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Differential expression of Yes-associated protein and phosphorylated Yes-associated protein is correlated with expression of Ki-67 and phospho-ERK in colorectal adenocarcinoma

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Summary. Yes-associated protein (YAP) is a transcriptional co-activator and functions as a nuclear downstream effector of the Hippo pathway. Differential expression of YAP and phosphorylated Yes-associated protein (pYAP), which are involved in the expression of Ki-67 and phosphorylated extracellular signal-regulated kinase (pERK) in colorectal adenocarcinoma (CRAC), is not clear. Herein, we hypothesized that nuclear expression of YAP could predict cell proliferation and poor prognosis, while cytoplasmic expression of pYAP would show a reverse correlation with cell proliferation. Paraffin-embedded samples from 144 CRAC patients were studied using immunohistochemistry for YAP, pYAP, Ki-67 and pERK. Frozen samples from 20 CRAC patients were examined for YAP mRNA in tumor and non-tumor tissues, using quantitative real-time PCR. High nuclear YAP expression coincided with high Ki-67 expression (P=0.002). The high nuclear YAP expression group tended to display a poor overall and disease-free survival (P=0.089 and P=0.089, respectively), but YAP mRNA levels in the 20 CRAC tissues were not significantly different in comparison with the 20 non-tumor tissues (P=0.929). We observed an inverse correlation between high cytoplasmic pYAP expression and high Ki-67 expression (P=0.001). Nuclear pERK expression was positively correlated with nuclear YAP expression, but negatively correlated with cytoplasmic pYAP expression (P=0.017 and P=0.020, respectively). Activated nuclear YAP and inactivated cytoplasmic pYAP in CRAC showed a positive correlation with Ki-67 and nuclear pERK expression, suggesting that the expression of YAP and pYAP is a possible predictor of tumor cell proliferation and prognosis in CRAC.

Key words: Colorectal adenocarcinoma, Yes-associated protein, Phosphorylated Yes-associated protein, Phosphorylated extracellular signal-regulated kinase, Ki-67

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Introduction

Yes-associated protein (YAP), the mammalian homologue of Drosophilia Yorkie, is the major downstream effector of the Hippo pathway (Zhao et al., 2008). YAP is a transcription co-activator, which induces the transcription of genes that promote cell proliferation and expression of negative regulators of apoptosis (Dong et al., 2007). The Hippo pathway, initially identified as a tumor suppressor pathway in Drosophilia, regulates cell growth and apoptosis (Huang et al., 2005; Dong et al.,

2007). The Hippo signaling pathway phosphorylates and inactivates YAP by promoting its cytoplasmic localization, where phosphorylated YAP at serine 127 is exported out of the nucleus, while inactivation of the Hippo pathway results in nuclear accumulation of YAP (Dong et al., 2007; Pan, 2010). The dysfunction of the Hippo pathway can lead to uncontrolled cell proliferation, and dysregulation of the Hippo/YAP signaling circuit could result in cancer cell proliferation and invasion (Xu et al., 2009).

YAP has been shown to be elevated in colonic adenocarcinoma, non-small cell lung carcinoma, ovarian serous cystadenocarcinoma, hepatocellular carcinoma, and esophageal squamous cell carcinoma (Steinhardt et al., 2008; Xu et al., 2009; Wang et al., 2010; Kim et al., 2011; Muramatsu et al., 2011). It is well documented that YAP expression is positively correlated with poor prognostic factors in diverse cancer patients. Overexpression of YAP in hepatocellular carcinoma was associated with poorer tumor differentiation and shown to be an independent prognostic marker for lower overall and disease-free survival (Xu et al., 2009). In non-small-cell lung carcinoma, YAP positivity is correlated with pathologic TNM stage and short overall survival (Wang et al., 2010). Nuclear expression of YAP is associated with shortened overall survival in esophageal squamous cell carcinoma and primary ovarian carcinoma (Hall et al., 2010; Muramatsu et al., 2011). However, to our knowledge, the clinicopathologic values of YAP and phosphorylated YAP (pYAP) in human colorectal adenocarcinoma (CRAC) have not yet been explored.

