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Serum soluble Fas ligand (sFasL) in patients with primary squamous cell carcinoma of the esophagus

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Abstract: Esophageal carcinomas have been shown to express Fas ligand (FasL) and down-regulate Fas to escape from host immune surveillance. Circulating soluble FasL (sFasL) has been suggested to provide protection from Fas-mediated apoptosis. The aim of this study was to assess serum sFasL levels in esophageal cancer. The pretreatment levels of sFasL in the serum of 100 patients with esophageal squamous cell cancer and 41 healthy volunteers were determined by ELISA. Probability of survival was calculated according to the method of Kaplan-Meier. The prognostic influence of high and low level of sFasL was analyzed with the log-rank test. The mean serum level of sFasL in patients with esophageal cancer was significantly higher than that in healthy donors (1.567±1.786 vs 0.261±0.435, $p<0.0001$). The levels of serum sFasL were significantly higher in advanced stages (II vs IV $p<0.034$; III vs IV $p<0.041$; except II vs III $p=0.281$), patients with lymph node (N0 vs N1 $p<0.0389$) or distant (M0 vs. M1 $p<0.0388$) metastases and significantly lower in patients with well differentiated tumors (G1 vs G2 $p<0.0272$). The serum levels of soluble FasL were not related to gender, age, tumor size, T-stage, tobacco smoking and history of chronic alcohol intake. The survival difference between pretreatment high and low level of sFasL in surgery and chemio- and/or radiotherapy group was not statistically significant ($p=0.525$; $p=0.840$). Our results indicate that elevated serum sFasL levels might be associated with a disease progression in patients with esophageal squamous cell carcinoma.

Keywords: Soluble Fas ligand - sFasL - Esophageal cancer

Introduction

Despite advances in diagnosis and staging, (neo-) adjuvant therapy and surgical technique, the overall survival of the esophageal carcinoma remains lower than other solid tumors [1,2]. Esophageal cancer is usually detected at an advanced stage, requiring a multimodal concept. Numerous patients develop locally recurrent tumors or distant metastases within a short period of time after curative surgery. Despite the presence of tumour specific cytotoxic T cells and natural killer cells, the immune system fails to contain esophageal carcinomas [3,4]. As in other malignant tumors, esophageal cancer cells actively suppress the immune defence of the host by secreting a number of immunosuppresive factors [5,6]. However, the mechanisms by which such factors overcome anti-tumour immunological responses are still poorly understood.

Fas ligand (FasL), a type II membrane-bound 40-kDa protein which belongs to the tumour necrosis factor (TNF) family, induces apoptotic death of sensitive lymphoid cells expressing its cell surface receptor (FasR) [7]. Activated T and B lymphocytes express FasR, and thus are sensitive to FasR-mediated apoptosis [7,8]. This has been proposed to be responsible for several regulatory functions of the immune system, including tolerance acquisition, down-regulation of immune reactions, and clonal deletion of peripheral lymphocytes [3,9-11]. The expression of FasL has been shown in colon carcinoma [12], gastric carcinoma [13], hepatocellular carcinoma [14], lung carcinoma [15], breast carcinoma [16], ovarian carcinoma [17], esophageal carcinoma and their lymph node metastases [18,19] that may trigger apoptosis of acti-

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vated lymphocytes. This process suggests that the FasL allow tumors to escape the host's immune surveillance [4]. However, the distribution of these molecules does not correlate simply with the degree of malignancy, and the biologic features of these molecules are not fully understood. The membrane-bound FasL (mFasL) may be cleaved by a specific matrix metalloproteinase-like enzyme and be presented in a soluble form (sFasL). Human sFasL is a 26-kDa glycoprotein consisting of an extracellular region for binding to FasR and it has been identified in the culture supernatant of FasL expressing cells [20]. Membrane form of FasL as well as sFasL binds to Fas and transduces an apoptotic signal in Fas-expressing cells [21]. Elevated serum sFasL concentrations have been observed in patients with various kinds of hematological malignancies [20,22]. However, to our knowledge there are few reports to date that demonstrate elevated serum sFasL levels in patients with solid tumours [12,23,24].

In the present study we measured the serum concentration of sFas-L in patients with esophageal carcinoma and examined whether the results were correlated with clinicopathologic feature and survival rates.

