The Hippo Coactivator YAP1 Mediates EGFR Overexpression and Confers Chemoresistance in Esophageal Cancer

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Abstract

Purpose: Esophageal cancer is an aggressive malignancy and often resistant to therapy. Overexpression of EGFR has been associated with poor prognosis of patients with esophageal cancer. However, clinical trials using EGFR inhibitors have not provided benefit for patients with esophageal cancer. Failure of EGFR inhibition may be due to crosstalk with other oncogenic pathways.

Experimental Design: In this study, expression of YAP1 and EGFR were examined in EAC-resistant tumor tissues versus sensitive tissues by IHC. Western blot analysis, immunofluorescence, real-time PCR, promoter analysis, site-directed mutagenesis, and in vitro and in vivo functional assays were performed to elucidate the YAP1-mediated EGFR expression and transcription and the relationship with chemoresistance in esophageal cancer.

Results: We demonstrate that Hippo pathway coactivator YAP1 can induce EGFR expression and transcription in multiple cell systems. Both YAP1 and EGFR are overexpressed in resistant esophageal cancer tissues compared with sensitive esophageal cancer tissues. Furthermore, we found that YAP1 increases EGFR expression at the level of transcription requiring an intact TEAD-binding site in the EGFR promoter. Most importantly, exogenous induction of YAP1 induces resistance to 5-fluorouracil and docetaxel, whereas knockdown of YAP1 sensitizes esophageal cancer cells to these cytotoxics. Verteporfin, a YAP1 inhibitor, effectively inhibits both YAP1 and EGFR expression and sensitizes cells to cytotoxics.

Conclusions: Our data provide evidence that YAP1 upregulation of EGFR plays an important role in conferring therapy resistance in esophageal cancer cells. Targeting YAP1-EGFR axis may be more efficacious than targeting EGFR alone in esophageal cancer.

Introduction

Esophageal cancer is a lethal illness with high incidence globally and significantly increased incidence in the United States with an estimated 18,170 new cases and an estimated death of 15,450 cases in 2014 (1), and the 5-year survival rate for patients with advanced esophageal cancer is <10% (2). Esophageal cancer is inherently resistant to therapy. Even localized esophageal cancer is frequently resistant (3). Considerable lack of understanding of molecular underpinnings of esophageal cancer has been an ongoing barrier for the development of effective strategies. EGFR and the Hippo pathway coactivator YAP1 play important roles in control of cell growth. Deregulation of these pathways may represent key elements for resistance in esophageal cancer.

EGFR is a transmembrane protein with intrinsic kinase activity (4). Activation of EGFR is due to the binding of its specific ligands such as EGF, TGFα, and amphiregulin and, abundance in EGFR protein levels per se results in dimerization of EGFR and activation of downstream signal cascades that regulate cell proliferation, invasion, and survival. EGFR overexpression or amplification has been reported in several human tumors including those of head and neck, breast, colon, lung, stomach, and esophagus (5–8). Increased EGFR expression has been associated with advanced stage, higher metastatic potential, and shorter survival of patients with breast, colon, lung, and esophageal cancer (8, 9). However, several clinical trials targeting EGFR either by antibodies (10–13) or kinase inhibitors (14, 15) have been disappointing in patients with gastrointestinal cancer. Therefore, inhibition of EGFR alone does not seem sufficient and it may be that EGFR is activated through other oncogenic signaling and targeting those pathways may be advantageous (16, 17).
The Hippo signaling pathway regulates organ size and cell proliferation. YAP1 is a key downstream effector of the Hippo signaling pathway and is tightly regulated by a number of upstream kinases and their adaptors such as Mst1/2, Sav1, and Lats1/2 which are tumor suppressors in several tumor types (18). Conditional deletion of these molecules in mice led to a dramatic proliferation. YAP1 is a key downstream effector of the Hippo signaling pathway and is tightly regulated by a number of upstream kinases and their adaptors such as Mst1/2, Sav1, and Lats1/2 which are tumor suppressors in several tumor types (18).

In this study, we provide novel information that YAP1 upregulates EGFR expression at the level of transcription through a TEAD-binding site. YAP1 upregulation of EGFR plays an important role in conferring therapy resistance in esophageal cancer cells. Verteporfin, a small-molecular inhibitor of YAP1, effectively diminishes both YAP1 and EGFR expression and sensitizes cells to cytotoxics. Therefore, targeting YAP-EGFR axis may be more promising than targeting EGFR alone in esophageal cancer.

