

Original Article

Clinical Significance of Tumor Cell-lined Vessel in Colorectal Cancer: Correlation with Tissue-Infiltrated Macrophage and Hypoxia Inducible Factor-1alpha Expression

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Key Words

Colorectal cancer;
Hypoxia;
Macrophage;
Tumor cell-lined vessel

Purpose. Recent studies had suggested tumor cell-lined vessel may involve in cancer progression. Our aim was to study the role of tumor cell-lined vessel (TCLV) in colorectal cancer (CRC) and the association of TCLV with tissue-infiltrated macrophage (TIM) and hypoxia inducible factor-1 alpha (HIF-1 α) expression.

Methods. Immunohistochemical staining in 58 patients with CRC were evaluated. Double-stain of CD34 and pan-cytokeratin was used to identify TCLV which are lack of endothelium lining. Hypoxia was evaluated by examining HIF-1 α expression in tumor cells. Monoclonal antibodies of CD68 were used to investigate the TIM. Their correlations with clinicopathologic features were analyzed with chi-square test.

Results. TCLVs were identified in 17/58 (29.3%) of tumor samples. Presence of TCLV correlated significantly with T stage (P = 0.001) and histologic differentiation (P = 0.038). In cancer tissues, the mean TIM (cells/mm²) was 143 \pm 49. There was significant correlation between TIM and T stage (P = 0.016), lymph node state (P = 0.034), and tumor metastasis (P = 0.002). Significant association between HIF-1 α expression and presence of TCVL was also noted (P = 0.004).

Conclusion. This study showed that cancer microenvironment could change tumor cells to a more aggressive behavior. The presence of TCVL, TIM, and HIF-1 α expression are potentially useful markers of tumor progression in CRC.

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Angiogenesis is essential for the development of human solid carcinomas. Many studies in various human tumors had demonstrated that tumor angiogenesis is a parameter that associated with carcinogenesis and tumor progression. However, controversial results of tumor angiogenesis had been reported in colorectal cancers (CRCs). There are several

studies that do not demonstrate the relationship between tumor angiogenesis and tumor aggressiveness or progression.¹⁻⁶ Recently, a non-angiogenesis, vasculogenic mimicary or tumor cell-lined vessels (TCLV), had been proposed as an alternative way for tumor circulation.^{7,8} By the formation of TCLV, some cancer cells participated in the formation of ves-

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sel-like structure that provide blood supply for tumor cells. While TCLV were observed in many types of cancers, its role in CRC and the mechanisms of TCLV formation are still unknown.

The microenvironment within tumors is important for tumor progression; in which circulation is compromised because of structurally disorganized blood vessels.⁹ In this sense, the tumor cell seems to adopt an ischemic (hypoxic) phenotype. The transcriptional response to hypoxia relies on multiprotein complexes to regulate several transcription factors, the most well studied of which is hypoxia inducible factor-1 α (HIF-1 α), which then enhances the expression of hypoxia-responsive gene and, thus, allows improved cell survival in conditions of limited oxygen availability. Thus HIF-1 α are very frequently over-expressed in human tumors. Although HIF-1 α expression were studied in many cancers, its effect and association with TCLV is not clear.

In addition, tumor microenvironment are composed of an array of cell types, including not only neoplastic cells but also fibroblasts, endothelial cells and inflammatory cells; in which macrophage orchestrated the major component. Generally, macrophages in cancer tissue are thought to exert antitumoural effects through immune surveillance. Recent study, however, indicates that inflammation in tumor tissues might stimulate growth and invasion.¹⁰ A role also has been suggested for infiltrated macrophages in the progression to malignancy in CRC. For example, a chronic inflammatory disease of the colon, ulcerative colitis, increases the cancer risk in human.^{11,12} Furthermore, patient with CRC accompanied by ulcerative colitis have a high incidence of invasive cancer.^{13,14} However, it's relation with presence of TCLV and HIF-1 α expression still unknown.

In the present study, we investigate the TCLV, HIF-1 α expression, and macrophage infiltration in CRC and their association with clinicopathologic features.

Materials and Methods

Patient and specimens

Surgical specimens were obtained from 58 pa-

tients with the diagnosis of CRC from the pathology archived at our institute between May 2006 and December 1994. Patient age ranged from 27-92 years. For further analysis, Tumor stage was subdivided into two groups according to their tumor growth (T1 + T2 vs T3 + T4), lymph node involvement (N0 vs N1 + N2), and distant metastasis (M0 vs M1).

From each tissue block, a series of 4 μ m sections were cut. A 4 μ m flanking section was stained with hematoxylin and eosin for pathological evaluation. A series of sections was used for immunohistochemistry.