The aim of the present study was to determine whether differential expression of YAP and pYAP can be an important factor for tumor cell proliferation in human CRAC. Here, we investigated the expression pattern of YAP and pYAP in CRAC, and we evaluated the relationships between YAP and pYAP expression and clinicopathologic variables including Ki-67 expression, phosphorylated extracellular signal-regulated kinase (pERK), histologic differentiation, pathologic TNM stage, and overall survival. We also studied mRNA levels of YAP in CRAC tissues and non-tumor tissues.

Materials and methods

Patients, tissue samples, and reagents

We investigated 144 paraffin-embedded samples from 144 CRAC patients for immunohistochemical study. Information on the patients and samples was obtained from surgical pathology files maintained in the pathology department of Chungbuk National University Hospital, South Korea between 1994 and 1998. All archival tissues were routinely fixed in 10% neutral-buffered formalin and embedded in paraffin. To create a tissue microarray, tissue cores (3.0 mm in diameter) were punched from original paraffin blocks and inserted into new paraffin blocks. Arrays were constructed using two 3-mm diameter cores for tissue.

Colon tissue samples of 20 patients with CRAC were included in the present study. Tumor tissues and non-tumor tissues of the mucosal and submucosal layers were collected at the time of surgery and frozen at -80°C for RNA extraction. These specimens were provided by National Biobank of Korea, Chungnam National University Hospital, Daejeon, Korea.

All enrolled patients underwent curative surgical resection with none having prior chemotherapy or radiation therapy. The study was approved by the Chungnam National University Hospital Institutional Review Board.

Immunohistochemical analysis

Embedded tissue sections on microslides were deparaffinized with xylene and hydrated in graded alcohol series. The sections were heated in a pressure cooker containing 10 mmol/L sodium citrate (pH 6.0) for 3 min at full power for antigen retrieval. Peroxide blocking was performed using 3% H_2O_2 in methanol at room temperature for 10 min, and non-specific protein-binding sites were blocked by incubation with serum-free protein for 20 min. The sections were incubated overnight at 4°C with the primary antibodies: rabbit polyclonal anti-YAP antibody (1:100, Cell Signaling Tech, Danvers, MA, USA); rabbit polyclonal anti-phospho-YAP (Ser127) antibody (1:100, Cell Signaling Tech), mouse monoclonal anti-Ki-67 antibody (1:100, Dako, Glostrup, Denmark) and rabbit polyclonal anti-phospho-ERK1/2 (1:50, Abcam, Cambridge, UK). After washing, samples were incubated in Dako REAL EnVision/horseradish peroxidase rabbit/mouse detection reagent for an additional 20 min at room temperature followed by additional washing. After rinsing, the chromogen was developed for 2 min. The slides were then counterstained with Meyer's hematoxylin, dehydrated and coverslipped.

Evaluation of 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. Nuclear YAP, cytoplasmic pYAP, nuclear pERK and cytoplasmic pERK expressions were observed in the tumor cells. In this study, we used the modified scoring method of Sinicrope et al. for evaluating both the intensity of immunohistochemical staining and the proportion of stained epithelial cells (Sinicrope et al., 1995). The staining intensity was further classified as follows: 1, weak; 2, moderate; and 3, strong. The positive cells were quantified as a percentage of the total number of epithelial cells and assigned to one of the following five categories (0, 0%; 1, <10%; 2, 10%-50%; 3, 51%-80%; and 4, >80%). The percentage of positivity of the tumor cells and the staining intensities were then multiplied to generate the immunohistochemistry score

for each of the tumor specimens. For categorical analyses, the immunoreactivity in tumor cells was graded as low or high from median values (high grade, more than median values; cut-off values were 7 for expression of nuclear YAP, 3 for expression of cytoplasmic pYAP, 5 for expression of cytoplasmic pERK and 4 for expression of nuclear pERK expression) (Fig. 1). We defined the median Ki-67 labeling index value as cut-off point. Cut-off value of 19% distinguishing high versus low Ki-67 labeling index was used in this study. Each sample was examined separately and scored by two pathologists (K. H. K. and D. H. K.). Discrepancies in scores were discussed to obtain a consensus.

