BMC Gastroenterology

RESEARCH

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Netrin-1-CD146 and netrin-1-S100A9 are associated with early stage of lymph node metastasis in colorectal cancer

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Abstract

Background The netrin-1/CD146 pathway regulates colorectal cancer (CRC) liver metastasis, angiogenesis, and vascular development. However, few investigations have yet examined the biological function of netrin-1/CD146 complex in CRC. In this work, we investigated the relationship between the netrin-1/CD146 axis and S100 proteins in sentinel lymph node, and revealed a possible new clue for vascular metastasis of CRC.

Methods The expression levels of netrin-1 and CD146 proteins in CRC, as well as S100A8 and S100A9 proteins in the sentinel lymph nodes were determined by immunohistochemistry. Using GEPIA and UALCAN, we analyzed netrin-1 and CD146 gene expression in CRC, their association with CRC stage, and their expression levels and prognosis in CRC patients.

Results The expression level of netrin-1 in N_{1a+1b} (CRC lymphatic metastasis groups, exculded N_{1c}) was positively increased with N_0 ($p=0.012$). The level of netrin-1 protein was positively correlated with CD146 protein ($p<0.05$). The level of S100A9 protein was positively correlated with CD146 protein ($r=0.492$, $p=0.007$). Moreover, netrin-1 expression was obviously correlated with S100A9 expression in the N_1 stage ($r=0.867$, $p=0.000$). CD146 level was correlated with S100A9 level in the N_2 stage ($r=0.731$, $p=0.039$). CD146 mRNA expression was higher in normal colorectal tissues than in CRC ($p<0.05$). Netrin-1 and CD146 expression were not significantly associated with the tumor stages and prognosis of patients with CRC ($p>0.05$).

Conclusions The netrin-1/CD146 and netrin-1/S100A9 axis in CRC tissues might related with early stage of lymph node metastasis, thus providing potential novel channels for blocking lymphatic metastasis and guiding biomarker discovery in CRC patients.

Keywords Netrin-1, CD146, S100 proteins, Lymph node metastasis, Colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, with 1.1 million new cases annually, and is the second leading-cause of cancer death [1]. Genetic alterations produce the dysregulation of signaling pathways, the inhibition of apoptosis and the induction of proliferation, invasion and migration, resulting in CRC development and metastasis [2]. Recently, netrin-1 and its receptor signaling have been increasingly determined to promote tumorigenesis in many types of cancers [3].

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Netrin-1 is a secreted glycoprotein that plays key roles in neuronal navigation, angiogenesis, and cell survival [4]. Moreover, netrin-1 has been implicated in numerous diseases, including cardiovascular disease, cancer, polycystic kidney disease, inflammatory disease, etc. [5–8]. Netrin-1 has been shown to be up-regulated in many cancers, and this up-regulation has been shown to constitute a selective mechanism that blocks apoptosis induced by the dependence receptors DCC and UNC5H [4]. Most of its activity has been found to occur through regulation of the signaling pathways combined with its main receptors [9].

In addition, CD146 is a highly glycosylated type I transmembrane protein, and was first identified as a specific cell-adhesion molecule for melanoma [10]. CD146 still plays a critical pro-migratory role in the vascular system, normal tissue development, and tumor progression model [11, 12]. Overexpression of CD146 has been discovered in numerous cancers, including CRC, intrahepatic cholangiocarcinoma, lung cancer, and breast cancer [13–16]. Accumulating evidence confirmed that the overexpression of CD146 could promote tumor progression and metastasis by altering the expression of genes in cancer cell proliferation, apoptosis, and angiogenesis [17, 18].

Notably, CD146 has been shown to act as a novel receptor for netrin-1 in promoting angiogenesis and vascular development [19, 20]. By regulating the activity of the miR-329-3p/netrin-1-CD146 complex, exosomal lncRNA PCAT1 promotes tumor circulating cell-mediated CRC liver metastasis [21]. However, the biological function of netrin-1-CD146 complex underlying the initiation and progression of CRC remains poorly defined.

S100A8 and S100A9 belong to the S100 multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles [22–24]. S100 proteins, the largest subgroup within the EF-hand protein family, are closely associated with cardiovascular disease, various types of cancer, inflammation, and autoimmune pathologies [25–28]. Primarily, S100A8 and S100A9 have been described in neutrophils and macrophages and were revealed to be involved in myeloid cell maturation [29, 30]. The two proteins were found to form S100A8/A9 heterocomplexes [31, 32], which were demonstrated to be secreted by activated monocytes via a tubulin-dependent pathway [33]. By forming a common heterodimer structure, S100A8/A9, S100A8, and S100A9 are widely reported to participate in multiple signaling pathways in tumor cells [34]. Meanwhile, through interfering with tumor metabolism and the microenvironment, S100A8/A9, S100A8, and S100A9, mainly as promoters, contribute to tumor development, growth, and metastasis [34]. Furthermore, during inflammation, S100A8/A9 is released actively, and exerts a critical effect in modulating the inflammatory response, and participates in cytoskeleton rearrangement and arachidonic acid metabolism [35]. Recently, S100A8/A9 was found to be a key protein between inflammation and cancer [36, 37].

Extant literature demonstrated that myeloid-derived suppressor cells (MDSC) induced by tumor-derived granulocyte colony-stimulating factor (G-CSF) express S100A8/A9 [38, 39]. Therefore, non-metastatic lymph nodes can be explained by MDSC-mediated premetastatic niche formation, in which proinflammatory factors, such as S100A8 or S100A9, are abundantly expressed [40, 41]. The premetastatic niche, an immunosuppressive and proinflammatory environment, is generated in premetastatic organs by MDSC to facilitate tumor cell metastasis [42, 43].

In aggregate, netrin-1-CD146 complex has been reported to promote CRC liver metastasis, angiogenesis, and vascular development [19–21]. S100A8 and S100A9 expression are closely related metastatic and non-metastatic lymph nodes [40, 41]. However, few investigations have yet examined the biological function of netrin-1-CD146 complex in CRC. In this study, we aimed to detect tissue levels of netrin-1-CD146 complex in patients with CRC, analyze the potential relationship between netrin-1-CD146 complex and S100A8/A9 expression in sentinel lymph nodes, and provide evidence to support their utilization as therapeutic targets.