Immunostaining procedure and quantification of HIF-1 α expression

Immunoperoxidase procedure was realized using polyclonal rabbit (1:400 dilution) antihuman HIF-1 α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactivity of HIF-1 α in CRC tissue was determined by assessing semiquantitatively the percentage of decorated tumour cells by experienced pathologists using a Zeiss Axioplan microscope as reported previously¹⁵ on the whole tissue section by examining all optical fields. Staining intensity was not incorporated in the scoring method because it was more or less constant.

Identification of TCLV

TCLV were identified as our previous report.¹⁴ Briefly, the sections were doubly stained to highlight tumor cells and endothelial cells, and a condensing lens was used to highlight RBCs. TCLV were defined as vessels with CK-positive tumor cells in obvious contact with the lumen, as indicated by the absence of detectable overlying CD34 immunoreactivity. Furthermore, to distinguish microhemorrhage from tumor-lined vessels, sections or area with severe hemorrhage were excluded in this study.

Macrophage index

Tissue-infiltrated macrophage (TIM) count was quantitatively determined as described previously.¹⁶ Briefly, macrophages were counted by eye in the five most confluent microscopic fields ("hot spots"). The

mean count was determined from the highest three counts of the five and considered as the TIM count. For cut-off point analysis the medians were used to categorize tumor into high and low groups.

Statistical analysis

Results from immunohistochemistry for determination of TCLV, HIF-1 α expression and TIM were examined in relationship to clinicopathologic features by the χ^2 test method for a table comparison. Statistical significance was accepted for $P < 0.05$.

Results

TCLV in CRC

In an attempt to identify TCLV, tissue sections were double labeled for cytokeratin associated with epithelium and CD34 expressed by endothelium. As shown in Fig. 1, in cancer tissues, some tumor cells composed a vessel-like structure with circulated RBC inside. In comparing to the nearby endothelium lined

vessels, TCLV were entirely lined by brown-colored tumor cells, or in part, by CD34-positive and CK-positive cells. They were identified in 17/58 (29.3%) cancer specimen and correlation with patient's features was showed in Table 1. The presence of TCLV significantly correlated with T stage and histologic differentiation ($P = 0.001$ and $P = 0.038$, respectively) (Table 1).

HIF-1 α expression in CRC

In normal colon epithelium, faint immunoactivity

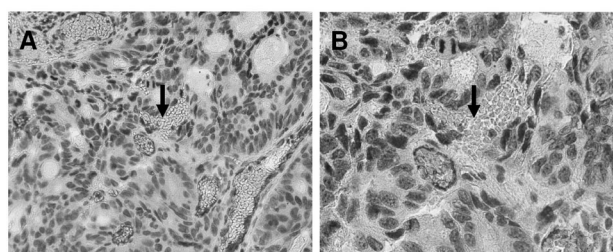


Fig. 1. Tumor cell-lined vessels in CRC. In CRC cancer tissues, some tumor cells composed a vessel-like structure with circulated RBC inside (arrow) (original magnification X 100 in A and X 200 in B).

Table 1. Correlation of HIF-1 α expression and TIM with clinicopathologic features of patients with CRC

Clinicopathologic features	TCLV		P value	HIF-1 α expression		P value	TIM count		P value
	Absent (n = 41)	Present (n = 17)		Negative (n = 34)	Positive (n = 24)		Low (n = 30)	High (n = 28)	
Gender									
Male (n = 23) ¹	19	4	0.092	13	10	0.502	13	10	0.373
Female (n = 35)	22	13		21	14		17	18	
T stage									
T1 + T2 (n = 26)	24	2	0.001	24	2	< 0.0001	18	8	0.016
T3 + T4 (n = 32)	17	15		10	22		12	20	
N stage									
N0 (n = 31)	20	11	0.207	19	12	0.430	20	11	0.034
N1 + N2 (n = 27)	21	6		15	12		10	17	
M stage									
M0 (n = 47)	36	11	0.051	33	14	< 0.0001	29	18	0.002
M1 (n = 11)	5	6		1	10		1	10	
Differentiation									
Well (n = 16)	15	1	0.038	9	7	0.973	6	10	0.277
Moderate (n = 20)	11	9		12	8		10	10	
Poor (n = 22)	15	7		13	9		14	8	

The expression of HIF-1 α is classified as positive if nuclear staining is greater than 11% of cell and/or with strong cytoplasmic staining and negative if nuclear staining is less than 10% of cells and/or with weak cytoplasmic staining. TIM, tissue-infiltrated macrophage. TCLV, tumor cell-lined vessel. LN, lymph node. The TIMs are classified as low if count value is ≤ 140 cells/mm² and high if counting value > 140 cells/mm².

