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ORIGINAL ARTICLE – COLORECTAL CANCER

Significance of mTOR Signaling and Its Inhibitor Against Cancer Stem-Like Cells in Colorectal Cancer

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ABSTRACT

Purpose. To determine the role of the mammalian target of rapamycin (mTOR) signaling in sustaining cancer stem-like cells and its clinical values in colorectal cancer (CRC).

Methods. mTOR expression in CRC patients was analyzed by immunohistochemistry and survival analysis was used to confirm the clinical value of mTOR. Colorectal cell lines were treated by mTOR inhibitors rapamycin and PP242, and sphere formation assay and aldehyde dehydrogenase (ALDH) assay were utilized to determine the impact of mTOR inhibition in CRC stem-like cells, combined or not combined with chemotherapeutic drug (fluorouracil and oxaliplatin).

Results. mTOR expression was associated with outcomes of CRC patients and predicted poor prognosis in stage II CRC patients. mTOR signaling was activated in stem-like colorectal cancer cells, and mTOR inhibitors (rapamycin and PP242) decreased the capacity of sphere formation as well as ALDH activity. Furthermore, mTOR inhibitors also were demonstrated to suppress the stimulation of stem-like cells by chemotherapy.

Conclusions. mTOR shared predictive significance in stage II CRC patients' outcomes and played a vital role in the maintenance of colorectal cancer stem-like cells. mTOR inhibitors might hold the potential to become a therapeutic target against CRC stem cells.

Zerong Cai and Jia Ke have contributed equally to this study.

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Colorectal cancer (CRC) is the second-leading cause of cancer death in the United States.[1] Although comprehensive treatment modalities, including surgery, chemotherapy, and radiotherapy have been developed, the prognosis of CRC patients with advanced stages remains poor, mainly due to tumor metastasis, recurrence, and resistance to chemotherapy.[2] Growing evidence suggests that cancer stem cells (CSCs) might play an important role in tumor initiation, drug resistance, and metastasis in CRC and current therapies show poor effects against CSCs.[3–6] Identifying and developing target therapy against CSCs might provide promising strategies in the treatment of colon cancer.

The mammalian target of rapamycin (mTOR), a serine/threonine kinase existing in two functionally distinct complexes (mTORC1 and mTORC2), is commonly dysregulated in human cancers.[7] mTORC1 activates S6K1 and 4EBP1 and regulates cell growth by controlling mRNA translation initiation and elongation. mTORC2 phosphorylates Akt^{Ser473} and participates in the regulation of multiple functions, including cell survival and cytoskeletal organization. Continuous activation of $p70S6K^{Thr389}$ and Akt^{Ser473} resulted by mTOR activation were found to be involved in the drug resistance in several types of cancers.[8–11] Rapamycin, an allosteric inhibitor of mTORC1, is clinically effective in limited types of tumors as a chemotherapy agent, and the therapeutic response to is highly variable, which probably due to the fact that rapamycin is

only an inhibitor against mTORC1 and mTORC2 resistance.[7,12,13] Recent data indicate that dual inhibition of mTORC1 and mTORC2 might achieve broader and more robust anticancer effects.[14–16]

In addition, epithelial-mesenchymal transition (EMT), a trans-differentiation program in embryonic development, is found to induce stem cell properties in various types of cancers.[17,18] Also, mTORC1 and mTORC2 were found to regulate EMT in colon cancer cells.[14] Thus, we hypothesized that mTOR signaling might take a part in the regulation of colorectal CSCs. In this study, we investigated the role of mTOR pathway and the effect of its inhibition in colorectal CSCs.

MATERIALS AND METHODS

Immunohistochemistry

A total of 365 primary CRC tissues were obtained from the tumor bank of the First Affiliated Hospital, Sun Yat-Sen University (Guangzhou, China). Tumor tissues of 33 CRC patients who underwent neoadjuvant chemotherapy were obtained from the tissue bank of the Sixth Affiliated Hospital, Sun Yat-Sen University (Guangzhou, China). FOLFOX6 regimen was employed in all neoadjuvant patients, which was consisted of 2-h infusion of oxaliplatin (100 mg/m^2) and 2-h infusion of leucovorin (400 mg/m^2) on day l, followed by 5-fluorouracil (400 mg/m^2) on day 1 and 46-h infusion (2.4 g/m^2). FOLFOX6 regimen was repeated every 2 weeks. All the patients received 12 intervals before tumor resection. Immunohistochemistry was performed to detect mTOR expression (1:800 dilution, Abcam, Cambridge, UK) as previously described.[19] Open-source software (TMAJ, Johns Hopkins TMA Core Facility, Baltimore, MD) was used to measure the mTOR expression index by researchers who were blinded to the corresponding clinicopathological information. The immunohistochemistry index cutoff point was established as 5.055 using X-tile software program (version 3.6.3, Yale University School of Medicine, CT USA).