Quantitative real-time PCR

Total RNA from 20 pairs of CRAC tissue and non-tumor tissue from 20 CRAC patients was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) and converted to cDNA. YAP mRNA levels were quantified in CRAC tissue and non-tumor tissue using the Rotor-Gene SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Quantitative real-time PCR (qRT-PCR) was run on Rotor-Gene Q (Qiagen, Hilden, Germany). The sequences of the primer pairs are YAP forward 5'-CGCTCTTCAACGCCGTCA-3' and YAP reverse 5'-AGTACTGGCCTGTCGGGAGT-3'. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. GAPDH was used as the reference gene (QT01192646, Qiagen). We used each non-tumor tissue control as the calibrator. The relative levels of gene expression were represented as ΔCt (ΔCt =Avg.YAP CT-Avg. GAPDH CT), and the fold change of gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Experiments were repeated in triplicate (Livak and Schmittgen, 2001).

Table 1. Association of nuclear YAP expression with clinicopathologic variables in colorectal adenocarcinoma.

Characteristics	Patients	nuclear YAP expression		
	No. (%)	Low	High	P
Age (years)				0.788
≤ 60	61 (42.4)	41 (43.2)	20 (40.8)	
>60	83 (57.6)	54 (56.8)	29 (59.2)	
Gender				0.181
Male	64 (44.4)	46 (48.4)	18 (36.7)	
Female	80 (55.6)	49 (51.6)	31 (63.3)	
Ki-67				0.002
Low	69 (50.7)	53 (60.9)	16 (32.7)	
High	67 (49.3)	34 (39.1)	33 (67.3)	
Nuclear pERK				0.340
Low	96 (71.6)	64 (74.4)	32 (66.7)	
High	38 (28.4)	22 (25.6)	16 (33.3)	
Cytoplasmic pERK				0.935
Low	76 (56.7)	49 (57.0)	27 (56.3)	
High	58 (43.3)	37 (43.0)	21 (43.8)	
Differentiation				0.421
Well	44 (30.6)	27 (28.4)	17 (34.7)	
Moderate	85 (59.0)	56 (58.9)	29 (59.2)	
Poor	15 (10.4)	12 (12.6)	3 (6.1)	
Pathologic stage				0.215
I and II (%)	75 (52.1)	53 (55.8)	22 (44.9)	
III and IV (%)	69 (47.9)	42 (44.2)	27 (55.1)	

Pearson's chi-square test.

Statistical analysis

For the statistical analysis of staining intensity, the data for each tissue were categorized as 'high' or 'low' from the median score for YAP nuclear expression, pYAP cytoplasmic expression and Ki-67 nuclear expression. The clinicopathologic variables were analyzed for statistical significance using the Mann-Whitney U test and Pearson's chi-square test. The strengths of association between two variables were accessed via Spearman's coefficient of rank correlation. For the evaluation of survival, Kaplan-Meier survival curves were constructed with the log rank test. Statistical significance was assumed when $P<0.05$ (SPSS 19, Chicago, IL, USA).

Results

Clinicopathologic features and the expression patterns of YAP and pYAP in CRAC

We investigated 144 samples of CRAC. The average age of the patients was 60.4 years, and the male:female ratio was 80:64 (53.0%:42.4%). YAP was generally expressed in both the nucleus and cytoplasm, but more strongly expressed in the nucleus in CRAC. pYAP was expressed more strongly and diffusely in the cytoplasm in CRAC.

Correlation of nuclear YAP and cytoplasmic pYAP expression with Ki-67, pERK and clinicopathologic variables

Correlations between YAP and pYAP expression and age, sex, pERK expression, Ki-67 expression, histologic grade, T (tumor) stage, nodal status, and pTNM staging for CRAC samples are summarized in Tables 1 and 2 (Hamilton et al., 2010). The relationships between high Ki-67 expression and high nuclear YAP expression or low cytoplasmic pYAP expression attained statistical significance (P=0.002 and P=0.001, respectively; Pearson's chi-square test). High cytoplasmic pERK expression showed a positive correlation with high cytoplasmic pYAP expression, but no correlation with high nuclear YAP expression (P<0 .001 and P=0.935, respectively; Pearson's chi-square test). The nuclear expression of pERK was positively correlated with YAP nuclear expression, but negatively correlated with pYAP cytoplasmic expression (P=0.017 and P=0.020,

respectively; Spearman's coefficient of rank correlation) (Table 3).