of HIF-1 α expression was observed mainly in superficial layer of colon mucosa (Fig. 2A). In cancer tissues, increasing intensity of HIF-1 α expression was found in most of CRC and frequently nuclear translocation of HIF-1 α expression were noted in addition to increased staining intensity (Fig. 2B). However, not all tumors stained positive with this antibody. Within positive tumors, the extent, intensity, intracellular location, and distribution of staining seen were heterogeneous. The staining intensity varied within a given section and between sections. HIF-1 α expression was semiquantitatively evaluated (percentage of decorated tumour cells). HIF-1 α positive staining was observed in 24/58 (41%) of cases as comparing to 34/58 (59%) of negative cases. Correlation of HIF-1 α expression with clinicopathologic features of colonrectal patient was listed in Table 1. Significant correlation was noted between HIF-1 α expression and T stage ($P < 0.0001$) and distant metastasis ($P < 0.0001$). Moreover, presence of TCLV also closely associated with HIF-1 α expression ($P = 0.004$, Table 2).

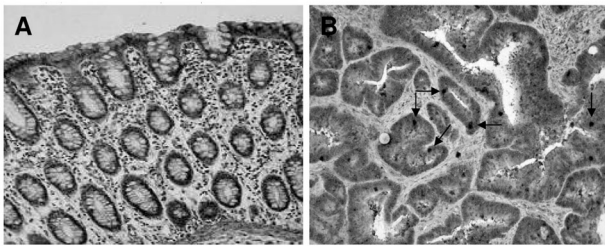


Fig. 2. HIF-1 α expression in normal (A) and cancer tissues (B) of CRC. A. Faint staining is found in some cell close to the lumen. B. Nuclear accumulation of HIF-1 α in tumor cells of CRC were frequently noted (arrow). (original magnification X 100 in A and B)

TIM in CRC

The immunohistochemical staining showed that the cytoplasm of TIM was stained brown by anti-CD68 antibodies (Fig. 3). CD68+ cells (macrophages) were observed in all of tumors that were stained. The TIM count varied from 40 to 242 counts/mm² (143.0 ± 49.1 counts/mm²). We used a median value of 140 (infiltrating macrophage density per 200X field) as the cutoff to separate tumors into those with high ($n > 140$) and low ($n \leq 140$) infiltrating macrophage counts. Association of TIM with patient's features was showed in Table 1. TIM count was significantly associated with T stage ($P = 0.034$), lymph node involvement ($P = 0.016$) and distant metastasis ($P = 0.002$). We also compared TIM count between patients with presence v.s absence of TCLV and positive v.s negative HIF-1 α expression. However, no significant correlation was noted (Table 2).

Discussion

Despite the association of tumor angiogenesis and

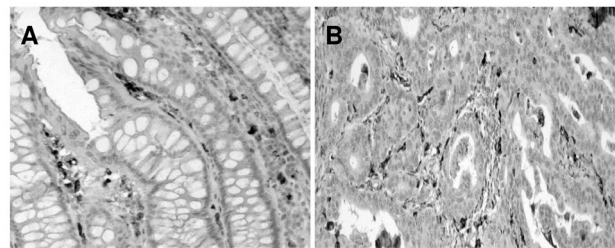


Fig. 3. Tissue-infiltrated macrophages (TIM) in normal (A) and cancer tissues (B) of CRC. Few TIM was noted in the stroma of colon mucosa (A) and increased TIM in CRC tissue (B). (original magnification X 200 in A and B)

Table 2. Correlation among TCLV, HIF-1 α expression, and TIM

	TCLV		P value	HIF-1 α		P value
	Absent (n = 41)	Present (n = 17)		Negative (n = 34)	Positive (n = 24)	
HIF-1 α						
Negative (n = 34)	29	5	0.004			
Positive (n = 24)	12	12				
TIM						
Low (n = 30)	23	7	0.228	21	9	0.060
High (n = 28)	18	10		13	15	

malignancy had been reported in various cancers, controversial results were reported in CRCs. This led us to hypothesize that the other alternative ways, for example TCLV, for tumor growth and progression is occurred in this type of cancer. We showed using dual immunostaining that TCLV do present in CRC. We further demonstrated that, in CRC specimens, the presence of TCLV closely associated with HIF-1 α expression and tumor progression. These results implicated that the interaction between cancer cell and micro-environment such as hypoxia and macrophage infiltration might promote tumor invasion and progression.