Cell Lines, mTOR Inhibitors, and Chemotherapy Drugs

The human colon cancer cell lines, CACO2, SW480, HT29, and DLD-1, were purchased from the Chinese Academy of Science Cell Bank (Shanghai, China) and routinely cultured. We chose mTORC1 inhibitor rapamycin and the second-generation mTOR inhibitor PP242, which targets both mTORC1 and mTORC2.[20] Rapamycin (Selleck Chemicals, USA), PP242 (Selleck Chemicals), 5-fluorouracil (5-FU; Sigma-Aldrich), and oxaliplatin (Selleck Chemicals) were dissolved to stock doses with DMSO and stored at −20 °C. Parallel cultures of cells in medium with DMSO were used as controls.

Western Blot Analysis

Total protein was extracted and separated by SDS-PAGE and transferred to NC membranes (Millipore Corp, MA, USA). Antibody incubations were performed and protein staining and quantification were performed by Odyssey Imaging System (LI-COR Biosciences, NE USA). The protein immunoblots were probed with the following antibodies: anti-β-actin (1:10,000, Proteintech Group, Chicago, IL), anti-phospho-mTOR (S2448) (1:500, Abcam, Cambridge, UK), anti-pan-Akt (1:2,000, Cell Signaling Technology, MA, USA), anti-phospho-Akt (Ser473) (1:1,000, Cell Signaling Technology), anti-phospho-p70S6 kinase (Thr389, 1A5) (1:1,000, Cell Signaling Technology), and anti-total-p70S6 kinase (1:1,000, Bioworld Technology, MN, USA).

ALDEFLUOR Assay

ALDEFLUOR kit (Stem Cell Technologies, Canada) was used to detect the aldehyde dehydrogenase (ALDH) enzymatic activity of the cells. Cells (5×10^5) were freshly harvested, suspended in ALDEFLUOR assay buffer containing 5 µl of ALDEFLUOR reagent, and incubated for 40 minutes at 37 °C. Negative control samples were incubated with 5 µl of the ALDH inhibitor dimethylaminobenzaldehyde (DEAB). ALDEFLUOR was excited at 488 nm, and fluorescence emission was detected via the FITC fluorochromes using a BD FACSCanto™ flow cytometer (BD Biosciences, NJ, USA). 7-Amino-Actinomycin D (7-AAD, BD Pharmingen™, NJ, USA) was used to establish the gates for cell viability. A total of 3×10^4 viable cell events were acquired and used to generate the parameters for analysis.

Sphere Formation Assay

The cells were suspended in serum-free DMEM/F12 (Gibco, China) supplemented with 20 ng/ml epidermal growth factor (EGF, PeproTech, NJ, USA), 20 ng/ml basic fibroblast growth factor (bFGF, PeproTech), 2 % B-27 supplement (Gibco), 20 mmol/l HEPES, 100 units/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco) at a density of 3,000 cells per well in ultralow attachment 24-well plates (Corning, MA, USA). Sphere formation was observed and images were captured using a Leica DMI 4000B inverted research microscope (Leica Microsystems, Wetzlar Germany).

