Inhibition of YAP reverses primary resistance to EGFR inhibitors in colorectal cancer cells

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Abstract. Mutant KRAS and BRAF are associated with primary EGFR inhibitor resistance in colorectal cancer (CRC). However, other biomarkers that could predict EGFR inhibitor resistance remain elusive. In the present study, immunoblotting and cell proliferation results revealed that yes-associated protein (YAP), a downstream effector of the Hippo pathway, was positively associated with primary cetuximab resistance in CRC cells. YAP knockdown enhanced the cytotoxicity of cetuximab in CRC cells. Simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor of the mevalonate pathway that inhibits YAP bioactivity through nuclear translocation and total YAP expression, increased the cytotoxicity of EGFR inhibitors (cetuximab and gefitinib) against CRC cells. The combination of simvastatin and EGFR inhibitors inhibited YAP and EGFR signaling more markedly than each agent alone. Adding back geranylgeranyl pyrophosphate (GGPP), a key product of the mevalonate pathway, reversed the YAP bioactivity inhibition induced by simvastatin and the cell proliferation inhibition induced by the combination of simvastatin and EGFR inhibitors. Collectively, these results revealed that YAP may be useful in identifying cetuximab resistance in CRC and indicated that targeting of both YAP and EGFR signals may present a promising therapeutic approach for CRC.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide (1). In recent years, >1.2 million patients have been diagnosed annually and ~600,000 patients succumb to CRC every year (2). Surgical resection is the main method for treatment of early-stage CRC with a favorable prognosis. However, most patients are diagnosed with metastatic cancer, which is not suitable for resection. Traditional chemotherapy is the main approach for metastatic CRC (mCRC) and the overall survival can reach 15-19 months (3). With the advances in therapies, targeted drugs, such as cetuximab and bevacizumab, can prolong the overall survival to 28.7-33.1 months in mCRC patients (4,5). Meanwhile, small EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, have been commonly used in non-small cell lung cancer and have improved progression-free survival, exhibiting great application potential. However, EGFR-inhibitor resistance has restricted their application, creating an urgent need to discover novel treatment strategies.

Yes-associated protein (YAP), a downstream effector of the Hippo pathway, is involved in tissue overgrowth and tumor formation (6). A study revealed that YAP could act as a transcriptional co-activator to promote the expression of genes involved in cell proliferation and apoptosis (7). A recent study revealed that YAP may be useful for identifying resistance to cetuximab (8). However, the role of YAP in cetuximab resistance remains elusive in CRC.

In the present study, we observed that the expression of YAP was negatively associated with cetuximab sensitivity in CRC cell lines, independent of KRAS mutation status, and YAP knockdown enhanced the cytotoxicity of cetuximab. Simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, has been reported to inhibit YAP bioactivity in multiple types of cancer cell lines (9,10). We determined that simvastatin increased the sensitivity of CRC cells to cetuximab both in vitro and in vivo. These findings indicated that YAP may be useful in identifying cetuximab resistance in CRC cancer.

Materials and methods

Cell culture and agents. The CRC cell lines COLO 320, HCT 116, HT 29, SW 48, SW 480 and SW 1116 were purchased from the American Type Culture Collection (ATCC; Manassas,
VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories; Thermo Fisher Scientific, Inc., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Gaithersburg, MD, USA). Cell cultures were maintained with 100 U/ml of penicillin G sodium and 100 µg/ml streptomycin sulfate (Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA) at 37°C in a humidified 5% CO2, 95% air incubator. Simvastatin was purchased from J&K Scientific Ltd. (Beijing, China). Cetuximab was purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA) and YM53601 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Gefitinib, FTI277 and GGTI298 were purchased from Selleck Chemicals (Houston, TX, USA) and EGF and GGPP were purchased from Sigma-Aldrich; Merck KGaA.

