Original Article
Circulating DNA and its methylation level in inflammatory bowel disease and related colon cancer

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Abstract: Both of chronic inflammation and abnormal immune in inflammatory bowel disease can induce colon cancer. Previous research showed that cell apoptosis and necrosis become the main source of circulating DNA in the peripheral blood during tumorigenesis that reduced along with methylation degree. However, its role in the process of colitis transforming to colon cancer is not clarified. Drinking 3% DSS was used to establish colitis model, while 3% dextran sodium sulfate (DSS) combined with azo oxidation methane (AOM) intraperitoneal injection was applied to establish colitis related colon cancer model. Circulating DNA and its methylation level in peripheral blood were tested. Morphology observation, HE staining, and p53 and β-catenin expression detection confirmed that drinking 3% DSS and 3% DSS combined with AOM intraperitoneal injection can successfully establish colitis and colitis associated colorectal cancer models. Circulating DNA level in colitis and colon cancer mice increased by gradient compared with control, while significant difference was observed between each other. Circulating DNA methylation level decreased obviously in colitis and colon cancer, and significant difference was observed between each other. Abnormal protein expression, circulating DNA and its methylation level in ulcerative colitis associated colorectal tissues change in gradient, suggesting that circulating DNA and its methylation level can be treated as new markers for colitis cancer transformation that has certain significance to explore the mechanism of human ulcerative colitis canercation.

Keywords: Circulating DNA, methylation, colitis, colon cancer

Introduction

Inflammatory bowel disease (IBD) is a type of intestinal inflammatory disease including Crohn’s disease and ulcerative colitis [1]. Its etiology is associated with genetic susceptibility, diet, and environment [2]. Currently, it was thought that mucous membrane barrier damaged by inflammation and immune dysfunction were the key to its refractory, whereas long-term inflammation and immune abnormalities induced smooth muscle hyperplasia also can increase the possibility of developing colon cancer [3]. In 1948, circulating DNA is a kind of free nucleic acid in extracellular that was first found by French scientists Mandel and Metais. At present, the main source of circulating DNA is believed to be derived from apoptosis or necrosis induced DNA fragments. DNA fragments length also has difference between normal tissue and tumor necrotic tissue. DNA methylation is an important epigenetic modification way. Studies confirmed that DNA methylation changes play an important role in tumor formation. Alu is one of the most abundant repeated sequence in human genome that accounts for almost 10%, and the copy number was more than a million [4]. Studies also found that Alu often stays in low methylation level in tumor cells [5, 6].

Cell apoptosis, tissue damage, and immune disorder all have important influence in both IBD and cancer by affecting circulating DNA and its methylation level [7]. Long-term IBD induced tissue damage aggravation and body repair will...
increase the risk of colon cancer, while circulating DNA and its methylation level changes in IBD were not clarified.

Therefore, in this study, we used C57/BL6 mice to induce colitis by feeding 3% dextran sodium sulfate (DSS), and induce colon cancer by intraperitoneal injection of azo oxidation methane (AOM) (10 mg/kg) combined with feeding 3% DSS. We further performed Western blot to detect proto-oncogene β-catenin and tumor suppressor gene p53 level and HE staining to confirm modeling success. Circulating DNA and its methylation level were tested to evaluate their roles in colitis canceration.

Materials and methods

Main reagents

C57/BL mice were bought from the laboratory animal center of Soochow University. QIAamp-MinElute Virus Spin kit was got from Qiangen (Suzhou, China). Methylation sensitive restriction enzyme Hpa II or BstU I was purchased from Kangcheng Biotechnology Company (Shanghai, China). P53 and β-catenin antibodies were from Abcam (Hong Kong, China). Rabbit anti-mouse IgG (H+L) was from Proteintech (Wuhan, China). DSS and AOM were from Sigma (Shanghai, China). Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Soochow University.

Modeling and grouping

C57 mice were randomly divided into four groups with 10 in each group: blank control group, AOM control group, colitis group, and colon cancer group. Blank control group received normal diet. According to the reference [8], the mice in colitis group received 3% DSS instead of water for 7 days and rested for 16 days with 3 cycles. The mice in colon cancer group received 3% DSS combined with AOM intraperitoneal injection for 7 days and rested for 16 days with 3 cycles. The mice in AOM control group received AOM (10 mg/kg) intraperitoneal injection. The mice in colitis group and colon cancer group were euthanatized at 10 days after the last cycle, whereas the mice in other groups were euthanatized at the same day. After anesthesia and laparotomy, the blood was extracted from aorta abdominis, and two colon samples were collected at distal end and proximal end. Body weight, fur, mental status, and defecation behavior of the mice were observed every day.

