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Original Article

Decreased peritherapeutic VEGF expression could be a predictor of responsiveness to first-line FOLFIRI plus bevacizumab in mCRC patients

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Abstract: Objective: Bevacizumab is the only anti-angiogenic agent approved in first-line therapy for metastatic colorectal cancer (mCRC). Although chemotherapy plus bevacizumab has led to improve outcomes for mCRC patients and is a common choice for first-line treatment of mCRC, previous research has established no prominent biomarker that can help to select patients who may benefit from bevacizumab in order to improve cost-effectiveness and therapeutic outcomes. The aim of this study was to compare pre- and post-therapeutic VEGF immunohistochemical (IHC) expression in mCRC patients treated with FOLFIRI plus bevacizumab to identify its potential role as a predictive biomarker. Methods: A total of 57 mCRC patients who underwent FOLFIRI combined with bevacizumab chemotherapy as a first-line neoadjuvant regimen were enrolled and clinical outcome data analyzed. Results: Low post-therapeutic VEGF expression ($P < 0.001$) and decreased peri-therapeutic VEGF expression ($P < 0.001$) were significantly predictive factors of responders. Furthermore, the 6-month progression-free survival (PFS) rate in mCRC patients with decreased peri-therapeutic VEGF expression was significantly better than the rate for those patients with no peri-therapeutic VEGF expression alterations ($P = 0.033$). Conclusions: Decreased peri-therapeutic VEGF expression in mCRC patients could probably be used to predict responsiveness to bevacizumab and subsequent PFS in clinical practice.

Keywords: Vascular endothelial growth factor, peri-therapeutic VEGF expression, metastatic colorectal cancer, bevacizumab, response

Introduction

Angiogenesis, the process of new blood vessel formation from endothelial precursors, is a complex process regulated by numerous endogenous factors that stimulate or inhibit the neovascularization of both healthy and pathological tissues [1]. Angiogenesis plays an important role in the delivery of oxygen and nutrients to growing tumors, and as such is a vital element for tumor growth and metastases [2]. The clinical course of neoplastic disease depends on both angiogenesis and the anticancer immune response. Several positive regula-

tors of tumor angiogenesis have been identified. Among these, vascular endothelial growth factor (VEGF) is the most potential angiogenic factor stimulating endothelial cell proliferation, survival, and vascular maturation. A randomized Phase III trial has demonstrated that bevacizumab, an anti-VEGF monoclonal antibody, in combination with cytotoxic agents improves overall survival (OS) and other outcomes in mCRC patients as compared with cytotoxic agents alone [3, 4].

Recently, we have shown that prospective analysis of uridine diphosphate glucuronosyl transferase 1A1 (*UGT1A1*) genotyping for irinotecan dose escalation (FOLFIRI regimen) with combination of bevacizumab as the first-line setting in mCRC patients. We have shown that mCRC patients with pretherapeutic *UGT1A1* genotyping and subsequent irinotecan does escalation can achieve a more favorable response and outcome without a significant increase in toxicity while using the FOLFIRI plus bevacizumab regimen [5].

With regard to cost-benefit analyses for biological agents, especially in light of the current economic climate, a study by Sawyers, et al has strongly encouraged clinicians and researchers to identify biomarkers in cancer patients that can predict the effectiveness of particular treatments [6]. Despite the numerous candidate angiogenesis biomarkers that have been investigated, however, previous research has yet to establish clinical biomarkers for monitoring angiogenesis or predicting response to anti-angiogenic drugs [7]. There is consequently an obvious need for predictive markers both with respect to efficacy and toxicity, and better selection of patients is a prerequisite for a more effective treatment of mCRC patients.

In many trials, a higher VEGF level has been found to indicate a poor prognosis, but high VEGF expression was not found to be predictive of the effects of antiangiogenic drugs, including bevacizumab [2, 8]. Previous studies have demonstrated that the presence of VEGFR-1 319 C/A single nucleotide polymorphisms (SNPs) [9], a high level of pre-treatment lactate dehydrogenase (LDH) [10], and high *EPHB4* gene expression [11] are potential predictive biomarkers in mCRC patients treated with bevacizumab. The aim of the present study was to identify a biomarker that could potentially be used in clinical practice as a predictor of response to bevacizumab as the first-line therapy for mCRC.

