CLINICAL RESEARCH

Stromal Expression of Vimentin Predicts the Clinical Outcome of Stage II Colorectal Cancer for High-Risk Patients

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ACD 1 **Li-guo Liu***
ABCE 2 **Xue-bing Yan***
BCD 3 **Ru-ting Xie***
AEG 1 **Zhi-ming Jin**
DEF 4 **Yi Yang**

1 Department of General Surgery, Shanghai Jiao Tong University Affiliated Sixth Peoples' Hospital, Shanghai, P.R. China
2 Department of Medicine, Soochow University, Suzhou, Jiangsu, P.R. China
3 Department of Pathology, Shanghai Tenth Peoples' Hospital, Tongji University School of Medicine, Shanghai, P.R. China
4 Department of Oncological Surgery, Kunshan Traditional Chinese Medicine Hospital Affiliated to Nanjing University of Chinese Medicine, Kunshan, Jiangsu, P.R. China

Corresponding Authors:
Source of support:

* These authors contributed equally to this manuscript
Zhi-ming Jin, e-mail: jzmgyp@aliyun.com; Yi Yang, e-mail: 344549149@qq.com
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Background: Increased expression of vimentin in tissue samples from patients with colorectal cancer (CRC) has been previously demonstrated, but its prognostic significance remains controversial, and the clinical significance for patients with stage II CRC is still unknown. The aim of this study was to evaluate the expression of vimentin in CRC and its potential prognostic significance.

Material/Methods: We analyzed vimentin expression in 203 CRC tissue samples from patients with stage II cancer using immunohistochemistry, and correlated the findings with clinicopathological patient features. CRC-specific survival (CSS) and disease-free survival (DFS) were analyzed using the Kaplan-Meier method. Univariate and multivariate analysis was performed using the Cox proportional hazards method for survival.

Results: Vimentin expression was significantly correlated only with tumor (T) stage (p=0.024). Kaplan-Meier survival analysis indicated that vimentin expression could stratify the CSS and DFS of patients with stage II CRC at high risk (p=0.029, p=0.042, respectively), but not those of low-risk stage II patients (p=0.208, p=0.361, respectively). Univariate and multivariate analysis further revealed that stromal vimentin expression is an independent prognostic factor for CSS and DFS of high-risk stage II patients (p=0.043, p=0.022, respectively). Moreover, high-risk stage II patients with low stromal vimentin expression benefitted more from standard adjuvant chemotherapy than those with high stromal vimentin expression (CSS: p=0.012 *vs.* p=0.407; DFS: p=0.017 *vs.* p=0.420).

Conclusions: Our study suggests that stromal vimentin expression is a promising indicator for survival prediction and adjuvant chemotherapy response in patients with stage II CRC with high-risk factors for recurrence.

MeSH Keywords: **Chemotherapy, Adjuvant • Colorectal Neoplasms • Vimentin**

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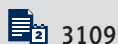
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Background

Colorectal cancer (CRC) is one of the most commonly diagnosed human malignancies worldwide [1]. In 2016, in the United States, there were estimated to be more than 1.4 million cases of CRC, of which, 134,490 cases were newly diagnosed in that year [2]. Radical surgery and adjuvant chemotherapy (AC) are the main treatment modalities for patients with CRC, mainly for those within stage II and stage III CRC. Although there have been advances in surgical techniques and chemotherapy, a considerable proportion of patients with CRC have a poor clinical outcome, largely due to adverse prognostic factors including local recurrence, distant/regional metastasis, and insensitivity to chemotherapy. Recently, accumulating studies have closely linked these factors to the molecular events in CRC development, such as specific mutations, signaling activation, and epigenetic modifications [3]. An understanding of the molecular events that occur in the progression of CRC is of practical prognostic significance and for treatment decisions for patients with CRC.