We analyzed the relationships between nuclear expression of YAP and overall and disease-free survival rates. Kaplan-Meier survival estimates showed a trend of higher nuclear immunoreactivity of YAP in CRAC being associated with shortened overall and disease-free survival (P=0.089 and P=0.089, respectively; Fig. 2A,B) but not on multivariate analysis (Table 4). Lower cytoplasmic immunoreactivity of pYAP in CRAC tended to show a shortened overall survival (P=0.119 ; Fig. 2C).

Relative YAP mRNA expression in 20 pairs of CRAC tissues and non-tumor tissues of the mucosal and submucosal layer

We analyzed relative expression levels of YAP mRNA by quantitative real-time PCR in 20 pairs of CRAC tissue and non-tumor epithelial tissue from 20 patients. YAP mRNA levels in the CRAC tissues were not significantly different in comparison with the non-tumor tissues (P=0.929). Seven CRACs had higher relative YAP mRNA levels while thirteen CRACs had lower levels than in the non-tumor epithelial tissue (Fig. 3). The mean value of the normalized YAP mRNA amount relative to non-tumor tissues from 20 CRACs was 1.012, while every value of normalized YAP mRNA

Table 2. Association of cytoplasmic pYAP expression with clinicopathologic variables in colorectal adenocarcinoma.

Characteristics	Patients	cytoplasmic pYAP expression		
	No. (%)	Low	High	P
Age (years)				0.511
≤ 60	58 (42.0)	33 (44.6)	25 (39.1)	
>60	80 (58.0)	41 (55.4)	39 (60.9)	
Gender				0.921
Male	61 (44.2)	33 (44.6)	28 (43.8)	
Female	77 (55.8)	41 (55.4)	36 (56.3)	
Ki-67				0.001
Low	68 (50.4)	27 (37.5)	41 (65.1)	
High	67 (49.6)	45 (62.5)	22 (34.9)	
Nuclear pERK				0.078
Low	96 (71.6)	47 (65.3)	49 (79.0)	
High	38 (28.4)	25 (34.7)	13 (21.0)	
Cytoplasmic pERK				<0.001
Low	76 (56.7)	52 (72.2)	24 (38.7)	
High	58 (43.3)	20 (27.8)	38 (61.3)	
Differentiation				0.595
Well	41 (29.7)	20 (27.0)	21 (32.8)	
Moderate	83 (60.1)	45 (60.8)	38 (59.4)	
Poor	14 (10.1)	9 (12.2)	5 (7.8)	
Pathologic stage				0.121
I and II (%)	70 (50.7)	33 (44.6)	37 (57.8)	
III and IV (%)	68 (49.3)	41 (55.4)	27 (42.2)	

Pearson's chi-square test.

Table 3. Correlation between nuclear pERK, cytoplasmic pERK, nuclear YAP and cytoplasmic pYAP final scores by immunohistochemical staining of colorectal adenocarcinoma.

Spearman's rho	Nuclear pERK	Cytoplasmic pERK	Cytoplasmic pYAP
Cytoplasmic pERK			
Correlation coefficient	0.277**		
Sig. (2-tailed)	0.001		
No.	134		
Cytoplasmic pYAP			
Correlation coefficient	-0.201*	0.467*	
Sig. (2-tailed)	0.020	<0.001	
No.	134	134	
Nuclear YAP			
Correlation coefficient	0.206*	-0.042	-0.095
Sig. (2-tailed)	0.017	0.630	0.266
No.	134	134	138

*P<0.05; **P<0.01.

Table 4. Cox Regression analysis of overall survival.

Variable	Frequency	Coefficient	P	Hazard ratio	95.0% CI
Nuclear YAP					
low	84			Reference group	
high	48	0.187	0.594	1.206	0.606-2.400
Cytoplasmic pYAP					
low	71			Reference group	
high	61	-0.192	0.647	0.825	0.363-1.877
Age					
≤ 60	56			Reference group	
>60	76	0.253	0.493	1.288	0.625-2.653
Gender					
male	57			Reference group	
female	75	-1.022	0.015	0.360	0.158-0.822
Ki-67					
low	65			Reference group	
high	67	-0.521	0.147	0.594	0.293-1.201
Nuclear pERK					
low	94			Reference group	
high	38	0.393	0.304	1.482	0.701-3.134
Cytoplasmic pERK					
low	74			Reference group	
high	58	-0.081	0.836	0.922	0.428-1.986
Differentiation					
well	40			Reference group	
moderate	79	-0.343	0.405	0.709	0.316-1.591
poor	13	1.483	0.011	4.405	1.410-13.765
Pathologic stage					
I and II	66			Reference group	
III and IV	66	3.371	<0.001	29.120	7.705-110.062
Radiotherapy					
No	112			Reference group	
Yes	20	1.040	0.016	2.828	1.216-6.576
Chemotherapy					
No	35			Reference group	
Yes	97	-1.544	0.007	0.213	0.069-0.662