Transfection. siRNAs against YAP and control siRNAs were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). We used two independent siRNAs to target YAP. The siRNA sequence for YAP-1 was 5'-GAC AUC UUC (Shanghai, China). The siRNA sequence for YAP-2 was 5'-AAU UCU CCG AAC GUG UCA CGU UU-3'. Cells were transfected with control siRNA and YAP siRNA for 48 h.

Cell proliferation assay. Cells were seeded (4x10^4 cells/well in 100 µl DMEM with 10% FBS) in a 96-well flat-bottomed plate (Corning Inc., Corning, NY, USA) 24 h before treatment. Following incubation, cell growth was determined with a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay following the manufacturer's instructions.

Hoechst 33342 staining. Cells were seeded (4x10^4 cells/well in 100 µl DMEM with 10% FBS) in a 96-well flat-bottomed plate 24 h before treatment. Apoptotic cells were determined using Hoechst 33342 (Sigma-Aldrich; Merck KGaA) DNA staining according to the manufacturer's protocol. Apoptotic cells were detected by fluorescence microscopy (Nikon, Tokyo, Japan), which revealed nuclear condensation and DNA fragmentation.

Quantitative real-time PCR. Cells were collected in TRIzol (Invitrogen) for total RNA extraction according to the manufacturer's protocol. Retrotranscription was performed with Reverse Transcriptase M-MLV (Takara Biotechnology, Co., Ltd., Dalian, China). Real-time PCR reactions were performed with a SYBR Premix Ex Taq™ kit (Takara Biotechnology) on an iQ5 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mixtures were incubated at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 20 sec, 55-65°C for 20 sec and 72°C for 30 sec. The primers used were as follows: YAP sense, 5'-GGT GCCACGTCCAAGGAAAG-3' and antisense, 5'-GTTGAGG CCACAGGAGTTGC-3'; CTGF sense, 5'-TGGGTGACGC AGAAAGCTC-3' and antisense, 5'-CCAATGACACGG CCTCTCCTG-3'; CYR61 sense, 5'-TTCTTCTCAGAGGCG GCACCTC-3' and antisense, 5'-AGCCCTGCATCTTATA CAACC-3'; GAPDH sense, 5'-CAAGGCAACCCAGGAA-3' and antisense, 5'-CCCTCTGATAGGGGGCAGT-3'. The data were analyzed with the 2^(-ΔΔCq) method (11).

Nuclear and cytoplasmic protein extraction. After the addition of reagents as indicated, nuclear and cytoplasmic proteins from cells and mouse xenograft tumors were extracted with a Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions.

Immunofluorescence. Approximately 4x10^5 cells were seeded on a 24-well culture dish. After the addition of reagents, the cells were fixed with PBS-buffered 4% paraformaldehyde for 10 min followed by permeabilization with 0.3% Triton X-100 for 10 min and blocking with 3% BSA for 30 min. The primary antibody (YAP) was added at a 1:75 dilution in PBS overnight at 4°C, followed by three PBS washes. The secondary FITC-labeled antibody (cat. no. 31568) (Invitrogen; Thermo Fisher Scientific, Inc.) at a 1:75 dilution in PBS was then incubated with cells for 2 h at 37°C. After three PBS washes, immunofluorescence was viewed with a fluorescence microscope (Eclipse TE300; Nikon, Tokyo, Japan). All procedures were performed at room temperature unless otherwise specified.

In vivo assay and ethical standards. Four-week-old female BALB/c nude mice obtained from Beijing HFK
Biotechnology Co., Ltd. (Beijing, China) were maintained under specific pathogen-free conditions. Mice were injected subcutaneously with SW 480 cells. When the xenografts reached a volume of 80-100 mm\(^3\), the mice (five mice per group) were assigned into four groups (control, cetuximab, simvastatin and cetuximab + simvastatin). The mice were treated with intraperitoneal injection of 20 mg/kg cetuximab in dimethyl sulfoxide (DMSO) once every 3-4 days and/or orally with 6 mg/kg simvastatin in DMSO once daily. Tumor diameters were determined with a digital caliper every three days following treatment. All animal experimental procedures used in the present study were approved by the Experimental Animal Manage Committee of West China Hospital, Sichuan University under contract 2016003A and were performed strictly according to the guidelines of the Animal Ethics Committee Guidelines of the Animal Facility of West China Hospital and the Animal Care and Use Committee of Sichuan University.

