FOXP3 Expression as a Biomarker in Metastatic Colorectal Cancer Treated with Irinotecan-based Therapy

Khallaf SM[1], Abd El Moatmed AM[1], Shabaan SH[2], Zaky AH[1], Abdalla AZ[1]

[1] Department of Medical Oncology, South Egypt Cancer Institute, Assiut University, Assiut, Egypt
[2] Pathological Oncology Department, South Egypt Cancer Institute Assiut University, Assiut, Egypt

Abstract:

Background: Forkhead box protein 3 (FOXP3) is one component of the colorectal tumor microenvironment had a crucial role in development of metastases and progression. Our study aims to investigate the role of FOXP3 as a prognostic biomarker in metastatic colorectal cancer (mCRC) patients received second line therapy (irinotecan-based chemotherapy in combination with anti-EGFR or anti-VEGF, when indicated).

Methods: The study included 53 patients with mCRC treated with irinotecan-based chemotherapy with anti-EGFR or anti-VEGF. FOXP3 was assessed by immunohistochemistry. We use the median of FOXP3 score as the cutoff value of its expression. We evaluate the correlation between the expression of FOXP3, response to therapy, and survival of the patients.

Results: Out of total 53 patients, about half of the cases had rectal site of primary tumor (52.83%). Most of them had T3 or T4 tumor (90.57%), positive nodal disease (79.25%), high tumor grade (84.91%) and synchronous metastases (60.37%). Approximately half of the patients (49.18%) had high FOXP3 expression while the other (50.91%) had a low expression.

There is a clinical significance of higher overall response rate (15.4% vs. 11.1%) and disease control rate (42.3% vs. 33.3%) in favor of high FOXP3 expression, but of statically insignificance.

Interestingly, high FOXP3 expression in mCRC patients was associated with significantly longer median progression-free survival compared with those having low FOXP3 expression (5.55 months vs. 3.71 months; P= 0.036). Also, there was a significant prolongation of 9.69 months in median overall survival in favor of patients with high FOXP3 expression (14.98, 95%CI: 11.96-18.00months vs. 5.29, 95%CI: 4.02-6.56 months; P= 0.045).

Conclusion: FOXP3 is a potential good prognostic marker in mCRC patients receiving irinotecan-based chemotherapy with target therapy, when indicated. This is a promising marker that may be incorporated into the prognostic panel for these patients.

Keywords: FOXP3 expression, Colorectal cancer, Overall survival, Progression free survival

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Authors Information:

Salah Mabrouk Khallaf
Medical Oncology Department, South Egypt Cancer Institute Assiut University, Assiut, Egypt
email: Salahmab76@yahoo.com

Amany Mohammed Abd El Moatmed
Medical Oncology Department, South Egypt Cancer Institute Assiut University, Assiut, Egypt
email: Amanymo29@yahoo.com

Shimaa Hassan Shabaan
Department of Pathological Oncology Department, South Egypt Cancer Institute Assiut University, Assiut, Egypt
email: Shimaashaban19@yahoo.com

Amen Hamdy Zaky
Medical Oncology Department, South Egypt Cancer Institute Assiut University, Assiut, Egypt
email: amenzaky74@yahoo.com

Ashraf Zeidan Abdalla
Medical Oncology Department, South Egypt Cancer Institute Assiut University, Assiut, Egypt
email: profzedan@yahoo.com

Corresponding Author:
Amany Mohammed Abd El Moatmed
Medical Oncology Department, South Egypt Cancer Institute Assiut University, Assiut, Egypt
email: Amanymo29@yahoo.com

Introduction:

The annual incidence of Colorectal cancer is about 10% of cancers around the world [1]. Metastatic colorectal cancer (mCRC) represents 20% of newly diagnosed colorectal cancer cases, approximately 80% of them being unresectable. They show improvement in median survival of about 30 months with the use of multimodality therapy as cytotoxic chemotherapy in addition to target therapy [2].

Fluoropyrimidines-based chemotherapy is well known as the main backbone of treatment, the addition of irinotecan showed prolongation of survival, enhancing response which is one of the approved regimens as first or second-line therapy in mCRC [3]. The available regimens for irinotecan - fluoropyrimidine-based therapy are FOLFIRI which consist of Irinotecan/5-FU/Leucovorin and CAPIRI which consist of irinotecan/oral capecitabine; however, modification of dose applied to CAPIRI as it shows more toxicity than FOLFIRI [4]. The inclusion of targeted therapy such as anti-EGFR (Epidermal growth factor receptor) monoclonal antibodies mAbs as cetuximab or panitumumab or anti-VEGF (Vascular endothelial growth factor) mAbs as bevacizumab,

ramucirumab, or aflibercept, in the treatment regimens have been shown to improve the survival [5].