Epithelial-mesenchymal transition (EMT) is the process by which epithelial cells acquire a mesenchymal cell phenotype, and EMT is now known to be involved in diverse biological processes, including embryonic development, wound healing, tissue fibrosis, and neoplastic transformation [4]. Cancer cells undergoing EMT may acquire enhanced proliferation, invasion, and cell migration abilities, and malignant neoplastic cells may acquire resistance to conventional chemoradiotherapy [5]. In CRC, EMT has been shown to stimulate tumor angiogenesis and cancer stem cell traits, which indicates that targeting EMT could be a promising therapeutic strategy for patients with CRC [5]. A recent study has shown that EMT induction in CRC cells is dependent on microsatellite instability, indicating that EMT may also contribute to the progression of hereditary forms of CRC [6]. However, despite emerging studies regarding the possible role of EMT in CRC, its prognostic value remains poorly investigated.

The EMT phenotype is also commonly characterized by molecular changes, including downregulation of epithelial cell markers and upregulation of mesenchymal markers [7]. E-cadherin is the most widely studied epithelial marker, and loss of E-cadherin expression is associated with lymph node metastasis and poor patient outcome in patients with CRC [8,9].

Mesenchymal cell markers include N-cadherin and vimentin [10]. We have previously demonstrated that high expression of N-cadherin promotes the development of CRC and is an independent prognostic factor for overall survival [11]. However, the clinical significance of vimentin remains controversial. For example, Ngan et al. suggested that vimentin expression could predict prognosis for patients with stage III CRC, whereas a recent study found no prognostic value of vimentin for these patients [12,13]. There are limited studies on the clinical significance of vimentin in stage II CRC. Therefore, we conducted a retrospective study of 203 patients diagnosed with stage II CRC to investigate the predictive value of vimentin for survival and response to chemotherapy for patients with stage II CRC.

Material and Methods

Ethical approval and patient consents

This study was reviewed and approved by the Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth Peoples' Hospital and Shanghai Tenth Peoples' Hospital. Written informed consents were obtained from patients for the use of their resected surgical specimens and use of their clinical data. Patient confidentiality was ensured during the study.

Patient selection, diagnosis, and staging of colorectal cancer (CRC)

Initially, a total of 391 patients were enrolled in the study. Patients were identified who had stage II CRC and who had received colorectal surgery between October 2005 and May 2014 in the Department of General Surgery, Shanghai Jiao Tong University Affiliated Sixth Peoples' Hospital and Shanghai Tenth Peoples' Hospital. Preoperative radiological examination was performed to identify regional and distant metastasis. Postoperative histopathological and clinical staging was conducted according to the Tumor Node Metastasis (TNM) classification of the Union for International Cancer Control (UICC) (7th edition). None of the patients studied received preoperative chemotherapy or radiotherapy.

A total of 188 patients were excluded based on the following criteria: (1) incomplete clinicopathological parameters or follow-up records (n=151); (2) death caused by infection, cardiovascular disease, or other noncancerous factors (n=17); (3) concomitant other cancer types (n=8); (4) emergency surgery for complete intestinal obstruction (n=7); (5) histopathologically confirmed positive surgical margin (n=5). Finally, 203 patients were enrolled for this study, and CRC tissues from these patients were prepared and analyzed.

The postoperative follow-up was routinely carried out according to the National Comprehensive Cancer Network (NCCN) guidelines with physical examination and serum tumor markers every six months, and computed tomography (CT) imaging was performed annually. Adjuvant chemotherapy (AC) included capecitabine, FOLFOX, and CapeOx, and was recommended for patients at high-risk of recurrence and carried out under standard conditions.

Table 1. Correlations between stromal vimentin expression and the clinicopathological characteristics of stage II CRC patients.

Characteristics	Total	Vimentin expression		P value
		Low	High	
Age				
≤60	83	23	60	0.724
>60	120	36	84	
Gender				
Male	124	40	84	0.209
Female	79	19	60	
Tumor location				
Colon	97	26	71	0.498
Rectal	106	33	73	
Tumor size				
≤5 cm	122	33	89	0.438
>5 cm	81	26	55	
Tumor differentiation				
Well/moderate	153	45	108	0.849
Poor	50	14	36	
T stage				
T3	82	31	51	0.024
T4	121	28	93	
Lymphovascular/perineural invasion				
Positive	38	9	29	0.418
Negative	165	50	115	
Lymph node sampling				
<12	38	14	24	0.241
≥12	165	45	120	
Serum CEA level				
≤5 ng/ml	131	37	94	0.729
>5 ng/ml	72	22	50	
Ki-67 expression				
<30%	109	34	75	0.472
≥30%	94	25	69	

Patients at high risk of tumor recurrence were defined as those with the histological features of poor tumor differentiation (high grade), lymphovascular or perineural invasion, and an insufficient number of lymph nodes examined at surgical resection (<12). Patients at low risk of tumor recurrence were defined as those without the above characteristics.

