Upregulation of nectin-4 is associated with ITGB1 and vasculogenic mimicry and may serve as a predictor of poor prognosis in colorectal cancer

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Abstract. Colorectal cancer (CRC) is one of the most common malignancies worldwide. Unlike endothelium-dependent vasculature, vasculogenic mimicry (VM) is an alternative type of blood supply in tumors that is frequently associated with poor patient outcome. Nectin-4 serves a vital role in the formation and maintenance of adherens junctions; integrin β-1 (ITGB1) promotes tumor invasion, metastasis and VM formation. In the present study, the analysis of nectin-4 mRNA expression in a database of The Cancer Genome Atlas (TCGA) was combined with that of another non-overlapping cohort of 68 patients with CRC. TCGA data were used to examine nectin-4 mRNA expression in CRC and its correlation with the clinicopathological features of patients. Data from the non-overlapping cohort of patients was used to determine nectin-4 and ITGB1 protein expression in CRC by immunohistochemical (IHC) staining. Cluster of differentiation 34/periodic acid-Schiff double staining was performed to validate the presence of VM formation. The association with, and significance of combining nectin-4, ITGB1 protein expression and VM formation for predicting patient prognosis was evaluated. The TCGA dataset demonstrated that nectin-4 mRNA was upregulated in CRC, which was significantly relate to lymph node metastasis (P=0.0017), distant metastasis (P=0.0045), and tumor-node-metastasis (TNM) stage (P=0.0015). Of the 68 patients analyzed by IHC staining, 48 (70.6%) were positive for nectin-4, 46 (67.6%) for ITGB1 and 17 (25%) for VM formation. Nectin-4 protein expression was associated with ITGB1 protein expression (P<0.01) and VM formation (P<0.05). Nectin-4, ITGB1 expression and VM formation were associated with distant metastasis stage (P<0.05) and TNM stage (P<0.05). Based on these findings it was concluded that nectin-4 was upregulated in CRC tissues compared with normal mucosal tissues, and was associated with ITGB1 expression and VM formation. Furthermore, nectin-4 and ITGB1 protein expression, together with VM formation may be used to predict poor prognosis in CRC.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide and the second most common cause of cancer-associated mortality (1). Despite advances in the treatment of CRC, the mortality rate of this disease remains high (2). Therefore, more reliable molecular prognostic markers are required to improve CRC diagnosis and treatment.

The nectin protein family belongs to the immunoglobulin superfamily, and its members are involved in the formation and maintenance of adherens junctions in cooperation with cadherin (3). At present, nectin-1, -2, -3 and -4 have been identified (3). Nectin-4 was originally described as a molecule homologous to the poliovirus receptor, also known as poliovirus receptor-like-4 (4). Unlike nectin-1 and -3, which are widely expressed in the tissues of adults, nectin-4 is largely restricted to embryonic and placental tissues (5). However, studies have indicated that tracheal tissue, skin and hair follicles express low levels of this protein (5,6). In addition, a number of studies have revealed that nectin-4 overexpression is fundamental for the invasion and metastasis of ovarian (7), breast (8), and lung cancer (9). The role of nectin-4 in cancer has not been extensively studied and its expression and prognostic value in CRC remains to be elucidated.

Integrins are cell membrane receptors that recognize and bind to the extracellular matrix and participate in multiple aspects of metastasis (10), including tumor angiogenesis (11). Furthermore, it has previously been reported that integrins are associated with another type of blood supply in tumors, vasculogenic mimicry (VM) (12-14). VM, which differs from classical tumor angiogenesis, is an alternative means of increasing the blood supply to tumors. In VM, tumor cells

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and a channel consisting of a basement membrane that stains positively to periodic acid-Schiff (PAS) are present; however this channel does not contain endothelial cells (15). It has been reported that VM, which is commonly observed in malignancies, is associated with poor differentiation, advanced clinical stage and poor prognosis (16). Further insight into the mechanisms of underlying the formation VM may provide novel insight for the development of anti-tumor therapy. A study demonstrated that short fibers and small pores in the matrix environment induce the formation of VM; specifically, an upregulation of the conserved transcription module has been reported in tumor cells, resulting in enhanced invasion and metastasis regulated by integrin β-1 (ITGB1) (14). Therefore, ITGB1 may serve a vital role in VM formation; however, further investigation is required.

