Methylation of promoter region of *CDX2* gene in colorectal cancer

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Abstract. The incidence of colorectal cancer is on the increase owing to changes in daily diet. In the present study, the methylation status of caudal type homeobox transcription factor 2 (*CDX2*) gene in lesion tissue of colorectal cancer (CRC) was investigated. Additionally, the correlation between the promoter methylation of *CDX2* gene, CRC and gene expression in patients with CRC and normal population was examined. Between April 2014 and May 2015 78 cases with CRC were enrolled in the study. Using methylation-specific polymerase chain reaction (PCR), the promoter methylation of CDX2 in normal tissues and colorectal tissues was examinned. Through the fluorescence quantitative PCR technique, the expression levels of *CDX2* gene were determined in a normal population and lesion tissue of patients with CRC. At the same time, we evaluated the levels of the *CDX2* gene product in the normal population and lesion tissue of patients with CRC. The results showed that the methylation rate of the promoter region of *CDX2* gene in normal colorectal tissue was 43.5%, whereas that in the lesion tissue of CRC was 78.5%. The result was statistically significant ($P<0.05$). The quantity of mRNA and protein expression of *CDX2* gene in colorectal and normal tissue was significantly different ($P<0.05$). In conclusion, the methylation of the *CDX2* gene promoter region was associated with risk of CRC, i.e., methylation of the promoter region of *CDX2* gene favors the occurrence of CRC.

Introduction

High fat diet and low fiber has become increasingly widespread in the daily diet, which to some extent, accelerates the incidence of colorectal cancer (1). Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. Its incidence rate in China ranks fourth and its mortality rate ranks third (2). Therefore, the prevention and treatment of CRC have important practical significance for improving quality of life of patients. In its early stage, CRC mainly infiltrates locally while at its advanced stage it is characterized by lymphatic spread, with surgical resection being the only viable treatment option (3). However, the postoperative recurrence rate of patients with CRC following surgical resection is approximately 45.3-73.5%, which challenges the survival rate within 5 years of surgery (4). Therefore, investigation into the pathogenesis of CRC is crucial. Caudal type homeobox transcription factor 2 (CDX2) in the human body is a type of transcription factor for regulating gene transcription and expression in gastrointestinal embryo pathways (5-7). *CDX2* gene plays an important role in some relevant digestive system cancers, including gastric and colon cancer (8). The downregulation of *CDX2* gene expression may lead to the loss of relevant differentiation function and the increase of value-added ability in the process of the occurrence of CRC, and it may also co-operate with other genes to participate in regulating the occurrence of relevant tumors (9). It is believed that the methylation of DNA promoter to a certain extent can regulate the expression of relevant genes (10). However, the transcriptional regulation of many genes in the human system specifically expressed in differentiation is de-methylated (11), but it is methylated in specific cells, not requiring the expression of genes.

Previous studies on *CDX2* gene were primarily focused on gastric and colon cancer. At present, few studies are available on the function of *CDX2* gene in CRC and on the methylation of the promoter region of *CDX2* gene in CRC. To the best of our knowledge, the present study, for the first time, examined the methylation of promoter region of *CDX2* gene in CRC to provide preliminary data on the correlation between the methylation of the promoter regions of CDX2 and CRC. We also aimed to determine strategies in the prevention and treatment of CRC in advanced stage.

Patients and methods

Patients. In total 78 cases with CRC (tested by relevant pathology) were selected at the Luoyang Central Hospital (Henan, China) from May 2014 to April 2015. The patietns comprised 43 men and 35 women, with an average age of 42±7.9 years. Additionally, a total of 52 (31 men and

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21women, with an average age of 45±9.3 years) participants who were diagnosed with colorectal polyps served as controls (normal population). The study subjects were not undergoing treatment in the form of surgery *in vitro*, chemotherapy and radiotherapy.

Reagents. The following reagents were used: Mouse anti-human CDX2 monoclonal antibody (Roche Diagnostics, Indianapolis, IN, USA), goat anti-mouse HRP secondary antibody (Roche Diagnostics); RT-polymerase chain reaction (PCR) kit (Takara Bio, Inc., Otsu, Japan); mouse anti-human immunohistochemistry kit (Roche Diagnostics); DBA staining kit (Thermo Fisher Scientific, Waltham, MA, USA); animal cell genomic extraction kit (Axygen Scientific Inc., Union City, CA, USA); CpG methylation enzyme (New England Biolabs, Ipswich, MA, USA); MethylDetector bisulfite modification kit (Active Motif, Carlsbad, CA, USA).