Materials and methods

Patients and tissue samples

Between June 2011 and August 2013, a total of fifty-seven mCRC patients who underwent FOLFIRI combined with bevacizumab as first-line neoadjuvant regimen were enrolled and followed up until January 2014. The enrolled 57 patients were proven mCRC patients via image studies from independent radiologists (e.g., CT, MRI, or PET). No surgical therapy as performed in our studies subjects between the time of colonoscopic biopsy and initiation of the bevzcizumab. The treatment consisted of bevacizumab (5 mg/m^2 as a 120-min intravenous infusion), followed by irinotecan (180 mg/m^2 as a 120-min intravenous infusion), leucovorin (400 mg/m^2 as an intravenous infusion over 2 hours), and 5-fluorouracil (2800 mg/m^2 as an intravenous infusion over a 46-hour period), repeated biweekly. FOLFIRI combined with bevacizumab was previously approved by the FDA in Taiwan as the first-line treatment for mCRC, with associated costs reimbursed for up to 6 months. Thereafter, the enrolled patients were changed to FOLFIRI regimen after the 6-month reimbursement period.

Tissue samples were obtained from each patient pre- and post-therapeutically. The pre-therapeutic samples consisted of tissues taken via colonofiberscope before administration of FOLFIRI combined with bevacizumab. The post-therapeutic samples consisted of tissues taken via colonofiberscope or surgical specimen after administration. All clinical samples were obtained with informed consent from each patient, and the study protocol was approved by the hospital's institutional review board.

Post-therapeutic surveillance

The response was assessed with computed tomography (CT) scans [12], magnetic resonance imaging (MRI) [13-15], bone scans, or positron emission tomography (PET) [16]. Usually CT remains the first choice of evaluated tool for therapeutic response; however, another image studies such as MRI, PET, or bone scan would be applicable if clinically in need. We