amount relative to non-tumor tissues from 20 non-tumor tissues was 1.000. High nuclear YAP expression was detected in eleven of 20 tumor tissues and positively correlated with YAP mRNA amount in 20 tumor tissues (P=0.046). In the 20 non-tumor epithelial tissues, epithelia in crypt zone generally showed nuclear YAP overexpression whereas surface tip epithelia did not.

Discussion

In this study, we investigated the expression patterns of YAP and pYAP in CRAC using immunohistochemistry. Our study indicated that YAP nuclear expression and pYAP cytoplasmic expression were significantly associated with tumor cell proliferation (P =0.002 and P=0.001, respectively). Higher nuclear expression of YAP showed a trend toward a correlation with a worse overall and disease-free survival (P=0.089 and P=0.089, respectively).

A previous report demonstrated that YAP nuclear staining was increased in human colonic adenocarcinomas and was seen in proliferative or regenerative epithelia (Steinhardt et al., 2008). Although YAP is expressed physiologically in the intestinal stem cell compartment and capable of driving stem cell proliferation, the activation of YAP in normal epithelia is insufficient to drive colonic tumorigenesis and is kept mostly inactive through the action of the Hippo pathway. YAP overexpression in colon cancer is needed to recruit a critical proliferative drive that is not engaged at normal physiologic YAP abundance (Avruch et al., 2012). YAP activity is regulated by phosphorylation. A serine/threonine kinase called large tumor suppressor-1 (Lats-1), which is in turn activated by another

