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Original Article

Correlation of KAI1, CD133 and vasculogenic mimicry with the prediction of metastasis and prognosis in hepatocellular carcinoma

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Abstract: Background: Hepatocellular carcinoma (HCC) is an aggressive type of tumor with high mortality and poor prognosis, KAI1 is a metastasis suppressor gene which was first found in prostate carcinoma and mapped to chromosome 11p11.2. Vasculogenic mimicry (VM) is a new blood supply phenomenon that exists in highly malignant tumors. CD133 is one of the most common CSC markers for cancer stem cells, and it is related to drug resistance. The purpose of this study was to verify the hypothesis that the above biomarkers have some association with metastasis and prognosis in HCC. Methods: The levels of KAI1, VM and CD133 in 108 whole tissue samples of HCC were detected by immunohistochemistry and histochemistry. Clinical data were also collected. Results: Levels of CD133 and VM were significantly higher, and the level of KAI1 was significantly lower in HCC tissues than that in normal liver tissues. Levels of CD133 and VM were positively associated with cirrhosis, grade, venous invasion, lymph node metastasis (LNM), intrahepatic metastasis, and tumor-node-metastasis (TNM) stages, and negatively with patients' overall survival (OS). The level of KAI1 was negatively correlated with cirrhosis, grade, venous invasion, lymph node metastasis (LNM), intrahepatic metastasis and TNM stages, and positively with patients' overall survival (OS). In a multivariate analysis, CD133, VM KAI1, and TNM stage were independently correlated with OS in patients with HCC. Conclusions: KAI1, CD133, and the existence of VM may have important impacts on metastasis and prognosis in HCC.

Keywords: Hepatocellular carcinoma, KAI1, VM, CD133, immunohistochemistry, prognosis

Introduction

Primary liver cancer is the most common malignant tumor, and it is the second leading cause of cancer deaths worldwide [1]. The incidence of liver cancer in China is 25.7/100,000, and it is only lower than lung cancer and stomach cancer, but the fatality rate of liver cancer is 23.7/100,000, ranked 2nd in cancer mortality rate in China [2]. Hepatocellular carcinoma (HCC) is the most common type of liver cancer and it is an aggressive type of tumor with a high mortality and poor prognosis. The symptoms are not obvious in patients in the early stages of HCC. Recurrence and metastasis are the main causes of treatment failure in advanced liver cancer [3, 4], and these may be related to downexpression or loss of the suppressor gene. KAI1, which was first found in prostate carcinoma, has the ability to suppress the metastasis of tumors, and it is located on human chromosome 11p11.2 [5]. It belongs to the tetraspanin superfamily, which is composed of four membrane-spanning domains and has the ability to control the regulation of cell migration, fusion, adhesion, differentiation and proliferation [6, 7]. Further research has demonstrated that the decreased expression or loss of the KAI1 gene is closely associated with metastasis and recurrence in different kinds of tumors, such as laryngeal carcinoma [8], breast carcinoma [9], lung carcinoma [10], gastric carcinoma [11] and colon carcinoma [12].

Classic angiogenesis theory suggests that angiogenesis mainly depends on vascular endothelial cells. In 1999, vasculogenic mimicry (VM) was firstly found by Maniotis et al. [13].

Table 1. Patients characteristics

Patients characteristics	Frequency (*n*)	Percentage (%)
Gender		
Male	62	57.4
Female	46	42.6
Age, yr		
≤ 60	77	71.3
> 60	31	28.7
Location		
Left	48	44.4
Right	60	55.6
Tumor size, cm		
≤ 5	46	42.6
> 5	62	57.4
Cirrhosis		
Absent	47	43.5
Present	61	56.5
Grade		
Well	42	38.9
Moderate	42	38.9
Poor	24	22.2
Intrahepatic metastasis		
Absent	50	46.3
Present	58	53.7
LNM		
Absent	76	70.4
Present	32	29.6
Venous invasion		
Absent	41	38.0
Present	67	62.0
TNM Stage		
I+II	42	38.9
III+IV	66	61.1

Vasculogenic mimicry (VM) is a new blood supply phenomenon. It is a vascular channel structure produced by some highly aggressive tumor cells that are present in malignant tumors, such as prostatic tumors [14] and gastrointestinal malignancy [15]. The presence of VM can explain the failure of anti-angiogenic therapy. VM is composed of three parts: tumor cells, the abundant extracellular matrix, and the vasculogenic-like channel [16-19]. A growing number of studies have shown that VM should be considered a valuable biomarker when predicting metastasis and prognosis of many cancers [20].