CRC-specific survival (CSS) was calculated from the date of surgery to the date of death caused by CRC. Disease-free survival (DFS) was calculated from the date of surgery to the date of CRC recurrence/metastasis. The baseline clinicopathological characteristics of patients are detailed in Table 1.

Immunohistochemical (IHC) staining and evaluation

Immunohistochemistry (IHC) was performed on CRC tissues as previously described [14,15]. Briefly, formalin-fixed, paraffin-embedded colorectal tissues were sectioned consecutively at 5-µm
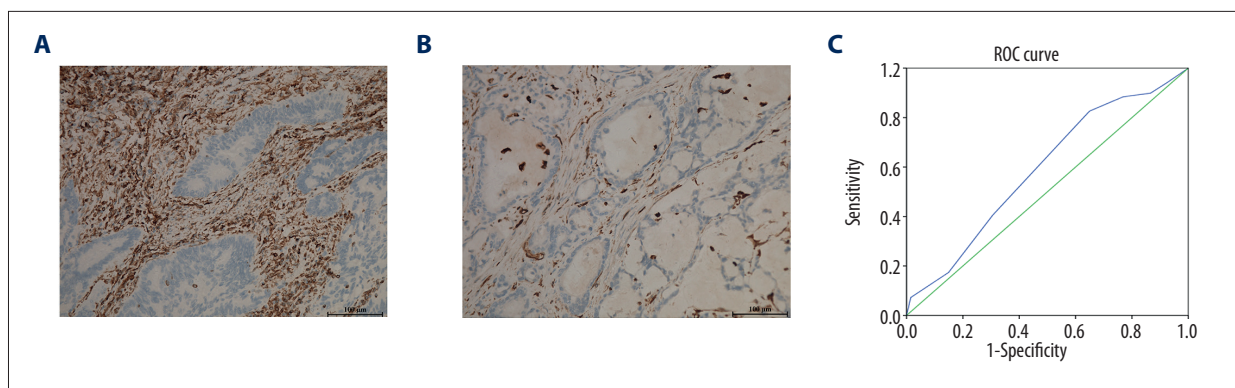
Figure 1. Stromal expression of vimentin in patients with stage II colorectal cancer (CRC). (**A**) High stromal expression of vimentin in CRC tissues (magnification: ×200). (**B**) Low stromal expression of vimentin in CRC tissues (magnification: ×200). (**C**) Receiver operating characteristic (ROC) curve of staining scores for stromal vimentin expression.

and immersed in xylene for dewaxing. Rehydration of sections was performed with a series of gradient alcohols. Tissue sections were then incubated with 3% hydrogen peroxide solution to inactivate endogenous peroxidase and heated in a microwave for antigen retrieval. Nonspecific antibody binding to the tissue sections was inhibited by blocking the sections in 10% fetal bovine serum (FBS) at 37°C for 30 min. After three washes with phosphate-buffered saline (PBS), the sections were incubated with primary antibody to vimentin (1: 200) (Bioworld, St. Louis Park, MN, USA) overnight at 4°C. Polyclonal goat anti-rabbit IgG (1: 250) (Abcam, Cambridge, MA, USA) was applied as the secondary antibody. Finally, the color reaction was developed using diaminobenzidine (DAB) reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and the sections were counterstained with hematoxylin. After dehydration and sealing, the sections were prepared for light microscopic examination.

