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Original Article

The correlation of KAI1, Slug and vasculogenic mimicry in the prediction of metastasis and prognosis in colorectal carcinoma

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Abstract: KAI1 and the epithelial-mesenchymal transition regulator Slug, as well as vasculogenic mimicry (VM), play a significant role in the process of metastasis and prognosis in many human cancers. This study investigated the relationship of KAI1, Slug and VM and their connection with clinicopathological features, metastasis and their prognostic value in colorectal carcinoma (CRC). Immunohistochemistry was performed to analyze the expression of KAI, Slug, VM, E-cadherin (E-cad) and vimentin in 134 human CRC and corresponding normal mucosal tissues. The results showed that the protein expressions of KAI1, Slug and VM in tumor tissues were significantly different from adjacent normal mucosal tissues. The expression of KAI1 was negatively related, while the expressions of Slug and VM are positively related with differentiation, lymph node metastasis, distant metastasis and TNM stage. KAI1 was negatively related, while VM was positively related with invasion. The expression of KAI1 showed a negative correlation with Slug and VM. Moreover, the expression of Slug was positively associated with VM. A Kaplan-Meier analysis showed that the expression of Slug and VM was negatively and KAI1 expression was positively correlated with overall survival. Low KAI1 expression and high Slug and VM expression showed a poorer prognosis in CRC. Multivariate Cox regression analysis showed that the expression of KAI1, Slug, VM and TNM stage were independent predictors of OS in CRC. The expression of KAI1, Slug and VM was correlated with metastasis and prognosis. These findings suggest that KAI1, Slug and VM can be novel therapeutic targets to predict metastasis and prognosis in CRC patients.

Keywords: CRC, KAI1, Slug, VM, prognosis

Introduction

CRC is the fourth most common malignancy and the third leading cause of death worldwide. It is estimated that 1 million new cases of CRC were reported globally, and among them 19100 were in China [1]. In China, the increase of risk factors, such as unhealthy diet, obesity, and smoking led to higher morbidity of CRC. Although the diagnosis and treatment of CRC has been improved over the past decades, on the whole, the prognosis for CRC patients still remained poor [2]. Hence, it is necessary to identify biomarkers associated with metastatic phenotype, which may act as new therapeutic strategies for CRC.

The membrane protein KAI1, also known as CD82, belongs to the tetraspanin superfamily that consists of four transmembrane domains, short N- and C-terminal cytoplasmic domains [3]. Tetraspanins are involved not only in extensive physiological processes, but also in the pathological situations such as cancer invasion and metastasis [4, 5]. KAI1 was first identified on chromosome 11P11.2 and demonstrated its role in the suppression of prostate metastasis. KAI1 promotes cell-cell or cell-extracellular matrix interaction to restrain cancer metastasis [5]. Previous studies have reported the metastatic suppressor function of KAI1 in a variety of malignant tumors [6-10].

Epithelial-mesenchymal transition (EMT) is a biological process wherein the polarized epithelial cells downregulate E-cadherin, which results in the dissolving of cell-cell adhesions, disruption of cell polarity, and upregulation of multiple

Table 1. Patient characteristics

Patient characteristic	Frequency (n)	Percentage (%)
Sex		
Male	77	57.5
Female	57	42.5
Age		
60 years	53	39.6
≥ 60 years	81	60.4
Diameter of tumor		
5.0 cm	79	59.0
≥ 5.0 cm	55	41.0
Location		
Rectum	70	52.2
Colon	64	47.8
Differentiation		
Well	31	23.1
Moderate	69	51.5
Poor	34	25.4
Depth of invasion		
Under serous membrane	76	56.7
To serous membrane	58	43.3
Lymph node metastasis		
Negative	79	59.0
Positive	55	41.0
Distant metastasis		
Negative	116	86.6
Positive	18	13.4
TNM stage		
I + II	79	59.0
III + IV	55	41.0

mesenchymal-associated proteins to become motile mesenchymal cells [11-13]. EMT accelerates cancer progression by allowing the primary tumor cells to break through the basal lamina and invade the adjacent tissues, leading to tumor metastasis [14]. Slug, also known as Snail2, plays a critical role in the Twist1-induced EMT process and cancer progression [15]. Moreover, studies have shown that Slug overexpression is associated with increased metastasis and a reduced postoperative survival of many cancers [16-21].

