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Prognostic Value of EGFR Expression for Patients With Stage III Colorectal Cancer Receiving Fluoropyrimidine Metronomic Maintenance Therapy After Radical Resection and Adjuvant Oxaliplatin-Based Chemotherapy

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This study evaluated the survival effects of metronomic maintenance therapy with oral fluoropyrimidine in patients with stage III colorectal cancer (CRC) according to epidermal growth factor receptor (EGFR) expression. We enrolled 197 patients with stage III CRC who had undergone radical resection and FOLFOX regimen adjuvant chemotherapy. The clinicopathological features and effects of metronomic maintenance therapy with oral capecitabine (daily dose of 850 mg/m^2, twice daily, on days 1–14 every 3 weeks for 6 months) on survival according to treatment group and EGFR expression were analyzed. By conducting an in vitro cell line study and in vivo study through knockout of the *EGFR* gene, we analyzed the capacities of cell proliferation and migration. Relapse and survival were significantly more common in the FOLFOX group. Metronomic maintenance therapy was a significantly independent associated factor of relapse and survival as well as a prognostic factor of disease-free survival and overall survival. Significant intergroup differences in survival were only observed in patients with positive EGFR expression. Thus, our findings suggest EGFR expression is a prognostic factor in patients with stage III CRC receiving metronomic maintenance therapy. Analysis of EGFR expression in these patients helps identify potential candidates who may receive the optimal survival benefit from metronomic maintenance therapy.

Key words: Metronomic maintenance therapy; Capecitabine; Epidermal growth factor receptor (EGFR); Oxaliplatin-based regimen; Stage III colorectal cancer (CRC)

INTRODUCTION

Colorectal cancer (CRC) is the second most common type of cancer and the third leading cause of cancer-related death worldwide. Approximately 1.7 million new diagnoses of CRC and 830,000 CRC-related deaths were reported in 2016[1]. In the US, CRC was the third most common cancer and the third leading cause of cancer death in 2016. Additionally, an estimated 145,600 new CRC diagnoses and 51,020 CRC-related deaths were

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reported in 2019[2]. In Taiwan, CRC is the most common cancer type, and its prevalence has increased rapidly since 2006. Moreover, CRC has been the third leading cause of cancer-related death since 1996. The incidence of CRC was 32.38 per 100,000 in 2000 (with 7,213 new diagnoses) and 66.32 per 100,000 in 2017 (with 15,579 new diagnoses)[3].

According to the Surveillance, Epidemiology, and End Results (SEER) data, 39% of CRC cases are diagnosed at the localized stage of the disease. The 5-year overall survival (OS) rates for localized-stage disease, regional-stage disease, and distant-stage disease of CRC were reported to be 89.8%, 71.1%, and 13.8%, respectively[4]. In Taiwan, the 5-year OS rates for stage I, II, III, and IV CRC in 2013 were revealed to be 80.9%, 71.2%, 59.9%, and 12.3%, respectively[3]. Furthermore, patients with locally advanced CRC (stage II + III) who have undergone adjuvant chemotherapy have a 26.7% risk of developing relapse in 5 years. However, postoperative adjuvant chemotherapy significantly improves survival in patients with stage III CRC after radical surgery[5–7]. The MOSAIC trials have demonstrated significant disease-free survival (DFS) and OS improvement in patients treated with the FOLFOX4 (oxaliplatin plus continuous-infusion fluorouracil plus leucovorin) regimen[8,9]. Therefore, an oxaliplatin-based regimen has become the gold standard in postoperative adjuvant chemotherapy treatment for patients with stage III colon cancer. According to an analysis by the ACCENT Group in an 8-year follow-up period, 32.9% of patients developed cancer recurrence. Moreover, 82% and 74% of recurrences occurred within the first 3 years in patients with stage III and stage II colon cancers, respectively[10,11]; the peak incidence of recurrence was between 1 and 2 years after initial treatment.[10] Because of their similar benefit to survival, most postoperative adjuvant chemotherapy regimens are administered for 6 months[7,12,13]. Therefore, in patients with stage III CRC, metronomic maintenance therapy with orally administered fluoropyrimidine following 6 months of an oxaliplatin-based regimen may decrease the risk of recurrence[14]. Capecitabine (Xeloda®; F. Hoffmann-La Roche Ltd., Basel, Switzerland) is an oral fluoropyrimidine carbamate prodrug of 5-fluorouracil (5-FU), which is an effective single agent or combined adjuvant chemotherapy for patients with stage III colon cancer[15–18]. Therefore, capecitabine is an ideal medicine for metronomic maintenance treatment for patients with stage III CRC.

Our previous study demonstrated that epidermal growth factor receptor (EGFR) expression has prognostic value, specifically in patients with metachronous metastatic CRC (mCRC)[19]. We also demonstrated that tumor EGFR expression is a significant independent negative predictive factor for relapse and a significant independent negative prognostic factor for DFS and OS in patients with stage III CRC who have undergone radical resection surgery and adjuvant FOLFOX chemotherapy[20]. We hypothesized that $EGFR^-$ tumor cells are less proliferative and less migratory than are $EGFR^+$ tumor cells. Therefore, we investigated the mechanistic connections between 5-FU and EGFR by conducting in vitro CRC cell line and in vivo animal studies. Moreover, cell proliferation and migration could be inhibited by fluoropyrimidine-based therapy. We used Caco2 cells because they express EGFR and exhibit no mutations in the oncogenic gene *KRAS*[20]. We showed that after CRISPR gRNA transfection, the EGFR protein level in the Caco2 cells decreased substantially. The proliferative and migratory capacities of the Caco2 cells decreased after *EGFR* knockout, and the proliferative and migratory capacities of the Caco2 cells with or without EGFR expression were inhibited by 5-FU. We determined that 5-FU administration and *EGFR* knockout had additive inhibitory effects on the proliferative and migration capacities of Caco2 cells. Accordingly, in this study, we evaluated the survival effects of metronomic maintenance therapy with oral capecitabine after adjuvant oxaliplatin-based regimen therapy in patients with stage III CRC who had undergone radical resection; this evaluation was conducted according to EGFR expression levels.

MATERIALS AND METHODS

Patients

We analyzed 197 consecutive patients with histologically confirmed stage III CRC who had received surgical treatment at a single institution between January 2008 and June 2012 and had received adjuvant chemotherapy with the FOLFOX regimen after surgery. To reduce the effect of neoadjuvant treatment on gene expression, patients were excluded if they had undergone neoadjuvant treatment with either chemotherapy or radiotherapy before surgery. The present study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUHIRB-E-20150003).