Statistical analysis. Values are presented as the means ± standard error of the mean (SEM). All statistical analyses were performed with SPSS 14.0 software for Windows (SPSS, Inc., Chicago, IL, USA). Correlation analysis between the expression of YAP and proliferation inhibition rate was performed by Spearman's correlation test as previously described (12). Student's t-test was performed when two groups were compared; one-way ANOVA and post hoc Tukey's test were performed when multiple comparisons were conducted. P<0.05 was considered to indicate a statistically significant result.

Figure 1. YAP expression is negatively associated with the sensitivity of colorectal cells to cetuximab. (A) Levels of YAP were determined by immunoblotting analysis in five cell lines and GAPDH was used as the control. Representative results are shown. (B) Relative levels of YAP compared with GAPDH were determined via immunoblotting analysis in five cell lines. (C) Relative proliferation rate: Cells treated with cetuximab were compared with each untreated cell line separately. Cell proliferation was determined with CCK-8 assays of five cell lines treated with 0.2 µM cetuximab for 48 h in 0.5% FBS. All the data are presented as the means ± SEM; n=3 biological replicates. YAP, yes-associated protein.

**Results**

YAP expression is negatively associated with the sensitivity of CRC cells to cetuximab. First, we examined YAP expression in five CRC cell lines (SW 1116, HCT 116, SW 480, COLO 320 and SW 48). Since KRAS mutation status is reported as an independent biomarker for cetuximab efficacy (13), we divided the cancer cells into two groups, wild-type KRAS CRC cell lines and mutant KRAS CRC cell lines. Subsequently, we investigated whether YAP participated in cetuximab resistance in vitro. We assessed the relative expression of YAP compared with the expression of GAPDH in the five cell lines. The relative YAP expression of the mutant KRAS CRC cell lines (SW 1116, HCT 116 and SW 480) were 0.64, 0.56 and 0.42, respectively; and that of the wild-type KRAS CRC cell lines COLO 320 and SW 48 were 0.96 and 0.24, respectively (Fig. 1A and B). Subsequently, we determined the relative proliferation rate of each cell line treated with cetuximab compared with each untreated cell line, separately. We observed that SW 1116 and COLO 320, which had the highest YAP expression in each group, demonstrated the lowest sensitivity to cetuximab (Fig. 1C). SW 480 and SW 48 cell lines had the lowest YAP levels and the highest sensitivity to cetuximab in each group (Fig. 1C). The relative proliferation rate of the mutant KRAS CRC cell lines was 0.96 vs. 0.85 for SW 1116 vs. SW 480, and 0.79 vs. 0.60 for the wild-type KRAS CRC cell lines COLO 320 vs. SW 48. We then conducted a correlation analysis to determine statistical relationships involving YAP expression and rate of proliferation inhibition against cetuximab. The correlation coefficients of the mutant KRAS
CRC cell lines and wild-type KRAS cell lines were 0.83 and 0.89 (P<0.05), respectively. Collectively, these results indicated that cetuximab sensitivity was negatively correlated with YAP expression independent of KRAS mutation status.

