Colon Cancer Cells Escape 5FU Chemotherapy-Induced Cell Death by Entering Stemness and Quiescence Associated with the c-Yes/YAP Axis

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Abstract

Purpose: Metastasis and drug resistance are the major limitations in the survival and management of patients with cancer. This study aimed to identify the mechanisms underlying HT29 colon cancer cell chemoresistance acquired after sequential exposure to 5-fluorouracil (5FU), a classical anticancer drug for treatment of epithelial solid tumors. We examined its clinical relevance in a cohort of patients with colon cancer with liver metastases after 5FU-based neoadjuvant chemotherapy and surgery.

Results: We show that a clonal 5F31 cell population, resistant to 1 μmol/L 5FU, express a typical cancer stem cell–like phenotype and enter into a reversible quiescent G0 state upon reexposure to higher 5FU concentrations. These quiescent cells overexpressed the tyrosine kinase c-Yes that became activated and membrane-associated upon 5FU exposure. This enhanced signaling pathway induced the dissociation of the Yes/YAP (Yes-associated protein) molecular complex and depleted nuclear YAP levels. Consistently, YES1 silencing decreased nuclear YAP accumulation and induced cellular quiescence in 5F31 cells cultured in 5FU-free medium. Importantly, YES1 and YAP transcript levels were higher in lower metastases of patients with colon cancer after 5FU-based neoadjuvant chemotherapy. Moreover, the YES1 and YAP transcript levels positively correlated with colon cancer relapse and shorter patient survival (P < 0.05 and P < 0.025, respectively).

Conclusions: We identified c-Yes and YAP as potential molecular targets to eradicate quiescent cancer cells and dormant micrometastases during 5FU chemotherapy and resistance and as predictive survival markers for colon cancer. Clin Cancer Res; 20(4); 837–46. ©2013 AACR.

Introduction

Treatment of colon cancer involves the surgical resection of the primary tumor. For patients with stage III disease, a survival advantage is obtained with 5-fluorouracil (5FU)/leucovorin-based adjuvant chemotherapy used in combination with oxaliplatin or irinotecan (1–3). In the last 8 years, cetuximab and panitumumab, two monoclonal antibodies that target the epidermal growth factor receptor, were shown to be effective in combination with chemotherapy or as single agents in patients with wild-type KRAS tumors (4–6). In addition, antiangiogenic therapy targeting vascular endothelial growth factor (bevacizumab) confers a benefit when used in combination with chemotherapy (7). However, the development of drug resistance remains a major limitation in the efficacy of the clinical response to chemotherapeutic and targeted therapy regimens.

A growing body of evidence suggests that the majority of tumors comprise a population of tumor-initiating or cancer stem cells (CSC) that are responsible for the development and maintenance of tumors and resistance to cytotoxic drugs (8). In breast cancer, studies using clinical tumor samples support the hypothesis that the residual disease after neoadjuvant chemotherapy is enriched with CSCs. Cell suspensions derived from chemotherapy-treated patients showed an increase in mammosphere formation, self-renewal, and enrichment in CD44+CD24−low stem-like cells (9). In murine models of colon cancer cell xenografts, the treatment...
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Translational Relevance
Metastasis, chemoresistance, and dormancy are the major limitations in the management of patients with cancer. Here, we show that 5-fluorouracil (5FU) chemoresistance in human colon cancer cells preselects two distinct drug-resistant clonal populations each enriched in cells expressing specific stem cell markers and differential self-renewing and metastatic potentials. The 5FU-induced quiescence is linked to activation of the c-Yes tyrosine kinase and nuclear depletion of the c-Yes–associated YAP oncogene. Consistently, YES1 silencing decreased nuclear YAP accumulation and induced cellular quiescence in 5FU-free conditions. We further demonstrate the prognostic relevance of our data in colon cancer liver metastases treated by neoadjuvant chemotherapy regimens based on 5FU. High YES1 and YAP transcript levels measured in residual liver metastases after adjuvant chemotherapy are observed in patients at risk for colon cancer relapse and shorter survival. Therefore, these two markers identify candidates for adapted treatment targeting the c-Yes/YAP axis in chemoresistant quiescent colon carcinomas and liver metastases.