Chemotherapy Treatment Groups

The adjuvant oxaliplatin-based regimen was mFOLFOX as follows: each cycle of FOLFOX consisted of oxaliplatin (Eloxatin®; 85 mg/m^2; Sanofi-Aventis, Paris, France) and folinic acid (Covorin®; 400 mg/m^2; Swiss Pharmaceutical Co. Ltd, Tainan, Taiwan) on day 1, and a 46-h infusion of 5-FU® (2800 mg/m^2; Nang Kuang Pharmaceutical Co. Ltd, Tainan, Taiwan) repeated every 2 weeks, biweekly for 12 cycles. Of 197 patients, 87 patients (44.7%) received only the adjuvant oxaliplatin-based regimen (FOLFOX group), and 110 patients (55.8%) received oral capecitabine after adjuvant oxaliplatin-based regimen (FOLFOXC group).

Oral capecitabine was administered at 850 mg/m^2/day, twice daily, on days 1–14 repeated with 3-week intervals for 6 months. After detailed information on potential benefits and disadvantages was explained to the patients, they provided oral consent to receive capecitabine.

Patient Follow-Up

Patients were regularly followed up for clinical outcomes and DFS and OS statuses. Clinicopathological variables included age at diagnosis, gender, tumor location, histological type, TNM classification, vascular invasion, perineural invasion, and preoperative and postoperative serum carcinoembryonic antigen (CEA) level. The TNM classification was defined according to the criteria of the American Joint Commission on Cancer/Union for International Cancer Control (AJCC/UICC)[21]. Right-sided colon cancers were defined as those located in the cecum, ascending colon, hepatic flexure, and transverse colon, and left-sided cancers were defined as those located in the splenic flexure, descending colon, sigmoid, and rectum. All patients were followed until their deaths, their last follow-up, or December 31, 2018. Relapse included the development of a new local recurrence (tumor growth restricted to the anastomosis or the region of the primary operation) or distant metastatic lesions (distant metastases or diffuse peritoneal carcinomatosis) after surgery. DFS was defined as the time from the date of primary treatment to the date of diagnosis for recurrence or metastatic disease or to the date of the last follow-up. OS was defined as the time from the date of primary treatment to the date of death from any cause or until the date of the last follow-up.

Immunohistochemical Analysis of EGFR Expression

The procedure for immunohistochemical (IHC) analysis of EGFR expression was based on those of our previous studies[19,20]. In brief, formalin-fixed and paraffin-embedded tissue blocks were cut into 3-μm sections to retrieve antigens. Endogenous peroxidase was blocked using 3% hydrogen peroxide. After washing, the sections were incubated with EGFR. Next, the Dako REAL EnVision Detection System-horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) was applied. Finally, the sections were incubated in 3 ,3 -diaminobenzidine, counterstained with Mayer's hematoxylin, dehydrated through two changes of 95% ethanol and two changes of 100% ethanol, cleared in three changes of xylene, and then mounted. Negative controls were obtained by replacing the primary antibody with nonimmune serum. The immunoreactivity of EGFR was evaluated by two independent researchers who were blinded to the patients' outcomes. The expression patterns of EGFR were determined in a semiquantitative manner through light microscopy. Immunoreactivity for EGFR (membrane staining) was categorized according to the presence of tumor cell staining and staining intensity, as mentioned in our previous studies[19,20].

Cell Culture and Antibodies

The human colon cancer cell line Caco2 was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin mixture, trypsin-EDTA, and fetal bovine serum (FBS) were obtained from Gibco Life Technologies (Milano, Italy). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). The protein assay kit was bought from Bio-Rad (Berkeley, CA, USA). An enhanced chemiluminescence kit, and rabbit monoclonal antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and EGFR were purchased from Proteintech (Chicago, IL, USA) and Abcam (Cambridge, UK), respectively. Goat anti-rabbit immunoglobulin G was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). MTS [3-(4,5-dimethylthiazol-2 -yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-*H*-tetrazolium] (Sigma-Aldrich, Gillingham, UK) and EGFR Human Gene Knockout Kit [clustered regularly interspaced short palindromic repeats (CRISPR)] were purchased from Sigma-Aldrich and OriGene (Rockville, MD, USA), respectively. The Caco2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37% and 5% CO_2 in humidified atmosphere. The culture medium was changed every other day, and the cells were subcultured using trypsin-EDTA. We obtained 5-FU from Sigma-Aldrich Co.

EGFR Knockout

EGFR knockout was performed as per the manufacturer's instructions with minor modifications. Before transfection, Caco2 cells were seeded in six wells at 1 10^5 cells per well. At 24 h, the cells were transfected with 1 μg of CRISPR gRNA vectors (gRNA sequence: 5 -TCCTCCAGAGCCCGACTCGC-3) and scrambled control (scrambled sequence: 5 -GCACTACCAGAGCT AACTCA-3) with Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). After 72 h of incubation, cells were split 1:10, grown for an additional 3 days, and then split the cells again. After the Caco2 cells were split seven times, puromycin was added for selection, and the knockout clones were identified with Western blot.

Western Blotting

Whole-cell lysates were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer (1 mM EDTA, pH 8.0; 100 mM NaCl; 20 mM Tris, pH 8.0, 0.5% Nonidet P-40; 0.5% Triton X-100), and protein concentration was determined using the Bio-Rad protein assay kit. Western blot was performed as previously described[20].

MTS Cell Viability Assay

Transfected Caco2 cells were seeded in 96 wells (5×10^4 cells/well) and incubated at 37°C. After cell adhesion (designated as 0 h), the transfected Caco2 cells were treated with 5-FU (Sigma-Aldrich; 10 μM/ml) and incubated at 37°C for 24, 48, and 72 h. MTS was added at 0, 24, 48, and 72 h. Thereafter, the cells were incubated at 37°C for 3 h and were then quantified spectrophotometrically using a 490-nm wavelength.

Migration Assay

Cell migration was assessed using a wound-healing assay[22]. In brief, the Caco2 cells were cultured as confluent monolayers and wounded with a 200-μl pipette tip. The detached cells were rinsed off carefully. At 0 and 24 h after wounding, for each wound, three pictures were taken of different areas under bright field microscopy. Each picture was measured with ImageJ software. Data are shown as percentage of wound closure compared with the initial wound.

In Vivo Animal Studies

Six-week-old Balb/c male nude mice were purchased from BioLasco Taiwan−(Taipei, Taiwan). At 7 weeks of age, scrambled control and EGFR-knockout Caco2 cells were subcutaneously implanted in the bottom left or right flank of each 7-week-old male nude mouse. The tumor size (cm^3) was measured thrice a week and calculated according to the formula: $(\text{length} \times \text{width})^2/2$. Four weeks after transplantation, 5-FU (10 mg/kg) was administered intraperitoneally thrice a week for 3 weeks. Animals were sacrificed at 8 weeks after the injection of tumor cells. For the in vivo study, we followed the protocols approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University (Approval No. 105229) per the Guiding Principles for the Care and Use of Laboratory.