So tumor recurrence and metastasis also have some association with a small number of tumor cells termed cancer stem cells (CSCs) or tumor initiating cells (TIC). CSCs have the abilities of proliferation, self-renewal and multilineage differentiation and maintain the growth of the tumor [21]. CD133 is one of the most common CSC markers for cancer stem cells in various cancers like melanoma [22], lung [23], colon [24], breast [25], and ovarian cancer [26]. CD133 was originally found as a marker of hematopoietic stem cells. It is a 120 kDa five transmembrane domain cell surface glycoprotein [27, 28]. As mentioned earlier, CD133-expressing cells have more tumorigenic and aggressive capabilities than CD133-free cells [29].

Overall, studies of KAI1, VM and CD133 showed that these biomarkers might have a great effect on tumor development. However, correlations among KAI1, CD133, and VM in HCC remain unclear. In this study, we explored the relationship between their expression and clinicopathological characteristics.

Patients and methods

Patients and tissue samples

Primary tumor tissues diagnosed with HCC and adjacent normal liver tissues were collected from the department of pathology of the First Hospital Affiliated of Bengbu Medical College from January 2011 to December 2011. Normal liver tissues were collected from the same patients, at least 5 cm away from the carcinoma margins. Patients who had received preoperative chemotherapy or embolization were excluded. The clinical, pathological, and follow-up data (8 months by phone, mail, or email) for all patients was incomplete. The study group includes 108 patients, 62 males and 46 females, ranging in age from 31 to 86 years; the average age is 54.87 ± 10.361 years. Overall survival (OS) time was collected from the surgery date to the death date or to December 2016 (mean OS time 26.8 months; range 2-72 months). The grade of tumor differentiation was defined according to the World Health Organization criteria. The clinical stages were defined according to the International Union Against Cancer's tumor-node-metastasis

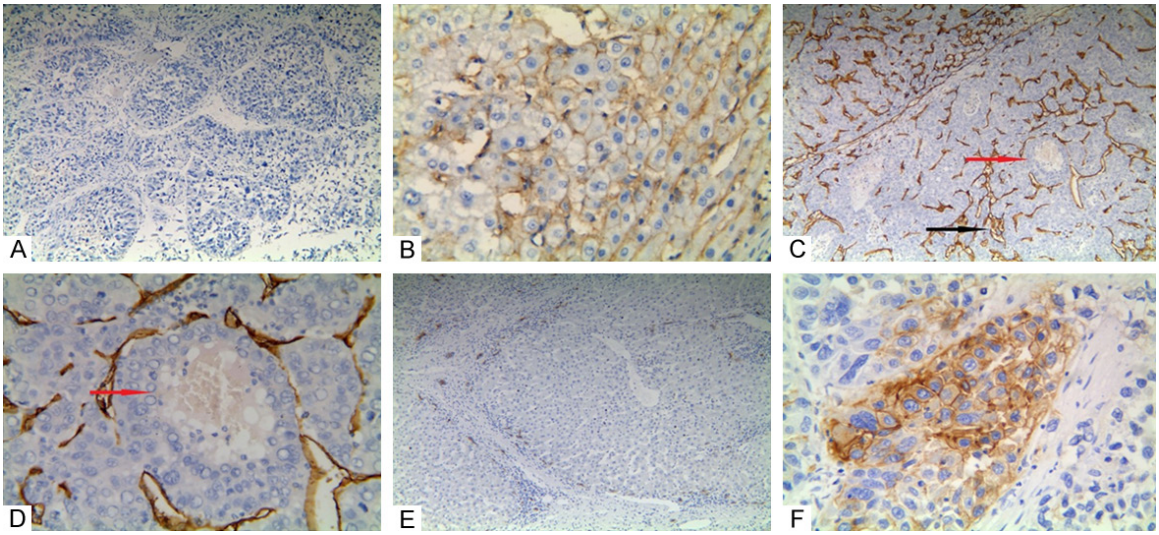

Figure 1. Immunostaining of KAI1, or VM or CD133 in HCC or the control tissues. A. Negative staining of KAI1 in the cancer cells (100 magnification). B. Positive staining of KAI1 in the membrane of the control tissue (400 magnification). C. Positive staining of VM in the HCC tissue (100 magnification, red arrow is a VM structure, black arrow is a microvessel). D. Positive staining of VM in the HCC tissue (400 magnification, red arrow is a VM structure). E. Negative staining of CD133 in the control tissue (100 magnification). F. Positive staining of CD133 in the membrane and cytoplasm of cancer cells (400 magnification).

classification. Other clinicopathological characteristics of the cases are provided in **Table 1**.

