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Article

Predictive Values of EGFR Expression for the Fluoropyrimidine Metronomic Maintenance Therapy in Patients with Stage III Colorectal Cancer after Radical Resection and Adjuvant Oxaliplatin-Based Chemotherapy

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Abstract: *Background:* This retrospective study evaluates the survival effects of metronomic maintenance therapy with fluoropyrimidine in patients with stage III colorectal cancer (CRC) according to epidermal growth factor receptor (EGFR) expression. *Methods:* 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 on survival according to treatment group and EGFR expression were analyzed. By conducting an i*n vitro* cell line study and in *vivo* study through knockdout of *EGFR* gene, we analyzed the capacities of cell proliferation and migration. *Results:* Postoperative relapse and mortality were significantly more common in the FOLFOX group. Metronomic maintenance therapy was a significantly independent predictive factor of postoperative relapse and mortality, as well as a prognostic factor of disease-free survival and overall survival. The significant differences of survival between the

two groups were only observed in patients with positive EGFR expression. *Conclusions:* The present study suggested EGFR expression as the prognostic factor in patients with stage III CRC receiving metronomic maintenance therapy. By analyzing EGFR expression, we can identify the potential candidates with optimal survival benefit from metronomic maintenance therapy in patients with stage III CRC.

Keywords: fluoropyrimidine; metronomic maintenance therapy; oxaliplatin-based regimen; stage III colorectal cancer; epidermal growth factor receptor

1. 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 an 830,000 CRC-related deaths were reported in 2016 [1]. In the United States, 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 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 SEER (Surveillance, Epidemiology, and End Results) data, 39% of CRC cases are localized-stage disease at diagnosis. 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 provides a significant survival improvement in patients with stage III CRC after radical surgery [5–7]. MOSAIC trials have demonstrated significant 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 postoperative adjuvant chemotherapy 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% of recurrences occurred within the first 3 years in patients with stage III colon cancers and 74% of recurrences occurred within the first 3 years in patients with stage II colon cancers [10,11]; the peak incidence of recurrence was between 1 and 2 years after initial treatment [10]. Because of their similar benefit on survival, most postoperative adjuvant chemotherapy regimens are administrated for 6 months [7,12,13]. Therefore, for patients with stage III CRC, metronomic maintenance therapy with orally administrated 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-FU which has been reported to be 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 a 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 postoperative 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 chemotherapy with the FOLFOX regimen [20]. Accordingly, we conducted the present retrospective study to evaluate the survival effects of metronomic maintenance therapy with capecitabine after

adjuvant oxaliplatin-based regimen therapy in patients with stage III CRC who had undergone radical resection; the conducted this evaluation according to EGFR expression levels. We also investigated the mechanistic connections between 5-FU and EGFR by conducting i*n vitro* CRC cell line and *in vivo* animal studies.

2. Materials and Methods

2.1. Patients

In the retrospective study, we analyzed 197 patients with histologically confirmed stage III CRC who had received surgical treatment from a single institution between January 2008 and June 2012. 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. All 197 patients with stage III CRC in the present study had received adjuvant chemotherapy with the FOLFOX regimen after radical surgery. The present study was approved by the institutional review board of Kaohsiung Medical University Hospital (KMUHIRB-E-20150003).

2.2. Chemotherapy Treatment Groups

The adjuvant oxaliplatin-based regimen was mFOLFOX as follows: each cycle of FOLFOX consisted of oxaliplatin (85 mg/m^2) on day 1, folinic acid (400 mg/m^2), and a 46-hour infusion of 5-FU (2800 mg/m^2) repeated every 2 weeks), biweekly for 12 cycles. Of 197 patients, 87 patients (44.7%) received only adjuvant oxaliplatin-based regimen (FOLFOX group), and 110 patients (55.8%) received oral capecitabine after adjuvant oxaliplatin-based regimen (FOLFOXC group). Oral capecitabine were administered at a total daily dose of 850 mg/m^2, twice daily, on days 1-14 days every 3 weeks for 6 months. After the detailed information on potential benefits or disadvantages were explained to the patients, they provided oral consent to receive capecitabine.

