Plasminogen Activator Inhibitor-1 as a Potential Marker for the Malignancy of Esophageal Squamous Cell Carcinoma

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ABSTRACT
Purpose and Experimental Design: To test whether plasminogen activator inhibitor-1 (PAI-1) can serve as a candidate marker for the malignancy of esophageal squamous cell carcinoma (SCC), we performed a quantitative reverse transcription-PCR for PAI-1 gene and evaluated the possible relationship between PAI-1 gene expression levels and clinicopathological findings in esophageal SCC.

Results: Significant increases in PAI-1 scores were observed in metastasis-positive esophageal SCCs (3.08 ± 0.80) compared with metastasis-negative ones (−0.31 ± 0.62; P = 0.0042). PAI-1 expression scores significantly increased with tumor stage (P = 0.05, ANOVA).

Conclusions: These results suggested that PAI-1 might serve as a new parameter for prediction of prognosis in esophageal SCC.

INTRODUCTION
Esophageal squamous cell carcinoma (SCC) is one of the most aggressive of all cancers. Accumulating evidence indicates that a series of genetic changes in dominant oncogenes such as cyclic D1 and hst/int 2 and tumor suppressor genes such as p53 and p16 are involved in the pathogenesis of human esophageal SCC (1–4). It is still important to search for novel genetic changes that might indicate the malignancy of esophageal SCC.

Plasminogen activator inhibitor-1 (PAI-1) is a multifaceted proteolytic inhibitor that not only functions as a fibrinolytic inhibitor but also plays an important role in signal transduction, cell adherence, and cell migration. There is clinical evidence implicating PAI-1 as a key factor in tumor invasion and metastasis (5). Moreover, PAI-1 has been linked to poor prognosis in several cancers (6–11). These results prompted us to examine the PAI-1 expression level in esophageal SCC.

To test the hypothesis that PAI-1 may serve as a candidate marker for the malignancy of esophageal SCC, we performed quantitative reverse transcription-PCR and evaluated the relationship between PAI-1 gene expression levels and clinicopathological findings in esophageal SCC.

MATERIALS AND METHODS
Patients and Tissue Specimens. The study group consisted of 49 esophageal SCC patients (mean age, 63.3 years; range, 50–77 years) who underwent surgery at Gastroenterological Surgery, Nagoya University Graduate School of Medicine between 1994 and 2002.

All tumors and corresponding normal tissues were collected at surgical resection and stored at −80°C. They were graded according to tumor-node-metastasis (TNM) stage as follows: 4 had stage I disease; 18 had stage II disease; 25 had stage III disease; and 2 had stage IV disease. The patients were classified into two groups according to age, sex, macroscopic type, pathological type, tumor location, tumor size, depth of tumor invasion, lymph node metastasis, and TNM stage.

RNA Preparation and Reverse Transcription. Total RNA was extracted from esophageal SCC and corresponding normal tissues with guanidinium thiocyanate as described previously (12). The amount of RNA was measured spectrophotometrically by absorbance at 260 nm. First-strand cDNA was generated from RNA as described previously (13).

Quantitative PCR. Quantitative PCR was performed in an ABI sequence detection system 7000 using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Thermocycling was done in a final volume of 50 μl containing 2.0 μl of the cDNA sample, 1.0 μl each of the PAI-1 primers (forward and reverse), and 25 μl of Mix SYBR Green I/Enzyme (including Taq DNA polymerase, reaction buffer, and deoxyribonucleotide triphosphate mixture). The PAI-1 primers for quantitative PCR were described previously (14). The PCR amplification consisted of 50 cycles (95°C for 15 s, 60°C for 60 s, and 72°C for 18 s) after an initial denaturation step (95°C for 10 min). To correct for differences in both quality and quantity between samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. GAPDH primers were purchased from Applied Biosystems. PAI-1 and GAPDH mRNA variability was determined from triplicate samples. The quantity of all triplicate samples was in error by <10%. We applied an average quantity of triplicate samples. The targets were obtained from the same mRNA preparations.

PAI-1 Expression Score. We calculated the relative amounts of esophageal SCC (T) and corresponding normal tissue (N) mRNA that were normalized to an internal control GAPDH mRNA. Because of the high ratio of the relative amount of T/relative amount of N, we applied the logarithmic scale to understand it more easily, as described previously (15, 16). We defined the PAI-1 expression score as follows: log2 (relative amount of T/relative amount of N).
**Statistical Analysis.** Differences between the means of analyzed variables observed were calculated by Student’s t test. The significance in correlations between tumor stages and variables was determined by one-way ANOVA. \( P < 0.05 \) (two-tailed) was considered significant. Survival rates were calculated by the Kaplan-Meier method for analysis of censored data.