Regarding TCLV, recent studies indicated that some tumors may be vascularised by using existing vessel, a process described as vascular cooption, or even by forming TCLV through a non-endothelial cell process. Previous studies had showed tumor cell-lined vessels in certain types of tumor.^{7,14,17,18} These TCLV had been described as vasculogenic mimicry.⁷ It was first described in aggressive melanoma with a novel process by which tumors develop a highly patterned microcirculation that is independent of angiogenesis. By this mechanism, aggressive tumor cells acquire some genotype and phenotypic characteristics of endothelial cells to form vascular channel that facilitate cancer cells into broad contact with the blood stream, which predisposes the tumor to metastasis and contributes to the poor outcome. Although challenge had been proposed that TCLV could be an artifact caused by extravasation of trauma, however, recent studies have provided evidence that TCLV connected to endothelial-lined vessels and circulation between these vessels do excite.^{17,19,20} Therefore, the evidence of non-angiogenetic mechanisms existing in cancer is continuing to grow. Our study supporting the notion that non-angiogenesis pathways are involved in the progression of CRC and presence of TCLV is an indicator of cancer aggressiveness.

Present study also showed a close association between presence of TCLV and HIF-1 α expression. The increased levels of HIF-1 α were found frequently around areas of necrosis, a consequence event of tissue hypoxia. It had been reported that presence of hypoxic area is a typical feature of malignant human tumors²¹ and the presence of these hypoxic areas often correlates with prognosis in cancer, with the presence

of widespread hypoxia in tumors being associated with reduced survival following surgery and radio- or chemotherapy.²² Molecular response to hypoxia is mediated by the stabilization of HIF-1 α . Once stabilized, HIF-1 α translocates to nuclear and dimerizes with the constitutive HIF-1 β to form HIF-1, which act as a master regulator in response to changes in oxygen tension. HIF-1 transcription factor that stimulates variety of genes response for angiogenesis, changes energy metabolism and iron homeostasis, and promotes cell survival in this detrimental microenvironment.²³ Upon HIF-1 activated genes, some may involve in driving cancer cell to express genes with endothelium phenotype and leading to form TCLV. Hence, hypoxia activation of HIF-1 α might involve in driving the formation of TCLV.

Although the association of TIM and HIF-1 α didn't demonstrate in our study, it had been implicated that hypoxia or HIF-1 α overexpression is the major cause of TIM accumulation in tumor tissue. For example, HIF-1 knockout mice exhibit a markedly diminished inflammatory response in inflammatory lesions, which, like tumors, are often oxygen-depleted.²⁴ Hypoxia had been shown to stimulate macrophage adhesion to endothelial cells, in part, by HIF-1 induction of CD18, the beta subunit of all beta2 integrins.²⁵ Recent studies also showed high numbers of macrophage accumulate in hypoxic/necrotic areas of tumors due to the hypoxic release of macrophage chemoattractants.^{26,27} In addition, a significant association between HIF-1 α expression and macrophage accumulation in cancer tissues was reported in other malignancy.²⁸ The discrepancies between these studies and our results may be due to a number of factors related to sample size. In our study, only 58 cases was analyzed that represented a relatively small group. It is possible that if a large sample size is chosen, the correlation between HIF-1 α and TIM might be established. Thus, further investigation to extend the study sample and to clarify the effect of hypoxia on TIM behavior is necessary.

In conclusion, HIF-1 α expression and TIMs may change cancer cell behavior to be a more invasive and aggressive phenotype. Additionally, TCLV, TIM, and HIF-1 α expression could be useful as progression indicators for patients with CRC.

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原 著

腫瘤細胞形成血管在大腸直腸癌的臨床意義與巨噬細胞及缺氧因子的關係

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目的 最近研究指出某些腫瘤細胞形成血管與癌症進展有關。本實驗主要目的在於研究腫瘤細胞形成血管在大腸直腸癌的臨床意義與巨噬細胞及缺氧因子的關係。

方法 共 58 位大腸直腸癌的病理手術後標本，我們用免疫化學染色 CD34 和 pan-CK 來染腫瘤細胞形成血管，細胞缺氧用缺氧因子 HIF-1 α 來表現，免疫化學染色 CD68 來染巨噬細胞，然則用統計學的方法來分析彼此的關係。

結果 腫瘤細胞形成血管在 29.3% 的腫瘤標本中可被發現。腫瘤細胞形成血管與腫瘤 T stage 有關 (P = 0.001)。在癌症組織中，巨噬細胞與腫瘤 T stage (P = 0.016)，淋巴腺轉移 (P = 0.034)，及遠端轉移 (P = 0.002) 等有關。另外腫瘤細胞形成血管與缺氧因子之間亦有相關性 (P = 0.004)。

結論 本實驗發現癌症細胞內部的細微環境本身可以改變讓癌細胞更具侵略性。而對於大腸直腸癌的預後來說，腫瘤細胞形成血管、巨噬細胞及缺氧因子可以是有用的指標。

關鍵詞 大腸直腸癌、腫瘤細胞形成血管、巨噬細胞及缺氧因子。