of mice with chemotherapeutic agents enriched the tumor xenografts in E2A^CD44^ and E2A^CD44^CD166^ CSCs (10). In other studies, CSCs were isolated from various types of tumors and analyzed for chemoresistance ex vivo. In human colon cancer, CD133-positive CSCs were highly resistant to 5FU and oxaliplatin (11). When the colon cancer cell line HT29 was treated continuously with 5FU or oxaliplatin, the emergent resistant subpopulations were highly enriched in cells expressing stem cell markers, including CD133 (16- to 30-fold) and CD44 (2-fold; ref. 12). These findings suggest that drug-resistant subpopulations may be enriched in CSCs.

The intrinsic resistance of CSCs to chemotherapeutic agents may be explained by high expression of ATP-binding cassette (ABC) multidrug transporters, antiapoptotic proteins, and by the resistance to DNA damage. Accumulating evidence has indicated that CSC quiescence may also account for a possible mechanism of resistance because the activity of several cytotoxic agents is dependent on cell-cycle progression (13–16). Cellular quiescence is a basic mechanism of clinical tumor dormancy, although angiogenic dormancy and escape from immune system control also play important roles (17).

We previously reported that chronic treatment of the HT29 colon cancer cell line with chemotherapeutic agents resulted in the emergence of drug-resistant HT29 subpopulations overexpressing the chemokine (C-X-C) motif receptor 4 (CXCR4). In addition, overexpression and autocrine activation of CXCR4 played a role in the metastatic spreading to the lungs in immunodeficient mice (18, 19). Here, we report the acquisition of a complex mechanism of chemoresistance to 5FU involving selection for colon CSCs and their quiescence linked to the activation of the c-Yes tyrosine kinase. We show that c-Yes controls the balance between quiescence and cycling of 5FU chemoresistant cells as well as the cytoplasmic/nuclear ratio of the YAP-activated transcription coactivator. Finally, we discovered a direct relationship between c-Yes/YAP expression levels and overall and disease-free survival of patients with colorectal liver metastases after 5FU-based neoadjuvant therapy.

Materials and Methods
Human colorectal carcinoma cell lines and patients’ tissue samples
HT29 parental cell line, 5FU (10^-6 and 10^-5 mol/L)-resistant HT29 subpopulations (HT29FU) and 5F7 and 5F31 clonal derivatives as well as 5FU-resistant (8 × 10^-6 mol/L) RKO colon cancer cell line (RKO) cells (RKO-FU) and oxaliplatin-resistant (2 × 10^-5 mol/L) RKO cells (RKO-oxaliplatin [OXA]) and HT29OXA cells subpopulations were cultured as previously described (20–22). Liver metastases of colon adenocarcinoma were resected from 49 patients and were processed and stored by the Tumor Cell and Tissue Bank of the Regional Reference Cancer Center of Lille. After hepatic resection, fragments were taken from macroscopic metastases, snap-frozen in liquid nitrogen, and stored at –80°C. The whole remaining tissue was fixed in 10% formalin and several other fragments were taken from the fixed metastases and embedded in paraffin. Hematoxylin, eosin, saffron, and Astra blue-stained sections were examined by an experienced pathologist to establish the histological diagnosis. Fragments used in this study contained at least 60% malignant cells. Informed consent was obtained from all patients. The curative microscopically complete R0 liver resections in all patients were performed between February 2002 and May 2009. Neoadjuvant chemotherapy was administrated for 28 patients before surgery according to the recommendations of the French Thesaurus of Digestive Cancerology: Folfirix in 17 patients, Folfiri in 1 patient, Folfri-bevacizumab in 10 patients. Information about sites of tumor recurrence or metastasis development after curative hepatectomy and delay of recurrence were collected for each patient.

