Correlation between expression and differentiation of endocan in colorectal cancer

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Abstract

AIM: To investigate the expression frequency of endocan in colorectal cancer and analyze the relationship between endocan expression and clinical parameters and to study the role of endocan in colorectal carcinogenesis.

METHODS: Expression of endocan in 72 tumor tissue samples of colorectal cancer as well as in 27 normal mucous membrane tissue samples was analyzed using in situ hybridization, immunohistochemistry on tissue microarray, Western blot and reverse-transcript polymerase chain reaction (RT-PCR).

RESULTS: The expression of endocan was higher in normal colon and rectum tissue samples than in cancerous tissue samples (mRNA = 92.6%, protein = 36%), and was lower in colorectal cancer tissue samples (mRNA = 70.4%, protein = 36.1%). No correlation was found between staining intensity and clinical parameters such as sex, age, tumor size and TNM stage. However, the expression of endocan was positively correlated with the tissue differentiation in colorectal cancer.

CONCLUSION: The expression of endocan is down-regulated in colorectal cancer and is positively correlated with the tissue differentiation in colorectal cancer, suggesting that the expression of endocan is associated with development and differentiation of colorectal cancer.

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Key words: Endocan; Colorectal cancer; Differentiation; Expression; In situ hybridization

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INTRODUCTION

Colon and rectum cancers accounted for about 1 million new cases in 2002 (9.4% of the world total)[1]. There is at least a 25-fold variation in the occurrence of colorectal cancer around the world. The incidence of colorectal cancer increases rather rapidly in countries where the overall risk was formerly low (especially in Japan, but also elsewhere in Asia)[2]. Although it has been found that many factors are correlated with genesis and development of colon and rectum cancers, it cannot explain all the clinical and pathological manifestations. It is critical to investigate new factors which are intimately correlated with initiation and development of colorectal cancer.

Endocan, previously called endothelial cell-specific molecule-1 (ESM-1)[3], is over expressed in human tumors, and its serum levels are elevated in late-stage lung cancer and experimental tumor, as measured by enzyme-linked immunoassay or by
immunohistochemistry. mRNA level of endocan is also recognized as one of the most significant molecular signatures with a poor prognosis of several types of cancer including lung cancer. Over expression of this dermatan sulphate proteoglycan is also directly involved in tumor progression as observed in mouse models of human tumor xenografts. These results suggest that endocan is a biomarker of inflammatory disorders and tumor progression as well as a validated therapeutic target in cancer.

We studied the expression of endocan in colon and rectum tissue samples. The results of this study indicate that endocan expression is down-regulated in colorectal cancer and positively correlated with the differentiation of colorectal cancer. Changes in endocan expression represent an important step in development and differentiation of colorectal cancer.

MATERIALS AND METHODS

Patients and samples
Seventy-two colorectal cancer patients, who consecutively underwent radical surgical resection at Anhui Medical University Hospital from the year 2001 to 2003, were recruited into this study. Tumor and mucosa samples were embedded in paraffin after 16 h formalin fixation. None of the patients (23 males, 49 females, mean age 54 years, range 17-87 years) received any anticancer therapy. According to the TNM classification, 43 cases were at stages I and II, 29 cases at stages III and IV. Well- and moderately- differentiated adenocarcinoma was found in 57 patients and poorly-differentiated adenocarcinoma was observed in 15 patients, and strong lymphoid infiltrate including lymphoid follicles with germinal centers was demonstrated in 39 patients.

In situ hybridization

cRNA probe labeling: The sequences of specific primers for endocan are as follows: sense, 5'-AGCTGGAATTTCATGAGAG (20 bp) and antisense, 5'-TCTCTCAGAAAGCTTAGCG (20 bp). PCR was performed to amplify endocan DNA, and the PCR product was ligated into the pGEM-T-Vector (Promega). The recombinant plasmid was transformed into E.coli, amplified and digested with the restriction endonuclease (EcoRI and HindIII). The objective gene (V-gene) was purified using a DNA gel extraction kit to obtain the probes for the following digoxigenin-labeling and hybridization was performed as previously described.

