event in the carcinogenesis of gastric TPAD. Additionally, using a three-dimensional culture model, we found that YAP overexpression in gastric adenocarcinoma cells promoted tumor cell invasion, while depletion of YAP reduced tumor cell invasion (Figure 3). These findings provide evidence that YAP expression plays a critical role in invasion of gastric adenocarcinoma.

The rate of β-catenin mutations in human gastric carcinoma tissue samples has been estimated to be 0-26% (15-17). Nuclear expression of β-catenin has been reported to occur in 12-37% of gastric carcinomas based on immunohistochemistry (16-18). In our cases of LD, HD and TPAD, we observed significant cytoplasmic accumulation of β-catenin and a loss of the membranous localization of β-catenin; nuclear expression of β-catenin was not significant. Nuclear expression of β-catenin was observed in only five out of the

148 TPAD cases (3%), which contrasts with previous reports of frequencies of 12-37% (16-18). However, a recent study showed that nuclear expression of β-catenin was present in only three out of 80 (4%) cases of gastric adenocarcinoma (19). The cytoplasmic accumulation of β-catenin in LD, HD and TPAD might result from the de-regulation of β-catenin, being insufficient for translocation into the nucleus. De-regulated cytoplasmic accumulation of β-catenin can perturb gastric homeostasis, including cell-cell adhesion. Our data showed a higher cytoplasmic expression of β-catenin in dysplastic gastric lesions (LD and HD) than in TPADs (Figure 2B). These data suggest that the de-regulation of β-catenin played a role in the progression of gastric dysplasia, which is an early event in gastric carcinogenesis (19-21).

Recent studies have shown that the interaction between the Wnt/β-catenin and the Hippo/YAP signaling pathways is critical for organ regeneration and carcinogenesis (22, 23). In HEK293T cells and Caco-2 colorectal cancer cells, activated YAP has been shown to suppress T-cell factor (TCF) transcriptional activity without suppressing the stability of β-catenin; indeed, activated YAP increases the level of β-catenin. Additionally, the overexpression of YAP suppressed the activity of a stabilized form of mutant β-catenin, and the stabilized form of mutant β-catenin exhibited a markedly enhanced TCF transcriptional activity. These results demonstrated that endogenous YAP negatively regulates the expression of Wnt-target genes, such as *TCF*, without suppressing β-catenin levels (23). Our results support the suggestion that the Hippo/YAP signaling pathways regulate β-catenin localization. It has been suggested that aberrant accumulation of β-catenin in the cytoplasm of dysplastic epithelia (LD and HD) provides evidence of the binding of stabilized β-catenin to the overexpressed YAP in the cytoplasm, which reduces its association with the cell membrane; however, β-catenin did not exhibit persuasive translocation into the nucleus. Thus, the overexpression of YAP might reduce a fraction of the β-catenin-mediated transcriptional activity and prohibit interactions with cell–cell adhesion molecules due to β-catenin binding in the cytoplasm. Our findings lead us to hypothesize that nuclear β-catenin has a weak effect on gastric carcinogenesis.

Gastric adenocarcinomas have been sub-classified into intestinal and diffuse types. Diffuse-type gastric adenocarcinomas correspond to poorly differentiated gastric carcinomas and include the SRCC and non-SRCC types (24). Meanwhile, it has been demonstrated the differences between SRCC and poorly-differentiated types of TPADs at the molecular features or malignant progression (25). Gastric SRCCs and TPADs are different in terms of clinical and pathological features, and these differences indicate different pathways of carcinogenesis and biological behaviors (26, 27). A comprehensive outline of the carcinogenesis of gastric SRCC has not been reported. The sequence 'gastritis-atrophy-metaplasia-cancer' is generally agreed upon as the primary course of carcinogenesis of gastric TPAD (28, 29). Our results show that the down-regulation of YAP and β-catenin in SRCC and their contrasting up-regulation in TPAD might be associated with the different carcinogenesis of SRCC and TPAD.

In conclusion, the present study suggests that nuclear localization of YAP is correlated with carcinogenesis of human TPAD from the early to late steps of induction, that a de-regulated over-abundance of cytoplasmic expression of β-catenin may occur in the early steps of the induction of human TPAD, and that the lack of nuclear YAP overexpression and cytoplasmic β-catenin accumulation observed in SRCC result from carcinogenesis that is different from that of TPAD.

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