Materials and Methods

Cells and reagents

The human Barrett's cell lines CPA and CPC and esophageal cancer cell lines Flo-1, SKGT-4, BE3, OE33, JHESO, OACP, YES-6, and KATO-TN have been previously described (21–23). Fetal liver cell line B299 and tumor cell lines from tumor tissues of Mst1/2−/− mice were generated by published methods. All human cell lines were tested and authenticated in the characterized cell line core facility of U.T.M D Anderson Cancer Center (Houston, TX). Verteporfin was purchased from United States Pharmacopeia. Doxycycline hyclate was obtained from Sigma-Aldrich. Antibody against YAP1 and phospho-EGFR, phospho-AKT (473), MCL-1 was purchased from Cell Signaling Technology. EGFR antibody was obtained from Santa Cruz Biotechnology. Antibodies against SOX-9 and Hes-1 were purchased from Chemicon. DNA plasmids that encode wild-type human YAP1 (hYAP1, CMV-YAP1) or a mutant protein that can no longer be phosphorylated at Ser127 (ref. 24; hYAP1 S127A, CMV-S127A-YAP1) and Tead2 cDNA vector (pcDNA2-TEAD2) were obtained from Addgene. Doxycycline inducible YAP1 lentivirus expression plasmid (PIN20YAP1) and lenti viral shRNA plasmids for knockdown YAP1 were previously described (25).

Primary mouse esophageal epithelial cells isolation and culture

Mouse primary esophageal cells were isolated according to published methods as described previously (26–28).

Protein extraction and Western blot analysis

Protein isolation and Western blot analyses were performed as previously described and immunoreactive bands were visualized by chemiluminescence detection (29).

 Luciferase reporter assays and transient transfection

The EGFR promoter (around 2.3k) containing an intact TEAD-binding site (TCATTGCC) was amplified using high fidelity PCR with primers (EGFRp23k.F5.Kpn and EGFRp.R1b.Xho) from genomic DNA extracted from SKGT-4 cells with the following sequences: EGFRp23k.F5.Kpn 5′ aagCTTACCCtgggtgacacacagaggtt 3′; EGFRp.R1b. Xho 5′ aacCTCGAgggctagtgggacctgc 3′. The native fragment of EGFR promoter (−2,286 bp to +102 bp) was digested with Kpn1 and Xho1 and then cloned into pGL4.22 (Promega) at the site of Kpn1 and Xho1.

The EGFR promoter-luciferase constructs with two mutant Tead-binding sites TCATTGCC on the EGFR 2.3k promoter (−2,178 bp to −2170 bp) were generated according to the site-directed mutagenesis kit (Stratagene). Mutant Mt1 replaced 3 bp from Tead-binding site TCATTGCC to TCTCGGCC, whereas the mutant Mt2 deleted internal 6 bp from Tead-binding site TCATTGCC. The fragments were verified by sequencing before cloning into pGL4.22 vector. The primers for mutant 1 and 2 EGFR promoters as followings: EGFR-p2.3k-Mt1.F 5′ agcaactgggccactattgTCTCGGCC tgtggtggtggcacacacacc-3′; EGFR-p2.3k-Mt1.R 5′ cttggggttggctaacacacAGGCGAGAcaaatagtggccagcgttgc-3′; EGFR-p2.3k-Mt2.F 5′ agcaactgggccactattgTCTCGGCC tgtggtggtggcacacacacc-3′; EGFR-p2.3k-Mt2.R 5′ cttggggttggctaacacacAGGCGAGAcaaatagtggccagcgttgc-3′;

Transient cotransfection with EGFR luciferase reporter either wide-type or mutants and Renilla vector was performed as previously described (30).

IHC

IHC staining for YAP1 and EGFR was performed on tissue microarray slides consisting of 113 esophageal adenocarcinoma and non-neoplastic esophageal tissue samples from patients who underwent esophagectomy without neoadjuvant therapy and has been described previously (9, 30). In addition, 10 cases of pretreatment biopsies with complete response tissues term as P2 using antibodies against YAP1 (1:100) and EGFR as described previously (30). The staining results were evaluated by two IHC experts from the service laboratory (N. Khalor and Q. Chen) and a scientist (S. Song) from the research laboratory at the same time to score the percentage of tumor cell nuclei stained (0, no staining; 1, ≤10%; 2, 10%–50%; and 3, >50%) and the staining intensity (0-negative, 1-weak, 2-moderate and 3-strong).