The purpose of the present study was to evaluate the expression and prognostic value of nectin-4, ITGB1 and VM in CRC by performing a retrospective study based on data from The Cancer Genome Atlas (TCGA) cohort, and data obtained from another cohort of 68 non-overlapping patients with CRC.

Materials and methods

Bioinformatics analysis of TCGA data. Data for nectin-4 mRNA expression in CRC and normal tissues was downloaded from TCGA (https://cancergenome.nih.gov/) to examine the role of nectin-4 expression in CRC and its association with the clinicopathological features of patients. A total of 372 CRC samples and 31 normal samples from TCGA database were downloaded using the R package TCGA-Assembler 2.0 (17). This cohort of 372 patients with CRC consisted of 204 male and 168 female patients; 199 cases ≥65 years old, 173 <65 years old; 282 were located in the colon, 90 were located in the rectum; 67 were Tumor-Node-Metastasis (TNM) I/TII stage, 304 were TNM III/TIV; 204 had no lymph node metastasis, 165 had lymph node metastases; 254 had no distant metastasis, and 50 had distant metastases.

Patients and tissue samples. The present study was approved by the Ethics committee of The First Affiliated Hospital of Guangxi Medical University (Nanning, China) and performed in accordance with the guidelines of the Declaration of Helsinki (no. BBMCEC2012063). All patients provided written informed consent to participate in the study. Between September 2013 and September 2016, a total of 68 CRC paraffin embedded tissues and 15 normal mucosal tissues were obtained from The First Affiliated Hospital of Guangxi Medical University. The cohort constituted 39 (57.4%) males and 29 (42.6%) females, and the median age of patients was 56 years (range, 26-81 years). Pathological staging was accorded to the National Comprehensive Cancer Network CRC classification (18). Detailed clinical and pathological data were also collected. Patients who had received preoperative chemo- or radiotherapy, or any other anti-cancer therapy were excluded from the study.

Immunohistochemical (IHC) staining and cluster of differentiation (CD)34/PAS dual staining. All tissue samples were fixed in 10% formalin for 24 h in room temperature and embedded in paraffin. Tissue sections (4-5 μm) were
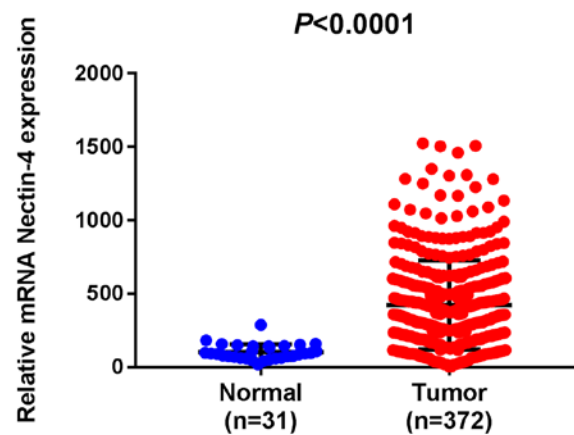

Figure 1. Nectin-4 mRNA expression in tumor and normal mucosal specimens, based on the data from The Cancer Genome Atlas database.

deparaffinized in xylene and rehydrated in ethanol (100, 95, 80 and 70%), and slides were soaked in methanol (98%), containing 3% H_2O_2 for 10 min to block endogenous peroxidase activity. For antigen retrieval the slides were heated in the microwave for 30 min in citric acid buffer (pH 6.4). The slides were blocked with 10% normal goat serum (OriGene Technologies, Inc.) in PBS for 30 min at room temperature, and further incubated with primary antibodies for nectin-4 (cat. no. 21903-1-AP, 1:100, Proteintech Group, Inc., Chicago, IL, USA), ITGB1 (cat. no. 12594-1-AP, 1:200, Proteintech Group) and CD34 (cat. no. ZM-0046, OriGene Technologies, Inc.) at 4°C overnight. The following day the slides were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (OriGene Technologies, Inc.) for 30 min at room temperature. Visualization of the IHC reaction was performed using 3,3'-diaminobenzidine for 5 min at room temperature. After IHC staining of CD34, the sections were washed with running distilled water for 5 min and incubated with periodic acid for 20 min and Schiff reagent for 8 min at room temperature. CD34/PAS double-positive staining was used to characterize VM structures. Following staining with hematoxylin for 1 min at room temperature the slides were examined under an Olympus BX53 light microscope (magnification, x200 and x400; Olympus Corporation, Tokyo, Japan).

