Yes-associated Protein 1 Exhibits Oncogenic Property in Gastric Cancer and Its Nuclear Accumulation Associates with Poor Prognosis

Wei Kang,1,2,§ Joanna H.M. Tong,1,2,§ Anthony W.H. Chan,1 Tin-Lap Lee,4 Raymond W.M. Lung,1,2 Patrick P.S. Leung,1,2 Ken K.Y. So,1,2 Kaichun Wu,5 Daiming Fan,4 Jun Yu,2,3 Joseph J.Y. Sung,2,3 Ka-Fai To1,2,*

1Department of Anatomical and Cellular Pathology, Sir Y. K. Pao Center for Cancer, State Key Laboratory in Oncology in South China, The Chinese University of Hong Kong, Hong Kong; 2Institute of Digestive Disease and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong; 3Department of Medicine, The Chinese University of Hong Kong, Hong Kong; 4The Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, USA. 5State Key Laboratory of Cancer Biology and Institute of Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xi'an, the People’s Republic of China. *Corresponding Author. §Equal contribution.

Correspondence:

Prof. Ka-Fai To, Department of Anatomical and Cellular Pathology, Institute of Digestive Diseases, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N.T., Hong Kong SAR, The P. R. China. Phone: (852) 26323334; Fax: (852) 26376274; E-mail:
kfto@cuhk.edu.hk
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Statement of Translational Relevance

Gastric cancer remains a worldwide common malignancy with high mortality. Further understanding of gastric carcinogenesis is critical in searching for potential prognostic biomarkers and therapeutic targets. The function of YAP1 in gastric cancer is unclear. In this study we demonstrated that nuclear accumulation of YAP1 correlated with poor disease specific survival. Especially for patients in early stage, YAP1 was an independent prognostic marker. The oncogenic property of YAP1 in gastric cancer was demonstrated by \textit{in vitro} and \textit{in vivo} study. We further investigated the downstream effectors of YAP1 by microarray analysis and confirmed experimentally. Our finding not only enhanced our understanding in the mechanisms of gastric carcinogenesis, but also provided a potential diagnostic marker and therapeutic target for this aggressive malignancy.
Abstract

Purpose: Yes-associated protein 1 (YAP1) is a multifunctional protein that can interact with different transcription factors to activate gene expression. The role of YAP1 in tumorigenesis is unclear. We aimed to investigate the functional role of YAP1 in tumorigenesis of gastric cancer.

Experimental Design: YAP1 expression in gastric adenocarcinoma was evaluated. The biological function was determined by proliferation assay, colony formation, cell invasion, flow cytometry analysis through knocking down or ectopic expressing YAP1 in gastric cancer cell lines coupled with in vivo study. The possible downstream effectors of YAP1 were investigated by expression microarray.

Results: YAP1 protein expression was up-regulated in gastric cancer. Nuclear accumulation of YAP1 was associated with poor disease specific survival (P = 0.021), especially in patients with early stage diseases (P < 0.001). Knockdown YAP1 resulted in a significant reduction in proliferation, anchorage-dependent colony formation, cell invasion and cell motility. Ectopic YAP1 expression promoted anchorage-independent colony formation, induced a more invasive phenotype and accelerated cell growth both in vitro and in vivo. Microarray analysis highlighted the alteration of MAPK pathway by YAP1. We confirmed a constitutive activation of RAF/MEK/ERK in YAP1-expressing MKN45 cells and further demonstrated that YAP1 enhanced serum/EGF induced c-Fos expression in gastric cancer cells.

Conclusions: Our findings supported YAP1 exhibits oncogenic property in gastric cancer. We provided the first evidence that YAP1 exerted the oncogenic function by enhancing the
capacity to activate the early response gene pathway. YAP1 could be a prognostic biomarker and potential therapeutic target for gastric cancer.
Introduction

Gastric cancer is one of the most common malignancies worldwide and is the second most frequent cause of cancer-related death with little improvement of long-term survival during the past decades. More than 700,000 people die from gastric cancer each year and more than 40% occur in China (1). Gastric cancer is thought to result from a combination of environmental and genetic factors. Various genetic, epigenetic and molecular alterations are found in gastric cancer that underlies the multi-step tumorigenic process. *H. pylori* infection, alterations in oncogenes, tumor-suppressor genes, cell cycle regulators, cell-adhesion molecules and DNA repair genes, and genetic instability as well as telomerase activation are implicated in gastric tumorigenesis (2).

