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RESEARCH

The Expression of Non-Mast Histamine in Tumor Associated Microvessels in Human Colorectal Cancers

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Abstract Angiogenesis is essential for the growth, expansion and metastasis of human colorectal cancers (CRCs). Histamine produced by mast cells is a potent proangiogenic factor. However, the significance of non-mast cell expressing histamine in the tumor microenvironment remains unknown. In this study, we evaluated the histamine positive microvessels with the specific marker for biosynthesis of histamine L-histidine decarboxylase (HDC) in the CRC tumor microenvironment. The relationship between HDC positive microvessel density (HDC-MVD) and clinical pathological parameters was assessed. The results revealed that HDC-MVD in the tumor microenvironment of CRCs was significantly increased as compared with the controls. CRC patients with lymph node invasion had a particularly higher density of HDC-MVD than those without. The density of HDC-MVD accounted for ~79 % of CD34 positive MVD in CRCs and double IHC analysis demonstrated that these HDC positive microvessels were mostly CD34 positive microvessels and with a high proliferative activity. Our results suggest that histamine expressed in microvessels could be an additional cellular source and involved in the cancer invasion through promoting angiogenesis in human CRCs.

Keywords Histamine · Angiogenesis · Colorectal cancer

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Introduction

Colorectal cancer (CRC) is one of leading cancers with high mortality worldwide. In many CRC patients, the metastasis is observed at the time of diagnosis and the prognosis becomes very poor [1]. Accumulative evidence has supported that angiogenesis is essential for the growth, expansion and metastasis of human cancers and anti-angiogenesis has become a new potential therapeutic approach in treating human cancers, particularly for those cancer patients with metastasis [2, 3]. Tumor associated angiogenesis is regulated by many factors produced by microenvironmental cells (both tumor cells and non-tumor cells) [2–4]. Histamine, the most common biogeneic amine widely distributed in human body and with different functions [5, 6], has recently been found to be one of the potent proangiogenic factors [7], the promoting effect of histamine on angiogenesis is via histamine-2 (H2) receptor and the H2 receptor antagonist cimetidine can inhibit histamine-induced angiogenesis [7, 8], such H-2 receptor antagonist treatment as an adjuvant approach has shown the improved survival in patients with gastrointestinal cancers [8–13]. In the tumor microenvironment, histamine can be produced by many types of cells and mast cell is the main cellular sources for histamine [14, 15]. However, histamine can also be synthesized by both tumor cells and non-mast cells [16]. Recently, several studies have demonstrated that histamine is observed in the blood vessel wall in many species [17–19]. It is well known that new blood vessel formation (angiogenesis) is critical for the cancer growth and metastasis and inhibition of angiogenesis

has been selected as a potent adjuvant approach [3, 20], Previous studies have revealed that the degradation of histamine is decreased in colonic adenoma tissues and expression of HDC (histamine) is increased in human colorectal adenomas and cancers [21–23]. Thus, histamine expressed in the tumor associated microvesells may play a pivotal role in modulating angiogenesis and then biological behaviors in CRCs.

In the studying of histamine expression, L-histidine decarboxylase (HDC) has been used as a specific marker for histamine biosynthesis, as it is the only enzyme responsible for the generation of histamine from histidine [6]. Therefore, the aim of this study was to examine the microenvironmental HDC positive mcirovessel density (MVD) in CRC specimens and their pathological significance. Our results indicate that the HDC positive MVD in the tumor microenvironment is significantly increased and associated with lymph node involvement, thus histamine from microvesells might serve as additional histamine source and contribute to the regulation of angiogenesis and then cancer invasion in CRCs.

Patients and Methods

A total of 30 cases of CRCs were collected from Department of Surgery, the Second Affiliated Hospital of Zhengzhou University. Baseline clinical information was summarized in Table 1. Demographic information: the mean age of patients with CRC was 55.6 years. Histologically, all the tumors were adenocarcinomas; 11 were poorly differentiated, 12 were moderately-differentiated and 7 were well-differentiated. According to the Dukes stage classifications, 6 were classified as A, 10 as B and 14 as C; TNM classification were present in Table 1. No one received radiotherapy and/or chemotherapy preoperatively. Resected specimens from patients with CRC were immediately fixed in 10 % formalin and embedded in paraffin after curative surgery. Normal colorectal biopsies from 10 subjects (male/female ratio 5/5; mean age 40 years; 5 from rectum and 5 from colon) without evidence of pathological abnormality were retrieved from our endoscope biopsy bank were used as the controls. This work was approved and partially supported by Local Research and Ethical Committee of the Second Affiliated Hospital of Zhengzhou University.