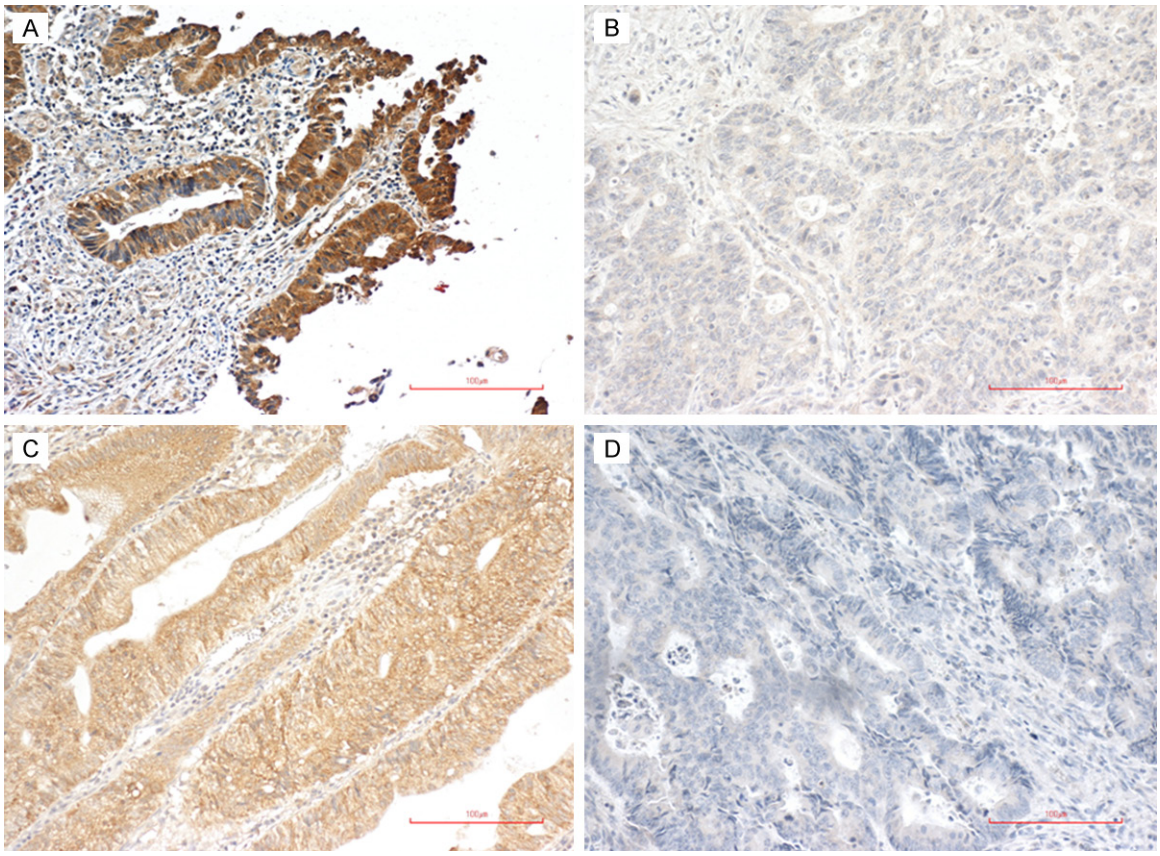


Figure 1. Immunohistochemical staining of vascular endothelial growth factor (VEGF) in colorectal cancer tissues. VEGF protein in the cytoplasm of tumor cells was observed as supranuclear staining (brown color). Reduction in both staining intensity and positive staining cells was required for an interpretation of decreased VEGF expression after bevacizumab treatment. A 64 year-old male colon cancer patient with liver metastases showed a decrease from the pre-therapeutic VEGF score (A, score 3, 200 ×) to the post-therapeutic score (B, score 0, 200 ×). A 40 year-old female rectal cancer patient with liver metastases showed a decrease in VEGF expression from the pre-therapeutic score (C, score 2, 200 ×) to the post- therapeutic score (D, score 0, 200 ×).

recorded the all responses, and then just identified which of them was the best for the prediction of therapy for mCRC [17]. The time for the first response assessment was usually after the sixth cycle of FOLFIRI combined with bevacizumab. Responses were classified according to the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1) [12]. A complete response (CR) was defined as the disappearance of all target lesions; a partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameter of all the target lesions, taking as a reference point the baseline sum's longest diameter. Progressive disease (PD) was defined as an at least 20% increase in the sum of the longest diameter of the target lesions, taking as a reference the smallest sum of longest diameters recorded before the patient started to receive treatment. PD was also defined as the identification of one or more new lesions. A stable disease (SD) was defined as neither having sufficient shrinkage to qualify as a PR nor a sufficient increase to qualify as PD. Moreover, we reported the best response for each patient, which was defined as the best one recorded by the investigators, since confirmatory image evidence for responses obtained after the fourth to sixth cycles of the chemotherapy was not consistently available.

The responders included those patients with CRs and PRs, whereas the non-responders included those with SDs and PDs. The primary efficacy endpoint was the crude rate of 6-month PFS, which was defined as 6 months after the treatment of a disease that a patient lives with the disease but it does not get worse (i.e., CRs or PRs or even SDs) at 6 months. If death

Table 1. Demographic data for the 57 metastatic colorectal cancer patients treated with FOLFIRI combined with bevacizumab

Variables	Case Number (%)
Gender	
Male/Female	36 (63.2)/21 (36.8)
Age (y/o)	
< 65/≥ 65	30 (52.6)/27 (47.4)
Tumor size (cm)	
< 5/≥ 5	40 (70.2)/17 (29.8)
Tumor site	
Colon/Rectum	28 (49.1)/29 (50.9)
Invasive depth	
T2/T3/T4	7 (12.2)/36 (63.2)/14 (24.6)
Vascular invasion	
No/Yes	44 (77.2)/13 (22.8)
Perineural invasion	
No/Yes	26 (45.6)/31 (54.4)
Tumor grade	
WD/MD/PD[1]	3 (5.3)/50 (87.7)/4 (7.0)
Tumor type	
A/M[2]	53 (92.9)/4 (7.1)
Pre-therapeutic VEGF[3] score	
Low/High[4]	20 (35.1)/37 (64.9)
Post-therapeutic VEGF[3] score	
Low/High[4]	43 (75.4)/14 (24.6)
Decreased VEGF[3] score peri-therapeutically	
No/Yes	20 (35.1)/37 (64.9)

