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World J Gastroenterol 2004;10(19):2800-2804
World Journal of Gastroenterology
 ISSN 1007-9327

• COLORECTAL CANCER •

Lymphatic metastasis and nm23H$_1$ genetic instability in Chinese colon cancer patients

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Received: 2004-02-02 **Accepted:** 2004-02-21

Abstract

AIM: To investigate the pathogenic mechanism of colon cancer at the molecular level and to elucidate the relationship between intercellular adhesion molecule-1 (ICAM-1) and nm23H$_1$ genes and Chinese patients with colon cancer.

METHODS: DNA was extracted from paraffin-embedded materials. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) was used to analyze MSI and LOH. Expression of ICAM-1 was detected by Envision immunohistochemistry. Experimental results were analyzed with Leica-Qwin computer imaging techniques and SPSS software of statistics.

RESULTS: ICAM-1 expression of lymphatic endothelium was negative in normal colon and positive in colon cancer respectively. The number of lymphatics positive for ICAM-1 was gradually increased with degree of cancer invasion (P<0.01). In the group with metastasis of colon cancer, the number of lymphatics positive for ICAM-1 in lymph nodes was more than that in the group with no metastasis (P<0.01). The frequency of MSI, LOH and nm23H$_1$ protein was 26.67%, 20.00% and 53.33% in colon cancer, respectively. In TNM staging, MSI (43.75%) and nm23H$_1$ protein (81.25%) in stages I+II were detected more easily than the corresponding indexes (MSI: 7.14%, P<0.05 and nm23H$_1$: 21.43%, P<0.01) in stages III+IV. By comparison, the frequency of LOH (35.71%) in stages III+IV was more than that of LOH (6.25%, P<0.05) in stages I+II. LOH exhibited a rising trend along with the Duke's staging. nm23H$_1$ protein in the group of tubular adenocarcinoma (60.00%) was higher expressed than that in the group of mucoid adenocarcinoma (20.00%) (P<0.01), and exhibited a rising trend with the differentiation degrees of tubular adenocarcinoma. nm23H$_1$ protein in MSI positive group was higher expressed (75%) than that in MSI negative group (45.45%, P<0.05).

CONCLUSION: The expression of ICAM-1 in lymphatic vessels is beneficial to the judgement of the invasion and metastasis ability of colon cancer and the anti-tumor immunity function, and shows an important clinical significance in predicting lymphatic metastasis of colon cancer. MSI and LOH may separately control the development of sporadic colon cancer with different pathways. LOH mostly arises in the late period of sporadic colon cancer and endows a high aggressive and poor prognostic phenotype. By compassion, MSI may be an early period molecule marker for sporadic colon cancer, enhanced expression of nm23H$_1$ protein can effectively inhibit colon cancer metastasis and improve prognosis of sporadic colon cancer patients.

Su ZH, Li JC. Lymphatic metastasis and nm23H$_1$ genetic instability in Chinese colon cancer patients. *World J Gastroenterol* 2004; 10(19): 2800-2804
http://www.wjgnet.com/1007-9327/10/2800.asp

INTRODUCTION

Colon cancer is one of the common malignant tumors. A series of investigations have revealed that the main reason why it gives rise to death of patients lies in its invasiveness and metastasis[1]. Therefore, preventing colon deterioration and decreasing the death rate of colon cancer is of great significance. So far, it has been known that many factors may have an influence on colon cancer, such as anti-oncogenes, adhesion molecular E-selectin and E-cadherin, vascular endothelial growth factor and matrix metal protein (MMP-2)[2-5]. Among these factors, anti-oncogene and adhesion molecules have become the "hot spots" in research of colon cancer[6-8].

Intercellular adhesion molecule-1 (ICAM-1), an important transmembrane glycoprotein, is negatively expressed on endothelial cells of lymphatic vessels in normal conditions[9, 10]. ICAM-1 could induce attachment of cells such as lymphocytes to endothelial cells of blood vessels, and traverse blood vessels, aggregate antigens and take part in immune and inflammatory reactions. When tumors appeared in the body, lymphatic vessels became the main pathway for tumor cells to be transferred as a result of their own permeability[11]. In the process, recognition, adhesion, morphologic change and penetration between cells were involved. Vasse *et al*.[12]. reported that breast cancer cells could over-express the specific ligands of ICAM-1 (lymphocyte function associated antigen 1, LFA-1). With the development of cancer, the expression of LFA-1 went to an ascending trend on cancer cells. All the results indicated that ICAM-1 might take part in the metastasis. How endothelial cells of lymphatic vessels express ICAM-1? Few reports are available about whether endothelial cells of lymphatics express ICAM-1 or not in cancer tissue and whether ICAM-1/LFA-1 take part in the process of cancer cells attached to endothelial cells of lymphatics and metastasis.