Methods

Patient sample collection

The tissue samples contained 78 colorectal tissues (10 normal, 8 adenomas, and 60 tumor tissues, including 56 colon cancer and 4 upper rectal cancer) and 29 sentinel lymph nodes collected from 60 tumor tissues (20 positive lymph nodes, 9 negative lymph nodes). Sentinel lymph node identification was performed using 1% methylene blue dye during surgery. The sentinel lymph node was separately harvested and sent to the Department of Pathology for further analysis. Normal tissue samples included those from 6 males and 4 females with a median age of 45, and adenomas included those from 4 males and 4 females with a median age of 56. The medical records of tumor clinic-pathologic data from our institutional database, including age, gender, differentiation, size, lymphatic metastasis, T-stage, N-stage, and M-stage, were reviewed retrospectively. The Union for International Cancer Control (UICC) tumour node metastasis (TNM) staging system was applied according to the 8th edition of the Cancer Staging Manual. The tumor clinic-pathologic data are reported in Table 2.

All patients' surgical specimens were diagnosed by experienced gastrointestinal pathologists. No radiotherapy or chemotherapy had been conducted before surgery. Exclusion criteria were: (1) familial colon adenoma; (2) patients with partial clinical data; (3) inflammatory bowel disease; (4) other malignant tumors; (5) connective tissue diseases; and (6) acute phase of inflammatory disease [44]. Specimens from CRC and benign colorectal lesions were obtained retrospectively from consecutive patients who underwent surgery at the Department of Anorectal Surgery, the Third People's Hospital of Hangzhou, Hangzhou, P.R. China, between June 2020 and October 2022. The study protocol was approved by the Ethics Committee of the Third People's Hospital of Hangzhou, and all study participants provided a written informed consent.

Immunohistochemical analysis

Netrin-1 and CD146 in the colorectal tissues, as well as S100A8 and S100A9 in the sentinel lymph nodes, were separately evaluated for protein expression using immunohistochemistry (IHC), which was performed based on the standard streptavidin-peroxidase method. Paraffin wax-embedded sections were deparaffinized through the application of 100% xylene and ethyl alcohol. The antigen was retrieved with 0.01 M citrate buffer solution (pH 6.0) and heated for 15 min at 95℃. The endogenous peroxidase activity was inactivated by incubating the slides in 3% hydrogen peroxide at room temperature for 10 min. After rinsing in phosphate-buffered saline (PBS), 10% bovine serum was applied for 20 min to block nonspecific reactions.

The slides were subsequently incubated with a goat polyclonal antibody against netrin-1(ab122903, 1:100, Abcam), a rabbit monoclonal antibody against CD146 (ab75769, 1:100, Abcam), and a mouse monoclonal antibody against human S100A8 (T-1030, 1:100, BMA Biomedicals) and S100A9 ((T-1026, 1:200, BMA Biomedicals). After rinsing in PBS, these slides were incubated with peroxidase-labeled anti-goat, anti-rabbit, and anti-mouse IgG secondary antibody (Kit II, ZSGB-BIO), respectively. The peroxidase reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with hematoxylin. Images of the tissues were snapped with a visible-light microscope. The intensity of specific staining was analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, U.S.A.). The intensities of the positive staining in the cytoplasm and nucleus were evaluated with the mean integrated optical density (mean IOD). Mean IOD was equal to IOD/area of the tumor Sect. [45].

Analysis of netrin-1 and CD146 gene expression and their association with CRC stage using GEPIA

GEPIA (http://gepia.cancer-pku.cn/index.html) is a commonly used interactive tool [46]. The GEPIA dataset, which comprises samples from 9,736 tumors and 8,587 normal tissues from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases, respectively, was used to determine the expression levels of netrin-1 and CD146, as well as their associations with CRC stage [45].

Analysis of prognosis associated with netrin-1 and CD146 expression using GEPIA and UALCAN

UALCAN (http://ualcan.path.uab.edu/) is a portal for facilitating tumor subgroup gene expression and survival analyses, and an update to the integrated cancer data analysis platform [47, 48]. UALCAN generates graphs depicting gene expression and survival curves, and performs pan-cancer gene expression analysis [45]. Here, we used UALCAN to mine survival data for patients with CRC samples that were associated with netrin-1 and CD146 expression. Meanwhile, GEPIA was used to validate the correlation between netrin-1 and CD146 expression in terms of either overall survival (OS) or disease-free survival (DFS). The log-rank test was performed, and the resultant hazard ratios (HRs) and p-values or Cox p-values from the log-rank test were plotted.

Statistical analysis

All results were represented as median ± standard deviation using SPSS software (IBM SPSS, Version 20.0). Independent- samples t-test was used to determine statistical significance for changes in two groups. One-way analysis of variance (ANOVA) was utilized to evaluate the findings of more than two groups. Correlations were detected using the Spearman's test. All tests were two-sided. A p-value of < 0.05 was considered to be statistically significant in all analyses.

Results

The expression characteristics of netrin-1 and CD146 in colorectal tissues

We collected tissue samples from 10 normal colorectal tissues, 8 colorectal adenoma, and 60 CRC for analyzing netrin-1 and CD146 expression. Positive staining of netrin-1 and CD146 protein in colorectal tissues were mainly observed in the nucleus, and a few were observed in the cytoplasm, as brown-yellow colour (Figs. 1 and 2). There was no significant difference in netrin-1 and CD146 expression among different groups based on

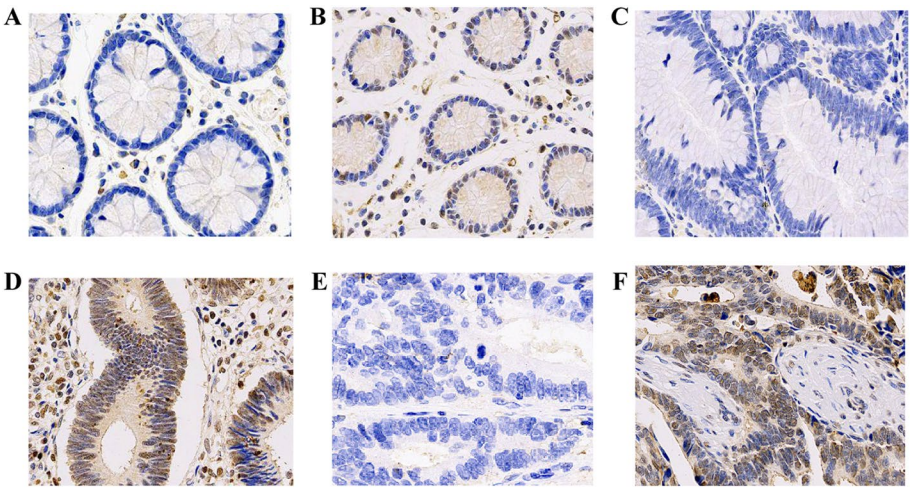

Fig. 1 Immunohistochemical analyses of netrin-1 expression. A Negative expression in normal tissues. **B** Positive expression in normal tissues. **C** Negative expression in adenoma. **D** Positive expression in adenoma. **E** Negative expression in colorectal cancer (CRC). **F** Positive expression in CRC (×400 magnitude)