IHC evaluation. The slides were independently evaluated by two pathologists, and IHC staining was quantified using the Remmele immunoreactive score (IRS). IRS=staining intensity (SI) x percentage of positive cells (PP). SI was defined as: i) 0, Negative; ii) 1, weak; iii) 2, moderate; and iv) 3, strong. PP was defined as: i) 0, Negative; ii) 1, <25% positive cells; iii) 2, 26-50% positive cells; iv) 3, 51-75% positive cells; and v) 4, >75% positive cells. A total of ten visual fields from different areas of each tumor were used for IRS evaluation. The staining scoring system was defined as follows: i) 0, Negative staining (-); ii) 1-4 as weakly positive (1+); iii) 5-8 as moderately positive (2+); and iv) 9-12 as strongly positive staining (3+). The negative and weakly positive categories (- and 1+) were defined as negative, and moderate and strong positive categories (2+ and 3+) were recorded as positive results (19).
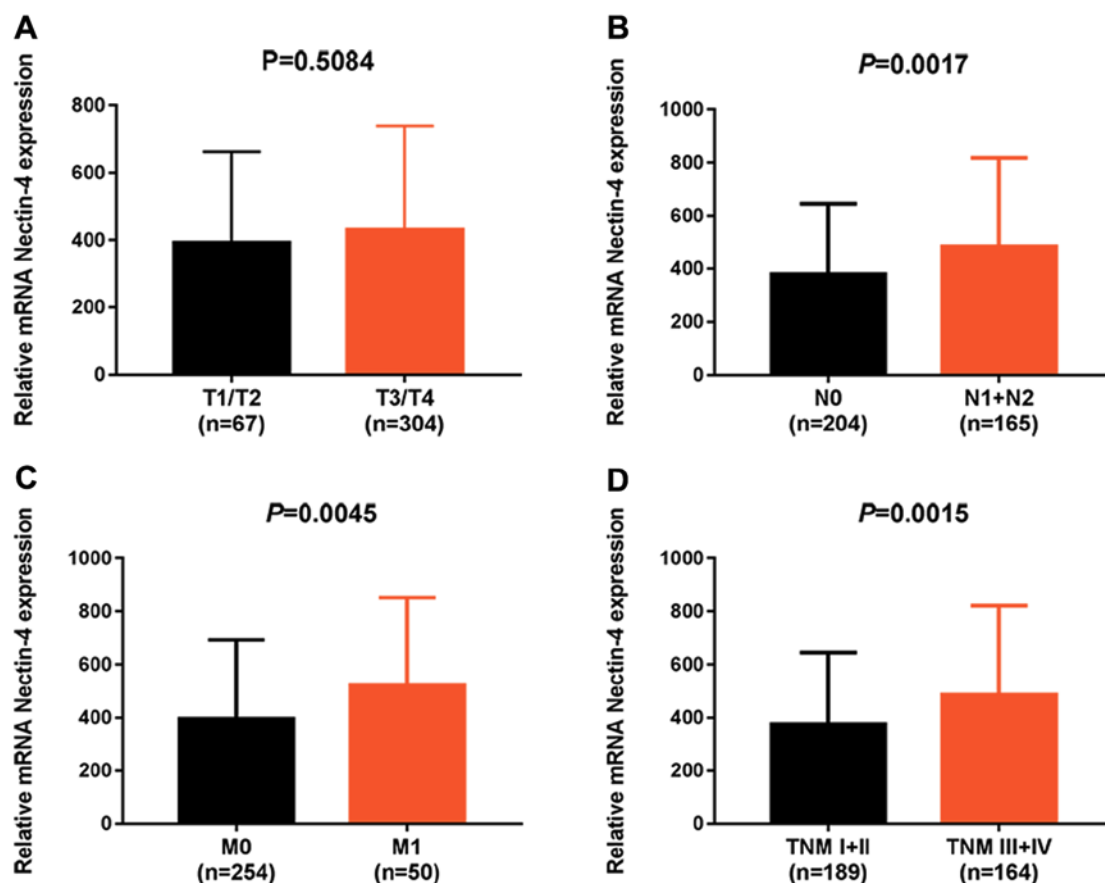
Figure 2. Nectin-4 mRNA expression is associated with various clinicopathological parameters. Based on the data from The Cancer Genome Atlas database, P-values were calculated using the t-test. Association between nectin-4 expression and (A) tumor stage (B) lymph node metastases, (C) distant metastasis stage, and (D) tumor node metastasis stage. T, tumor stage; N stage, lymph node metastasis stage; M stage, distant metastasis stage; TNM stage, Tumor-Node-Metastasis stage.