Yes-associated protein 1 (YAP1) is a 65 kDa proline-rich phosphoprotein located on chromosome 11q22.1. It is originally identified due to its interaction with the Src family tyrosine kinase Yes (3). It contains a WW domain, a PDZ interaction motif, a SH3 binding motif, and a coiled-coil domain (4). YAP1 has been found amplified in breast cancer and liver cancer, where it promotes tumorigenesis (5-6). Over-expression of YAP1 induces epithelial-to-mesenchymal transition, growth factor-independent proliferation, and suppression of apoptosis, suggesting a role as oncoprotein. Recent studies have established YAP1 as a transcription co-factor and a downstream effector of the MST2-WW45–Lats1/2 (Hpo–Sav–Wts in Drosophila) signaling, a pathway that controls cell growth and organ size (7-8). In contrast, other studies identify that YAP1 can stabilize p73 post-translationally and therefore activate the transcription of proapoptotic genes in response to apoptotic stimuli (9). Together with the evident that up-regulation of YAP1 correlates with better survival in
a cohort of breast cancer patients (10), its activity is thought to favor tumor suppression. It has been proposed that whether YAP1 is anti- or pro-tumorigenic is dependent on cell context and type of stimuli. Post-translational modifications and binding affinity of protein-protein interaction domains are also critical in determining the biological role of YAP1 (9). Given that little is known about the function of YAP1 in gastric cancer, we aimed to examine its potential role in tumorigenesis of gastric cancer by both ectopic expression and knockdown approaches.

Materials and Methods

Cell lines. Nine human gastric cancer cell lines, MKN45, MKN28, KATO-III, AGS, NCI-N87, SNU1, SNU16, MKN1 and MKN7, were obtained from either the American Type Culture Collection (Rockville, MD, USA) or RIKEN Cell Bank (Tsukuba, Japan).

Primary gastric adenocarcinoma specimens. A total of 30 primary gastric adenocarcinomas (GACs) and their paired non-cancerous gastric mucosal tissues were obtained from the Endoscopy Centre of the Prince of Wales Hospital. Another 10 GACs and paired non-cancerous gastric mucosal tissues from patients who underwent curative surgery were collected. All specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C until processing. A cohort of 129 formalin-fixed paraffin-embedded tissues of GACs diagnosed between 1998 and 2002 in the Prince of Wales Hospital, Hong Kong was retrieved. The clinicopathologic information was summarized in Supplementary Table 1A. In brief, the median age of the patients was 69 years (range 38-88 years) and male to female ratio was 1.8:1. The median follow-up time was 17.2 months (range 0.3-143.4 months). The study protocol was approved by the Joint CUHK-NTE Clinical Research
Ethics Committee, Hong Kong.

**Quantitative real-time PCR (QRT-PCR).** Total RNA was prepared using Trizol reagent (Invitrogen) and reverse transcription was done by using High-capacity cDNA Reverse Transcription Kit (Appliedbiosystems). QRT-PCR was run on ABI 7500 real time fast PCR system (Appliedbiosystems) using primers listed in Supplementary Table 2. All reactions were done in triplicate. The relative expression level was normalized with beta-actin and calculated using the $2^{\Delta\Delta Ct}$ method.

**Immunohistochemistry.** Immunohistochemistry was performed on the Ventana Nex ES automated stainer (Ventana Corporation, Tucson, AZ) using anti-YAP1 antibody (1:100, Abcam). Antigen retrieval was performed by using pressure cooker with 10nM citrate buffer (pH 6.0) for 4 minutes. The nuclear expression of YAP1 was scored by estimating proportion of tumor cells with positive nuclear staining (0, none; 1, $\leq10\%$; 2, 10 to $\leq50\%$; 3, $>50\%$). The cytoplasmic expression of YAP1 was assessed by assigning a proportion score and an intensity score. The proportion score was according to proportion of tumor cells with positive cytoplasmic staining (0, none; 1, $\leq10\%$; 2, 10 to $\leq25\%$; 3, $>25$ to $50\%$; 4, $>50\%$). The intensity score was assigned for the average intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; 3, strong). The cytoplasmic score of YAP1 was the product of proportion and intensity scores, ranging from 0 to 12. The cytoplasmic expression was categorized into low (score 0 to 3), intermediate (score 4-6), and high (score 7-12). The scoring was independently assessed by two pathologists (K.F.T. and A.W.H.C.)