[1]WD: Well-differentiated; MD: Moderately-differentiated; PD: Poorly-differentiated. [2]A: Adenocarcinoma; M: Mucinous carcinoma. [3]VEGF: Vascular endothelial growth factor. [4]Low: The immunochemical score (IHC) of VEGF was 0 and 1; high: the IHC score of VEGF was 2 and 3.

occurred, it meant that patients were considered as progression. With progression being judged according to the independent radiologists' tumor assessments at 6 months by the image study. Secondary endpoints included objective response rate (evaluated by RECIST) and OS, which was defined as the given patient without an event were censored at the time of last follow-up.

Clinicopathological features

The analyzed clinicopathological features included the patients' gender, age, tumor size, tumor location, tumor invasive depth, vascular invasion, perineural invasion, tumor differentiated grade, tumor type, and pre- and post-therapeutic, as well as peri-therapeutic, VEGF IHC scores.

Immunohistochemical (IHC) staining

The archival slides were reviewed, and representative formalin-fixed and paraffin-embedded tissue blocks containing tumor cells were selected for immunohistochemical staining. The detailed procedures are described in our previous study [18].

Evaluation of VEGF expression after bevacizumab treatment

The interpretation of immunohistochemical staining was performed in consideration of both staining intensity and percentage of positive cancerous cells. Cytoplasmic VEGF immunostaining was considered positive. The VEGF expression was compared in paired samples from tissues before and after bevacizumab treatment. The staining intensity was graded as absent, weak, moderate, or strong. A reduction of more than 20% in positive cells was regarded as a decrease in positive cells. To avoid sampling bias, reduction in both staining intensity and positive staining cells was required for interpretation as a decrease in VEGF expression after bevacizumab treatment (**Figure 1**).

In evaluating the expression of VEGF, scoring of the immunohistochemical staining was performed. Score 0 was given for samples completely absent of staining or with less than fifty percent weak staining. Score 1 was given for samples with more than fifty percent positive cells with weak staining or for moderate staining in less than fifty percent positive cells. For cases with moderate positive staining in more than fifty percent of cells, or with strong staining in less than fifty percent of cells, a score of 2 was assigned. When strong positive staining in more than fifty percent of cells was obtained, a score of 3 was assigned. VEGF overexpres-

Table 2. Analysis of the correlation between clinicopathologic factors and responders/non-responders

Variables	Responders (N = 44) (%)	Non-responders (N = 13) (%)	*P*-value
Gender			
Male/Female	28 (63.6)/16 (36.4)	8 (61.5)/5 (38.5)	0.890
Age (y/o)			
< 65/≥ 65	24 (54.5)/20 (45.5)	6 (46.2)/7 (53.8)	0.594
Size (cm)			
< 5/≥ 5	29 (65.9)/15 (34.1)	11 (84.6)/2 (15.4)	0.195
Site			
Colon/Rectum	23 (52.3)/21 (47.7)	5 (38.5)/8 (61.5)	0.381
Depth			
T2/T3/T4	5 (11.4)/29 (65.9)/10 (22.7)	2 (15.4)/7 (53.8)/4 (30.8)	0.731
Vascular invasion			
No/Yes	34 (77.3)/10 (22.7)	10 (76.9)/3 (23.1)	0.979
Perineural invasion			
No/Yes	18 (40.9)/26 (59.1)	8 (61.5)/5 (38.5)	0.189
Grade			
WD + MD/PD[1]	42 (95.5)/2 (4.5)	11 (84.6)/2 (15.4)	0.179
Type			
A/M[2]	42 (95.5)/2 (4.5)	11 (84.6)/2 (15.4)	0.179
Pre-therapeutic VEGF[3] score			
Low/High[4]	15 (34.1)/29 (65.9)	5 (38.5)/8 (61.5)	0.772
Post-therapeutic VEGF[3] score			
Low/High[4]	38 (86.4)/6 (13.6)	5 (38.4)/8 (61.5)	< 0.001
Decreased peri-therapeutic VEGF[3] score			
No/Yes	7 (15.9)/37 (84.1)	13 (100.0)/0 (0.0)	< 0.001

