Deregulation of the PP2A Inhibitor SET Shows Promising Therapeutic Implications and Determines Poor Clinical Outcome in Patients with Metastatic Colorectal Cancer

Ion Cristóbal, Raúl Rincón, Rebeca Manso, Cristina Caramés, Sandra Zazo, Juan Madoz-Gúrpide, Federico Rojo, and Jesús García-Foncillas

Abstract

Purpose: SET is an endogenous PP2A inhibitor that might represent a novel molecular target for antitumor therapy. The aim of this study was to evaluate the molecular effects of SET deregulation and its potential clinical significance in metastatic colorectal cancer (mCRC).

Experimental Design: We studied the biologic effects of SET on cell growth, colonsphere formation, caspase activity, PP2A activation status, and sensitivity to oxaliplatin and FTY720 treatments. Moreover, we analyzed SET expression by immunostaining in 242 patients with mCRC.

Results: SET deregulation promotes cell growth and colonsphere formation and inhibits PP2A, thereby impairing its antitumor effects. Moreover, SET reduces sensitivity to oxaliplatin in colorectal cancer cell lines, which is restored after FTY720 treatment. SET overexpression was detected in 24.8% (60 of 242) of patients with mCRC and determined significantly shorter overall (8.6 vs. 27 months; \( P < 0.001 \)) and progression-free survival (7.1 vs. 13.7 months; \( P < 0.001 \)), and poor response to oxaliplatin-based chemotherapy (\( P = 0.004 \)). Interestingly, its prognostic value was particularly evident in patients younger than 70 years and in those harboring KRAS mutations.

Conclusions: SET overexpression is a frequent event in mCRC that plays a potential oncogenic role associated with worse outcome and resistance to oxaliplatin. Moreover, this alteration defines a subgroup of patients who could benefit from therapies containing PP2A activators such as FTY720. Clin Cancer Res; 21(2); 1–10. © 2014 AACR.

Introduction

Colorectal cancer is the gastrointestinal cancer with the highest incidence, and the stage of the disease at the time of diagnosis is the most critical factor for patient outcomes (1). Moreover, progression to metastatic disease affects a large number of cases and it represents the subgroup of patients with the worst prognosis. Despite progressive clinical advances that have been carried out in the last decade to decrease or prevent metastasis, patient outcomes are still very poor (2). It is therefore a clinical challenge to develop alternative therapeutic strategies to improve the survival of these patients.

The protein SET is a PP2A inhibitor (3) that participates in the regulation of a wide variety of molecular processes (4–10). Of importance, SET plays an oncogenic role modulating signaling pathways with high relevance in human cancer (11). For instance, it has been reported that SET activates the transcription factor AP-1, deregulates AKT signaling, inhibits the DNase activity of the tumor-suppressor NM23-H1, or negatively regulates p53 acetylation, thus repressing its activity (12–16). Moreover, SET is overexpressed in several neoplasms (17–21) and it has been proposed as a novel molecular target for anticancer therapy (20–23). The transcription factor EVI-1 and the miR199b have been described to regulate SET expression in acute myeloid leukemia and choriocarcinoma (18, 24).

As indicated above, SET strongly inhibits PP2A, a tumor suppressor that regulates many signaling pathways, and whose loss of function is involved in cell transformation (11, 25–27). Different molecular strategies to inhibit PP2A have been described in transformed cells, including the overexpression of endogenous inhibitors such as SET. In fact, the inactivation of PP2A in human cancer seems to be a very recurrent and relevant event in human cancer and the potential therapeutic benefits of its pharmacologic activation has been investigated in the last years with very promising results (28–30). Interestingly, it has been reported that the antitumor effects showed by the PP2A activator FTY720 are mediated by SET in lung cancer (31). Moreover, our group has recently described that PP2A inhibition is a common event in colorectal cancer and that its restoration using FTY70 or forskolin induces promising antitumor effects (32, 33).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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In this report, we further investigated the potential relevance of SET in colorectal cancer. Western blot analysis confirmed SET overexpression in colorectal cancer cell lines. Moreover, we observed that SET promotes cell growth, colonosphere formation, and restores the reduced cell viability induced after PP2A overexpression. Furthermore, we identified that SET induces a resistance in colorectal cancer cells to oxaliplatin that is impaired after FTY720 treatment. To determine its clinical relevance, we quantified SET in a series of 242 patients with metastatic colorectal cancer (mCRC), observing that SET overexpression is a recurrent molecular event that predicts shorter overall survival (OS), disease-free survival (DFS), and response to oxaliplatin-based chemotherapy.

