Molecular Detection of p16 Promoter Methylation in the Serum of Patients with Esophageal Squamous Cell Carcinoma

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ABSTRACT

Purpose and Experimental Design: Recent evidence shows that the presence of promoter hypermethylation of tumor suppressor genes has been demonstrated in the serum DNA of patients with various cancers such as lung, liver, and head and neck cancer. We have examined promoter hypermethylation of the p16 gene in esophageal squamous cell carcinoma (SCC) using methylation-specific PCR to detect tumor DNA in the serum.

Results: Aberrant promoter methylation of the p16 gene was detected in 31 of 38 (82%) esophageal SCCs. Subsequently, we tested for promoter methylation in the paired serum DNA of 31 patients with a p16 alteration in the primary tumor. We found that 7 of these 31 (23%) patients had the same methylation changes in the serum DNA.

Conclusions: This result indicates that promoter methylation present in the tumors of esophageal SCC patients can be detected in the serum of the same patient and that this approach can potentially be used for the screening and monitoring of the disease.

INTRODUCTION

Esophageal SCC is one of the most aggressive cancers in the world (1). To save the lives of patients with this fatal disease, surgery, subsequent chemotherapy, and radiotherapy are used. Methods to detect surgically resectable tumors could significantly reduce deaths from this disease. Accumulating evidence indicates that a series of genetic changes in dominant oncogenes such as cyclin D1 and hst1/int2 and tumor suppressor genes such as p53 and p16 are involved in the pathogenesis of human esophageal SCC (2–5). The identification of these genetic changes at sites away from the primary tumor may help to assess the extent of disease and overall tumor burden at the time of initial diagnosis. Previous studies have proposed that tumor DNA is released into the circulation and enriched in plasma and serum (6, 7). Radioimmunoassays have revealed that the serum of cancer patients contains approximately four times the amount of free DNA compared with normal control (8). Based on these observations, studies have shown that it is possible to detect tumor-specific DNA in the serum of colorectal cancer patients using a mismatch ligation assay (9).

Recently, a tumor suppressor gene, p16, has been found to harbor promoter hypermethylation associated with loss of protein expression in cancer cells (10). The presence of epigenetic methylation might be useful as a molecular target for tumor cell detection. In fact, the presence of gene promoter hypermethylation has been demonstrated in the serum DNA of patients with various cancers such as lung, liver, or head and neck cancer (11–13).

These results prompted us to examine p16 methylation in the serum DNA of esophageal SCC patients because p16 inactivation is an important factor for tumorigenesis in the esophagus (14), and many esophageal SCCs would have aberrant p16 promoter methylation. In this study, we have examined the methylation status of the CpG island in exon 1 of the p16 gene in esophageal SCC using MSP because this area is functionally relevant to the promoter of the p16 gene and is frequently methylated (10). As expected, most esophageal SCCs (82%) showed aberrant methylation. Of the 31 cases with hypermethylation in tumors, 7 cases exhibited the same alteration in the matched serum DNA. This result indicates that promoter methylation present in the tumors of esophageal SCC patients can be detected in the serum of the same patient and that this approach can potentially be used for cancer monitoring of this deadly disease.

MATERIALS AND METHODS

Sample Collection and DNA Preparation. Thirty-nine primary tumors were collected at the Nagoya University School of Medicine from esophageal cancer patients who had been diagnosed histologically. All esophageal cancers were SCCs. 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 samples were digested overnight by proteinase K, and DNA was prepared by extraction with phenol.

Bisulfite Modification. DNA from tumor and serum specimens was subjected to bisulfite treatment as described previously. One μg of DNA was denatured by NaOH and modified by sodium bisulfite (15, 16). DNA samples were then purified using the Wizard purification resin (Promega Corp.), treated again with NaOH, precipitated with ethanol, and resuspended in water.

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2 The abbreviations used are: SCC, squamous cell carcinoma; MSP, methylation-specific PCR.
MSP. The modified DNA was used as a template for MSP. Primer sequences used for amplification of p16 have been described previously (10). The primers for the unmethylated reaction were: (a) p16UMS (sense), 5'-TTATAGGGTG-GGGTGATTGT; and (b) p16UMAS (antisense), 5'-CAAC-CCCAAACCACAACCATAA. The primers for the methylated reaction were: (a) p16MS (sense), 5'-TTATAGGGTG-GGGGGGATCGC; and (b) p16MAS (antisense), 5'-GAC-CGCCAGAACCACCTGAAA. 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; and 1 cycle of 72°C for 5 min. DNA from L132 (embryonic lung cell line) and H1299 (lung cancer cell line) cells was used as a positive control for unmethylated and methylated alleles, respectively. Controls without DNA were performed for each set of PCR. Ten μl of each PCR product were loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. Each MSP was repeated at least three times.