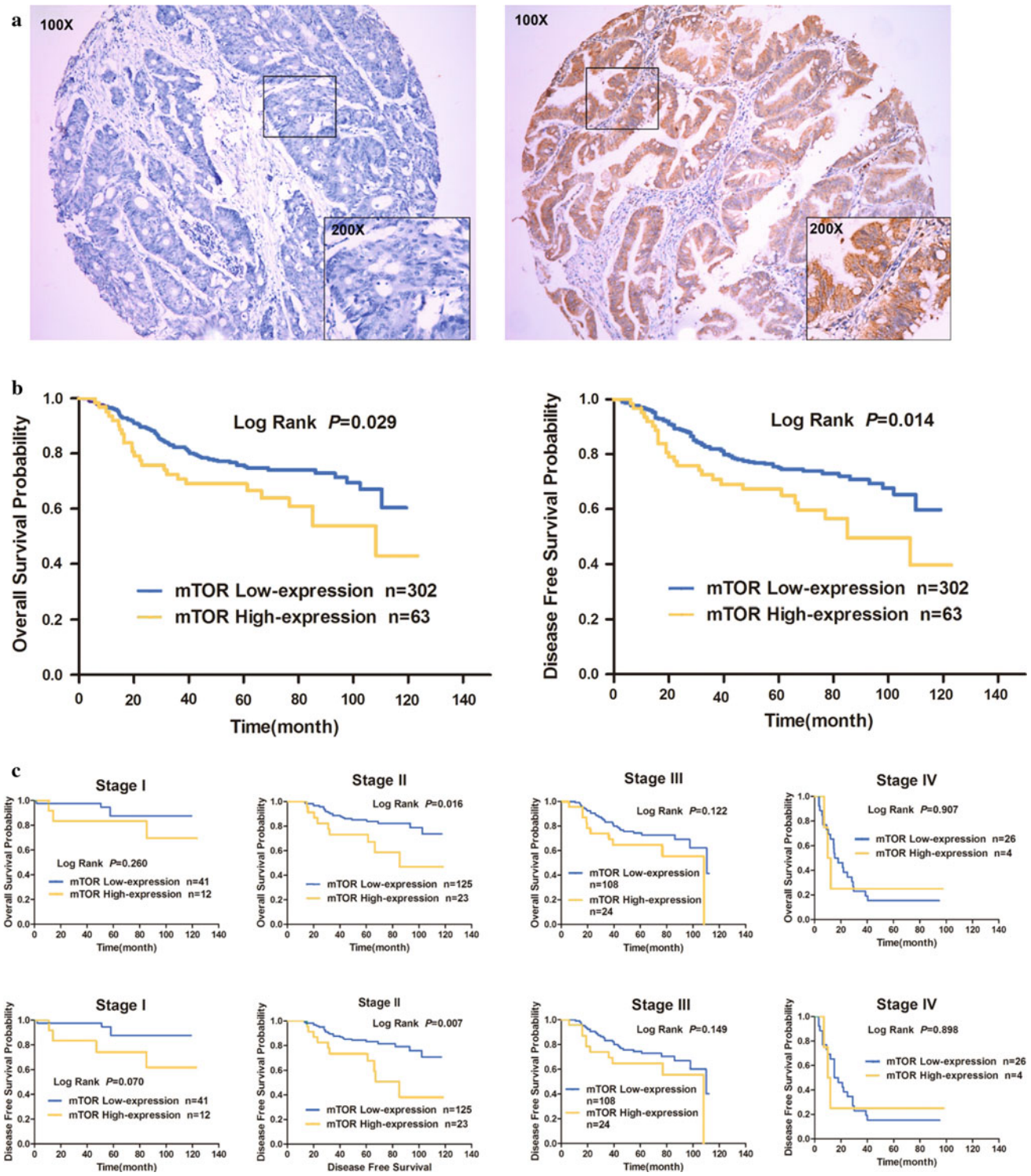
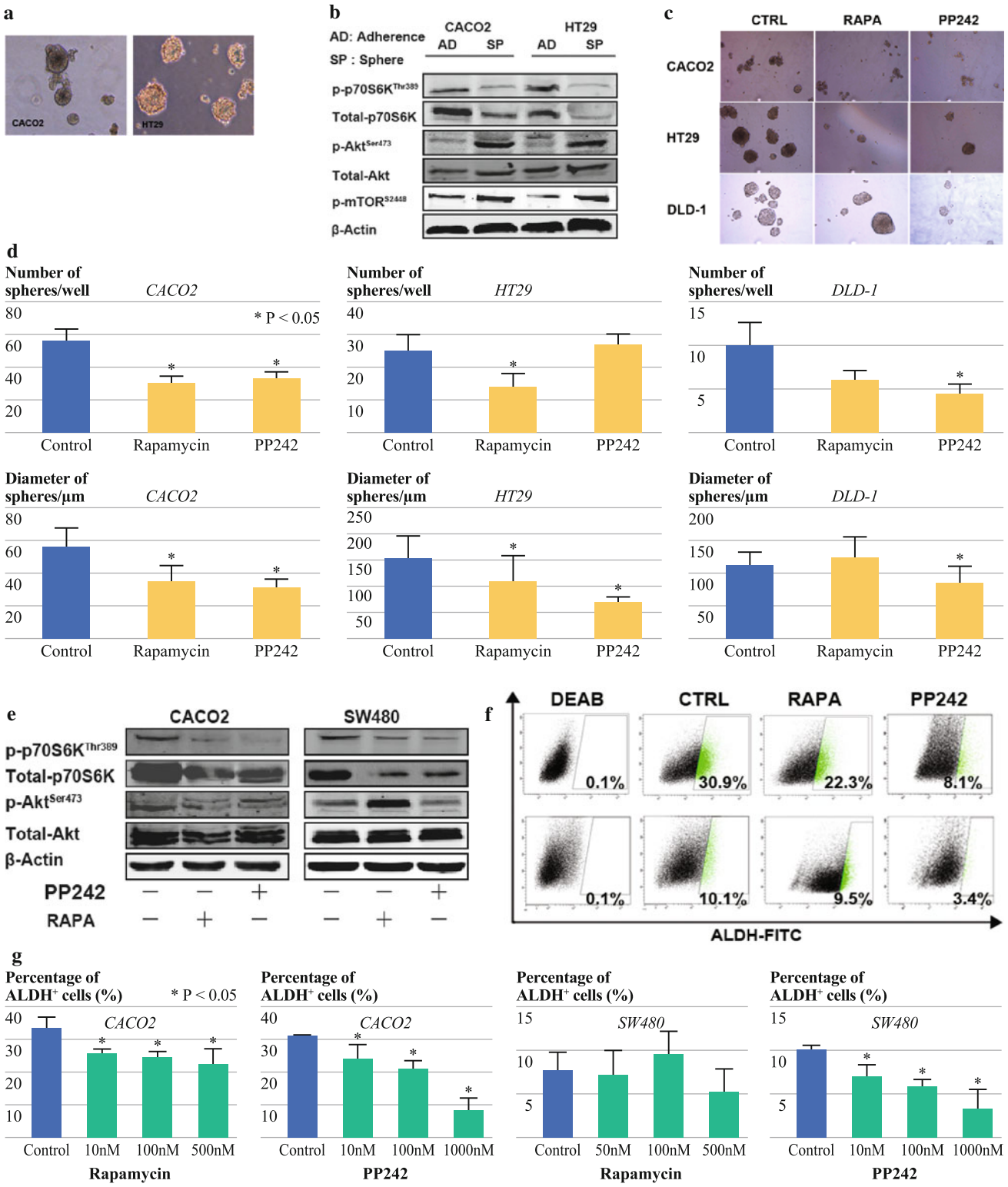