The staining results were scored by at least two independent investigators who were blinded to the patient data. Any discrepancy in the histological evaluation was resolved by consensus. All sections were scored according to the evaluation method described previously [16]. Briefly, the scoring method was based on the percentage of positive stromal regions (PR) and staining intensity (SI). PR was scored as follows: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (>75%). SI was scored as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). A final score was calculated as the product of SI and PR. For statistical analysis, a cut-off point of the final score was determined by the receiver operating characteristic (ROC) curve. Sections that scored more and less than the cut-off point were regarded as high expression and low expression, respectively.

Statistical analysis

All statistical analysis was performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). The cut-off value of

the ROC curve was determined using the Youden index. The correlation of vimentin expression with the patient clinicopathological parameters was examined using the chi-squared test. CSS and DFS curves were depicted using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) based on the Kaplan-Meier model and intergroup differences were examined by the log-rank test. Significant independent factors affecting CSS and DFS were evaluated by univariate and multivariate using the Cox proportional hazards method for survival. A p-value less than 0.05 indicated statistical significance.

Results

Expression of vimentin in patients with stage II CRC

The expression of vimentin protein in CRC tissues was detected by immunohistochemical (IHC) staining. Positive expression of vimentin was predominantly located in the tumor stroma (Figure 1A, 1B), and 144 of 203 tumor samples from patients with stage II CRC demonstrated high levels of stromal expression of vimentin with a mean staining score of 5.98±2.59.

ROC curve analysis demonstrated that the optimal cut-off point of IHC staining scores for vimentin was 5 (Figure 1C). Therefore, we used this cut-off point to assign the patients to a high expression group (staining score ≥6, n=144) and a low expression group (staining score <6, n=59).

Correlation between vimentin expression and the clinicopathological characteristics of patients with stage II CRC

The results of the chi-squared test, performed to determine whether stromal expression of vimentin correlated with the clinical traits of patients with stage II CRC are summarized in Table 1. Stromal expression of vimentin was significantly
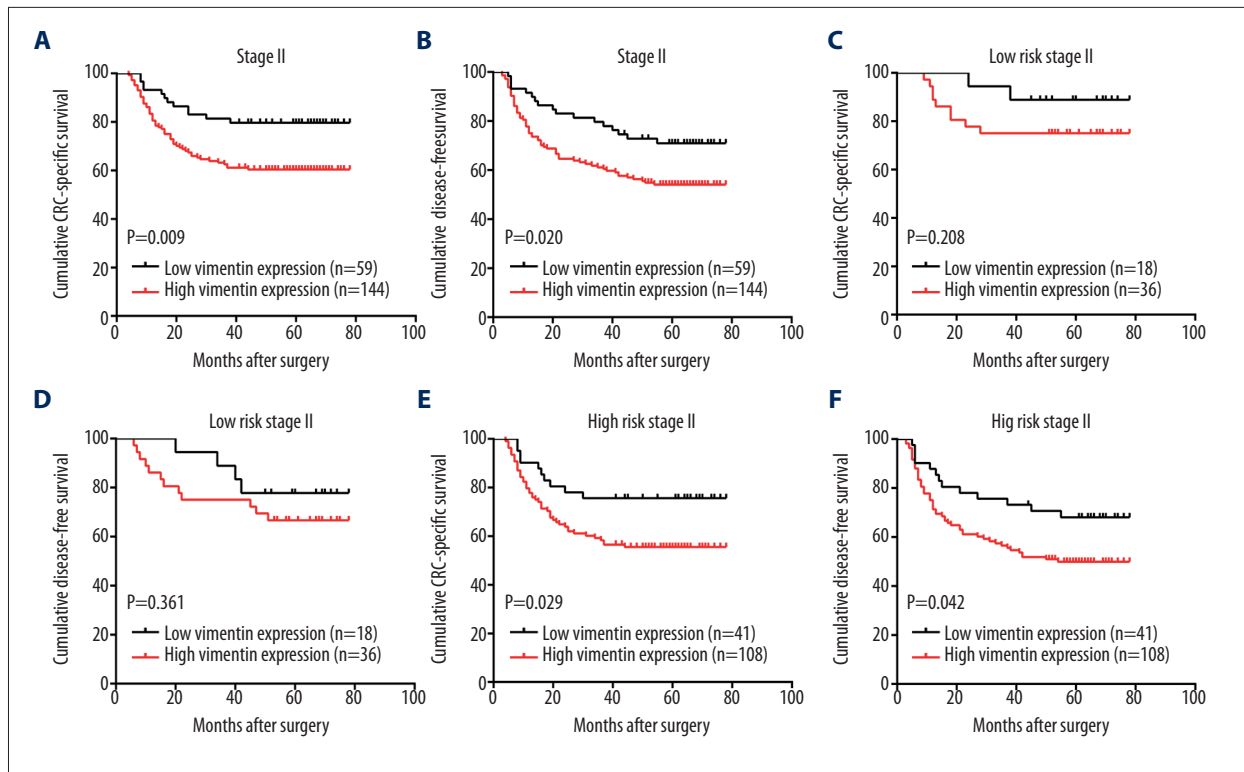
Figure 2. Prognostic stratification of stromal vimentin expression in patients with stage II colorectal cancer (CRC). (**A, B**) CRC-specific survival (CSS) and disease-free survival (DFS) curves of the whole study cohort. (**C, D**) CSS and DFS curves of patients with stage II CRC with no high-risk factors. (**E, F**) CSS and DFS curves of patients with stage II CRC with high-risk factors.