Translational Relevance
The SET oncogene represents a novel potential therapeutic target in human leukemia, but its potential relevance in solid tumors as colorectal cancer remains mostly unknown. Here, we show that SET deregulation is a recurrent alteration that contributes to inactivate PP2A and enhances tumor malignant properties of colorectal cancer cells. Interestingly, together with its prognostic value, SET is predictive of response to oxaliplatin-based chemotherapy backbone, which could have an important relevance in the current clinical practice. Thus, SET could differentiate a subgroup of patients who could benefit by the future incorporation of PP2A-activating drugs in anticancer protocols, alone or in combination with standard chemotherapy.

Materials and Methods
Cell cultures and transfection
The human colorectal cancer cell lines SW480 (ATCC CCL-228), WiDr (ATCC CCL-218), DLD-1 (ATCC CCL-221), HT-29 (ATCC HTB-38), SW620 (ATCC CCL-227), HCT-116 (ATCC CCL-247) and LS513 (ATCC CRL-2134) were purchased from the American Type Culture Collection (ATCC). Of note, the SW480 and SW620 cell lines are derived from the same patient. Authenticity of the ATCC catalogue was previously described (32). Primary colorectal tissues were surgical resection specimens obtained from Fundacion Jimenez Diaz Biobank (BFJD, Madrid, Spain). The study comprised fresh-frozen samples of 14 patients with colorectal cancer with paired normal mucosa and tumor obtained from surgical specimens, consecutive FFPE tumor samples of 145 patients with colorectal cancer without mCRC, and 242 patients with metastatic disease who were retrospectively selected from 2001 to 2012 according to the following criteria: adenocarcinoma, operable disease, no neoadjuvant therapy, enough available tissue, clinical follow-up data, and metastatic disease. TNM (tumor–node–metastases) staging was classified using the 7th American Joint Committee on Cancer (AJCC) staging system for colorectal cancer. Clinical data were collected from medical clinical records by oncologists (J. García-Foncillas and C. Caramés). KRAS mutational status was determined by the Cobas KRAS Mutation Test Kit (Roche Molecular Diagnostics) following the manufacturer’s procedures. Tissue microarrays (TMA) were constructed. Representative areas of each tumor were carefully selected and three tissue cores (1-mm diameter) were obtained using a TMA workstation (T1000 Chemicon). Samples were taken anonymously. The ethical committee and Institutional Review Board approved the project.

Western blot analysis
Protein extracts were isolated using TRIzol Reagent (Invitrogen) following the manufacturer’s indications, clarified (12,000 × g, 15 minutes, 4°C), denatured, and subjected to SDS-PAGE and Western blot analysis. Antibodies used were rabbit polyclonal anti-SET (Abcam) and mouse monoclonal anti-β-actin (Sigma). Proteins were detected with the appropriate secondary antibodies conjugated to alkaline phosphatase (Sigma) by chemiluminescence using Tropix CSPD and Tropix Nitro Block II (Applied Biosystems).

Cell viability assay
Cell proliferation was measured in triplicate wells by the MTS assay in 96-well plates using the CellTiter 96 AQuesous One Solution Cell Proliferation Assay (Promega), following the manufacturer’s indications. IC50 was calculated using the SigmaPlot 11.0 bioinformatic tool.

PP2A phosphatase activity assays
PP2A assays were performed with cell lysates (50μg) using a PP2A immunoprecipitation phosphatase assay kit (Millipore) as previously described (28).