Statistical Analysis

All data were statistically analyzed using the Statistical Package for the Social Sciences, version 22.0 (SPSS Inc., Chicago, IL, USA). The correlation between clinicopathological features and treatment group was examined using the chi-square test for categorical variables and Student's *t*-test for continuous variables. Univariate and multivariable logistic regression models were used to evaluate the independent factors of relapse and survival. A Cox proportional hazard model was used to identify independent prognostic factors for OS and DFS. DFS and OS were evaluated using the Kaplan–Meier method, and the log-rank test was used to compare time-to-event distributions. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Clinical and Pathological Characteristics of Patients With Stage III CRC Between the Two Treatment Groups

The clinical and pathological characteristics of the 197 patients (Fig. 1) with stage III CRC are summarized in Table 1. Of the 197 patients, 118 (59.9%) were men and 79 (40.1%) were women. The median age of the 197 patients was 62 years (range, 30–82 years). Among all patients, 87 (44.2%) received only an adjuvant oxaliplatin-based

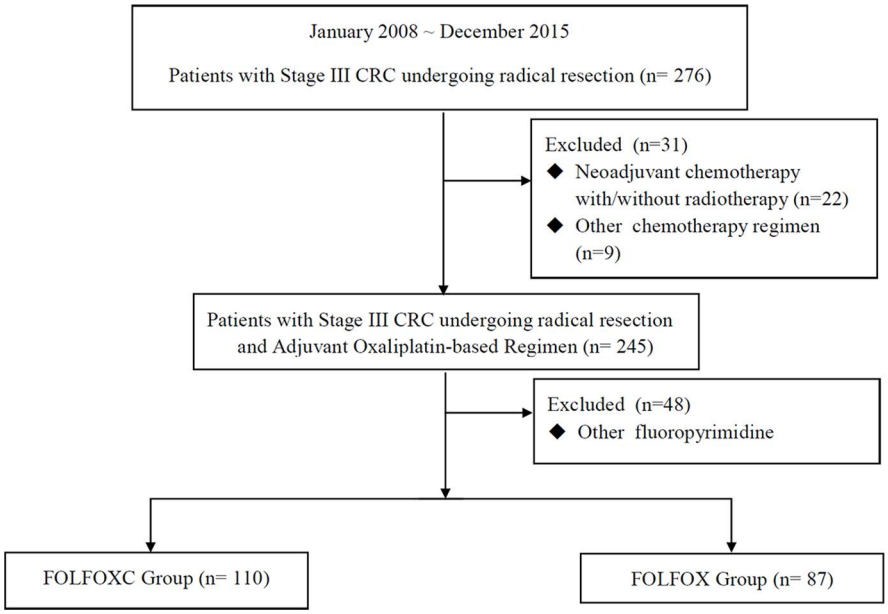

Figure 1. CONSORT diagram showing the inclusion and exclusion criteria in the present study.

Table 1. Baseline Characteristics of Patients With Stage III Colorectal Cancer According to Treatment Group (FOLFOX vs. FOLFOXC)

Characteristic	FOLFOX Group (n = 87) [n (%)]	FOLOFXC Group (n = 110 [n (%)]	p Value
Age			0.745
<65 years	51 (58.6%)	67 (60.9%)	
65 years	36 (41.4%)	43 (30.1%)	
Gender			0.152
Female	30 (34.5%)	49 (44.5%)	
Male	57 (65.5)	61 (55.5)	
Tumor size			0.447
<5 cm	54 (62.1%)	74 (67.3%)	
5 cm	33 (37.9)	36 (32.7)	
EGFR expression			0.540
Positive	59 (67.8%)	70 (63.6%)	
Negative	28 (32.2)	40 (36.4)	
Tumor location			0.991
Right-sided colon	23 (26.4%)	29 (26.4%)	
Left-sided colon	64 (73.6)	81 (73.6)	
Histology			0.813
Well differentiated	11 (12.6%)	2 (1.8%)	
Moderately differentiated	74 (85.1%)	97 (88.2%)	
Poorly differentiated	2 (2.3%)	11 (10.0)	
Tumor depth			0.293
T1 + T2	9 (10.3%)	17 (15.5%)	
T3 + T4	78 (89.7)	93 (84.5)	
Lymph node metastasis			0.685
N1	57 (65.5%)	69 (62.7%)	
N2	30 (34.5)	41 (37.3)	
Vascular invasion			0.023*
No	59 (67.8%)	57 (51.8%)	
Yes	28 (32.2)	53 (48.2)	
Perineurial invasion			0.770
No	52 (59.8%)	58 (61.8%)	
Yes	35 (40.2)	42 (38.2)	
Pre-op serum CEA level			0.065
<5 ng/ml	42 (51.9%)	71 (65.1%)	
5 ng/ml	39 (48.1)	38 (34.9)	
Post-op serum CEA level			0.344
<5 ng/ml	70 (81.4%)	95 (86.4%)	
5 ng/ml	16 (18.6)	16 (13.6)	
Relapse			<0.001*
No	41 (47.1%)	81 (73.6%)	
Yes	46 (52.9)	29 (26.4)	
Survival			0.002*
Yes	57 (65.5%)	93 (84.5%)	
No	30 (34.5)	17 (15.5)	
Disease-free survival (mean ± SD, months)	40.18 ± 40.21	54.87 ± 28.61	0.003*
Overall survival (mean ± SD, months)	55.02 ± 36.06	64.09 ± 25.53	0.040*

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; CEA, carcinoembryonic antigen.
*$p < 0.05$.

regimen (FOLFOX group), and 110 (55.8%) received oral capecitabine as metronomic maintenance therapy after the adjuvant oxaliplatin-based regimen (FOLFOXC group). The median age in the FOLFOX group was 62 years (range, 30–81 years), and that in the FOLFOXC group was 63 years (range, 35–82 years). For all 197 patients, the median follow-up duration was 61.2 months (range, 8.1–128.7 months). IHC analysis of EGFR expression was performed for all patients, of which 129 (65.5%) showed positive EGFR expression ($EGFR^+$); this EGFR expression pattern was did not differ significantly between the FOLFOX and FOLFOXC groups ($p = 0.540$) (Table 1).

Lymphovascular invasion was more common in the FOLFOXC group than in the FOLFOX group (48.2% vs. 32.2%, $p = 0.023$). In the FOLFOX group, 46 patients (52.9%) developed relapse; by contrast, in the FOLFOXC group, only 29 patients (26.4%) developed relapse. These results indicate a statistically significant difference in relapse between the groups ($p < 0.001$). In addition, 57 patients (65.5%) in the FOLFOX group and 93 patients (84.5%) in the FOLFOXC group survived, indicating a significant difference in survival between the groups ($p = 0.002$). Age, gender, tumor size, tumor location, histological type, tumor depth, lymph node metastasis (N1 or N2), perineural invasion, EGFR expression, and preoperative and postoperative serum CEA levels did not differ significantly between the FOLFOX and FOLFOXC groups (all $p > 0.05$).