YES1 silencing
YES1 silencing was performed by retroviral infection of 5F31 cells using a pRETRO-Super vector as previously described (23). Short hairpin RNAs (shRNA) targeting YES1 transcript sequences were also previously described (24). The selection of the YES1–silenced populations was performed with puromycin (1 μg/mL).

Statistical analyses
All data were expressed as mean (standard deviation) and categorical variables by percentage (frequency). Overall survival (OS) and disease-free survival (DFS) were calculated by the Kaplan–Meier method, and the differences between groups were compared using the log-rank test. To identify predictive variables of OS or DFS, continuous
variables were analyzed by a Cox proportional hazards model and qualitative variables using the log-rank test. Expectation maximization algorithm was used to determine the cutoff values of Yes and YAP. All analyses were performed using the SAS software version 9.2 (SAS Institute Inc.). A P value of <0.05 was considered significant.

**Results**

**5FU-resistant HT29 clones exhibited heterogeneity in their chemoresistance**

Previous studies have reported the emergence of stable HT29 cell subpopulations resistant to 5FU that were capable to differentiate into goblet cell–like or enterocyte-like phenotypes (20). Cloning of the HT29 subpopulation resistant to $10^{-6}$ mol/L 5FU (HT29FU) by limiting dilution gave rise to several HT29 cell clonal subpopulations (HT29 5F clones; ref. 21). The analysis of IC$_{50}$ values in HT29 5F clones toward newly added 5FU revealed differential intrinsic levels of chemoresistance to this drug. As shown in Fig. 1, the 5F31 clone was highly 5FU resistant (IC$_{50}$ = $19.6 \times 10^{-6}$ mol/L 5FU), whereas 5F7 clone was much less resistant (IC$_{50}$ = $1.9 \times 10^{-6}$ mol/L) but still more resistant than the parental HT29 cell line (IC$_{50}$ = $1.1 \times 10^{-6}$ mol/L). The highly 5FU-resistant 5F31 cells were able to recover proliferative capacity after drug withdrawal.

**Drug resistance is associated with a diverse expression pattern of stem cell markers**

5FU-resistant clones highly expressed several colon CSC surface markers as shown by flow cytometry using a quadruple labeling of CD24, CD44, CD133, and CXCR4 (Supplementary Fig. S1A). In contrast, parental HT29 cell population contained a low percentage of cells expressing stem cell markers, ranging from 4% (CXCR4) to 32% (CD24; Supplementary Table S1). The two 5FU-resistant clones contained more than 90% of cells expressing at least one marker. The 5F31 cell population mainly contained CD24+/CD44+ cells (55%), CD24+/CD44+/CD133+ cells (14%), and CD24+ cells (14%). The 5F7 cell population was composed of CD24+/CD44+/CD133+/CXCR4+ cells (50%) and of CD24+/CD44+/CXCR4+ cells (31%). The 5FU-resistant (HT29FU) and oxaliplatin-resistant (HT29OXA) subpopulations (22) also contained an important proportion of CD24, CD44, CD133, and CXCR4-positive cells (Supplementary Table S1). Moreover, the percentages of CD133- and CXCR4-positive cells varied substantially in 5F7 and 5F31. This heterogeneity was further confirmed using the CSC marker aldehyde dehydrogenase (ALDH)1A3, an isof orm of ALDH (Supplementary Fig. S1B; 25). ALDH1A3 was strongly downregulated (74-fold) in 5F7 cells and conversely upregulated in 5F31 cells (3-fold), as compared with the parental HT29 cells. Total ALDH activity was also higher in 5F31 versus 5F7 cells. Altogether, our data show that resistant clones were enriched in stem cell–like cancer cells that differ in the expression pattern of stem cell markers.