**Western blot analysis.** Western blot analysis was performed as previously described (11). The primary antibodies used were YAP1 (Abcam), ERK1/2, phosphorylated-ERK1/2, c-Fos
(all from Cell Signaling). All horseradish peroxidase-conjugated secondary antibodies used were purchased from DAKO.

**Vector construction and transfection.** Full length YAP1 was amplified by PCR and cloned into pcDNA3.1(+) (Invitrogen) to produce YAP1 expression vector and transfected into MKN45 cells using Genejuice (Novagen). Stable YAP1-expressing clones were selected using G418. Restoration of YAP1 expression was confirmed by western blot analysis. HP Validated siRNAs targeting YAP1 were purchased from Qiagen (Catalog No.S102662954) and transfected into AGS and MKN1 cells using Lipofectamine 2000 (Invitrogen). Successful knockdown was confirmed by qRT-PCR and Western blot.

**Cell proliferation and colony formation assays.** Cell proliferation was assessed using CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) (Promega, Madison, WI) according to the manufacturer’s instructions. Monolayer colony formation assay and anchorage-independent soft agar assay were performed as described previously (12).

**Cell invasion.** The invasive capacity of cells was determined using the BD BioCoat Matrigel invasion chambers (BD Biosciences). Transfection cells were seeded on the top chamber of each insert with complete medium added to the bottom chamber. After 24 hours, cells on the membrane were wiped off with a cotton swab. Fixed and stained with 1% toluidine blue, cells on the underside of the membrane were counted from 5 microscope fields (original magnification, ×400). Each experiment was performed in triplicate and the mean value expressed from two independent experiments.

**Cell cycle analysis.** Cell cycle analysis was performed using flow cytometry as described previously (13).
**Apoptosis assay.** Early apoptosis was detected using Annexin V-FITC apoptosis detection kit (BioVision) according to manufacturer’s instruction.

**In vivo tumorigenicity.** MKN45 cells (1×10^6 cells suspended in 0.1ml PBS) transfected with YAP1 expression vector or empty vector were injected subcutaneously into the dorsal flank of five 4-week-old male Balb/c nude mice (YAP1-expressing clones on the right and vector control clones on the left). Tumor diameter was measured and documented every 3 days until the end of week 3. Tumor volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumor and calculating as follows: volume = (shortest diameter)^2 × (longest diameter) × 0.5. The experiment was repeated for three times. The animal handling and all experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

**Expression microarray.** Human genome U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA) was applied to identify the transcript expression profiles. It provides comprehensive genome wide expression analysis over 47,000 transcripts and variants. The raw data were quantile normalized by robust multiarray average (RMA) algorithm and analyzed in Partek Genomics Suite 6.4 (Partek, St. Charles, MO). Differential gene expression was evaluated using one-way ANOVA. A fold change cutoff of 1.5 and false discovery rates less than 5% were set to identify differentially expressed gene between YAP1-expressing MKN45 cells to vector controls, or siYAP1 knockdown AGS cells to the scramble controls. The integrated gene network analysis on the gene set with significant expression changes were generated by Ingenuity Pathways Analysis (IPA, Ingenuity Systems, www.ingenuity.com). Each gene identifier was mapped to its corresponding gene
object in the Ingenuity Pathways Knowledge Base. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity. Fischer's exact test was used to calculate a $P$-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. A $P$-value of less than 0.01 was considered significant.

**Statistical analysis.** The Mann–Whitney U test was used to compare the difference in biological behavior between YAP1-expressing MKN45 cells and empty vector-transfected MKN45, or siYAP-knockdown AGS cells and scramble siRNA-transfected AGS cells. Correlations between YAP1 nuclear/cytoplasmic stain and clinicopathologic parameters were assessed by the non-parametric Spearman’s rho rank test. The Kaplan-Meier method was used to estimate the survival rates for each variable. The equivalences of the survival curves were tested by log-rank statistics. For those variables being statistically significant found in the univariate survival analysis ($P < 0.05$), the Cox proportional hazards model with the likelihood ratio statistics was employed to further evaluate them for multivariate survival analysis. All statistical analyses were carried out by using statistical program SPSS version 16.0. A two-tailed $P$-value of $< 0.05$ was regarded as statistically significant.

