Investigation of methylation and protein expression of the Runx3 gene in colon carcinogenesis

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Abstract. In the present study, the methylation and protein expression of the runt-related transcription factor 3 (Runx3) gene was detected in sporadic colorectal cancer, colonc adenoma and normal colon tissue to evaluate their clinical significance in colorectal carcinogenesis. A total of 34 colon cancer specimens, 34 colonc adenoma specimens and 34 normal colon tissue specimens were used in the study. The CpG island methylation status of the Runx3 gene was detected by methylation-specific polymerase chain reaction and the protein expression of Runx3 was detected by immunohistochemistry. The results showed that the rates of methylation of the Runx3 gene in colon cancer and colonc adenomas were significantly higher than that in the normal colon tissue (23.5, 20.6 vs. 0.0%；P<0.05). There was no significant difference in the percentage of methylation of the Runx3 gene between colonc adenoma and colon cancer (P>0.05). The positive percentage of Runx3 protein expression was significantly lower in colon cancer compared with colonc adenoma and normal tissue (17.7 vs. 61.8, 76.5%；P<0.05). Methylation of the promoter CpG islands of the Runx3 gene is an important genetic event of colon carcinogenesis and may be associated with an altered protein level of Runx3.

Introduction

DNA methylation is one of the most common types of epigenetic change, whose methylation is provided by S-adenosyl methionine. With the catalytic potential of DNA methyltransferase, the cytosine of DNA at the C-5 position is converted to 5-methylcytosine, occurring at the transcriptional level, and this does not change the primary structure of DNA base modifications (1,2). The methylation position is often a 5'-CpG-3' dinucleotide. Extensive gene methylation exist in human primary tumor-suppressor gene (3-5).

Key words: Runx3, methylation, colorectal cancer

Materials and methods

Tissue sample collection. The study was approved and registered by the Ethics Committee of Sichuan Provincial People's Hospital (Sichuan, China) in July 2008. The Ethics Committee approved the screening, treatment and data collection of these patients, and all the subjects provided written informed consent. All the studies were undertaken following the provisions of the Declaration of Helsinki.

In total, 68 surgical resection of colon cancer and adenomatous tissue samples were collected from Sichuan Provincial People's Hospital between October 2008 and February 2009. The 68 patients were all first diagnosis and underwent treatment with preoperative radiotherapy, chemotheraphy or immunotherapies, in which 34 cases were of colorectal adenoma and 34 cases were of colon cancer. A total of 34 healthy cases were normal tissue biopsy specimens under colonoscopy, and used as the negative control. The collection of new fresh tissue specimens was stored in liquid nitrogen within 20 min at -183°C. A total of 8 males and 16 females provided the colon cancer samples, and were aged from 38-78 years (mean age, 58±2.5 years). A total of 7 males and...
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17 females, aged from 28-73 years (mean age, 53±2.7 years), provided the colonic adenoma samples. The normal colon mucosa samples were provided from 18 males and 16 females, aged from 38-78 years (mean age, 58±2.2 years).

The TIANamp Genomic DNA kit was purchased from Tiangen Biotechnology Co., Ltd., (Beijing, China), and the EZ DNA Methylation-Gold™ kit was from Rimo Science and Technology Development Co., Ltd., (Beijing, China). DNA markers λDNA/HindIII, 50-bp DNA Labber and 2X Taq polymerase chain reaction (PCR) MasterMix were from Tiangen Technology Co., Ltd. The rabbit anti-human monoclonal antibody Runx3 was from Abcam (ab40278; Cambridge, MA, USA); and the DAB kit was from Zhongshan Golden Bridge Biological Technology Co., Ltd. (Beijing, China).

### Primer synthesis and preparation

The DNA sequences were as reported in GenBank, and the PCR primers were designed according to the study by Issa (12). The primers were as follows: Runx3 methylation front guide sequences [methylated-specific forward (MF)], 5'-TTACGAGGGGCGGTCGTACCGGGG-3' and methylated reverse primer sequence [methylated-specific reverse (MR)], 5'-AAAACGACCGACGCGAACGCCTCC-3'; Runx3 unmethylated front guide sequences (unmethylated-specific forward primers), 5'-TTAGGGGTGGTTGTATGTGGG-3' and reverse primer sequence (unmethylated-specific reverse primers), 5'-AAAACCAACACAAACACCTCC-3'.

### DNA extraction

The present study referred to Herman et al (13) for the MSP methods. The TIANamp Genomic DNA kit was used to extract tissue sample DNA. The EZ DNA Methylation-Gold kit was used for the methylation-extracted tissue. The DNA Runx3M system included 12.5 µl 2X Taq PCR MasterMix, 1 µl Runx3 MF and 1 µl Runx3 MR methylation. Following the treatment, 2.5 µl DNA and 8 µl ddH2O underwent the following Runx3 gene PCR conditions: denaturation at 94°C for 10 min, annealing at 65°C for 45 sec, and extension at 72°C for 10 min. The PCR products were applied to 1% agarose gel electrophoresis and gel images were captured using the Gel Doc 1000 imager (Bio-Rad, Hercules, CA, USA).