All experimental work with the tissue specimens was approved by the patients. The ethical approval of this study was obtained from the Ethics Committee of Bengbu Medical College and was conducted according to the moral code of the Helsinki Declaration.

Immunohistochemistry

Immunohistochemistry was conducted on the basis of the Elivision™ Plus detection kit instructions (Lab Vision, USA). All specimens were fixed in 10% buffered formalin, embedded in paraffin and sectioned (thickness, 4 μm). Sections were then deparaffinized and dehydrated with xylene and graded alcohol. Subsequently, the sections were washed in phosphate-buffered saline (PBS, pH 7.2) for 10 min. The endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 at room temperature for 10 min, then placed in a citrate buffer (pH 6.0) and heated to 95°C for 30 min for antigen repair. After washing in PBS three times, the sections were blocked in goat serum and incubated with mouse monoclonal antibody against human KAI1 (Abcam, USA) or CD34 (Abcam, USA), or CD133 (Abcam, USA) for 1 h at 37°C. All samples underwent periodic acid-Schiff (PAS)-CD34 dual staining to determine the endothelial cells in the glycosylated basement membranes of the vessels, as well as vessel-like (VM) structures [30]. Furthermore, we found no necrosis and hemorrhage in tumor tissue near the VM structures. All sections were counterstained with hematoxylin, dehydrated, air-dried, and mounted.

Immunohistochemical evaluation

Slides were read by two pathologists who were blinded to all information about the cases. The positive expressions of KAI1 and CD133 were both found mainly on the membranes and cytoplasms of the HCC cells and normal liver tissues. They were presented as a brown granular material under the microscope. The intensity of the positive results was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong. The extent of positivity was scored according to the percentage of cells that were stained positive: < 10% is 1; 11 to 50% is 2; 51 to 75% is 3; > 75% is 4. The final score was determined by multiplying the intensity of positivity and the extent of positivity scores, which ranged from 0 to 12. The expressions of KAI1 and CD133 were considered positive when the scores were

Table 2. The correlations between VM, KAI1, CD133 and clinicopathological characteristics in hepatocellular carcinoma

Variable	VM		*p*	KAI1		*p*	CD133		*p*
	-	+		-	+		-	+	
Gender			0.868			0.785			0.540
Male	30	32		38	24		32	30	
Female	23	23		27	19		21	25	
Age, yr			0.073			0.309			0.738
≤ 60	42	35		44	33		37	40	
> 60	11	20		21	10		16	15	
Location			0.322			0.965			0.830
Left	21	27		29	19		23	25	
Right	32	28		36	24		30	30	
Tumor size, cm			0.579			0.503			0.182
≤ 5	24	22		26	20		26	20	
> 5	29	33		39	23		27	35	
Cirrhosis			< 0.001			< 0.001			< 0.001
Absent	39	8		15	32		35	12	
Present	14	47		50	11		18	43	
Grade			< 0.001			< 0.001			< 0.001
Well	39	3		10	32		38	4	
Moderate	14	28		31	11		10	32	
Poor	0	24		24	0		5	19	
Intrahepatic metastasis			< 0.001			< 0.001			0.004
Absent	37	13		16	34		32	18	
Present	16	42		49	9		21	37	
LNM			< 0.001			0.004			< 0.001
Absent	45	31		39	37		47	29	
Present	8	24		26	6		6	26	
Venous invasion			< 0.001			< 0.001			< 0.001
Absent	38	3		10	31		38	3	
Present	15	52		55	12		15	52	
TNM Stage			< 0.001			< 0.001			< 0.001
I+II	36	6		13	29		35	7	
III+IV	17	49		52	14		18	48	

Table 3. Correlations among KAI, VM and CD133 in HCC

Variable	KAI1		*r*	*p*	CD133		*r*	*p*
	-	+			-	+		
VM			-0.677	< 0.001			0.592	< 0.001
-	14	39			42	11		
+	51	4			11	44		
CD133			-0.526	< 0.001				
-	18	35						
+	47	8						

≥ 3. A modified Yue's method was used to evaluate the VM of HCC [31].