Univariate and Multivariable Analyses of Associated Factors for Relapse and Survival

To identify independently associated factors for relapse and survival in patients with stage III CRC, we used a logistic regression model to perform univariate and multivariable analyses (Table 2). According to the univariate analysis of the correlation between relapse and clinicopathological features, the $EGFR^+$ patients had a 2.2-fold higher risk of relapse than did the $EGFR^-$ patients ($p = 0.016$). Moreover, the patients in the FOLFOX group had a 3.3-fold higher risk of relapse than did those in the FOLFOXC group ($p < 0.001$). Multivariate analysis of relapse indicated that metronomic maintenance therapy with capecitabine was an independently associated with relapse [$p = 0.001$; odds ratio (OR), 3.026; 95% confidence interval (CI), 1.554–6.678] (Table 2). Furthermore, univariate analysis of survival revealed that $EGFR^+$ patients had a 3.9-fold higher risk of death than did the $EGFR^-$ patients ($p = 0.002$). Multivariate analysis of survival also indicated that EGFR expression and capecitabine metronomic maintenance therapy were independently associated with survival ($p = 0.008$; OR, 3.529; 95% CI, 1.399–8.905; and $p = 0.010$; OR, 2.735; 95% CI, 1.2.7–5.884, respectively) (Table 2).

Univariate and Multivariable Analyses of Survival of Patients With Stage III CRC

To investigate the independent prognostic factors for OS and DFS in patients with stage III CRC, we used a Cox proportional hazards model to perform univariate and multivariable analyses (Table 3). EGFR expression was revealed to be an independent prognostic factor for both DFS [$p = 0.027$; hazard ratio (HR), 1.914; 95% CI, 1.076–3.405] and OS ($p = 0.001$; HR, 4.417; 95% CI, 1.813–10.761). Similarly, metronomic maintenance therapy with capecitabine was revealed to be an independent prognostic factor for both DFS ($p < 0.001$; HR, 3.351; 95% CI, 2.000–5.614) and OS ($p = 0.001$; HR, 3.186; 95% CI, 1.631–6.222).

A Kaplan–Meier survival analysis indicated that the patients in the FOLFOX group had significantly worse DFS ($p < 0.001$) and OS ($p = 0.001$) compared with those in the FOLFOXC group (Fig. 2A and B). The median DFS periods of the patients in the FOLFOX and FOLFOXC groups were 16.7 and 57.9 months ($p < 0.001$), respectively, whereas the median OS periods of the patients in the FOLFOX and FOLFOXC groups were 50.3 and 68.7 months ($p = 0.001$), respectively. The 5-year DFS rates were 43% and 71% for the FOLFOX and FOLFOXC groups, respectively. Furthermore, 16 of 46 patients (34.8%) with relapse in the FOLFOX group and 4 of 29 patients (13.8%) with relapse in the FOLFOXC group experienced relapse between 6 and 12 months postoperatively. However, 45 of 46 patients (97.8%) with relapse in the FOLFOX group and 24 of 29 patients (82.7%) with relapse in the FOLFOXC group experienced relapse within 3 years postoperatively. The 5-year OS rates were 61% and 88% for the FOLFOX and FOLFOXC groups, respectively. We also performed subgroup analyses according to EGFR expression and treatment group, and we found no significant differences in the DFS and OS of the $EGFR^-$ patients between the FOLFOX and FOLFOXC groups (Fig. 3A and B); however, we observed significant differences in the DFS (Fig. 3C) and OS (Fig. 3D) of the $EGFR^+$ patients between the FOLFOX and FOLFOXC groups. Specifically, the $EGFR^-$ patients in the FOLFOX and FOLFOXC groups exhibited similar DFS (median DFS, 79.6 vs. 64.3 months, $p = 0.588$) (Fig. 3A) and OS (median OS, 90.9 vs. 80.8 months, $p = 0.290$) (Fig. 3B) periods. The 5-year DFS rates were 69% and 72% for the FOLFOX and FOLFOXC groups, respectively, and the 5-year OS rates were 92% and 90% for the FOLFOX and FOLFOXC groups, respectively. However, we found that the $EGFR^+$ patients in the FOLFOX had a significantly poorer DFS than did those in the FOLFOXC group (13.1 vs. 52.3 months, $p < 0.001$) (Fig. 3C). Furthermore, of 38 patients with relapse in the FOLFOX group, 14 $EGFR^+$ patients (36.8%) experienced relapse between 6

Table 2. Univariate and Multivariable Analysis of Factors Associated With Relapse and Survival in Patients With Stage III Colorectal Cancer

	Relapse				Survival			
Parameters	Univariate Analysis [OR (95% CI)]	*p* Value	Multivariable Analysis [OR (95% CI)]	*p* Value	Univariate Analysis [OR (95% CI)]	*p* Value	Multivariable Analysis [OR (95% CI)]	*p* Value
Age (years)								
65 vs. <65 (79/118)	0.757 (0.419–1.369)	0.358	0.728 (0.371–1.428)	0.356	0.906 (0.462–1.774)	0.773	0.959 (0.444–2.070)	0.915
Gender								
Male vs. female (118/79)	1.448 (0.798–2.625)	0.223	1.221 (0.620–2.405)	0.563	1.243 (0.631–2.449)	0.529	0.849 (0.383–1.883)	0.687
Location								
Right vs. left (52/145)	1.023 (0.533–1.962)	0.946	0.985 (0.469–2.067)	0.968	1.250 (0.605–2.583)	0.546	1.191 (0.515–2.754)	0.683
Tumor size								
5 cm vs. <5 cm (69/128)	0.805 (0.438–1.480)	0.486	0.547 (0.2634–1.136)	0.998	0.945 (0.474–1.883)	0.871	0.786 (0.345–1.78)	0.565
Tumor depth								
T3 + T4 vs. T1 + T2 (171/26)	1.792 (0.759–5.288)	0.160	1.117 (0.397–3.148)	0.106	2.656 (0.760–9.280)	0.126	1.942 (0.478–7.890)	0.353
Lymph node metastasis								
N2 vs. N1 (57/121)	1.596 (0.715–4.493)	0.214	0.997 (0.495–2.009)	0.994	1.615 (0.828–30150)	0.159	1.521 (0.688–3.363)	0.301
Histology								
PD vs. MD + WD (22/175)	1.734 (0.712–4.226)	0.226	1.481 (0.530–4.141)	0.454	1.575 (0.601–4.130)	0.356	1.011 (0.311–3.290)	0.986
Vascular invasion								
Yes vs. no (81/116)	1.109 (0.619–1.987)	0.729	1.421 (0.702–2.874)	0.328	0.963 (0.494–1.877)	0.912	1.062 (0.477–2.366)	0.883
Perineurial invasion								
Yes vs. no (77/120)	1.524 (0.847–2.740)	0.160	1.369 (0.684–2.740)	0.375	1.356 (0.698–2.632)	0.369	0.938 (0.429–2.052)	0.872
Pre-op CEA (ng/ml)								
5 vs. <5 (77/113)	1.762 (0.966–3.214)	0.065	1.547 (0.744–3.217)	0.242	1.752 (0.883–3.476)	0.108	1.404 (0.6618–3.190)	0.418
Post-op CEA (ng/ml)								
5 vs. <5 (31/165)	1.684 (0.778–3.649)	0.186	1.074 (0.409–2.828)	0.885	2.043 (0.895–4.661)	0.090	1.404 (0.496–3.978)	0.523
EGFR expression								
Positive vs. negative (129/68)	2.19 (1.158–4.175)	0.016*	1.947 (0.965–3.927)	0.063	3.917 (1.646–9.316)	0.002*	3.529 (1.399–8.905)	0.008*
Chemotherapy group								
FOLFOX vs. FOLFOXC (87/110)	3.314 (1.724–5.696)	<0.001*	3.026 (1.554–5.892)	0.001*	2.879 (1.458–5.685)	0.002*	2.735 (1.271–5.884)	0.010*