Analysis of caspase activation
Quantification of caspase-3/7 activities was carried out using the caspase Glo-3/7 assay kit (Promega Corp.). Briefly, 5 × 103 cells were plated in a white-walled 96-well plate, and the Z-DEVDFMK reagent, the luminogenic caspase-3/7 substrate containing a tetrapeptide Asp–Glu–Val–Asp, was added with a 1:1 ratio of reagent to sample. After 90 minutes at room temperature, the substrate cleavage by activated caspase-3 and -7, and the intensity of a luminescent signal was measured by a FLUOstar OPTIMA luminometer (BMG Labtech). Differences in caspase-3/7 activity are expressed as fold-change in luminescence.

Cell-cycle analysis
Cells were harvested, pelleted, and fixed in 70% ethanol on ice for 1 hour. After two washes in PBS, cell were treated with RNaseA for 30 minutes at 37°C and stained with propidium iodide (25 μg/mL; BD Pharmigen) for 10 minutes at room temperature in dark conditions before the flow analysis.

Colonospheres
For the generation of colonospheres, 10,000 cells were plated in 6-well ultra-low attachment plates (Corning). Colorectal cancer
cell were grown in serum-free medium DMEM/F12+GlutMAX-I (Gibco) containing 1% N2 (Gibco), 2% B27 (Gibco), 20 ng/mL human FGF (Sigma), and 50 ng/mL EGF (Sigma). After 7 days, plates were analyzed for colonosphere formation. For quantification of the number of cells per colonosphere, colonospheres were collected and dissociated with trypsin to give single-cell suspensions. Viable cells were counted in a Neubauer chamber using a Trypan Blue exclusion test.

**Immunohistochemistry**

Tissue sections (3 μm) were placed on plus charged glass slides. After deparaffinization in xylene and graded alcohols, heat antigen retrieval was performed in pH9 EDTA-based buffer (Dako). Endogenous peroxidase was blocked by 0.03% hydrogen peroxide for 5 minutes. Slides were incubated with same primary antibody against SET as described for 60 minutes at room temperature, followed of appropriate anti-lg horseradish peroxidase–conjugated polymer (Flex+; Dako). Sections were visualized with 3,3′-diaminobenzidine as a chromogen. All stainings were performed in a Dako Autostainer. Sections incubated with normal nonimmunized rabbit immunoglobulins were used as negative controls. As positive control, a section of colorectal tumor with immunoglobulins were used as negative controls. The histoscore was calculated by estimation of the percentage of tumor cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The following formula was used:

\[
\text{histoscore} = (\text{low}\%) \times 1 + (\text{medium}\%) \times 2 + (\text{high}\%) \times 3
\]

and the results ranged from 0 to 300.

**Statistical analysis**

Statistical analyses were performed using SPSS 20 for windows (SPSS Inc.). OS was defined as the time from the date of surgery to the date of death from any cause or last follow-up. DFS was defined as the time from surgery until any primary, regional, or distant recurrence, appearance of a secondary tumor or death. Clinical benefit to oxaliplatin-based chemotherapy was defined as any response or prolonged stable disease (>12 weeks). The Kaplan–Meier method and survival comparisons were done with the log-rank test if proportional hazard assumption was fulfilled and Breslow otherwise. The Cox proportional hazards model was adjusted taking into consideration significant parameters in univariate analysis. A P value less than 0.05 was considered statistically significant. Receiver operating curve (ROC) was used to determine the optimal cutoff point based on progression endpoint for SET expression as previously described (35). This work was carried out in accordance with Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines (36).