Statistical analysis

The relationships between the expressions of the above biomarkers and clinicopathological parameters were compared using Fisher's exact test or chi-square test. The correlations among KAI1, VM, or CD133 were compared using Spearman's coefficient test. The effects of KAI1, VM, or CD133 on survival were determined by univariate and multivariate analysis. Independent prognostic factors were determined using the Cox regression model for multivariate analysis. The Kaplan-Meier method with log-rank test for univariate OS analysis

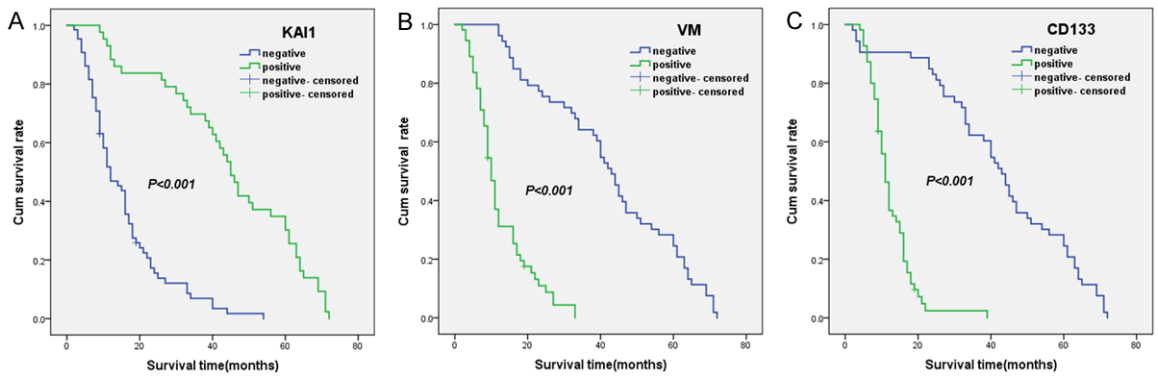

Figure 2. Kaplan-Meier analysis of the survival rate of patients with HCC. (A) Overall survival of all patients in relation to KAI1 expression (log-rank = 56.769, P < 0.001). (B) Overall survival of all patients in relation to VM (log-rank = 78.447, P < 0.001). (C) Overall survival of all patients in relation to CD133 (log-rank = 77.921, P < 0.001). In (A-C) analyses, the green line represents the positive staining of factors and the blue line represents negative staining factors.

was used to evaluate associations between KAI1+, VM+, CD133+ and clinical characteristics using SPSS (IBM, New York, NY, USA, version 24). A value of $P < 0.05$ was statistically significant.

Results

Expression of KAI1, VM and CD133 in HCC and their association with clinicopathology

In order to assess the function of KAI1, VM and CD133 in HCC, their expression levels were evaluated in both HCC and normal liver tissue slides by immunohistochemistry. These data were then compared to patients' clinical characteristics. The rates of KAI1 in HCC tissue were 39.8% (43/108) and 88.9% (96/108) in control tissues (**Figure 1A** and **1B**). This difference was statistically significant (P < 0.001). The expression level of KAI1 was clearly lower in HCC tissues than that in the control normal tissues. The expression level of KAI1 in HCC patients was inversely correlated with cirrhosis, tumor-node-metastasis (TNM), grade, intrahepatic metastasis, lymph node metastasis (LNM), venous invasion ($P < 0.05$), but not with the patients' gender, age, location or tumor size ($P > 0.05$, **Table 2**).

In contrast to the KAI1 expression, the rate of VM+ findings (small vessel, which is like a channel in HCC, the channel is PAS-positive but CD34-negative. The VM structure pattern included tubular, linear, and network, etc.) in the HCC samples was significantly higher than that in the control tissues. VM+ was found in 55 of 108 HCC samples (50.9%), and no VM structure exists in normal liver tissues (**Figure 1C**, **1D**). This difference was statistically significant (P < 0.001). VM in HCC was positively associated with tumor cirrhosis, tumor-node-metastasis (TNM), grade, intrahepatic metastasis, lymph node metastasis (LNM), and venous invasion (P < 0.05), but not with the patients' gender, age, location or size of tumor (P > 0.05, **Table 2**).