Statistical analysis. An independent sample t-test was used to compare continuous variables between CRC tissues and normal tissues. The χ^2 test was used to determine the association between nectin-4, ITGB1 and VM formation. The association between nectin-4, ITGB1, VM formation and the clinicopathological parameters of patients was analyzed using the two-tailed χ^2 test. All statistical analyses were performed using the SPSS software package (version 17.0; SPSS, Chicago, IL, USA), and P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of nectin-4 mRNA expression is associated with aggressive CRC. Nectin-4 mRNA expression data from 372 patients with CRC, and 31 normal cases were downloaded from the TCGA database. P-values were calculated using the t-test. Nectin-4 mRNA expression in CRC tissues and normal mucosal tissues was analyzed. As presented in Fig. 1, nectin-4 mRNA expression was upregulated in CRC tissues compared with that in normal mucosal tissues (P<0.0001; Fig. 1). The association between nectin-4 mRNA expression and patients' clinicopathological parameters were also assessed. Nectin-4 mRNA expression in tumor tissues was significantly associated with lymph node metastasis (N stage; Fig. 2B), distant metastasis (M stage, Fig. 2C) and advanced clinical stage

(TNM stage, Fig. 2D). By contrast, no significant differences were identified between nectin-4 mRNA expression levels and tumor (T) stage (T stage, Fig. 2A), patient's age, tumor type, tumor site, presence of polyps (data not shown). Collectively these results suggested that the overexpression of nectin-4 mRNA was associated with the clinical progression of CRC.

Nectin-4 protein expression is higher in CRC tumors compared with normal mucosal tissues. To further confirm the results obtained from TCGA database analysis, a non-overlapping cohort of 68 patients with CRC were recruited, and nectin-4 protein expression levels were determined using IHC staining. A total of 68 CRC and 15 normal mucosal tissue samples were collected. According to the National Comprehensive Cancer Network CRC classification, 7 patients were classified as stage I, 25 as stage II, 19 as stage III and 17 as stage IV. Consistent with the results obtained from the TCGA cohort, high nectin-4 protein expression was more frequently observed in tumor tissues when compared with normal tissues (Fig. 3). Only 3 (20%) normal mucosal samples displayed high nectin-4 protein expression levels, while 48 (70.6%) of the CRC tissues displayed high levels (Table I, P<0.01).