Indirect immunofluorescence

Indirect immunofluorescence staining was performed as described (29). Expression and localization of the proteins were
observed under a confocal microscope system (FluoView FV500; Olympus) and analyzed by CellQuest PRO software (BD Biosciences).

Tumor sphere formation assay

Sphere culture was performed as previously described (30). Briefly, single cell suspension of KATO-TN cells with (DOX+) or without (DOX−/) YAP1 induction and JHESO cells with or without knockdown YAP1 were seeded in triplicate onto a 6-well ultra-low attachment plate (2,500 cells/well; Corning) in serum-free DMEM/F-12 supplemented with 20 ng/mL EGF, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 2% B27 supplement w/o vitamin A, and 1% N2 Supplement (Invitrogen). After 10 to 20 days of culture, the number of tumor spheres formed (diameter >100 μm) was counted under microscope.

Establishment of 5-FU–resistant subclones from esophageal cancer cells

To establish 5-fluorouracil (5-FU)–resistant subclones, Flo-1 parent esophageal cancer cells were cultured with various concentrations of 5FU for 3 to 5 weeks, and the surviving cells were collected. This collection procedure was repeated four times. The establishment of these 5FU-resistant subclones took 3 to 6 months and newly derived 5FU-resistant clones, designated as Flo-1RF.

In vivo xenograft mouse model

JHESO cells were subcutaneously injected with $1 \times 10^6$ cells in nude mice. $n = 5$ each group. After around 10 days invocation, VP was applied by intraperitoneal (i.p.), 50 mg/kg/mouse, 5-FU was applied by i.p., 30 mg/kg/mouse and their combination, three times a week for total 3 weeks. Control group was applied same volume of PBS (100 μL/mouse). The tumor size and volume were measured as previously (28). All the measurements were compared using unpaired Student $t$ test.

Statistical analysis

Data were analyzed using the Student $t$ test and Fisher exact test (for IHC). A $P$ value of <0.05 was required for statistical significance, and all tests were two sided as previously described.

Results

YAP1 and EGFR are overexpressed in esophageal cancer tumor tissues and are associated with therapy resistance

Both EGFR and YAP1 play important role in control growth and tumor maintenance. Previously, we have shown that EGFR is upregulated in both EAC and ESCC and increased EGFR expression correlates with a shorter survival (9). To determine whether both YAP1 and EGFR expressions are associated in EAC, immunoblotting was performed in two benign Barrett’s cell lines CPA and CP-C and six EAC cell lines. Results in Fig. 1A showed that expression of both YAP1 and EGFR were determined by IHC in sensitive EAC tumor tissues (P0/pCR), relative sensitive tumor tissues (P1), and resistant tumor tissues (P2). Both EGFR and YAP1 highly expressed in majority of P2 tissues.

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Figure 1.

YAP1 and EGFR are overexpressed in esophageal cancer tumor tissues and associated with therapy resistance. A, expressions of YAP1 and EGFR were determined by immunoblotting in Barrett’s and EAC cell lines as described in Materials and Methods. B, expressions of YAP1 and EGFR were determined by IHC using antibodies against YAP1 and EGFR in EAC TMA tissues. Representative YAP1 and EGFR staining is shown in normal and EAC tissues. C, expression of EGFR significantly correlated the poor survival of EAC patients. Cox regression for OS analysis; $P < 0.01$. D, expression of YAP1 and EGFR were determined by IHC in sensitive EAC tumor tissues (P0/pCR), relative sensitive tumor tissues (P1), and resistant tumor tissues (P2). Both EGFR and YAP1 highly expressed in majority of P2 tissues.
pretreatment biopsies tissues (P0/P1) and found that expression of both YAP1 and EGFR in resistant tumor tissues (P2) is correlated and much higher than in sensitive tumors (P0 or P1; Fig. 1D). Fifty percent of resistant tissues (P2) have strong staining (3+) for both EGFR and YAP1, whereas only 20% of sensitive tumors (P0/P2) have weak staining (1+) for both EGFR and YAP1. These data support the notion that both YAP1 and EGFR are involved in esophageal cancer tumor progression as well as therapy resistance.