CD133 protein was expressed positively in 50.9% (55/108) of HCC and 4.6% (5/108) of normal liver tissues. There was a significant difference between the HCC group and the normal liver tissues (P < 0.001, **Figure 1E** and **1F**). There was a positive relationship between the expression of CD133 and tumor cirrhosis, tumor-node-metastasis (TNM), grade, intrahepatic metastasis, lymph node metastasis (LNM), venous invasion (P < 0.05), but not with the patients' gender, age, location or size of tumor (P > 0.05, **Table 2**).

Association among KAI1, VM and CD133 in HCC

Spearman correlation coefficient analysis suggested a negative association between the positive expression of KAI1 and that of VM (r = -0.677, P < 0.001), or CD133 (r = -0.526, P < 0.001). Expressions of VM and that of CD133 were positively correlated (r = 0.592, P < 0.001, **Table 3**).

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

Variable	*n*	Mean OS (Mouths)	Log-rank	*P-value*
CD133			77.921	< 0.001
-	53	42.2 ± 19.6		
+	55	12.1 ± 5.9		
KAI1			56.769	< 0.001
-	65	15.0 ± 10.8		
+	43	44.7 ± 19.6		
VM			78.447	
-	53	42.3 ± 18.9		< 0.001
+	55	12.0 ± 7.4		
Gender			1.878	0.171
Male	62	29.3 ± 21.8		
Female	46	23.5 ± 19.2		
Age, yr			0.006	0.937
≤ 60	77	27.0 ± 21.2		
> 60	31	26.6 ± 20.1		
Location			1.306	0.253
Left	48	24.7 ± 20.4		
Right	60	28.6 ± 21.1		
Tumor size, cm			1.396	0.237
≤ 5	46	25.5 ± 17.9		
> 5	62	27.8 ± 22.8		
Cirrhosis			34.430	< 0.001
Absent	47	40.7 ± 19.6		
Present	61	16.2 ± 14.5		
Grade			111.322	< 0.001
Well	42	48.5 ± 15.7		
Moderate	42	15.6 ± 7.8		
Poor	24	8.6 ± 5.4		
Intrahepatic metastasis			44.169	< 0.001
Absent	50	39.5 ± 21.9		
Present	58	15.9 ± 11.7		
LNM			71.217	< 0.001
Absent	76	34.1 ± 20.6		
Present	32	9.5 ± 4.8		
Venous invasion			91.271	< 0.001
Absent	41	49.3 ± 15.1		
Present	67	13.1 ± 7.7		
TNM Stage			75.108	< 0.001
I+II	42	46.8 ± 18.0		
III+IV	66	14.2 ± 9.4		

Univariate analysis

Follow-up data showed that OS time was longer in HCC patients with a positive expression of KAI1 (44.7 ± 19.6 months) compared with those who were KAI1-negative (15.0 ± 10.8 months; log-rank = 56.769, P < 0.001; **Figure 2A**), and the OS time of VM-positive patients (12.0 ± 7.4 months) was lower than that of the VM-negative patients (42.3 ± 18.9 months; log-rank = 78.447, P < 0.001; **Figure 2B**). Similarly, the OS time was significantly shorter in HCC patients with a positive expression of CD133 (12.1 ± 5.9 months) compared with those who were CD133-negative (42.2 ± 19.6 months; log-rank = 77.921, P < 0.001; **Figure 2C**). In the univariate analysis, OS time was significantly related to clinical characteristics, such as cirrhosis (P < 0.001, log-rank = 34.430), intrahepatic metastasis (P < 0.001, log-rank = 44.169), venous invasion (P < 0.001, log-rank = 91.271), LNM (P < 0.001, log-rank = 71.217), and TNM stage (P < 0.001, log-rank = 75.108) (**Table 4**).

Multivariate analysis

Multivariate analysis demonstrated that positive expressions of KAI1 and CD133, VM, and LNM, as well as the TNM stage, were independent prognostic indicators for HCC (**Table 5**).