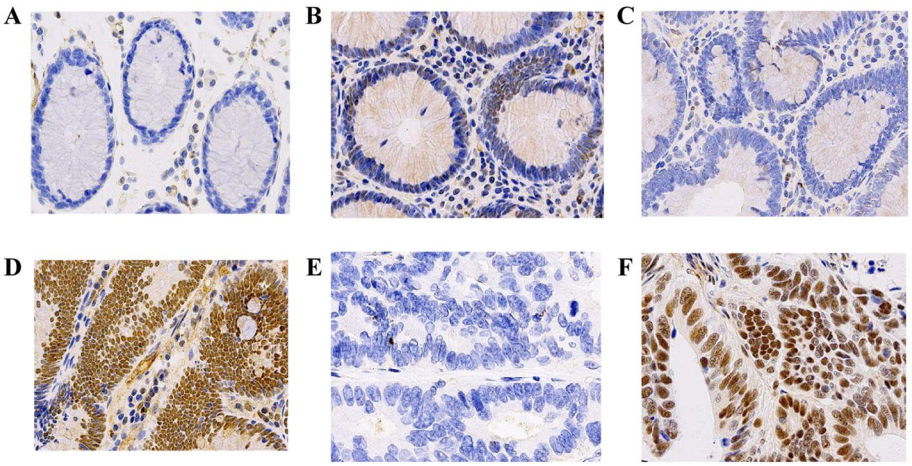

Fig. 2 Immunohistochemical analyses of CD146 expression. **A** Negative expression in normal tissues. **B** Positive expression in normal tissues. **C** Negative expression in adenoma. **D** Positive expression in adenoma. **E** Negative expression in colorectal cancer (CRC). **F** Positive expression in CRC (×400 magnitude)

Table 1 Mean IOD values of 78 colorectal tissues

Variables	n	Netrin-1 mean IOD	*F*-value	*p*-value	CD146 mean IOD	*F*-value	*p*-value
Normal colorectal tissue	10	0.01 ± 0.00	1.046	0.357	0.02 ± 0.01	0.502	0.607
Colorectal adenoma	8	0.01 ± 0.02			0.03 ± 0.02		
Colorectal cancer	60	0.15 ± 0.42			0.04 ± 0.08		

IOD Integrated optic density

Data presented as mean ± SD

normal colorectal tissue, colorectal adenoma, and CRC ($p > 0.05$; Table 1). However, netrin-1 mean IOD values in CRC were obviously higher than normal colorectal tissue and colorectal adenoma.

Clinicopathological characteristics of netrin-1 and CD146 in CRC

We explored the expression of netrin-1 and CD146 protein in CRC. The detailed clinicopathological

Table 2 Clinicopathological characteristics of netrin-1 and CD146 in CRC

Variables	n	Netrin-1 mean IOD	*p*-value	CD146 mean IOD	*p*-value
Gender					
Male	41	0.19 ± 0.49	0.177	0.05 ± 0.09	0.346
Female	19	0.17 ± 0.15		0.03 ± 0.02	
Age					
≤ 60 years	13	0.10 ± 0.18	0.589	0.02 ± 0.02	0.286
> 60 years	47	0.17 ± 0.46		0.05 ± 0.09	
Differentiation					
Low	9	0.09 ± 0.21	0.778	0.03 ± 0.02	0.928
Medium	45	0.18 ± 0.47		0.04 ± 0.09	
High	6	0.08 ± 0.17		0.04 ± 0.02	
Size					
≤ 5 cm	46	0.18 ± 0.47	0.318	0.05 ± 0.09	0.566
> 5 cm	14	0.05 ± 0.13		0.03 ± 0.01	
Lymphatic metastasis					
Yes	32	0.24 ± 0.55	0.065	0.05 ± 0.11	0.360
No	28	0.05 ± 0.11		0.03 ± 0.02	
TNM stage					
I	10	0.12 ± 0.16	0.196	0.05 ± 0.02	0.648
II	17	0.02 ± 0.01		0.02 ± 0.01	
III	28	0.27 ± 0.58		0.05 ± 0.11	
IV	5	0.02 ± 0.02		0.05 ± 0.04	

CRC Colorectal cancer, *TNM* Tumor, node, metastasis, *IOD* Integrated optic density

Data presented as mean ± SD

Table 3 Lymphatic metastasis stages of netrin-1 in CRC

Variables	n	Netrin-1 mean IOD	*F*-value	*p*-value
Lymphatic metastasis				
N_1	22	0.32 ± 0.64	3.092	0.053
N_2	10	0.06 ± 0.16		
N_o	28	0.05 ± 0.11		

CRC Colorectal cancer, *IOD* Integrated optic density

Data presented as mean ± SD

characteristics of netrin-1 and CD146 mean IODs in CRC are presented in Table 2. There was no significant difference between netrin-1 and CD146 expression and gender, age, differentiation, size, lymphatic metastasis, and TNM stage ($p > 0.05$). Afterwards, we found that the *p*-value of the lymphatic metastasis group in netrin-1 protein was 0.065. We, therefore, analyzed lymph node metastases in netrin-1 protein by stages (Table 3). It was found that netrin-1 protein expression was markedly increased in the N_1 stage group, and the *p*-value was 0.053. To further elucidate the characteristics of netrin-1 protein expression in lymphatic metastasis, we excluded only three cases of ectopic tumor deposit, i.e., three N_{1c} cases, and conducted reanalysis

Table 4 Lymphatic metastasis stages (excluding N_{1c}) of netrin-1 in CRC

Variables	n	Netrin-1 mean IOD	*F*-value	*p*-value
Lymphatic metastasis				
N_{1a+1b}	19	0.37 ± 0.69*	3.759	0.03
N_2	10	0.06 ± 0.16		
N_o	28	0.05 ± 0.11		

CRC Colorectal cancer, *IOD* Integrated optic density

Data presented as mean ± SD

* N_{1a+1b} versus N_o ($p = 0.012$)

(Table 4). We finally found a significant difference between the groups ($p = 0.03$). Afterwards, multiple comparisons were performed in the groups, a significant difference was identified between N_{1a+1b} and N_o groups ($p = 0.012$).

