Detection of Tumor DNA in Serum of Colorectal Cancer Patients

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We previously found p16 promoter methylation in DNA in the sera of 13 colorectal cancer patients out of 44 (30%) whose tumor DNA exhibited the methylation, using methylation-specific PCR (MSP). To examine whether the cancer detection rate could be improved by using a different tumor marker, we examined the K-ras status in 90 colorectal cancer patients using a mismatch ligation assay. Among the 31 patients showing K-ras gene mutations in their tumors, the same mutations were observed in serum DNA of 11 patients (35%). Among the 90 patients, 63 showed tumors positive for K-ras mutation or p16 promoter methylation, or both, and 22 had serum DNA positive for one or both. K-ras mutation was found even in serum DNA of patients with Dukes A cancer, suggesting that colorectal cancer might be detected even in patients without symptoms by using this ligation assay.

Key words: K-ras — Colorectal cancer — Mismatch ligation assay

Colorectal cancer is among the most common and fatal cancers throughout the world. Since this kind of cancer can be eliminated by surgical excision if diagnosed at an early stage, developing screening methods to detect these cancers is an urgent matter. Clinically, invasive examinations such as colonography and colonoscopy have been performed, but they are burdensome and expensive. Fecal occult blood has been used as a noninvasive test for the detection of this disease, but the appearance of hemoglobin in stool is not specific for neoplasm. Therefore, we are interested in developing reliable and specific molecular genetic tests for the detection of colorectal cancer.

Recently, several reports have indicated that p16 methylation is a tumor-specific epigenetic change, and that the tumor DNA can be detected in bodily fluids. We then examined p16 methylation in the sera of 94 colorectal cancer patients using a methylation-specific PCR (MSP) method, and found that 13 of 44 (30%) patients with p16 promoter methylation in tumor DNA also exhibited abnormal methylation in their serum DNA. Moreover, abnormal methylation was found in the sera of patients in all clinical stages, suggesting that early colorectal cancer could be detected, as proposed previously. Our results suggested that p16 methylation is a useful marker for colorectal cancer detection. To increase the cancer detection rate, we needed to combine other tumor markers or detection methods. Hibi et al. previously showed that either K-ras or p53 mutation was detected in the serum in 40% of 25 patients by using a mismatch ligation assay. In their small study, p53 mutation was used as a target for tumor detection, but this approach is limited by the large number of different mutations identified in the p53 gene. On the other hand, it is not necessary to know the K-ras gene status in primary tumors in advance, because K-ras mutation is mostly limited to within codon 12 or 13. Therefore, we applied K-ras mutation as a tumor marker in our larger study.

In this report, we first examined K-ras mutation in 90 colorectal cancer samples using a mismatch ligation assay. We next examined whether the same K-ras mutation could be detected in the serum DNA of these cancer patients. The results obtained were compared with the clinicopathological features.

MATERIALS AND METHODS

Sample collection and DNA preparation Ninety primary tumors were collected at the Nagoya University School of Medicine from 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. Corresponding serum samples were obtained from the same patients about 1 week before surgery and stored at −80°C. Tumor and serum DNAs were prepared as described previously. We confirmed that the prepared DNA was of good quality by PCR amplification of the β-actin gene.

Mismatch ligation assay Mismatch ligation assay was performed as described previously. Briefly, the first exon of K-ras was amplified, and used as the template for mismatch ligation assay in all tumor samples. For each ligation assay, 50 ng of PCR product was mixed with 8 ng each of three mutation-specific oligomers, 200 ng of blocking oligomer, and 8 ng of a common 32P-labeled oligomer in a volume of 20 µl containing 150 mM NaCl, 10

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mM MgCl₂, 100 mM Tris-HCl (pH 7.5), 1 mM spermidine, 1 mM dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP), and 3 μg of T4 gene 32 protein (Boehringer Mannheim, Mannheim, Germany). This mixture was denatured at 95°C for 5 min and allowed to cool at room temperature for 15 min, at which time 1 unit of T4 ligase was added. The ligation was carried out at 37°C for 1 h and terminated by heat inactivation at 68°C for 10 min. The [32P]phosphate on the unligated 3′ oligomer was removed by the addition of 1 unit of alkaline phosphatase and subsequent incubation at 37°C for 30 min. The ligation products were separated on 12% denaturing polyacrylamide gel. The presence and the nature of mutations were determined based on the relative migration of the ligation products formed in control experiments using templates with known K-ras mutations. Oligonucleotide sequences used for the ligation assays were: 12b-Ala, 5′-TGGAGCTGC-3′; 12b-Asp, 5′-TGGAGCTGA-3′; 12b-Val, 5′-GTTGAGCTGT-3′, and 12b-common 5′-TGGCGTAGG-3′. The 11-mer spanning the ligation junction used as a blocking oligomer was 5′-TGGTGCGTAG-3′(12N). When a plasma sample was found to be positive, DNA extraction and PCR reaction were repeated at least once from the original plasma samples to confirm the presence of the mutant allele.

