

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 carcinoma 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 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 $\Delta Np63$ (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 $\Delta Np63$ protein, suggesting that $\Delta Np63$ might be a good marker for detection of squamous cell carcinoma. Therefore, we postulated that RT-PCR² 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 $\Delta Np63$ 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 $\Delta Np63$ -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 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 $\Delta Np63$ 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.

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² The abbreviations used are: RT-PCR, reverse transcription-PCR; CEA, carcinoembryonic antigen; SCC, squamous cell carcinoma-associated antigen; TNM, tumor-node-metastasis.

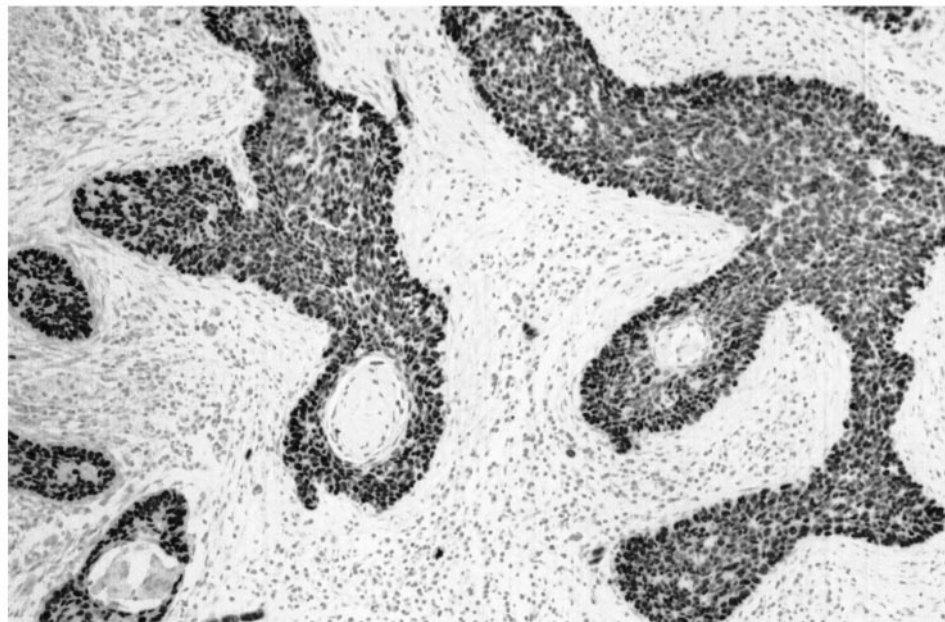


Fig. 1 $\Delta Np63$ expression in esophageal squamous cell carcinoma. $\Delta Np63$ protein was highly expressed in the nucleus of cancer cells, whereas no expression was observed in the surrounding normal cells. Original magnification, $\times 100$.

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% H_2O_2 . 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'-TGAATTCACGGCTCAGCTCAT. The predicted size of the PCR product from the $\Delta Np63$ 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 χ^2 test was used to examine the association between $\Delta Np63$ expression and various clinicopathological features.

RESULTS

We first investigated the presence of $\Delta Np63$ protein in primary esophageal cancer using immunohistochemistry. Interestingly, $\Delta Np63$ was positively stained in all 23 (100%) surgically resected specimens of primary esophageal cancer (Fig. 1).

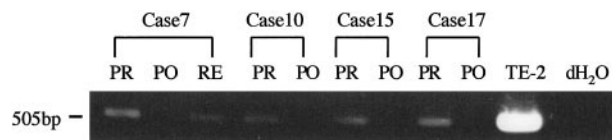


Fig. 2 Representative autoradiographs detecting $\Delta Np63$ gene expression in the peripheral blood by RT-PCR. All four patients had $\Delta Np63$ gene expression in their preoperative specimens (PR). After surgical resection, this expression diminished in their postoperative specimens (PO). In case 7, $\Delta Np63$ expression was detected again in the blood specimens (RE) when the recurrent tumor appeared in the upper mediastinum 10 months after surgery. In each case, cDNA from TE-2 cells and distilled H_2O ($d\text{H}_2\text{O}$) were used as positive and negative controls, respectively.

This result suggests that $\Delta Np63$ 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 $\Delta Np63$ expression.

We next assayed for $\Delta Np63$ gene expression in peripheral blood cells, which might include tumor cells, by $\Delta Np63$ -specific RT-PCR. Of 33 blood samples from patients with primary esophageal squamous cell carcinoma, 17 (52%) exhibited $\Delta Np63$ expression, suggesting that $\Delta Np63$ is a good marker to detect esophageal squamous cell carcinoma cells in the blood (Fig. 2). Additionally, $\Delta Np63$ 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 $\Delta Np63$. We observed no $\Delta Np63$ expression from these samples, suggesting that our RT-PCR assay is highly specific for this purpose.

Table 1 Clinicopathological features and $\Delta Np63$ expression in the blood of primary esophageal squamous cell carcinoma patients

Clinicopathological feature	Variable	No. of cases	$\Delta Np63$ RNA expression		P^a
			+	-	
Sex	Male	25	12	13	NS ^b
	Female	8	5	3	
Age	≤60	15	9	6	NS
	>60	18	8	10	
Tumor size	≤5 cm	13	7	6	NS
	>5 cm	20	10	10	
Depth	T ₂	4	2	2	NS
	T ₃	16	7	9	
	T ₄	13	8	5	
Lymph node metastasis	+	13	6	7	NS
	-	20	11	9	
TNM stage	Stage II	4	2	2	NS
	III	16	7	9	
	IV	13	8	5	
SCC	+	7	4	3	NS
	-	26	13	13	
CEA	+	2	1	1	NS
	-	31	16	15	

^a P was determined by χ^2 test.

^b NS, not significant.

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 $\Delta Np63$ gene expression preoperatively, 9 had no detectable $\Delta Np63$ 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 $\Delta Np63$ 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 $\Delta Np63$ gene expression preoperatively.

The clinicopathological data of the 33 patients with primary esophageal cancers were correlated with $\Delta Np63$ expression in their blood. There was no significant difference in the distribution of the clinicopathological features of the patients with or without $\Delta Np63$ 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, $\Delta Np63$, 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 $\Delta Np63$ 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 $\Delta Np63$ (AIS) gene is amplified and plays an oncogenic role in human cancers (8). Moreover, we found that the $\Delta Np63$ gene is overexpressed in esophageal cancers. We first confirmed the protein expression of $\Delta Np63$ in all primary esophageal cancer specimens by an immunohistological method. These results suggested that the $\Delta Np63$ 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 $\Delta Np63$ -specific RT-PCR. On the other hand, no blood specimens from control patients had detectable $\Delta Np63$ 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 $\Delta Np63$ 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 $\Delta Np63$ -specific RT-PCR than with SCC or CEA; therefore, RT-PCR for $\Delta Np63$ 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 $\Delta Np63$ in the blood and various clinical factors. This result may 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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