The expression characteristics of S100A8 and S100A9 in sentinel lymph nodes

A total of 29 sentinel lymph node specimens from 60 CRC patients were investigated. Of the 29 cases, there were 22 male cases and 7 female cases. Male patients' age ranged between 52 and 93 years (mean value,

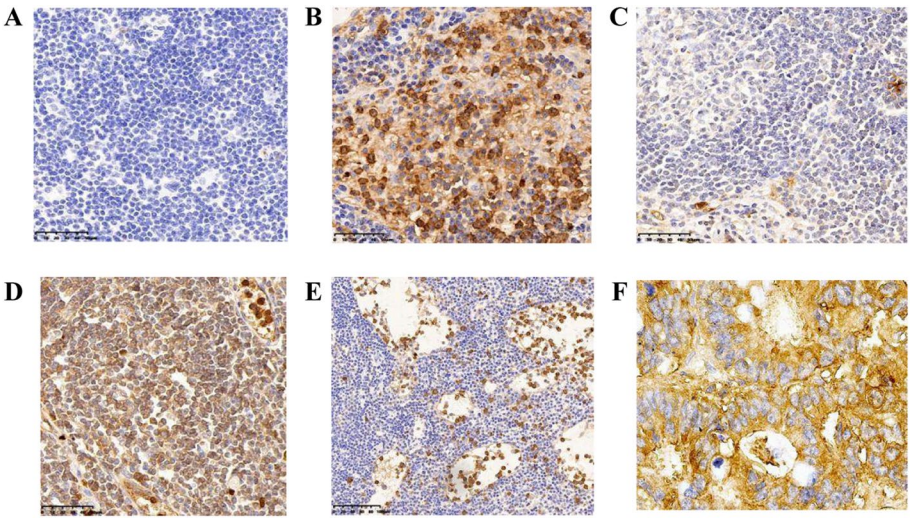

Fig. 3 Immunohistochemical analyses of S100A8 and S100A9 expression. **A** S100A8 negative expression in sentinel lymph node, scale bar: 50 μm. **B** S100A8 positive expression in sentinel lymph node, scale bar: 50 μm. **C** S100A9 weak staining in sentinel lymph node, scale bar: 50 μm. **D** S100A9 positive expression in sentinel lymph node, scale bar: 50 μm. **E** S100A8 expression in the lumen of sentinel lymph nodes, scale bar: 100 μm. **F** S100A9 positive expression in CRC (×400 magnitude)

69.77 ± 10.13), and female patients' age ranged between 44 and 84 years (mean value, 69.43 ± 17.32). There were 9 cases without lymph node metastasis and 20 cases with lymph node metastasis (including N_1-stage, 12 cases and N_2-stage, 8 cases). Meanwhile, there were 10 out of 29 CRC specimens with vascular infiltration. According to the TNM classification system, 4 (13.8%) were stage I, 5 (17.2%) were stage II, 18 (62.1%) were stage III, and 2 (6.9%) were stage IV. Signals for S100A8 and S100A9 in sentinel lymph nodes were detected predominantly in the nucleus, but cytoplasm staining was also observed, as brown-yellow colour (Fig. 3). For a comparative study, S100A8 and S100A9 expression in three cases of CRC were mainly detected in the cytoplasm (Fig. 3F).

There was no significant difference in S100A8 and S100A9 expression among different groups based on lymph node metastasis, and there was no lymph node metastasis in CRC ($p > 0.05$) (Table 5). No significant difference was also found in S100A8 and S100A9 expression between vascular infiltration group and non-vascular infiltration group ($p > 0.05$).

Table 5 Sentinel lymph node characteristics of S100A8 and S100A9 in CRC

Variables	n	S100A8 mean IOD	*p*-value	S100A9 mean IOD	*p*-value
Lymphatic metastasis					
Yes	20	0.03 ± 0.05	0.542	0.08 ± 0.20	0.770
No	9	0.02 ± 0.02		0.06 ± 0.03	
Vascular infiltration					
Yes	10	0.04 ± 0.06	0.138	0.04 ± 0.03	0.427
No	19	0.02 ± 0.02		0.10 ± 0.20	

Data presented as mean ± SD. CRC: colorectal cancer; IOD: integrated optic density

Table 6 Correlations among netrin-1, CD146, S100A8, and S100A9 in CRC

Variable	*r*	*p*-value
60 cases		
Netrin-1 and CD146	0.414	0.001
29 cases		
Netrin-1 and CD146	0.430	0.020
Netrin-1 and S100A8	0.098	0.614
Netrin-1 and S100A9	0.155	0.421
CD146 and S100A8	0.055	0.777
CD146 and S100A9	0.492	0.007
S100A8 and S100A9	0.175	0.364

CRC Colorectal cancer patients from mono-factor analysis

Correlations among netrin-1, CD146, S100 A8, and S100 A9 in CRC patients

Monofactor analysis found that netrin-1 expression was associated with CD146 expression (60 cases, $p = 0.001$; 29cases, $p = 0.02$; Table 6). We also determined that CD146 level was associated with S100A9 level ($p = 0.007$). There were no significant correlations among netrin-1 and S100A8, netrin-1 and S100A9, CD146 and S100A8, and S100A8 and S100A9

($p=0.614$, 0.421, 0.777, and 0.364, respectively). To further evaluate the characteristics between netrin-1/CD146 and sentinel lymph nodes metastasis, we, therefore, reanalyzed their correlations in CRC progression by N stage (Table 7). We found that netrin-1 expression was obviously correlated with S100A9 expression in the N_1 stage ($p=0.000$). We also found that CD146 level was correlated with S100A9 level in the N_2 stage ($p=0.039$).

Table 7 Correlations between netrin-1/CD146 and S100A8/S100A9 in CRC

Variable	*r*	*p*-value
N_o		
Netrin-1 and S100A8	-0.409	0.274
Netrin-1 and S100A9	-0.362	0.339
CD146 and S100A8	0.170	0.663
CD146 and S100A9	0.630	0.069
N_1		
Netrin-1 and S100A8	0.125	0.700
Netrin-1 and S100A9	0.867	0.000
CD146 and S100A8	-0.288	0.364
CD146 and S100A9	0.031	0.923
N_2		
Netrin-1 and S100A8	-0.277	0.507
Netrin-1 and S100A9	0.060	0.887
CD146 and S100A8	0.024	0.955
CD146 and S100A9	0.731	0.039

Progression by N stage

The expression of netrin-1 and CD146 mRNA between normal tissues and CRC

When using GEPIA to analyze netrin-1 and CD146 mRNA expression, 550 cases of colon adenocarcinoma (COAD), 184 cases of rectal adenocarcinoma (READ), and 1,334 samples of normal colorectal tissues were included, respectively. This analysis revealed that netrin-1 mRNA expression did not exhibit any significant difference, and CD146 mRNA expression was significantly higher in normal colorectal tissues than in CRC ($p<0.05$) (Fig. 4).