YAP1 induces EGFR overexpression in esophageal cancer tumor cells

EGFR is overexpressed in many tumor types; and tumor cells utilize EGFR signaling to maintain their growth advantage; however, how EGFR is upregulated is not well defined. We have previously demonstrated that conditional deletion of the core Hippo signaling components Sav1, Mst1/2 result in tumors of the mouse liver through deregulation of YAP1 (18). A transposon mutagenesis screen in a Sav1-mutant background revealed activation of EGFR is a frequent co-occurring event found in 50% to 60% of tumors. This observation led to the hypothesis that YAP1 might further activate EGFR signaling by increasing EGFR expression. To determine this possibility and to gain further insight into the relationship between YAP1 and EGFR expression, we first transduced the esophageal cancer cells SKGT-4, YES-6 and KATO-TN cells with a doxycycline-inducible human flag-tagged YAP1S127A cDNA (PIN20 YAP1S127A). Successful YAP1 induction in SKGT-4, YES-6, and KATO-TN cells by doxycycline at 1 μg/mL increased expression of EGFR in concert with increased YAP1 (Fig. 2A, left), whereas expression of IGFR was not affected (Fig. 2A). In contrast, shRNA-mediated knockdown of YAP1 in JHESO cells greatly reduced EGFR protein levels (Fig. 2A, right). Moreover, in SKGT-4 (PIN20YAP1) cells, YAP1-induced EGFR expression was diminished by knockdown of YAP1 in doxycycline-induced SKGT-4 cells (Fig. 2B) confirming the direct regulation of EGFR expression by YAP1. Furthermore, immunofluorescence demonstrates that induction of YAP1 by doxycycline at 1 μg/mL increases EGFR expression in SKGT-4 cells (Fig. 2C).

Figure 2. YAP1 upregulates EGFR expression in both normal and malignant esophageal cancer cells. A, SKGT-4, YES-6, and KATO-TN cells were transduced with lentiviral plasmid containing inducible YAP1 cDNA (PIN20YAP1). YAP1 expression was induced by doxycycline to the culture medium at 1 μg/mL. Immunoblotting using antibodies against YAPI, EGFR, and IGFR was performed (left). Immunoblotting of YAPI, EGFR was performed in JHESO cells with two independent YAPI shRNAs (YAPI sh2 and YAPIsh3) clones (right). B, immunoblotting of YAPI, EGFR was performed in SKGT-4 (PIN20YAP1) cells with (DOX+) or without (DOX−) YAPI induction and knockdown YAPI in SKGT-4(PIN20YAP1) DOX−induced cells. C, immunofluorescent staining of YAPI and EGFR in SKGT-4 cells that were transduced with inducible YAPI (PIN20YAP1) with or without doxycycline induction at 1μg/mL. D, YAPI and EGFR were detected by immunoblotting in 293T cells transfected with either mutant YAPIS127A or wt YAPI expression vectors (left). YAPI and EGFR were detected by immunoblotting in primary murine esophageal cells transduced with lentiviral plasmid containing inducible YAPI cDNA (PIN20YAP1, middle). EGFR expression was examined by immunoblotting in B299 cells and tumor cell lines isolated from Sav1−/− or Mst1/2−/− mouse tumor tissues (right).
To determine whether EGFR expression is regulated by YAP1 in primary esophageal cells and in other cell types, transfection of human embryonic kidney (HEK293T) cells with constitutively active mutant YAP1S127A cDNA or with wild-type YAP1 induced EGFR expression (Fig. 2D, left). In addition, primary murine esophageal cells that expressed YAP1S127A (DOX +) demonstrated higher EGFR expression than cells without YAP1 induction (Fig. 2D, middle). In addition, Western blot analysis in hippo deregu- lated mice tumor cells lines isolated from hippo mutant (Sav/C0/C0 and Mst1/2/C0/C0) mice tumor tissues demonstrated elevated EGFR expression compared with the immortalized liver B299 cells (Fig. 2D, right), while there is no change in the level of IGFR. Hence, EGFR can be elevated in multiple cell types by expression of a constitutively active mutant form of YAP1 or by activation of endogenous YAP1 protein that occurs following the deletion of Hippo pathway signaling components.