**Drug-resistant HT29 clones differed in their proliferation and metastatic potentials**

Stem cells are defined by their combined ability to self-renew and to differentiate. We investigated the self-renewal potential of 5F7, 5F31, and HT29 cells and their 5FU-treated counterparts using anchorage-independent growth (Fig. 2A). 5F31 cells formed well-delimited regular spheres, whereas 5F7 and parental cells produced irregular spheres with a strong tendency to form aggregates. The self-renewal potential of spheres was evaluated by an increase ratio in the number of spheres formed over three consecutive generations (Fig. 2B). The ratio between third and first generation of spheres was significantly enhanced in 5FU-treated 5F7 and 5F31 cells (1.9- and 5.8-fold, respectively) as compared with their untreated counterparts (1.3- and 2.5-fold, respectively). Also, the HT29FU and HT29OXA subpopulations were enriched in the sphere-forming, self-renewing cells when compared with their parental cell line.

The 5F7 and 5F31 clones were then assessed for their tumorigenic and metastatic potential using orthotopic xenografts. Both clones produced fairly well-differentiated adenocarcinomas with cell layers and glands containing mucus in their lumen (Fig. 2C). The presence of lung micrometastases was observed in both 5F7- and 5F31-xenografted mice; however, the number of lung metastases was higher in 5F7 xenografts in comparison with 5F31 xenografts (9 vs. 2 per mouse). In addition, only 5F31 cells generated metastases in lymph vessels around the liver, demonstrating that these two clones have similar tumorigenic but differential metastatic abilities.

**Variations in drug resistance of HT29 clones are connected to different phases of the cell cycle**

Cellular responses to 5FU treatments using $1/2$IC$_{50}$ and $2 \times$IC$_{50}$ 5FU concentrations for 5F7 cells (1 and 4 μmol/L) and 5F31 cells (10 and 40 μmol/L) were examined by the flow cytometry cell-cycle analysis (Supplementary Table S2).
Treatment of HT29 parental cells by high 5FU concentrations induced typical cell accumulation in the S-phase as previously shown (26). Similarly, 5F7 cells accumulated at the S-phase in response to graded high 5FU concentrations, as well as at the G2–M-phase. In contrast, subsequent exposure of 5F31 cells to 5FU induced a selective and concentration-dependent accumulation of cells at the G0-phase of the cell cycle, from 2.9% in control cells to 12.3 and 50.4% in 5F31 cells exposed to 10 and 40 μmol/L 5FU, respectively. In addition, the percentage of quiescent cells in the 5FU-treated 5F31 cell line gradually increased over time, from 8.0% (1 hour) to 14.7% (4 hours), 32.5% (72 hours), and 50.4% (96 hours). Because nondividing cells are not targeted by chemotherapeutic drugs targeting proliferating cells, our data suggest that 5F31 cells evade 5FU treatment through entry in a quiescent G0 state. Importantly, a high percentage of quiescence was observed in other drug-resistant colon cancer cell subpopulations, i.e., RKO-OXA (8.5%), -FU (9.8%) versus control RKO cells (3.1%), and HCT-116-OXA (11.1%), -FU (21.8%) versus control HCT-116 cells (4.7%). As these 5FU- and OXA-resistant cells are enriched in stem cell markers (our data and ref. 12), we concluded that the entry into quiescence is a more general mechanism regulating colon cancer stem cell survival and chemoresistance.

**5FU resistance of 5F7 and 5F31 HT-29 clones was connected to differential activation of Chk-2 and c-Yes kinases**

Control- and subsequently 5FU-treated 5F7 and 5F31 resistant cells were analyzed by phosho-kinase array and Western blotting (Fig. 3A and B). Treatment of 5F7 cells with 5FU primarily induced checkpoint kinase 2 (Chk-2) phosphorylation at the threonine 68 residue. As shown previously, Chk-2 is activated by the ataxia telangiectasia mutated (ATM)-mediated responses to DNA damage (27). Activation of this DNA damage-sensing pathway prevents progression through the cell cycle and recruits the DNA repair machinery. Our result showing accumulation of 5F7 cells in the S- and G2–M-phase upon 5FU treatment is consistent with these observations. In contrast, the level of phosphorylated Chk-2 remained unchanged in 5FU-treated 5F31 cells (Fig. 3A and B). Instead, they had higher levels of phosphorylated c-Yes. This finding was confirmed by Western blotting of c-Yes in antiphosphotyrosine precipitates (Fig. 3C). The c-Yes phosphorylation level was increased by 3.8-fold upon 5FU exposure. c-Yes phosphorylation was further confirmed by ELISA assay (Fig. 3D). Control- and 5FU-treated 5F31 cells showed higher c-Yes protein levels than the parental HT29 or 5F7 cells (4.5- and 4.9-fold, respectively, \( P < 0.01 \); Fig. 3B). Also, they expressed 7.7-fold higher levels of YES1 transcripts, whereas 5F7 cells showed only 3-fold higher than parental HT29 cells (Fig. 3E).