The relationship between netrin-1 and CD146 mRNA expression and CRC stage

The relationship between the mRNA expression levels of netrin-1 and CD146, and the tumor stages of patients with CRC were analyzed using GEPIA. Netrin-1 and CD146 expression were not significantly associated with the tumor stages of patients with colon or rectal cancers ($p=0.338$, $p=0.423$, $p=0.101$, and $p=0.235$, respectively (Fig. 5).

The expression of netrin-1 and CD146 mRNA related to the prognosis of patients with CRC

After using UALCAN to analyze the data, it was revealed that netrin-1 expression was not significantly associated with the survival of patients with colon or rectal cancers ($p=0.50$ and $p=0.84$, respectively) (Fig. 6A, B). When using GEPIA to evaluate the relationship between netrin-1 mRNA expression and CRC prognosis, no significant differences were observed in OS and DFS between low and high expression patients with colon or rectal cancer ($p=0.80$ and $p=0.33$, and $p=0.91$ and $p=0.82$, respectively) (Fig. 6C-F).

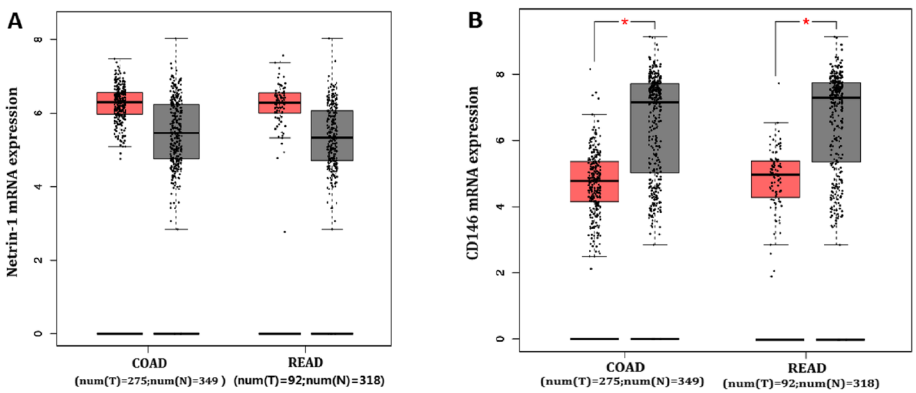

Fig. 4 Expression of netrin-1 and CD146 mRNA related to normal tissues and colorectal cancer. **A** Expression of netrin-1 in normal tissues and colon adenocarcinoma (COAD), and normal tissues and rectum adenocarcinoma (READ) patients. **B** Expression of CD146 in normal tissues and COAD, and normal tissues and READ patients ($p<0.05$)

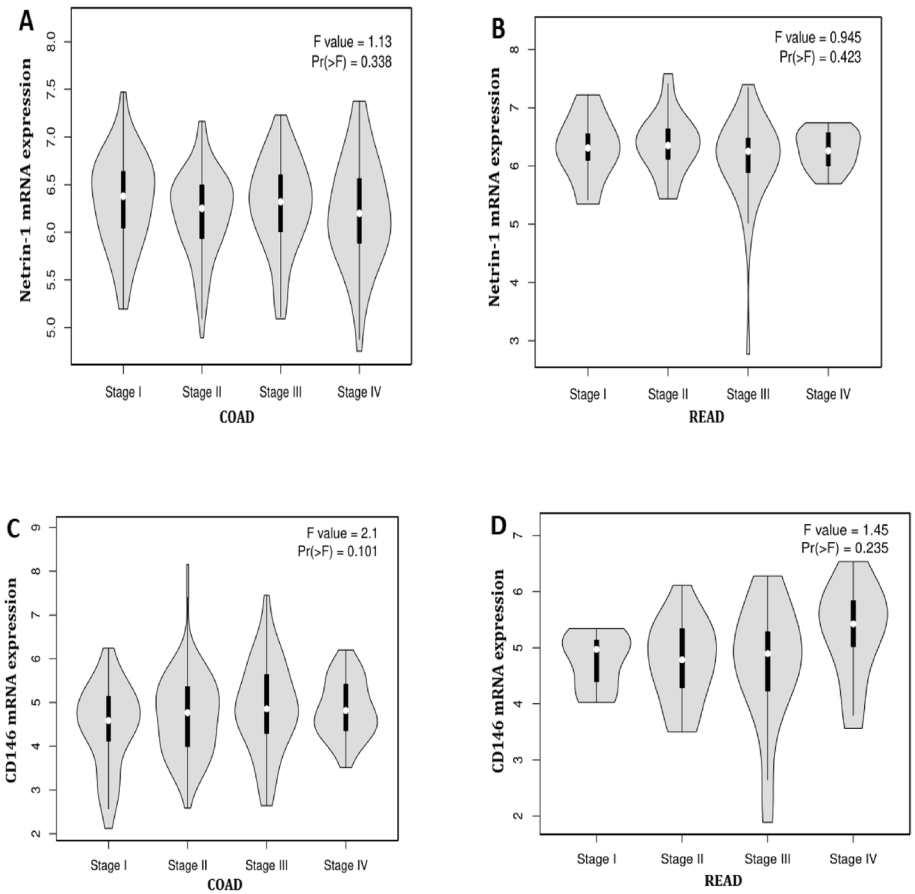

Fig. 5 **A** and **B** Correlation between netrin-1 expression and the tumor stages of patients with COAD and READ, respectively. **C** and **D** Correlation between CD146 expression and the tumor stages of patients with COAD and READ, respectively

Likewise, we found that CD146 expression was not significantly associated with the survival of patients with colon or rectal cancer ($p=0.16$ and $p=0.36$, respectively) (Fig. 7A, B). No significant differences were identified in OS and DFS between low and high expression patients with colon or rectal cancer ($p=0.86$ and $p=0.46$, and $p=0.57$ and $p=0.50$, respectively) (Fig. 7C-F).

Discussion

In recent years, netrin-1 has been shown to be up-regulated in many cancers, and this up-regulation has been proposed to act as a selective mechanism that blocks apoptosis induced by its dependence receptors DCC and UNC5H [4]. Further research data revealed that netrin-1 is upregulated not only in cancer cells, but also in cancer-associated stromal cells, which produce netrin-1 to control cancer cell plasticity [49]. CD146, an endothelial transmembrane protein of the immunoglobulin superfamily, is a previously unknown receptor for netrin-1, demonstrating the involvement of netrin-CD146 signaling in angiogenesis during vertebrate development [20]. Meanwhile, the miR-329-3p/netrin-1-CD146 complex promotes tumor circulating cell-mediated CRC liver metastasis [21]. However, the expression pattern and mechanism of netrin-1-CD146 complex in CRC remain largely undetermined.