correlated only with tumor (T) stage (p=0.024); no significant correlation was found with other clinicopathological characteristics including age (p=0.724), gender (p=0.209), tumor location (p=0.498), tumor size (p=0.438), tumor differentiation (p=0.849), the presence of lymphovascular or perineural invasion (p=0.418), lymph node sampling (p=0.241), preoperative carcinoembryonic antigen (CEA) levels (p=0.729), and Ki-67 positivity rate for the tumor cells (p=0.472).

Prognostic significance of stromal vimentin expression in stage II CRC

The correlation between stromal vimentin expression and postoperative survival for patients with stage II CRC using the Kaplan-Meier model is shown in Figure 2A, 2B. Patients with stage II CRC with high stromal expression of vimentin had significantly lower CRC-specific survival (CSS) and disease-free survival (DFS) rates than those with low stromal vimentin expression (p=0.009; p=0.020, respectively).

In subgroup analysis, based on concomitant high-risk factors, stromal vimentin expression had no prognostic significance for the CSS and DFS of patients with no high-risk factors (Figure 2C, p=0.208; Figure 2D, p=0.361). However, for patients with stage II CRC and high-risk factors, increased expression

of stromal vimentin was associated with a worse clinical outcome when compared with reduced expression of stromal vimentin (Figure 2E, p=0.029; Figure 2F, p=0.042). Furthermore, for stage II patients with high-risk factors (shown in Tables 2 and 3), univariate analysis suggested that stromal vimentin expression (p=0.034), tumor differentiation (p<0.001), and tumor (T) stage (p=0.045) were significantly correlated with CSS; multivariate analysis indicated that these were all independent factors for CSS (p=0.043, p<0.001, and p=0.023, respectively). Similarly, univariate analysis showed that vimentin expression (p=0.047), tumor differentiation (p<0.001), and lymphovascular or perineural invasion (p=0.035) were significantly correlated with DFS; multivariate analysis confirmed that these were also independent factors for DFS (p=0.022, p<0.001, and p=0.016). Taken together, these findings collectively showed that stromal expression of vimentin was an independent prognostic factor for patients with stage II CRC and high-risk factors.