FIG. 1 **a** Representative images of mTOR staining in CRC tissues with low mTOR expression (*left*) and high mTOR expression (*right*) (magnification: ×100, inserts: ×200). The immunohistochemistry index cutoff point was established at 5.055 using X-tile software. **b** Kaplan–Meier survival curves of all CRC patients grouped by mTOR expression showed significant differences in overall survival ($P = 0.029$) and disease-free survival ($P = 0.014$). **c** Kaplan–Meier survival curves of 363 CRC patients from different TNM stages grouped by mTOR expression showed that significant difference in overall survival and disease survival was observed in stage II patients ($P < 0.05$), whereas no significant difference was observed in stage I, III, or IV patients ($P > 0.05$)



Statistical Analysis

The statistical significance of the correlation between the mTOR expression index and patient survival was estimated using the Mantel-Cox log-rank test. Correlation analyses between the mTOR expression index and clinicopathological variables were analyzed using the *t* test, the χ^2 test, or Fisher's exact test (two-tailed). Kaplan–Meier curves were depicted to estimate patients' survival. The expression dynamics of mTOR between the nonchemotherapy group

◀**FIG. 2** **a** Representative images of spheres from the CACO2 and HT29 cell lines cultured in a serum-free medium system (Magnification ×200). **b** Western blot analysis of p-mTOR^{S2448}, p-Akt^{Ser473}, and p-p70S6K^{Thr389} in adherent (AD) and spheroid (SP) cells from the CACO2 and HT29 cell lines. **c** Representative images of spheroid cells from the CACO2, HT29, and DLD-1 cell lines after treatment with IC50 dose of rapamycin (100 nM), PP242 (500 nM), and DMSO control (magnification ×100). **d** The number and diameter of the spheres per well in the CACO2, HT29, and DLD-1 cell lines after treatment with rapamycin and PP242 for 1–2 weeks. The numbers shown are the mean ± SD from three independent experiments. The diameters were calculated and displayed as the mean ± SD. **e** Western blot analysis of p-Akt^{Ser473} and p-p70S6K^{Thr389} in CACO2 and SW480 cells after treatment with rapamycin (100 nM), PP242 (500 nM), and DMSO for 72 h. **f** Flow cytometer analysis of the ALDEFLUOR assay in CACO2 and SW480 cells treated with rapamycin (100 nM), PP242 (500 nM), and DMSO for 72 h. Cells incubated with the ALDEFLUOR substrate (BAAA) and the specific ALDH inhibitor DEAB were used to determine the baseline fluorescence (0.1 %) of these cells and to define the ALDH$^+$ region. **g** The percentages of ALDH$^+$ cells after treatment of CACO2 and SW480 cells with increasing doses of rapamycin and PP242 for 72 h. The data shown in the *bar graph* are the mean ± SD of three independent experiments

and the chemotherapy group were evaluated using the Mann–Whitney *U* test. For continuous variables, the data were expressed as the mean ± SD. The data from the sphere formation assay and the ALDEFLUOR assay were analyzed by the *t* test or the Mann–Whitney *U* test. All statistical analyses were performed in SPSS version 13. *P* values <0.05 were considered statistically significant.