Examination of CD34 Positive and HDC Positive Microvessel Density (MVD) with Immunohistochemistry (IHC)

Four μm paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol routinely, IHC with primary antibodies CD34 and HDC was performed with ABC kit according to our published procedures. The primary antibodies were incubated overnight 4 °C respectively with (1) mouse anti-CD34 monoclonal antibody (working dilution 1:50, purchased from DAKO, Carpinteria, CA, USA) (2) rabbit anti-HDC polyclonal antibody (working dilution 1:2000, purchased from Eurodiagnostic, Malmo, Sweden). 3-Amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) was used as chromogen and slides were counterstained with Mayer's hematoxylin. The negative control slides for IHCs were performed routinely: primary antibodies were substituted with the isotype-matched control antibodies. Sections from corpus mucosa were used as positive controls. The stained slides were observed under light microscope.

Double IHCs to Identify HDC Expressed in CD34 Positive Microvessels and Proliferative Activity in HDC Positive Microvessels in the Tumor Microenvironment

CD34 combined with morphological features has been commonly used as one of the general markers for the tumor associated microvessels. In this study, to examine whether HDC positive microvessels were the tumor associated microvessels, double IHC with HDC/CD34 was performed with EnVision Doublestain System kit (DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions and our published method [24]. 3-amino-9-ethylcarbazole substrate kit for peroxidase (AEC, Vector Laboratories, Burlingame, CA, USA) was used for the visualization of CD34 immunoreactivity and Vector blue alkaline phosphatase substrate kit III (Vector blue, Vector Laboratories, Burlingame, CA, USA) for HDC immunoreactivity respectively. Additionally, to examine the proliferative capacity of HDC positive microvesels, double IHC with Ki67/HDC antibodies were performed as well. AEC kit was used for the visualization of Ki67 immunoreactivity and vector blue kit for Ki67 immunoreactivity

Table 1 Clinicopathological information of CRC patients

	Male/female	Position		TNM			Lymph node involvement	
		Colon	Rectum	I	II	III	Positive	Negative
CRC	17/13	11	19	5	10	15	9	21

respectively. The sections were slightly counterstained with Mayer's hematoxylin.

Morphometric Evaluation

All the stained slides were examined under light microscope. CD34 labeled total microvessel density (MVD) and HDC positive MVD in the CRC tumor stroma between cancerous epithelium and the controls were counted in 3 independent high-power fields (×200) with abundant distribution by the method described by Weidner et al [25] and our previous publication [24]. The average values were used for statistical analysis.

Statistics

The CD34 positive MVD and HDC positive MVD in the tumor stroma of CRCs expressed mean ± SEM (mean of standard error) and the variability in associating with clinical histological parameters was evaluated with the Mann–Whitney test between two groups and the ANOVA Kruskal-Wallis test among three groups. Values of $P<0.01$ or $P<0.05$ were considered statistically significant.

Results

HDC Positive Microvessel Density (HDC-MVD) was Increased in CRC Tumor Stroma

CD34 combined with morphological features has been commonly used as a commonest used marker for the identification of tumor associated microvessels. As expected, our CD34 IHC results showed that CD34 positive microvessels were greatly increased in the CRC tumor stroma (Fig. 1b) as compared with the normal controls (Fig. 1a). Our CD34-MVD results confirmed that CD34-MVD in CRC tumor stroma was higher than that in the control lamina propria (see Fig. 2b, $P<0.01$ as compared with the controls, the Mann–Whitney test).

In the investigation of histamine biosynthesis, HDC has been accepted as a specific marker [6]. It has been previously reported that the expression of HDC immunoreactivity is observed in both the epithelial cells and mast cells in the lamina propria [6, 26]. In this study we could also able to find that HDC immunoreactivity in colonic epithelial cells (see arrow heads in Fig. 1c) and cancer cells (see arrow heads in Fig. 1d). However, since the frequency of HDC immunoreactivity in CRC cancer cells has been well studied and reported a 90 % positive rate in CRCs by Boer and colleagues [14], our focus in this study was the expression of HDC in tumor associated microvessels. In this study, a specific HDC antibody combined with the morphology was used to label the histamine expressed in the microvessels. The results revealed that HDC positive microvessels could be observed in both the normal lamina propria and tumor stroma. In the normal lamina propria, HDC positive microvessels were found in the lamina propria between colorectal crypts (Fig. 1c); quantitative data showed that HDC positive microvessel density (HDC-MVD) was at a low level (Fig. 2a). In CRC sections, the HDC positive microvessels were observed (Fig. 1d), the positive sections showed that the whole microvessel wall was labeled with HDC immunoreactivity in the tumor stroma (Fig. 1d) and the HDC-MVD was higher than that in the controls (see Fig. 2b; $P<0.01$ as compared with the controls, the Mann–Whitney test). The ratio of HDC-MVD/CD34-MVD was changed from the controls to CRCs. In the controls, the rate of HDC-MVD/CD34-MVD was ~73 %, it was significantly increased in CRCs with a rate ~79 % (CRC vs. Control: 79.10±1.45 vs. 73.03±1.90, $P<0.05$, the Mann–Whitney test).