Predictive role of stromal vimentin expression for chemotherapy benefits in patients with stage II CRC with high-risk factors

To determine whether stromal vimentin expression correlated with the benefit of adjuvant chemotherapy (AC) in patients with stage II CRC with high-risk factors, survival analysis was

Table 2. Univariate analysis and multivariate analysis for prognostic factors in CRC-specific survival of high-risk stage II CRC patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age	1.184	0.693–2.022	0.537			
Gender	0.605	0.336–1.089	0.094			
Tumor location	0.883	0.528–1.478	0.637			
Tumor size	1.022	0.601–1.737	0.935			
Serum CEA level	0.851	0.498–1.454	0.555			
Ki-67 expression	1.094	0.654–1.830	0.733			
Lymphovascular/perineural invasion	0.646	0.373–1.119	0.119			
Lymph node sampling	1.163	0.628–2.157	0.631			
Tumor differentiation	2.665	1.589–4.470	<0.001	3.105	1.839–5.241	<0.001
T stage	2.371	1.018–5.521	0.045	2.694	1.143–6.349	0.023
Vimentin expression	2.092	1.058–4.135	0.034	2.028	1.021–4.029	0.043

Table 3. Univariate analysis and multivariate analysis for prognostic factors in disease-free survival of high-risk stage II CRC patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age	1.134	0.691–1.861	0.618			
Gender	0.629	0.366–1.080	0.093			
Tumor location	0.855	0.529–1.381	0.522			
Tumor size	0.929	0.564–1.530	0.772			
T stage	1.762	0.873–3.558	0.114			
Lymph node sampling	1.075	0.613–1.885	0.801			
Serum CEA level	0.962	0.589–1.573	0.877			
Ki-67 expression	0.990	0.613–1.599	0.966			
Tumor differentiation	3.236	1.995–5.247	<0.001	3.484	2.142–5.666	<0.001
Vimentin expression	1.847	1.007–3.386	0.047	2.032	1.106–3.734	0.022
Lymphovascular/perineural invasion	0.581	0.351–0.963	0.035	0.534	0.322–0.888	0.016

performed based on the Kaplan-Meier model. According to follow-up data, 93 of 149 patients received standard AC and these patients had no better CSC and DFS than those who received no AC (Figure 3A; p=0.058) (Figure 3B; p=0.067). For patients with stage II CRC at high-risk and with low stromal vimentin expression, AC treatment was significantly associated with a more favorable clinical outcome than for those patients who did not receive AC (Figure 3C, CSS: p=0.012; Figure 3D, DFS: p=0.017). However, this association was not statistically significant in patients with high stromal vimentin expression (Figure 3E, CSS: p=0.407; Figure 3F, DFS: p=0.420).

