Molecular Detection of Circulating Esophageal Squamous Cell Cancer Cells in the Peripheral Blood

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ABSTRACT

Purpose and Experimental Design: To detect surgically resectable tumors earlier and improve the prognosis of esophageal squamous cell carcinoma patients, we examined ΔNp63 expression that was specific for squamous cell carcinoma in blood samples obtained from 43 esophageal cancer patients.

Results: Seventeen of 33 (52%) patients with primary esophageal squamous cell carcinoma and 6 of 10 (60%) patients with postoperative recurrent squamous cell carcinoma had detectable ΔNp63 expression in their peripheral blood using ΔNp63-specific reverse transcription-PCR. Furthermore, ΔNp63 is a more sensitive marker compared with other commonly used tumor markers such as squamous cell carcinoma-associated antigen and carcinoembryonic antigen.

Conclusions: This approach would be potentially useful for the monitoring of patients with this aggressive disease.

INTRODUCTION

Esophageal squamous cell carcinoma remains an aggressive cancer with a poor prognosis despite the recent progress in diagnostic procedures and multidisciplinary treatments (1). To detect surgically resectable tumors earlier and improve the prognosis of patients with this disease, new diagnostic procedures may be needed. Accumulating evidence indicates that a series of genetic changes in dominant oncogenes, such as cyclin D1 and int2/hst1, and tumor suppressor genes, such as p53 and p16, are involved in the pathogenesis of esophageal squamous cell carcinoma (2–5). The identification of these genetic changes at sites away from the primary tumor may help with early diagnosis of disease and assessment of the overall tumor burden at the time of initial diagnosis.

Previously, we demonstrated the usefulness of blood screening tests to detect tumor-specific DNA in serum using sensitive molecular biology techniques (6). A tumor screening test with blood samples is convenient and less invasive compared with other conventional methods such as barium swallow X-rays. With regard to squamous cell carcinoma of the esophagus, we have examined promoter hypermethylation of the p16 gene using methylation-specific PCR and found this methylation change in the serum DNA of 23% of esophageal squamous cell carcinoma patients with the same methylation in the primary tumors (7).

Recently, we have shown that the ΔNp63 (AIS) gene, a p53 homologue, is overexpressed and plays an oncogenic role in squamous cell carcinomas of the lung, head and neck, and esophagus (8, 9). Preliminary studies have revealed that all esophageal squamous cell carcinomas express the ΔNp63 protein, suggesting that ΔNp63 might be a good marker for detection of squamous cell carcinoma. Therefore, we postulated that RT-PCR2 of ΔNp63 should be a useful assay for the detection of cancer cells circulating in the peripheral blood.

In this study, we assayed blood samples obtained from 43 esophageal squamous cell carcinoma patients for ΔNp63 expression. Seventeen of 33 (52%) patients with primary esophageal squamous cell carcinoma and 6 of 10 (60%) patients with postoperative recurrent squamous cell carcinoma had detectable circulating squamous cancer cells in their peripheral blood using ΔNp63-specific RT-PCR. This detection rate was higher than that of the more commonly used tumor markers, SCC and CEA. This approach could be potentially useful for the monitoring of patients with this aggressive disease.

MATERIALS AND METHODS

Sample Collection. Blood samples were obtained from 33 consecutive patients with primary esophageal squamous cell carcinoma (including 23 surgically resected cases and 10 non-resected cases) and 10 postoperative patients with recurrent esophageal squamous cell carcinoma. These samples were collected at the time of admission to the Second Department of Surgery, Nagoya University School of Medicine from October 2000 to October 2001. Other blood samples were collected from the 23 resected cases 1 week after surgery. Tumor samples were also collected from these 23 cases to assess ΔNp63 expression by immunohistochemistry. As a control, tumor and blood samples were collected from 30 age-matched patients with advanced gastric or colorectal cancer who underwent a surgical resection of the tumor over the same period. Pathological TNM stages of control patients were as follows: 4 had stage I disease; 13 had stage II disease; and 13 had stage III disease. Oral or written informed consent, as indicated by the institutional review board, was obtained from all patients.

