Increased serum cell-free DNA levels in relation to inflammation are predictive of distant metastasis of esophageal squamous cell carcinoma

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Abstract. Distant metastasis hinders a favorable outcome for patients with esophageal squamous cell carcinoma (ESCC) by limiting the surgical cure. The levels of cell-free DNA (cfDNA) in the blood have served as a predictor for metastasis and recurrence in distant organs in liver cancer. Thus, this study tested the clinical efficacy of serum cfDNA levels as a predictive marker for distant metastasis of ESCC. We investigated cfDNA levels in a cohort of 101 ESCC patients and 46 age- and gender-matched control patients with benign disease. We found that serum cfDNA levels were significantly higher in the ESCC patients than in the control patients (P=0.034). In the ESCC patients, serum cfDNA levels were positively associated with tumor size and cytokeratin 19 fragment (CYFRA 21-1) expression (r=0.416 and r=0.573, respectively). An increase in cfDNA levels was also associated with host inflammation status including C-reactive protein levels and neutrophil and monocyte numbers in the peripheral blood. Serum cfDNA levels tended to be higher in advanced tumors when compared to early stage tumors. We found that serum cfDNA levels were significantly higher in ESCC patients with distant metastasis than in those without (P=0.011). Logistic regression analysis showed that serum cfDNA levels represented only one independent risk factor for distant metastasis among the five factors tested including gender, age, cfDNA levels, CYFRA 21-1 and squamous cell carcinoma antigen levels (P=0.0414). These results suggest that increased serum cfDNA levels may serve as a useful predictor for distant metastasis of ESCC.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is a predominant histological subtype of esophageal cancer. ESCC ranks as the sixth most common cancer among males and the ninth most common cancer among females (1). Worldwide, ESCC is also one of the most fatal malignancies, as progression occurs without symptoms and many tumors are significantly advanced by the time of diagnosis (2,3). Despite recent advances in surgical techniques and therapy combined with chemotherapy or chemo-radiotherapy (4-6), many ESCC patients develop metastatic disease. In particular, distant metastasis hinders a favorable outcome for patients with ESCC by limiting the surgical cure (7,8). As a result, ESCC patient prognosis remains poor, with a 5-year survival rate of less than 20% worldwide (9,10). In order to improve the poor prognosis of ESCC, it is essential to identify an easy-to-use marker for predicting the spread of ESCC cells into distant organs such as the lung and the abdominal lymph node.

Cell-free DNA (cfDNA) circulating in the blood has recently received much attention as an easy-to-use tool for the evaluation of the malignant potential of various cancers (11-13). Tomita *et al* showed for the first time, the diagnostic efficacy of cfDNA circulating in the blood of 24 patients with ESCC (14). However, they used only a very small cohort and failed to investigate the relationship between cfDNA levels and distant metastasis of ESCC. This prompted us to examine whether circulating cfDNA levels in the blood may serve as a prognostic indicator in a larger cohort of ESCC patients.

Materials and methods

Patients and samples. Between April 1998 and October 2006, 101 patients underwent surgical treatment for ESCC at Yamaguchi University Hospital. The clinical characteristics of these patients were based on the TNM classification (15) of the Union Internationale Contra le Cancer (UICC) and are presented in Table I. To explore the relationship between serum cfDNA levels and patient outcome, we excluded 10 patients who exhibited residual tumors. The remaining 91 patients were entered into our study and followed up as

Table I. Patient characteristics and serum cell-free DNA level.