RESULTS

mTOR Expression was Associated with Outcomes of CRC Patients and Predicted Poor Prognosis in Stage II CRC Patients

To explore the prognostic value of mTOR, a total of 365 CRC tissues were obtained and evaluated for mTOR expression using immunohistochemistry. As shown in Fig. 1a, mTOR exhibited both membranous and cytoplasmic staining in primary tumor tissues. Based on the cutoff point, 63 patients (17.3 %) had high expression of mTOR and 302 patients (82.7 %) had low expression. The chi-square test showed that mTOR expression was significantly associated with patients' gender ($P = 0.026$) and preoperative serum CEA ($P = 0.034$) but not patients' age, CA-199, pathological type, tumor differentiation, TNM stage, or tumor location (Table 1). Survival analysis revealed that patients with high mTOR expression had poorer disease-free survival ($P = 0.015$, hazard ratio (HR) 1.715, 95 % confidence interval (CI) 1.108–2.655) and overall survival ($P = 0.031$, HR 1.645, 95 % CI 1.047–2.585) than patients with low mTOR expression (Fig. 1b; Table 2). In multivariate Cox regression analysis, mTOR expression was confirmed to be a significant risk factor associated with CRC patients' disease-free survival ($P = 0.049$, HR 1.689, 95 % CI 1.002–2.848) but not overall survival ($P = 0.081$, HR 1.659, 95 % CI 0.915–3.088) after adjusting for other potential risk factors (Supplementary Table). Furthermore, mTOR expression was found to be specifically associated with poorer survivals in stage II CRC patients when subgroup analysis was performed based on the TNM stages (Fig. 1c). Multivariate analysis revealed that mTOR expression was an independent prognostic factor for both disease-free survival ($P = 0.01$) and overall survival ($P = 0.027$) in stage II CRC patients (data not shown).

mTOR Inhibitor Suppressed Colorectal CSCs

CACO2 and HT29 spheroid cells that maintain the stemness of differentiation and tumor initiation were cultured in a serum-free medium system (Fig. 2a).[21] After 2 weeks of cultivation, spheroid cells were harvested and their mTOR expression was analyzed using Western blot. mTOR and its downstream protein phospho-Akt^{Ser473} (mTORC2) were upregulated in the spheroid cells compared with the adherent cells, whereas phospho-p70S6^{Thr389} (mTORC1) was downregulated in spheroid cells (Fig. 2b). To determine the effect of mTOR inhibition on colon CSCs, colon cancer spheres formed by the CACO2, HT29, and DLD-1 were treated with rapamycin and PP242 separately. After 72 h, the number (RAPA (rapamycin): $P = 0.005$; PP242: $P = 0.003$) and diameter (RAPA: $P = 0.017$, PP242: $P = 0.008$) of spheres formed by CACO2 cell line were all significantly reduced by both rapamycin and PP242. For the spheres formed by HT29 cell line, the number was decreased by rapamycin ($P = 0.041$), and the diameter was reduced by PP242 ($P = 0.002$). While for the spheres formed by DLD-1 cell line, both the number ($P = 0.038$) and the diameter ($P = 0.025$) were significantly reduced by PP242, not by rapamycin (Fig. 2c, d).

ALDH enzyme activity test was applied to confirm the effect of mTOR inhibition on colorectal CSCs because of its proven role in identifying CSCs.[22,23] ALDHFLUOR assay was initially done in a panel of colon cancer cell lines, including HT29, HCT116, DLD-1, CACO2, and SW480. CACO2 and SW480 were chosen for further experiments, because the cells from these two cell lines were nicely distributed on FACS dot plot analysis (data not shown). Secondly, the inhibition ability of rapamycin and PP242 on mTORC1 and mTORC1/2 signaling were confirmed in both CACO2 and SW480 cell lines. Both rapamycin and PP242 were found to downregulate the expression of phospho-Akt and phospho-p70S6 kinase in CACO2 cell line. However, upregulation of phosphor-Akt

TABLE 1 Clinicopathological characteristics of the 365 CRC patients grouped by mTOR expression