Detection of methylation. DNA extracted from the sample tissue was in accordance with the instructions of the animal cell genomic extraction kit, purchased from Axygen Scientific Inc. The modification of DNA bisulfite was performed in accordance with the instructions of the MethylDetector bisulfite modification kit (Active Motif).

The methylation primer was designed according to the supplier's protocol (12). The primers are listed in Table I. The reaction system of methylation-specific PCR was 25 μl, i.e., ddH_2O 8.5 μl, DNA 2 μl, Premix Ex Taq DNA polymerase mixture 12.5 μl, methylation- or nonmethylation-specific primer 2 μl. The PCR reaction conditions were: denaturation at 95˚C for 3 min, annealing at 95˚C for 30 sec, 58˚C for 30 sec, and extension 72˚C for 30 sec, for a total of 30 cycles. A final extension was conducted at 72˚C for 10 min, and final heat preservation was at 4˚C. The PCR products was recovered and sequenced using gel electrophoresis (Invitrogen Life Technologies, Carlsbad, CA, USA).

Fluorescence quantitative PCR (qPCR). RNA extraction was performed as described previously (13). Fluorescence qPCR was performed according to the manufacturer's instructions (Takara fluorescent qPCR; Takara Bio, Inc.).

Enzyme-linked immune reaction. The procedure was performed according to the instructions of ELISA kit. An assay buffer of PBST (Biosharp, Hefei, China) was used to prepare dilutions of 1:25, and a standard curve was then designed. Firstly, the sample to be measured was diluted at a 1:100 ratio, and 100 μl of sample to be measured was mixed with 50 μl of detection solution in each well. The samples were kept for 2 h at 20˚C, followed by the addition of TMB to measure light absorption at 495 nm using a microplate reader ((Model 3550; Bio-Rad Labs,Hercules, CA, USA). The concentration of CDX2 in every sample was measured according to the standard curves.

Western blotting. The animal cell protein extraction kit (Roche Diagnostics) was used to extract the total proteins. Western blot analysis was performed as described previously (13). After protein quantitation using the Lowery protein assay, equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three-buffer system. The membranes were incubated with primary antibody at a dilution of 1:500 (anti-CDX2, RayBiotech, Inc., Norcross, GA, USA; cat no.: 119-14329) overnight at 4˚C. The membrane was washed with TBST and incubated with a peroxidase-conjugated secondary polyclonal goat-anti-mouse antibody (1:1000) (Santa Cruz Biotechnology, Inc. CA, USA; cat no.: sc-395763) for 1 h. Western blot film was scanned and the membrane was stripped and reprobed with antibody against β-actin (1:1500) (Santa Cruz Biotechnology, Inc.; cat no.: sc-8432) to confirm equal sample loading

Table I. Primer of methylation-specific polymerase chain reaction.

Primers	Length of products (bp)
Methylation forward primer 5'-CGAAAATAAATCACTACGACG-3'	200
Non-methylation forward primer 5'-ATTCAAAATAAAAATCACTACAACA-3'	204
Common reverse primer 5'-AAAGGATATTGGAGAGTATTTTAG-3'	

Immunohistochemical staining. The procedure was performed according to the mouse anti-human immunohistochemistry kit (Roche Diagnostics). After staining, the nucleus appeared yellow, which was considered positive. The staining criteria were classified as: (-) negative, unstained cells or stained very lightly and there was no noticeable difference between the cell and the background; (+) extensively weakly colored after staining, the color of cell was slightly higher than that of the background; (++) extensively moderately colored after staining, the color of cell was markedly higher than that of the background; and (+++) extensively strong colored after staining, where the cells were dark brown. In the study, all the (+) (++) (+++) were considered positive. The final score was evaluated by two experts independently, and the average score was considered as final.

Statistics analysis. The SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) was used to process the obtained data. Measurement data were presented as mean ± standard deviation and the measurement data was tested using the χ^2 test.