Materials and methods

Patients. One hundred esophageal cancer patients (89 men and 11 women, with a median age of 59.1 years, range from 34 to 81 years) diagnosed and treated at the Bialystok Medical University in the Department of Thoracic Surgery were enrolled in this study. The inclusion criteria were no previous history of malignancy, primary squamous cell carcinoma of the esophagus confirmed histopathologically, no previous radiotherapy, chemotherapy, immunotherapy and no anti-inflammatory drugs during the preceding 2 weeks. Patients with liver function failure such as viral hepatitis, alcohol hepatitis, and fatty liver were excluded. Patients who smoked at least 1 pack of cigarettes a day (smokers) and drank at least 20g of ethanol daily for 5 or more years were assessed separately. We also studied 41 age-matched healthy subjects, all of whom were infection free and none of whom had taken any drugs in the 2 weeks preceding blood sampling. The clinicopathological stage was determined according to the TNM classification system of the International Union Against Cancer [25]. A relatively large number of patients had advanced stage disease (71 patients, 71%). Seventy-three patients (73%) had lymph node metastases, and 23 patients (23%) had distant metastases. The study population had the following performance status (PS): PSO, 61 patients; PS1, 31 patients; and PS2, eight patients. Forty two patients underwent primary surgery and 58 received chemio- and/or radiotherapy. In operated patients and patients treated with chemio- and/or radiotherapy, mean follow-up duration was 18 months (range, 5-90) and 8 months (range, 3-24), respectively. The clinicopathological data of all patients are shown in Table 1. Informed consent was obtained from all healthy volunteers and all patients.

Measurement of serum sFasL concentration. Venous blood samples were drawn into sterile vacuum tubes when esophageal carcinoma was detected before surgery or anticancer therapy. The tubes were centrifuged at 3000 rpm and separated sera were stored at -80°C for future use.

The level of soluble Fas Ligand (sFasL) in the sera was determined with enzyme ligand immunosorbent assay (ELISA) kit for the quantitative detection of human sFasL (Bender MedSystem, Vienna, Austria) according the producer's protocol. Briefly, 50 µl of a serum together with the same amount of Sample Diluent were added to the corresponding sample well of the microwell plate. After the sFasL present in the sample had bound to the anty-sFasL coating antibodies adsorbed to the microwells, 50 µl of diluted Biotin Conjugate solution was added. During the microplate incubation at room temperature for 2 hours biotin-conjugated monoclonal anti-sFasL antibodies present in Biotin conjugate Solution bound to sFasL captured by the first antibody. After the removal

Table 1. Clinicopathological data of patients with primary esophageal squamous cell carcinoma

	N	(%)
Gender		
Male	89	89%
Female	11	11%
Age		
< 64	69	69%
> 64	31	31%
Location of the esophageal cancer		
Upper	4	4%
Middle	53	53%
Lower	43	43%
Tumour size		
< 5cm	74	74%
> 5cm	26	26%
T-stage		
T1	5	5%
T2	23	23%
T3	35	35%
T4	37	37%
N-stage		
N0	27	27%
N1	73	73%
M-stage		
M0	77	77%
M1	23	23%
Differentiation		
G1	21	21%
G2	41	41%
G3	38	38%
TNM-stage		
I	5	5%
II	24	24%
III	48	48%
IV	23	23%
Tobacco		
(+)	72	72%
(-)	28	28%
Alcohol		
(+)	62	62%
(-)	38	38%