Characteristic	mTOR		All cases	*P* value
	High expression	Low expression		
No. of patients	63	302	365	NS
Age (year)	61.6 ± 12.7	58.4 ± 14.3	58.9 ± 14.1	0.119
Gender, no. of patients (%)			365	0.026
Male	42 (66.7 %)	155 (51.3 %)	197	
Female	21 (33.3 %)	147 (48.7 %)	168	
BMI (kg/m^2)	21.4 ± 3.3	21.0 ± 4.0	21.1 ± 3.9	0.494
CEA (ng/ml), no. of patients (%)			339*	0.034
<5	43 (78.2 %)	180 (63.4 %)	223	
≥5	12 (21.8 %)	104 (36.6 %)	116	
Tumor location, no. of patients (%)			363**	0.118
Colon	25 (39.7 %)	151 (50.3 %)	176	
Rectum	38 (60.3 %)	149 (49.7 %)	187	
Tumor diameter (cm)	4.8 ± 1.8	5.0 ± 2.1	4.9 ± 2.1	0.15
Histopathology, no. of patients (%)			365	0.063
Adenocarcinoma	53 (84.1 %)	277 (91.7 %)	330	
Mucinous carcinoma	10 (15.9 %)	25 (8.3 %)	35	
Differentiation, no. of patients (%)			364***	0.413
Well/moderate	52 (83.9 %)	264 (87.7 %)	316	
Poor	10 (16.1 %)	37 (12.3 %)	47	
TNM stage, no. of patients (%)			365	0.188
Tis	0 (0 %)	2 (7 %)	2	
I	12 (19.0 %)	41 (13.6 %)	53	
II	23 (36.5 %)	125 (41.4 %)	148	
III	24 (38.1 %)	108 (35.8 %)	132	
IV	4 (6.4 %)	26 (8.6 %)	30	

CRC colorectal cancer, *mTOR* mammalian target of rapamycin, *CEA* carcinoembryonic antigen, *BMI* body mass index, *NS* not significant

* Twenty-six cases were not tested for serum CEA level

** Two cases of tumor location information were lost

*** One case of differentiation information was lost

was noticed when SW480 cells were treated by rapamycin. And downregulation of phospho-p70S6 kinase by rapamycin, reduction of phospho-Akt, and phospho-p70S6 kinase by PP242 also was seen in SW480 cell line (Fig. 2e). After that, the CACO2 and SW480 cell lines were treated with increasing doses of mTOR inhibitors for 72 h and the presence and size of the cell population with ALDH enzymatic activity were assessed. Similar to the results in sphere assay, increasing doses of rapamycin and PP242 reduced the percentage of ALDH$^+$ cells in CACO2 cell line in a dose-dependent manner ($P < 0.05$), whereas in SW480 cell line, the percentage of ALDH$^+$ cells was only decreased by PP242 ($P < 0.05$; Fig. 2f, g). These results suggest that mTORC2 inhibition might serve as a satisfactory treatment strategy against colon cancer stem-like cells.

mTOR Inhibitor Suppressed the Stimulation of Colorectal CSCs Induced by Chemotherapy

Tumor samples from another 33 stage II or III rectal cancer patients who underwent neoadjuvant therapy were analyzed by immunohistochemistry to evaluate the impact of chemotherapy on the expression of mTOR before surgery. We found that mTOR expression was significantly higher in the tumor tissues from patients with preoperative chemotherapy than those without preoperative chemotherapy ($n = 280$; $P < 0.0001$; Fig. 3a, b). To investigate the relationship between mTOR signaling and CSCs stimulation induced by chemotherapy, CACO2 and SW480 cell lines were treated with 5-FU or oxaliplatin. After 72 h, Western blot analysis revealed that mTOR downstream targets phospho-Akt and phospho-p70S6 kinase were

upregulated ($P < 0.05$), and ALDHFLUOR assay showed that the percentages of $ALDH^+$ cells were increased under the pressure of chemotherapeutic drugs (Fig. 3c–e). To examine whether the stimulation of stem-like cells by chemotherapy could be suppressed by mTOR inhibitors, chemo-treated colon cancer cells were also co-treated with rapamycin or PP242. Western blot analysis revealed that the stimulation of phospho-Akt and phospho-70S6 kinase by 5-FU or oxaliplatin was suppressed by both rapamycin and PP242 (Fig. 3c). The ALDHFLUOR assay showed that only mTORC1/2 inhibitor PP242 suppress the enrichment of $ALDH^+$ cells induced by 5-FU or oxaliplatin (Fig. 3d, e; $P < 0.05$), whereas rapamycin showed little effect (data not shown).