In our study, we found that netrin-1 mean IOD values in CRC were obviously higher than those in normal colorectal tissue and adenoma, although this result was not statistically significant. It was also determined that CD146 mean IOD values exhibited no distinct increase. Netrin-1 is preferentially expressed at the base of intestinal crypts; whereas, its receptor DCC is evenly distributed throughout the villi. Netrin-1binding to DCC in crypt cells contributes to cell survival [50]. The even distribution of netrin-1 on the villus surface prevents apoptotic cell death, thus promoting tumour growth [50]. These results indicate that a higher distribution of netrin-1 could exist in adenomas and CRC, and this was confirmed by our findings. As a multi-functional molecule, CD146 participates in various biological processes, including angiogenesis, tumor metastasis, lymphocyte

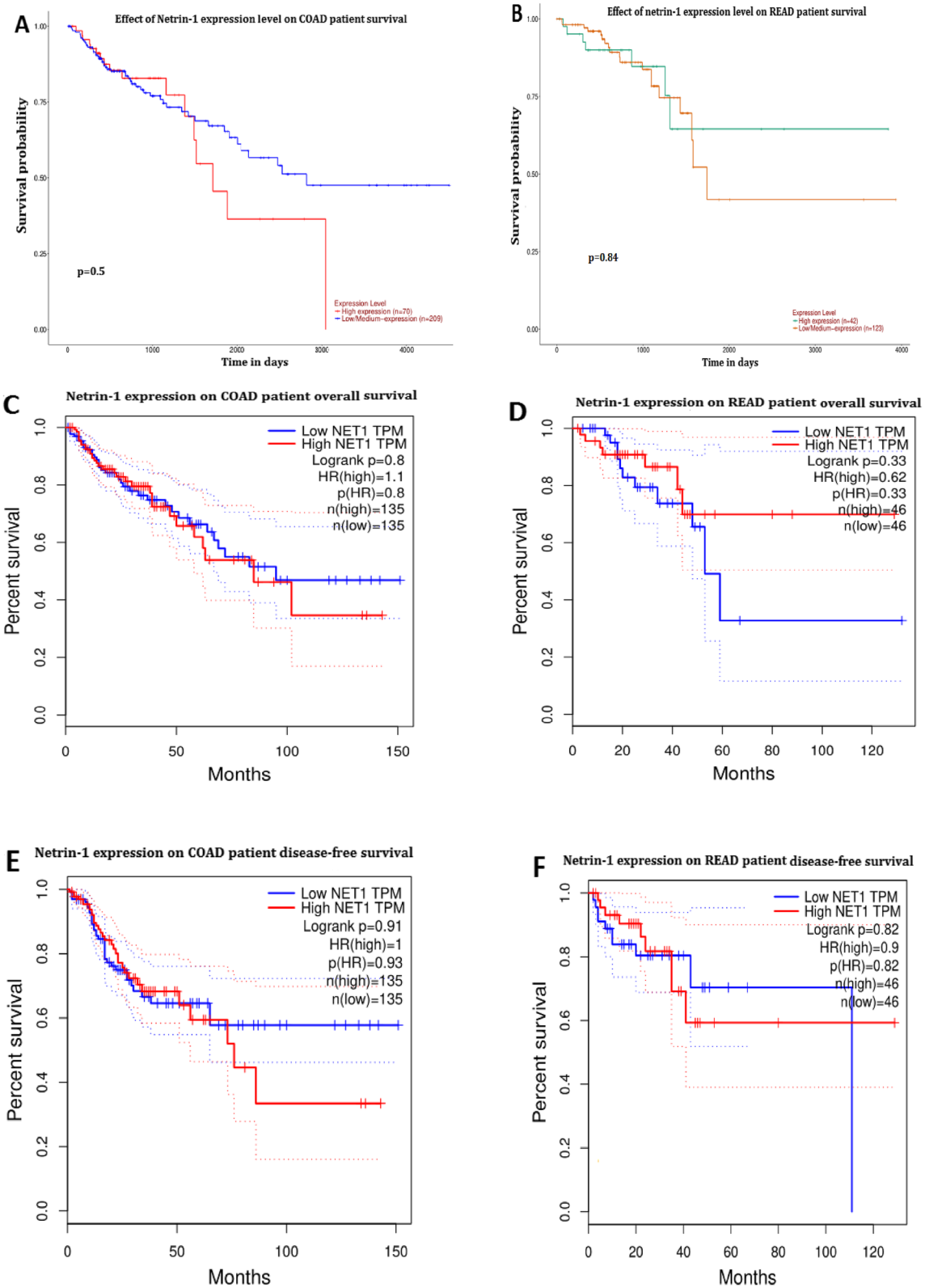

Fig. 6 **A** and **B** Correlation between netrin-1 mRNA expression and the survival of patients with COAD and READ, respectively. **C** and **D** Correlation between netrin-1 mRNA expression and the OS of patients with COAD and READ, respectively. **E** and **F** Correlation between netrin-1 mRNA expression and the DFS of patients with COAD and READ, respectively