	Cell-free DNA amount		
	Low (≤135 ng/ml)	High (>135 ng/ml)	P-value
Gender[a]			0.034
Male (n=85)	56	29	
Female (n=16)	15	1	
Age (year)[b]	62.9±8.9	60.3±10.4	0.202
Tumor differentiation[a]			0.565
Well (G1) (n=12)	10	2	
Moderately (G2) (n=74)	50	24	
Poorly (G3) (n=15)	11	4	
UICC TNM stage[a]			0.057
I (n=27)	22	5	
IIA/IIB (n=26)	21	5	
III (n=24)	16	8	
IVA/IVB (n=24)	12	12	

To evaluate the high and low cfDNA levels, we used a cut-off value of 135 ng/ml. [a]Difference was analyzed by the Fisher's exact test. [b]Data represent mean ± SD, and difference was analyzed by the Student's t-test.

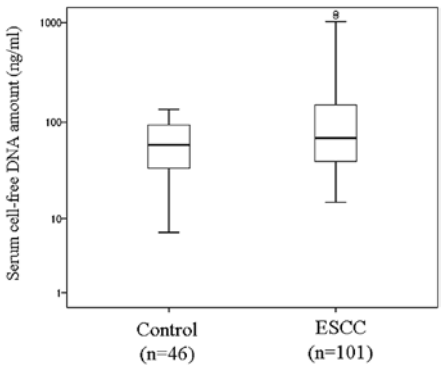

Figure 1. Box and whisker plot of cell-free DNA (cfDNA) levels in sera from patients with esophageal squamous cell carcinoma (ESCC) and control patients without ESCC. Serum cfDNA levels were significantly higher in ESCC patients (median, 67.5 ng/ml; range, 15-1236 ng/ml) than in control patients without known ESCC (median, 59 ng/ml; range, 7-135 ng/ml) (P=0.034).

described previously (4). Briefly, all 91 patients were appraised at least once every three months postoperatively by routine X-ray, ultrasonography (US), computed tomography (CT) or magnetic resonance imaging, and the levels of tumor markers such as squamous cell carcinoma (SCC) antigens and cytokeratin 19 fragments (CYFRA 21-1) were examined.

As controls, we used 46 serum samples taken from 46 age- and gender-matched patients with benign disease who were recruited from outpatient clinics at the Yamaguchi University School of Medicine between April 1998 and October 2006. Laboratory tests and imaging studies including US and CT did not reveal any ESCC in the 46 control patients.

The study protocol was approved by the Institutional Review Board for the Use of Human Subjects at the Yamaguchi University School of Medicine, and written informed consent was obtained from each patient prior to their entry into the study.

Extraction and quantification of DNA in sera. Blood samples were collected as described previously (16). After clotting, which occurred within 1 h of collection, samples were centrifuged at 3000 rpm (1600 x g) for 10 min at room temperature. Sera was then stored at -80˚C until required. DNA was extracted from 1 ml of serum using the DNA Extractor SP Kit for Serum and Plasma (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions. DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Reagent and Kit (Invitrogen, Paisley, UK).

Statistical analysis. The Student's t-test, the Mann-Whitney U test and the Fisher's exact test were used to analyze differences between the two groups. To identify independent factors for distant metastasis, five factors including gender, age, cfDNA levels, CYFRA 21-1 and SCC antigen levels were analyzed using logistic regression analysis. The values for cfDNA were transformed logarithmically prior to the statistical analysis. All analyses were performed using SPSS 11.0J software (SPSS, Inc., Chicago, IL, USA). P-values of <0.05 were considered statistically significant.

Results

Serum cfDNA levels were significantly higher in the ESCC patients (median, 67.5 ng/ml; range, 15-1236 ng/ml) than in control patients without diagnosed ESCC (median, 59 ng/ml; range, 7-135 ng/ml; P=0.034, by the Mann-Whitney U test; Fig. 1).

