

## RUNX3 Methylation Status in Colonic Carcinoma and Adenoma

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**Background :** RUNX3 is expressed throughout the luminal gastrointestinal tract. RUNX3 is on chromosome 1p36, a location considered to carry an important tumor suppressor for many types of cancers. Epigenetic silencing of RUNX3 is causally related to human gastric cancer. **Methods :** Colorectal cancers, adenoma, and the corresponding normal mucosa were obtained from 26 individual patients. To identify methylation of RUNX3 in colonic carcinogenesis, methylation-specific PCR was performed. **Results :** RUNX3 methylation was found in one case of colonic carcinoma. The normal mucosa and tubular adenoma of this case had no methylation. No other cases were found to have methylations. **Conclusions :** These results are very different from the findings of gastric carcinomas, where frequent DNA methylation in the vicinity of the RUNX3 promoter is found. Although, the possibility of a role of RUNX3 methylation in the colon can not be completely ruled out, these results suggest that methylation of the RUNX3 promoter region might not contribute to the adenoma-carcinoma sequence of the colon.

**Key Words :** RUNX3-Methylation-Colon

Colorectal cancer is the fourth most common world-wide malignancy of both sexes.<sup>1</sup> Most colorectal cancers develop from adenomatous polyps, and the adenoma-carcinoma sequence has, in recent years, become the most widely accepted concepts in colorectal carcinogenesis. The morphological and genetic progression in the adenoma-carcinoma sequence has been well described. There are multiple genetic events, including tumor-suppressor genes, oncogenes and DNA mismatch repair genes, contributing to the development of colorectal cancer. Defects in DNA methylation may also play an important role in colon cancer. A potential alternative mode for alternating a gene during tumorigenesis is an epigenetic process, which includes DNA methylation abnormalities. DNA methylation abnormalities are believed to be involved in early in the pathway by which normal tissue undergoes neoplastic transformation. Hypermethylation of CpG islands around promoter regions have been shown to be an important mechanism for gene silencing in some tumor-suppressor genes.<sup>2</sup> The importance of RUNX3, as a tumor suppressor gene, has recently been reported in gastric carcinogenesis and hypermethylation of RUNX3 is related to the genesis and progression of gastric cancer.<sup>3</sup>

RUNX3 is expressed throughout the luminal gastrointestinal tract and is on chromosome 1p36, a location considered to carry an important tumor suppressor of many types for cancers including gastric cancer, hepatocellular carcinomas, malignant melanomas, colon carcinomas, pancreatic and bile duct cancers and neuroblastoma.<sup>4</sup> It might also function as a tumor suppressor in variable types of cancers, where mutations or deletions are often found, in either TGF- $\beta$  receptors or Smads. Colon cancer is related to mutations of the TGF- $\beta$  receptor and Smads. Inactivation of the RUNX3 gene, by promoter hypermethylation, in colon cancer has, to our knowledge, never been studied. The aim of our study was to examine the role of RUNX3 promoter methylation in the colonic adenoma-carcinoma sequence, and determine its correlation with the clinicopathological variables.

## MATERIAL AND METHODS

### Tumor samples

Colorectal adenocarcinomas, adenomas, and their corresponding

normal mucosa were separately obtained from 26 individual patients at the Ewha Womans University Medical Center, Korea. Among these 26 cases, 21 were obtained from surgically resected specimens and 5 from colonoscopic biopsies. The normal mucosae were sampled from the surgical margin of the resected specimens. The adenomas were sampled at a distance from the cancer to avoid contamination with cancer cells.

**Microdissection and DNA extraction**

For each case, normal, adenoma and carcinoma samples were separately microdissected from two to three H&E-stained slides. DNA extraction was performed using a modified single-step DNA extraction method.<sup>5</sup> The procured cells (50 cells/ $\mu$ L) in 30  $\mu$ L of DNA extraction buffer, containing 100 mM Tris-HCl (pH8.0), 0.1% Tween 20 and 2 mg/mL proteinase K, were incubated at 52°C for 1 or 2 days. The mixture was then boiled for 10min to inactivate the proteinase K.