2.3. Patient follow-up

Patients were regularly followed up with clinical outcomes and survival statuses. Cliniopathological variables included age at diagnosis, sex, 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) [34]. Right-sided colon cancers were defined as those located in the cecum, ascending colon, hepatic flexure, and transverse colon, whereas 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.

The postoperative 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. Disease-free survival (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. Overall survival (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.

2.4. Immunohistochemical (IHC) analysis of EGFR expression

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. Then, DAKO REAL EnVision Detection System-HRP (Dako) was applied. Finally, the sections were incubated in 3′,3′-diaminobenzidine, before being counterstained with Mayer's hematoxylin, dehydrated through two changes of 95 % ethanol and

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two changes of 100 % ethanol, and 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 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].

2.5. In Vitro Cell Line Experiments and in Vivo Experiments

2.5.1. Cell culture and Antibodies

The human colon cancer cell line Caco-2 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). Protein assay kit was bought from Bio-Rad (Berkeley, CA, USA). An enhanced chemiluminescence kit, 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)-2H-tetrazolium) (Sigma-Aldrich) and EGFR Human Gene Knockout Kit (Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR) were purchased from Sigma-Aldrich (Gillingham, UK) and OriGene (Rockville, MD, USA), respectively. The Caco-2 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. (Gillingham, UK).

2.5.2. EGFR knockout

The processes of *EGFR*-knockout were performed following the instruction of manufacture with little modification. Before transfection, Caco2 cells were seeded 1×10^5 per well of 6 wells. 24 hours later, Caco2 cells were transfected with 1μg of CRISPR gRNA vectors (gRNA sequence-5'TCCTCCAGAGCCCGACTCGC3') and scrambled control (scrambled sequence-5'GCACTACCAGAGCTAACTCA3') with Lipofectamine 3000 ® (ThermoFisher Scientific). After 72 hours of incubation, cells were spited 1:10, grown for additional 3 days; and then spited the cells again. After the Caco2 cells were split for 7 times, puromycin was added for selection and the knockout clones were identified by western blot.

2.5.3. Western blotting

Whole cell lysates were prepared using RIPA lysis buffer RIPA 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 were perform as previously described [20].

2.5.4. MTS 3 - (4,5 – dimethylthiazol – 2 -yl) – 5 - (3 - carboxymethoxyphenyl) – 2 - (4 - sulfophenyl) - 2Htetrazolium) cell viability assay.

Transfected Caco2 cells were seeded in 96 wells (5×10^4 cells per well) and incubated at 37°C. After cells adhesion (as 0h), the transfected Caco2 cells were treated with 5-FU (Sigma-Aldrich, Gillingham, UK) (10uM/ml) and incubated at 37°C for 24 hours, 48 hours, and 72 hours. MTS was added at hour 0, hour 24, hour 48, and hour 72. Thereafter, the cells were incubated in 37°C for 3 hours and, then, were quantitated spectrophotometrically by using wavelength of 490 nm.

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2.5.5. Migration assay

Cell migration was assessed using a wound-healing assay [35]. 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 hour 0 and 24 after being wounding, each wound was taken three pictures on different areas under a bright field microscopy. Each picture was measured with image J software. Data were showed as a percentage of wound closure compared to initial wound.

2.5.6. In vivo Animal Studies

Six-week-old Balb/c male nude mice were purchased from BioLasco Taiwan Co., Ltd (Taipei, Taiwan). At 7 weeks of age, each nude mouse was implanted subcutaneously with scrambled control and *EGFR* knockout Caco2 cells in the bottom left or right flanks of 7-week old male nude mouse. The tumor size (cm^3) was measured thrice a week and calculated according to the formula: (length×width ^{2})/2. Following 4 weeks after transplantation, 5-FU (10 mg/Kg) was administrated by intraperitoneal injection thrice a week for 3 weeks. Animals were scarified at 8 weeks after injection of tumor cells. To perform the in vivo study, we followed the protocols approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University (IACUC Approval No: 105229) in according with the Guiding Principles with the Care and Use of Laboratory.