HDC Positive Microvessles Were Mostly CD34 Positive Microvessels in the CRC Tumor Stroma Showing Proliferative Activity

To identify the HDC positive microvessels in the tumor storma, double IHC with CD34/HDC were performed (see Fig. 1e & f). The results showed that most CD34 positive microvessels were also positive for HDC in both the controls (Fig. 1e) and CRCs (Fig. 1f). Further double IHC with Ki67/HDC antibodies revealed that the proliferative activity labeled by Ki67 immunoreactivity was rarely found in HDC positive microvessels in the lamina propria of controls (Fig. 1g); however it was frequently observed in HDC positive microvessels in the tumor stroma of CRCs (Fig. 1h).

The Density of HDC-MVD was Associated with the TNM Stage and Lymph Node Involvement in Patients with CRCs

When the analysis of HDC positive MVD with pathological parameter were performed, it revealed that the density of HDC-MVD in CRC patients with high TNM stages (III + IV) was significantly increased as compared with those with low stage (I or II) (see Fig. 3a), which was confirmed when analysis of HDC-MVD in different Dukes stage groups (Dukes A/B/C: 27.10±2.80 vs. 27.216±4.02 vs. 53.18±3.33, $P<0.01$, one-way ANOV, the Kruskal-Wallis test). However, the density of HDC-MVD was significantly higher in CRC patients with lymph node involvement than those without (see Fig. 3b, $P<0.05$). It was hard to find a correlation between cancer differentiation degree and the HDC-MVD (data not shown).