RESULTS

We first examined the K-ras status in colorectal cancers using mismatch ligation assay. Of 90 specimens of colorectal cancer DNA, 31 (34%) exhibited K-ras mutations. This result confirms that many colorectal cancers have K-ras gene mutations that may play an important role in the tumorigenic pathway of colorectal cancer, as previously described. The results are also consistent with the finding that K-ras mutation is an early event in tumorigenesis. Subsequently, we tested the sera of the 90 colorectal cancer patients for the presence of K-ras mutation. Of the 31 patients showing K-ras gene mutations in their tumors, the same mutations were observed in the sera of 11 patients (35%; Fig. 1). We also examined serum samples of 59 patients whose primary tumors had no K-ras gene mutations, and found that their serum DNA exhibited no mutations.

After completion of the mismatch ligation assay of all specimens, clinicopathological data were correlated with the results of molecular analysis (Table I). There was no significant difference in the distribution of patients positive or negative for K-ras mutation in serum DNA in terms of Dukes stage, tumor size, extent of tumor, lymph node metastasis, or vital status. Though no parameter was correlated with the presence of K-ras mutation in serum, this mutation was found even in the serum DNA of patients with Dukes A cancer, suggesting that colorectal cancer might be detected in patients without symptoms by using this ligation assay.

DISCUSSION

The results of this study show that mutant DNA circulates in the plasma of some patients with colorectal cancer, and can be detected sensitively using the mismatch ligation assay. Several investigators have reported the detection of mutant K-ras DNA in plasma or sera of patients with colorectal cancer. However, most dealt with a small number of samples and employed PCR, with the attendant risk of a false-positive result. Mismatch ligation assay, depending on a simple ligase-mediated reaction, can reduce this risk and the sensitivity of the assay, with radioisotope labeling, is comparable to that of PCR. However, we should bear in mind the possibility that K-ras mutations detected might be derived from other diseases, such as lung cancer and chronic pancreatitis.

Previous studies in cancer patients have suggested that those with genetic alterations in serum or plasma DNA are

| Clinicopathological feature | Variable | Mutation in serum DNA | P value<sup>a</sup> |
|-----------------------------|----------|-----------------------|-------------------|
| Dukes stage                 | A        | 3                     | 0.9364            |
|                             | B        | 4                     |                   |
|                             | C        | 4                     |                   |
| Size (max.)                 | <5 cm    | 7                     | 0.4578            |
|                             | 5 cm≤    | 4                     | 0.4524            |
| Extent of tumor             | ≤m.t.<b> | 5                     |                   |
|                             | m.t.<    | 6                     | 14                |
| Lymph node metastasis       | +        | 4                     | 0.6828            |
| Vital status                | alive    | 8                     | >0.9999           |
|                             | dead     | 3                     | 6                 |
| Total                       |          | 11                    | 20                |

<sup>a</sup> Fisher’s exact test.

<sup>b</sup> Muscular tunic.
Table II. Number of Positive Cases and Detection Rate of Colorectal Cancer DNA in Serum of 90 Colorectal Cancer Patients

| Genetic alterations     | No. of positive cases in tumor | No. of positive cases in serum | Detection rate (%) | Specificity (%) |
|------------------------|-------------------------------|-------------------------------|--------------------|-----------------|
| K-ras                  | 31                            | 11                            | 35                 | 100             |
| p16 methylation        | 43                            | 13                            | 30                 | 100             |
| Either of the above    | 63$^b$                       | 22$^a$                        | 35                 | 100             |

$^a$ Ref. 7.
$^b$ Eleven cases showed both alterations in tumor.
$^c$ Two cases showed both alterations in serum.

more likely to develop metastases and die of their disease. In this small study, we could not detect any association between the existence of tumor DNA in serum and clinicopathological features. Further studies are needed to determine the clinical relevance of identifying specific genetic alterations in the serum DNA of colorectal cancer patients for the prognosis and monitoring of the disease.

It should be possible to increase the detection rates of cancer DNA in serum by combining tumor markers or detection methods. Previously, we examined p16 methylation in the sera of colorectal cancer patients using the MSP method and found that 13 of 44 (30%) patients with this methylation in tumor DNA exhibited the same methylation in their serum DNA (Table II). In this paper, we examined the mutant K-ras DNA in the sera of colorectal cancer patients using a mismatch ligation assay. The detection rate of cancer DNA in the serum was increased to 22 of 90 colorectal cancer patients (Table II). Another possible approach to increase the detection rate is to improve the sensitivity of the detection methods. Recently, Traverso et al. reported that a digital-PCR-based method detected mutational DNA derived from colorectal cancer. Surprisingly, colorectal cancer DNA with genetic alterations was detected from fecal DNA samples of more than 90% of colorectal cancer patients. This approach could be applied to our MSP and mismatch ligation assay to increase the sensitivity.

Although much work remains to be done, molecular detection of genetic alterations in cancer DNA can be used to identify early cancers. Further, it may be possible for this assay to be converted to a quantitative format to measure circulating tumor DNA. It would be interesting to study the variation in circulating tumor DNA levels in relation to cancer treatment and patient prognosis.

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