2.6. 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 t test for continuous variables. Univariate and multivariable logistic regression models were used to evaluate the independent predictors of postoperative relapse and postoperative mortality. A Cox proportional hazard model was used for univariate and multivariable analyses 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 p value less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical and Pathological Characteristics of Patients with Stage III CRC Between The Two Treatment Groups

The clinical and pathological characteristics of the 197 patients (Figure 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 (range, 30–82) years. Among all patients, 87 (44.2%) received only an adjuvant oxaliplatin-based 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 (range, 30–81) years, and that in the FOLFOXC group was 63 (range, 35–82) years. For all 197 patients, the median follow-up duration was 61.2 (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-positive); this EGFR expression pattern was did not differ significantly between the FOLFOX and FOLFOXC groups (p = 0.540; Table 1).

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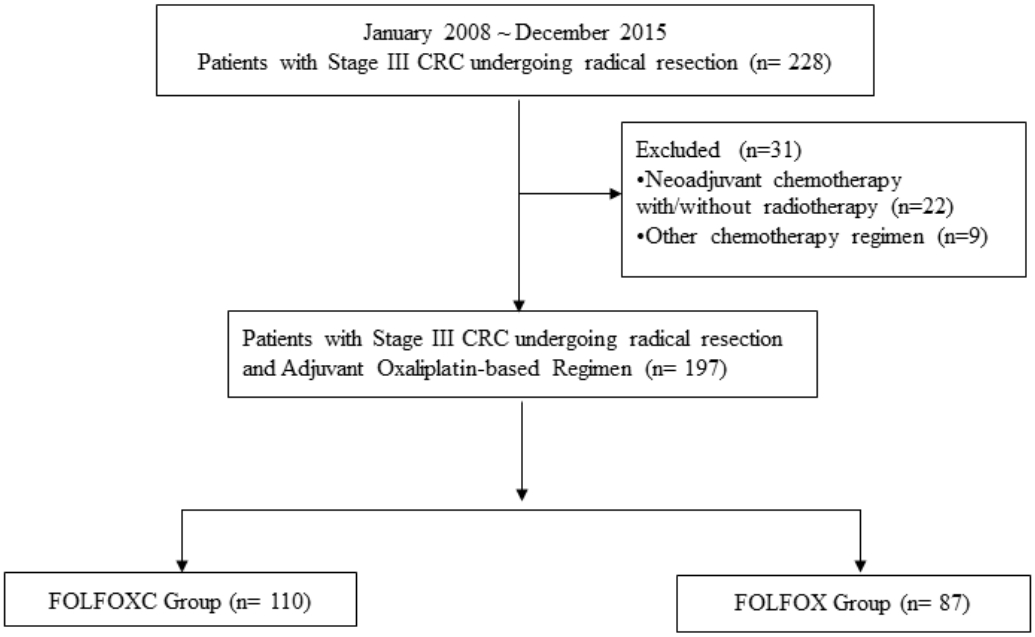

Figure 1. CONSORT diagram showing the inclusion and exclusion criteria in the present study.

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 postoperative relapse; by contrast, in the FOLFOXC group, only 29 patients (26.4%) developed postoperative 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, sex, 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 $p > 0.05$).

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Table 1. Baseline Characteristics of Patients with Stage III Colorectal Cancer According to Treatment Group.