In the ESCC patients, serum cfDNA levels were positively associated with tumor size and CYFRA 21-1 levels (r=0.416 and r=0.573, respectively; P<0.0001 for both; Fig. 2), but not with SCC antigen levels (r=0.050, P=0.642). In addition, serum cfDNA levels were associated with the host inflammation status such as C-reactive protein (CRP) levels (r=0.430, P<0.0001) and the number of neutrophils and monocytes present in the peripheral blood (r=0.378 and r=0.544, respectively; P<0.0001 for both; Fig. 2). Serum cfDNA levels tended to be higher in advanced stage tumors than in the early stage tumors (P=0.057 by the Fisher's exact test; Table I). Serum cfDNA levels were also significantly higher in male than female ESCC patients (P=0.034 by the Fisher's exact test; Table I). Serum cfDNA levels were not associated with gender in the control patients without ESCC (data not shown).

We also identified that serum cfDNA levels were significantly higher in ESCC patients with distant metastasis (median, 83 ng/ml; range, 30-1236 ng/ml) than in those without distant metastasis (median, 74 ng/ml; range, 15-1152 ng/ml; P=0.011, by the Mann-Whitney U test; Fig. 3). Serum cfDNA levels were not associated with additional clinicopathologic factors including disease-free periods and overall survival (data not shown).

Logistic regression analysis revealed that serum cfDNA levels represented only one independent risk factor for distant metastasis from the five factors tested, which included gender,

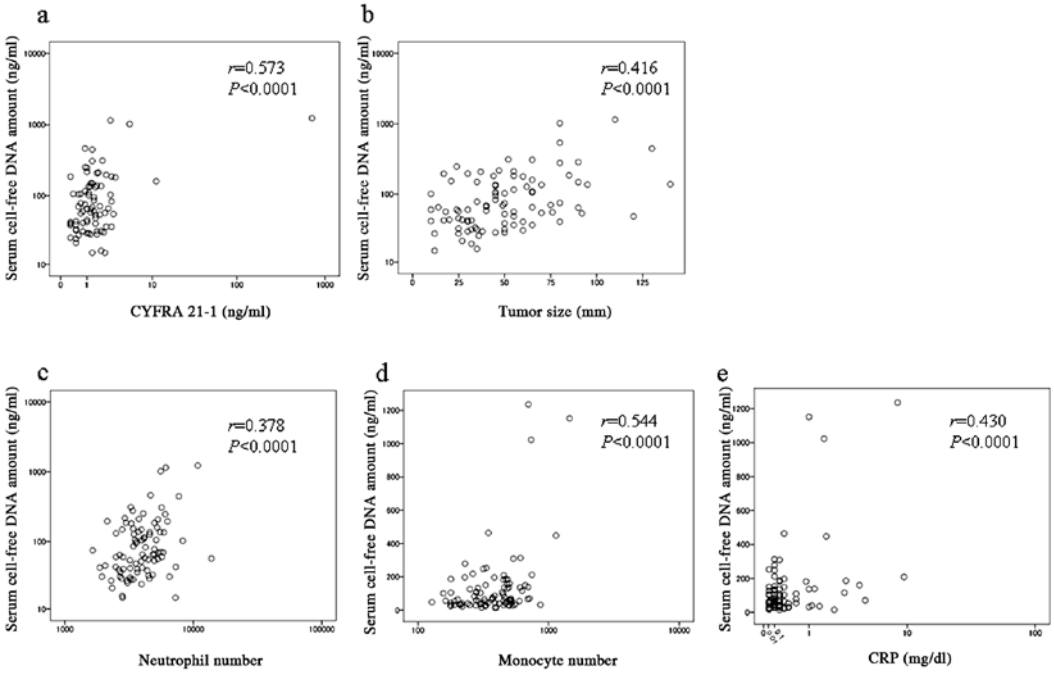

Figure 2. Association between serum cell-free DNA (cfDNA) levels and CYFRA 21-1 levels (a), tumor size (b), the numbers of neutrophils (c) and monocytes (d), and c-reactive protein level (e) in patients with esophageal squamous cell carcinoma. Note that cfDNA levels were associated positively with the five factors.