**YES1 silencing in 5F31 cells induced quiescence and restricted YAP nuclear accumulation**

To investigate whether c-Yes is involved in the 5FU-induced cellular quiescence of 5F31, we silenced YES1 in these cells using shRNA. Two silenced sh1 and sh2 5F31 populations showing differential levels of YES1 silencing were obtained, as compared with control transfected cells...
with the scrambled shRNA sequence (Scr; Fig. 4A). Consistent with recent data published on YES1–silenced HT29 cells (24), YES1 silencing in 5F31 cells was associated with increased β-catenin levels (Fig. 4A). Most interestingly, YES1 silencing levels were directly correlated with the emergence of G0 quiescent 5F31 cells (Fig. 4B), as observed in sh2 (50% silencing, 10% in G0 state) and sh1 cells (86% silencing, 29% cells in G0 state) as compared with Scr cells (4.6% in G0 state). Next, we compared the proliferation capacity of 5FU-treated sh1 cells versus control Scr cells after 5FU treatment (40 μmol/L, 5 days) and subsequent drug withdrawal. The population doubling time of silenced sh1 cells was 57 hours, whereas the control Scr cells divided every 36 hours. These data demonstrate that depletion of c-Yes arrests 5F31 cells in the G0-phase of the cell cycle and suggest that c-Yes is required for the reentry of 5F31 quiescent cells into the proliferative state. As YAP is a growth-promoting transcriptional coactivator that promotes stem cell self-renewal and progenitors expansion (28), we analyzed the consequences of YES1 silencing on its fate. As shown in Fig. 4A, total YAP levels remained unchanged upon YES1 silencing, however, the YAP nuclear/cytoplasmic ratio decreased in sh1 cells as compared with control Scr cells (39%–61% vs. 48%–52%; Fig. 4C), implying that c-Yes controls levels of nuclear YAP.

The nuclear accumulation of YAP was restricted in 5FU-treated quiescent 5F31 cells
Because the majority of 5F31 cells submitted to high 5FU concentration enter quiescence, we examined the effect of

Figure 3. 5FU-resistant 5F7 and 5F31 clones selectively activate different signaling pathways following subsequent exposure to 5FU. A, total cell lysates from control- and 5FU-treated 5F7 and 5F31 cells (4 days) were analyzed using phospho-kinase arrays. Chk-2 serine/threonine kinase, c-Yes tyrosine kinase (arrows). Other kinases explored in these phospho-antibody arrays are involved in cancer cell proliferation and cell-cycle controls, survival, adhesion, and transcription (see the phospho-array coordinates in Materials and Methods). F17, F18, G5, G6: negative controls; A1, A2, A17, A18, G1, G2: positive controls. B, Western blots of the total cell lysates using specific antibodies against P-Chk-2 (T68), Chk-2, and c-Yes. Actin was used as loading control. The data are representative of two independent experiments. C, analysis of c-Yes phosphorylation by immunoprecipitation using the antiphosphotyrosine antibody followed by Western blotting with the anti-c-Yes antibody. D, analysis of c-Yes phosphorylation using the phospho-c-Yes (Y426) ELISA assay. Error bars, mean ± SD for three replicates. E, analysis of c-Yes mRNA levels in 5F7 and 5F31 cells in comparison with HT29 cells.
5FU treatment upon the nuclear distribution of YAP. As shown in Fig. 4D and E, the nuclear pool of YAP decreased markedly upon 5FU exposure in 5F31 cells (27% vs. 55%), whereas total YAP level was not significantly altered. Our data support a possible link between nuclear YAP depletion and cellular quiescence. As stated above, c-Yes becomes phosphorylated in 5F31 cells reexposed to 5FU. A confocal microscopy analysis showed that 5FU exposure induced the recruitment of cytoplasmic c-Yes to the cell membrane (Fig. 4F). This shift in the c-Yes subcellular distribution is consistent with its activation because the c-Yes kinase inhibitor SU6656 prevents c-Yes interaction with integral membrane proteins (29, 30). Thus, we hypothesized that 5FU treatment induces c-Yes phosphorylation and dissociates the c-Yes/YAP complex in 5F31 cells and that the released c-Yes is targeted to the plasma membrane. To test this hypothesis, YAP coimmunoprecipitation experiments were performed using the anti-YAP antibody. Interestingly, c-Yes was found in YAP immunoprecipitates prepared from control 5F31 cells but not from 5FU-treated 5F31 cells, showing that the c-Yes/YAP complex was lost upon 5FU exposure (Fig. 4G).