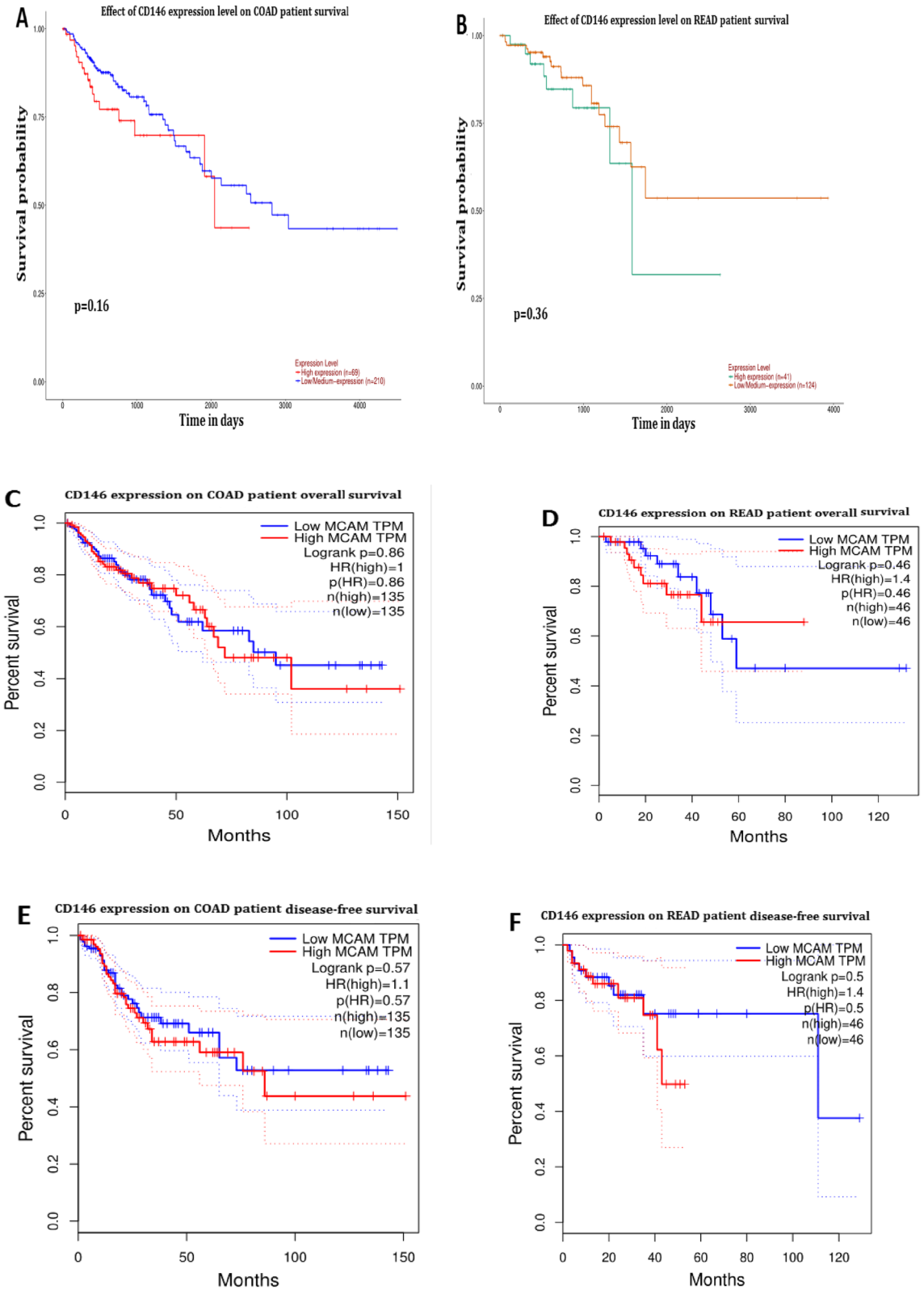

Fig. 7 **A** and **B** Correlation between CD146 mRNA expression and the survival of patients with COAD and READ, respectively. **C** and **D** Correlation between CD146 mRNA expression and the OS of patients with COAD and READ, respectively. **E** and **F** Correlation between CD146 mRNA expression and the DFS of patients with COAD and READ, respectively

activation, and morphogenesis during development and tissue regeneration [51, 52]. Expressed in endothelial cells, CD146 is required for endothelial cell proliferation, migration and tube formation, and plays critical roles in angiogenesis [53–56]. Regarding the different expression sites of CD146 and other netrin-1 receptors, CD146 mean IOD values exhibited no significant differences among groups.

It was also found that netrin-1 and CD146 levels were not correlated with clinicopathological characteristics in CRC, respectively. However, the netrin-1 level of the lymph node metastasis group was only close to, but did not reach, a statistically significant difference ($p=0.065$). We continued to analyze lymph node metastases in netrin-1 protein by stages, and determined that netrin-1 level was obviously increased in the N_1 stage group, and the *p-* value was 0.053. Because lymph node metastasis and tumor deposit formation have different mechanisms [57], we excluded only three N_{1c} cases of ectopic tumor deposit. It was finally revealed that netrin-1 level in N_{1a+1b} patients was positively correlated with N_o ($p=0.012$), but not correlated with N_2 ($p>0.05$). Previous research demonstrated that lymphangiogenesis plays a crucial role in promoting cancer metastasis to sentinel lymph nodes and beyond [58], and plays other roles in tumor pathogenesis, such as the niche function of tumor stem cells and regulatory functions of antitumor immune responses [59]. We speculated that netrin-1 high level in N_{1a+1b} patients may be likely related with lymphangiogenesis and early lymph node metastasis.

There was no significant difference in S100A8 and S100A9 mean IOD of CRC between lymph node metastasis and no lymph node metastasis ($p>0.05$). Furthermore, no significant difference was also found in S100A8 and S100A9 mean IOD between vascular invasion group and non-vascular invasion group ($p>0.05$). Non-metastatic lymph nodes can be regarded as the MDSC-mediated premetastatic niche formation, in which proinflammatory factors, such as S100A8 or S100A9, are abundantly expressed [40, 41]. In our study, the expression of S100A8 and S100A9 in TNM stage I ($pT_2N_0M_0$) occurred in 4 cases (13.8%). In order to further explore the expression of proinflammatory factors, we need to collect more and earlier cases need to be collected in the future. Monofactor analysis found that netrin-1 expression was associated with CD146 expression ($p<0.05$), and CD146 level was associated with S100A9 level ($p=0.007$). Moreover, we found that netrin-1 expression was obviously correlated with S100A9 expression in the N_1 stage ($p=0.000$), and CD146 level was correlated with S100A9 level in the N_2 stage ($p=0.039$). CD146 is critically involved in S100A8/A9-mediated cancer metastasis and inflammation when expressed at high levels [60]. Previous results demonstrated that the S100A8/A9-CD146 signaling axis plays a critical role in metastatic onset of melanoma cells [61], and regulates a novel transcription factor ETV4, which leads to epithelial-mesenchymal transition (EMT) through ZEB1 and thereby to metastasis in breast cancer cells [17]. Interestingly, exMCAM-Fc, a Fc fusion protein with the extracellular region of CD146, that could prevent the interaction of S100A8/A9 with CD146, efficiently reduced the number of circulating tumor cells that appeared in the blood flow [62]. According to the above research results, we speculated that the netrin-1-CD146 signaling axis is likely related with lymphangiogenesis for forming lymphatic metastasis passage, but netrin-1-S100A9 and CD146-S100A9 signaling axis are likely correlated with premetastatic niche formation for driving cancer cell dissemination to sentinel lymph nodes and beyond. Netrin-1may forms a complex with S100A9 in the N_1 stage, and then binds to the transmembrane protein CD146 in cancer cells. However, with the down-regulation of netrin-1 in the N_2 stage, S100A9 directly binds to the transmembrane protein CD146 in cancer cells. These complex communication network may be related to the different factors secreted by the secretome in tumor microenvironment at different time periods [63]. S100A8/A9 complex, S100A8, and S100A9 might be serve different functions in tumor-related inflammatory responses.