**Results**

**Up-regulation of YAP1 protein expression in gastric cancer.** Over-expression of YAP1 mRNA was found in 6 out of 9 gastric cell lines (Fig. 1A). The up-regulation of YAP1 protein in these cell lines was confirmed by Western blot analysis (Fig. 1B). In primary GAC,
11 out of 30 cases (36.7%) showed more than 1.5-fold up-regulation of YAP1 mRNA expression in tumor tissues compared to paired non-cancerous gastric mucosal tissues. However, the mean level of YAP1 mRNA expression in 30 tumor tissues was not significantly higher than that in the normal tissues (3.1 (0.19 – 16.43) vs. 2.5 (0.16 -8.46), \( P = 0.134 \), Fig. 1C). By Western blotting, YAP1 protein was found up-regulated in 9 out of 10 gastric cancers compared to their non-cancerous counterparts (Fig. 1D).

Immunohistochemistry was performed to assess the YAP1 protein expression in tissue microarrays containing 129 formalin-fixed paraffin-embedded GACs. Gastric carcinoma cells often exhibited cytoplasmic and nuclear expression of YAP1 (Fig. 1E). Since the subcellular localization is critical in determining the functions of YAP1, the cytoplasmic and nuclear staining were scored separately. Marked nuclear immunoreactivity was seen in 42% (54/129) of the GACs. Intermediate and low nuclear stain were noted in 31% (40/129) and 19% (25/129), respectively. Ten GAC samples (8%) showed negative nuclear stain of YAP1. For the cytoplasmic localization of YAP1, strong and moderate immunoreactivity were seen in 32% (41/129) and 42% (54/129) of GACs, respectively. Weak and negative YAP1 cytoplasmic immunoreactivity accounted for 26% (34/129) of the tumor samples.

**Association of YAP1 expression with clinicopathologic characteristics.** Up-regulation of YAP1 nuclear expression in gastric cancer associated with a poorer disease specific survival by univariate analysis (\( P = 0.021 \), Fig. 2A). Univariate analysis indicated that other factors including female gender (\( P = 0.027 \)), histology with diffuse component (\( P = 0.021 \)), higher tumor grade (\( P = 0.037 \)) and advance stage (\( P < 0.001 \)) also correlated with poor survival (Supplementary Table 1A). By multivariate Cox proportional hazards regression
analysis, only stage was independently associated with disease specific survival ($P < 0.0001$).

However, in patients with early stage diseases (stage I & II), a strong association for higher nuclear YAP1 expression (score 2 or above) with shorter disease specific survival ($P < 0.001$, Fig. 2B) was demonstrated. To examine the clinical significance of YAP1 as a predictive marker for disease specific survival in early stage gastric cancer, we recruited another 65 cases of stage I and II gastric cancers for further analysis. In a cohort of 101 early stage gastric cancers, the YAP1-low expression subgroup (score 0 or 1; $n=23$) had a significantly better prognosis than YAP1-high expression subgroup (score 2 or 3; $n=78$) by Kaplan-Meier analysis ($P = 0.040$, Supplementary Fig. 1). Multivariate Cox regression showed that YAP1 was an independent predictor of disease specific survival for patients in early stage ($P = 0.042$) in addition to T stage ($P = 0.038$). Nuclear expression of YAP1 did not associate with age, gender, histological type, grading, staging, or the presence of *H. pylori* (Supplementary Table 1B and Table 1C). Cytoplasmic expression of YAP1 did not associate with survival or other clinicopathologic parameters.