Association between nectin-4, ITGB1 and VM formation in CRC. In addition to nectin-4 protein expression, the expression of ITGB1 and VM formation were also determined. Of
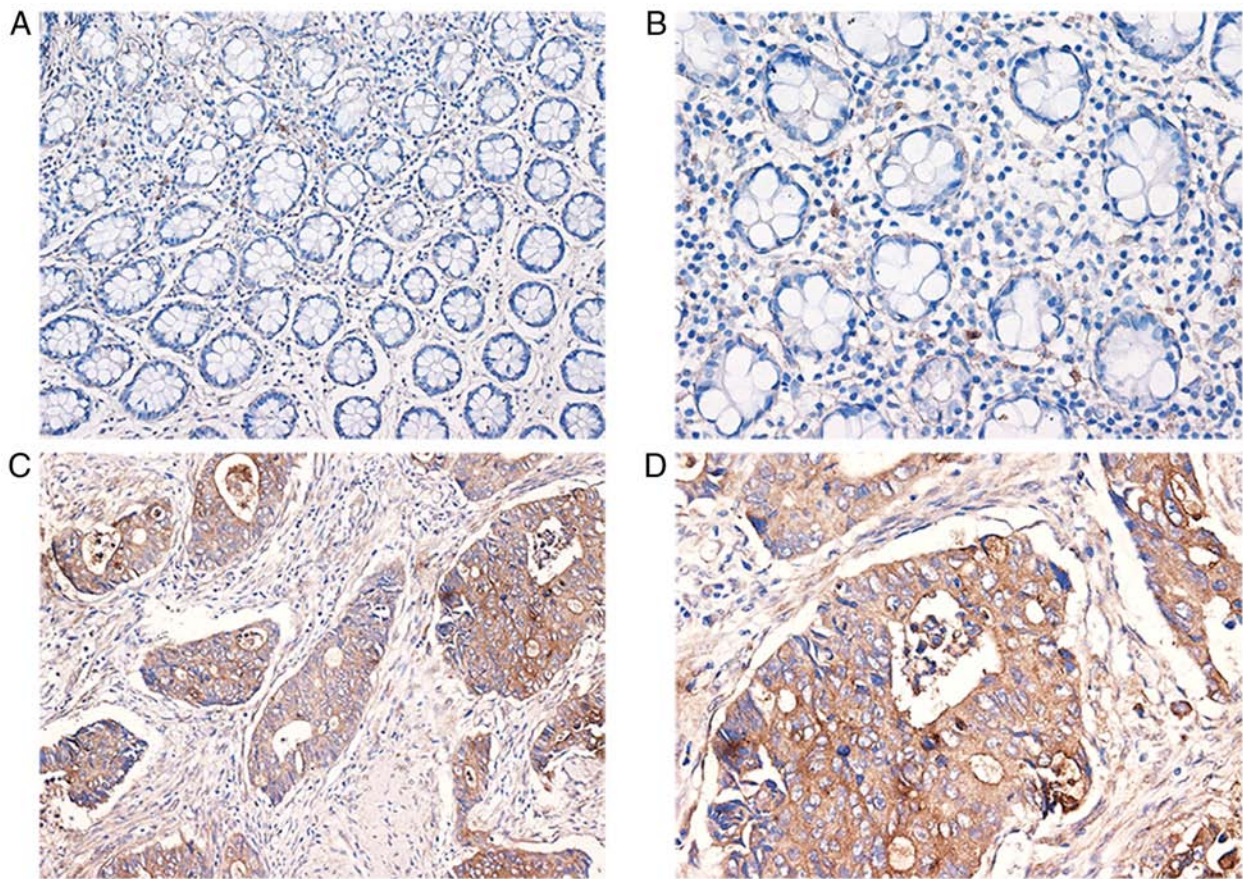
Figure 3. Nectin-4 protein expression in normal colorectal mucosal tissue, and colorectal cancer tissue. Normal colorectal mucosal tissue: Magnifications, (A) x200 and (B) 400. Colorectal cancer tissue: Magnifications, (C) x200 and (D) 400.

the 68 cases analyzed, 48 (70.6%) were positive for nectin-4 protein expression, 46 (67.6%) were positive for ITGB1 protein expression and in 17 (25%) cases, VM formation was observed (Table II). Nectin-4 and ITGB1 protein expression were higher in later TNM stages (TNM III and IV) compared with early cancer stages (TNM I and II; Fig. 4). Notably, statistical analysis revealed that nectin-4 was positively associated with ITGB1 expression (Table II). CD34/PAS double staining was used to detect VM formation. Structures characterized by CD34$^-$/PAS$^+$ staining, with red blood cells in the vascular-like tube, and surrounded by tumor cells, were identified as VM (Fig. 5, red arrow). Structures with CD34$^+$ and PAS$^+$ staining were identified as endothelium dependent vasculature (Fig. 4, black arrow). Statistical analysis revealed that nectin-4 protein expression was positively associated with VM formation (Table II).