Hybridization: All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. A series of 5-μm thick sections were cut for analysis. In situ hybridization was performed as previously described with certain modifications, using digoxigenin-labeled antisense cDNA probes. Briefly, the sections were dried at 60°C for 4 h, de-waxed and rehydrated and pretreated with DEPC-treated PBS containing 100 mmol/L glycine and 0.3% Triton X-100, respectively. The sections were then permeabilized with 20 μg/mL RNase-free proteinase K (booster, Wuhuan, China) for 20 min, incubated at 37°C for at least 20 min with prehybridization buffer. Each section was overlaid with 30 μL hybridization buffer containing a 10 ng digoxigenin-labeled cDNA probe and incubated at 42°C overnight. After hybridization, the section was incubated with digoxigenin antibody (75 mU/mL) for 2 h. The positive signal for endocan mRNA was detected using DAB as a substrate. The presence of brown staining in the cytoplasm was considered positive.

Protein extraction from paraffin-fixed tissue

Paraffin-fixed tissue was cut into 50 5-μm thick sections for protein extraction and mounted onto plain glass slides. Three 5-μm thick sections for protein extraction were deparaffinized in xylene, rehydrated in graded ethanol, immersed in distilled water, and air-dried. To exclusively collect 5 mm × 5 mm cancer tissues, the targeted areas were cut microscopically with a fine needle for observation of the morphology of HE-stained sections under a microscope. After the tissue sections on the glass slide were immersed in distilled water, only the targeted areas of cancer tissue were separated from the glass slide and recovered. Adenoma tissue was also cut into sections and collected in the same manner. Normal mucosa was recovered from 5 cm-long sections of full-depth colorectal wall with a fine needle as previously described.

Immunohistochemistry

The pathology of colorectal carcinoma was performed on 5-μm thick sections of 10% formalin-embedded samples with a S-P kit. Slides were boiled in 10 mmol/L citrate buffer (PH 6.0) for 10 min to allow antigen retrieval before a 12-h incubation at 4°C with primary antibody against endocan (Santa Cruz). The mean percentage of positive tumor cells was determined in ten areas at a high magnification (× 400) and graded from 0 to 4 (0 = negative, 1-4 = positive). Negative controls were obtained by omitting the primary antibody. Each normal mucosa sample, as an internal positive control, was simultaneously analyzed. Slides were read by two observers blinded to the clinical data.

Reverse-transcript polymerase chain reaction (RT-PCR)

Two micrograms of total RNA was prepared from colon and rectal tissues, randomly primed, and reverse transcribed with Superscript II (Gibco). The sequences of specific primers used for endocan are as follows: sense, 5'-CTCAGCGCATGGATGCGATACTG-3'; antisense, 5'-GAGACCCGGCAGCACTTCTTCA-3'; and β-actin: sense, 5'-ACTCTTCCAGCCTTCTCC-3' and antisense: 5'-ATCTCTTCTCGCATTCTGTC-3'. After a hot start at 94°C, 35 PCR cycles were performed, each cycle consisting of annealing at 57°C for 45 s and extension at 72°C for 45 s.

Western blot analysis

Twenty micrograms of protein was incubated in a
loading buffer (125 mmol/L Tris-HCl, pH 6.8, 10% β-mercapto-ethanol, 4.6% SDS, 20% glycerol and 0.003% bromophenol blue) for 5 min at 100°C, separated by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electroblotted to PVDF membrane (BioRad). After non-specific binding sites were blocked for 1 h with 5% nonfat milk in TPBS (PBS contained 0.05% Tween 20), the membrane was incubated overnight at 4°C with primary antibody. After washing 3 times in TPBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 2 h at room temperature, and washed twice with TPBS. Immunoblot was detected by autoradiography using an enhanced chemoluminescence detection kit.

Statistical analysis
Chi-square test and F-test were used to compare the categorical data. SPSS 11.0 was used to analyze the data.

RESULTS
Expression of endocan in colon mucous membrane and colorectal cancer specimens
In situ hybridization analysis showed that endocan mRNA was expressed in the cytoplasm of epithelial cells in mucous membrane of colon and rectum and in well- and moderately-differentiated colorectal cancer. However, endocan mRNA expression was down-regulated in poorly-differentiated colorectal cancer (Figures 1 and 2).

Meanwhile, we performed immunohistochemical staining for endocan protein with a monoclonal antibody against human endocan. The endocan protein expression was concordantly regulated by mRNA.

The statistical results demonstrated that endocan was differentially expressed in normal colon mucosa and carcinoma tissue samples. The expression rate of endocan was 92.6% (25/27) in normal colon mucosa tissue samples and 36.1% (24/72) in colorectal cancer tissue samples, and was significantly lower in cancerous tissue samples than in normal tissue samples ($P = 0.001$, Table 1). Endocan protein was identically expressed as mRNA; The expression rate was 70.4% (19/27) in normal colon and rectum mucosa tissue samples and 36.1% (26/72) in colorectal cancer tissue samples. Endocan was also differently expressed in normal and colorectal cancer tissue samples ($P = 0.005$, Table 2).