Angiogenesis plays a crucial role in tumor progression because tumors are supplied with oxygen and nutrients and tumor cells are associated with pathways for circulation. Vasculogenic mimicry (VM) was first reported in 1999 by Maniotis et al. who found that uveal melanoma cells resembled the endothelial phenotype and formed vascular networks in the absence of endothelial cells [22]. VM channels are vascular channel-like structures that are lined by tumor cells in which no endothelial cells can be seen on their inner walls [23]. VM-forming tumor cells change their cell markers and form vessel-like structures that are similar to the pattern of embryonic vasculogenesis. Current anti-angiogenic treatments cannot inhibit the VM process and could even promote VM formation [24]. These discoveries explain why anti-angiogenic drugs are less effective than hoped [25]. VM has been involved in the invasion, metastasis and poor prognosis in many malignant tumors [26-30].

Overall, the studies of KAI1, Slug and VM showed that these biomarkers are involved in tumor progression. However, the relationships among KAI1, Slug and VM in CRC metastasis and prognosis is currently unknown. Hence, in this study, we tried to detect the expression of KAI1, Slug and VM in CRC to identify the relationships among the protein expression, metastatic potential, and survival of CRC.

Material and methods

Patients and tissue samples

A total of 134 CRC tissues (77 men and 57 women; median age: 63.7 years; range: 20-83 years) who underwent radical resection and peripheral mesenteric lymph node dissection of CRC were randomly selected from the department of pathology of the First Hospital Affiliated of Bengbu Medical college from January 2007 to December 2008. Of these, 134 CRC tissues along with 134 surrounding normal mucosa were removed from each patient, and from the surrounding colorectal mucosal tissues at least 5 cm away from the cancer edge to obtain samples of CRC. Patients who received preoperative chemotherapy or radiotherapy were excluded. We collected the complete clinicopathological and follow-up data of all the patients (at 6 months interval by mail, phone or social application). All patients had no history of hereditary CRC and other malignancies. Written informed consent was obtained from all patients. The study was approved by the ethics committee of the Bengbu Medical College and performed in accordance with the guidelines of the Declaration of Helsinki. Overall

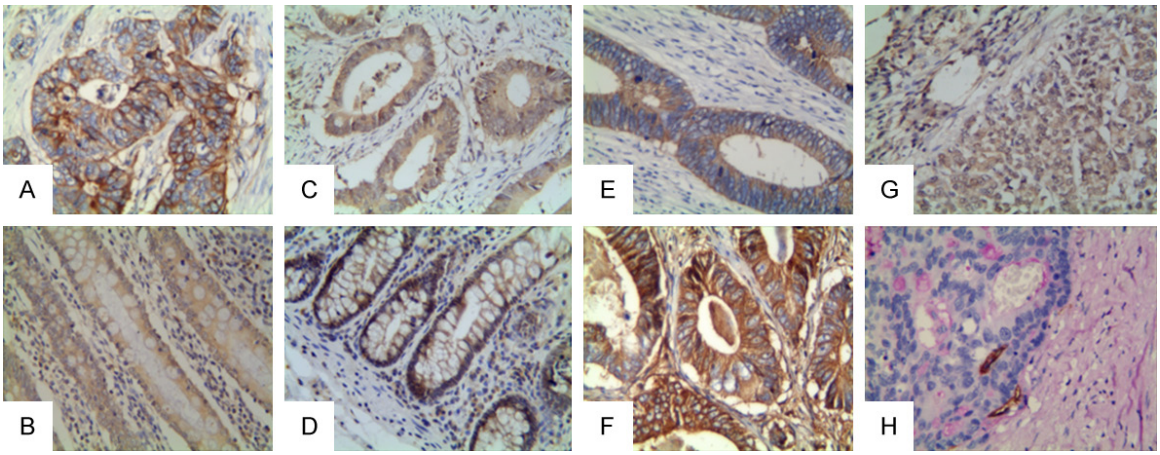

Figure 1. Immunohistochemical staining results of KAI1, Slug, E-cad, vimentin and VM in CRC and control tissues (400 × magnification). A. Positive expression of KAI1 in the membrane of cancer cells. B. Positive expression of KAI1 in the membrane of control tissues. C. Positive expression of slug in the cytoplasms as well as in the nuclei of cancer cells. D. Positive expression of Slug in the cytoplasms of the control tissues. E. Positive expression of E-cad in the membranes of cancer cells. F. Positive expression of E-cad in the membranes and cytoplasms of cancer cells. G. Positive expression of vimentin in the cytoplasms as well as in the nuclei of cancer cells. H. Positive staining of VM in CRC tissues by CD34/PAS double staining.

survival (OS) time was evaluated from the patients' operation date till death, and the data were censored if the patients died due to disease uncorrelated with CRC, accident and those who were lost to follow-up in December 2015 (mean OS: 51.92 months; range: 6-106 months). The histological types of CRC were assessed according to the World Health Organization classification. CRC stages were defined according to the International Union Against cancer/American Joint Committee on Cancer TNM criteria. Other pathological data are shown in **Table 1**.