**YES1 and YAP expression levels were increased by chemotherapy in human colon liver metastases and are correlated with colon cancer relapse**

To examine the clinical significance of our data, we analyzed the level of YES1 and YAP transcripts in a cohort of 49 patients with colon cancer who underwent surgical resection of liver metastases. Twenty-eight patients received the chemotherapeutic regimens prior surgery. As shown in Fig. 5A, quantification of YES1 and YAP transcript expressions by quantitative real-time PCR showed enhanced YES1 (4.2-fold) and YAP (1.7-fold) transcript levels in patients treated by chemotherapy ($P = 0.035$ and $0.026$, respectively; Fig. 5A). Consistently, increased c-Yes protein levels were observed in 5FU- and OXA-resistant HT29 and RKO colon cancer cells, respectively. Of note, YAP protein levels were strongly elevated in OXA-resistant HT29 and RKO cells (Supplementary Fig. S2). This indicates that chemotherapy may preselect for quiescent colon CSCs harboring a deregulation of the c-Yes/YAP axis. Most importantly, YES1 expression negatively correlated with the OS (threshold of 2.25, $P = 0.0231$) and DFS (threshold of 1.35, $P = 0.0433$; Fig. 5B). Interestingly, YAP expression also correlated with shorter OS (threshold of 2.62, $P = 0.025$) and DFS (threshold of 2.75, $P = 0.0088$). The neoadjuvant chemotherapy treatment is administered when liver metastases are initially unresectable or marginally resectable ($\geq 5$ bilateral nodules). Thus, the treated patients have generally a more severe disease. As expected, in the studied population there was no difference in OS and DFS between the groups of treated and nontreated patients because of the advantages provided by neoadjuvant chemotherapy. In the nontreated group, there was no correlation between YES1 and YAP transcript levels and the clinical outcomes (DFS and OS). In the group of treated patients, YES1 overexpression was negatively correlated with OS, with statistical significance ($P = 0.022$) and with near significance with DFS ($P = 0.071$). About YAP transcript, a trend was observed for both OS and DFS ($P = 0.092$ and $P = 0.1$, respectively). Thus, the subgroup of treated patients with liver metastases expressing high YES1 transcript levels is associated with poorer
outcomes. These clinical data are consistent with our experimental study showing a direct correlation between c-Yes expression and the quiescence of chemoresistant HT29 colon cancer cells treated with 5FU. Altogether, these results show that a poorer clinical outcome segregates with the increased YES1 and YAP transcript levels and concerns patients who received 5FU-based neoadjuvant chemotherapy.