Characteristic	FOLFOX Group (N=87, %)	FOLFOXC Group (N=110, %)	*p* value
Age			
< 65 years	51 (58.6)	67 (60.9)	0. 745
≥ 65 years	36 (41.4)	43 (30.1)	
Gender			
Female	30 (34.5)	49 (44.5)	0.152
Male	57 (65.5)	61 (55.5)	
Tumor size			
<5 cm	54 (62.1)	74 (67.3)	0.447
≥5 cm	33 (37.9)	36 (32.7)	
EGFR expression			
Positive	59 (67.8)	70 (63.6)	0.540
Negative	28 (32.2)	40 (36.4)	
Tumor location			
Right-sided colon	23 (26.4)	29 (26.4)	0.991
Left-sided colon	64 (73.6)	81 (73.6)	
Histology			
WD	11 (12.6)	2 (1.8)	0.813
MD	74 (85.1)	97 (88.2)	
PD	2 (2.3)	11 (10.0)	
Tumor depth			
T1 + T2	9 (106.3)	17 (15.5)	0.293
T3 + T4	782 (89.7)	93 (84.5)	
Lymph node metastasis			
N1	57 (65.5)	69 (62.7)	0.685
N2	30 (34.5)	41 (37.30)	
Vascular invasion			
No	59 (67.8)	57 (51.8)	0.023*
Yes	28 (32.2)	53 (48.2)	
Perineural invasion			
No	52 (59.8)	58 (61.8)	0.770
Yes	35 (40.2)	42 (38.2)	
Preoperative Serum CEA level			
<5 ng/ml	42 (51.9)	71 (65.1)	0.065
≥5 ng/ml	39 (48.1)	38 (34.9)	
Postoperative Serum CEA[a] level			
<5 ng/ml	70 (81.4)	95 (86.4)	0.344
≥5 ng/ml	16 (18.6)	16 (13.6)	
Postoperative relapse			
No	41 (47.1)	81 (73.6)	< 0.001*
Yes	46 (52.9)	29 (26.4)	
Mortality			
No	59 (67.8)	96 (87.3)	0.001*
Yes	28 (32.2)	14 (12.7)	

Abbreviations: CEA: carcinoembryonic antigen; EGFR: epidermal growth factor receptor; MD: moderately differentiated; PD: poorly differentiated; SD: standard deviation; WD: well differentiated.

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3.2. Univariate and Multivariable Analyses of Predictive Factors for Postoperative Relapse and Postoperative Mortality

To identify independent predictive factors for postoperative relapse and postoperative mortality in the 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 postoperative relapse and clinicopathological features, the EGFR-positive patients had a 2.2-fold higher risk of postoperative relapse than did the EGFR-negative patients (p = 0.016). Moreover, the patients in the FOLFOX group had a 3.3-fold higher risk of postoperative relapse than did those in the FOLFOXC group ($p < 0.001$). The multivariate analysis of the correlation between postoperative relapse and clinicopathological features indicated that metronomic maintenance therapy with capecitabine was an independent predictive factor for postoperative relapse (p = 0.001; odds ratio [OR], 3.026; 95% confidence interval [CI], 1.554–6.678; Table 2). Furthermore, according to the univariate analysis of the correlation between postoperative mortality and clinicopathological features, the EGFR-positive patients had a 3.9-fold higher risk of postoperative mortality than did the EGFR-negative patients (p = 0.002). The multivariate analysis of the correlation between postoperative relapse and clinicopathological features also indicated that EGFR expression and capecitabine metronomic maintenance therapy were independent predictive factors for postoperative mortality (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).

3.3. Univariate and Multivariable Analyses of Survival of Patients with Stage III CRC

To investigate the independent predictive factors for OS and DFS in the 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 2B). 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. The 5-year OS rates were 58% and 84% 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-negative patients between the FOLFOX and FOLFOXC groups (Fig. 3A and 3B); however, we observed significant differences in the DFS (Fig. 3C) and OS (Fig. 3D) of the EGFR-positive patients between the FOLFOX and FOLFOXC groups. Specifically, the EGFR-negative 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-positive 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). 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 39% and 79% for the FOLFOX and FOLFOXC groups, respectively.