Associations between nectin-4, ITGB1 and VM, and the clinicopathological parameters of patients with CRC. To evaluate the influences of nectin-4, ITGB1, and VM on CRC, the obtained results were further compared with patient clinicopathological characteristics. The expression of nectin-4 (48/68, 70.6%) and ITGB1 (46/68, 67.6%), in addition to VM formation 17/68, 25%), were all positively associated with distant metastasis stage (M stage; P=0.031, P=0.017, and P=0.034, respectively), TNM stage (P=0.033, P=0.020, and P=0.023, respectively), but not with patient sex, age, tumor size or lymph node metastasis stage (N stage; Table III). Compared with the early stages (TNM I and II), the expression of nectin-4 and ITGB1, and VM formation were more frequently observed in the later stages of CRC (TNM III and IV). These results indicated that nectin-4 and ITGB1 protein expression, and VM formation were positively associated with CRC progression and may be indicators of poor prognosis.

Discussion

CRC is a common gastrointestinal malignancy with a high incidence of metastasis. Once metastasis occurs, the outcomes of surgery, radiotherapy or chemotherapy on patient prognosis are unsatisfactory, and the mortality rate remains high. Therefore the identification of novel molecular prognostic and predictive markers is required.

Nectin-4, a cell adhesion molecule that interacts with the cadherins, serves a key role in the formation and maintenance of adherens junctions (6,20). Nectin-4 consists of three immunoglobulin-like domains, which constitute transmembrane and extracellular domains, and a short cytoplasmic tail (20). It has been reported that nectin-4 promotes the anchorage-independent growth of human mammary epithelial cells by driving cell-to-cell attachment and activating integrin β4/Src homology region 2-containing protein tyrosine phosphatase 2/c-Src signaling (21). In addition, nectin-4 regulates epithelial-mesenchymal transition, tumor invasion

Table I. Nectin-4 expression in colorectal cancer and normal mucosal tissues.

Immunohistochemical staining	Colorectal cancer tissues n=68 (%)	Normal tissues n=15 (%)	P-value
Negative (-)	20 (29.4)	12 (80)	0.001[a]
Positive (+)	48 (70.6)	3 (20)	

[a]P<0.01, as determined by a χ^2 test.

Table II. Correlation between nectin-4 and ITGB1 or VM in colorectal cancer.

	ITGB1 (n)				VM (n)			
Nectin-4 (n)	-	+	χ^2 value	P-value	-	+	χ^2-value	P-value
-	17	3	32.556	<0.001[b]	19	1	4.628	0.014[a]
+	5	43			32	16		

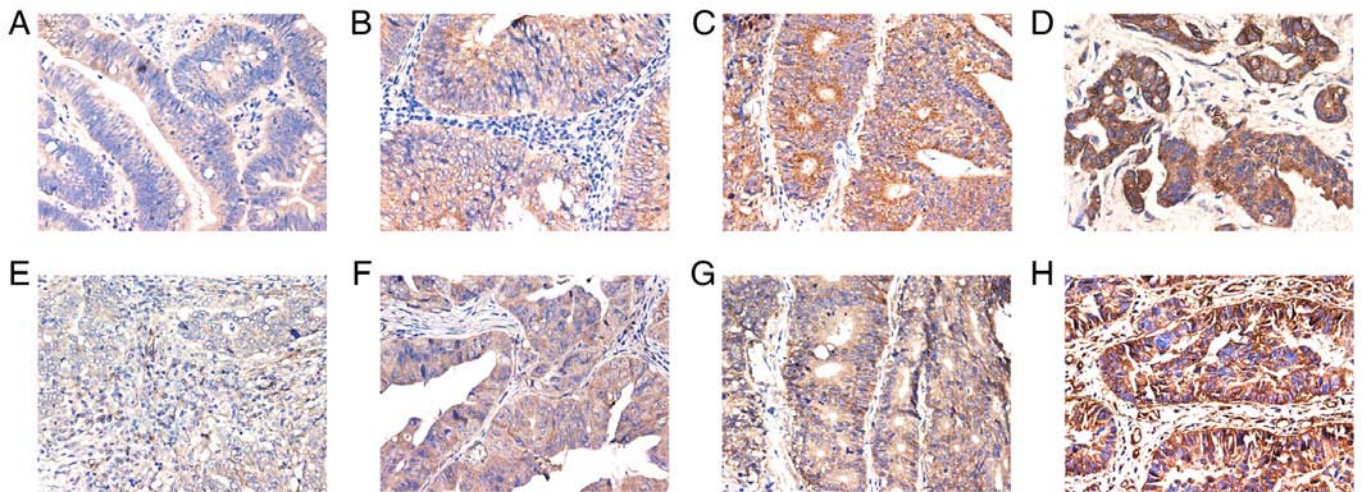[a]P<0.05 and [b]P<0.01, as determined by a χ^2 test.