OR, odds ratio; CI, confidence interval; PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated; CEA, carcinoembryonic antigen.
*$p < 0.05$.

Table 3. Univariate and Multivariable Analysis of Prognostic Indicators for Disease-Free Survival and Overall Survival in Patients With Stage III Colorectal Cancer

	Disease-Free Survival				Overall Survival			
Parameters	Univariate Analysis [OR (95% CI)]	*p* Value	Multivariable Analysis [OR (95% CI)]	*p* Value	Univariate Analysis [OR (95% CI)]	*p* Value	Multivariable Analysis [OR (95% CI)]	*p* Value
Age (years)								
65 vs. <65 (79/118)	0.753 (0.470–1.207)	0.239	0.666 (0.399–1.111)	0.119	0.815 (0.452–1.470)	0.498	0.777 (0.409–1.474)	0.440
Gender								
Male vs. female (118/79)	1.447 (0.899–2.330)	0.128	1.349 (0.798–2.279)	0.264	1.316 (0.723–2.397)	0.369	0.886 (0.442–1.777)	0.734
Location								
Right vs. left (52/145)	1.021 (0.612–1.704)	0.936	0.981 (0.562–1.712)	0.946	1.224 (0.655–2.287)	0.527	1.146 (0.558–2.353)	0.710
Tumor size								
5 cm vs. <5 cm (69/128)	0.853 (0.525–1.386)	0.521	0.643 (0.367–1.126)	0.122	0.876 (0.479–1.605)	0.669	0.632 (0.308–1.298)	0.212
Tumor depth								
T3 + T4 vs. T1 + T2 (171/26)	1.858 (0.853–4.047)	0.119	1.314 (0.565–3.056)	0.526	2.692 (0.835–8.673)	0.097	2.283 (0.626–8.325)	0.211
Lymph Node metastasis								
N2 vs. N1 (71/126)	1.246 (0.783–1.984)	0.353	1.137 (0.664–1.947)	0.641	1.829 (1.026–3.261)	0.041*	1.828 (0.919–3.637)	0.085
Histology								
PD vs. MD + WD (22/175)	1.646 (0.868–3.122)	0.127	1.525 (0.724–3.212)	0.267	1.685 (0.754–3.763)	0.203	1.328 (0.477–3.699)	0.588
Vascular invasion								
Yes vs. no (81/116)	1.051 (0.665–1.661)	0.832	1.304 (0.754–2.255)	0.342	1.034 (0.577–1.853)	0.911	1.010 (0.502–2.031)	0.977
Perineurial invasion								
Yes vs. no (67/120)	1.391 (0.883–2.192)	0.155	1.198 (0.715–2.008)	0.492	1.475 (0.829–2.622)	0.186	1.008 (0.516–1.970)	0.981
Pre-op CEA (ng/ml)								
5 vs. <5 (77/113)	1.540 (0.960–2.469)	0.073	1.296 (0.743–2.262)	0.361	1.589 (0.873–2.894)	0.130	1.322 (0.638–2.738)	0.453
Post-op CEA (ng/ml)								
5 vs. <5 (31/165)	1.617 (0.917–2.852)	0.097	1.271 (0.640–2.526)	0.493	1.968 (0.997–3.884)	0.051*	1.463 (0.609–3.515)	0.395
EGFR expression								
Positive vs. negative (129/68)	1.951 (1.148–3.317)	0.014*	1.914 (1.076–3.405)	0.027*	4.203 (1.861–9.493)	0.001*	4.417 (1.813–10.761)	0.001*
Chemotherapy group								
FOLFOX vs. FOLFOXC (87/110)	2.995 (1.878–4.778)	<0.001*	3.351 (2.000–5.614)	<0.001*	2.759 (1.516–5.020)	0.001*	3.186 (1.631–6.222)	0.001*

OR, odds ratio; CI, confidence interval; PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated; CEA, carcinoembryonic antigen.
*$P < 0.05$.