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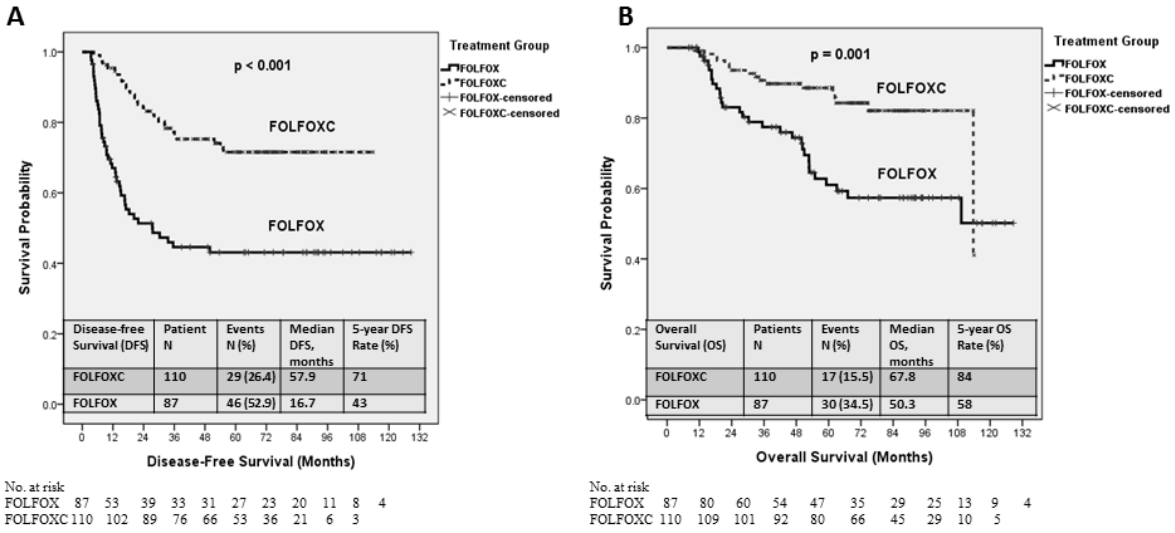

Figure 2. Kaplan–Meier survival curve for patients with stage III colorectal cancer stratified by treatment group. (A) Disease-free survival ($p < 0.001$). (B) Overall survival ($p = 0.001$).