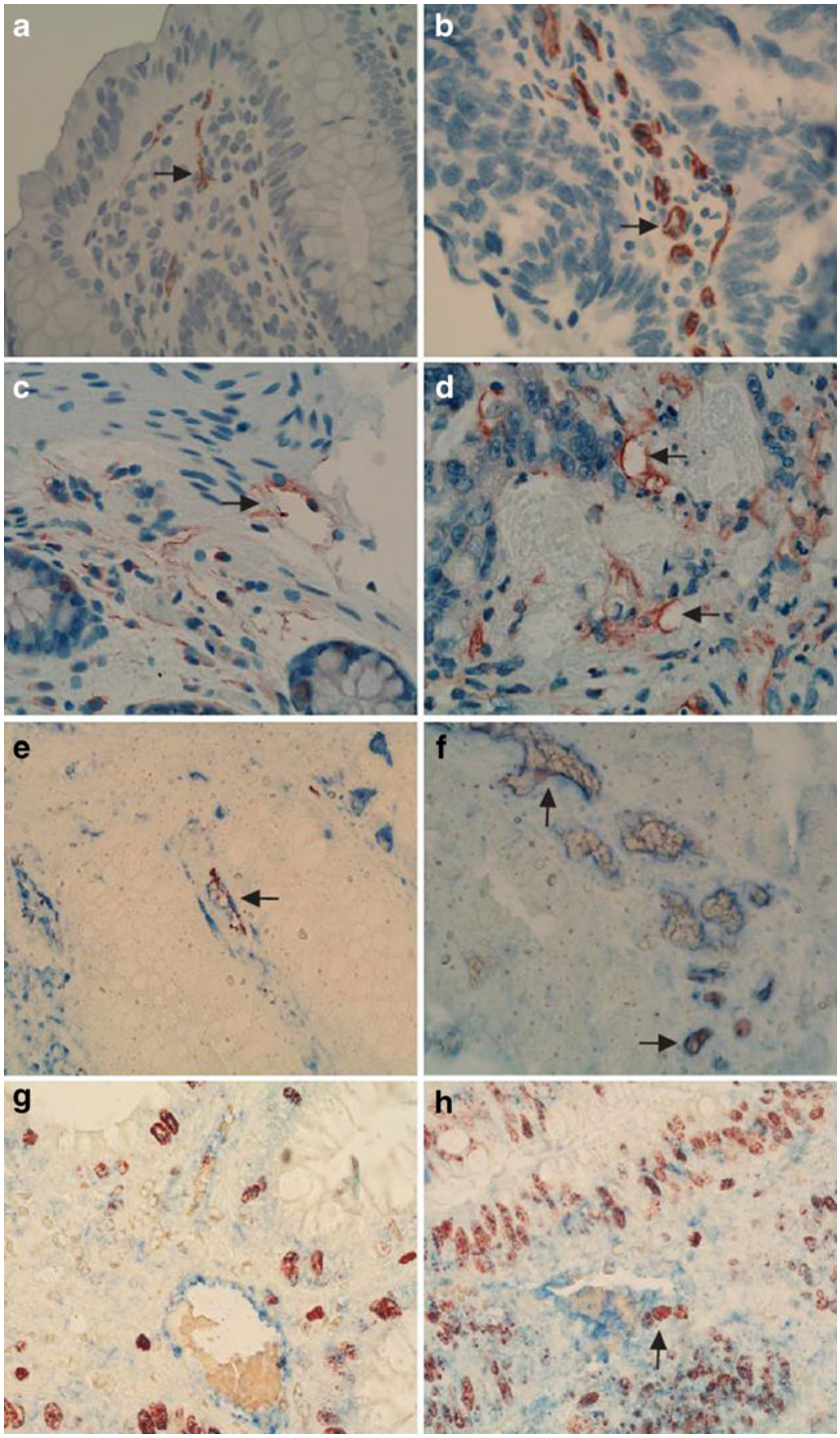

Fig. 1 Immunohistochemical examinations of CD34 positive and HDC positive tumor associated microvessls, and the co-localization of CD34/HDC and Ki67/HDC in the CRC tumor stroma. Low density of CD34 positive microvessels were observed in the lamina propria of normal controls (*arrow* pointed in **a**), but the density of CD34 positive tumor associated microvessel was greatly increased in the CRC tumor stroma (*arrow* pointed in **b**) compared with the normal controls. A very similar expression pattern of HDC positive microvessel was also observed in both the normal lamina propria (*arrow* pointed in **c**) and the CRC tumor stroma (*arrow* pointed in **d**). Double IHC with CD34/HDC antibodies revealed a co-localization of HDC (HDC visualized by vector blue, *blue colour*) with CD34 (CD34 visualized by AEC, *red colour*) in both the control (**e**) and the CRC (**f**), and confirmed the HDC positive microvessels were CD34 positive microvessels. Double IHC with Ki67/HDC antibodies further demonstrated that the HDC positive microvessels in the normal controls were with a low proliferative capacity (Ki67 was visualized by AEC, *red colour*; HDC was visualized by vector blue, *blue colour*. See **g**), but they were with a higher proliferative capacity in the CRC tumor stroma (see **h**). (**a**–**d**: single IHCs and counterstained with hematoxylin, **e**–**h**: double IHCs and counterstaining was not applied. All the magnifications are 400×)

Discussion

In this study, we were able to demonstrate the expression of histamine in the microvessels through their HDC immunoreactivity in both the normal lamina propria and CRC tumor stroma.

One of the critical steps for cancer growth and metastasis is the enhanced formation of new blood vessels (angiogenesis) to establish a supportive tumor microenvironment. Angiogenesis is modulated by many factors. Histamine produced by the mast cells has been shown to be an important stimulator for tumor associated angiogenesis [27]. However, histamine has also been found in blood vessels in many species including human [17–19] and the importance of non-mast cell histamine in modulating angiogenesis has been addressed