Results

Methylation status of CDX2 gene in tissue of CRC and normal population. As shown in Fig. 1, the controls had a lower degree of CDX2 promoter methylation, but there was a high degree of methylation for *CDX2* gene in the lesion tissue of patients with CRC. Table II shows the rate of CDX2 promoter methylation in the colorectal tissue of the normal

Table II. Relative expression quantity of mRNA of CDX2 in colorectal tissue in normal population and patients with colorectal cancer (use GAPDH as internal control).[a]

Group	Cases	*CDX2* gene Methylation	Promoter methylation Non-methylation	χ^2	P-value
Normal population	63	27	36	5.8855	0.023[b]
Patients with colorectal cancer	78	61	17		

[a]State of promoter methylation of CDX2 in tissue of normal colorectal and colorectal cancer. [b]P<0.05 is of noticeable difference.

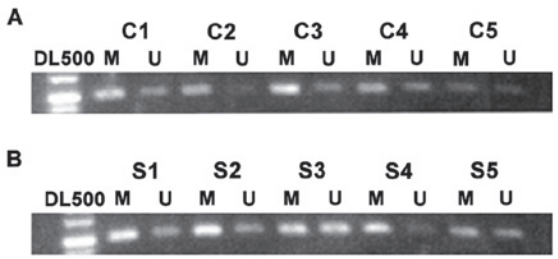

Figure 1. Methylation of promoter region of *CDX2* gene in (A) normal colorectal and (B) colorectal cancer tissue. C1-C5 are samples of colorectal tissue of different normal population; S1-S5 are samples of colorectal tissue of patients with colorectal cancer. M indicates methylation of promoter; U indicates Un-methylation of promoter; appearance of both M and U simultaneously indicates the methylated promoter.

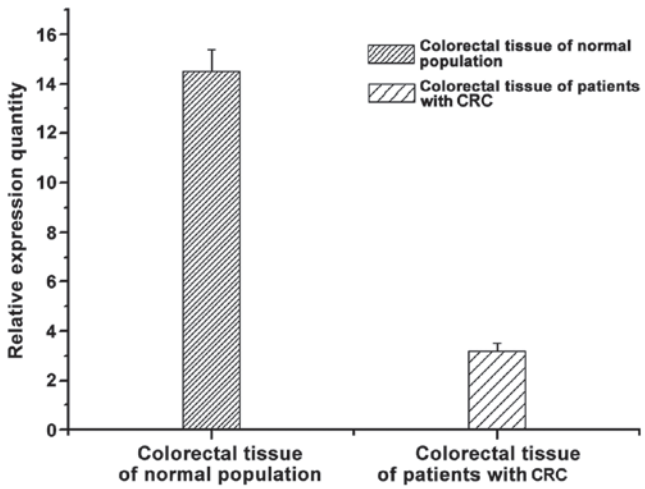

Figure 2. Fluorescence quantitative polymerase chain reaction for the relative expression of CDX2 protein in controls and patients with colorectal cancer.

population, which was ~43.5%, but the rate in the lesion tissue of CRC was ~78.5%. The results were statistically significant.

Fluorescence qPCR. By comparing the levels of *CDX2* gene in controls and patients with CRC, we found that the level of *CDX2* gene was higher in colorectal tissue of the normal population and lower in colorectal tissue of the patients with CRC

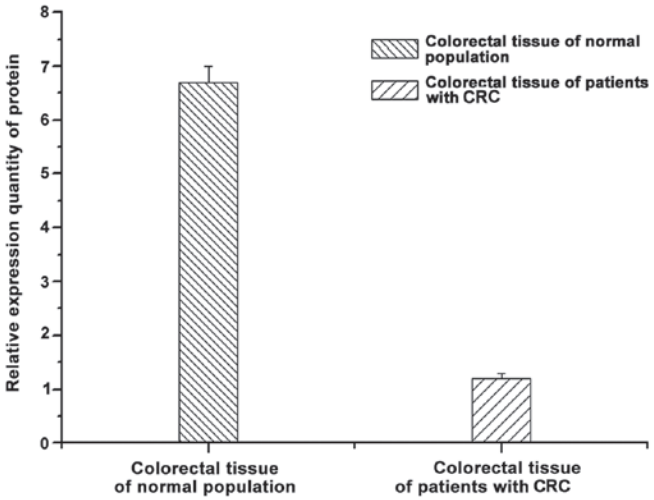

Figure 3. ELISA for the relative expression of CDX2 protein in controls and patients with colorectal cancer.