Correlation between expression of endocan and differentiation of colorectal cancer
The expression of mRNA and protein in colorectal cancer tissue samples was not correlated with age, gender, clinical stage, tumor size or lymph node metastasis, but positively correlated with the differentiation of tumors (Table 3).

RT-PCR and Western blot were performed to further observe the relationship between the expression levels of endocan and differentiation of colorectal cancer (Figure 3). Both endocan transcript and translation were detected in colon mucous membrane and in well- and moderately-differentiated colon carcinoma, but scarcely detected in poorly-differentiated carcinoma.

DISCUSSION
Endocan was originally cloned from a human endothelial
The expression of endocan in normal mucous membrane and different differentiation colon carcinoma tissues. It had a high expression in well and moderately-differentiated colon carcinoma tissues, but a weak expression in poorly differentiated carcinoma tissues. 

A: The expression of endocan in normal mucous membrane; B: The expression of endocan in well-differentiation colon carcinoma tissue; C: The expression of endocan in well-differentiation colon carcinoma tissue; D: The expression of endocan in poorly differentiated colon carcinoma tissue; E: Negative control. Left: in situ hybridization; Right: immunohistochemistry.
The expression of endocan in colon and rectum tissues.

**A**

RT-PCR analysis of endocan mRNA in colon and rectum tissues. Endocan mRNA was highly expressed in normal colon and rectum tissue and well and moderately differentiated colorectal cancer tissues, but was down regulated in poorly differentiated colorectal cancer tissues. Endocan expression (up) and β-actin expression (down). 1: 100 bp Marker; 2: Normal colon and rectum mucous membrane; 3: Well-differentiated differentiated colorectal cancer tissue; 4: Moderately colorectal cancer tissue; 5: Poorly differentiated colorectal cancer tissue.

**B**

Expression of endocan by Western blot. Endocan was detected at high expression levels in normal colorectal and well-differentiated tissues; Moreover, there was a down regulation in the poorly differentiated colorectal cancer tissues. Lane 1, 3, 5: Normal mucus, well-differentiated tissues; lane 2, 4, 6: Poorly-differentiated tissues.

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**Table 3**  Correlation of *endocan* mRNA and protein expression with clinical pathological parameters

| Group        | n   | Endocan mRNA |     | P     | Endocan protein |     | P     |
|--------------|-----|--------------|-----|-------|-----------------|-----|-------|
| Age ≤ 54     | 34  | 10           | 24  | 29.4  | 0.676 (χ² = 0.174) | 11  | 23    | 32.4  | 0.702 (χ² = 0.146) |
| Age > 54     | 38  | 14           | 24  | 36.8  | 0.019 (χ² = 0.01)  | 15  | 23    | 39.5  | 0.530 (χ² = 0.395)  |
| Sex Male     | 23  | 9            | 14  | 39.1  | 0.099 (χ² = 0.146) | 10  | 13    | 43.5  | 0.031 (χ² = 0.395)  |
| Sex Female   | 49  | 17           | 32  | 34.7  | 0.643*           | 16  | 33    | 32.7  |
| Size < 3     | 11  | 3            | 8   | 27.3  | 0.031 (χ² = 0.01)  | 5   | 6     | 45.5  | 0.483*         |
| Size > 3     | 61  | 21           | 40  | 34.4  | 0.791*           | 21  | 40    | 34.4  |
| Infiltration |       |              |     |       |                  |     |       |
| Full-thickness| 64  | 21           | 43  | 32.8  | 0.919            | 21  | 43    | 32.8  | 0.099*         |
| Non-full-thickness| 8  | 3            | 5   | 37.5  | 0.796 (χ² = 0.067) | 9   | 18    | 33.3  | 0.899*         |
| Metastasis   |       |              |     |       |                  |     |       |
| Nonmetastatic| 27  | 8            | 19  | 29.6  | 0.031 (χ² = 4.642) | 26  | 31    | 45.6  | 0.003 (χ² = 8.824) |
| Metastatic   | 45   | 16           | 29  | 35.6  | 0.324 (χ² = 0.973) | 18  | 25    | 41.9  | 0.324 (χ² = 0.973) |
| Grade        |       |              |     |       |                  |     |       |
| Differentiated| 57  | 23           | 34  | 40.4  | 0.031 (χ² = 4.642) | 26  | 31    | 45.6  |
| Poorly differentiated| 15 | 1            | 14  | 6.7   | 0.324 (χ² = 0.973) | 18  | 25    | 41.9  |
| TNM stage    |       |              |     |       |                  |     |       |
| I and II     | 43   | 18           | 25  | 41.9  | 0.324 (χ² = 0.973) | 18  | 25    | 41.9  |
| III and IV   | 29   | 8            | 21  | 27.6  | 0.324 (χ² = 0.973) | 8   | 21    | 27.6  |

*P < 0.05 vs controls.