DISCUSSION

Cancer stem cells, which only constitute a small fraction of tumor components but drive the tumorigenesis and differentiation of solid cancers, are thought to be important causes for tumor initiation and progression.[3] In this study, mTOR high-expression was identified to be an independent

TABLE 2 Univariate analysis of clinicopathological variables and mTOR expression in 365 CRC patients

Characteristic	Overall survival			Disease-free survival		
	RR	95 % CI	*P* value	RR	95 % CI	*P* value
Gender						
Male	0.963	0.652–1.421	0.848	0.902	0.617–1.319	0.595
Female						
Age (year)						
<60	1.011	0.996–1.026	0.141	1.41	0.959–2.073	0.08
≥60						
BMI (kg/m^2)						
<21.22	0.790	0.533–1.171	0.241	0.809	0.551–1.187	0.809
≥21.22						
CEA (ng/ml)						
<5	2.032	1.33–3.103	0.001	1.835	1.214–2.772	0.004
≥5						
CA-199 (U/ml)						
<37.5	1.985	1.264–3.119	0.003	1.836	1.176–2.866	0.008
≥37.5						
Pathological type						
Adenocarcinoma	1.393	0.762–2.546	0.282	1.311	0.719–2.392	0.377
Mucinous/others						
Differentiation						
Well/moderate	2.315	1.428–3.751	0.001	2.172	1.345–3.51	0.002
Poor						
Tumor location						
Colon	0.753	0.517–1.095	0.138	0.797	0.553–1.148	0.223
Rectum						
Colon and rectum						
TNM stage						
I/II	2.789	1.862–4.177	<0.001	2.552	1.727–3.771	<0.001
III/IV						
Obstruction/perforation						
Present	1.975	1.139–3.425	0.015	1.864	1.078–3.224	0.026
Absent						
mTOR expression						
High	1.645	1.047–2.585	0.031	1.715	1.108–2.655	0.015
Low						

CRC colorectal cancer, *CEA* carcinoembryonic antigen, *CI* confidence interval, *BMI* body mass index, *RR* relative risk, *mTOR* mammalian target of rapamycin