HE staining

After fixed with formalin for 24 h, the colon tissue sample was washed and gradient dehydrated by ethanol. And then the sample received conventional paraffin embedding and slicing after xylene hyalinization. The slice was baked for 1 h at 60°C before dyeing, and then it received dimethyl benzene dewaxing, gradient ethanol hydration before hematoxylin and eosin staining.

Western blot

1 ml lysis buffer containing protease inhibitor was added to 100 mg colon tissue in EP tube. After cutting up and homogenization, the tissue was cracked at 4°C for 5 min. It was then centrifuged at 12000 rpm under 4°C for 15 min and the supernatant was protein. After quantified by BCA, the protein was degenerated after 5 min boiling and separated by 10% SDS-PAGE. After protein was transferred to PVDF membrane at 300 mA for 1 h, it was incubated with p53 antibody (1:1000) at 4°C overnight. After washed by TTBS for three times, it was incubated with secondary antibody (1:1000) at 37°C for 2 h and developed by chemiluminescence.

Circulating DNA extraction

The blood sample was centrifuged at 3500 rpm under room temperature for 10 min, and the serum was separated. Circulating DNA was extracted by QIAampMinElute Virus Spin kit according to the manual. Circulating DNA was extracted and stored at -80°C.

Methylation sensitive restriction enzyme digestion

We adopt two types of DNA methylation sensitive restriction enzyme Hpa II or BstU I to test DNA methylation in whole blood. Firstly, the sample was digested by Hpa II or BstU I (reaction system includes 300 ng DNA, 1 × NE Buffer + 5 U Hpa II or 1 × NE Buffer 4 + 5 U BstU I) under the optimum temperature of two enzymes for 16 hours. Then the sample was heated at 65°C for 30 min to inactivate endonuclease.
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Control group received same manipulation except for inactivated enzyme. PCR was applied after digestion. DNA samples still could be amplified by PCR after digestion represented methylated Alu, while DNA in control that could be amplified represented total Alu. Methylation level was expressed as the ratio of methylated Alu and total Alu.

Alu methylation level detection

Alu amplification sequence was as follows: forward, 5'-CTGTAATCCGACACTTTG-3'; reverse, 5'-CCAGGCTGGAGTGCA-3'. BstU I sequence was as follows: forward, 5'-TTGTTGGTTTTCGGAACTGAGG-3'; reverse, 5'-GGCATCGTTTATGGTGGAA-3'. Hpa II sequence was as follows: forward, 5'-CGAGGCCCTGTAATTGGAATG-3'; reverse, 5'-CACTCAGCTAAGAGCATCGAGG-3'. Real-time PCR was performed on ABI PRISM 7500 system (CA). PCR reaction contained 95°C for 2 min, followed by 40 cycles including 95°C for 30 s and 62°C for 40 s. Melting curve was verified for specificity and the result was calculated by 2ΔΔCt method.

Statistical analysis

All statistical analyses were performed using SPSS15.0 software. Results were presented as means and standard deviation. Differences between multiple groups were analyzed by one-way ANOVA or SNK-Q test.

Results

General physical sign

All the mice in colitis group and colon cancer group appeared diarrhea and macroscopic bloody stool on the 7th day after drinking 3% DSS, whereas the mice in control group showed normal faeces. After euthanasia, colon cancer group (AOM+DSS) and colitis group (DSS) presented significantly lower mean weight than blank control (P < 0.05). Control group showed normal colon tissue. Colitis mice presented intestinal wall thickening, occasional blood in the lumen, villus drop after clean, and visible ulcer. Colon cancer group showed adenoma in the lumen with blood and visible ulcer. No mice formed tumor except in the colon cancer group.

HE staining

Normal control group showed intact mucosa epithelium, regular glandular arrangement, integrate structure, and no inflammatory cell infiltration. Colitis group presented widespread mucous glands destruction, epithelial shedding, ulcer formation, and a large number of inflammatory cells infiltration mainly as lymphocytes. Colon cancer group manifested large amount of inflammatory cells infiltration in mucous layer, disorganized glands, disordered tumor glandular tube that protrude over mucous membrane surface (Figure 1).