The use of anti-EGFR has certain molecular and pathologic limitations since mutant RAS mCRC is associated with resistance to the anti-EGFR mAbs [6], while the anti-EGFR agents showed improvement in response rate (RR), progression free survival (PFS) and overall survival (OS) when compared with doublet chemotherapy alone in patients with wild type KRAS as proven in several trials [7]. Furthermore, the tumor location is essential before deciding anti-EGFR mAbs in wild RAS mCRC where left-sided tumor showed prolonged PFS, OS, and improving RR [8], while, in right-sided mCRC, there was no benefit seen in PFS or OS except for RR, so the use of antiVEGF mAbs in addition to chemotherapy may be the proper choice independent on RAS mutational state in right sided tumor [9].

Despite the great advances in treatment strategies for metastatic colorectal cancer to improve therapy outcomes, mCRC mortality still accounts for about 9.4% of cancer-related deaths [10]. CRC progression was affected by many factors such as tumor biology, tumor microenvironment, and immune response [11]. The tumor microenvironment (TME) has an important role in tumors' initiation, promotion, progression, and development of metastases through different mechanisms of action such as stimulation of angiogenesis and suppression of the immune system [12].

TME is composed of many cells as tumor cells, stromal, infiltrating inflammatory, and immune cells [11]. Forkhead box protein 3(FOXP3) as a transcription factor had a main role in the development of regulatory T-cells (Treg cells) and its' function, which are expressed in many types of cancer [13]. The FOXP3 role in cancer is different in various tumors where it is correlated with worse outcomes in the lung, breast, hepatocellular, ovarian cancer, and melanoma, while its role in CRC is controversial [14,15,16,17].

Some studies supported the theory that high FOXP3 expression antagonizes the antitumor immune response and permits the tumor cells to escape from the effector T cell response resulting in tumor progression [18].

Other studies show that high FOXP3 infiltrating tumors antagonize the tumor immune effect through suppression of proto-oncogenes and stimulating transcription of tumor suppressor gene [19].

Another study by Miyara M, et al describes that FoxP3 CD4 T cells have 3 subpopulation cells including naive Treg, effector Treg, and Fr-III (FOXP3loCD45RA−) cells with different proportions according to age or comorbidity of the person. They show phenotypical & functional heterogensity [20].

Our study investigated the relation between FOXP3 expression in one side and response to treatment, and survival in other side, in mCRC patients receiving second-line chemotherapy with irinotecan-fluoropyrimidine-based chemotherapy with anti-VEFG or anti-EGFR, when indicated.

Patients and Methods:

This retrospective study compromised 53 patients diagnosed with mCRC at South Egypt cancer institute, Assiut university, Egypt, in the duration between 2012 to 2020. All patients were aged more than 18 years, both sexes included. All patients received second-line chemotherapy with FOLFIRI or CAPIRI with or without target agents for at least 2 cycles and assessment was done once. All patients had proven diagnoses of metastases.

We exclude those who received oxaliplatin-based chemotherapy, those with anal cancer, patients with double malignancy, or patients with inadequate tumor block for further investigation.

The protocol was approved by the Institutional review board and ethical committee under IRB approval No: 544 on (20th June 2021). Written informed consent was obtained from all patients.

The patients in this study received either FOLFIRI or CAPIRI as second line, twenty-six patients (49%) received monoclonal antibodies (17 patients received anti-EGFR and 9 patients received anti-VEGF) with the following protocols:

FOLFIRI regimen:

Irinotecan 180 mg/m2 IV over 30–90 minutes, day 1

Leucovorin 400 mg/m2 IV infusion to match duration of irinotecan infusion, day 1

5-FU 400 mg/m2 IV bolus day 1, followed by 1200 mg/m2/day x 2 days

(Total 2400 mg/m2 over 46–48 hours) continuous infusion

Repeat every 2 weeks.

CAPIRI regimen:

Irinotecan 200 mg/m2 IV over 30–90 minutes, day 1

Capecitabine 1000 mg/m2 twice daily PO for 14 days, to be repeated every 3 weeks.