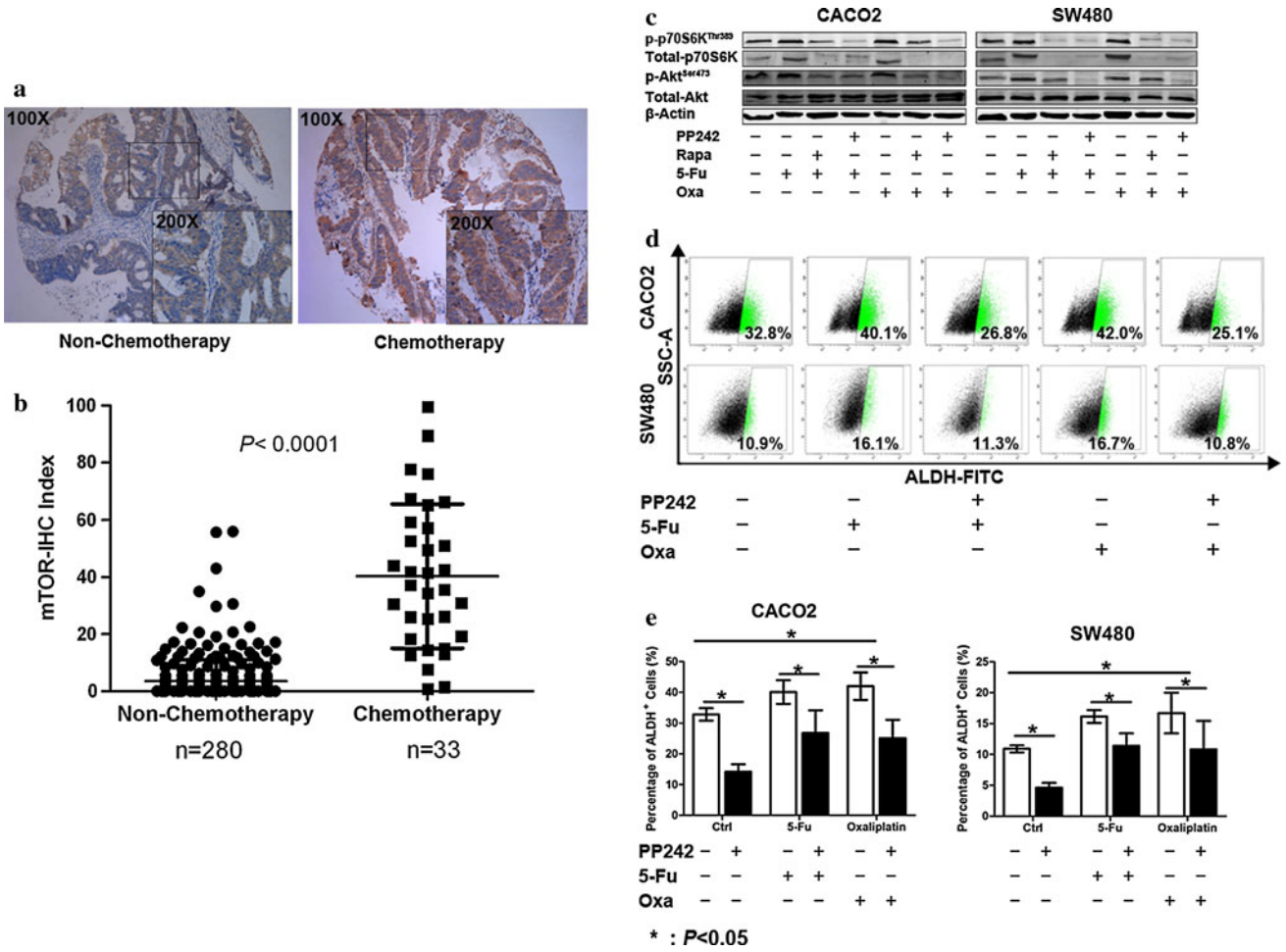


FIG. 3 **a** Representative images of mTOR staining in tumor tissues from CRC patients who were treated with (*right*) or without (*left*) chemotherapy before surgery (magnification ×100, inserts ×200). **b** mTOR staining index of patients from the non-chemotherapy group ($n = 280$) and the chemotherapy group ($n = 33$). A significant difference ($P < 0.0001$) was observed between the two groups using the Mann–Whitney *U* test. **c** Western blot analysis of p-Akt^{Ser473} and p-p70S6K^{Thr389} in CACO2 and SW480 cells after treatment with 5-FU (0.1 μM) or oxaliplatin (0.01 μM) in the presence or absence of PP242 (500 nM). **d**, **e** ALDEFLUOR assay after treatment of CACO2 and SW480 cells with 5-FU (0.1 μM) or oxaliplatin (0.01 μM), with or without PP242 (500 nM) for 72 h. Data shown in the *bar graph* represent three independent experiments and indicate mean ± SD

risk factor for the prognosis of stage II CRC patients and mTOR inhibition suppressed colorectal CSCs, even after being activated by chemotherapy.

Previous study showed that the mTOR pathway members, such as the p70S6 kinase and Akt, were highly expressed in primary CRC tissues, but no further prognostic value of mTOR in CRC patients was reported.[24] In our study, we started by detecting mTOR expression in a large number of colorectal patients ($n = 365$) and demonstrated that mTOR expression was inversely associated with the prognosis of CRC patients. In the subgroup analysis, mTOR expression was found specifically to be an independent risk factor for the poor survival of stage II CRC patients. The prognosis of stage II CRC patients varied, and previous studies showed that ~20–30 % of stage II CRC patients would develop local or distant recurrences after surgery.[25] Although the exact mechanisms underlying the phenomenon remained unclear, our results indicate mTOR signaling might contribute to this, and help to compensate the shortage of TNM stage system in predicting the outcomes of CRC patients.

Chemotherapy-resistant CSCs activation is believed to be the main source for the tumor recurrence after current therapy in CRC.[3,5] In this article, we found that mTOR signaling was involved in the regulation of colorectal CSCs. mTOR signaling was activated in cancer stem-like cells enriched by suspension culture, a nice in vitro cancer stem cell model.[21] mTOR inhibitors diminished the capacity of sphere formation. In addition, ALDH1 activity, which is a marker for cancer stem and progenitor cells of CRC, also was decreased after mTOR suppression.[22,23] Both findings highlighted an essential role of the mTOR signaling in the

maintenance of CRC stem cells, which in part explained the clinical value of mTOR expression. Furthermore, it has been revealed that surviving breast cancer cells after chemotherapy possess increased tumor-initiating capacity and therefore probably promote metastasis afterwards.[26,27] In our study, chemotherapy stimulated ALDH activity in CRC cell lines. At the same time, we also found that chemotherapy elevates the mTOR expression in the primary tumors of CRC patients and mTOR inhibitor PP242 were capable of suppressing the activation of CSCs induced by chemotherapy. This result suggests that mTOR inhibition might be a good therapeutic strategy against colorectal CSCs, because it also inhibits colon cancer proliferation.[28]

In our results, two generations of mTOR inhibitors, the rapamycin for mTORC1 and PP242 for mTORC1/2, showed distinct effects on colorectal CSCs. These heterogeneous results also were found in the studies of antiproliferation effect of mTORC1 and mTORC2 inhibitors in CRC cells. Recently, mTORC2 were also found to have more impact in CRC tumorigenesis and proliferation than mTORC1, because it can directly regulate Akt activity.[28–30] That might explain our results that PP242 showed stronger inhibition against colon CSCs than rapamycin. Taken together with our previous results that demonstrate the predictive significance of mTOR in CRC patients' outcomes and the relationship between mTOR inhibition and colorectal CSCs, our findings provide the rationality to include mTOR inhibitors against CSCs as part of the therapeutic regimen for CRC patients.

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