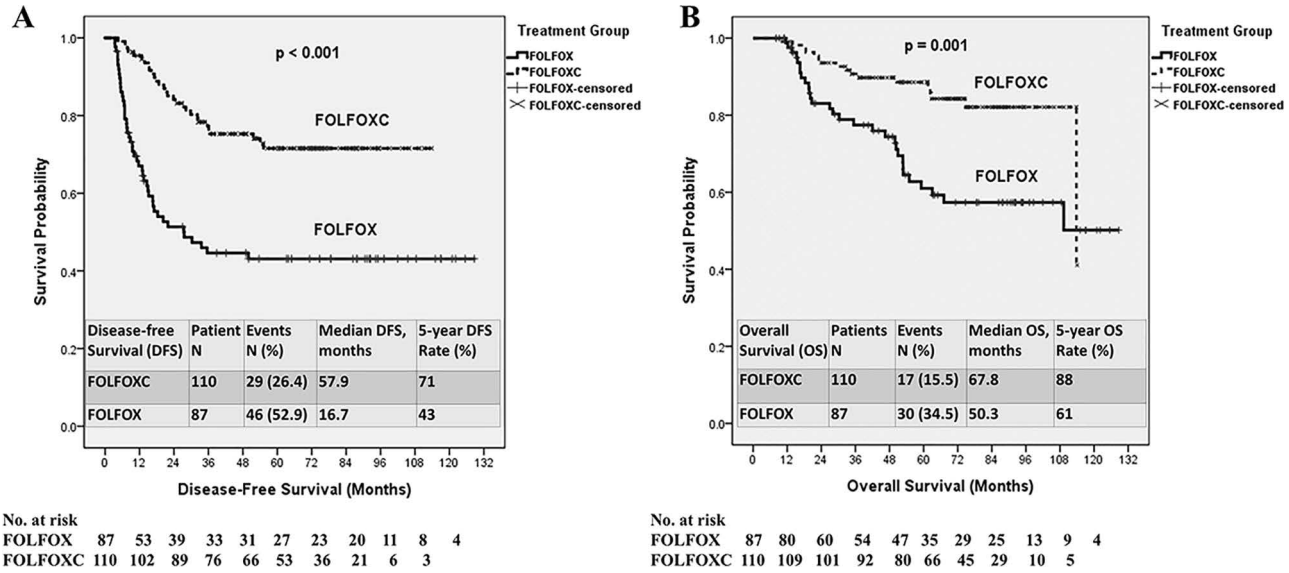

Figure 2. Kaplan–Meier survival curve for patients with stage III colorectal cancer stratified by treatment group. (A) Disease-free survival ($p < 0.001$). (B) Overall survival ($p = 0.001$).

and 12 months postoperatively; by contrast, of 19 patients with relapse in the FOLFOXC group, 4 $EGFR^+$ patients (21.1%) experienced relapse between 6 and 12 months postoperatively. However, 37 of 38 patients (97.4%) and 16 of 19 $EGFR^+$ patients (84.2%) with relapse in the FOLFOX and FOLFOXC groups, respectively, experienced relapse within 3 years postoperatively. The patients in the FOLFOX group also had significantly poorer OS than did those in the FOLFOXC group (42.0 vs. 61.5 months, $p < 0.001$) (Fig. 3D). The 5-year DFS rates were 31% and 71% for the FOLFOX and FOLFOXC groups, respectively, and the 5-year OS rates were 45% and 87% for the FOLFOX and FOLFOXC groups, respectively.

Characterization of EGFR-Knockout Caco2 Cell Lines

In this study, we used CRISPR gRNA vectors (OriGene) to target the EGFR protein and generate truncated EGFR mutants in Caco2 cells. After screening, we identified one clone with heterozygous deletion. The heterozygous knockout status was confirmed using Western blotting (Fig. 4A).

Effect of 5-FU on Caco2 Cells Proliferation and Viability

To analyze the suppressive effects of 5-FU (Sigma-Aldrich) on the proliferation of the control and *EGFR*-knockout Caco2 cells, we performed the MTS assay to determine the in vitro viability of scrambled control and *EGFR*-knockout Caco2 cells at 0, 24, 48, and 72 h after 5-FU (Sigma-Aldrich) treatment. We observed that the *EGFR*-knockout Caco2 cells exhibited significantly lower viability at 24 h ($p < 0.05$; −11.3%), 48 h ($p < 0.001$; −28.6%), and 72 h ($p < 0.001$; −32%) after 5-FU treatment compared with the control cells (Fig. 4B). These results indicate that the *EGFR*-knockout Caco2 cells were more sensitive to the antiproliferative effects of 5-FU than the scrambled control Caco2 cells.

Effect of 5-FU on the Migration of Caco2 Cells

A wound-healing assay was performed to examine the effects of 5-FU on the migration of Caco2 cells. The results revealed that the *EGFR*-knockout Caco2 cells exhibited significantly lower migration abilities 24 h after 5-FU treatment compared with the scrambled control cells (Fig. 4C). These results signify that the *EGFR*-knockout Caco2 cells were more sensitive to the migration inhibitory effects of 5-FU than the scrambled control Caco2 cells.

Inhibiting Effects of 5-FU on Tumor Growth in Xenograft Mouse Model

To evaluate the inhibitory effects of 5-FU on tumor growth in vivo, the *EGFR*-knockout and scrambled control Caco2 cells were implanted subcutaneously in 7-week-old male nude mice at the bottom left or bottom right flanks (Fig. 4D). The tumors were palpable at 28 days after inoculation and were allowed to grow for 61 days (Fig. 4E and F). On day 35, scrambled control and *EGFR*-knockout groups were randomly divided into 5-FU-treated and 5-FU-nontreated groups. The mice were treated according to their allocated treatment groups, and tumor burden was quantitated. We found that the mice injected with the *EGFR*-knockout Caco2 cells had significantly smaller tumors than did those injected with