**Methylation-Specific PCR**

Methylation-specific PCR was performed as reported previously.<sup>6</sup> Briefly genomic DNA was modified by treatment with sodium bisulfite, and then purified. The pure DNA was subjected to PCR, using specific primer sequences for the methylated and unmethylated forms of the RUNX3. The primer set used for detecting methylated DNA were Rx3-5M (5'-TTACGAGG-GGCGGTTCGTACGCGGG-3'), and Rx3-3M (5'-AAAACGA-CCGACGCGAACGCCTCC-3'), and those for detecting unmethylated DNA were Rx3-5U (5'-TTATGAGGGGTGGTTG-

TATGTGGG-3'), and Rx3-3U (5'-AAAACAACCAACACA-AACACCTCC-3').

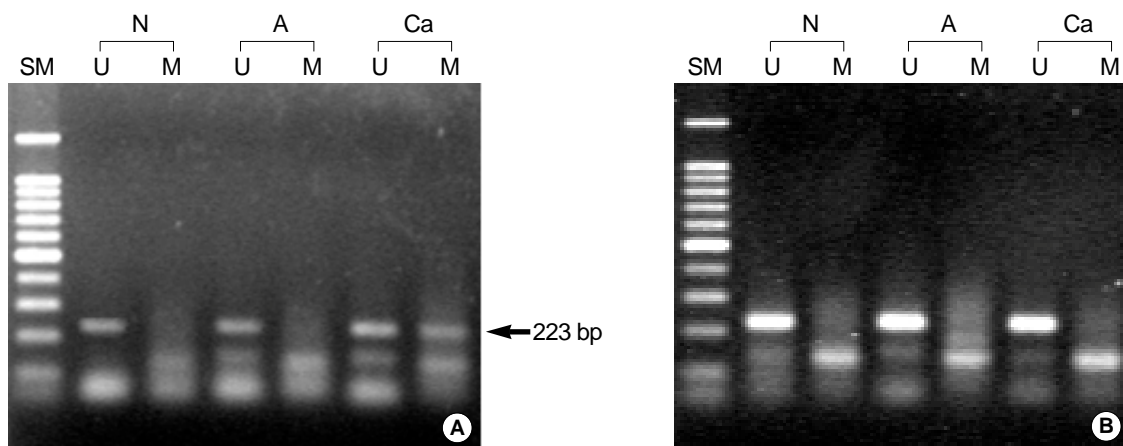
**RESULTS**

The pathological findings of the 26 cases were reviewed. The average age of the patients was 66.9 ranging from 32 to 92 years with 18 males and 8 females. The locations of their cancers were: the left-sided colon in 20 cases and the right-sided colon in the other 6. Among the 21 resected cases, the primary tumor size varied as follows : <2 cm in 6 cases, 2-5 cm in 11, and >5 cm in 4. The Modified Dukes' stages were: A in 13 cases, B and C in 4 cases each (Table 1). The methylation status of the RUNX3 in the colon were analyzed. Among the 26 cases, 1 (4%) had methylation in the colonic carcinoma. This was biopsied from the left sided tumor of an 80 years old female patient. The normal mucosa and tubular adenoma of this case had no methylation, but the RUNX3 was methylated in the carcinoma (Fig. 1). Methylation of the RUNX3 was detected in no other cases.

**Table 1.** Findings of clinicopathologic variables and results of methylation

Variables & methylation	Data
Age	32-92 years (average: 66.9 years)
Male:Female	18:8
Left:Right (side)	20:6
Tumor size	<2 cm: 6, 2-5 cm: 11, >5 cm: 4
Modified Dukes' stage	A: 13, B: 4, C: 4
Methylation	NI:0 (0/26), Ad:0 (0/26), Ca:1 (1/26)

Ad, Adenoma; Ca, Carcinoma; NI, Normal.



**Fig. 1.** Methylation analysis of RUNX3 in colon. The 223 bp product is indicative of both unmethylated and methylated RUNX3 alleles. (A) A cancer of colon has methylation, whereas the normal mucosa and adenoma have no methylation. (B) All have no methylation. A, Adenoma; Ca, Carcinoma; NI, Normal; M, Methylated allele; U, Unmethylated allele; SM, Size marker, 100 bp DNA ladder marker.