**Results**

**SET is overexpressed and affects cell growth and colonosphere formation in colorectal cancer cells**

We first confirmed SET deregulation by Western blot analysis in a set of primary colorectal cancer patients with paired normal mucosa and tumor samples, observing SET deregulated in 6 out of the 14 cases analyzed (Supplementary Fig. S1A). To investigate its biologic relevance as a potential oncogene in colorectal cancer, we assessed the effects of SET silencing on cell growth using two different specific siRNAs. Interestingly, we observed a decreased proliferation in SW480, HT-29, and LS513 cells transfected with any of the siRNAs against SET in comparison with cells transfected with a negative control siRNA (Fig. 1A). Of note, the effect in LS513 cells was less significant probably due to their lower SET expression levels. Similar results were obtained with the DLD-1, SW620, and HCT-116 cell lines (Supplementary Fig. S1B). In concordance with these results, SET silencing in SW480, HT-29, and LS513 cells led to a decreased colonosphere formation ability in both number (Fig. 1B) and size of colonospheres formed (Fig. 1C), indicating that SET is involved in colonosphere formation and self-renewal of colorectal cells. These observations were confirmed in DLD-1, SW620, and HCT-116 cells (Supplementary Fig. S1C and S1D). Validation of SET silencing was performed by both real-time PCR and Western blot analysis (Supplementary Fig. S1E). Altogether, these results would indicate that SET overexpression is a common event that plays an oncogenic role in colorectal cancer.

**SET overexpression leads to PP2A inhibition**

Because SET has been reported as an endogenous PP2A inhibitor, we analyzed whether SET deregulation can alter the effects of the tumor-suppressor PP2A in colorectal cancer cells. Thus, we first confirmed by PP2A assays that ectopic expression of SET and PP2A leads to changes in the PP2A activity. As expected, we observed a PP2A inhibition after SET transfection, whereas SW480, HT-29, and LS513 cells transfected with PP2A showed increased PP2A activity levels. Moreover, overexpression of SET counteracted PP2A activation in cells ectopically expressing PP2A, and those cells showed PP2A activity levels similar to the corresponding controls (Fig. 2A). These observations were confirmed performing experiments with SET silencing (Supplementary Fig. S2A).

**SET restores cell viability after PP2A overexpression**

To analyze whether SET deregulation can alter the action of PP2A on cell growth, we next studied the effect of SET overexpression after PP2A activation. We observed an increased proliferation in SW480, HT-29, and LS513 cells transfected with SET in comparison with cells transfected with an empty vector (Fig. 2B). On the contrary, the activation of PP2A induced by its overexpression resulted in a decreased cell growth. Interestingly, we also observed that SET restored cell proliferation in SW480 cells ectopically expressing PP2A. Similar results were obtained in the HT-29 and LS513 cell lines (Fig. 2B). These observations were confirmed carrying out experiments with SET silencing (Supplementary Fig. S2B).

To further investigate the biologic effects of SET deregulation in colorectal cancer, we assessed apoptosis in SW480, HT-29, and LS513 cells ectopically expressing SET, PP2A, or both SET and PP2A. In concordance with its ability to impair cell proliferation, PP2A showed a caspase-dependent proapoptotic effect that was markedly reduced after SET overexpression (Supplementary Fig. S2C). Validation of SET overexpression was performed by both real-time PCR and...
Western blot analysis (Supplementary Fig. S2D). Thus, these results suggest that SET overexpression promotes cell proliferation and inhibits the antitumor effects of PP2A in colorectal cancer cells.

SET induces a decreased oxaliplatin sensitivity that is restored by FTY720 treatment

To assess a potential therapeutic role of SET affecting sensitivity of colorectal cancer cells to standard induction chemotherapy drugs used in this disease, we treated SW480 cells with oxaliplatin, alone or after transfection with a specific siRNA against SET. Interestingly, we observed that SET silencing enhanced sensitivity of SW480 cells to oxaliplatin treatment. These results were confirmed in the HT-29 and LS513 cell lines (Fig. 3A). When we examined 5-FU alone and in combination with oxaliplatin, higher sensitivity was also found after SET silencing (Supplementary Fig. S3A). Moreover, higher apoptosis was observed in SW480, HT-29, and LS513 cells treated with 5-FU combined with oxaliplatin when SET was silenced (Supplementary Fig. S3B). We also evaluated the sensitivity to oxaliplatin and 5-FU, observing a higher sensitivity to these drugs after SET silencing (Supplementary Fig. S3C). In concordance with these results, SW480, HT-29, and LS513 cells ectopically expressing SET showed an increased resistance to oxaliplatin. Interestingly, this effect was impaired when these cells were treated with oxaliplatin in combination with FTY720, a drug which showed marked antitumor properties (Fig. 3B). Effects of FTY720 alone or in combination with 5-FU or 5-FU and oxaliplatin were also evaluated observing similar results (Supplementary Fig. S4A and S4B).