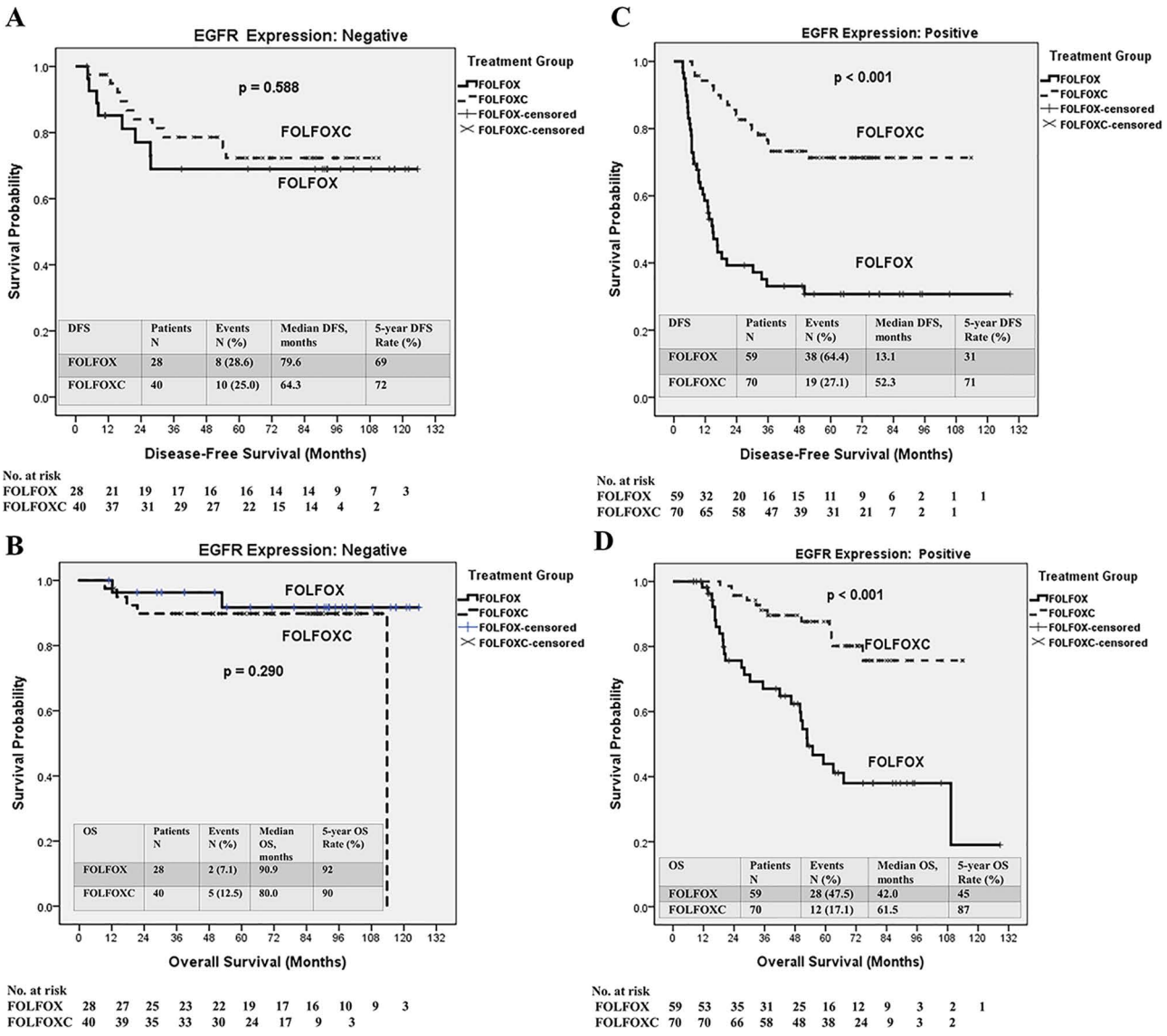

Figure 3. Kaplan–Meier survival curve for patients with stage III colorectal cancer stratified by treatment group and epidermal growth factor receptor (EGFR) expression. (A) Disease-free survival of patients with negative EGFR expression stratified by treatment group ($p = 0.588$). (B) Overall survival of patients with negative EGFR expression stratified by treatment group ($p = 0.290$). (C) Disease-free survival of patients with positive EGFR expression stratified by treatment group ($p < 0.001$). (D) Overall survival of patients with positive EGFR expression stratified by treatment group ($p < 0.001$).

the scrambled control Caco2 cells ($p = 0.033$) on day 38. The tumors were the smallest in the 5-FU-treated *EGFR*-knockout group on day 61 (Fig. 4E and F). These results provide evidence that *EGFR*-knockout enhanced the anti-proliferative effects of 5-FU in vivo.

DISCUSSION

Postoperative adjuvant chemotherapy can improve the survival of patients with stage III CRC, especially when such a chemotherapy regimen is combined with oxaliplatin[5–7,9,10]. However, most patients with stage III CRC develop local recurrences or distant metastases within the first 3 years after radical resection[10,11]. Therefore, whether administering maintenance chemotherapeutic agents after 6 months of postoperative adjuvant chemotherapy with an oxaliplatin-based regimen can decrease the risk of local recurrence or distant metastasis in such patients is an appealing topic. In this regard, metronomic maintenance therapy using orally administered fluoropyrimidine agents, such as capecitabine, would be a feasible option for such patients. Although studies on capecitabine metronomic therapy for patients with CRC are limited (most are given to patients with mCRC or elderly patients with advanced CRC), capecitabine has been shown to be