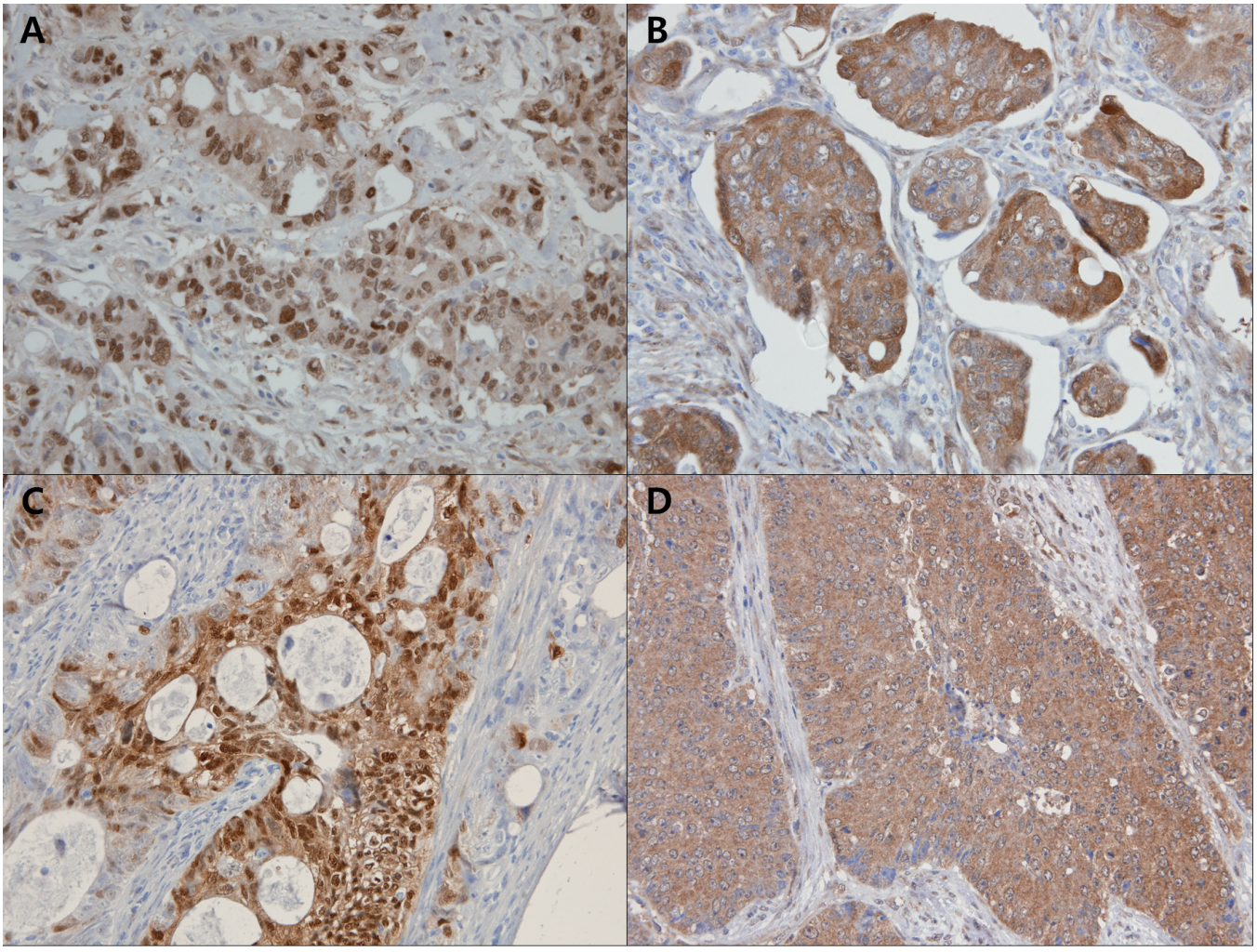

Fig. 1. Representative expression of YAP, pYAP, and pERK in colorectal adenocarcinoma, revealed by immunohistochemical staining. **A.** High-grade nuclear expression of YAP. **B.** High-grade cytoplasmic expression of pYAP. **C.** High-grade nuclear expression of pERK. **D.** High-grade cytoplasmic expression of pERK. x 400

serine/threonine kinase called Mst, phosphorylates YAP at serine 127 (Septer et al., 2012). Ablation of the kinases Mst1 and Mst2, orthologs of the Drosophila anti-proliferative kinase Hippo, from mouse liver caused an abrupt loss of YAP phosphorylation and enhanced YAP nuclear accumulation accompanied by hyper-proliferation, anti-apoptosis, and the subsequent emergence of hepatocellular carcinoma (Zhou et al., 2011). In mouse intestinal epithelia, elimination of the protein kinases Mst1 and Mst2 results in a marked increase in the abundance of YAP and its intense nuclear localization with a decrease in the extent of YAP phosphorylation (Zhou et al., 2011). Phosphorylation of YAP promotes YAP nuclear exit, translocation to the cytoplasm and subsequent degradation (Zhao et al., 2010). In our study, higher nuclear expression of YAP correlated with tumor proliferation, which was based on Ki-67 expression, while lower cytoplasmic expression of