Discussion

Currently, treatment of synchronous or metachronous colorectal liver metastases requires neoadjuvant chemotherapy before hepatic surgery. Chemotherapeutic regimens consisting of 5FU and folinic acid combined with oxaliplatin (Folfox), irinotecan (Folfiri), or irinotecan and bevacizumab (Folfiri–bevacizumab) are scheduled every 15 days during 3 months (1–3). Drugs are withdrawn 1 month before surgery. Although temporarily efficient, this treatment rarely cures cancer and disease relapses from the drug-resistant cells. To investigate chemoresistance mechanisms occurring upon sequential chemotherapy regimens, 5FU-resistant clones propagated in drug-free medium were reexposed to 5FU. We show that 5FU-resistant cells could use different strategies to survive rechallenge with 5FU. On the one hand, 5F7 cells that show a weak chemoresistant potential respond to the drug by activating a typical ATM/Chk-2 pathway that prevents cell-cycle progression until DNA repair is completed (27). On the other hand, 5F31 cells although derived from the same parental chemoresistant subpopulation resist high 5FU concentrations by entering a protective quiescent state, reversible upon drug withdrawal. The escape into quiescence may recapitulate clinical observations linked to tumor dormancy and cancer relapse following chemotherapy. This is an important observation that should be investigated further to determine therapeutic regimens targeting also quiescent CSCs. Interestingly, both 5FU- and OXA-resistant cells expressed colon CSC markers, implying that 5FU-based chemotherapy fails to eradicate quiescent CSCs. The variability in the expression pattern of several stem cell markers identified in 5FU-resistant 5F7 and 5F31 cells suggests that the resistant clones emerge from different CSC subtypes. This notion reflects, again, clinical observations that a tumor may contain heterogeneous populations of CSCs (31). Almost an exclusive expression of CSC markers CXC4 by 5F7 cells and ALDH1A3 by 5F31 cells might be predictive of the metastatic potential (32, 33). This is consistent with our previous finding that CXC4 overexpression stimulates metastatic spreading of 5F7 subcutaneous xenografts to the lungs (19). Using orthotopic murine xenografts, we found here that 5F31 cells expressing high ALDH1A3 levels disseminated to more metastatic sites than 5F7 cells overexpressing CXC4, emphasizing a strong link between the tumorigenic and metastatic potential and the CSC phenotype. We argue that such a mechanism responsible for such a link may involve c-Yes/YAP signaling pathways.

The YES1 gene is amplified in 5FU-resistant cancer cell lines due to its chromosomal localization close to the thymidylate synthase gene, a 5FU target (19, 34, 35). 5F31 cells, characterized by the high chemoresistance potential and the ability to enter quiescence upon 5FU exposure, displayed a particularly elevated level of c-Yes. We have therefore explored the possible implication of c-Yes and YAP in driving 5FU resistance and quiescence of 5F31 cells. YAP stimulates the proliferation of mouse intestinal stem cells and maintains their pluripotency (28, 36, 37). YAP is kept under an inactive state in differentiated cells through the Hippo pathway that phosphorylates YAP at the 127 serine residue and induces its cytoplasmic sequestration and degradation (38). Here, we found that the entry of 5FU-resistant 5F31 cells in quiescence upon a 5FU rechallenge induced the repression of nuclear YAP levels, suggesting that this repression contributes to cancer cell dormancy. In agreement, we observed that the 5FU-induced c-Yes phosphorylation and its translocation to