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Inflammatory disorders[10-15] and tumor progression, and in the control of fundamental cellular processes, such as adhesion[16], migration and angiogenesis. Inflammatory cytokines, such as TNF-α and LPS[17], and pro-angiogenic growth factors, such as VEGF[18], HGF/SF[19,23] and FGF-2[21,24], strongly stimulate the expression and secretion of endocan in human endothelial cells.

Endocan has been identified as a potential novel endothelial cell marker and a new target for cancer therapy. It was reported that high endocan mRNA levels correlate with a poor prognosis and metastasis of several types of cancer, including breast, renal and lung cancer[1,25,26]. A study of 78 breast cancer patients, with the aim to define the optimal prognosis classifier, was performed on 70 genes according to standard prognostic criteria, showing that endocan over expression in breast cancer is associated with a higher risk of metastasis and death within 5 years[27]. Furthermore, 1234 genes that have been identified are differentially expressed in renal cell carcinoma, and endocan mRNA level is 3-fold higher in renal cell carcinoma samples than in normal tissue samples[28]. This up-regulation of endocan expression also correlates with increased tumor vasculature and inflammation in renal cancer, which is actually the ninth most common malignancy in Western countries, and no effective treatment is available for it. Similarly, a recent extensive hybridization study showed that the endocan gene is one of the most highlighted genes, with at least a 2-fold increase in all the 8 renal cell carcinoma samples analyzed, compared to normal tissue samples[29]. Interestingly, a parallel up-regulation was also revealed for VEGF and e-Met proto-oncogene receptor for HGF/SF, both of which are heavily implicated in angiogenesis. A comparable study, by dot blotting and hybridization showed that endocan is dramatically up-regulated in several (5/14) renal cell carcinoma biopsies, and is correlated with both VEGF and VEGF receptor gene expressions[30]. A gene profiling study of tissues from 23 lung cancer patients demonstrated that endocan is one of the significant poor prognosis classifiers among the 42

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genes associated with a high risk of cancer-related death.

Endocan was less reported in colon and rectum tissues. Moreover, little is known about its molecular mechanism. We mapped the regulation of endocan expression in normal membrane mucosa and colorectal cancer tissue samples. Our results reveal that endocan was significantly expressed at transcriptional and translational level in normal colorectal mucous membrane and in well- and moderately-differentiated colorectal cancer, but weakly expressed in poorly-differentiated colorectal cancer. Meanwhile, RT-PCR and Western blot also showed that the expression of endocan was upregulated in normal colon and rectum tissue samples, and down-regulated in poorly-differentiated colorectal cancer tissue samples.

All these data show that endocan is differently expressed in colon and rectum tissue and other tissues. According to the previous results, endocan is almost not expressed in normal human tissue except in lung tissue. Our study showed that endocan was also expressed in normal colon and rectum tissue, but its expression was down-regulated in colorectal cancer, suggesting that regulation may be complex in colon and rectum. As we know, there are a lot of germs in human colon. Most of the outer germs are killed by gastric acid when they get into the stomach through the mouth. In the upper part of the small intestine, the number of germs is also small. However, this number increases gradually at the end of the ileum and reaches its maximum in the colon, where the contents is neutral or alkaline and movement is slower, thus making the germs propagate at a fast pace. There are $10^{6}-10^{11}$ germs per gram of colon contents. However, these germs can decompose protein, which is called degradation. In this process, the germs also produce some virulent substances, amino acids, peptide, amine, and hydrogen sulphide and proper indole, all of which can activate macrophages and monocytes to secrete a large number of cell factors, such as IL-1 and TNF-α, which can stimulate expression of endocan. That is why we can detect a high expression of endocan in normal colon and rectum tissue. However, the expression of endocan was down-regulated in poorly-differentiated colorectal cancer, suggesting that endocan may be closely related with differentiation and development of colorectal cancer.

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