[1]WD: Well-differentiated; MD: Moderately-differentiated; PD: Poorly-differentiated. [2]A: Adenocarcinoma; M: Mucinous carcinoma. [3]VEGF: Vascular endothelial growth factor. [4]Low: The immunochemical score (IHC) of VEGF was 0 and 1; high: the IHC score of VEGF was 2 and 3.

sion was defined as a score of 2 or 3, while VEGF non-overexpression was defined as a score of 0 or 1. The pre-treatment VEGF IHC scoring was performed in approximately one week before the administration of FOLFIRI plus bevacizumab. However, the "post-treatment" VEGF IHC scoring was performed when the regimen was used for 6 cycles. We defined the "peri-therapeutic" as the alternation of VEGF IHC scoring between the pre-treatment and the post-treatment. Decreased peri-therapeutic VEGF expression was defined as a decrease in the scoring of VEGF by at least one point (e.g., a decrease from a score of 2 to a score of 1, or from a score of 2 to a score of 0).

Statistical analysis

The continuous variables are presented as mean ± standard deviation (SD), and dichotomous variables are presented as number and percent values. All statistical analyses were performed using the Statistical Package for the Social Sciences, version 18.0 (SPSS, Inc., Chicago, IL). A *P* value less than 0.05 was considered statistically significant. The correlations between clinicopathologic features and responders/non-responders were compared using the Chi-square test. The sensitivity, specificity, and accuracy of peri-therapeutic VEGF scores for prediction of response to bevacizumab combined with FOLFIRI were evaluated. The 6-month PFS and OS were calculated by the Kaplan-Meier method, and differences in survival rates were analyzed by the log-rank test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Demographic data for the 57 mCRC patients are shown in **Table 1**. We followed the enrolled

Table 3. The sensitivity, specificity, and accuracy of decreased vascular endothelial growth factor (VEGF) immunohistochemical (IHC) score peri-therapeutically vs responder for prediction of response of FOLFIRI combined with bevacizumab

	Responder (N)	Non-responder (No.) (N)	*P* value	Odds ratio (95% CI[1])
Decreased peri-therapeutic VEGF score			< 0.001	2.857
Yes	37	0		(1.572-5.192)
No	7	13		
Total	44	13		

[1]CI: Confidence interval. Sensitivity: 84.1% (71.6-96.6%). Specificity: 100%. Positive predictive value: 100%. Negative predictive value: 65.0% (48.7-81.3%). Accuracy: 87.7% (76.5-99.0%).

patients until January 2014, with a median follow-up period of 13.8 ± 5.9 median months (range, 6-32 months). Thirty-seven patients (64.9%) were deemed to have high pre-therapeutic VEGF scores. After treatment, there were only fourteen patients (24.6%) still categorized as having high VEGF scores. Thirty-seven patients (64.9%) were classified as having decreased peri-therapeutic VEGF scores.

Correlation between clinicopathologic features and responders by statistical analyses

There were forty-four patients (77.2%) who were found to be responders (**Table 2**). Based on a univariate analysis of the correlation between responders and clinicopathologic features, we found that low post-therapeutic VEGF scores ($P < 0.001$) and decreased peri-therapeutic VEGF scores ($P < 0.001$) were significantly predictive factors of responders (**Table 2**). However, there were no significant differences in gender, age, tumor size, tumor location, tumor invasive depth, vascular invasion, perineural invasion, tumor grade, tumor type, and pre-therapeutic VEGF score (all $P > 0.05$). Notably, high pre-therapeutic VEGF scores were not a significant predictor of responders ($P = 0.772$).

Predictive accuracy of peri-therapeutic VEGF score for response to bevacizumab

As shown in **Table 3**, a decreased peri-therapeutic VEGF score was a predictor for response to bevacizumab ($P < 0.001$; odds ratio, 2.857; 95% confidence interval, 1.572-5.192). The sensitivity was 84.1% (71.6-96.6%); specificity was 100%; positive predictive value was 100%; negative predictive value was 65.0% (48.7-81.3%); and accuracy was 87.7% (76.5-99.0%).