Target therapy

Cetuximab 500 mg/m2 IV over 2 hours, day 1, every 2 weeks

Panitumumab 6 mg/kg IV over 60 minutes, day 1, repeat every 2 weeks.

Bevacizumab 5 mg/kg IV, day 1, repeat every 2 weeks.

Patients continue chemotherapy till progression, unacceptable toxicity, or patient request.

We collected information from recorded data sheets about all patients' demographics, clinicopathological features, response to therapy and survival data.

Immunohistochemistry:

According to USA Bioss INC for immunohistochemistry, steps were done on formalin fixed paraffin embedded tissue (FFPET), chopped into a section of 3-micron thickness on a slide and immersed on positively charged glass slides. then deparaffinization and rehydration through graded alcohols to distilled water. Immersion of the slides was

done into unsealed plastic container (Coplin jars) filled with enough antigen retrieval solution (Tris EDTA) in a water bath heated at 90 for 45 minutes. Then application of hydrogen peroxide block and incubation for 10-15 minutes at room temperature to decrease endogenous activity of peroxidase. Ultra V Block was applied to the slides and incubated for 5 minutes at room temperature in a humid chamber to avoid unneeded background staining.

Then Primary rabbit monoclonal anti-forkhead-box protein P3 antibody (Catalog bsm-52079R, Bioss Inc, USA) at 1:200, was applied and incubation was done overnight at 4°C in a humid chamber, then washing of the sides for 2-3 times using phosphate buffer solution (PBS).

Then immunostaining was done applying a universal staining kit "Ultra Vision Detection System Anti-Polyvalent, HRP/DAB (Ready-To-Use) (BIOCYC Gesellschaft für Biotechnologie, Kosmetik und Recyclingverfahren mbH & Co. Entwicklungs KG Am Mühlenberg 11, 14476 Potsdam, Germany) following the instructions of manufacturer. Biotinylated Goat Anti-Polyvalent was added to the slides and incubated for 5 minutes at room temperature, then rinsed and washed with PBS 2 times. Streptavidin was applied for 10 minutes also to the slides and incubated at room temperature then washed as before. Diaminobenzidine (DAB solution) chromogen was applied to the slides for 5 minutes and then washed in distilled water. Sections were then counterstained using Mayer's hematoxylin, then tap water washing, dehydration into ascending alcohols then cleared in Xylene, and lefted to dry in air. DPX is applied to each slide and the cover is slipped.

Sections from reactive lymph node were used as positive control for FOXP3 expression on nucleus or cytoplasm. The positivity was identified as brown cytoplasmic staining of acinar cells (according to the datasheet of USA Bioss Inc), and sections of tissue-specific positive controls were stained using the same protocol but with omitting the primary antibody using it as a negative control.

The slides were evaluated by pathologist without previous knowledge of clinicopathologic features of the lesions. In each case, the immune-stained section was examined histologically at a lower magnification (X4 and X10) to detect the positive cells and percentage of positive cells (PP %). The FOXP3 positivity was identified as brown cytoplasmic staining.

We use the median FOXP3 expression of 72% as the optimal cut-off value of our study where low expression is defined as FOXP3 expression less than or equal to the median, while high FOXP3 expression is more than median.

Study endpoints

The primary endpoints were therapy response rate including duration of response and duration of clinical benefit (DoCB) and progression-free survival (PFS). The secondary endpoint was overall survival (OS).

Statistical analysis and tests

The cut-off date for our data collection is December 30, 2022.

Qualitative variables were declared as frequencies (percentages), and compared using chi-square or Fisher's test when applicable, while quantitative variables were described as median(range) or mean (standard deviation or 95% confidence interval (95%CI) according to its normality of distribution. Quantitative variables were compared with the use of a parametric Student's t-test or a nonparametric (Mann-Whitney U test) test, accordingly.

The reversed Kaplan–Meier method was used for the calculation of median follow-up time.

Kaplan-Meier methods were used for the estimation of the survival curve and compared using the log-rank test. P-value (two-sided) < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 22.

Results:

Relation between FOXP3 expression and demographics and clinicopathological data of the patients.

The mean age of our patients was 42.6 years and about 67% of them were diagnosed before reaching 50, slightly more than half of the patients were females. majority of the patients (96%) had a good performance state (PS: 0-1).