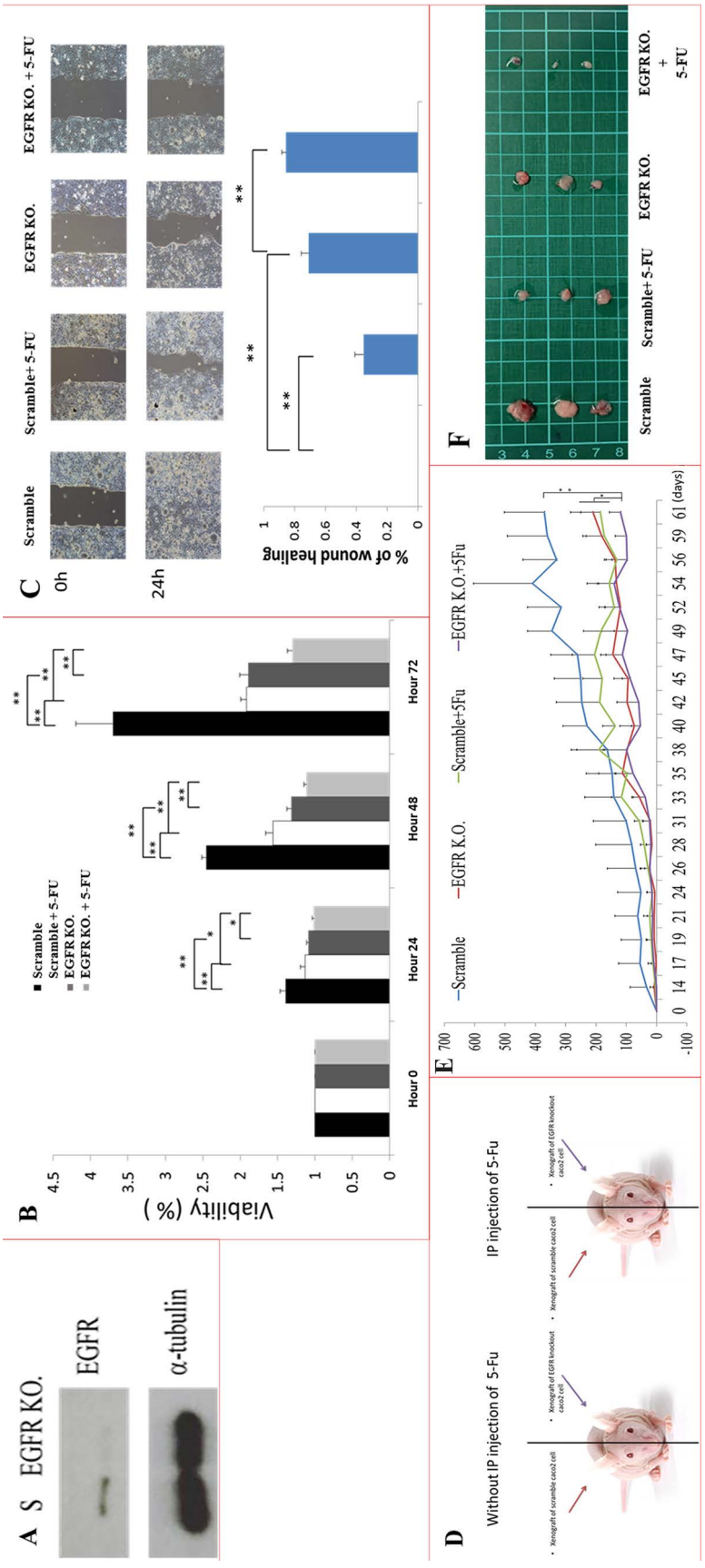

Figure 4. Effects of 5-fluorouracil (5-FU; Sigma-Aldrich) on the proliferation, viability, and migration abilities of Caco2 cells. (A) The protein level of EGFR in Caco2 cells decreased after CRISPR knockout. Protein level was detected by Western blotting. (B) The viability of the Caco2 cells decreased significantly in 5-FU-treated *EGFR*-knockout Caco2 cells at 24 h ($^*p < 0.05$; −11.3%), 48 h ($^{**}p < 0.001$; −28.6%), and 72 h ($^{**}p < 0.001$; −32%). (C) The migration ability of the Caco2 cells decreased significantly in 5-FU-treated *EGFR*-knockout Caco2 cells at 24 h. $^*p < 0.05$; $^{**}p < 0.001$. (D) Scrambled control and *EGFR*-knockout Caco2 cells was implanted subcutaneously in the bottom left or right flank of each 7-week-old male nude mouse. The 5-FU was injected intraperitoneally at day 35 after the implantation of Caco2 cells. (E) The tumor volume was measured thrice a week for 61 days. The tumor growth curve is shown for the scramble control group (scramble; blue line), *EGFR*-knockout group (red line), 5-FU-treated scramble control group (green line), and 5-FU-treated *EGFR*-knockout group (purple line). (F) Compared with the control group, tumor lumps were smaller in the 5-FU-treated scramble control group and the EGFR-knockout group; the smallest tumor lumps were in the 5-FU-treated scramble control group. S: Scrambled control Caco2 cells; EGFR KO: EGFR-knockout Caco2 cells.

effective when used in a postoperative adjuvant manner for patients with stage III colon cancer[16–18,23].

Of the 197 patients enrolled in the present study, 87 received only an adjuvant oxaliplatin-based regimen (FOLFOX group) and 110 received oral capecitabine as metronomic maintenance therapy after the adjuvant oxaliplatin-based regimen (FOLFOXC group). IHC analysis revealed that 129 (65.5%) patients exhibited positive EGFR expression. No significant difference in EGFR expression was observed between the FOLFOX and FOLFOXC groups. However, the FOLFOX group had a significantly higher proportion of patients who developed postoperative relapse compared with the FOLFOXC group. Most cases of relapse (92.0%, 69/75) occurred within 3 years postoperatively, which is consistent with the literature.[10] However, a higher proportion of patients experienced relapse in the FOLFOX group than in the FOLFOXC group within 3 years postoperatively (97.8% vs. 82.7%). The disparity in the number of censored patients is responsible for an artificial separation here (a more heavily censored group will have fewer patients at risk for each subsequent interval, and thus, each subsequent event will produce a much larger interval or steeper curve). Therefore, in the FOLFOX group, the 3-year DFS was 45%, which was lower than that reported in the literature[8–11], but the 5-year DFS was 43%. In the FOLFOXC group, the 3-year DFS was 77% and the 5-year DFS was 71%, consistent with those reported in previous reports[8–11,24]. Huang et al.[24] also reported 62.3% 5-year DFS in the comparison group (without UFUR) and 69.1% 5-year DFS in the UFUR group. Furthermore, the mortality rate was significantly higher in the FOLFOX group than in the FOLFOXC group. Using univariate and multivariable analyses, we observed that metronomic maintenance therapy with capecitabine was an independent and favorable predictive factor for reduced postoperative relapse and mortality ($p = 0.001$ and $p = 0.013$, respectively). Using Kaplan–Meier survival analysis, we also observed that metronomic maintenance therapy with capecitabine was an independent prognostic factor for both DFS and OS ($p < 0.001$ and $p = 0.001$, respectively). Furthermore, we observed significant differences in DFS and OS between the two groups in patients with positive EGFR expression, but not in those with negative EGFR expression. However, in patients with positive EGFR expression, a higher proportion of patients experienced relapse in the FOLFOX group than in the FOLFOXC group within 3 years postoperatively (97.4% vs. 84.2%).

Lymphovascular invasion is a major poor prognostic factor in patients with CRC.[25–29] Although lymphovascular invasion was more common in the FOLFOXC group than in the FOLFOX group, our results reveal that the FOLFOXC group had significantly fewer patients who developed postoperative relapse compared with the FOLFOXC group. Moreover, we demonstrated that metronomic maintenance therapy with capecitabine was independently associated with relapse and DFS. These results suggest that metronomic maintenance therapy with capecitabine can inhibit postoperative relapse. Simkens et al. conducted a phase 3 randomized controlled trial (CAIRO3) and demonstrated that metronomic maintenance treatment with capecitabine plus bevacizumab significantly improved the progression-free survival (PFS) of patients compared with the PFS of an observation group[30]. Another randomized controlled trial conducted by Luo et al. revealed a significantly longer PFS in the capecitabine maintenance group compared with another group[31]. Similarly, several in vivo and in vitro studies have demonstrated the inhibitory effects of metronomic maintenance therapy with capecitabine on the proliferation and metastasis of gastric cancer cells[32], colon cancer cells[33,34], and breast cancer cells[34,35]. In the present study, we noted that the 5-year OS rate was significantly lower in the patients in the FOLFOX group than in those in the FOLFOXC group. We also observed that metronomic maintenance therapy with capecitabine was an independent prognostic factor for OS. Therefore, metronomic maintenance therapy with capecitabine resulted in better DFS and OS. Our results are in line with those reported by Huang et al.[14,24], although these two studies have used tegafur-uracil (UFUR; TTY Biopharm Co., Taiwan) as metronomic maintenance therapy instead of capecitabine.

We performed subgroup analyses according to tumor EGFR expression and treatment group to determine the predictive factors for postoperative relapse and mortality. According to our results, significant differences in the 5-year DFS and OS rates between the FOLFOX and FOLFOXC groups were evident in $EGFR^+$ patients, not in $EGFR^-$ patients. Therefore, although the $EGFR^+$ patients had worse prognoses, capecitabine metronomic maintenance therapy could effectively compensate and improve their prognoses to the same level as that of the $EGFR^-$ patients. We found that the $EGFR^-$ patients did not benefit from capecitabine metronomic maintenance therapy in terms of survival. Thus, we determined that only the $EGFR^+$ patients could benefit from metronomic maintenance therapy, which has not been reported in previous studies[14,35].

The present study has some limitations. First, this was a single-center study with a relatively small sample size and a selection bias of treatment regimen. Second, we categorized EGFR expression based on the results of IHC analysis, but we did not evaluate the mRNA expression levels in patients. Third, we did not measure the toxicity of capecitabine treatment in the two groups. Nevertheless, our study provided several important findings.

In summary, we demonstrated that metronomic maintenance therapy with capecitabine can significantly improve the prognoses of patients with stage III CRC

following radical resection and FOLFOX adjuvant chemotherapy. Moreover, the extent of prognosis improvement is substantial in patients with positive EGFR expression. However, a prospective, randomized clinical trial is necessary to verify our results.

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