To further investigate the antitumor effects induced by the PP2A activation after FTY720 treatment or SET silencing, we carried out some cell-cycle analyses in SW480 and HT-29 cells, observing a reduction in S-phase together with an accumulation in G0–G1. As expected, these results were more evident after FTY720 treatment than after SET silencing (Supplementary Fig. S4C) probably because FTY720 acts via both SET and CIP2A inactivation (31, 32). Moreover, we observed lower sensitivity of colorectal cancer cell lines to oxaliplatin and 5-FU after ectopic expression of SET (Supplementary Fig. S4D). Therefore, these observations suggest that SET is involved in modulating sensitivity of colorectal cancer cells to oxaliplatin treatment, and that FTY720 impairs the SET-induced resistance to oxaliplatin.

Prevalence of SET overexpression in mCRC and its association with molecular and clinical parameters

To study the prevalence and clinical significance of SET overexpression, we quantified the expression of SET by immunohistochemistry in a cohort of 242 patients with mCRC. Patient characteristics are presented in Supplementary Table S1 and immunohistochemical detection of SET is shown in Supplementary Fig. S5. SET was overexpressed in 60 of 242 cases (24.8%). We found this alteration associated with worse...
Eastern Cooperative Oncology Group (ECOG) performance status (38.1% vs. 20%; $P = 0.013$), development of liver metastasis (29.3% vs. 15.4%; $P = 0.019$), and with the presence of synchronous metastasis at diagnosis (24.5% vs. 12.8%; $P = 0.018$). Moreover, the prevalence of SET overexpression was markedly higher in patients with colon primary tumors than in those with rectal primary tumors (32.1% vs. 11.8%; $P = 0.001$). Association between SET overexpression and molecular and clinical parameters are included in Table 1.

To further investigate the significance of SET deregulation in colorectal cancer, we next evaluated its expression in earlier stages of colorectal cancer, studying a cohort of 145 patients with colorectal cancer without metastatic disease. Interestingly, SET overexpression had a lower prevalence in this cohort (13.8%) compared with the mCRC cohort (24.8%). Moreover, we observed SET overexpression associated with those patients with rectal tumors although significance was not achieved ($P = 0.056$; Table 2).

**Clinical significance of SET overexpression in mCRC**

Clinical follow-up data were available for all the 242 patients with mCRC, 149 male and 93 female, with a median of age of 70 years (range, 29–93). The median OS of the global cohort was 21.9 months [95% confidence interval (CI), 17.2–26.5]. We found that the subgroup of patients with SET overexpressed showed a substantially shorter OS (median OS, 8.6 vs. 27 months; $P < 0.001$; Fig. 4A) and progression-free survival (PFS; median PFS, 7.1 vs. 13.7 months; $P < 0.001$; Fig. 4B). Whereas the prognostic impact of SET in OS was significant in both subgroups of patients younger (median OS, 10.2 vs. 34.6 months; $P < 0.001$) and older than 70 years (median OS, 6.5 vs. 18.1 months; $P = 0.015$), in PFS significance was only
achieved in the subgroup of younger patients (median PFS, 9.7 vs. 18.1 months; \(P < 0.001\); Supplementary Fig. S5C). In addition, the prognostic impact of SET overexpression was independent of the KRAS mutation status, and was associated with shorter OS and PFS in both the KRAS wild-type (median OS, 9.7 vs. 25.3 months, \(P = 0.009\); median PFS, 8.8 vs. 14.4 months, \(P = 0.031\)) and KRAS-mutated subgroups (median OS, 5.9 vs. 30.7 months, \(P < 0.001\); median PFS, 4.5 vs. 12.5 months, \(P = 0.002\); Supplementary Fig. S6A). Interestingly, we observed that SET overexpression was predictive of clinical benefit in those patients who received oxaliplatin-based chemotherapy (\(N = 101\); \(P = 0.004\); Supplementary Fig. S6B). Moreover, SET overexpression also determined worse OS (median OS, 17.4 vs. 34.6 months; \(P < 0.001\)) and PFS (median PFS, 11.3 vs. 14.5 months; \(P = 0.024\)) in this subgroup of cases (Fig. 4B). Of importance, multivariate analysis demonstrated that SET overexpression was an unfavorable independent factor associated with OS and PFS (Table 3) in mCRC. As indicated above, this study was performed in accordance with the REMARK guidelines.