Figure 4. Immunohistochemical analysis of nectin-4 and ITGB1 protein expression in CRC tissues (magnification, x400). (A-D) Nectin-4 and (E-H) ITGB1 protein expression were higher in the later stages of CRC (TNM III and TNM IV, C-D, G-H) compared with the early stages (TNM I and TNM II, A-B, E-F). CRC, colorectal cancer; ITGB1, Integrin β-1; TNM, tumor node metastasis.

and metastasis in breast cancer through its influence on the Wnt/β-catenin signaling pathway and the phosphoinositide 3-kinase/protein kinase B signaling axis (22). In non-small cell lung cancer, nectin-4 promotes tumor invasion and metastasis by activating the Rho-related protein racL (10). Furthermore, it has been reported that VM, as an alternative blood supply to tumors, is associated with a malignant phenotype and poor patient prognosis (23-25). Traditional anti-angiogenic therapy is aimed at endothelium-dependent blood vessels. Although this treatment delays the progression of tumors in a short period of time, recurrence and metastasis are issues for a number of patients (26). Therefore, further insight into the mechanisms of VM may aid developments in the field of anti-tumor therapeutics. Recent studies have reported that

ITGB1 may promote proliferation, invasion and metastasis in a variety of tumor types, and may therefore be associated with poor prognosis (27,28). Additional studies have also revealed that ITGB1 is crucial for the formation of VM, where ITGB1 promoted migrational persistence and influenced the shape of VM structures by regulating specific aspects of the transcriptional module associated with the VM network-forming phenotype (15).

In the present study, nectin-4 mRNA expression data from 372 patients with CRC were downloaded from a TCGA database. In this cohort, the overexpression of nectin-4 mRNA was strongly associated with lymphatic metastasis, distant metastasis and TNM classification. Collectively these findings suggest that nectin-4 overexpression is associated with

Table III. Relationship between nectin-4, ITGB1, VM and clinicopathological features in colorectal cancer.

Characteristics	Nectin-4			ITGB1			VM		
	-	+	P-value	-	+	P-value	-	+	P-value
Sex									
Male	10	28	0.528	11	27	0.499	24	11	0.207
Female	10	20		11	19		27	6	
Age									
≥65	5	15	0.403	6	14	0.789	16	4	0.760
<65	17	31		16	32		35	13	
Size (cm)									
≥5.0	10	25	0.876	9	26	0.344	25	8	0.889
<5.0	10	23		13	20		26	9	
N stage									
N0	13	27	0.504	14	26	0.577	30	10	0.909
N1 + N2	7	21		8	20		21	7	
M stage									
M0	19	32	0.031[a]	21	30	0.017[a]	42	9	0.034[a]
M1	1	16		1	16		11	8	
TNM stage									
I + II	14	20	0.033[a]	16	18	0.020[a]	30	4	0.023[a]
III + IV	6	28		6	28		21	13	