### Immunohistochemical detection of the expression of Runx3 proteins

The Runx3 antigen retrieval underwent a high-pressure hot fix. Phosphate-buffered saline solution was used instead of a primary antibody for the negative control. A known positive plate was used as a positive control.

### Criteria

Noticeable methylated-specific PCR products at 250 bp indicated positive methylation. Negative was the presence of a 240-bp unmethylated band, but no band for the methylated-specific PCR product (13). Following IHC staining, when >10% of the whole section was brown/yellow, this was deemed as positive.

### Statistical analysis

Statistical analysis was used to compare the percentage of Fisher's exact probability. Statistical analysis was performed using statistical software SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Data processing used a test level of α=0.05, and P<0.05 was considered to indicate a statistically significant difference.

### Results

Runx3 gene promoter CpG island methylation. The percentage of Runx3 promoter CpG island methylation positive expression in the colon carcinomas and adenomas were 23.5 (8/34) and

![Figure 1. Expression level of runt-related transcription factor 3 (Runx3) in colon tissue in (A) normal mucosa adenoma tissue, (B) adenoma tissue and (C) colon cancer tissue. Immunohistochemical assay was used to detect the expression of Runx3 (magnification, x100).](image-url)
20.6% (7/34), and there was no detection in the normal tissue. The methylation of the Runx3 gene in the colon cancer and colon adenoma groups was significantly different from normal colon mucosa (P<0.05). However, there was no significant difference between colon cancer and colon adenoma (P>0.05, Table 1).

**Runx3 protein.** Runx3 protein expression percentages were 17.6 (6/34), 61.8 (21/34) and 76.5% (26/34) in the colorectal cancer, adenoma and normal groups, respectively. There was a significant difference between the colon cancer and the colon adenoma and normal groups (P<0.05, Table II, Fig. 1).

**Discussion**

DNA methylation is closely associated with tumorigenesis, which is one of the inactivation mechanisms of the tumor-suppressor genes (14). The Runx3 gene is a newly discovered suppressor oncogene, which is the most primitive Runx alkylene type in the mammalian gene family, and it has a regulatory effect on epithelial cell growth and differentiation. It also plays an important role in the spinal ganglia development and differentiation of T cells. The Runx3 protein is involved in the TGF-β signal transduction pathway of a transcription factor, which is directly combined with Smad receptor binding. The protein guides the TGF-β1/Smad signaling pathway to activate Smad (one type of apoptotic factor). The complex moves from the cytoplasm into the nucleus of the target site, and the TGF-β1 Smad complexes and target sites combine to promote TGF-β1 signaling to mediate apoptosis in normal cells. Synergistic action of TGF-β1 on epithelial cell growth has a negative regulation, which occurs in the TGF-β1 signaling pathway and plays a key role (15). A previous study found that for the Runx3 gene colon cancer hypermethylation, the Runx3 CpG island methylation guides Runx3-induced gene inactivation, which is closely associated with colon cancer (16). Runx3 protein deficiencies can lead to the TGF-β1 signaling pathway blocking TGF-β1-induced cell growth inhibition, which reduces the sensitivity to apoptosis. β-catenin accumulates in the cytoplasm resulting in signal pathway activation, cell proliferation and apoptosis imbalance. The genetically unstable cancerous cell clonal expansion promotes the occurrence of tumors (17). Runx3 gene-knockout mice, which have decreased Runx3 protein expression, significantly increased the incidence of tumors in mice (18). Ku et al (19) applied the MSP technique for detecting methylation in human colon cancer cell lines and found that 50% of human colon cancer cell line expression of Runx3 was decreased or there was no expression. The study also found that compared with the positive expression percentage of corresponding normal tissue, the percentage of Runx3 protein expression was significantly decreased in human colon cancer, liver cell cancer, bile duct cancer, pancreatic cancer, lung cancer, esophageal cancer, endodermal sinus tumors, breast (20-27) and other tumors. In the present study, 8 cases of Runx3 CpG island methylation were found in 34 cases of colon cancer. Methylation was therefore 23.5% (8/34), and Ku et al (19) reported that there is an 18.4% methylation rate on the Runx3 CpG island in colon cancer, while there is no Runx3 methylation in normal colon tissue. Goel et al (28) also identified 20.8% (19/91) of the presence of Runx3 CpG island methylation in colon cancer cases, and no methylation was found in the normal group; these two studies show close and similar results with our present study. The present study identified that the Runx3 protein expression percentage was 76.5% (26/34) in normal colon mucosa, 61.8% (21/34) in the adenoma group, and 17.6% (6/34) in the colon cancer group. The colon adenoma group was significantly lower than the colon cancer group, and there was a significant difference (P<0.05). Chen et al (27) reported similar results, which suggests that Runx3 CpG island methylation may occur in normal colon mucosa transforming into colon gland adenoma. This may be an early stage of development in the cancer sequence, and the colon cancer early onset molecular events and low expression of Runx3 protein may be associated with colon Runx3 CpG island methylation.

**References**