nm23H$_1$ is one of the main anti-oncogenes. A great number of experiments indicated that inactivation of these genes, that is, genetic instability, resulted in metastasis[13,14]. However, there were few researches of these genes on colon cancers[15]. In order to further investigate the pathogenic mechanism of colon, we examined the instability of D17S396 of nm23H$_1$ in unrelated patients with the single strand conformation polymorphism analysis of polymerase chain reaction products (PCR-SSCP).

MATERIALS AND METHODS

Case selection and extraction of DNA

Thirty-two specimens were obtained during 2000 to 2002. There were 21 males and 11 females, aged 27-77 years. Twenty-seven cases were patients with tubular adenocarcinoma, 5 cases were

patients with mucoid adenocarcinoma in histological types. A senior pathologist made the final diagnosis on the basis of histological examination. No patient received radioactive therapy, chemotherapy before operation. Fresh surgical tissue samples were fixed immediately in formaldehyde solution for 12-24 h and paraffin-embedded for PCR-SSCP and immunohistochemical assay.

DNA extraction

DNA was extracted according to the standard protocols.

PCR amplification

Designed primers were synthesized by Shanghai Shengyou Biology Company. The primer sequences were (sense) 5'- TTGA CCGGGGTAGAGAACTC -3', (antisense) 5'- TCTCAGTACTT CCCGTGACC -3'. PCR mixture contained 200 ng of template-DNA and PCR reaction buffer containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.4), 1.5 mmol/L $MgCl_2$, 0.5 μmol/L each of two fragment-specific primers, 100 μmol/L each of dATP, dGTP, dTTP and dCTP, and 2 units of Taq DNA polymerase (Shanghai Shengyou Biology Company) for a reaction volume of 50 μL. The conditions for all PCR amplifications were at 94 ℃ for 5 min for pre-denaturation, at 94 ℃ for 45 s, at 62 ℃ for 45 s and at 72 ℃ for 45 s. Amplification was carried out for 35 cycles with a final extension for 10 min at 72 ℃. The amplified fragments were run in 1% agarose gel.

SSCP analysis

SSCP analysis of fragments was performed on a mini electrophoresis Unit (Bio-Rad Company, USA). Ten microlitre of the PCR product was diluted with 10 μL of sample buffer containing 90% formamide, 0.05% bromphenol blue dye and 0.05% xylene cyanol. The samples were heated at 100 ℃ for 8 min, transferred into an ice-cold water bath for 3 min, and analysed by 8% PAGE in 45 mmol/L-Tris-borate (pH8.0)/1 mmol/L-EDTA (TBE) buffer under 13 v/cm at 10 ℃.

DNA silver staining

Gels were stained with silver as follows. Gels were firstly fixed in 100 mL/L alcohol for 10 min and then oxidized in 100 mL/L nitric acid for 3 min. After washed for 1 min with double distilled water, they were stained in 2 g/L silver nitric acid for 5 min and washed for 1 min with double distilled water. Gels showed appropriate color in 15 g/L anhydrous sodium carbonate and 4 mL/L formalin and then the reaction was terminated by 7.5 mL/L glacial acetic acid. Finally they were washed with double distilled water.

Immunohistochemical assay

Immunohistochemical study was performed using Envision method. Briefly, 5 μm thick sections of the tissue were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. After three times of rinsing with 0.01 mol/L phosphate-buffered saline (PBS) (pH = 7.4), the slides were incubated with 10% normal goat serum at room temperature for 10 min to block the nonspecific reaction, and incubated for two hours with anti-ICAM-1 antibody. After rinsed in PBS for five min, they were incubated with Envision complex for two hours at room temperature, and stained with DAB after washed in PBS.

Statistical analyses

The experimental results were expressed as mean±SD. The correlation was analyzed with SPSS 8.0 software. *P* value less than 0.05 was regarded as statistically significant.