**YAP knockdown enhances the sensitivity of CRC cell lines to cetuximab.** To further investigate the effect of YAP on the sensitivity of cancer cell lines to cetuximab, we transfected cells with two independent RNA interference constructs (siYAP-1 and siYAP-2) (Fig. 2A and B, E and F). We selected two cell lines with primary cetuximab resistance, namely, HCT 116 and SW 480 (Fig. 1C), to conduct the following study. The results revealed that the combination of cetuximab and siYAP-1 significantly reduced cell proliferation compared with cetuximab or siYAP-1 alone (HCT 116, 0.7 µM cetuximab vs. 0.7 µM cetuximab + siYAP-1, mean relative cell proliferation rate: 83.5 vs. 49.9%, P<0.01; SW 480, 0.7 µM cetuximab vs. 0.7 µM cetuximab + siYAP-1, mean relative cell proliferation rate: 78.3 vs. 57.9%, P<0.01) (Fig. 2C and D). In addition, results involving siYAP-2 revealed a similar trend (Fig. 2G and H). Furthermore, colony formation assays revealed that knockdown of YAP-1 promoted colony inhibition caused by cetuximab (Fig. 2I and J). These results indicated that YAP may be related to primary resistance to cetuximab.

**Simvastatin inhibits YAP bioactivity through total YAP expression and nuclear translocation.** Simvastatin, an HMG-CoA reductase inhibitor, is widely used to lower cellular cholesterol levels in patients with hypercholesterolemia (10). To investigate the effects of simvastatin on YAP bioactivity, we first detected the total YAP protein level (Fig. 3A and B). Furthermore, real-time PCR results revealed that simvastatin treatment did not change the YAP mRNA level (Fig. 3C and D). YAP subcellular localization is also important for its bioactivity, as only nuclear YAP can interact with TEAD1-4 transcription factors and execute its function as a transcriptional co-activator. Thus, we analyzed the effects of simvastatin on YAP localization via immunofluorescence, and the results indicated that simvastatin treatment caused a significant decrease in YAP presence in the
nucleus (HCT 116, DMSO vs. simvastatin, mean percentage of nuclear YAP was 73.6 vs. 15.2%, P<0.01; SW 480, DMSO vs. simvastatin, mean percentage of nuclear YAP was 75.8 vs. 11.2%, P<0.01) (Fig. 3E, F and G). Following the addition of geranylgeranyl pyrophosphate (GGPP), the effects of simvastatin on nuclear YAP were partly reversed (Fig. 3E, F and G). Western blot analysis also revealed that the nuclear YAP level significantly decreased following simvastatin treatment (Fig. 3H and I). In addition, experiments revealed that simvastatin inhibited the downstream targets of YAP, namely, CYR61 and CTGF, at the mRNA level (Fig. 3J and K). Collectively, these results indicated that simvastatin could act as a YAP inhibitor through the inhibition of the expression of YAP and nuclear translocation.

Simvastatin increases the antitumor activity of EGFR inhibitors in CRC cell lines. To investigate the antitumor effects of simvastatin and (or) cetuximab, cell proliferation was assessed.
Compared with single agents, cetuximab + simvastatin demonstrated more significant proliferation inhibition (HCT 116, 0.7 µM cetuximab vs. 0.7 µM cetuximab + 3 µM simvastatin, mean relative cell proliferation rate = 83.4 vs. 61.8%, P<0.01; SW 480, 0.7 µM cetuximab vs. 0.7 µM cetuximab + 3 µM simvastatin, mean relative cell proliferation rate = 78.4 vs. 52.5%, P<0.01) (Fig. 4A). Similar results were observed in COLO 320 and HT 29 cell lines (Fig. 4B). Additionally, colony formation assays demonstrated that the combination of simvastatin and cetuximab achieved greater colony inhibition than single agents (Fig. 4C). Subsequently, Hoechst staining indicated that cetuximab + simvastatin induced apoptosis more strongly than each agent alone (Fig. 4F and G). Collectively, these results revealed that simvastatin increased the cytotoxicity of cetuximab against CRC cells. To investigate whether simvastatin could enhance the cytotoxicity of gefitinib, a further combination of simvastatin with gefitinib was examined, and the results revealed greater cytotoxicity and colony inhibition compared with each drug alone (HCT 116, 6 µM gefitinib vs. 6 µM gefitinib + 1 µM simvastatin, mean relative cell proliferation rate: 40.3 vs. 19.0%, P<0.01; SW 480, 12 µM gefitinib vs. 12 µM gefitinib + 1 µM simvastatin, mean relative cell proliferation rate: 48.7 vs. 28.1%, P<0.01) (Fig. 4D and E). These results indicated that simvastatin could increase the cytotoxicity of EGFR inhibitors (cetuximab and gefitinib) against CRC cells. To further investigate the effect of the combined strategy on tumor growth, we implanted SW 480 cells in nude mice and divided these mice into four groups (control, cetuximab, simvastatin and cetuximab + simvastatin). Consistent with the in vitro results, the combination of cetuximab and simvastatin, and the mean tumor volumes [Volume = (L x W²)/2, L=length and W=width] were 648.1±143.3 mm³ (mean ± SEM) vs. 310.3±67.7 mm³, P<0.01) (Fig. 5C and D). Simvastatin had no obvious impact on the daily movements or the weights of mice.
In addition, we did not observe any severe side effects in the mice.