Figure 5. Correlations between YES1 and YAP expression and patient with colon cancer survival. A, comparison of YES1 and YAP transcript levels in colon liver metastases resected from patients who have received or not neoadjuvant chemotherapy. B, Kaplan–Meier survival analysis of patients with colon cancer according to the expression of YES1 and YAP transcripts.
the plasma membrane are associated with a loss of the c-Yes/YAP complex and depletion of nuclear YAP. In addition, we have shown that YES1 silencing induced the accumulation of 5F31 cells at the G0-phase of the cell cycle as well as depletion of nuclear YAP levels. Consequently, the c-Yes–dependent regulation of YAP activity in 5FU-resistant 5F31 cells can be considered as a potential mechanism involved in the control of the quiescence/proliferation balance of these cells. Taken together, our data support the notion that the c-Yes/YAP signaling pathway contributes to 5FU resistance through the combined acquisition of both cellular quiescence and stem cell–like phenotype. In support of this conclusion, recent data indicate that the Yes/YAP-TEA domain2 (TEAD2) signaling cascade downstream of the leukemia inhibiting factor (LIF) is necessary for the YAP nuclear translocation and self-renewal of mouse embryonic stem cells (39). YAP growth-promoting activity is mediated by the interaction between its N-terminal domain and the TEAD family of DNA-binding proteins (40). Oncogenic YAP is overexpressed in most colon tumors and promotes the proliferation of colon cancer cell lines (37, 41, 42). Consistently, YAP was recently incriminated in the metastatic potential of breast cancer and melanoma cells through its TEAD interaction domain (43). In cancer-associated fibroblasts, YAP promotes matrix stiffening, cancer cell invasion, and angiogenesis (44). YAP and its paralog transcriptional co-activator with PDZ-binding motif (TAZ) are two downstream targets of the Hippo pathway (45). Accordingly, several survival effectors are known to act through G-protein receptors and tyrosine phosphorylations, including the src family kinases. Further studies are needed to determine the possible connections of the c-Yes/YAP axis with the core components of the Hippo pathway. In our study, it is also plausible that the oxidative stress response induced by 5FU is implicated in c-Yes activation (46). Finally, the c-Yes/cdk42 pathway was shown to repress the transcription factor nuclear factor of activated T cells member (LIF) is necessary for the YAP nuclear translocation and self-renewal of mouse embryonic stem cells (39). YAP growth-promoting activity is mediated by the interaction between its N-terminal domain and the TEAD family of DNA-binding proteins (40). Oncogenic YAP is overexpressed in most colon tumors and promotes the proliferation of colon cancer cell lines (37, 41, 42). Consistently, YAP was recently incriminated in the metastatic potential of breast cancer and melanoma cells through its TEAD interaction domain (43). In cancer-associated fibroblasts, YAP promotes matrix stiffening, cancer cell invasion, and angiogenesis (44). YAP and its paralog transcriptional co-activator with PDZ-binding motif (TAZ) are two downstream targets of the Hippo pathway (45). Accordingly, several survival effectors are known to act through G-protein receptors and tyrosine phosphorylations, including the src family kinases. Further studies are needed to determine the possible connections of the c-Yes/YAP axis with the core components of the Hippo pathway. In our study, it is also plausible that the oxidative stress response induced by 5FU is implicated in c-Yes activation (46). Finally, the c-Yes/cdk42 pathway was shown to repress the transcription factor nuclear factor of activated T cells member (NFAT1) by inducing the formation of a cytoplasmic complex with casin kinase 1α (CK1α), and Lats-induced YAP phosphorylation primes YAP for CK1-induced phosphorylation (47, 48).

We have shown that 5FU-based neoadjuvant chemotherapy of patients with metastatic colon cancer increased levels of both YES1 and YAP transcripts in liver metastases, thus providing one explanation for the higher aggressiveness and metastatic potential of relapsed drug-resistant tumors. Furthermore, we found that YES1 and YAP transcript levels were correlated with the reduced DHS and OS, supporting the potential implication of the c-Yes/YAP signaling cascade in tumor relapse. Our data demonstrate that 5FU chemotherapy in the H129 colon cancer cell line preselects two distinct drug-resistant clonal populations each enriched in cells expressing their specific set of stem cell markers and different self-renewing potential, suggesting that in clinic, 5FU chemoresistant cancer cells may require clone-selective and adapted therapeutic strategies to be eradicated. Our data point to the c-Yes/YAP axis as signaling elements involved in the acquisition and maintenance of cellular quiescence linked to 5FU resistance in colon cancer. Taken together with our clinical study, we demonstrated here that the c-Yes/YAP signaling pathway should be considered as a potential therapeutic target to kill drug-resistant quiescent cancer cells. Alternatively, the identification of new compounds targeting CSCs in a quiescent state might prove to be beneficial for patients with cancer with colon liver metastases and detectable alterations of the c-Yes/YAP signaling axis.

Disclosure of Potential Conflicts of Interest

I.M. Ellis is a consultant and advisory board member of Genentech/Roche, Lilly/Imclone, and Amgen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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