2 The abbreviations used are: RT-PCR, reverse transcription-PCR, CEA, carcinoembryonic antigen, SCC, squamous cell carcinoma-associated antigen; TNM, tumor-node-metastasis.
Immunohistochemistry. Sections 6-μm thick were cut from paraffin tissue blocks, and the slides were dried at 60°C for 30 min, treated with xylene, and then dehydrated in alcohol. Endogenous peroxidase was blocked with 0.3% H2O2. After blocking with normal goat serum, the slides were incubated with a mouse monoclonal antibody against p63 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:400 dilution for 1 h at room temperature. A Vectastain ABC Kit and DAB Substrate Kit (Vector Laboratories, Burlingame, CA) were used to visualize the antibody binding.

RNA Preparation and RT-PCR. From the blood cells obtained from 10 ml of peripheral blood from the esophageal squamous cell carcinoma patients, total RNA was isolated using Trizol reagent (Life Technologies, Inc., Bethesda, MD). First-strand cDNA was generated from RNA as described previously (10). The PCR amplification consisted of 35 cycles (95°C for 30 s, 55°C for 1 min, and 72°C for 1 min) after an initial denaturation step (95°C for 2 min). The primers used were as follows: AIS-S (sense), 5'-GCAGCATTGATCAATCTTAGCAG; and AIS-AS (antisense), 5'-TGAATTCACGGCTCAT. The predicted size of the PCR product from the Np63 gene was 505 bp. Each RT-PCR was repeated at least three times. Ten μl of each PCR product were directly loaded onto nondenaturing 1% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

Statistical Analysis. The χ2 test was used to examine the association between ΔNp63 expression and various clinicopathological features.

RESULTS

We first investigated the presence of ΔNp63 protein in primary esophageal cancer using immunohistochemistry. Interestingly, ΔNp63 was positively stained in all 23 (100%) surgically resected specimens of primary esophageal cancer (Fig. 1).

This result suggests that ΔNp63 may be expressed ubiquitously in esophageal squamous cell carcinomas and might be a marker for the detection of this tumor cell. On the other hand, no control tumors (15 gastric cancers and 15 colorectal cancers) had ΔNp63 expression.

We next assayed for ΔNp63 gene expression in peripheral blood, which might include tumor cells, by ΔNp63-specific RT-PCR. Of 33 blood samples from patients with primary esophageal squamous cell carcinoma, 17 (52%) exhibited ΔNp63 expression, suggesting that ΔNp63 is a good marker to detect esophageal squamous cell carcinoma cells in the blood (Fig. 2). Additionally, ΔNp63 mRNA was also detected in blood samples from 6 of 10 (60%) patients with recurrent esophageal cancers. As a control study, we tested blood samples from 30 gastric or colorectal cancer patients for expression of ΔNp63. We observed no ΔNp63 expression from these samples, suggesting that our RT-PCR assay is highly specific for this purpose.
Approximately 30 years ago, the possibility of detecting circulating neoplastic cells in the blood was explored with the hope of establishing a sensitive and noninvasive tumor surveillance test (11, 12). However, the sensitivity and specificity of these early detection methods were poor; therefore, they could not be used clinically. Recent progress in biomolecular techniques may have changed this situation. PCR assays in particular have been shown to be able to detect minimal amounts of neoplastic cells. To date, several molecular targets, such as CEA and cytokeratins, have been reported to be biological markers for squamous cell carcinomas of the esophagus (13, 14). These proteins are specifically expressed in esophageal squamous cell carcinoma and were postulated to be useful for detecting tumor burden at the time of initial diagnosis. However, the expression of these molecules was so weak that nested PCR analysis was needed for their detection. Although the nested PCR assay is an extremely sensitive method, several studies have reported a high false positive rate when using this assay for the detection of these molecular targets (15–17). Therefore, other molecular targets abundantly expressed in tumor cells need to be explored so that nested PCR assay may not be required.