YAP1 induced EGFR transcription via TEAD binding site in the promoter of EGFR

Having established that YAP1 regulates EGFR expression in multiple cellular contexts, we next examined that this regulation occurs at the transcriptional or posttranslational level. First, EGFR mRNA level was upregulated in esophageal cancer cells stably transfected with YAP1 by qPCR as shown in Fig. 3A (right) which is in concert with increased YAP1 mRNA level (left). Analysis of the human EGFR proximal promoter region reveals an intact TEAD (CATTCC) binding site located around -2,178 to -2,170 of base pairs upstream of the transcription start site. The 2.3-k EGFR promoter containing this TEAD binding from the transcription start site was cloned and fused to a luciferase cDNA and cloned to the pGL4.22 vector and then were transfected into SKGT4 esophageal cancer cells contain a stably integrated doxycycline-inducible YAP1S127A cDNA. Upon YAP1S127A induction by doxycycline (DOX +) administration, more than 3-fold induction of EGFR luciferase activity was observed (Fig. 3B, left). As YAP1 is known to bind to TEAD transcription factors, we investigated whether YAP1 and TEADs can transactivate EGFR promoter-luciferase construct in esophageal cancer cells. Hence, the 2.3-k EGFR promoter was cotransfected with either YAP1 or YAP1 and TEAD into 293T cells. EGFR Luciferase activities were increased more than 2-fold by YAP1, while cotransfected both YAP1 and TEAD, the EGFR transcriptional activity was increased by 5-fold (Fig. 3, right). This indicates that YAP1 and TEAD cooperate to induce EGFR transcription. To determine whether the TEAD-binding site in the EGFR promoter is crucial for induction of EGFR by YAP1, either a mutation (ATT-TCG) or deletion of the TEAD-binding site was generated in the EGFR promoter using site-directed mutagenesis as

Figure 3.

YAP1 increases EGFR transcription through an intact Tead-binding site. A, mRNA levels of YAP1 and EGFR were determined by qPCR in SKGT-4 cells transduced with lentiviral plasmid containing inducible YAPI cDNA (PIN20YAP1) and induce YAPI with or without doxycycline at 1 μg/mL. B, transient transfection of EGFR luciferase promoter reporter into SKGT-4 (PIN20YAP1) cells with or without induced YAPI by doxycycline at 1 μg/mL. EGFR luciferase reporter activity was measured after 48 hours (left). Cotransfection of EGFR luciferase promoter reporter with YAPI or YAPI plus TEAD into 293T cells; EGFR luciferase reporter activity was detected after 48 hours (right). C, wide-type Tead-binding site and a mutation (ATT-TCG) or deletion of the TEAD-binding sites were depicted (left). Cotransfection of EGFR luciferase promoters (wide-type or mutated or deleted in the Tead-binding site) with YAPI or control vector into 293T cells; EGFR luciferase reporter activity was detected after 48 hours (right). D, cotransfection of EGFR luciferase promoters (wide-type or mutated or deleted in the Tead-binding site) with YAPI or control vector into SKGT-4 (PIN20YAP1) with (DOX +) or without (DOX −) YAPI induction; EGFR luciferase reporter activity was detected after 48 hours. For all experiments, values shown represent the mean and SD of at least triplicate assays (**, P < 0.01).
depicted in Fig. 3C (left). Induction of EGFR transcriptional activity by YAP1 was greatly diminished, when mutation or deletion of the TEAD-binding site in the EGFR promoter was introduced into 293T cells (Fig. 3C, right panel). Similarly, in SKGT4 esophageal cancer cells, induction of EGFR transcriptional activity by YAP1 induction upon doxycycline treatment was significantly reduced, when mutation or deletion of the TEAD-binding site in the EGFR promoter was introduced (Fig. 3D). These data indicate that YAP1 induces EGFR transcription and requires an intact TEAD-binding site in the EGFR promoter.

YAP1 activates EGFR signaling and mediates cell survival and CSCs properties

Activation of EGFR downstream signaling depends on both increased abundance of EGFR level as well as its phosphorylation. Having shown that YAP1 increases EGFR expression, next we sought to determine whether YAP1 also increases its phosphorylation. Increased expression of YAP1 by doxycycline induction in three esophageal cancer cell lines-SKGT-4, YES-6, and KATO-TN significantly induced phosphorylation of EGFR at pY1068 in concert with the increase in antiapoptotic protein MCL-1 although not so dramatic change in its protein level (Fig. 4A). Furthermore, YAP1 increased and sustained EGF-induced phospho-EGFR at pY1068 and its downstream AKT phosphorylation (Fig. 4B). To determine the functionality of YAP1 induction in esophageal cancer cells, we used several assays and found that YAP1 induction in SKGT-4 and KATO-TN cells increased esophageal cancer cell proliferation (Fig. 4C, left and middle), and tumor sphere forming capacity (Fig. 4D, left). In contrast, downregulation of YAP1 by lentivirus shRNA in JHESO cells decreased tumor sphere formation capacities (Fig. 4D, right). These indicate that YAP1 is required for tumor cell survival and maintenance, which probably involves activation of EGFR signaling.