Six-month PFS and OS based on peri-therapeutic VEGF score

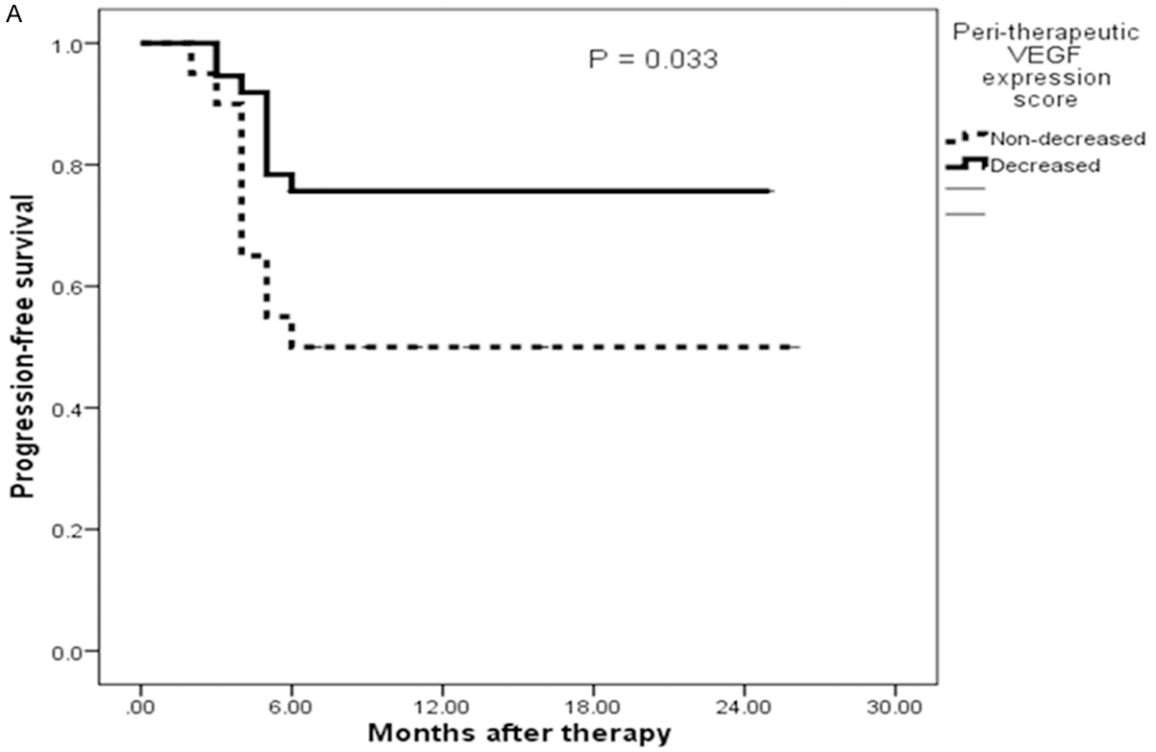
The 6-month PFS rate in mCRC patients with decreased peri-therapeutic VEGF scores was significantly higher than that without decreased peri-therapeutic VEGF scores ($P = 0.033$; **Figure 2A**). Moreover, the OS rate for mCRC patients with decreased peri-therapeutic VEGF scores tended to be higher than that without decreased peri-therapeutic VEGF scores, despite the fact that the difference was not statistically significant ($P = 0.094$; **Figure 2B**).

Discussion

In the present study, we demonstrated that (1) the tissue VEGF IHC scores before bevacizumab administration could not predict the response to bevacizumab; (2) decreased peri-therapeutic VEGF scores predicted better responses than did non-decreased ones; (3) decreased peri-therapeutic VEGF scores may result in a better prognosis in terms of the 6-month PFS but not OS.