**YAP1 knockdown in gastric cancer cells.** On siRNA-mediated suppression, a significantly decreased proliferation was observed in gastric cancer cell lines MKN1 and AGS compared with scramble siRNA and mock control groups ($P < 0.001$, Fig. 3A). Monolayer colony formation assay indicated that YAP1 knockdown significantly reduced colony formation in these cell lines ($P < 0.001$, Fig. 3B). In cell invasion assay, a significant reduction in the invasive phenotype through the Matrigel-coated Boyden chamber was noted on YAP1 knockdown ($P < 0.001$, Fig. 3C). Since a growth inhibitory effect was observed in siYAP1 transfected cells, we analyzed the transfectants for cell cycle parameters using flow
cytometry. Twenty-four hours after transfection, accumulation of G1 cells increased in siYAP1 transfectant compared to the scramble siRNA controls (Fig. 3D, 72% vs 55% in MKN1 and 49% vs 37% in AGS cells). Cell apoptosis, as determined by sub-G1 phase, was increased from 0.56% to 17% in AGS cells. However, siYAP1 did not change the percentage of sub-G1 cells in MKN1 (Fig. 3E). Similar finding was observed by Annexin V assay (data not shown). Thus, the apoptotic response appeared to be cell context-dependent. MKN1 cells harbor the TP53 mutation at the DNA-binding (core) domain (V143A), whereas AGS cells carry the wild type TP53 (Supplementary Fig. 2). It is uncertain whether the different TP53 status might contribute to the different apoptotic response to YAP1 knockdown between the two cell lines. Taken together, siRNA knockdown of YAP1 in MKN1 and AGS cells resulted in a significant reduction in proliferation, anchorage-dependent colony formation and cell invasion. G1 arrest was observed in both cell lines upon YAP1 knockdown yet the apoptotic response appeared to be cell context-dependent.

**YAP1 Overexpression in MKN45.** The expression of YAP1 was completely lost in gastric cancer cell line MKN45 due to an intragenic homozygous deletion (Supplementary Fig. 3). It therefore provided an *in vitro* model to study the YAP1 ectopic expression. We transfected pcDNA3.1(+)/YAP1 or empty vector into MKN45 cells and the clones that stably expressing YAP1 were selected by G418. Re-expression of YAP1 was confirmed by immunohistochemistry and Western blot (Fig. 4A & 4E). Transfection of YAP1 into MKN45 cells increased cell proliferation in both high and low serum conditions (Fig. 4A). A significant increase in the number of invading cells through the Matrigel coated membrane in pcDNA3.1(+) /YAP1 transfected group was demonstrated when compared...
with the vector alone group ($P < 0.001$, Fig. 4B). We further examined the effect of YAP1 on the ability to form colonies in soft agar, a more stringent parameter of oncogenic property. YAP1-expressing MKN45 cells formed larger colonies in soft agar by 14 days and the number of colonies was significantly increased by 44 folds ($P < 0.001$) compared to the vector controls (Fig. 4C), suggesting that YAP1 promoted anchorage-independent growth. The effect of YAP1 expression on \textit{in vivo} growth of tumor was studied by subcutaneous injection of YAP1-expressing MKN45 clones into nude mice. The tumor growth in YAP1-expressing clones was significantly enhanced compared with the vector control clones ($P < 0.001$, Fig. 4D). In summary, over-expression of YAP1 accelerated cell growth both \textit{in vitro} and \textit{in vivo}, promoted anchorage-independent colony formation, and induced a more invasive phenotype in MKN45 cells.

\textbf{Identification of cellular networks and pathways regulated by YAP1.} To gain insight into the mechanisms by which YAP1 exerts the oncogenic function, we compared gene expression profiles from YAP1-expressing MKN45 cells (YAP1-MKN45) versus vector controls (vector-MKN45), and YAP1-knockdown AGS cells (siYAP1-AGS) versus scramble controls (scramble-AGS). Differential gene expression was evaluated using one-way ANOVA. Genes that had greater than 1.5-fold differences in expression levels over the controls with false discovery rates (FDR) <5% were selected for Ingenuity pathway analysis. Selected up and down regulated genes were validated by qRT-PCR (Supplementary Table 3). A total of 27 partially overlapped networks with IPA score >11 were linked to YAP1 regulation. All networks involved more than 11 focus genes. Top functions of these genes were related to cancer, cell cycle, digestive system development and function, small
molecule biochemistry and lipid metabolism. The ten networks with the highest rank are
showed in Supplement Table 4.