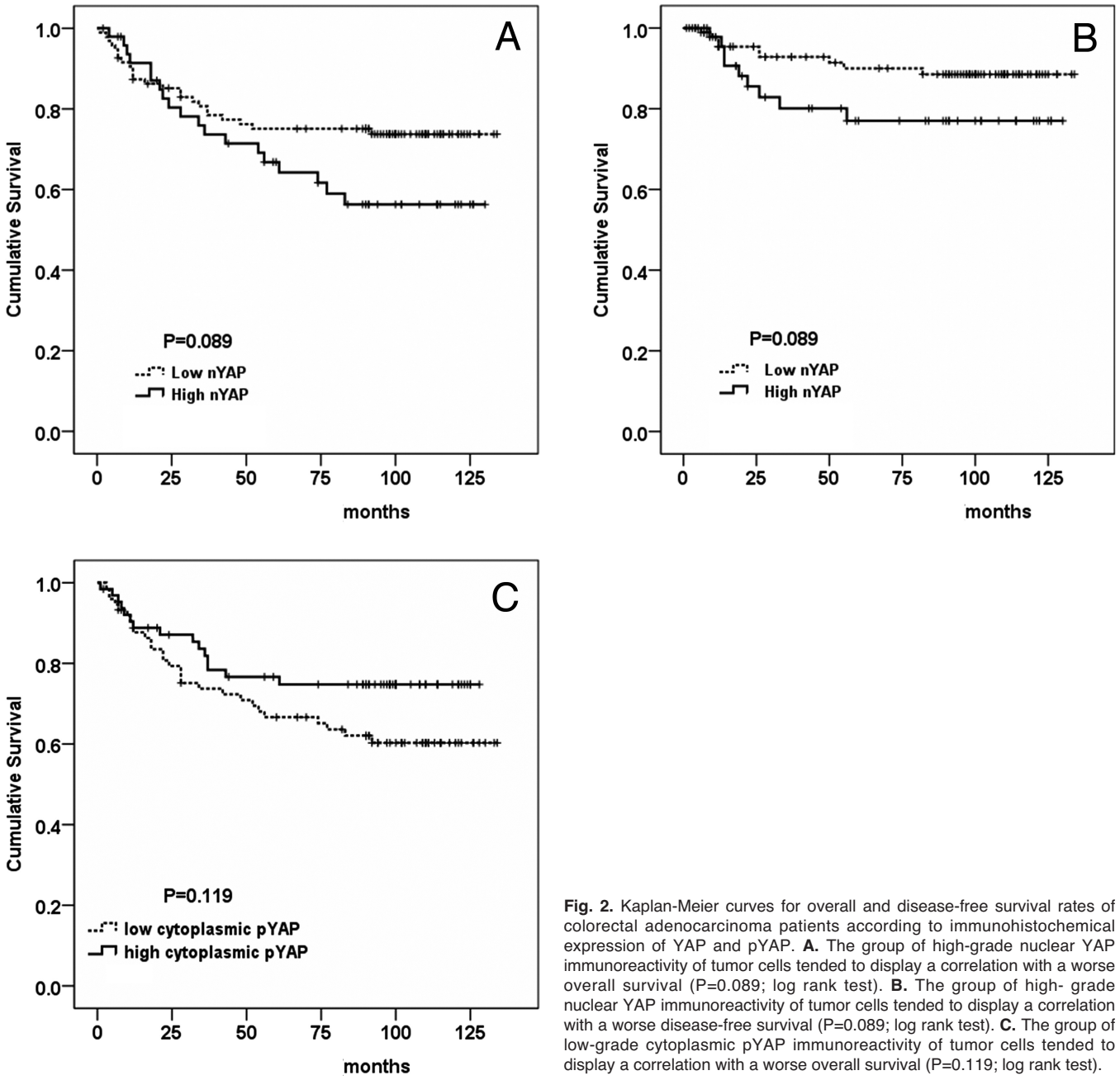

Fig. 2. Kaplan-Meier curves for overall and disease-free survival rates of colorectal adenocarcinoma patients according to immunohistochemical expression of YAP and pYAP. **A.** The group of high-grade nuclear YAP immunoreactivity of tumor cells tended to display a correlation with a worse overall survival (P=0.089; log rank test). **B.** The group of high- grade nuclear YAP immunoreactivity of tumor cells tended to display a correlation with a worse disease-free survival (P=0.089; log rank test). **C.** The group of low-grade cytoplasmic pYAP immunoreactivity of tumor cells tended to display a correlation with a worse overall survival (P=0.119; log rank test).

pYAP correlated with Ki-67 expression. Diffuse cytoplasmic expression of pYAP could occur if the Hippo pathway is preserved, and was inversely correlated with Ki-67 expression in CRAC. These results support the proposal that YAP overexpression in colon cancer is crucial to the ability of YAP to drive cell proliferation and that the Hippo pathway is the major regulator of YAP polypeptide degradation (Avruch et al., 2012). Recent evidence indicates that aberrant Wnt/,-catenin signaling results in nuclear import of the ,-catenin transcriptional co-activator human colorectal cancer cell lines. ,-catenin/TCF4 complexes in nucleus directly regulate YAP gene expression which is required for growth of established human colorectal cancer cell lines (Konsavage et al., 2012). For esophageal squamous cell carcinoma, gastric adenocarcinoma, hepatocellular carcinoma and non-small cell lung carcinoma, YAP has been associated with a poor prognosis for patients with these diseases (Xu et al., 2009; Wang et al., 2010; Kang et al., 2011; Muramatsu et al., 2011). In this study, we revealed that high nuclear immunoreactivity of YAP in CRAC tended to show shortened overall and disease-free survival (P=0.089 and P=0.089, respectively) (Fig. 2A,B), whereas low cytoplasmic immunoreactivity of pYAP in CRAC tended to show shortened overall survival (P=0.119), although the data did not show statistical significance (Fig. 2C).