YAP1 mediates constitutive and acquired therapy resistance in esophageal cancer cells

Expression of YAP1 and EGFR is increased in residual resistant tumor tissues in most post treated tumor tissues as shown in Fig. 1C; we next sought to determine whether YAP1-mediated EGFR is responsible for constitutive or acquired chemoresistance in both EAC and ESCC cells. SKGT-4 and KATO-TN have constitutively high or low YAP1 and EGFR expression; and SKGT-4 cells with high YAP1 and EGFR expression have more invasive capacity than KATO-TN cells with low YAP1 and EGFR (Supplementary Fig. S1A). When treating with 5-FU in these cells, SKGT-4 demonstrated more resistance than KATO-TN cell on 5-FU treatment in different dosages (Supplementary Fig. S1B and S1C). To further confirm the direct relationship between YAP1 and chemoresistance, induction of YAP1 in both EAC cell line SKGT-4 and ESCC cell line KATO-TN by doxycycline demonstrated more resistant to either 5-FU (Fig. 5A) or docetaxel (Fig. 5B) than esophageal cancer cells without YAP1 induction (DOX−). In addition, downregulation of YAP1 in JHESO cells in two individual clones greatly increased cell sensitivities to 5-FU than its parental cells JHESO (Fig. 5C). Furthermore, in the established chemoresistant esophageal cancer cells Flo-1RF, there is high expression of YAP1 and EGFR compared with their parental cells that is in concert with significant resistance to 5-FU treatment (Fig. 5D). Moreover, as shown in Supplementary Fig. S2, when we knocked down EGFR in YAP1-induced esophageal cancer cells (SKGT-4 DOX+) using Lenti-Crisp system, esophageal cancer cells become more sensitive to 5-FU treatment which phenocopies the effects by knocking down YAP1 as shown in Fig. 5C. These data suggest that YAP1 induction of EGFR is associated with constitutive and acquired chemoresistance in esophageal cancer cells.

YAP1 inhibitor inhibits YAP1 and EGFR expression and sensitizes cytotoxic drugs in vitro and in vivo

Increased EGFR expression is associated with poor clinical outcome for esophageal cancer patients and clinical trials based on EGFR inhibition have not been successful so far which may be due in part to the sustained increased EGFR expression and activation by YAP1. Therefore, alternative means of inhibition of EGFR signaling should be highly considered. We have demonstrated that YAP1 is responsible for sustained EGFR overexpression and activation, and hence targeting YAP1 may be an effective means to utmost inhibit EGFR signaling. Recently, verteporfin has been identified as a small-molecule inhibitor of YAP1 and TEAD association and a means of inhibiting YAP1’s oncogenic activity (31). As demonstrated in Fig. 6A, expression of EGFR and YAP1 was dramatically reduced by VP in a dose-dependent manner in both JHESO and OACPC cells. In addition, phosphorylation of AKT and other stem cell markers such as SOX9 and Hes-1 are reduced as well. Importantly, VP treatment alone at nontoxic concentration of (1 μmol/L) has minimal inhibition on JHESO cells, whereas in combination with 5-FU or docetaxel, the inhibitory effects on esophageal cancer cells are dramatically increased (Fig. 6B, left). This indicates that VP sensitizes the toxicity of 5-FU or docetaxel on esophageal cancer cells. In addition, to test whether the combination of VP and the EGFR inhibitor, erlotinib, synergistically inhibits esophageal cancer cell growth, low dosage of VP (0.5 μmol/L) or Erl (1 μmol/L) or 5-FU (5 μmol/L) either alone or in combination was applied in JHESO cells. Interestingly, the combination of a low dosage of VP (0.5 μmol/L) and erlotinib (1 μmol/L) has minimal inhibition on JHESO cells; however, the triple combination of VP (0.5 μmol/L) and erlotinib (1 μmol/L) and 5-FU has best effects to inhibit esophageal cancer cell survival (Supplementary Fig. S3). To further determine whether the combination of VP and 5-FU depends on the inhibition of YAP1, SKGT-4 stably YAP1 expression cells with (DOX+) or without (DOX−) YAP1 induction were treated with 5-FU and VP. The result in Fig. 6B (right) demonstrated that YAP1 induction (DOX+) made SKGT-4 cells more resistant to 5-FU; however, VP in combination of 5-FU dramatically increases the response of SKGT-4 cells on 5-FU especially in inducible YAP1 expression SKGT-4 (DOX+) cells. Results from in vitro xenograft model further confirmed that mice treated with VP greatly reduced tumor growth in vivo, whereas in the mice treated with VP in combination with 5-FU, the significant reduction of tumor weights and tumor volumes were observed compared with 5-FU alone (Fig. 6C). In addition, the level of YAP1, EGFR, and proliferation marker Ki-67 in mice tumors was dramatically diminished by the combination treatment of VP and 5-FU (Supplementary Fig. S4). Thus, VP through inhibition of both EGFR and YAP1 can overcome the acquired chemoresistance and sensitize 5-FU effects on esophageal cancer tumors.