Recently, Hibi et al. (9) demonstrated that the ΔNp63 (AIS) gene is amplified and plays an oncogenic role in human cancers (8). Moreover, we found that the ΔNp63 gene is overexpressed in esophageal cancers. We first confirmed the protein expression of ΔNp63 in all primary esophageal cancer specimens by an immunohistological method. These results suggested that the ΔNp63 gene might be a useful molecular marker for esophageal cancer. On the basis of these findings, we tried to detect circulating tumor cells and found them frequently in the blood of esophageal cancer patients using ΔNp63-specific RT-PCR. On the other hand, no blood specimens from control patients had detectable ΔNp63 gene expression.

For monitoring of squamous cell carcinoma in the esophagus, SCC and CEA have been used as tumor markers in the serum. To evaluate the usefulness of ΔNp63 as a tumor marker, SCC and CEA were also investigated in this series, and the detection rates of esophageal cancer by these tumor markers were compared. Our results indicated that the detection rate of esophageal squamous cell carcinoma was higher with ΔNp63-specific RT-PCR than with SCC or CEA; therefore, RT-PCR for ΔNp63 in blood cells might be a useful method for tumor diagnosis. Although studies with a large cohort of patients are required to confirm its usefulness, this method was sensitive and may be specific enough for the monitoring of esophageal cancer.

Besides the diagnosis and monitoring of cancer, the detection of circulating tumor cells may also reflect the biological activity of the tumor in the primary site. Some reports have indicated that the presence of circulating tumor cells was correlated with clinical outcome (14, 18, 19), but other reports have disagreed with this conclusion (20, 21). In this study, we could not find a significant correlation between the presence of ΔNp63 in the blood and various clinical factors. This result may have

### Table 1

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*NS was determined by χ² test.

### DISCUSSION

Subsequently, we examined postoperative blood samples from 23 patients who underwent surgical resection of esophageal squamous cell carcinoma (Fig. 2). Of the 12 patients whose blood samples were positive for ΔNp63 gene expression preoperatively, 9 had no detectable ΔNp63 expression in their postoperative blood specimen, indicating that the esophageal squamous cell carcinomas were successfully removed from these patients. However, it is possible that there were micrometastases not shedding tumor cells because advanced esophageal cancer has generally shown poor prognosis. The other three patients still had detectable ΔNp63 expression in their blood samples. Two of these patients had only a palliative resection, but the third patient, whose primary esophageal squamous cell carcinoma was pathologically diagnosed as TNM stage II, underwent a curative resection. This patient possibly had residual tumor elsewhere, and close follow-up is certainly indicated for this patient to detect any recurrence of esophageal squamous cell carcinoma. Until now, 5 of 23 surgically resected patients exhibited recurrent tumors, and 4 of these 5 patients were positive for ΔNp63 gene expression preoperatively.

The clinicopathological data of the 33 patients with primary esophageal cancers were correlated with ΔNp63 expression in their blood. There was no significant difference in the distribution of the clinicopathological features of the patients with or without ΔNp63 gene expression, suggesting that tumor cells may be released into the blood from esophageal squamous cell carcinoma even during the early clinical stages (Table 1).

Finally, we compared this novel marker, ΔNp63, with two more commonly used tumor markers; SCC and CEA. SCC was positive in the serum of 7 of 33 (21%) patients with primary esophageal squamous cell carcinoma and 4 of 10 (40%) patients with recurrent esophageal squamous cell carcinoma. CEA was positive in the serum of 2 of 33 (6%) patients with primary esophageal squamous cell carcinoma and 1 of 10 (10%) patients with recurrent esophageal squamous cell carcinoma. These results suggested that ΔNp63 may be a more sensitive marker of esophageal cancer than these two other tumor markers.
have been due to sampling bias from differences in tumor cell density in the blood because some investigators clearly indicate that tumor cells are intermittently shed into the peripheral blood (22, 23). Additional studies using a larger study population and quantitative RT-PCR for determining the number of circulating tumor cells might be needed to determine the relevance of identifying specific genetic alterations in the blood of esophageal squamous cell carcinoma patients and their correlation with clinicopathological features and/or prognosis.

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REFERENCES


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