**RESULTS**

We analyzed PAI-1 expression levels in 49 esophageal SCC samples using a quantitative reverse transcription-PCR. The mRNA concentrations were determined after extensive optimization of PCR conditions, including MgCl\(_2\) concentrations, reaction temperature, and cycling times. This provided us with a highly sensitive, specific, and reproducible real-time reverse transcription-PCR for specific detection of these mRNAs.

Fig. 1 shows the histogram of the PAI-1 expression score described in “Materials and Methods.” The average was 1.77 ± 0.59. Fig. 2 shows the differences in PAI-1 expression scores according to lymph node metastasis. A significant increase in the PAI-1 score was observed in metastasis-positive esophageal SCCs (3.08 ± 0.62; \( P = 0.0042 \)). As shown in Fig. 3, the PAI-1 expression score increased significantly with tumor stage (stage I, −2.09 ± 2.16; stage II, −0.83 ± 0.79; stage III, 3.99 ± 0.65; stage IV, 5.05 ± 0.65 (\( P = 0.05 \), ANOVA)). These results are summarized in Table 1.

We then examined the cumulative survival of patient groups according to PAI-1 expression score (\( > 2 \) or \( \leq 2 \)). Interestingly, the high PAI-1 expression score group showed significantly worse survival rates than the low PAI-1 expression score group (Fig. 4; \( P = 0.002 \)).

To confirm the prognostic significance of the PAI-1 expression score, other clinicopathological variables that might affect survival were further analyzed by Cox regression analysis. In univariate analysis, the pathological type (\( P = 0.0015 \)), depth of tumor invasion (\( P = 0.0028 \)), lymph node metastasis (\( P < 0.0001 \)), and PAI-1 expression score (\( P = 0.0007 \)) were significantly correlated with survival (Table 2).

**DISCUSSION**

The plasminogen activation system plays a role in cancer progression, presumably via extracellular matrix degradation and tumor migration (17). It is generally believed that serine protease, a urokinase-type plasminogen activator, at the cell surface initiates a proteinase cascade and promotes tumor invasion and angiogenesis. Urokinase-type plasminogen activator is frequently overexpressed in several cancers and is a strong prognostic indicator for decreased patient survival rates (18–20). Prognostic studies have indicated that the protease inhibitor, PAI-1, is also a clinical marker for a poor prognosis in a variety of human cancers (21–23). No clear explanation has been found for this apparent paradox to date. This discrepancy could be due to a difference in tumor histology, or it may merely reflect the biological tumor features of different types of cancer.

Angiogenesis (24), a fundamental process by which new blood vessels are formed, is essential in reproduction, develop-
ment, and wound repair. Tumor growth and metastasis are angiogenesis dependent. A tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow. Furthermore, angiogenesis is required for tumor cells to enter the circulation and metastasize to a distant site, such as the liver, lung, or bone. Tumor cells simultaneously secrete proteases (urokinase-type plasminogen activator) and their inhibitors (PAI-1), and the balance between the two precisely regulates the level of extracellular proteolysis, thus promoting or suppressing angiogenesis (25). Recently, it has been reported that deficient PAI-1 expression in host mice prevented local invasion and tumor vascularization (26). When this PAI-1 deficiency was circumvented by i.v. injection of a replication-defective adenoviral vector expressing human PAI-1, the invasion and its associated angiogenesis were restored. This experimental evidence demonstrates that host-produced PAI-1 is essential for cancer cell invasion and angiogenesis.

In this study, we demonstrated for the first time that PAI-1 expression increased with esophageal SCC stage and was associated with poor prognosis. In univariate analysis, the PAI-1 expression score was significantly correlated with survival of esophageal SCC. These results suggested that PAI-1 might serve as a new parameter for prediction of prognosis in esophageal SCC.

This study provides a solid basis for additional studies on the molecular mechanism of PAI-1 expression in esophageal SCCs. Because esophageal SCC is one of the most aggressive of all cancers, we may not be able to change the overall survival rate using information about PAI-1 expression levels. However, PAI-1 expression could be used as a marker for predicting the outcome of resection-treated esophageal SCC patients.

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