Immunohistochemistry analysis

All specimens were fixed in 10% buffered formalin and embedded in paraffin. The tissue was then cut into 4 μm thickness. All specimens were then deparaffinized and dehydrated with xylene and graded alcohol. Subsequently, the sections were washed in PBS (phosphate buffer solution, PH 7.2) for 10 min. Endogenous peroxidase activity was blocked by incubating the samples in methanol containing 3% H_2O_2 at room temperature (RT) for 10 min. The samples were then placed in a citrate buffer (PH 6.0) and heated to 95°C for 30 min for antigen retrieval. After washing with PBS three times, all the samples were blocked with goat serum for 20 min at RT, then incubated with rabbit monoclonal antibody against human KAI1 (Santa Cruz Biotechnology, CA, USA), CD34 (Santa Cruz Biotechnology), Slug (Cell Signaling Technology, Boston, MA), E-cad (Abcam Epitomics, CA, USA) and vimentin (Abcam Epitomics, CA, USA) at 4°C overnight. Then the slides were incubated with polymer enhancer (reagent A), goat anti-mouse antibody (reagent B), and developed in a freshly prepared 3,3'-diaminobenzidine (DAB) substrate. Finally, all the slides were counterstained with hematoxylin, dehydrated, air-dried and mounted. As for CD34/PAS double staining, CD34 immunohistochemical staining was performed. Then, the slides were treated with 0.5% periodic acid solution for 15 min first and then incubated with PAS solution for 15-30 min in the dark, rinsed with distilled water, and finally counterstained with hematoxylin. Negative controls were prepared by taking out the primary antibodies from the staining procedure.

Evaluation of immunostaining

All slides were investigated by two independent pathologists who were blinded to the clinical, pathological, and follow-up data. To avoid intratumoral heterogeneity of antibody expression, immunostaining was measured in 10 fields (× 100 magnification) for each slide. To evaluate KAI1, Slug, E-cad and vimentin expression, immuno-histochemistry results were graded in terms of both extent and intensity. The intensity

Table 2. The relationship between expression of KAI1/CD82, Slug, VM and clinicopathogical characteristics of colorectal carcinoma (CRC)

Variables	KAI1/CD82 expression		P	Slug expression		P	VM expression		P
	Negative	Positive		Negative	Positive		Negative	Positive	
Sex			0.913			0.867			0.363
Male	52	25		43	34		61	17	
Female	39	18		31	26		48	9	
Age			0.255			0.538			0.566
60 years	39	14		31	22		45	9	
≥ 60 years	52	29		43	38		64	17	
Diameter of tumor			0.535			0.895			0.458
5.0 cm	52	27		44	35		63	17	
≥ 5.0 cm	39	16		30	25		46	9	
Location			0.569			0.640			0.799
Rectum	46	24		40	30		58	13	
Colon	45	19		34	30		51	13	
Differentiation			< 0.001			< 0.001			< 0.001
Well	4	27		24	4		30	2	
Moderate	54	15		44	25		63	6	
Poor	33	1		3	31		16	18	
Depth of invasion			0.004			0.078			0.036
Under serous membrane	44	32		47	29		67	10	
To serous membrane	47	11		27	31		42	16	
Lymph node metastasis			< 0.001			< 0.001			< 0.001
Negative	74	42		63	16		78	2	
Positive	17	1		11	44		31	24	
Distant metastasis			0.010			< 0.001			< 0.001
Negative	74	42		73	43		101	16	
Positive	17	1		1	17		8	10	
TNM stage			< 0.001			< 0.001			< 0.001
I + II	43	36		62	17		75	4	
III + IV	47	8		12	43		33	22	