[a]$P<0.05$, as determined by two-tailed χ^2 test. N stage, lymph node metastasis stage; M stage, distant metastasis stage; TNM stage, tumor node metastasis stage; ITGB, integrin β-1; VM, vasculogenic mimicry.
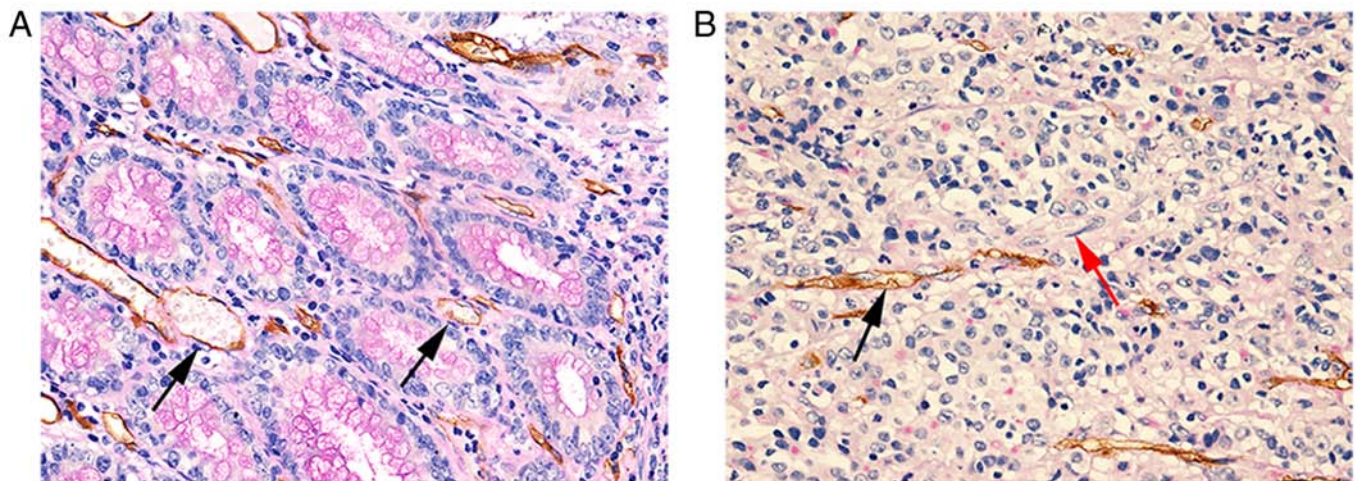

Figure 5. Identifying endothelium-dependent vessels and VM by cluster of differentiation 34/periodic acid-Schiff double staining. (A) VM negative staining in CRC tissue (magnification, x400). The black arrow indicates an endothelium-dependent vessel. (B) VM positive staining in CRC tissue (magnification, x400. The red arrow indicates a VM structure, and the black arrow indicates endothelium-dependent vasculature. VM, vasculogenic mimicry; CRC, colorectal cancer.

the progression of CRC, which is consistent with findings for nectin-4 expression in other tumor types (8,29). To further confirm the results obtained from the TCGA cohort, nectin-4 protein expression was assessed using IHC staining in a separate cohort of patients. The results revealed that nectin-4 protein expression was significantly upregulated in CRC tissues compared with normal mucosal tissues. In addition, an association between nectin-4, and ITGB1 protein expression and VM formation was observed in this cohort. Positive IHC staining results for these parameters were associated with M and TNM stage, but not patient sex, age, tumor size or N stage. Therefore, the results of the present study suggested that the

expression of nectin-4 and ITGB1, and VM formation may facilitate the progression of CRC.

In conclusion, we aimed to combine the data obtained from a TCGA database with that obtained from a separate patient cohort in order to examine nectin-4 expression at the mRNA and protein levels. The results concluded that nectin-4 was upregulated in CRC tissues compared with normal mucosal tissues, and that nectin-4 expression was positively associated with ITGB1 expression and VM formation. Furthermore, all three parameters (nectin-4, ITGB1 and VM) were significantly associated with M and TNM stage, characteristic features of highly invasive CRCs. Therefore, nectin-4, ITGB1 and VM combined may be useful in identifying the progression of CRC I patients, and those with poor prognosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ contributed to the study design, data analysis and drafted the manuscript. KL and YS performed the experiments and the bioinformatics analysis. PP and ShL contributed to the clinical data acquisition and analysis. SiL and ZY performed the experiments. MQ and JH contributed to the study design, reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics committee of the First Affiliated Hospital of Guangxi Medical University, and performed in accordance with the guidelines of the Declaration of Helsinki (no. BBMCEC2012063). All patients admitted to the study provided written informed consent for their participation.

Patient consent for publication

All patients admitted to the study provided informed consent for their participation and publication of the data.

Competing interests

The authors declare that they have no competing interests.

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