Colorectal Cancers with both p16 and p14 Methylation Show Invasive Characteristics

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Recent studies indicated that p16 and p14 inactivation owing to promoter methylation was important for colorectal tumorigenesis. In this study, we examined the methylation status of these genes in 86 primary colorectal cancers using methylation-specific PCR (MSP) and correlated the results with the clinicopathological features of the patients. Aberrant promoter methylation of p16 and p14 genes was detected in 43 of 86 (50%) and 25 of 86 (29%) colorectal cancers, respectively. Next, we examined the correlation of methylation status with the clinicopathological features. We found a significant difference in maximal tumor size (P=0.022) when patients with both p16 and p14 methylation were compared to other patients. On the other hand, there was no significant difference in other factors, such as the extent of tumor and Dukes stage. These results suggested that colorectal cancer with both p16 and p14 methylation has the same invasiveness at a smaller size compared to that of the cancer with neither p16 nor p14 methylation. Inactivation of both p16 and p14 genes may result in a malignant change in colorectal cancer cells, leading to advanced cancers with a smaller size than those with p16 or p14 activity.

Key words: p16 — p14 — Methylation — Colorectal cancer

There is now good evidence that a series of genetic alterations in both dominant oncogenes and tumor suppressor genes is involved in the pathogenesis of human colorectal cancer. Activation of oncogenes such as the ras gene, and inactivation of tumor suppressor genes such as the APC and p53 genes, have been identified in colorectal cancer.1–3) In addition, we found that several other genes are related to the pathogenesis of colorectal cancer.4, 5) An investigation of genetic changes is important to clarify the tumorigenic pathway of colorectal cancer.6)

It has recently become clear that alterations in DNA methylation are very common and are capable of directly modifying carcinogenesis.7) First, a tumor suppressor gene, p16, was found to harbor promoter hypermethylation associated with the loss of protein expression in cancer cells.8) Though homozygous deletions of the p16 locus are not present,9) p16 promoter methylation was detected in colorectal cancer.10) Subsequently, it has been found that human p14 was also silenced by promoter hypermethylation in colorectal cancer.11) p14 interacts with the MDM2 protein and neutralizes the MDM2-mediated degradation of p53. Thus, p14 acts as a tumor suppressor gene via inhibition of p53 degradation. These studies indicated that p16 and p14 inactivation due to promoter methylation was important for colorectal tumorigenesis.

These results prompted us to examine whether p16 and/or p14 methylation was related to the malignant pathway of colorectal cancer. In this study, the methylation status of these genes was first confirmed in primary colorectal cancers using methylation-specific PCR (MSP). Next, we correlated the results with the clinicopathological features of the patients.

MATERIALS AND METHODS

Sample collection and DNA preparation Eighty-six primary tumors and corresponding colorectal epithelial tissues were collected at the Nagoya University School of Medicine from Japanese colorectal cancer patients who had been diagnosed histologically. These samples were obtained during surgery. All tissues were quickly frozen in liquid nitrogen and stored at −80°C until analysis. Tumor and normal tissue samples were digested overnight with proteinase K, and DNA was prepared by extraction with phenol.

Bisulfite modification DNA from tumor and normal tissue specimens was subjected to bisulfite treatment as described previously.12) Briefly, 1 µg of DNA was denatured with NaOH and modified with sodium bisulfite. DNA samples were then purified using the Wizard purification resin (Promega Corp., Madison, WI), again treated with NaOH, precipitated with ethanol, and resuspended in water.

MSP The modified DNA was used as a template for MSP. Primer sequences of p16 for amplification were described previously.12) The primers of p14 for the unmethylated reaction were: p14UMS (sense), 5′-TTTT-TGGTGTTAAAGGGTGGTGTAGT, and p14UMAS (anti-
sense), 5’-CACAAAAACCCCTCACTCAAACAA, which amplify a 132 bp product. The primers of p14 for the methylated reaction were: p14MS (sense), 5’-GTTGT-TAAAGGGCCGGTAC, and p14MAS (antisense), 5’-AAACCCCTCCTCAGCAGA, which amplify a 122 bp product. The PCR amplification of modified DNA samples consisted of 1 cycle of 95°C for 5 min; 33 cycles of 95°C for 30 s, 69°C for 1 min, and 72°C for 1 min; 1 cycle of 72°C for 5 min. DNAs from L132 (embryonic lung cell line) and H1299 (lung cancer cell line) were used as positive controls of unmethylated and methylated alleles, respectively. Controls without DNA were used as positive controls of p16 and p14 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution for 1 h at room temperature. A Vectastain ABC Kit and a DAB Substrate Kit (Vector, Burlingame, CA) were used to visualize the antibody binding.