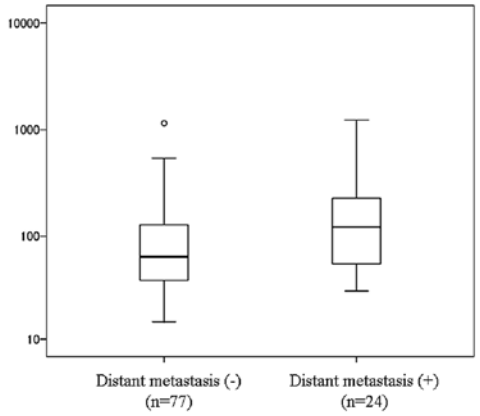

Figure 3. Box and whisker plot of cell-free DNA (cfDNA) levels in sera from patients with distant metastasis and those without. The serum cfDNA level was significantly higher in patients with distant metastasis (median, 83 ng/ml; range, 30-1236 ng/ml) than in those without (median, 74 ng/ml; range, 15-1152 ng/ml) (P=0.011).

age, cfDNA levels, CYFRA-21-1 and SCC antigen levels (P=0.0414, relative risk of 1.700; 95% CI, 1.021-2.831).

Discussion

Distant metastasis hinders a favorable outcome in ESCC patients by limiting the surgical cure (7,8), and accounts for the poor 5-year survival rate of less than 20% worldwide (9,10). In order to improve the poor prognosis for ESCC, it is essential to identify an easy-to-use marker for predicting metastasis and recurrence of ESCC in distant organs including the abdominal lymph node. In a large cohort of ESCC patients, we showed for the first time that serum cfDNA levels were significantly higher in patients with distant metastasis than in those without. Using logistic regression analysis, we also revealed that serum cfDNA levels represented only one independent risk factor for distant metastasis among the five factors tested.

In 1863, Rudolf Virchow proposed that inflammation may be a hallmark of cancer after he identified leukocytes within cancer tissues. Since then, it has been generally accepted that if a genetic abnormality is the 'match that lights the fire' of cancer, inflammation corresponds to the 'fuel that feeds the flames' (17). Thus, host inflammation status contributes greatly to cancer progression. As shown in our previous study (3), increased serum levels of inflammatory cytokines such as interleukin-6 in ESCC patients support this hypothesis. In addition, our present data demonstrated positive associations between increased levels of serum cfDNA and the total number of neutrophils and monocytes present in the peripheral blood of ESCC patients. This result is consistent with the results of our previous work, where we found that increased serum cfDNA levels were related closely to cancer-specific inflammation in patients with hepatocellular carcinoma (HCC) associated with hepatitis C virus (HCV) infection (18). Interestingly, that study revealed no association between the levels of serum cfDNA and CRP in HCC patients. In contrast, our current findings show a positive association between the levels of serum cfDNA and CRP in ESCC patients. This discrepancy is reasonable, given that CRP values in HCC patients may be largely affected by liver dysfunction rather than inflammation, as all of the HCC patients also exhibited HCV-related chronic liver disease.

CYFRA 21-1 belongs to the intermediate filament protein family and serves as a particularly useful diagnostic tool for many cancers including ESCC (19). It has been shown that increased levels of CYFRA 21-1 are linked to cancer

progression and poor outcome of ESCC (20,21). Therefore, our present finding that serum levels of cfDNA were positively associated with CYFRA 21-1 levels and tumor size gives strength to the clinical efficacy of cfDNA as an easy-to-use assay for monitoring ESCC. This hypothesis is further supported by our finding that serum cfDNA levels represent only one independent risk factor for distant metastasis in ESCC patients.

Collectively, the findings arising from the present study suggest that enhanced inflammation may easily destroy cancer cells, thus releasing higher levels of cellular DNA and CYFRA 21-1 into the blood stream. Therefore, cfDNA levels may serve as a predictive marker for distant metastasis in ESCC. Further investigation is required to precisely define the molecular basis underlying cfDNA release into the blood and to evaluate its efficacy as a biomarker for ESCC in a routine clinical setting.

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