Discussion

Although patients with stage II colorectal cancer (CRC) who are diagnosed histologically and who have been confirmed as negative for lymph node metastasis, should have a relatively good prognosis. However, increasing numbers of studies have reported that up to 20% of patients with stage II CRC will undergo tumor recurrence following resection [17,18]. There is a subgroup of patients with stage II CRC who have a worse clinical outcome than patients with stage III CRC presenting with up to two positive lymph nodes (stage IIIA) [17,18]. Also, there
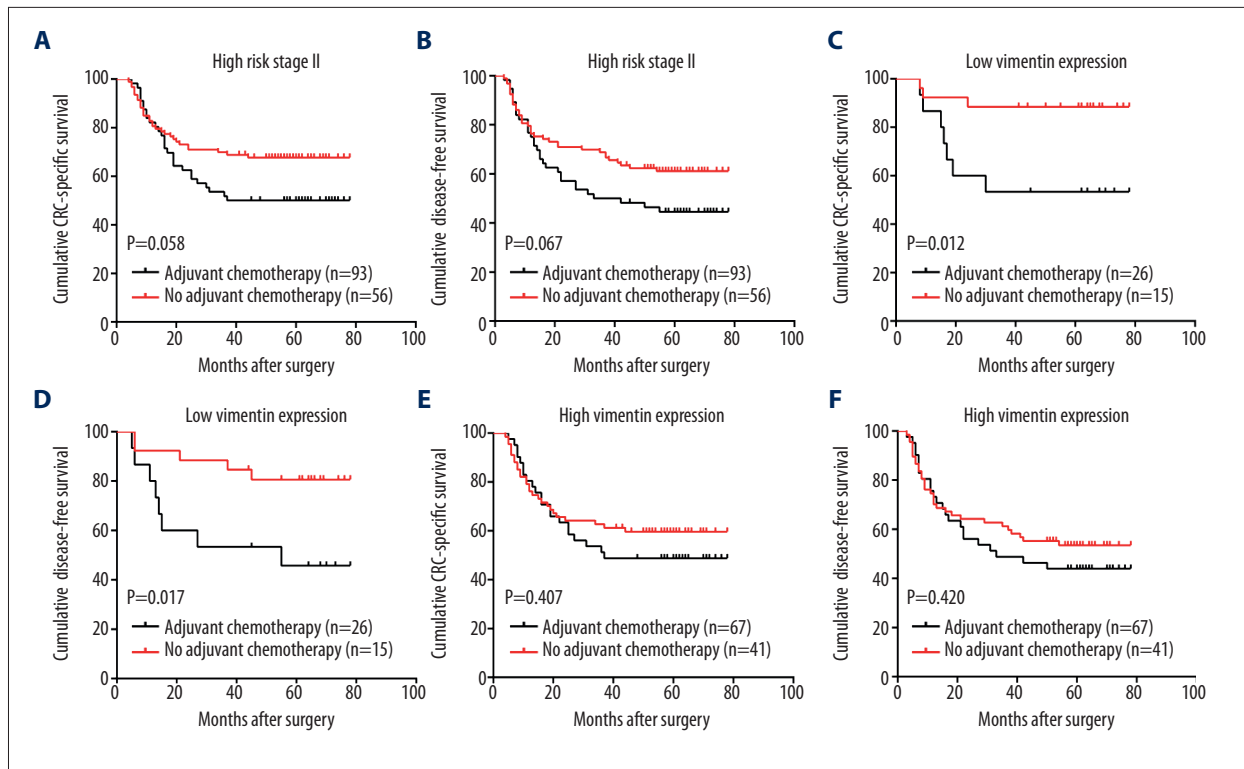
Figure 3. Correlations between stromal vimentin expression and adjuvant chemotherapy (AC) benefits in high-risk stage II patients with colorectal cancer (CRC). (**A, B**) CRC-specific survival (CSS) and disease-free survival (DFS) curves of all high-risk stage II patients with or without AC. (**C, D**) AC stratification for CSS and DFS curves of high-risk stage II patients with low stromal vimentin expression. (**E, F**) AC stratification for CSS and DFS curves of high-risk stage II patients with high stromal vimentin expression.

is still controversy regarding the role of treatment with adjuvant chemotherapy (AC) for patients with stage II CRC who are at high risk of tumor recurrence [19–21]. Current TNM staging and treatment guidelines may not provide recommendations that are relevant to these high-risk populations, and so reliable prognostic indicators for more accurate patient classification are needed.

Recent studies have indicated the potential predictive role of perineural invasion and microsatellite instability in disease progression and for chemotherapy benefit for patients with stage II CRC, but no definite conclusive statements have been made due to limited supporting data [22,23]. Epithelial-mesenchymal transition (EMT) as an oncogenic molecular event has been extensively investigated in the CRC field during the past decade. EMT-related markers or regulators have been proved to be involved in CRC development through various mechanisms. However, only a few of these findings have been translated into clinical benefit, and most of the promising EMT-related biomarkers remain are still undergoing research.

Vimentin is a canonical marker of EMT, and a novel study based on computational modeling recently proposed vimentin as a promising biomarker for CRC [24]. We conducted a retrospective study to validate its clinical significance in patients with stage II CRC. Our results showed that vimentin expression was mainly detected in the tumor stroma and 70.9% (144/203) of patients with stage II CRC, high vimentin expression was found. This observation is similar to findings of a recent study demonstrating increased expression of vimentin in the majority of patients with CRC (67.3%, 140/208) [16]. Statistical analysis suggested that stromal vimentin expression, was significantly correlated only with tumor (T) stage among all clinicopathological parameters tested, implying its potential involvement in tumor invasion. This finding also suggested that vimentin expression might serve as an early biomarker for disease progression in patients with early-stage CRC. This finding is partly supported by recent work by Akhtar et al., who found that vimentin expression increased from oral carcinoma *in-situ* to invasive carcinoma, and proposed its predictive role in the early diagnosis of tumor microinvasion [25].