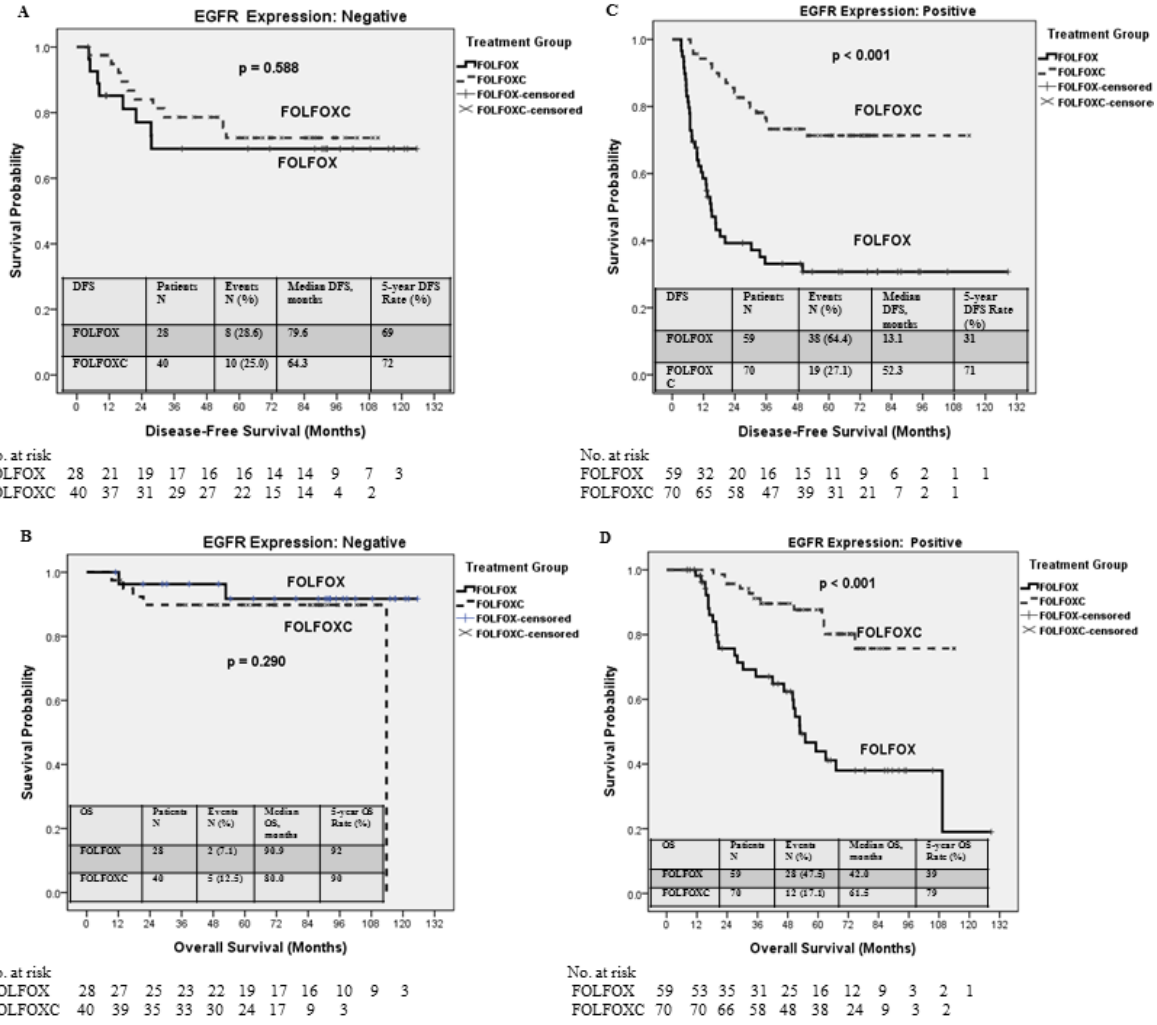

Figure 3. Kaplan–Meier survival curve for patients with stage III colorectal cancer stratified by treatment group and EGFR expression. (A) Disease-free survival of patients with negative EGFR expression stratified by treatment group ($p = 0.588$). (B) Overall survival of patients with negative EGFR expression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

3.4. In Vitro Cell Line and In Vivo Experiments

3.4.1. Characterization of EGFR knockout Caco2 cell lines

In this study, we used CRISPR gRNA vectors (OriGene, Rockville, MD, USA) 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. 3A).

3.4.2. Effect of 5-FU on Caco2 cells proliferation and viability

To analyze the suppressive effects of 5-FU (Sigma-Aldrich, Gillingham, UK) 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 hours after 5-FU (Sigma-Aldrich, Gillingham, UK) treatment. We observed that the *EGFR* knockout Caco2 cells exhibited significantly lower viability at 24 (*$p < 0.05$; -11.3%), 48 (**$p < 0.001$; -28.6%) and 72 (**$p < 0.001$; -32%) hours after 5-FU treatment compared with the control cells (Fig. 3B). 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.

3.4.3. 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 hours after 5-FU treatment compared with the scrambled control cells (Fig. 3C). 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.

3.4.4. 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. 3D). The tumors were palpable at 28 days after inoculation and were allowed to grow for 61 days (Fig. 3E and 3F). On day 35, scrambled control and *EGFR* knockout groups were randomly divided into 5-FU-treated and 5-FU-non-treated 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 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. 3E and 3F). These results provide evidence that *EGFR* knockout enhanced the antiproliferative effects of 5-FU *in vivo*.

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Table 2. Univariate and Multivariable Analysis of Predictive factors on Relapse and Mortality in Patients with Stage III Colorectal Cancer.

Arameters	Postoperative Relapse				Postoperative Mortality			
	Univariate analysis		Multivariable analysis		Univariate analysis		Multivariable analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value	OR (95% CI)	*p* value	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 <5cm (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*

Abbreviations: AJCC: American Joint Commission on Cancer; PD: poorly differentiated, MD: moderately differentiated, WD: well differentiated; CEA: carcinoembryonic antigen; OR: odd ratio; CI: confidence interval, *$p < 0.05$.

Table 3. Univariate and Multivariate Analyses of the Prognostic Indicators for Disease-free Survival and Overall Survival in Patients with Stage III Colorectal Cancer.

Parameters	Disease-free Survival				Overall survival			
	Univariate analysis		Multivariable analysis		Univariate analysis		Multivariable analysis	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (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 <5cm (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 (51/121)	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*

Abbreviations: AJCC: American Joint Commission on Cancer; PD: poorly differentiated, MD: moderately differentiated, WD: well differentiated; CEA: carcinoembryonic antigen; OR: odd ratio; CI: confidence interval, *$p < 0.05$.