**Effects of simvastatin and EGFR inhibitors on EGFR signaling and cyclin D1.** We then detected the effects of simvastatin and EGFR inhibitors on EGFR pathway signaling. Western blot analysis revealed that simvastatin treatment inhibited pEGFR and pAKT. The combination of cetuximab with simvastatin caused additional inhibition of pEGFR and pAKT compared with cetuximab alone (Fig. 5A). Additionally, the combination of simvastatin and gefitinib revealed a similar trend in pEGFR and pAKT inhibition (Fig. 5B). Subsequently, we analyzed the expression levels of cyclin D1, a protein related to proliferation. The results revealed that the combination of simvastatin and EGFR inhibitors caused an additional decrease in cyclin D1 compared with single agents (Fig. 5A and B). Consistent with the *in vitro* results, we observed a similar trend in the expression of pEGFR, pAKT and cyclin D1 in SW 480 xenograft tumors (Fig. 5E). Cetuximab+simvastatin treatment markedly reduced nuclear YAP levels compared with cetuximab alone *in vivo* (Fig. 5F).

**Simvastatin inhibition of EGFR-AKT signaling may be mediated through GGPP.** To determine the internal mechanism of pAKT inhibition caused by simvastatin, we hypothesized that simvastatin could inhibit the expression of pAKT through EGFR, which acts upstream of AKT and (or) acceleration of phosphatase and tensin homolog (PTEN), which negatively regulates the PI3K/AKT signaling pathway. We observed that EGF-activated pEGFR and pAKT signaling was markedly decreased following simvastatin treatment compared with the EGF control group, while the expression of PTEN was nearly unchanged (Fig. 6A and B). GGPP, a key product of the mevalonate pathway, reversed the decrease in pEGFR and pAKT caused by simvastatin (Fig. 6C and D, lanes 5 and 8). Since simvastatin is an inhibitor of the mevalonate pathway and...
simvastatin treatment inhibits synthesis of GGPP (9,10), these results indicated that the simvastatin-induced inhibition of the mevalonate pathway product GGPP.

**EGF promotes YAP bioactivity in some CRC cells.** Recent studies have revealed that crosstalk between the Hippo pathway and other signaling pathways plays a vital role in carcinogenesis and drug resistance (13,14). In the present study, we particularly studied the interaction between YAP, an effector of the Hippo pathway, and EGFR in CRC cells, and the data indicated that YAP knockdown nearly had no effect on the expression of pEGFR (Fig. 2A and B). Notably, EGF promoted YAP expression and that of its direct downstream target CYR61 in a dose-dependent manner in SW 48 and HCT 116 cells (Fig. 6E). When we blocked EGFR signaling with cetuximab, we observed that cetuximab reversed the EGF-induced increase in YAP and CYR61 (Fig. 6F, lanes 1, 2 and 5). Furthermore, when we combined cetuximab and simvastatin, YAP and CYR61 were inhibited more markedly than with cetuximab alone following EGF activation (Fig. 6F, lanes 5 and 8). These results indicated that activation of EGFR signaling can promote YAP signaling in some CRC cells, and a combination of cetuximab and simvastatin can inhibit YAP signaling more thoroughly than single treatment.