Discussion

In this study, we demonstrated for the first time that YAP1 upregulates EGFR expression at the level of transcription through
YAP1 mediates EGFR overexpression and chemoresistance in esophageal cancer.

The Hippo signaling pathway is gaining recognition as an important player in both organ size control and tumorigenesis because the disruption of several important components (Mst1/2, Sav1 and Lats1/2 and YAP1) in this pathway can lead to tumorigenesis (18, 19, 32). YAP1, an effector of the Hippo signaling pathway, has been reported as an oncogene in several tumor types. YAP1 activates EGFR signaling and mediates cell survival and CSCs properties. A, phospho-EGFR and MCL-1 were detected by immunoblotting in SKGT-4, YES-6, and KATO-TN cells transduced with YAP1S127 cDNA (PIN20YAP1) with (DOX+) or without (DOX-) doxycycline induction; B, phospho-EGFR and phosphor-AKT were detected by immunoblotting in SKGT-4 cells transduced with YAP1S127 cDNA (PIN20YAP1) with (DOX+) or without (DOX-) doxycycline induction and treated with EGF at 50 ng/mL. C, cell growth of SKGT-4 (PIN20YAP1) and KATO-TN (PIN20YAP1) with (DOX+) or without YAP1 induction was determined using MTS as described in Materials and Methods to determine the rate of proliferation at 3 and 6 days. **, P < 0.05 (left and middle). Cell growth of JHESO control and YAP1 knockdown cells (YAP1 sh2 and sh3) were determined using MTS to determine the rate of proliferation at 3 days. ** *, P < 0.05 (right). D, representative images of spheres in KATO-TN (PIN20YAP1) cells with (DOX+) or without YAP1 induction (DOX-; top). Representative bar graph demonstrating the sphere numbers in KATO-TN (PIN20YAP1) cells with (DOX+) or without YAP1 induction (DOX-; low; left). Representative images of spheres in JHESO cells with control and its knockdown (YAP1 sh) cells (top). Representative bar graph demonstrating the sphere numbers in JHESO cells with control and its knockdown (YAP1 sh) cells (low; right). Data are represented as mean and SD from three experiments. ** *, P < 0.001.
such as HCC and breast cancer and ESCC (33–35). EGFR overexpression or amplification has been reported in many human tumors and increased EGFR expression has been associated with advanced disease, development of metastasis, and poor clinical prognosis in a subset of tumors including esophageal ESCC and EAC. Both EGFR and YAP1 play important role in cell proliferation, survival, and tumor maintenance, perhaps chemoresistance. The cross-talk between these two pathways is merging. Zhang and colleagues first identified that EGFR ligand-amphiregulin (AREG) is a transcriptional target of YAP1, whose induction contributes to YAP1-mediated cell proliferation and migration (17). Anterior gradient homolog 2 (AGR2) induction of EGFR ligand, AREG is mediated by activation of the Hippo signaling pathway coactivator, YAP1 (36). Similarly, TAZ, a paralog of YAP1 also induces AREG production and activation of EGFR signaling (37). Vice versa, EGFR ligands HB-EGF, AR, and EGFR transactivator TGFβ stimulate expression of YAP1 target CTGF in hepatocellular carcinoma cell lines through upregulation of YAP1 (38). A recent study from Reddy and colleagues demonstrated that the EGFR-RAS-MAPK branch of EGFR signaling activates YAP1 via promoting phosphorylation of Ajuba family protein WTIP and enhancing WTIP binding to Lats1/2 (39). Hong and colleagues found that transforming activity of oncogenic RasV12 depends on its ability to downregulate SOCS-box proteins and thereby stabilize YAP1. Thus, the transforming potential of the Ras pathway appears to be mediated in part at the level of YAP1 protein turnover (40). In our study, we identified that YAP1 upregulates EGFR protein expression at the level of transcription. Mutation or deletion of the TEAD-binding site in the EGFR promoter diminished EGFR transcriptional induction by YAP1 indicating that an intact TEAD-binding site is necessary for YAP1 induction of EGFR. Thus, Hippo signaling influences EGFR signaling by two distinct mechanisms by directly upregulation of EGFR expression and upregulation of its ligand amphiregulin identified previously (17). Both mechanisms can sustain robust activation of EGFR signaling, thereby increasing tumor cell survival and therapy resistance (Fig. 6D).