Biologically targeted therapies are so named because their activity is restricted to impeding one or several pathways within tumor cells. Decisions regarding treatment options were dependent on performance status, age, organ function, and previous treatment decisions in the past [19]. *KRAS* gene mutation in the EGFR pathway is predictive for cetuximab treatment and has changed the approach from universal chemotherapy to more personalized treatments. Numerous retrospective studies have shown the prognostic value of VEGF expression in CRC. High VEGF expression in tumor tissue has been reported to indicate shorter relapse-free survival and OS [18, 20]. Conversely, some investigations did not find any clinical signifi-

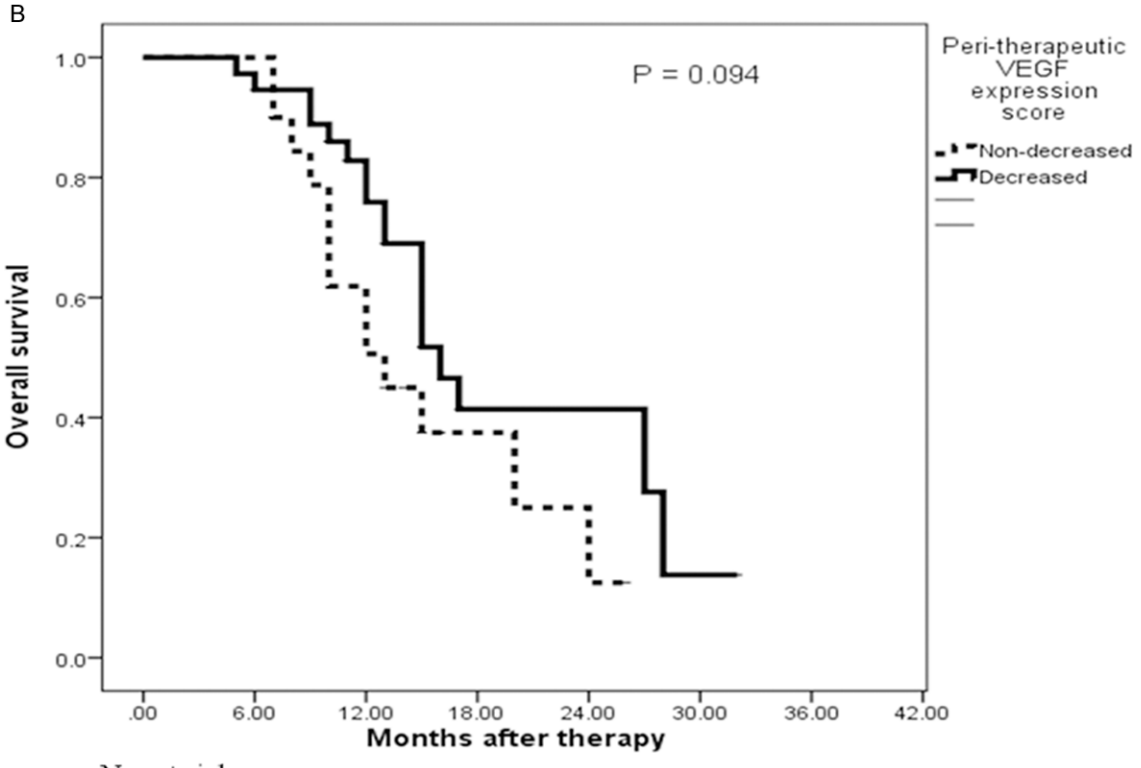
A



No. at risk

20	11	4	1	1
37	29	14	4	2

B



No. at risk

20	20	11	3	2
37	36	24	7	4

Figure 2. Cumulative survival rates of the 57 enrolled metastatic colorectal cancer patients (mCRC) undergoing FOLFIRI combined with bevacizumab as assessed by the Kaplan-Meier method. The differences in survival were analyzed by the log-rank test. A. The 6-month progression-free survival rate in mCRC patients with decreased peri-therapeutic VEGF scores was significantly higher than that in mCRC patients without decreased peri-therapeutic VEGF scores (P = 0.033); B. The overall survival rate in mCRC patients with decreased peri-therapeutic VEGF scores was not significantly better than that in mCRC patients without decreased peri-therapeutic VEGF scores (P = 0.094).