**Induction of proto-oncogene c-Fos by YAP1.** Close examination of the networks
identified that mitogen-activated kinases (MAPK) family genes were enriched in several
networks. For example, the network associated with “cancer, cellular movement and
neurological disease”, incorporating 23 focus genes, was centered on ERK (Supplementary
Fig. 4A). Another network built on 22 focus genes was mostly centered on MAPK, and
associated with lipid metabolism, molecular transport and small molecule biochemistry
(Supplementary Fig. 4B). MAPK pathway is involved in a wide variety of cellular
processes such as proliferation, differentiation, transcription regulation and development.
The pathway is also a key target of cell transformation in tumor development. We
compared the expression of MAPK family genes in YAP1-MKN45 cells to vector-MKN45.
Activation of MAPK pathway by YAP1 as indicated by elevated
phosphorylated-c-Raf/MEK1/2/ERK1/2 and c-Fos was observed in MKN45 cells stably
expressing YAP1 (Fig. 4E).

To further address the effect of YAP1 expression on the activation of MAPK signaling,
we stimulated YAP1-MKN45 and vector-MKN45 cells with serum after starvation for 24
hours. Western blot analysis was then performed to assess the level of
phosphorylated-ERK1/2 and c-Fos (Fig. 5A). Stronger ERK1/2 activation at 1 minute post
serum stimulation was observed in YAP1-expressing cells compared with the
vector-transfected cells. C-Fos was induced by serum at 30 minutes and maximal at 60
minutes. Ectopic expression of YAP1 resulted in a much stronger c-Fos induction in gastric
cancer cells. The MEK inhibitor PD98095 inhibited ERK activation and c-Fos induction (Fig. 5C), indicating that YAP1 regulated the serum-induced c-Fos induction though MAPK pathway. The enhanced capacity on c-Fos induction was also observed when stimulated the cells with epidermal growth factor (EGF, Fig. 5B). C-Fos is an immediate early gene whose expression is a key switch in cellular regulation. Together with c-Jun, it forms the AP-1 complex required for the transcription of many genes important for cell growth, differentiation and transformation. Our data suggested that c-Fos is a target for the function of YAP1. The early gene responses are representative of the events that initiate progression through the cell cycle. YAP1 might exert its growth promoting effect at least partly by up-regulating c-Fos in gastric cancer cells.

Discussion

In this study, we observed an over-expression of YAP1 in gastric cancers. This result was in keeping with a previous report that over-expression of YAP1 was found in 48% of the gastric cancer (14). Up-regulation of YAP1 was also found in colon cancer, lung adenocarcinoma, ovarian cancer, hepatocellular carcinoma and prostate cancer (8, 15-16). The findings suggested a potential oncogenic role of YAP1 in multiple human cancers. More importantly, we demonstrated that YAP1 nuclear accumulation significantly associated with poorer disease specific survival, particularly in patients with early stage gastric adenocarcinoma. Our data provided the first evidence that YAP1 could be used as a potential prognostic biomarker, especially for patients with early stage disease.

YAP1 functions as a transcription coactivator to regulate gene expression in nucleus (17-22). The Lats tumor suppressor kinase phosphorylates YAP1 at S127, enhances its
interaction with 14-3-3 proteins, resulting in YAP1 cytoplasmic sequestration and thereby rendering it unable to function as a transcription coactivator (7). Therefore, it’s believed that the subcellular localization is important in determining the biological significance of YAP1. Although we observed an overall up-regulation of cytoplasmic YAP1 expression in gastric cancer cells, the cytoplasmic expression of YAP1 did not correlate with any of the clinicopathological parameters. Our finding reiterated the functional significance of YAP1 nuclear localization.