of staining was graded as follows: 0, none; 1, weak; 2, moderate; and 3, strong. The extent of staining was graded as follows: 1, < 11%; 2, 11-50%; 3, 51-75%; and 4, > 75%. Finally, the intensity and extent were multiplied to produce an immunostaining score ranging from 0 to 12. The immunostaining scores ≥ 3 were considered positive. VM-like channels lined by tumor cells were PAS-positive and CD31-negative and had red blood but not endothelial cells. The number of VM channels was observed under 400 × magnification, and the average number was recorded in five fields.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 software. Fisher's exact or Pearson chi-square test was used to compare the correlations between clinicopathological variables and protein expression. Univariate analysis was used to compare clinicopathological features, lymph node metastases and distant metastases using Fisher's exact or Pearson chi-square test. The correlations among the expressions of these factors were compared using Spearman's correlation analysis. The relative factors of metastasis were illuminated using a multivariate logistic regression analysis. OS refers to the time from surgery till death or the end of follow-up period. The univariate survival analysis of OS was based on Kaplan-Meier method with log-rank tests. The Cox regression model for multivariate analysis was used to analyze the independent prognostic factor for OS. Covariates included were sex, age, tumor diameter, location, differentiation, depth of invasion, lymph node metastasis, distant met-

Table 3. Correlation between expression of KAI/CD82, Slug and VM in CRC

Variables	KAI1/CD82		r	P	Slug		r	P
	Negative	Positive			Negative	Positive		
KAI1/CD82							-0.426	< 0.001
Negative					37	54		
Positive					37	6		
VM			-0.263	0.002			0.545	< 0.001
Negative	66	24			74	34		
Positive	42	2			0	26		

Table 4. Results of univariate analyses of overall survival (OS) time

Variables	n	Mean OS (months)	*P*-value	Log-Rank
KAI1/CD82			< 0.001	35.286
Negative	90	41.9±22.2		
Positive	44	72.4±22.5		
Slug			< 0.001	50.779
Negative	74	67.6±19.8		
Positive	60	32.5±20.2		
VM			< 0.001	52.800
Negative	108	58.7±23.7		
Positive	26	23.5±17.3		
Sex			0.841	0.078
Male	77	48.2±25.9		
Female	57	56.9±26.8		
Age			0.290	3.822
60 years	53	56.6±25.8		
≥ 60 years	81	48.8±26.7		
Diameter of tumor			0.807	0.060
5.0 cm	79	52.8±26.3		
≥ 5.0 cm	55	50.6±27.0		
Location			0.096	2.767
Rectum	70	55.1±27.3		
Colon	64	48.4±25.4		
Differentiation			0.618	0.924
Well	31	55.2±24.9		
Moderate	69	51.2±23.8		
Poor	34	48.9±23.6		
Depth of invasion			0.449	3.518
Under serous membrane	76	57.8±25.3		
To serous membrane	58	45.2±24.4		
Lymph node metastasis			< 0.001	26.489
Negative	79	60.3±24.2		
Positive	55	35.4±23.1		
Distant metastasis			0.004	9.781
Negative	116	55.1±24.1		
Positive	18	35.3±22.1		
TNM stage			< 0.001	59.875
I + II	79	63.1±18.9		
III + IV	55	34.5±21.8		

astasis, and the expression of KAI1, VM, Slug, E-cad and vimentin. β-coefficient and 95% confidence intervals (CI) were analyzed. $P < 0.05$ was defined as statistically significant.

Results

Expression of KAI1, Slug and VM in tumor and normal tissues

To evaluate the contributions of KAI1, Slug and VM in CRC, we evaluated the results of CRC and normal colorectal mucosal tissue samples by immunohistochemical staining. KAI1 positive staining was mainly confined to the membranes and cytoplasms of cancer cells and normal tissues (**Figure 1A**, **1B**). The positive staining of Slug was mainly confined to the cytoplasm as well as in the nucleus of cancer cells and normal tissues (**Figure 1C**, **1D**). VM positive expression (Vessel-like channels which were PAS positive, but CD34 negative were considered VM) was confined to cancer cells and normal tissues (**Figure 1H**). In our study, the positive expression of KAI1 was 32.0% (43/134) in CRC and 55.2% (74/134) in