The ERK family of mitogen-activated kinase is activated by mitogenic factors through the Ras/Raf/mitogen-extracellular signal regulated kinase/ERK cascade (Pearson et al., 2001). ERK is activated in the cytoplasm and capable of affecting gene expression, but to exert many functions, ERK must translocate from the cytoplasm to the nucleus (Howe et al., 2002). According to this notion, a recent study has shown a positive correlation between nuclear pERK expression and histological grade or tumor stage in primary colon adenocarcinomas (Levidou et al., 2012). We found that nuclear pERK expression was positively correlated with nuclear YAP expression, but negatively correlated with cytoplasmic pYAP expression (P=0.017 and P=0.020, respectively; Spearman's coefficient of rank correlation) (Table 3). The results are in agreement with the hypothesis that subcellular localization of ERK is a critical factor in determining the responses of the ERK pathway (Howe et al., 2002; Chen et al., 2005), and that the expression of YAP and pYAP contributes to tumor cell proliferation.

YAP mRNA in CRAC was studied by quantitative real-time PCR analysis. We investigated 20 pairs of CRAC and non-tumor tissue from 20 patients. The mean value of the normalized YAP mRNA amount relative to non-tumor tissue in 20 CRAC tissues was not significantly different from that of the paired non-tumor tissues. A previous study indicated that increased YAP protein level was not due to increased transcription, since YAP mRNA was slightly decreased in the regenerating crypts (Cai et al., 2010). The result of our study indicated that overexpression of YAP in CRAC was not due to increased transcription but other factors.

In conclusion, the results of this study indicate that increased nuclear expression of YAP and decreased cytoplasmic expression of pYAP in CRAC are important for malignant cell proliferation. Moreover, nuclear YAP and cytoplasmic pYAP expression contributes to determining the prognosis of patients with CRAC. This study is limited by the lack of elucidation of the mechanisms underlying overexpression of nuclear YAP and cytoplasmic pYAP in CRAC, and thus, further studies are needed to confirm our findings and make

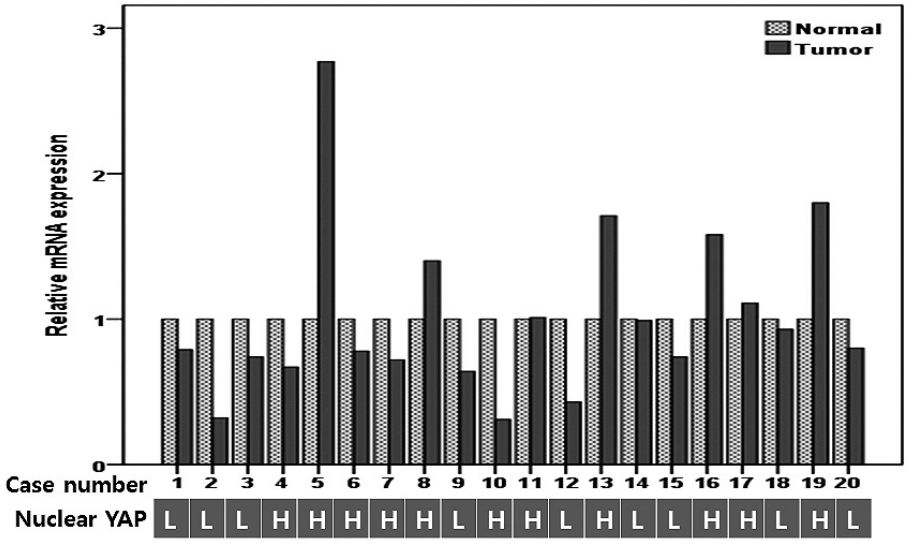

Fig. 3. Relative mRNA levels of Yes-associated protein (YAP) in colorectal adenocarcinoma. Twenty pairs of tumor and non-tumor tissues were assayed by quantitative real-time PCR. The fold change of YAP mRNA expression in tumor compared with normal mucosa was calculated by the $2^{-\Delta\Delta Ct}$ method. nuclear YAP: YAP nuclear expression in tumor; H: high nuclear YAP expression; L: low nuclear YAP expression.

YAP a target protein for cancer therapeutics.

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