**Discussion**

The SET oncogene has been reported to be upregulated in several types of cancer and it has been proposed as a candidate to develop novel molecular targeted therapies (21–23). Our previous results showed that PP2A inactivation is a common event in colorectal cancer and we identified SET deregulation as a possible contributing mechanism to inhibit PP2A in a set of 21 samples of patients with primary colorectal cancer (32). These results led us to hypothesize that SET could be playing an important oncogenic role in colorectal cancer. Although alterations affecting SET have been described in human cancer, its potential significance in colorectal cancer...
remains unexplored. In this report, we show that SET is deregulated in colorectal cancer and plays an oncogenic role promoting cell proliferation and colonosphere formation, impairing PP2A antitumor activities, and modulating sensitivity of colorectal cancer cells to oxaliplatin treatment. Furthermore, our in vitro and clinical data provide evidences that SET overexpression is a recurrent event (24.8%) that predicts adverse outcome and PFS in patients with mCRC (Fig. 4). In addition, we analyzed SET in a series of 145 patients with colorectal cancer without metastatic disease, observing a lower prevalence of SET overexpression in this cohort than in the metastatic cohort (13.8% vs 24.8%; Tables 1 and 2).

The high prevalence of this alteration suggests that SET overexpression is a key mechanism to inhibit PP2A in colorectal cancer cells, which could discriminate a subgroup of patients who might benefit from future therapies with PP2A activators. Furthermore, in concordance with the fact that SET silencing induced an increased sensitivity to oxaliplatin treatment in colorectal cancer cell lines (Fig. 3A), we observed that those patients without SET overexpression showed better response to oxaliplatin-based chemotherapy and longer OS and PFS (Fig. 4 and Supplementary Fig. S6B).

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**Table 1.** Association between SET and clinical and molecular parameters in 242 patients with mCRC

<table>
<thead>
<tr>
<th>SET</th>
<th>Cases (n)</th>
<th>SET+ (n, %)</th>
<th>SET- (n, %)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>145</td>
<td>125 (86.2)</td>
<td>20 (13.8)</td>
<td>0.233</td>
</tr>
<tr>
<td>Female</td>
<td>87</td>
<td>77 (88.6)</td>
<td>10 (11.4)</td>
<td></td>
</tr>
<tr>
<td>MSI</td>
<td>58</td>
<td>48 (82.3)</td>
<td>10 (17.7)</td>
<td></td>
</tr>
<tr>
<td>Age &gt;70</td>
<td>145</td>
<td>125 (86.2)</td>
<td>20 (13.8)</td>
<td>0.077</td>
</tr>
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<td>ECOG</td>
<td>145</td>
<td>125 (86.2)</td>
<td>20 (13.8)</td>
<td></td>
</tr>
<tr>
<td>Site of primary tumor</td>
<td>145</td>
<td>125 (86.2)</td>
<td>20 (13.8)</td>
<td>0.056</td>
</tr>
<tr>
<td>Colon</td>
<td>109</td>
<td>97 (89)</td>
<td>12 (11)</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>33</td>
<td>25 (75.8)</td>
<td>8 (24.2)</td>
<td></td>
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</tbody>
</table>