## DISCUSSION

The human runt-related transcription factor 3 gene (RUNX3) is one of three mammalian homologues of Runt, a gene important for segmentation in *Drosophila*.

RUNX3 was originally cloned as AML2,<sup>7</sup> and localized on human<sup>7,8</sup> and mouse<sup>9</sup> chromosomes 1p36.1 and 4, respectively. The 1p36 region of the gene locus is frequently deleted in many types of cancer. Therefore, it is postulated that the locus contains important tumor suppressor genes.<sup>4</sup>

Three RUNX genes have been identified in humans and mice: RUNX1, RUNX2 and RUNX3. Their gene products share many structural similarities, but have distinct biological activities. RUNX1 is critical for mammalian hematopoiesis,<sup>10</sup> RUNX 2 for osteogenesis,<sup>11,12</sup> and RUNX3 is strongly expressed in the gastrointestinal tract of mice,<sup>3</sup> and is an important target for the signaling of the TGF- $\beta$  superfamily. In general, RUNX gene products function as transcriptional regulators of the Smad gene family, which transmit TGF- $\beta$  and its homologues.<sup>13</sup> RUNX3 appears to be an important component of the TGF- $\beta$  induced tumor suppressor pathway and directly interacts with R-Smad. The TGF- $\beta$  signal transduction pathways are interrupted in many types of cancers, including those of the gastrointestinal tract. The TGF- $\beta$  receptor type II is altered in colon and gastric cancers and mutations in Smad2 are found in colon cancers. Yoshiaki *et al.*<sup>14</sup> suggested that if RUNX3 is an integral part of the TGF- $\beta$  induced signaling pathway, and contributes to its tumor suppressive activities, it might also function as a tumor suppressor in various cancers, where mutations or deletions are often found, in either the TGF- $\beta$  receptors or Smads. In many previous studies, the mutations of the TGF- $\beta$  receptor and Smad have been found in colorectal tumors.<sup>15-20</sup> In humans, one major mechanism for the suppression of tumor suppressor gene function is the aberrant methylation of the promoter region, resulting in down-regulation of gene expression.<sup>21</sup>

Recently, Li *et al.*<sup>3</sup> reported that RUNX3-null mice showed gastric mucosal hyperplasia, and were particularly insensitive to the apoptosis normally stimulated by TGF- $\beta$ . In gastric cancer, RUNX3 has frequently been found to be silenced by hypermethylation of the CpG islands in the exon 1 region and important as a tumor suppressor in gastric carcinogenesis. They suggested a possible involvement of RUNX3 in the very early stages of carcinogenesis. RUNX3 is expressed throughout the luminal gastrointestinal tract, but what is its function in the colon? Since RUNX3 is an important growth regulator of gastric epithelial cells, does it also play a role in growth of colonic epithelial cells

and colonic carcinogenesis? Therefore, it was presumed that DNA methylation in the RUNX3 promoter region might play particular roles in the tumorigenesis of colon cancers, and have a close relationship with the TGF- $\beta$  and Smad genes. To our knowledge, methylation of the RUNX3 promoter region in colorectal carcinomas has never been previously studied. In the present study, the methylation status of RUNX3 were analyzed in colonic carcinogenesis. However, only 1 case (4%) of colon cancer was found to have promoter region hypermethylation. In this case, the normal mucosa and tubular adenoma had no promoter region hypermethylation. These results are quite different from those of previous reports of gastric carcinoma, where frequent DNA methylation in the vicinity of the RUNX3 promoter was found.<sup>3</sup> It is known that in gastric cancer, RUNX3 play a role in the early stage of carcinogenesis. However, in this study of colonic carcinomas, the normal mucosa and adenoma had no methylation, whereas the carcinoma did. Although our sample size was small, the results suggest that RUNX3 in the colon is involved at the late stage of carcinogenesis. It could be assumed that loss of RUNX3, due to methylation, may not be a major pathway for colorectal carcinogenesis.

In conclusion, although a possible role for RUNX3 methylation in the colon can not be ruled out, our results suggest that its role might not contribute to the adenoma-carcinoma sequence, but might play a role in the late, rather than the early, stage in carcinogenesis.

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