Approximately 52.8% of the patients had rectal cancer, while the others had colon tumors (47.2%). About two-thirds of the patients had moderately differentiated tumors (G2), and thirty-three (62.3%) patients had adenocarcinoma histology. About 60% of the patients were diagnosed with synchronous metastases while the other 40% had metachronous metastases. Almost half of the patients (50.9%) had low FOXP3 expression (Figure .2) while the others had high FOXP3 expression (Figure .1). There was no significant correlation between FOXP3 expression and any of the previous patients' categories. The data are shown in Table (1).

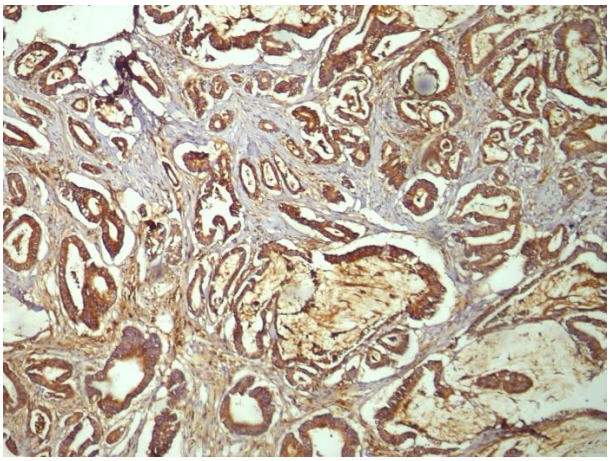

Figure 1. High FOXP3 expression

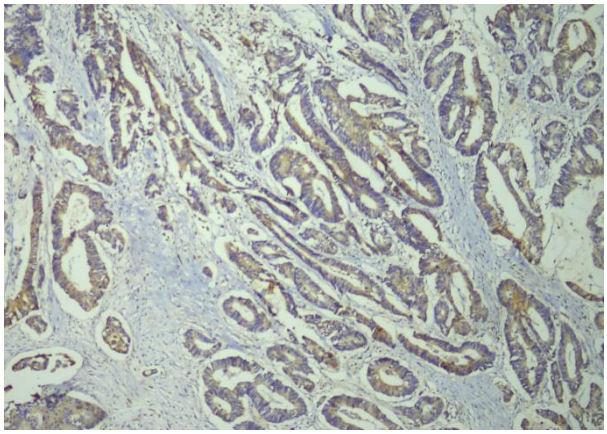

Figure 2. Low FOXP3 expression

Relation between FOXP3 expression and response to therapy

The number of patients who received target therapy was too small to give statistically significant results so patients with chemotherapy were added to them and calculated as a whole.

Patients with high FOXP3 expression had higher disease control rate (DCR) (42.3%) than those with low expression (33.3%) as shown in Figure 1, progression occurred in 15 patients with high FOXP3 expression and 18 patients with low expression, however, there was no statistically significant correlation between the expression and response to therapy with P value more than 0.05. Other values are listed in Table 2, Figure 3.

The median duration of response (DoR) was 11.4 months with a range from 4.7 to 82.5months, with slightly higher 3 months in patients with high FOXP3 expression than those with low expression, but only 6 patients were achieving this, making it too small number to be reliable for static calculation. Table (3)

Duration of clinical benefit (DoCB) was also calculated with a median of 11.3 months, with nearly the same duration in both groups of about 11 months and no significant correlation with FOXP3 with a P value of 0.9.

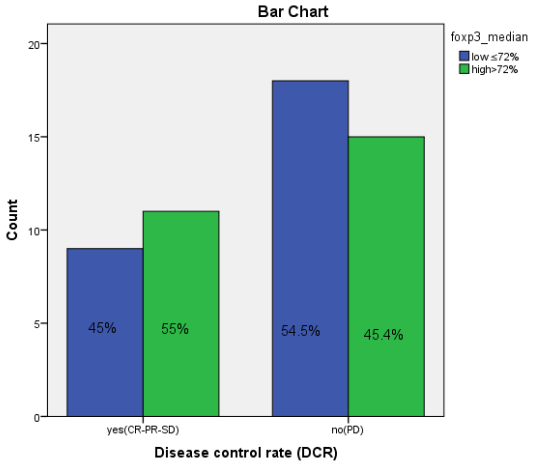

Figure 3. Disease control rate of Irinotecan -based therapy

Relation between FOXP3 expression and survival analysis.

After median follow up of 11 months, we found that median progression free survival was higher in those with high FOXP3 than those with low expression (5.5months versus 3.7months respectively with a P value of 0.036. Table 4 and figure 4.