Significance of SET Overexpression in Colorectal Cancer

Our previous results showed SET deregulation in a short set of 21 patients with primary colorectal cancer but we did not analyze the molecular or clinical relevance of this finding. Therefore, we analyzed the potential significance of SET in a series of 242 cases with colorectal cancer stage IV, the subgroup of patients with colorectal cancer that represents the highest therapeutic challenge. In fact, the colorectal cancer stage IV is defined by the presence of metastatic disease and constitutes the subgroup of patients with the worst prognosis. Although the therapeutic advances implemented in the last years have affected the clinical outcome, unfortunately, their prognosis is still very poor. Therefore, it is necessary to develop alternative therapeutic strategies that improve the survival of these patients. Importantly, we observed that SET overexpression is a recurrent event (24.8%) that predicts adverse outcome and PFS in patients with mCRC (Fig. 4). In addition, we analyzed SET in a series of 145 patients with colorectal cancer without metastatic disease, observing a lower prevalence of SET overexpression in this cohort than in the metastatic cohort (13.8% vs 24.8%; Tables 1 and 2).

The high prevalence of this alteration suggests that SET overexpression is a key mechanism to inhibit PP2A in colorectal cancer cells, which could discriminate a subgroup of patients who might benefit from future therapies with PP2A activators. Furthermore, in concordance with the fact that SET silencing induced an increased sensitivity to oxaliplatin treatment in colorectal cancer cell lines (Fig. 3A), we observed that those patients without SET overexpression showed better response to oxaliplatin-based chemotherapy and longer OS and PFS (Fig. 4 and Supplementary Fig. S6B).

**Table 2.** Association between SET and clinical and molecular parameters in 145 colorectal cancer patients without metastatic disease

<table>
<thead>
<tr>
<th>SET</th>
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<th>SET low, n (%)</th>
<th>SET high, n (%)</th>
<th>P</th>
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<tr>
<td>Sex</td>
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<td>125 (86.2)</td>
<td>20 (13.8)</td>
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<tr>
<td>Male</td>
<td>87</td>
<td>77 (88.6)</td>
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<tr>
<td>Female</td>
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<td>125 (86.2)</td>
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<td>0.077</td>
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<tr>
<td>ECOG</td>
<td>145</td>
<td>125 (86.2)</td>
<td>20 (13.8)</td>
<td></td>
</tr>
<tr>
<td>Site of primary tumor</td>
<td>145</td>
<td>125 (86.2)</td>
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<tr>
<td>Colon</td>
<td>109</td>
<td>97 (89)</td>
<td>12 (11)</td>
<td></td>
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<tr>
<td>Rectum</td>
<td>33</td>
<td>25 (75.8)</td>
<td>8 (24.2)</td>
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</tbody>
</table>
Interestingly, the observation that the PP2A activator FTY720, a drug with a promising preclinical antitumor efficacy in several cancer models including colorectal cancer (32, 37) that acts blocking SET (31), is able to impair the resistance induced by SET (Fig. 3B) would suggest that those patients with SET overexpression could improve their outcomes with a future inclusion of an oxaliplatin-based treatment in combination with FTY720 in the clinical protocols. In addition, the fact that the prognostic impact of SET was more evident in the subgroup of patients younger than 70 years (Supplementary Fig. S5C) is very interesting because this subgroup includes those cases susceptible of a more aggressive therapy that could benefit from the treatment with PP2A activators (e.g., FTY720).

The KRAS-mutated status is a very high prevalent alteration in mCRC that determines resistance to the monoclonal antibody cetuximab. Interestingly, it has recently reported that FTY720 could resensitize colorectal cancer cells to cetuximab, indicating a potential therapeutic relevance for FTY720 in mCRC (38). Of importance, when we stratify our series by the KRAS mutation status, the SET prognostic impact was particularly strong in those patients with KRAS-mutated (P < 0.001; Supplementary Fig. S6A). Our results would suggest that the presence of SET overexpression could cooperate inducing a higher resensitization to cetuximab after FTY720 treatment.