RESULTS

Expression of ICAM-1 in lymphatic endothelial cells

In the submucosa of normal colon, there existed a few lymphatic vessels with large, irregular cavities and thin walls. Simple squamous epithelia lined on them had no expression of ICAM-1 (Figure 1A). But in the submucosa and peripheral area of colon cancer, the number of lymphatic vessels was increased. Significant differences existed between them ($P<0.01$) (Table1). The expression of lymphatic endothelial cell was positive for ICAM-1 in colon cancer (Figure 1B). With the degree of cancer invasion, lymphatic vessels positive for ICAM-1 showed an increasing trend ($P<0.01$) (Table 2). When cancer metastasis appeared in the peripheral lymph node, ICAM-1 was strongly expressed in endothelial cells of their peripheral lymphatic vessels, and the number of lymphatic vessels positive for ICAM-1 was to the highest (Table 2).

Table 1 Comparison of ICAM-1 expression between colon cancer and normal colon (mean±SD)

Group	Cases	ICAM-1/15HPF
Normal colon	5	11.001±1.58
Colon cancer	32	26.131±9.19[b]

[a]$P<0.05$, [b]$P<0.01$, *vs* normal colon group.

Genetic instability at D17S396 of nm23H1

Microsatellite fragments of D17S396 were amplified. The positive rate of D17S396 MSI (Figures 2A, B), LOH (Figure 2C) and nm23H$_1$ protein (Figure 2D) was 26.67%, 20.00% and 53.33% respectively in 30 cases of colon cancer (Table 3).

MSI and LOH were independent of the histological type of colon cancer, the degree of differentiation and Duke's stage were related to the clinical TNM stage. In TNM staging, the frequency of MSI (43.75%) in stages I+II was more than that in stages III+IV (7.14%, $P<0.05$). In contrast, LOH (35.71%) in stages III+IV was detected more easily than that (6.25%, $P<0.05$) in stages I+II. In addition, LOH exhibited an ascending trend with the Duke's stage ($P<0.01$).

Expression of nm23H$_1$ protein

The positive rate of nm23H$_1$ protein was related with the histological type of colon cancer, differentiation degree and

Table 2 ICAM-1 expression in colon cancer (mean±SD)

Dukes stage	Cases	ICAM-1		ICAM-1/15HPF	*P* Value
		Low-expression	High-expression		
A	5	3	2	19.671±5.59	
B	15	6	9	23.571±9.65	
C	10	3	7	25.591±8.07	<0.01
D	2	0	2	35.681±2.51	
Metastasis	12	3	9	28.091±8.33	
No metastasis	20	11	9	22.621±8.99	

Table 3 Relationship between clinical pathological parameter and nm23H$_1$ genetic instability in colon cancer (mean±SD)

	Cases	MSI(%)	LOH(%)	nm23(%)	nm23 expression
Histological types	30	8 (26.67)	6 (20.00)	16 (53.33)	40.21±3.29
Tubular adenocarcinoma	25	7 (28.00)	5 (20.00)	15 (60.00)	40.76±2.74
High differentiation	8	2 (25.00)	1 (12.50)	8 (100.00)	41.49±2.01
Intermediate differentiation	13	5 (38.46)	2 (15.38)	6 (46.15)	40.41±1.98
Poor differentiation	4	0 (0.00)	2 (50.00)	1 (25.00)[d]	40.18±2.17
Mucoid adenocarcinoma	5	1 (20.00)	1 (20.00)	1 (20.00)[b]	39.53±2.61
TNM stage					
Stage I+II	16	7 (43.75)	1 (6.25)	13 (81.25)	42.42±1.08
Stage III+IV	14	1 (7.14)[e]	5 (35.71)[e]	3 (21.43)[f]	39.49±2.57
Dukes stage					
A	5	2 (40.00)	0 (0.00)	4 (80.00)	41.32±2.18
B	15	5 (33.33)	3 (20.00)	8 (53.33)	40.69±2.11
C	8	1 (12.50)	1 (12.50)	4 (50.00)	42.32±1.66
D	2	0 (0.00)	2 (100.0)[h]	0 (0.00)	39.24±2.32

[a]P<0.05, [b]P<0.01, *vs* tubular adenocarcinoma group; [c]P<0.05, [d]P<0.01 *vs* high differentiation group; [e]P<0.05, [f]P<0.01, *vs* stage I+II group; [g]P<0.05, [h]P<0.01 *vs* A, B, C groups respectively.