Chromosome 11q22, where YAP1 is located, is a recurrent amplicon in hepatocellular carcinoma and esophageal squamous cell carcinoma (6, 23). Amplification of this region, although infrequent, has been implicated in various human cancers (24-29). Our array CGH analysis revealed amplification of YAP1 in 1 out of 9 gastric cancer cell lines only (NCI-N87) (Supplementary Fig. 3). Consistently, high YAP1 mRNA and protein levels were detected in this cell line. However, over-expression of YAP1 was also seen in cell lines without gene amplification, e.g. MKN28 and AGS. Mechanisms other than gene amplification must contribute to the YAP1 overabundance in gastric cancer cells, for example, transcription activation, or protein stabilization. Up-regulation of YAP1 mRNA expression was observed in a subset (36.7%) of GAC. But the mean level of YAP1 expressions in tumor tissues was not significantly higher than that in paired non-cancerous gastric tissues (Fig. 1C). By immunohistochemistry, YAP1 protein expression was found in most GACs. Whereas in normal gastric mucosa, only scanty cells were positive. In addition, no YAP1 mutation was detected in 8 gastric cancer cell lines and 10 primary gastric tumors (Supplementary methods). We speculated that a major mechanism for YAP1 accumulation
in gastric cancer cells might related to post-translational modification or interaction with other cellular proteins that stabilize YAP1. YAP1 is a negatively regulated downstream target of the Hippo signaling pathway. The mammalian Hippo orthologs MST1/MST2 protein kinases suppress the oncogenic activity of YAP1 by promoting YAP1 S127 phosphorylation and subsequent cytoplasmic retention. Dysregulation of Hippo pathway has been implicated in tumorigenesis (7, 8). We demonstrated the loss of cleaved activated MST1/2 in gastric cancer tissues (Supplementary methods and Supplementary Fig. 5A), suggesting that the MST-YAP1 pathway is disrupted in a substantial fraction of gastric cancers. The finding was in keeping with previous studies that down-regulation of MST1/2 was observed in sarcomas and various cancers (30-31). In addition, in vitro studies demonstrated that ectopic expression of MST1 suppressed cell proliferation, induced apoptosis in gastric cancer cells (Supplementary Fig. 5B). We further showed that MST1 promoted the phosphorylation of YAP1 on S127, enhanced its retention in the cytoplasm and therefore quenched its oncogenic function in the nucleus (Supplementary Fig. 5C). The data indicated that activation of YAP1 in gastric cancer might be related to the disruption of Hippo pathway.

There is no DNA binding domain on YAP1 protein. It interacts with several transcription factors, including RUNX2, SMAD7, p73, p53BP2 and TEAD family members and modulates their transcriptional activity. The TEAD proteins are major partners of YAP1 and are required for the YAP1-mediated gene expression that promotes proliferation and inhibits apoptosis (22). On the other hand, YAP1 physically interacts with p73α, p73β and p63α and promotes apoptosis following DNA damage (32). The precise biological function...
of YAP1 is not clear. The choice of its partner transcription factors, and consequently the final outcome might be determined by multiple factors, e.g. cell context, type of stimuli, and regulation of upstream pathways. We showed in this study the up-regulation of YAP1 in gastric cancers. The accumulation of YAP1 in tumor cells suggests that it might be a candidate oncoprotein. Functional studies also supported a tumorigenic role of YAP1 in gastric cancer. Knockdown YAP1 in gastric cancer cell lines leaded to a dramatic decrease in proliferation and colony formation, whereas ectopic YAP1 expression significantly increased cell growth both in vitro and in vivo. This is in keeping with previous studies that implicating YAP1 as an oncogene (5-6). Being an adaptor protein with the capacity to interact with multiple transcription factors, YAP1 is placed at the crossroads of multiple signaling pathways. It has been proposed that YAP1 regulates the balance between cell proliferation and apoptosis to maintain homeostasis (8). Further investigation therefore has to be done to clarify the role it plays in physiological condition and during tumorigenesis.
References


21. Espanel X, Sudol M. Yes-associated protein and p53-binding protein-2 interact through their WW and


Figure Legends

Fig. 1. Over expression of YAP1 in gastric adenocarcinoma. A, YAP1 mRNA level was measured by qRT-PCR in gastric cancer cell lines. The experiment was done in triplicate. The error bars represented the standard deviations (SDs). The normal tissue corresponds to Human Stomach Total RNA commercially available from Ambion (AM7996). B, YAP1 protein level was determined by Western blot analysis in gastric cancer cell lines and normal gastric mucosa. (Normal 1 & 2 samples were the normal gastric mucosa obtained from weight reduction gastric surgery.) C, the box plot showed the YAP1 mRNA expression level from 30 cases of gastric adenocarcinoma (GAC) and paired non-cancerous gastric mucosa, no significance ($P = 0.134$). D, Expression of YAP1 protein in paired gastric cancer (T) and non-cancerous gastric mucosa (N) was determined by Western blot analysis. E, Immunohistochemical analysis of YAP1 expression in gastric adenocarcinoma. Case 49 – an example of intestinal type gastric adenocarcinoma. Case 102 – an example of diffuse type gastric adenocarcinoma (original magnification ×100, insert –original magnification ×400).