The GGPP pathway mainly mediates the combined effect of simvastatin and EGFR inhibitors. The mevalonate pathway plays an important role in the tumorigenesis of many cancers (14). To determine detailed information about which signaling branch of the mevalonate pathway, primarily mediates the combined therapy effect, we used three different inhibitors, FTI277, YM53601 and GGTI298, to inhibit farnesyl transferase, squalene synthase and geranylgeranyl transferase, respectively (Fig. 7A). The combination of FTI277 or YM53601 with EGFR inhibitors had no significant effect on cell proliferation compared with EGFR inhibitors alone (Fig. 7C, E, H and J). Adding back squalene, the product of squalene synthase, could not reverse the inhibitory effects on cell proliferation. These results indicated that activation of EGFR signaling can promote YAP signaling in some CRC cells, and a combination of cetuximab and simvastatin can inhibit YAP signaling more thoroughly than single treatment.
the cell proliferation inhibition mediated by simvastatin and EGFR inhibitors (Fig. 7B, D, G and I), while a combination of GGTI298 and cetuximab (or gefitinib), achieved approximately the same effect as the combination of simvastatin and cetuximab (or gefitinib) (Fig. 7C, E, H and J). In addition, after adding back GGPP, the expression of YAP exhibited no obvious change, but the expression of CYR61 was markedly increased, which indicated that YAP bioactivity was recovered (Fig. 7F and K, lanes 4 and 8), consistent with this, the inhibition of cyclin D1 and cell proliferation caused by the combination of simvastatin and EGFR inhibitors was rescued (Fig. 7F and K, lanes 5 and 8; lanes 6 and 10; Fig. 7B and D; G and I). Collectively, the aforementioned data indicated that the simvastatin-induced inhibition with EGFR inhibitors was mainly mediated through the GGPP pathway but not through the farnesyl pyrophosphate (FPP) pathway or squalene pathway (Fig. 7A).

**Discussion**

Resistance to EGFR inhibitors, such as cetuximab and gefitinib, has become an urgent issue for both basic science and clinical investigators (15-17). Driver genes, such as mutant KRAS, BRAF, PTEN and PIK3CA, are closely related to this resistance. In the present study, we observed that YAP may be useful in identifying cetuximab resistance in CRC cells. At present, the reported YAP inhibitors include verteporfin (18), statins and zoledronic acid (9,10). In the present study, we primarily used simvastatin as a YAP inhibitor. Simvastatin could not only inhibit total YAP protein expression but it also inhibited YAP translocation into the nucleus. However, simvastatin did not inhibit YAP mRNA levels, indicating that simvastatin promoted YAP protein degradation at the post-transcriptional level. Several studies have demonstrated...
that statins mainly inhibit YAP nuclear translocation to decrease its bioactivity, while the total YAP protein expression is not affected (9,19). We thought this discrepancy may be due to differences in the cell types used in each study.

The in vitro and in vivo study results demonstrated that the combination of simvastatin and EGFR inhibitors caused synthetic inhibition of cell proliferation. These results were consistent to the findings of Lee et al but did not reveal the function of YAP in cetuximab resistance (20). Compared with the cetuximab group, cetuximab + simvastatin markedly decreased the nuclear YAP levels in vivo. Furthermore, both pEGFR and pAKT were inhibited more markedly with the combination treatment than with single agents in vitro and in vivo. The aforementioned results were consistent with the cell proliferation and xenograft tumor growth assay results (Figs. 4 and 5).

Simvastatin induced pAKT downregulation in the present study. We hypothesized that this decrease may be caused by EGFR and (or) PTEN. In addition, our investigation revealed that simvastatin inhibited pEGFR but not PTEN, which indicated that simvastatin-induced AKT signaling was downregulated through EGFR. While EGF caused pEGFR and pAKT upregulation, this could be reversed by simvastatin, which verified this hypothesis. Furthermore, addition of GGPP reversed simvastatin-induced inhibition of EGFR-AKT signaling may occur through GGPP.