cance for VEGF expression in CRC patients [21, 22]. As has been previously established, bevacizumab targets the angiogenesis needed for tumor growth by inhibition of VEGF pathways [23]. The mechanism of action of bevacizumab has been shown to involve its capacity to bind to the VEGF protein and the resulting inhibition of angiogenesis [24]. Bevacizumab was approved by the FDA in 2004 as a first-line treatment for mCRC in combination with cytotoxic chemotherapy, with proof of principle for anti-angiogeneic therapy also provided [25]. Although VEGF expression in the tumor microenvironment should be expected to determine sensitivity to bevacizumab, no conclusive evidence yet exists that VEGF is a predictive biomarker of efficacy for antiangiogenic therapy [19]. Therefore, identification of alternative biomarkers to select those patients who are more likely to benefit from bevacizumab treatment would improve cost-effectiveness and therapeutic outcomes [26]. The current study offers a biological plausibility to support the role of VEGF alterations in response of bevacizumab, with a high sensitivity of 84.1% and a specificity of 100%. Our findings demonstrate that decreased post-therapeutic VEGF protein levels are an important predictor of patient response to bevacizumab, and can further predict 6-month PFS.

The identification of predictive markers of bevacizumab efficacy is a fascinating area of research [27, 28] and may lead to a further tailoring of anticancer treatments, thus optimizing their efficacy. As an example, a high dose trial of bevacizumab might be designed to improve treatment efficacy in subjects genetically predisposed to overexpress VEGF [29]. In 2012, Kara, et al reported that pre-therapeutic tissue VEGF scores were not a predictive factor for bevacizumab therapy [30]. This finding was consistent with our current results and those of previous studies [28, 31, 32]. In 2012, Bates, et al demonstrated that a low $VEGF_{165}b$, VEGF-A splice form/$VEGF_{total}$ ratio may be a predictive marker for bevacizumab therapy in mCRC, and concluded that patients with high relative levels may not benefit from bevacizumab [32]. They also reported that low/high $VEGF_{165}b$: $VEGF_{total}$ isoform ratios and the interaction test of such ratios were significant for PFS, but did not attain statistical significance for OS [32]. Similarly, we showed that the mCRC patients with low post-therapeutic and decreased peri-therapeutic VEGF scores have better responses to bevacizumab. Meanwhile, the mCRC patients with decreased peri-therapeutic VEGF scores have significantly better rates of PFS, but not OS. Compared with a study by Bates, et al [32], our study seemed to be simpler and easier for the prediction of the response to bevacizumab by using the altered VEGF IHC score as a potential surrogate predictor peri-therapeutically.

To the best of our knowledge, this is the first study to identify peri-therapeutic VEGF IHC scores as a potential predictive biomarker of responsiveness to bevacizumab in mCRC. We have mentioned some possible factors that may influence the response in mCRC patients to bevacizumab treatment. Meanwhile, the 6-month PFS rate was better in patients with decreased peri-therapeutic VEGF scores.

There are, however, a number of limitations in our study. First, only 57 mCRC patients were enrolled and FOLFIRI combined with bevacizumab therapy was only administered for 6 months. Thus, the ability to detect meaningful differences in all the clinically relevant endpoints, such as OS, was reduced. Second, the use of an IHC scoring system is subjective, and future validation using an automated quantitative system should be considered. Third, tissue-based biomarker studies are highly valuable because they give direct information regarding tumor tissues; however, there are some limitations for pharmacodynamic biomarkers in routine practice. Lastly, our studied patients were administered only 6 months of bevacizumab treatment under the reimbursement regulation of National Health Insurance Administration,

and this might have led to the fact that a significant effect was found for 6-month PFS but not for OS. Therefore, an extended therapeutic duration of bevacisumab administration would be mandatory for the confirmation of the decreased peri-therapeutic VEGF score for predicting OS in mCRC patients.

Although there were some limitations in the current study, it offered an easy, simple, and highly accurate method for predicting patient response to bevacizumab. As such, it may be possible to use the series of changes in VEGF IHC scores during the treatment period to validate the treatment effects. Certainly, there remains a need for large, well-designed prospective clinical trials to better define the significant predicting factors.

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Disclosure of conflict of interest

None.

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