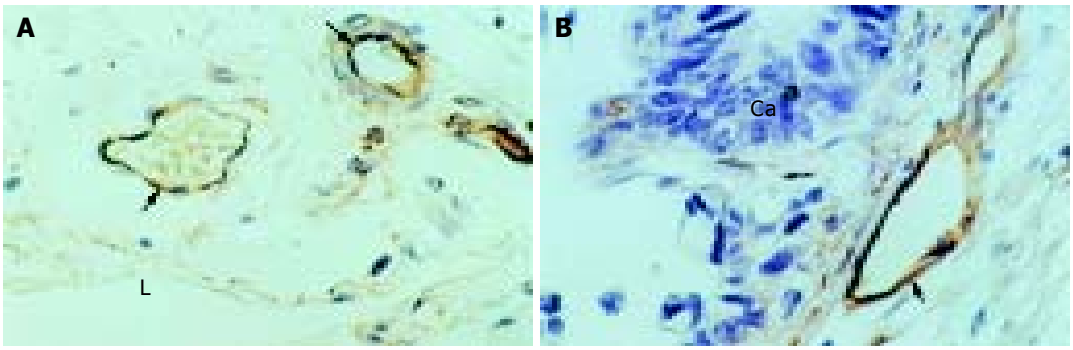

Figure 1 Expression of ICAM-1 in lymphatic endothelial cells. A: Negative expression of ICAM-1 in the lymphatic endothelium (L) of normal colon and positive expression of ICAM-1 in blood vessel endothelium (arrow) (Envision, original magnification ×400); B: Positive expression of ICAM-1 in the lymphatic endothelium (arrow) of colon cancer (Ca) (Envision, original magnification ×400).

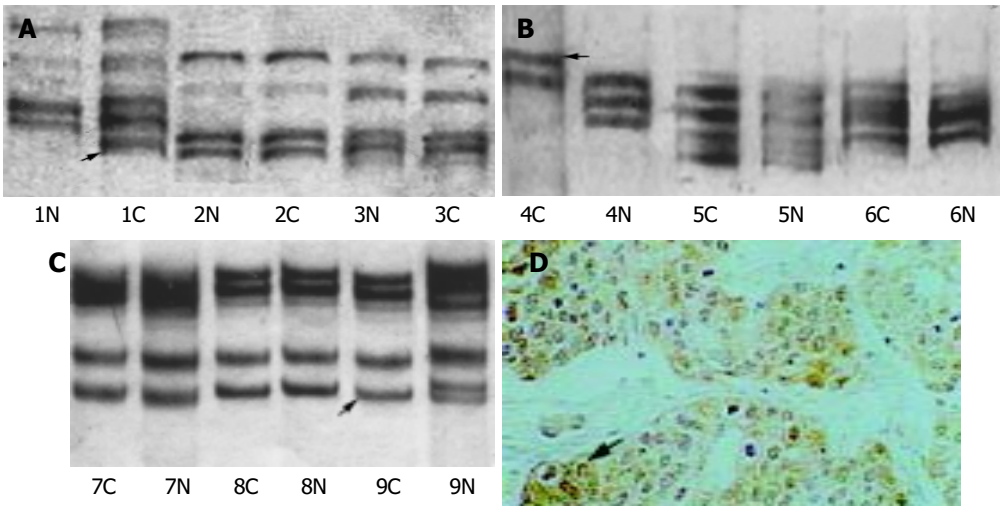

Figure 2 Positive D17S396 MSI, LOH and nm23H$_1$ in 30 cases of colon cancer. A: Positive MSI (arrow-headed) with an additional allele band (1C) compared with normal tissue (1N); B: Positive MSI (arrow-headed) with a removed allele band (4C) compared with normal tissue (4N); C: Positive LOH (arrow-headed) with a lacked allele band (9C) compared with normal tissue (9N); D: Positive nm23H$_1$ protein (arrow-headed) in cytoplasm, and nucleoli and membranes (Envision, original magnification ×200).

clinical stage. The expression of nm23H$_1$ protein in the group of tubular adenocarcinoma (60.00%) was apparently higher than that in the group of mucoid adenocarcinoma (20.00%, P<0.01), and exhibited a rising trend with the differentiation degrees of tubular adenocarcinoma (P<0.01). The positive rate of nm23H$_1$ in stages I+II (81.25%) was greater than that in stages III+IV (21.43%) (P<0.01). The same phenomenon occurred between the group positive for MSI (75%) and the group negative for MSI (45.45%) (P<0.05) (Table 4). However, LOH had no effect on the expression of nm23H$_1$ protein (Table 4). Computer imaging analysis showed that there was a difference among the groups in nm23H$_1$ protein expression level.