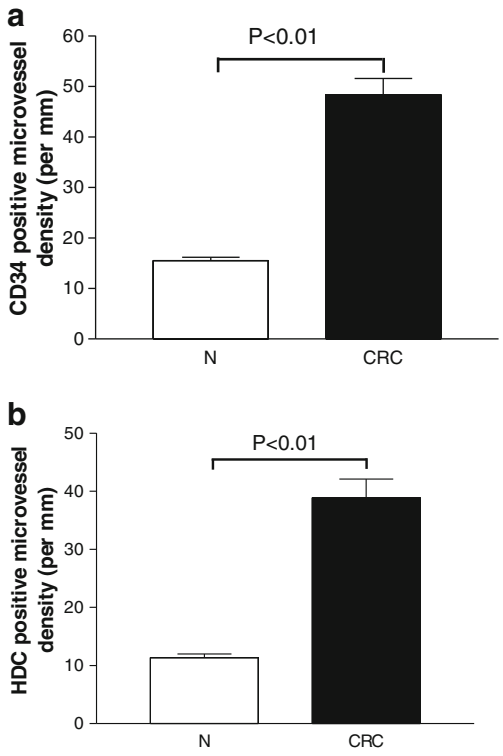

Fig. 2 Graphic analysis showed remarkably increased CD34 positive microvessel density (CD34-MVD) (**a**) and HDC-MVD (**b**) in the tumor stroma of CRCs as compared with the controls (both *P* values obtained from the Mann–Whitney test)

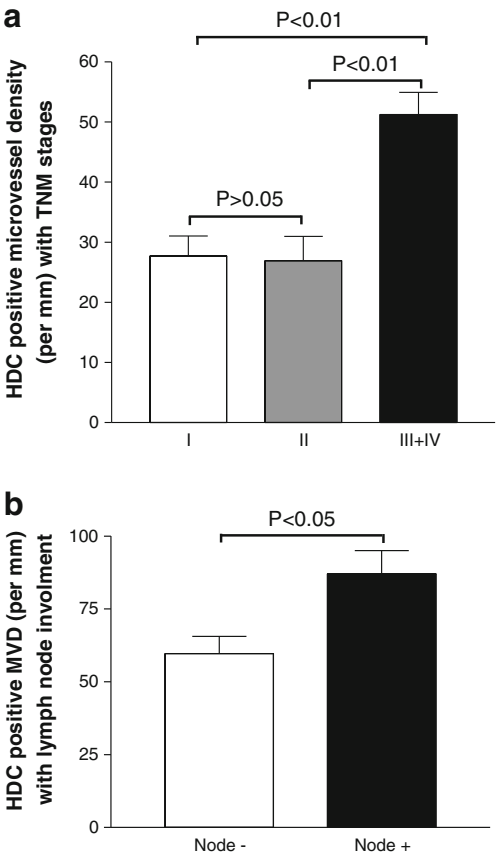

Fig. 3 The increase of HDC positive microvessel density (HDC-MVD) in the CRC tumor stroma is associated with TNM stages and the lymph node involvement. When the HDC-MVD in CRCs with different TNM stage groups was evaluated, it showed that CRC patients with TNM III + IV stage had a higher HDC-MVD than those with TNM I or II stages (see **a**; *P* values obtained from the Mann–Whitney test), and it was also significantly higher in CRC patients with lymph node involvement than those without (see **b**, $P<0.05$, obtained from the Mann–Whitney test)

in mice [28]. We have recently demonstrated an increased expression of HDC in the tumor associated microvessels in human esophageal squamous cell carcinomas, which suggests a pathological significance of non-mast cell histamine in regulating tumor angiogenesis. Our current study performed in human CRCs confirmed that the expression of HDC in the tumor associated microvessels in human cancers is a common phenomenon, because the HDC positive microvessels accounted for ~79 % CD34 positive microvessels in the CRC tumor stroma. Our data also showed the increased proliferative capacity of the HDC positive microvessels, which suggests an active angiogenesis in the CRC tumor microenvironment. When the HDC-MVD was analyzed in CRCs with different TNM stages, a significant increased tendency was shown in CRCs with high TNM stages. Particularly, the HDC-MVD was significantly higher in CRC patients with node involvement than those without; such result may indicate a close connection between angiogenesis and tumor invasion in CRCs.

It has been shown that the stimulation of histamine on angiogenesis is through H2 receptors [29, 30]; administration of H2 receptor blocking agents has been considered as a strategy to inhibit the growth of CRC. In fact, it has been demonstrated that administration of H_2 receptor antagonists in human cancers had a potential inhibition effect on angiogenesis and improved survival in cancers derived from digestive tract [8, 10, 13, 31]. Since the expression of histamine has been demonstrated in the endothelial cell of microvessels, it is reasonable to postulate that the inhibiting effect of H2 receptor antagonist on angiogenesis is partially through the blocking effect on non-mast histamine.

In summary, our study demonstrated the increase of histamine expressing microvessels in the CRC tumor stroma, which may have a pathophysiological significance. It can serve as an additional non-mast histamine source and participate in the regulation of angiogenesis in CRCs.

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