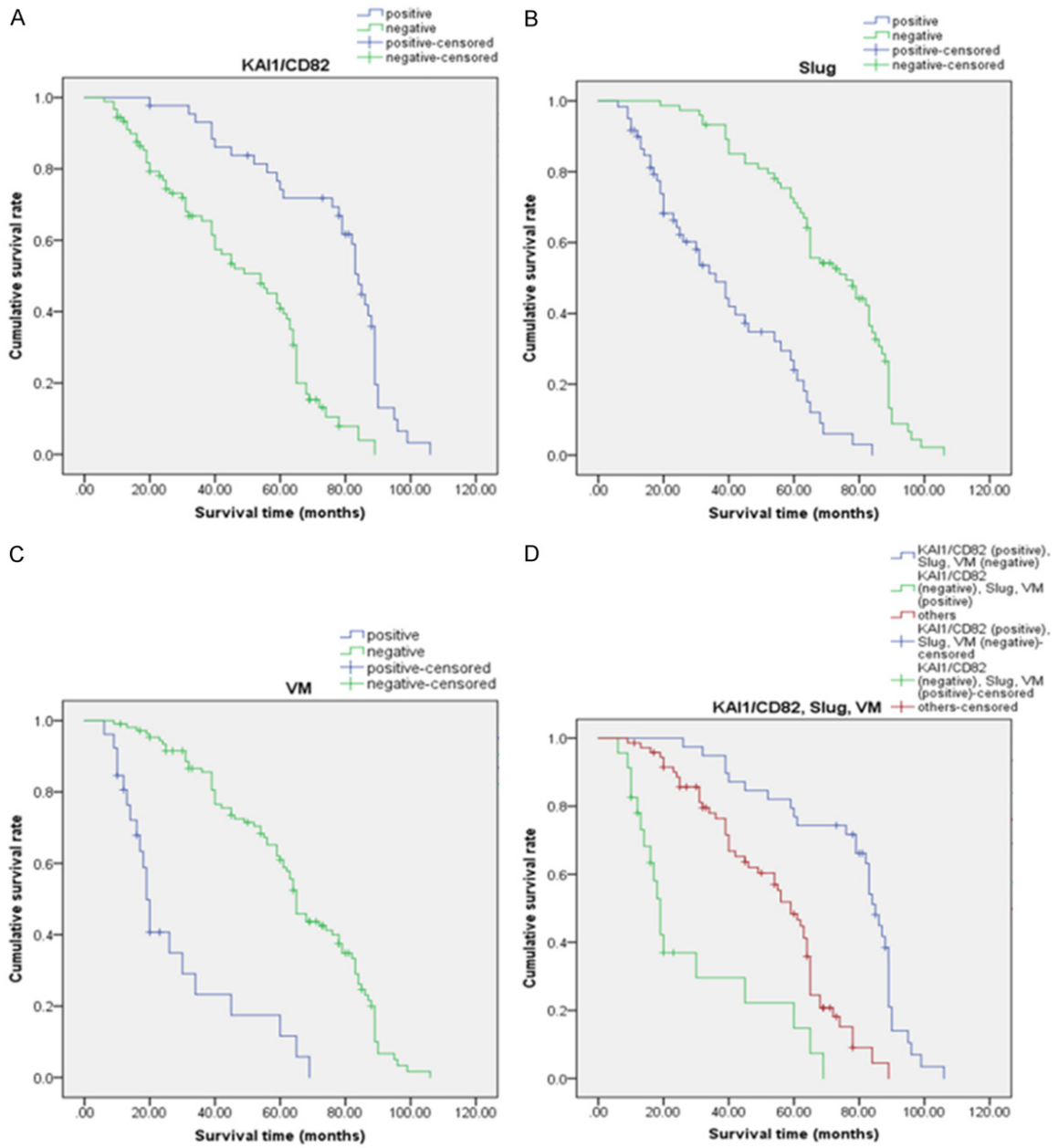

Figure 2. A Kaplan-Meier analysis of the survival rate of patients with colorectal carcinoma. (A) Overall survival of all patients in relation to KAI1 expression (log-rank = 35.286, P < 0.001). (B) Overall survival of all patients in relation to Slug expression (log-rank = 50.779, P < 0.001). (C) Overall survival of all patients in relation to VM expression (log-rank = 52.800, P < 0.001). In these analyses (A, B and C), the blue line represents positive expression of proteins and the green line represents negative expression of proteins. (D) Overall survival of all patients in relation to KAI1, Slug and VM expression (log-rank = 66.438, P < 0.001). The blue line represents positive expression of KAI1 and negative expression of Slug and VM, and the green line represents negative expression of KAI1 and positive expression of Slug and VM. The red line represents other positive or negative expressions of the proteins. In all analyses, + represents censored observation.

normal tissues. The positive expression of Slug was 44.7% (60/134) in CRC and 17.9% (24/134) in normal tissues. The positive expression of VM was 19.4% (26/134) in CRC and 0% in normal tissues. The positive expression of KAI1, Slug and VM were statistically different between CRC and normal tissues (P < 0.05).