Fig. 2. Kaplan-Meier plots of disease free survival according to YAP1 nuclear expression status. A, YAP1 nuclear accumulation associated with poor disease specific survival in patients with gastric adenocarcinoma. B, YAP1 nuclear accumulation (score 2 or higher) also significantly predicted poor survival in patients with early stage diseases, (stage I/II).

Fig. 3. Knockdown of YAP1 by RNA interference in gastric cancer cell lines MKN1 and AGS. A, MTT assay suggested that knockdown YAP1 by siRNA significantly suppressed proliferation in MKN1 and AGS cells. The mean and SD obtained from seven experiments were plotted (**, $P < 0.001$). B, Monolayer colony formation assay showed that YAP1
knockdown reduced anchorage-dependent colony formation (**, \( P < 0.001 \)). The experiment was done in triplicate and the error bars represented SDs. C, A significant reduction in the invasive ability was demonstrated on YAP1 knockdown compared with scramble siRNA treated cells. Representative images of cells invaded through the Matrigel-coated membrane to the underside of micropores were shown (**, \( P < 0.001 \)). D, Flow cytometric analysis suggested the accumulation of cells in G1 phase 24 hours after siYAP1 treatment. Representative data from three independent experiments was shown. E, Knockdown YAP1 increased sub-G1 population in AGS but not in MKN1 cells, suggested that inhibition of apoptosis by YAP1 was cell context-dependent. The representative data from three independent experiments was shown.

**Fig. 4.** Ectopic expression of YAP1 in MKN45 cells. A, Re-expression of YAP1 in MKN45 cells after transfection was confirmed by immunohistochemistry. Ectopic expression of YAP1 increased proliferation in both high and low serum conditions (*, \( P < 0.05 \); **, \( P < 0.001 \)). The mean and SD obtained from three experiments were plotted. B, A significant increase of invasion ability of MKN45 cells through the Matrigel-coated membrane was observed in YAP1 transfected cells compared with vector controls (**, \( P < 0.001 \)). The experiment was done in triplicate and the error bars represented SDs. C, YAP1-expressing cells formed larger colonies in soft agar by 14 days and the number of colonies was significantly increased compared with the vector controls (**, \( P < 0.001 \)). The experiment was done in triplicate and the error bars represented SDs. D, YAP1-expressing MKN45 cells and vector-transfected controls were injected subcutaneously to the right and left dorsal flank of nude mice, respectively. Three weeks after injection, the tumor size of
YAP1-MKN45 was significantly larger than vector-MKN45 (**, \( P < 0.001 \)). The mean and SD obtained from five mice were plotted. Representative results from 3 independent experiments were shown. The YAP1 expression in xenograft tumors was confirmed by immunohistochemistry. E, Western blot analysis showed up-regulation of MAPK pathway in MKN45 cells stably expressing YAP1.

**Fig. 5.** YAP1 enhanced serum/EGF stimulated c-Fos induction. A & B, YAP1-MKN45 (+) and vector-MKN45 cells (-) were cultured without serum for 24 hours and then stimulated with serum or epidermal growth factor (EGF) for 0, 1, 5, 15, 30, 60 and 90 minutes. Cells were immediately washed with cold phosphate-buffered saline and collected by scraping. The cell lysates were analyzed for MAPK pathway by western blot analysis using antibodies against phosphorylated-ERK1/2 and c-Fos. Representative of three independent experiments was shown. C, YAP1-MKN45 and vector-MKN45 cells were seeded onto 35-mm plates and cultured without serum for 24 hours. By the end of the 24-hour incubation, MEK inhibitor PD98059 (20\( \mu \)m, Calbiochem) was added to one set of cells for 2 hours. The cells were then stimulated with 10% serum for 0, 5, 15, 30, 60 and 90 minutes. The cell lysates were analyzed for MAPK activation and c-Fos expression by Western blot analysis. Treatment with PD98059 suppressed ERK1/2 phosphorylation and c-Fos induction in MKN45 cells.
Fig. 1.
Fig. 2.
Fig. 5.
Yes-associated Protein 1 Exhibits Oncogenic Property in Gastric Cancer and Its Nuclear Accumulation Associates With Poor Prognosis

Wei Kang, Joanna Tong, Anthony WH Chan, et al.

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