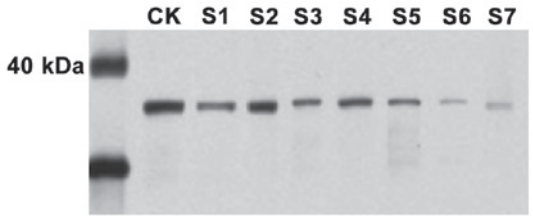

Figure 4. Western blotting, the data shows the expression of CDX2 protein in controls and patients with colorectal cancer. CK is the colorectal tissue of normal population; S1-S7 are colorectal tissue of different patients with colorectal cancer.

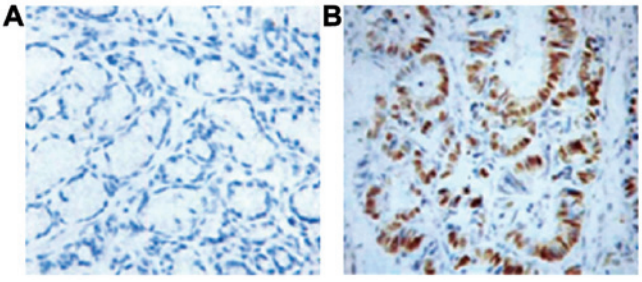

Figure 5. Immunohistochemical staining for CDX2 protein in colorectal tissue of patients with colorectal cancer and normal population. (A) Tissue of colorectal cancer; (B) normal tissue.

(Fig. 2). The result indicates that there may be a correlation between *CDX2* gene and the disease of CRC.

Enzyme-linked immune response and western blotting. The protein in controls and patients with CRC were determined by ELISA and western blotting (Figs. 3 and 4). In comparison to CRC patients, the expression of CDX2 was higher in the controls and a similar observation was made with the western blotting and fluorescence qPCR results.

Immunohistochemistry. By comparing the results of colorectal tissue staining in CRC patients and controls, we found that the positive expression of CDX2 primarily exists in colonic epithelial goblet cells (Fig. 5). Staining showed brownish yellow granules. We observed that CDX2 protein mainly exists in the colorectal tissue of the normal population and has a lower expression in CRC tissue.

Discussion

The occurrence and aggravation of CRC involves numerous factors and is not only influenced by physical conditions but also by diet, and mental state (14). The increase of studies of genomics with a focus on the occurrence of CRC have led to investigations at gene level. CDX2, a tail-type homologous box gene family, is a type of transcription factor of intestinal epithelial cell specificity in the human body (15). *CDX2* gene in human body is involved in the regulation of the expression of intestine-specific genes, intestinal development, and the differentiation and proliferation of intestinal epithelial cells (16). *CDX2* gene has also been found to be involved in the occurrence of some tumors and cancers in the human body (17). It has been suggested that *CDX2* gene has a higher expression in intestinal metaplasia associated with chronic atrophic gastritis and is expressed in gastric cancer (18). CDX2 may be associated with gastric mucosal epithelial transformation and gastric mucosal carcinogenesis (19-22). Previous findings have shown that transfection of CDX2 cDNA, and human HT29 CRC cell line to express CDX2 protein, indicated the oncogenic potential of the abovementioned cells, and metastasis of related cells markedly decreased while cell sensitivity for apoptosis significantly increased (23).

In summary, to the best of our knowledge, for the first time, we examined a correlation between methylation of the promoter region of CDX2 in CRC and CRC. The results have shown that in comparison to the normal population, the degree of methylation of the promoter region of CDX2 in lesion tissue of patients with CRC was higher than that of the normal population. The protein expression in the control and lesion sections of CRC patients showed that the expression level of CDX2 in the lesion section of patients with CRC was lower. This finding suggested that there was a certain correlation between CDX2 and CRC, or the decrease in the degree of *CDX2* gene promoter methylation to a certain extent, promotes the risk of CRC.

Acknowledgements

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