**Immunohistochemical analysis**  Immunohistochemical analysis was performed as previously described. The specimens were fixed with 10% formalin, embedded in paraffin, and cut into 3 µm sections. The slides were dried at 60°C for 30 min, treated with xylene, and dehydrated in alcohol. Endogenous peroxidase was blocked with 0.3% H₂O₂. Microwave treatment was performed for 4 min in Antigen Retrieval Citra Solution (Biogenex, San Roman, CA), because it has been shown that the immunoreactivity of p16 and p14 can be remarkably enhanced by this method. The slides were blocked with normal horse serum for 20 min, then incubated with monoclonal mouse antibody against p16 or p14 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution for 1 h at room temperature. A Vectastain ABC Kit and a DAB Substrate Kit (Vector, Burlingame, CA) were used to visualize the antibody binding.

**Statistical analysis**  The χ² test and Student’s t test were used to examine the association between p16 and/or p14 promoter methylation and clinicopathological features.

**RESULTS**

We first examined the methylation status of p16 promoter in tumors using the MSP technique. Aberrant promoter methylation of p16 gene was detected in 43 of 86 (50%) colorectal cancers. Subsequently, we tested for promoter methylation of p14 in the same tumors. We found that 25 of 86 (29%) patients had aberrant promoter methylation of the p14 gene. These results indicated that p16 and/or p14 aberrant methylation may play an important role in colorectal cancers. A representative MSP analysis of p16 and/or p14 gene promoter methylation in tumors is shown in Fig. 1. As a control, we screened for aberrant methylation in the DNA of 86 corresponding normal tissues, and no methylation of p16 or p14 was found in this control group. Under the control of p14 promoter, located approximately 20 kb centromeric to that of p16, exon 1β splices into exon 2 of p16 in an alternative reading frame, producing a different pattern than p16. Therefore, p14 promoter methylation would be related to promoter methylation of the adjacent gene, p16. However, methylations at the p16 and/or p14 promoters do not seem to be directly related (Table I). This result is consistent with a previous report. To confirm the p16 and p14 expression in colorectal cancers in terms of methylation status of these genes, we performed immunohistochemical analysis. As shown in Fig. 2, the expression of p16 and p14 was diminished if these genes were methylated.

To determine the role of p16 and/or p14 inactivation in colorectal cancer, we examined the correlation of methylation status with the clinicopathological features. There was no significant difference in the distribution of patients with positive or negative methylation of either p16 or p14 in

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**Table I. Methylation Status of p16 and p14 in Colorectal Cancer**

|        | p16 methylation | Total | P-value<sup>a</sup> |
|--------|-----------------|-------|---------------------|
| p16    |                 |       |                     |
| +      | 12              | 13    | 25                  | >0.9999 |
| −      | 31              | 30    | 61                  |         |
| Total  | 43              | 43    | 86                  |         |

<sup>a</sup> χ² test.
Fig. 2. Representative immunohistochemical staining of colorectal cancers for \textit{p16} and \textit{p14}. A. Case 80. \textit{p16} staining was present in cancer cells that showed no \textit{p16} methylation. B. Case 83. \textit{p16} staining was absent in cancer cells that showed \textit{p16} methylation. C. Case 41. \textit{p14} staining was present in cancer cells that showed no \textit{p14} methylation. D. Case 76. \textit{p14} staining was absent in cancer cells that showed \textit{p14} methylation.