We also found that high FOXP3 expression is significantly associated with longer overall survival (OS) of about 14.9 months when compared with low FOXP3 expression which is only 5.3 month, with P value of 0.045. Table 5 and figure 5.

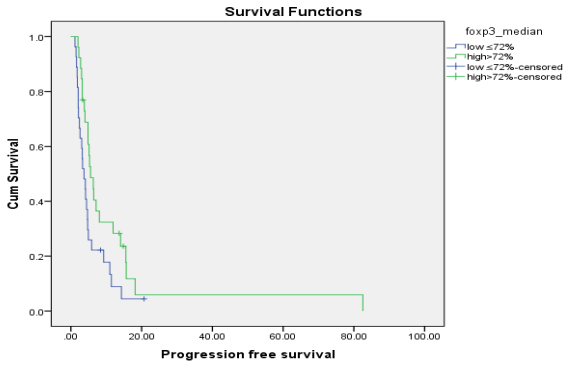

Figure 4. FOXP3 expression and Progression free survival

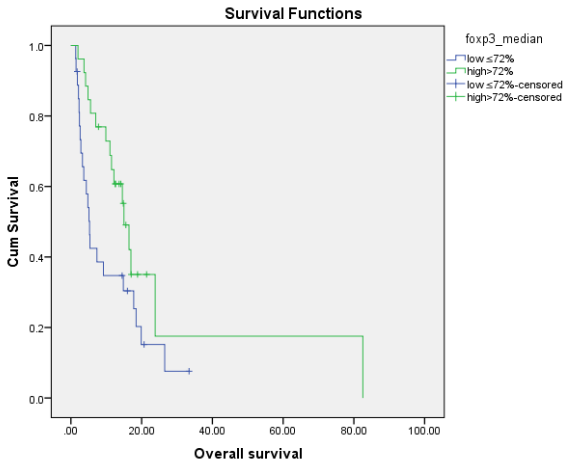

Figure 5. FOXP3 expression and Overall survival

Table 1. Demographics and Clinicopathological of 53 patients with mCRC

Characteristics	Number (n=53)	Percentage (%)
Age		
Mean (±SD)	42.6 (±14.61)	
95% CI	38.5-46.6	
≤50 years	36	67.9
>50years	17	32.1
Gender		
Male	23	43.4
Female	30	56.6
Performance state		
0-1	51	96.2
2	2	3.8
Tumor location		
Colon	25	47.2
Right	14	26.4
left	11	20.8
Rectal	28	52.8
Degree of differentiation (G)		
G1	8	15.1
G2	37	69.8
G3	8	15.1
Tumor histology		
Adenocarcinoma	33	62.3
Mucinous adenocarcinoma	17	32.1
signet ring carcinoma	3	5.7
Primary tumor(T)		
T2	5	9.4
T3	39	73.6
T4	9	17
Nodal stage(N)		
N negative	11	20.8
N positive	42	79.2
status at diagnosis		
Synchronous metastases	32	60.4
Metachronous metastases	21	39.6
Number of metastatic sites		
Less than 3 sites	37	69.8
More than or equal 3sites	16	30.2
KRAS status		
Wild	37	69.8
mutated	16	30.2
FOXP3 expression		
Low (≤median)	27	50.9
High (>median)	26	49.1

Abbreviations: BMI, body mass index; CI, confidence interval; FOXP3, forkhead box protein 3; KRAS, Kirsten rat sarcoma virus; SD, standard deviation.

Table 2. FOXP3 expression and response to therapy

Category	Low FOXP3 (n=27)		High FOXP3 (n=26)	
	No	**%**	**No**	**%**
Overall response				
Complete response	1	3.7	2	7.7
Partial response	2	7.4	2	7.7
Stationary disease	6	22.2	7	26.9
Progressive disease	18	66.7	15	57.7
Total	27	50.9	26	49.1
Objective response rate	**3**	**11.1**	**4**	**15.4**
Disease control rate	**9**	**33.3**	**11**	**42.3**

Table3. FOXP3 expression and duration of response (DoR)

Variable	Number	Percentage (%)	Median DoR(95%CI)	P value
Low FOXP3	3	42.9	11.0(0.91-21.1)	0.09
High FOXP3	3	42.9	14.0(0.0- 64.3)	
Overall	6	11.3	11.4(10.3-12.5)	

Table 4. FOXP3 expression and Progression free survival

Variable	Number	Percentage (%)	Median PFS (95%CI)	P value
Low FOXP3	25	52.1	3.7(2.3-5.2)	0.036
High FOXP3	23	47.9	5.5(3.6-7.4)	
Overall	48	90.6	4.7(3.8-5.6)	