![Fig. 1](Representative MSP of the p16 promoter in esophageal SCC and the corresponding serum samples. The presence of a visible PCR product in Lanes U indicates the presence of unmethylated genes; the presence of product in Lanes M indicates the presence of methylated genes in all three cases (cases 4, 9, and 32). In each case, modified DNA from L132 and H1299 cells was used as a positive control for unmethylated and methylated alleles, respectively.){/reference}

**Statistical Analysis.** Fisher’s exact test was used to examine the association between p16 promoter methylation and clinicopathological features.

**RESULTS**

We first examined the methylation status of the p16 promoter in tumors using the MSP technique. Aberrant promoter
methylated the p16 gene was detected in 31 of 38 (82%) esophageal SCCs. This result indicated that most esophageal SCCs examined had the p16 aberrant methylatation that may have an important role in SCC of the esophagus. It also indicated that p16 methylation may be a good marker to detect esophageal SCC DNA in serum because of the high methylation rate in tumors.

Subsequently, we tested for promoter methylation in the paired serum DNA of 31 of the patients with a p16 alteration in the primary tumor. We found that 7 of the 31 (23%) patients had the same methylation changes in their serum DNA. A representative MSP analysis for p16 gene promoter methylation from tumors and paired sera is shown in Fig. 1. As a control, we screened for aberrant methylation in the serum DNA of 7 patients with esophageal SCC and 40 patients with colorectal cancers whose corresponding tumor DNA had no methylation in the p16 promoter. No methylation was found in the serum DNA of this group control.

After completion of MSP analysis in all specimens, clinicopathological data were correlated with the molecular analysis (Table 1). We found no association between overall aberrant methylation in the tumor and serum DNA and the size of the tumor, stage of the disease, presence of lymph node metastasis, or prognosis. These results are summarized in Table 2.

DISCUSSION

In a previous study, Hibi et al. (9) suggested that K-ras or p53 mutation could be detected in the circulating tumor DNA from the serum of colorectal cancer patients using a mismatch ligation assay. Based on the method described in that study, such screenings are limited by the large number of different mutations identified in the p53 gene. Therefore, we searched for a technique that would allow detection of tumor DNA in serum without requiring advance knowledge of the position of genetic alterations.

Several tumor suppressor genes contain CpG islands in their promoters, prompting many studies investigating the role of methylation in silencing these genes. Many tumor suppressor genes show evidence of methylation silencing, providing a new potential pathway for tumor suppressor gene inactivation (17). Recent studies have demonstrated the presence of gene promoter methylation in the serum DNA of lung, liver, and head and neck cancer patients (11–13). These studies commonly used p16 promoter methylation as a tumor marker for MSP analysis because no abnormal methylation was found in serum DNA if this alteration was not present in the primary tumor. Moreover, MSP has sufficient sensitivity to detect scanty tumor DNA in serum; it can detect nearly 1 methylated gene copy/1000 unmethylated copies in dilution experiments (11).

Our results have two potential clinical applications: (a) patients at high risk for esophageal SCC could be screened for the presence of tumor cells in the serum by MSP technique. p16 methylation may be helpful in monitoring of multiple tumor types because inactivation of p16 by methylation is a common feature in human neoplasia (18); and (b) screening could also be used in the follow-up of patients diagnosed with esophageal SCC.

Given the facts that esophageal SCC is the most aggressive cancer in the world and there is no noninvasive test available, our preliminary study yielded a promising result: a tumor-associated DNA alteration could be detected in the serum of 18% of esophageal SCC patients (7 of 38 patients) using p16 methylation as a target. Moreover, the clinical sensitivity of this assay can be potentially improved by incorporating other possibly methylated targets such as MGMT, GSTP1, and DAP kinase genes. Previous studies in cancer patients have suggested that those with genetic alterations in serum or plasma are more likely to develop metastases and die of their disease (13, 19). In this small study, we could not detect any association between the existence of tumor DNA in the serum and clinicopathological features. Additional studies are needed to determine the clinical relevance of identifying specific genetic alterations in the serum of esophageal SCC patients for the prognosis and monitoring of the disease.

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