CD146 mRNA expression was higher in normal colorectal tissues than in CRC ($p<0.05$). This result may be associated with increasing aberrant promoter methylation to be silent CD146 gene expression in CRC [64]. Netrin-1 mRNA expression was not found to be statistically different. A recent study showed that netrin-1 DNA methylation is significantly higher in CRCs (24.6%) than in the adjacent normal intestinal mucosa (4.0%) [65]. Meanwhile, we found that the protein expression of netrin-1 and CD146 was not significantly associated with CRC stage. Furthermore, GEPIA analysis revealed that the levels of netrin-1 and CD146 mRNA in CRC were also independent of disease stage. However, an association between netrin-1 expression and renal clear cell carcinoma stage was observed [66]. Another investigation did identify a negative association between CD146 and thyroid cancer stage ($r=-0.231$, $p=0.010$) [67]. Therefore, netrin-1 and CD146 exhibit diverse biological characteristics in different solid tumors.

Our present study of CRC using UALCAN and GEPIA analysis did not find a significant association between netrin-1 expression and prognosis. Netrin-1 expression is associated with worse outcomes in poorly differentiated pancreatic adenocarcinomas [68]. Moreover, netrin-1

expression is still an independent prognostic factor for poor patient survival in brain metastases and aggressive neuroblastoma [69, 70]. We also did not find a significant association between CD146 expression and prognosis. Yet, CD146 is regarded as an independent prognostic factor for osteosarcoma patients [71]. High CD146 expression was associated with poor prognosis in patients with clear cell renal cell carcinoma [72]. CD146 also predicts poor prognosis in hepatocellular carcinoma [73], gastric cancer [74], and gallbladder adenocarcinoma [75].

Our study possesses certain limitations. First, our sample size small, and only the sentinel lymph node of partial specimens was provided. Second, to further analyze the expression of pro-inflammatory factors in sentinel lymph node, the samples ($T_{is}N_0M_0$ and $T_1N_0M_0$) should be obtained by means of animal experiments. Third, we did not follow-up on the survival of patients who supplied CRC tissues to compare with UALCAN and GEPIA analysis. Furthermore, we did not investigate the mechanism of netrin-1-CD146, netrin-1-S100A9 and S100A9-CD146 complex in lymphatic metastasis of CRC. Further studies are, therefore, required that address these issues.

In this study, we systemically analyzed the expression and prognostic value of netrin-1-CD146 complex, and which correlated with proinflammatory factors, in colorectal tumors. We speculated that netrin-1 high level in N_{1a+1b} patients may likely be related with lymphangiogenesis and early lymph node metastasis. A possible explanation for this could be as follows: cancer-associated stromal cells produce netrin-1 to control cancer cell plasticity [49], resulting in netrin-1 high level in N_{1a+1b} patients. After inducing by tumor-derived G-CSF, MDSC expressed S100A8/A9 and mediated premetastatic niche formation in sentinel lymphatic node [38, 39], where are abundantly imbued with S100A8 and S100A9 [40, 41]. Meanwhile, tumor cells also support MDSC expansion and recruitment by secreting multiple growth factors and cytokines [76]. Netrin-1 further promotes the immunosuppressive activity of MDSCs and high netrin-1 in plasma correlated with MDSCs in CRC [76]. Moreover, peritumoral lymphangiogenesis plays a prominent role in lymph node metastasis [77]. Netrin-1-CD146 might be related with lymphatic metastatic passage, where lymphatic vessels have a larger size with a hollow, oval shape [78]. On the other hand, netrin-1-S100A9 and S100A9-CD146 might be related with driving force in different N stages [61], which drives tumor cells from the primary focus to the sentinel lymph node and beyond. Netrin-1 has been shown to be up-regulated in the N_1 stage, which increases the chance of forming a complex with S100A9. In addition, our integrated bioinformatic analyses revealed that netrin-1 and CD146 levels were independent of CRC stage and prognosis.

Conclusions

Our findings revealed that the netrin-1/CD146 and netrin-1/S100A9 axis in CRC tissues might be related with early stage lymph node metastasis, thus providing potential novel channels for blocking lymphatic metastasis in CRC patients. In the future, we will continue to expand the clinical sample size to verify our clinical results and explore the potential mechanism. We believe that the netrin-1-CD146, netrin-1-S100A9, and CD146-S100A9 pathways should be investigated more deeply.

Abbreviations

CRC	Colorectal cancer
MDSC	Myeloid-derived suppressor cells
G-CSF	Granulocyte colony-stimulating factor
UICC	The Union for International Cancer Control
IHC	Immunohistochemistry
PBS	Phosphate-buffered saline
IOD	Integrated optical density
TCGA	The Cancer Genome Atlas
GTEx	The Genotype-Tissue Expression
OS	Overall survival
DFS	Disease-free survival
ANOVA	One-way analysis of variance
COAD	Colon adenocarcinoma
READ	Rectal adenocarcinoma
EMT	Epithelial-mesenchymal transition
HRs	Hazard ratios

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12876-024-03401-w.

Supplementary Material 1.

Acknowledgements

We thank the patients who provided the precious human colorectal tissues used in this study.

Authors' contributions

J.C. and J.H. performed the experiments and wrote the manuscript; J.Q. and G.Y. collected tissues samples; D.W. performed statistical analyses; J.C., J.H., J.Q. and Z.S. analyzed and interpreted the data; G.Y. and D.W. contributed to scientific discussions; J.C., J.H. and Z.S. designed the experiments, provided useful advice on the manuscript, and modified the manuscript. All authors read and approved the final manuscript.

Funding

This research was supported by grants from the Health, Science and Technology Plan Project of Hangzhou (grant no. A20200024), the Science and Technology Development Plan Project of Hangzhou (grant no. 20201203B201), and the Zhejiang Provincial Natural Science Foundation of China (grant no. LGF20H270001).

Availability of data and materials

Data is provided within the supplementary information files.

Declarations

Ethics approval and consent to participate

The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by the Institutional Ethics Committee of the Third People's Hospital of Hangzhou (Approval number: Y-KL2021009). Informed consent was obtained from all patients for this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 27 March 2024 Accepted: 3 September 2024
Published online: 11 September 2024

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