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
\(\Delta Np63\) 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 carci-
noma had detectable \(\Delta Np63\) expression in their peripheral
blood using \(\Delta Np63\)-specific reverse transcription-PCR.
Furthermore, \(\Delta Np63\) is a more sensitive marker compared
with other commonly used tumor markers such as squa-
mous cell carcinoma-associated antigen and carcinoembry-
onic antigen.

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

INTRODUCTION

Esophageal squamous cell carcinoma remains an aggres-
sive 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 prog-
nosis 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 diag-
nosis 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 com-
pared with other conventional methods such as barium swallow
X-rays. With regard to squamous cell carcinoma of the esopha-
gagus, we have examined promoter hypermethylation of the p16
gene using methylation-specific PCR and found this methyla-
tion change in the serum DNA of 23\% of esophageal squamous

cell carcinoma patients with the same methylation in the pri-
mary tumors (7).

Recently, we have shown that the \(\Delta Np63\) (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 \(\Delta Np63\) pro-
tein, suggesting that \(\Delta Np63\) might be a good marker for detec-
tion of squamous cell carcinoma. Therefore, we postulated that
RT-PCR\(^2\) of \(\Delta Np63\) 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 \(\Delta Np63\) expres-
sion. 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
\(\Delta Np63\)-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 col-
lected 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 \(\Delta Np63\) expression
by immunohistochemistry. As a control, tumor and blood sam-
ple 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′-GCAGCATTGATCAATCTTACAG; and AIS-AS (antisense), 5′-TGAATTCCAGGCCTCAGCTCAT. 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 cells, 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.
Blood samples were positive for esophageal squamous cell carcinoma (Fig. 2). Of the 12 patients whose operative blood specimen indicated that the esophageal squamous cell carcinomas were successfully removed from these patients. However, it is possible that there were micrometastases not shielding 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 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.

### DISCUSSION

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 Clinicopathological features and ΔNp63 expression in the blood of primary esophageal squamous cell carcinoma patients

<table>
<thead>
<tr>
<th>Clinicopathological feature</th>
<th>Variable</th>
<th>No. of cases</th>
<th>ΔNp63/RNA expression</th>
<th>+</th>
<th>–</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>25</td>
<td>12</td>
<td>13</td>
<td>NS&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td>Female</td>
<td>8</td>
<td>5</td>
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<tr>
<td>Age</td>
<td>≤60</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>NS</td>
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<tr>
<td></td>
<td>&gt;60</td>
<td>18</td>
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<tr>
<td>Tumor size</td>
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<td>13</td>
<td>7</td>
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<td>&gt;5 cm</td>
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<td>Depth</td>
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<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Lymph node metastasis</td>
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<tr>
<td>TNM stage</td>
<td>Stage II</td>
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<tr>
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<td>III</td>
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<td>7</td>
<td>9</td>
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<td>IV</td>
<td>13</td>
<td>8</td>
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<td>SCC</td>
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<td>26</td>
<td>13</td>
<td>13</td>
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<td>CEA</td>
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<td>NS</td>
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<td>31</td>
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<sup>a</sup> P was determined by χ² test.  
<sup>b</sup> NS, not significant.
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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