Discussions

Hepatocellular carcinoma (HCC) is a highly heterogeneous tumor. This heterogeneity has an effect on biomarker assessment. Therefore, it is urgent to find novel and effective biomarkers for predicting HCC patients' progression, metastasis and prognosis. The KAI1 gene is a metastasis suppressor for many types of cancers, such as prostate cancer, and the level of KAI1 in normal tissue is higher when compared to tumor tissue, and the overexpression of the KAI1 gene suppressed the metastatic ability of rat AT6.1 prostate cells [5]. The KAI1 gene encodes the integral membrane protein CD82 and is composed of four transmembrane domains and one large extracellular domain, suggesting that it belongs to TM4SF. The TM4SF consists of at

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

Variable	B	SE	P	RR	95% CI
TNM stage	1.246	0.494	0.012	3.477	1.320-9.161
LNM	0.970	0.349	0.005	2.638	1.331-5.228
CD133	1.306	0.494	0.008	3.691	1.402-9.713
VM	0.835	0.396	0.035	2.305	1.060-5.012
KAI1	-0.734	0.340	0.031	0.480	0.246-0.934

least 15 members, such as CD81, CD9, CD82, CD63, and CD151. Many of these proteins have the ability to associate with other molecules, including lineage-specific proteins, integrins, and other tetraspanins. They also play important roles in various biochemical activities, such as cell activation and proliferation, adhesion and motility, differentiation, and cancer [32]. There was an inverse relationship between KAI1 expression level and tumor cirrhosis, tumor-node-metastasis (TNM), grade, intrahepatic metastasis, lymph node metastasis (LNM), and venous invasion.

The blood provides nutrition for the growth of the tumor, and the formation of micro-vessels makes the tumors have more channels for dissemination and metastasis. The more microvessels there are, the more malignant the tumor is [33-38]. Previous studies show that portal vein invasion (PVI) usually happens in the early stage of HCC and has some association with the progress of the tumor [39-41]. Recently studies show that vasculogenic mimicry (VM) makes a contribution to PVI in HCC [40]. The existence of VM was first found in melanoma by Maniotis and his colleagues [13], and since then more reports about VM have emerged, with researchers finding it in different tumors, including tumors occurring in the breast, liver, colon, lung. and so on. Resent explorations suggested that the occurrence of VM often means a poor prognosis [42]. VM channels not only support the growth of tumors but also facilitate tumor cells and their metastasis to surrounding or distant tissues. Vasculogenic mimicry occurs when aggressive tumors need more blood during tumor growth and invasion, and tumor cells have greater plasticity. After the blood supply for tumor growth and invasion has been satisfied by VM, the endothelial cells can grow into the space made by tumor cells and then angiogenesis and vasculogenesis are produced consequently. In addition, VM in HCC was positively associated with tumor cirrhosis, tumor-node-metastasis (TNM), grade, intrahepatic metastasis, lymph node metastasis (LNM), and venous invasion.

Cancer stem cells (CSCs), also called tumor initiating cells (TICs), have been highlighted lately. Recently, a study suggested that CSCs may be derived from the malignant transformation of normal stem cells or arise from restricted progenitors of more differentiated cells [43]. CSCs have the capability of self-renewal, proliferation, and multilineage differentiation; therefore, they are considered to be the culprits in therapeutic resistance, metastasis and the recurrence of the tumors. The expression of the CD133 protein in 108 HCC specimens with follow-up data and the results suggested that the expression level of CD133 was positively correlative with cirrhosis, tumor-node-metastasis (TNM), grade, intrahepatic metastasis, lymph node metastasis (LNM) and venous invasion.

CSCs play an important role in the initiation and progression of HCC. Some studies showed that CSCs had the capability of differentiation along tumor and endothelial cells [44, 45]. This evidence has demonstrated that CSCs mimicked endothelial cells to form a vasculogenic-like network to convey nutrients and oxygen. At the same time, KAI1 could inhibit the process of epithelial-mesenchymal transition (EMT) to prevent angiogenesis [46]. Our study suggests that there is a negative association between the expression of KAI1 and VM or CD133. The low expression of KAI1 means losing the function of prohibiting tumor cell invasion. The combined detection of CD133 and VM often means a high rate of recurrence and metastasis. In summary, this study preliminarily demonstrated that the combined effect of KAI1, VM, and CD133 is valuable for the diagnosis and prognosis of HCC.

Conclusions

Our results suggest that KAI1 and CD133 and the existence of VM have significant impacts on the progression of HCC. Research on them can provide new ideas for clinical treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

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