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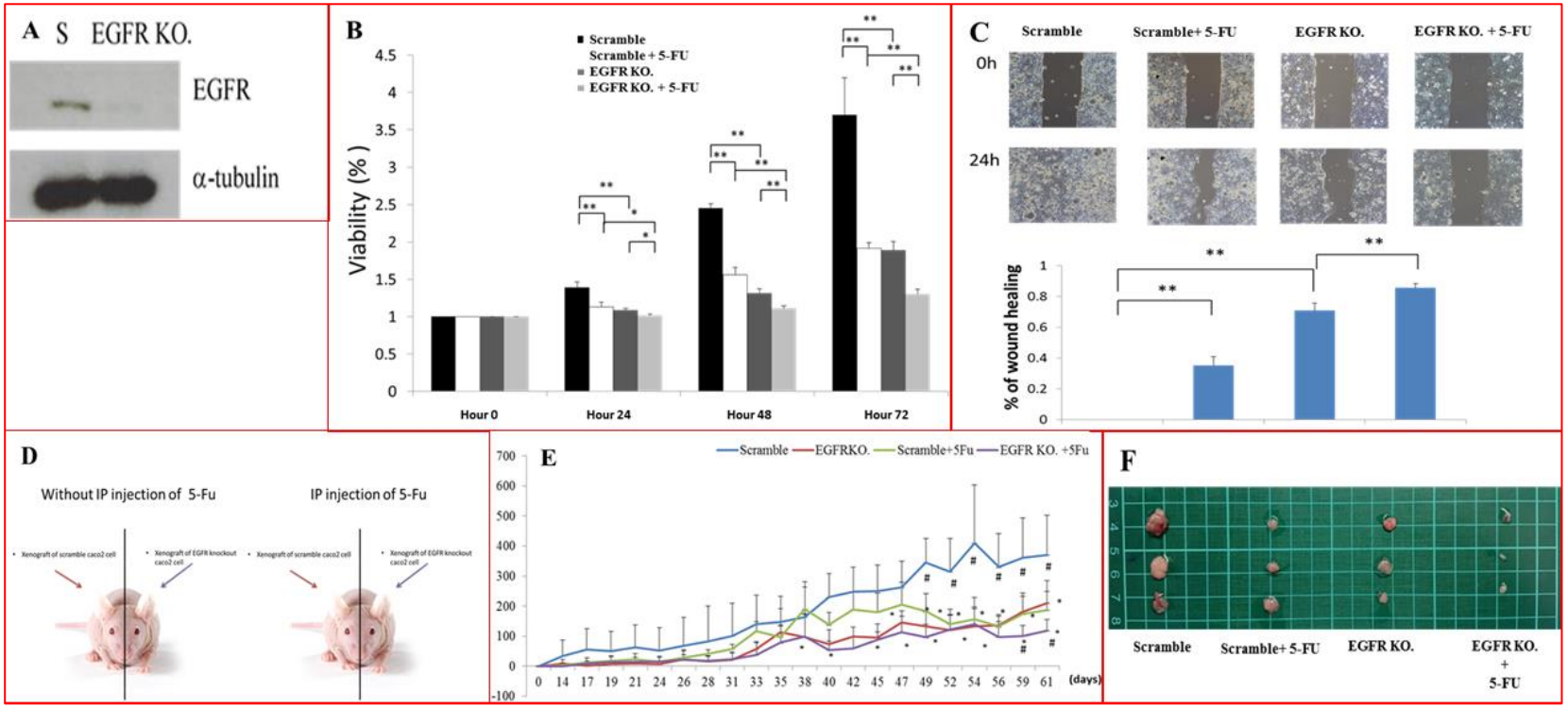
Figure 4. The effects of 5-Fluorouracil (5-FU) (Sigma-Aldrich, Gillingham, UK) on the proliferation, viability, and migration abilities of Caco2 cells. (A) The protein level of EGFR in Caco2 cells decreased after CRISPR Knockout. Protein level was detected by western blotting. (B) The viabilities of the Caco2 cells decreased significantly in 5-FU treated *EGFR* knockout Caco2 cells at hour 24 (*p<0.05; -11.3%), hour 48 (**p<0.001; -28.6%) and hour 72 (**p<0.001; -32%). (C) The migration abilities of the Caco2 cells decreased significantly in 5-FU treated *EGFR* knockout Caco2 cells at hour 24. (D) Each 7 weeks old male nude mouse was implanted subcutaneously with scrambled control and *EGFR* knockout Caco2 cells in the bottom left or right flanks. 5-FU was injected intraperitoneally at one side of the bottom at day 35 after implantation of Caco2 cells. (E) The tumor volume was measured thrice a week for 61 days. The tumor growth curve was showed for scramble control group (scramble; blue line), *EGFR* knockout group (red line), 5-FU treated scramble control group (green line), and 5-FU treated *EGFR* KO Group (purple line). (F) Compare to control group, the tumor lumps were smaller in 5-FU treated scramble control group and EGFR KO group; the smallest tumor lumps were in 5-FU treated scramble control group. (S: Scrambled control Caco2 cells; EGFR KO: EGFR knockout Caco2 cells. * ***p*<0.05;** ** ***p*<0.001**