Crosstalk between the Hippo signaling and other pathway signals is involved in tumorigenesis, cancer progression and drug resistance (21-24). In the present study, YAP knockdown had nearly no effect on EGFR signaling, while EGF treatment promoted the activation of YAP and CYR61. These results could partly explain some epidemiological studies, which reported that no benefit was observed in simvastatin users for cancer therapy (25,26), and the reason may be inadequate inhibition of YAP. In addition to EGFR signaling, YAP is also regulated by other signals, such as RHOA, LATS and G protein-coupled receptors (10,27). Therefore, it is difficult to fully inhibit YAP bioactivity, and multiple inhibition approaches may be an effective and feasible method, as demonstrated in our study (Fig. 6F, lanes 5, 6 and 8).

Recent studies have revealed that the antitumor effects of simvastatin are controversial (25,26,28-30). As an HMG-CoA reductase inhibitor, simvastatin can inhibit the mevalonate pathway specifically. The mevalonate pathway is involved in biosynthesis of squalene, GGPP and farnesyl pyrophosphate (FPP) (31). Squalene is an upstream product of cholesterol synthesis, and GGPP and FPP are critical for prenylation of small G proteins, such as those in the RAS, RHO and RAB families (Fig. 7A). In the present study, GGPP rescued CYR61 expression inhibited by simvastatin, but did not markedly alter YAP expression. The immunofluorescence analysis results revealed that GGPP partly reversed the effects of simvastatin on nuclear YAP levels. This may be because GGPP rescued YAP bioactivity primarily through YAP protein transportation to the nucleus and not by increasing YAP protein expression. Consistent with this, GGPP reversed the synthetic cell proliferation inhibition caused by simvastatin and the EGFR inhibitors. All of the aforementioned results revealed the effect of YAP bioactivity on the resistance to EGFR inhibitors. This was in accordance with the study of Lee et al, in which a significant association between the oncogene YAP1 and cetuximab resistance was reported (8). However, their analysis was mainly focused on clinical patients. In addition, combined treatment with GGTI298 and EGFR inhibitors caused proliferation inhibition similar to that of the combination of EGFR inhibitors and simvastatin. However, combining FTI277 (or YM53601) and EGFR inhibitors had little effect compared with EGFR inhibitors alone. These results demonstrated that the GGPP pathway mainly mediated the combined effect of simvastatin and EGFR inhibitors (Fig. 8).
Notably, the concentration of simvastatin used in vitro was much higher (>10-fold) than that typically used for lowering cholesterol, a dosage of approximately 0.2-1.5 mg/kg/day and thus, toxic effects at such concentrations should be evaluated clinically.

In the present study, we found that YAP knockdown enhanced the sensitivity of CRC cells to cetuximab, and inhibition of YAP bioactivity by simvastatin increased the cytotoxicity of cetuximab and gefitinib. The combination of simvastatin and EGFR inhibitors synthetically inhibited YAP and EGFR signals. Our findings revealed a new promising therapeutic strategy to enhance the efficacy of CRC treatment through combined YAP and EGFR targeting (Fig. 8) and indicated that further studies of YAP inhibitors and the effects of YAP on cetuximab resistance should be performed.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BSL and HWX designed and performed the experiments, analyzed the data and prepared the manuscript. SZ, QL and NXB contributed to studies related to Hoechst 33342 staining, real-time PCR and in vivo experiments. QLT and JTZ conducted immunoblotting to examine the EGFR pathway and colony formation assays. QYG and YZN analyzed the data and contributed to the manuscript. FB supervised and designed the experiments, assays. QYG and YZN analyzed the data and contributed to the manuscript. FB supervised and designed the experiments, assays. Authors' information

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