| Clinicopathological feature | Variable | No. of cases | \textit{p16} status | \textit{p14} status | \textit{p16} status | \textit{p14} status | \textit{p16} status | \textit{p14} status | \textit{p16} status | \textit{p14} status | P-value$^a$ |
|-----------------------------|----------|--------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|-----------|
| Age                         | 22–83    | 86           | 58.9±10.7$^{a1}$    | 59.2±12.0           | 60.5±11.0           | 61.3±11.9           | 0.71$^{a1}$         |
| Sex                         | male     | 51           | 8                   | 18                  | 9                   | 16                  | 0.58$^{b}$          |
|                             | female   | 35           | 4                   | 13                  | 4                   | 14                  |                     |
| Max. tumor size             | 15–100 mm| 86           | 37.5±13.7           | 51.5±19.5           | 51.7±21.0           | 61.3±18.8           | 0.022$^{a}$         |
| Extent of tumor             | ≤mt$^d$  | 22           | 5                   | 7                   | 3                   | 7                   | 0.31$^{b}$          |
|                             | mt$<$    | 64           | 7                   | 24                  | 10                  | 23                  |                     |
| Lymph node metastasis       | +        | 34           | 4                   | 13                  | 6                   | 11                  | 0.80$^{b}$          |
|                             | –        | 52           | 8                   | 18                  | 7                   | 19                  |                     |
| Dukes stage                 | A, B     | 53           | 9                   | 19                  | 8                   | 17                  | 0.48$^{b}$          |
|                             | C, D     | 33           | 3                   | 12                  | 5                   | 13                  |                     |
| Total                       |          | 86           | 12                  | 31                  | 13                  | 30                  |                     |

$^a$ Student’s \textit{t} test, $^b$ $\chi^2$ test, $^c$ mean±SD.
$^d$ Muscular tunic.
$^e$ \textit{p16}+\textit{p14}+ vs. others.
terms of sex, age, location, lymph node metastasis, the extent of tumor, or Dukes stage. However, we found a significant difference in maximal tumor size ($P = 0.022$) when we compared the patients with both $p16$ and $p14$ methylation to other patients (Table II). On the other hand, there was no significant difference in other factors such as the extent of tumor or Dukes stage. To confirm this result, we examined the relationship between tumor size and methylation status in a group of tumors with lymph node metastasis ($P = 0.026$; Table III). The results suggested that colorectal cancer with both $p16$ and $p14$ methylation has the same invasiveness at a smaller size, compared to that of the cancer without either $p16$ or $p14$ methylation.

**DISCUSSION**

Colorectal cancer, one of the most aggressive cancers, occurs with a high incidence in most countries. The usual treatment is surgery and subsequent chemotherapy and radiotherapy. It is important to determine genetic alterations in the cancer as an approach to predicting the malignancy of the cancer.

Several tumor suppressor genes contain CpG islands in their promoters, a fact which has prompted many studies to investigate the role of methylation in silencing these genes. Many tumor suppressor genes show evidence of methylation silencing, which represents a new potential pathway for the deactivation of these genes.

Aberrant methylation of $p16$ associated with a loss of expression was first reported by Herman et al. This phenomenon may be analogous to homozygous deletion, leading to a lack of $p16$ expression and a selective growth advantage to tumor cells. $p14$ promoter was also methylated in colorectal cancers and cell lines while normal tissues, including colon, were completely unmethylated. In these cell lines, expression of the $p14$ transcript was assessed by RT-PCR and found to be lacking. Treatment of these cell lines with the demethylating agent 5-aza-2′-deoxycytidine restored the expression of the $p14$ transcript just as well as $p16$ gene expression.

Zheng et al. reported that $p14$ methylation was associated with female gender, greater age, proximal anatomic location, and poor differentiation, but not with stage at diagnosis. On the other hand, Shannon and Iacopetta examined the methylation status of 7 cancer-related genes including $p16$ and the association with clinicopathological features. They could find no significant correlation of methylation status with Dukes stage. In this study, there was no significant difference in the distribution of positive or negative methylation in either $p16$ or $p14$. However, we found that cancers with both $p16$ and $p14$ methylation showed a significantly smaller size compared to cancers without it, while other factors such as the extent of the tumor and lymph node metastasis were not correlated to the status of $p16$ and/or $p14$ methylation. Inactivation of both $p16$ and $p14$ genes may result in a malignant change in colorectal cancer cells that later become advanced cancers of a smaller size compared to cancers with $p16$ or $p14$ activity.

Recent studies have shown that it is possible to reverse epigenetic changes and to restore gene function to a cell. Treatment with DNA methylation inhibitors can restore the activities of $p16$ gene and decrease the growth rate of cancer cells. Thus, it might soon be possible to restore the function of tumor suppressor genes and to slow the rate of colorectal cancer progression by administration of drugs such as cytosine analogues.

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