P53 and β-catenin expression in distal end of colon

P53 level increased significantly in colitis group and colon cancer group compared with normal control (P < 0.05). It was obviously higher in colon cancer group compared with colitis group (P < 0.05). Furthermore, AOM group showed higher p53 level than the control but lack of statistical difference (Figure 2).

Compared with normal control, colitis group and colon cancer group showed higher level of β-catenin, and they showed statistical difference with each other (P < 0.05). Similarly, AOM

Figure 1. HE staining results.
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Figure 2. p53 and β-catenin expression level comparison in colitis group and colon cancer group. **P < 0.05, compared with normal control; ##P < 0.05, compared with each other.

Figure 3. Circulating DNA level comparison. **P < 0.05, compared with normal control.

group presented no significant difference with control (P > 0.05) (Figure 2).

Circulating DNA level in plasma

After successfully establishing colitis and colitis related colon cancer model, we tested circulating DNA changes in normal control, colitis group, and colon cancer group, respectively. Compared with normal control, colitis group showed significantly higher level of circulating DNA (P < 0.05). Circulating DNA level was obviously higher in colon cancer group than that of colitis group (P < 0.05) (Figure 3).

Alu methylation level in whole blood

Alu methylation can increase its movement in genome and make genome unstable that can promote tumor occurrence and metastasis [9, 10]. Our study applied methylation specific PCR analysis to detect methylation level. Compared with normal control, the methylation level of two restriction enzyme cutting sites in colitis group and colon cancer group decreased significantly (P < 0.05). Colitis group also showed obviously difference with colon cancer group (P < 0.05) (Figure 4).

Discussion

Colorectal cancer is the most worried long-term complication of IBD patients [11], while atypical hyperplasia is one of the important link. IBD canceration mode can be summarized as follows: inflammation → atypical hyperplasia → cancer [12]. Carcinogenesis is mainly caused by the imbalance of cell proliferation and apoptosis [13]. Wnt signaling pathway can regulate cell proliferation, and its abnormal regulation may lead to tumor occurrence. β-catenin is a proto-oncogene that is also the key regulatory protein in the Wnt signaling pathway [14]. Most of the β-catenin can combine with transmembrane protein E-cadherin in normal cells, whereas small amount of free β-catenin in cytoplasm can form complex with APC, GSK-3β and axin that degrading β-catenin by phosphorylation. β-catenin degradation obstruction may lead to it accumulates in cytoplasm and enters nucleus, and further cause cell excessive proliferation and carceration by binding with LEF/TCF to activate downstream gene transcription [15]. It was found that β-catenin level increased in the T cells from long-term ulcerative colitis patients and colon cancer patients. Elevating β-catenin in T cells in mice made mice susceptible to colon cancer. It was thus considered as a marker of colitis transforming to colon cancer [16]. P53 can regulate cell cycle as a tumor suppressor gene. P53 gene can transcript to p53 protein to restrain G1/G0 stage and prohibit cell into S phase to inhibit cells excessive proliferation [17].

In this study, we applied classical DSS feeding to establish colitis model and AOM combined
with DSS to establish colon cancer model. We confirmed modeling success by morphology observation, p53 and β-catenin level detection, and HE staining. Furthermore, we tested circulating DNA and Alu methylation level in normal control, colitis and colon cancer mice.

The source and content of circulating DNA were both different in normal and colon cancer [18]. Circulating DNA level may also increase in inflammation following neutrophil proliferation. In our study, we successfully established colitis model and colon cancer model. By detecting circulating DNA in peripheral blood, we found that its level increased obviously in colitis group and colon cancer compared with normal control, while it showed significant difference between colitis and colon cancer. It suggested that circulating DNA elevation had indicating meaning in the process of colitis transforming to colon cancer, and circulating DNA microarray analysis may provide more information.

At the same time, we also detected DNA methylation level since the latter is the most aspect of epigenetic modification [19]. DNA methylation can silence some genes, while abnormal methylation can lead to gene copy number increase and induce tumor. Current study revealed that DNA methylation plays an important role in autoimmune related chronic inflammatory diseases [20]. Alu is the most abundant repeat sequence in genome and its methylation level reduced in cancer cells. Whether circulating DNA maintained methylation level diversity between colitis and colitis related colon cancer arouse our interest. We used dual-enzyme digestion and real time PCR to detect circulating DNA methylation level, and found it presented gradient increased trend in colitis and colon cancer.

Abnormal protein, circulating DNA, and its methylation level in ulcerative colitis related colon cancer may have certain significance for investigation of ulcerative colitis cancerration.

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Disclosure of conflict of interest

None.

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