Correlation between KAI1, Slug and VM expression and clinicopathological variables

No relationship between the expression of KAI1, Slug and VM, and sex, age, tumor diameter, and location was observed (P > 0.05). The positive expression rate of KAI1 showed a neg-

Table 5. Results of multivariate analyses of overall survival (OS) time

Variable	B	SE	P	RR	95% CI
Invasion	0.362	0.180	0.044	1.436	1.010-2.042
TNM stage	0.583	0.201	0.002	1.804	1.309-2.923
Slug	0.544	0.232	0.009	1.768	1.132-2.674
VM	0.891	0.202	< 0.001	2.325	1.564-3.526
KAI1	-1.221	0.284	< 0.001	0.295	0.169-0.515

ative correlation with differentiation, invasion, LNM, distant metastasis and TNM stage ($P < 0.05$; **Table 2**). The positive expression rate of Slug showed a positive correlation with differentiation, LNM, distant metastasis and TNM stage ($P < 0.05$). The positive expression rate of VM showed a positive correlation with differentiation, invasion, LNM, distant metastasis and TNM stage ($P < 0.05$; **Table 2**).

Correlations among KAI1, Slug and VM in CRC

There was a negative association between KAI1 expression and the expressions of Slug and VM ($r = -0.426$; $r = -0.263$, respectively; $P < 0.001$; $P = 0.002$; **Table 3**). There was a positive association between the expressions of Slug and VM ($r = 0.545$; $P < 0.001$; **Table 3**).

Univariate and multivariate analysis

The univariate analysis revealed that the OS time was significantly correlated with clinicopathological factors, including lymph node metastasis ($P < 0.001$, log-rank = 26.489), distant metastasis ($P = 0.004$, log-rank = 9.781), and TNM stage ($P < 0.001$, log-rank = 59.875; **Table 4**). The OS time of KAI1-positive patients (72.4±22.5 months) was significantly longer than those of KAI1-negative patients (41.9± 22.2 months; log-rank = 35.286, $P < 0.001$; **Figure 2A**). The OS time of Slug-positive patients (32.5±20.2 months) was significantly shorter than Slug-negative patients (67.6± 19.8 months; log-rank = 50.779, $P < 0.001$; **Figure 2B**). The OS time of VM-positive patients (23.5±17.3 months) was significantly shorter than VM-negative patients (58.7±23.7 months; log-rank = 52.800, $P < 0.001$; **Figure 2C**). The combination of negative KAI1 expression and the positive expression of Slug and VM demonstrated poor prognosis than the reverse combination (log-rank = 66.438, $P < 0.001$; **Figure 2D**). Multivariate analysis suggested that the expression of KAI1, Slug, VM and TNM stage were independent prognostic factors for CRC ($P < 0.05$; **Table 5**).

Correlation of KAI1, VM, E-cad and vimentin

According to the results mentioned above, we determined that the expression of KAI1 and VM may be related with EMT. Therefore, we examined the expression of E-cadherin and vimentin, which are considered the classic markers and critical transcription factors of EMT in tumors as well as normal tissues. It was observed that KAI1 expression was positively associated with E-cadherin and negatively associated with vimentin ($r = 0.233$, $P = 0.007$; $r = -0.342$, $P < 0.001$). VM expression was negatively associated with E-cadherin and positively associated with vimentin ($r = -0.313$, $P < 0.001$; $r = 0.355$, $P < 0.001$). We also found that the lower expression of KAI1 and E-cadherin, and the higher expression of VM and vimentin tended to cause a poorer prognosis.

Discussion

Tumor metastasis is considered as a significant factor that affects the prognosis of CRC, and it also remains a major obstacle for successful treatment. Thus, the need to assess the candidate bio-markers of CRC metastasis and prognosis becomes increasingly important. In this study, we analyzed the tumor prognosis related factors KAI1, Slug and VM to provide a new direction for investigating the metastasis and prognosis of CRC.