In conclusion, we show that SET overexpression is a recurrent molecular event that plays an oncogenic role in colorectal cancer contributing to inactivate the tumor-suppressor PP2A. Moreover, SET overexpression predicts worse outcome and response to oxaliplatin-based therapy, and its prognostic value is particularly significant in patients younger than 70 years and in those harboring KRAS mutations. Of importance, our results indicate that SET could serve to define a subgroup of patients with mCRC with worse outcome that could benefit by the future incorporation of PP2A-activating drugs, such as FTY720, in anticancer protocols.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: I. Cristóbal, C. Caramés, F. Rojo, J. García-Foncillas
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Cristóbal, R. Rincón, R. Manso, S. Zazo, F. Rojo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Cristóbal, R. Rincón, R. Manso, C. Caramés, F. Rojo, J. García-Foncillas
Writing, review, and/or revision of the manuscript: I. Cristóbal, R. Rincón, J. Madoz-Gúrpide, F. Rojo, J. García-Foncillas
Study supervision: I. Cristóbal, J. Madoz-Gúrpide, F. Rojo
Table 3. Univariate and multivariate Cox analyses in the cohort of 242 patients with mCRC

<table>
<thead>
<tr>
<th>Univariate OS analysis</th>
<th>Significance</th>
<th>Multivariate OS Cox analysis</th>
<th>Significance</th>
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<td></td>
<td></td>
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<tr>
<td>&lt;70</td>
<td>1.000</td>
<td>-0.001</td>
<td>1.000</td>
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<td>≥70</td>
<td>1.803 (1.301–2.499)</td>
<td>1.272 (0.871–1.857)</td>
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<tr>
<td>Gender Male</td>
<td>1.000</td>
<td>0.475</td>
<td>-</td>
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<tr>
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<td>-</td>
</tr>
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<td>Synchronous No</td>
<td>1.000</td>
<td>1.318 (0.938–1.852)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td>1.381 (0.938–1.852)</td>
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<tr>
<td>ECOG 0–2</td>
<td>1.000</td>
<td>&lt;0.001</td>
<td>1.000</td>
</tr>
<tr>
<td>3–4</td>
<td>1.906 (1.573–2.311)</td>
<td>1.706 (1.371–2.123)</td>
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</tr>
<tr>
<td>Number of metastatic sites 1–2</td>
<td>0.084</td>
<td>-</td>
<td>0.001</td>
</tr>
<tr>
<td>&gt;2</td>
<td>1.000</td>
<td>1.242 (0.971–1.590)</td>
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<tr>
<td>SET No</td>
<td>1.000</td>
<td>-0.001</td>
<td>1.000</td>
</tr>
<tr>
<td>Yes</td>
<td>2.490 (1.770–3.504)</td>
<td>2.097 (1.451–3.029)</td>
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</tr>
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</table>

Univariate PFS analysis

<table>
<thead>
<tr>
<th>Univariate PFS analysis</th>
<th>Significance</th>
<th>Multivariate PFS analysis</th>
<th>Significance</th>
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<tr>
<td>Age, y</td>
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<tr>
<td>&lt;70</td>
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<td>0.002</td>
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<td>≥70</td>
<td>1.852 (1.243–2.760)</td>
<td>1.533 (0.972–2.417)</td>
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<tr>
<td>Gender Male</td>
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<td>0.595</td>
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<tr>
<td>Female</td>
<td>0.897 (0.601–1.339)</td>
<td>0.082</td>
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<tr>
<td>Synchronous No</td>
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<td>1.473 (0.952–2.281)</td>
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<tr>
<td>Yes</td>
<td></td>
<td>1.590 (1.050–2.391)</td>
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<td>ECOG 0–2</td>
<td>1.000</td>
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<td>1.000</td>
</tr>
<tr>
<td>3–4</td>
<td>1.556 (1.224–1.978)</td>
<td>1.369 (1.045–1.793)</td>
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<tr>
<td>Number of metastatic sites 1–2</td>
<td>0.029</td>
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<td>0.004</td>
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<tr>
<td>&gt;2</td>
<td>1.374 (1.033–1.827)</td>
<td>1.547 (1.154–2.073)</td>
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<tr>
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<td>1.000</td>
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<tr>
<td>Yes</td>
<td>2.323 (1.486–3.633)</td>
<td>2.192 (1.357–3.541)</td>
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</tbody>
</table>

Abbreviation: HR, hazard ratio. Statistically significant values are in bold.

Acknowledgments

The authors thank Cristina Chamizo for technical assistance.

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