Because previous studies highlighted the prognostic significance of stromal vimentin expression in human malignancy, we performed a Kaplan-Meier model to investigate the correlation between vimentin expression and postoperative survival

in patients with stage II CRC [26,27]. The findings of this study showed that patients with high stromal vimentin expression had worse CRC-specific survival (CSS) and disease-free survival (DFS) when compared with patients with low stromal vimentin expression. However, when subgroup analysis was conducted, based on high-risk factors, we found no significant correlation between stromal vimentin expression and the clinical outcome of low-risk patients, suggesting that stromal vimentin expression may not be an effective prognostic predictor for all stage II patients. We then performed univariate and multivariate analysis only for high-risk patients with stage II CRC, whose clinical outcome could be stratified by stromal vimentin expression. Current studies have demonstrated that numerous conventional high-risk clinicopathological factors, such as histologic differentiation, lymphovascular or perineural invasion, and number of harvested lymph nodes, have controversial roles in predicting patient outcome, indicating that novel molecular classification should be considered [28,29].

The findings of this study indicate that stromal vimentin expression is an independent prognostic factor for CSS and DFS for patients with stage II CRC who are at high risk of recurrence, indicating its potential role in supplementing and improving upon the current clinical prognosis systems based mainly on conventional clinicopathological factors.

Although the National Comprehensive Cancer Network (NCCN) guidelines have proposed adjuvant chemotherapy (AC) for patients with stage II CRC at high risk, the benefits remain controversial [19,20]. This situation may largely reflect the lack of effective criteria for identifying suitable patients who are most likely to benefit from conventional AC [30]. In this study, patients receiving AC had no better outcome than those receiving no AC, consistent with the observations of a recent large-scale clinical study enrolling 2,488 patients with stage II CRC [31]. However, when subgroups were classified by stromal vimentin expression we found that AC could improve outcome in high-risk stage II patients with low vimentin expression when compared with those with high vimentin expression, suggesting that AC might be an effective therapeutic option for high-risk stage II patients with low stromal vimentin expression. This finding is similar to the proposal of Loree et al. that AC might only improve the outcome in selected high-risk stage II patients [19]. Moreover, inspired mainly by emerging evidence that vimentin plays a crucial role in resistance to other CRC treatments such as histone deacetylase inhibitor, we hypothesize that this finding might be associated with the chemotherapy resistance induced by high vimentin expression [32]. Considering the crucial role of tumor stroma in therapy resistance, we further speculate that a higher level of vimentin staining may indicate more tumor stroma, and as a consequence

more barriers preventing chemotherapy agents from accessing the tumor cells [33]. However, whether upregulated vimentin has any impact on CRC cell sensitivity to conventional chemotherapy drugs, and the relevant molecular events, remain to be further investigated.

There are several limitations in this study. First, the study cohort was relatively small, and we were unable to employ an additional independent validation cohort to confirm the results. Furthermore, there were some inherent and inevitable factors, such as ethnic differences and non-standardized experiment conditions and evaluation criteria, that could have influenced the assessment of the clinical value of a single biomarker. Therefore, further studies, including a larger patient study size, multiple centers, and adequately controlled studies, are advised to support and extend this preliminary study. Also, the expression of stromal vimentin may be evaluated using objective and quantitative analysis, with bioinformatics methods. Finally, we have not investigated the clinical significance of circulating vimentin in patients with stage II CRC, although the value of this approach has been highlighted in a recent study [34]. Further efforts should also be made to identify whether vimentin may be developed as a novel noninvasive prognostic biomarker for CRC.

Conclusions

The findings of this study have shown that stromal expression of vimentin may be a potential prognostic factor in patients with stage II colorectal cancer (CRC) with high-risk factors. This study has also shown that patients with low expression of stromal vimentin appear to benefit more from adjuvant chemotherapy (AC) than those with high expression of stromal vimentin. Therefore, expression of stromal vimentin may be a promising predictive indicator for patient survival and response to chemotherapy in patients with stage II CRC who have high-risk factors.

Conflict of interest

None.

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