In the previous study, the expression of KAI1 was reduced in the progression of CRC [8]. In our study, KAI1 positive expression was significantly associated with differentiation, invasion, LNM, distant metastasis and TNM stage (**Table 2**). In addition, our data indicated that KAI1 expression was down-or-lost in CRC metastasis [7, 8], while Yang et al. indicated that KAI1 expression was recaptured in CRC associated with metastasis [31]. Furthermore, a Kaplan-Meier survival analysis indicated that survival time was longer in KAI1-positive CRC patients than in KAI1-negative patients with CRC. Although KAI1 expression remained controversial in the progression of CRC, we concluded that KAI1 expression was significantly associated with the metastasis and prognosis of CRC.

Slug (Snail2), a zinc-finger transcription factor, is an essential mediator of EMT [16]. Slug protein expression was up-regulated in the progression of CRC [21]. In addition, the expression of Slug was significantly correlated with tumor differentiation, LNM and distant metastasis (**Table 2**). Furthermore, a Kaplan-Meier survival analysis indicated that the survival time was shorter in CRC patients with Slug-positive expression than in Slug-negative expression patients. Our results are in line with previous studies of CRC, which demonstrated that Slug plays a vital role in CRC metastasis and prognosis [21].

Previous studies have demonstrated that VM was associated with the progression and metastasis of many cancers [26-28]. In our study, we found that VM was significantly correlated with tumor differentiation, invasion, LNM, distant metastasis and TNM stage (**Table 2**). Furthermore, a Kaplan-Meier survival analysis indicated that survival time was significantly shorter in VM-positive patients than in VM-negative patients. These findings indicate that VM acts as an effective bio-maker for predicting the progression and metastasis of CRC. Our findings are consistent with other immunohistochemical studies, indicating the metastasis and prognostic significance of VM in CRC [29, 32].

Our study demonstrated that KAI1 expression is negatively correlated with Slug. This was in accordance with a study by Jaeseob et al. [33], which found that KAI1 inhibited EMT by repressing the associated integrin signaling. In addition, our study showed that KAI1 expression was negatively correlated with VM, which was consistent with the Zhu et al. study [34], where the expression of KAI1 and VM are closely related to the prognosis of CRC. Furthermore, our study showed that Slug expression was positively correlated with VM and was consistent with the Dan San et al. study [35], which found that Slug contributes to the formation of VM by inducing and maintaining CSCs subpopulation. Based on the results of these studies, we concluded that the interaction of these factors was related to the metastasis and prognosis of CRC.

Based on our analysis, we found that KAI1 expression was positively correlated with E-cad expression and negatively correlated with vimentin expression, which was consistent with the findings of previous studies [34]. Moreover, these results also illustrated that KAI1 may inhibit tumor metastasis and invasion by inhibiting the EMT process. Furthermore, Slug was considered as an essential mediator for inducing EMT [16], which was supported by our observation of the positive correlation between Slug and vimentin, as well as its negative correlation with E-cad. In our study, we found that VM was positively associated with vimentin and negatively associated with E-cad. These results are consistent with previous studies indicating that EMT has been associated with the formation of VM in various cancers [35]. Therefore, we speculated that KAI1 may inhibit the process of EMT to prevent VM formation. Overall, these results indicate that there is a complex relationship between KAI1, Slug and VM in the progression of CRC and may be mutually modulated by EMT processes. Our survival analysis showed that reduced KAI1 expression and increased Slug and VM expression are indicators of poor prognosis in CRC patients (**Figure 2**). Our multivariate analysis suggests that the expressions of KAI1 and Slug are independent prognostic factors for CRC patients (**Table 5**), which is consistent with the findings of previous studies [21, 35]. Thus, our study demonstrated KAI1, Slug and VM as reliable biomarkers to predict the progression, metastasis, and prognosis of CRC.

Although we only used IHC to investigate the relationship between these factors, our findings still suggest a complex association between KAI1, Slug and VM expression with EMT processes in the progression and prognosis of CRC. In the future, we will investigate the relationship between the EMT process and KAI1 and Slug in tumor progression at the molecular level. In conclusion, low expression of KAI1 is associated with high expression of Slug and VM and is associated with metastasis and poor clinical outcomes of CRC. Thus, the combined detection of KAI1, Slug and VM may be used as biomarkers for predicting the metastasis and prognosis of CRC patients.

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Disclosure of conflict of interest

None.

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