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4. 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 effective when used in a postoperative adjuvant manner for patients with stage III colon cancer [16–18,21].

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. 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 the significant differences of DFS and OS between the two groups only in patients with positive EGFR expression, but not in those with negative EGFR expression.

Lymphovascular invasion is a major poor prognostic factor in patients with CRC [22–26]. 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 an independent predictive factor for postoperative 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 the PFS of an observation group [27]. Another randomized controlled trial conducted by Luo *et al*. revealed a significantly longer PFS in the capecitabine maintenance group compared with another group [28]. 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 [29], colon cancer cells [30,31], and breast cancer cells [32]. 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 predictive 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] and Huang *et al*. [33], 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. We revealed that the

significantly differences of the 5-year DFS and OS rates between the FOLFOX group and FOLFOXC group were only noted in EGFR-positive patients, but not in EGFR-negative patients. Therefore, although the EGFR-positive patients had worse prognoses, capecitabine metronomic maintenance therapy could effectively compensate and improve their prognoses to the same level as that of the EGFR-negative patients. We found that the EGFR-negative patients did not benefit from capecitabine metronomic maintenance therapy in terms of survival. Thus, we determined that only the EGFR-positive patients could benefit from metronomic maintenance therapy, which has not been reported in previous studies [14, 33].

On the basis of our results, we hypothesize that EGFR-negative tumor cells are less proliferative and less migratory compared with EGFR-positive tumor cells. Moreover, cell proliferation and migration could be inhibited by fluoropyrimidine-based therapy. In this study, we used Caco-2 cells to perform the *in vitro* and *in vivo* experiments 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 Caco-2 cells decreased substantially. The proliferative and migratory capacities of the Caco-2 cells decreased after *EGFR* knockout, and the proliferative and migratory capacities of the Caco-2 cells with or without EGFR expression were inhibited by 5-FU. We determined that 5-FU administration and *EGFR* Knockout had synergistic inhibitory effects on the proliferative and migration capacities of Caco-2 cells. Notably, these *in vitro* results were verified using *in vivo* experiments.

The present study has some limitations. First, this study involved a single-institution retrospective design 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.

5. Conclusions

In conclusion, we demonstrated that metronomic maintenance therapy with capecitabine could significantly improve the prognoses of patients with stage III CRC following radical resection and FOLFOX adjuvant chemotherapy. Moreover, the effects of prognosis improvement are noteworthy in patients with positive EGFR expression. However, a prospective, randomized clinical trial is necessary to verify the results of the present study.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Immunohistochemical staining of EGFR in CRC. A. Negative expression (magnification, 100×). B. 1+ expression (weak intensity of membrane staining) (magnification, 100×). C. 2+ expression (moderate intensity of membrane staining) (magnification, 100X). D. 3+ expression (strong intensity of membrane staining) (magnification, 100×).

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