Although the current literature suggests that EGFR is overexpressed and activated in tumor cells and tissues, few reports discuss how EGFR is upregulated or activated in tumor tissues. Our study sheds more light on one mechanism by which EGFR is deregulated in tumor tissues and indicates that YAP1 is responsible for the EGFR sustained overexpression in tumor cells.

Esophageal cancer is a lethal illness and an inherently resistant to therapy even if it is diagnosed as localized cancer. Following preoperative chemoradiation, residual cancer is noted in 70% of surgical specimens (41). However, the tumor resistant mechanisms are still unclear. We have previously reported that hedgehog (Hh) pathway is often upregulated in esophageal cancer and mediates therapy resistance (42). mTOR activates Hh signaling...
in EAC by phosphorylation of S6K and the crosstalk between mTOR and Hh signaling confers tumor cell growth advantage and resistant to therapy (23). Our current data suggest that YAP1-mediated EGFR upregulation in esophageal cancer cells might be the driver for constitutive or acquired resistance. First, esophageal cancer cells with high YAP1 and EGFR are more invasive and more resistant to cytotoxic drugs. Second, both YAP1 and EGFR are upregulated in posttreatment resistant tumor tissues (P2) compared with sensitive tissues (P0 or P1); and 5-FU-resistant esophageal cancer cell lines have more YAP1 and EGFR expression compared with their parental cells. Furthermore, the direct evidence for YAP1-mediated resistance is seen by introducing YAP1 into esophageal cancer cells by doxycycline induction and cells with YAP1 induction have more growth advantage when treated with cytotoxic drugs. In contrast, knockdown of YAP1 by lentivirus shRNA in esophageal cancer cells decreases the tumor cell growth advantage and increases 5-FU sensitivity in esophageal cancer cells which is consistent with the recent finding of Huang and colleagues that knockdown of YAP1 sensitizes ovarian cancer cells to cisplatin and survivin inhibitors (43). This indicates that YAP1 confers the tumor cell growth advantage and mediates chemoresistance. Targeting YAP1 may be a means to overcome chemoresistance.

Although EGFR is overexpressed and amplified in esophageal cancer, inhibition in the clinic has failed (11, 14). YAP1-mediated sustained EGFR upregulation and activation may be one explanation for this phenomenon. Although inhibition of EGFR signaling at the baseline by EGFR inhibitor or its monoclonal antibody, YAP1 keeps activation and upregulation of EGFR that seems to confer therapy resistance as we have demonstrated in this report. Therefore, inhibition of YAP1 or in combination of EGFR and plus cytotoxics may be the best way to gain advantage. In fact, our data demonstrate that the YAP1 inhibitor VP effectively inhibits both YAP1 and EGFR protein levels and its downstream signaling and synergistically inhibit tumor cell growth when in combination with a cytotoxic in vitro and in vivo.

In conclusion, we have identified that YAP1 positively regulates EGFR expression at the level of transcription through an intact TEAD-binding site at the EGFR promoter. Expression of both YAP1 and EGFR was increased in resistant esophageal cancer tumors (P2) compared with sensitive tumors (P0/P1) and mediated therapy resistance. Thus, the YAP1-EGFR axis could be an important therapeutic target in esophageal cancer (Fig. 6D). Future preclinical and clinical studies targeting YAP1-EGFR axis and in combination of chemotoxics are warranted.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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