Table 5. FOXP3 expression and overall survival

Variable	Number	Percentage (%)	Median OS (95%CI)	P value
Low FOXP3	22	57.9	5.3(4.0-6.6)	0.045
High FOXP3	16	42.1	14.9(11.9-18.0)	
total	38	71.7	11.5(5.6-17.4)	

Discussion:

CRC is a heterogenous disease with various responses to therapy, although there is improvement in median survival with combined therapy (chemotherapy in addition to targeted therapy against EGFR and angiogenesis), the response to anti-EGFR ranges from 40 to 60% [21]. FOXP3 as a transcription regulatory factor, is an important component of tumor microenvironment. It plays an important part in the immune tolerance transfer and improvement of the immunosuppressive tumor microenvironment, however, there is growing evidence that this role is disturbed in cancer cells [22]. We here evaluate the role of FOXP3 expression as a predictive and prognostic marker in metastatic colorectal cancer patients.

Our study includes patients with mCRC with a mean age of 42 years which is relatively like a study by Kassem, N. M., et al. 2019, 68% of them were younger than 50 years old and slightly more than half of them (56.6%) were females [23]. More than half of our patients (52.83%) had rectal site tumors, while the others had colon cancer. Almost 85% of the population studied had moderate to high-grade tumors while the others had low-grade tumors. Most of our patients had high risk tumor features as 90% had T3/T4, 79.3% had positive nodal stage, which is matched with study by Sun X, et al.2017 [24].

A greater number of the patients (60.4%) had synchronous metastases, with 68.1% of the patients having more than 3 sites of metastases. Wild KRAS was predominant in about 69.8% of our patients while only 30% had mutated KRAS. approximately 50.9% of the population studied had low FOXP3 expression while the others had high expression of FOXP3. We found that there was no significant correlation between FOXP3 and age, sex primary tumor site, degree of differentiation, depth of invasion, nodal stage, Kras status, synchronous or metachronous metastases or site of and FOXP3 expression (P value >0.05) which is similar to study by Ganapathi, S. K., et al.2014 who described that there is no relation between FOXP3 expression and depth in invasion(T)[25], however, our study is different from study by Sun X et al 2017 who describes that FOXP3 expression was decreasing as increasing T stage or with poorly differentiated tumor and there was a negative correlation between its

expression and nodal stage; but it was similar to our study in that there was no correlation with age, sex or tumor location [24].

When we investigated the predictive and prognostic role of FOXP3 expression in our study, we found that ORR and DCR were modestly higher in patients with high FOXP3 expression 15.4% and 42.3% respectively than those with low FOXP3 expression 11.1% and 33.3% respectively. We also found that progressive disease occurs more frequently in those with low FOXP3 66.7% than those with high expression 57.7%. However, there was no statistically significant correlation between FOXP3 expression and response to therapy with a P value of more than 0.05. Our results were different from a study by Oshi M, et al.2022 who reported that increased Treg cells (which are marked by FOXP3) are associated with higher response with chemotherapy without bevacizumab. This could be explained by the small sample size in our study and heterogenesity in tumor location which had a significant influence on response to therapy and aggressiveness of the tumor [26].

Although there was a mild increase in PFS of only 1.84 months in those with high FOXP3 expression than in patients with low FOXP3 expression, it was statistically significant with P value of 0.036.

As regards, Overall survival, and expression of FOXP3, we found median OS was significantly prolonged by 9.7 months more in patients with high FOXP3 expression than patients with low expression of FOXP3(P value 0.045). Our study results were similar to the study by Sun X, et al. 2017 who reported that high FOXP3 expression was associated with longer disease-free survival and overall survival in CRC patients [24].

This may be explained by that high FOXP3 expression is possibly associated with an increased the number of anti-tumor immune cells in the microenvironment and associated with activation of several immune-related genes such as IFN-α response, IL6/JAK/STAT signaling, and inflammatory response, leading to suppression of the tumor [26]. But different from the study by Kim M, et al .2013 who reported that there was no significant correlation between median OS and high or low FOXP3 expression which could be explained by heterogenesity in treg cell function in various tumors [18].

FOXP3 is a potentially good prognostic marker in mCRC patients receiving irinotecan-based chemotherapy with target therapy, when indicated. This is a promising marker that may be incorporated into the prognostic panel for these patients.

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