Table 4 Relationship between LOH, MSI and nm23 protein expression (mean±SD)

Groups	Cases	Expression of nm23 protein(%)	Intensity of nm23 protein
Positive to MSI	8	6/8 (75.00)	39.06±2.14
Negative to MSI	22	10/22(45.45)	41.14±2.36
Positive to LOH	6	2/6 (33.33)	41.23±2.27
Negative to LOH	24	14/24 (58.33)	39.44±2.52

DISCUSSION

Metastasis, the spread of cells from primary neoplasms to distant sites and their growth at that location, is the most harmful aspect of cancer. Despite great improvements in early diagnosis, surgical techniques, general patient care, local and systemic adjuvant therapies, most deaths from cancer are attributable to metastases that are resistant to conventional therapies. During metastatic cascade, tumor cells interact with various host cells as well as extracellular matrices and basement membrane components including laminin, fibronectin, and type I collagen through certain adhesion molecules such as integrins. Such adhesive interactions may lead to the enhancement of survival, arrest, or invasiveness of tumor cells and is one of the most important events in the metastatic process[14-17].

Intercellular adhesion molecule-1 (ICAM-1) is a single transmembrane glycoprotein and has two patterns in the body. One is located on the endothelial cells of blood vessels and is consisted of outmembrane region, transmembrane region and cytoplasmic region. The other (sICAM-1) is soluble in serum and is consisted of extracellular domains and originates from leucocytes, endothelial cells and hepatocytes[18-20]. The specific ligand of ICAM-1, LFA-1, can be expressed on the surfaces of leucocytes and lymphocytes. In normal conditions, ICAM-1/LFA-1 plays an important role in various immune responses.

How ICAM-1 expresses when tumors appear in the body? In the present case, ICAM-1 was expressed on endothelial cells of lymphatic vessels in colon cancer. With the degree of cancer invasion, lymphatic vessels positive for ICAM-1 showed an increasing trend. When cancer metastasis appeared in peripheral lymph nodes, ICAM-1 was strongly expressed on endothelial cells of peripheral lymphatic vessels, and the number of lymphatic vessels positive for ICAM-1 was the highest. The results might give a hint that cancer cells can be transferred into lymphatic vessels in combination with LFA-1. Furthermore, sICAM-1 transformed from ICAM-1 could inhibit natural killer cells, which could activate lymphocytes and restrict major histocompatibility complex (MHC) to react with T cells and tumor cells, thus promoting tumor cells to escape[21-23]. Therefore, sICAM-1 could strengthen and promote cancer cells to survive and transfer in lymphatic vessels when tumor metastasis occurred.

Genetic instability is the main reason why tumors appear and transfer[24-31]. MSI and LOH could induce canceration in the body. MSI was firstly found in hereditary non-polyposis colorectal cancer (HNPCC), and then in some kinds of sporatic tumors such as colon cancer, gastric cancer, uterus cancer, breast cancer, prostate cancer and pancreatic cancer. Our results showed that DNA from thirty Chinese patients at the site of D17S396 appeared microsatellite instability, the incidence was 26.67%. Subsequent experiments indicated that the incidence of MSI at the site of D17S396 in the stage of TNM I+II was greater than that in the stage of TNM III+IV, suggesting that MSI might be one of the markers for early colon cancer.

In contrast to MSI, the incidence of LOH at the site of D17S396 increased with the degree of Duke stage. Therefore, our results made it clear that LOH of nm23H$_1$ appeared at the later stage of colon cancer, which endowed colon cancer with a high invasiveness and a poor prognosis.

The expression of nm23H$_1$ has a negative relationship with tumor metastasis. Leone *et al*.[32] found that nm23H$_1$ had a function on the prevention of tumor metastasis by inhibiting the ability of cancer cells to clone. With the degree of tumor stage, the expression of nm23H$_1$ decreased. Our results indicate that with the development of colon cancer, the expression of nm23H$_1$ decreases.

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Edited by Wang XL and Zhang JZ **Proofread by** Xu FM