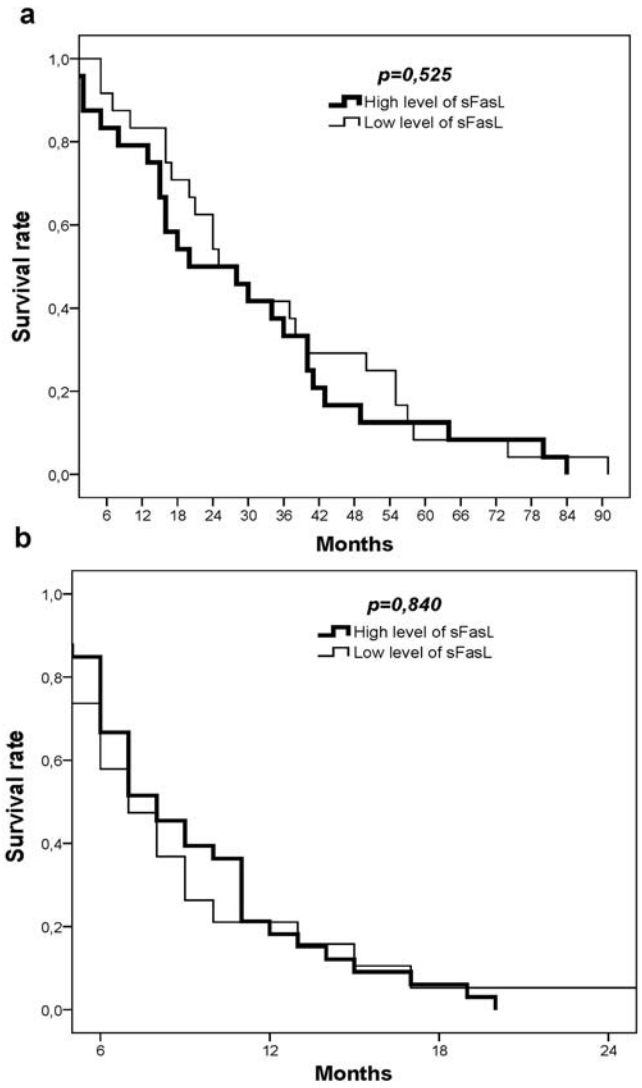

Fig. 1. Kaplan-Meier survival curves demonstrate no statistical difference: **a**. (p=0.525; long-rank test) between low level and high level of sFasL esophageal cancer patients undergoing surgery; **b**. (p=0.840; long-rank test) between low level and high level of sFasL esophageal cancer patients undergoing chemio- and/or rediotherapy.

unbound biotin-conjugated anti-sFasL during four washing steps, each with approximately 300 µl of Wash Buffer, 100 µl of diluted Streptavidin-HRP was added. Following incubation at room temperature for 1 hour unbound Streptavidin-HRP was removed during four washing steps with approximately 300 µl of Wash Buffer each, and 100 µl of TMB Substrate Solution reactive with HRP was added. After incubation at room temperature for 20 minutes the reaction was stopped by adding 100 µl of Stop Solution (1 M phosphoric acid), and the absorbance was measured at 450 nm with a microplate reader (ETI System Sorin Biomedica).

sFasL sample concentration was determined on the base of a standard curve prepared from seven sFasL standard dilutions. All the sera samples were analyzed in duplicate, and the limit of detection was determined to be 0.1 ng/ml.

The patients were divided into two groups according to the level of sFasL in the serum. A level of sFasL greater than the mean level of sFasL plus 3 standard deviations in healthy controls was regarded as a high level, and a level less than the mean level of sFasL plus 3 standard deviations in healthy controls was regarded as a low level.

Statistical analysis. The U-Mann-Whitney test for two independent probes were used to analyze the correlation between serum sFasL and patients parameters, including histopathologic findings. The Kaplan-Meier method was used to generate survival curves. Survival differences were analyzed with the log-rank test, based on the status of level sFasL (high level and low level). Probability values <0.05 were considered statistically significant. All of the analyses were performed using statistical analysis software (SPSS version 13.0 for Windows).

Results

Associations between sFasL serum levels and clinical factors are shown in Table 2. The mean serum sFasL levels in the healthy individuals and the squamous cell esophageal cancer patients were significantly different (0.261 ± 0.435 vs 1.567 ± 1.786; p<0.0001). The serum levels of soluble FasL were not related to gender (p=0.343), age (p=0.694) and tumor size (p=0.649). No statistically significant correlation between levels sFasL and T-stage was noted. No correlation was found, either, between serum levels and tobacco smoking (p=0.068) and history of chronic alcohol intake (p=0.318).

Serum sFasL levels had a statistically significant relationship to the N-stage (p<0.0389) and M-stage (p<0.0388). The sFasL concentrations in stage IV were significantly higher than they were in both the stage II (p<0.034) and III (p<0.041). Statistically significant difference was observed with respect to histopathologic grading. The sFasL concentration in well differentiated tumors was significantly lower than in patients with moderate differentiated (p<0.027).

Kaplan-Meier survival curves for operated patients and patients treated with chemio- and/or radiotherapy categorized according to serum sFasL high level and low level are shown on Fig. 1a and Fig. 1b. Survival of patients with high level was not statistically different to those with low level according to the long-rank test in operated patients (p=0.525) and received oncological treatment (p=0.840).

Discussion

Recent studies indicating that most of the tumors escape from the host immune attack by imitating themselves as immune-privileged sites by either overexpressing FasL or down-regulating Fas [4,12,13]. It has emerged that esophageal cancers can also counterattack the immune system. Expression of FasL by esophageal carcinoma cells in vivo enables them to induce Fas-mediated apoptosis of activated tumor-infiltrating lymphocytes. This counterattack model suggests that the FasL may offer a survival advantage to tumors [6]. FasL proteins were detected in tumors

Table 2. Correlation between serum soluble Fas ligand (sFasL), healthy group and clinicopathologic data of patients with primary esophageal squamous cell carcinoma

Variable	sFasL level (ng/ml), mean ±SD	P-value
Esophageal cancer Healthy group	1.567±1.786 0.261±0.435	<0.0001
Gender Male Female	 1.532±1.760 1.248±2.496	 0.343
Age < 64 > 64	 1.645±1.932 1.393±1.421	 0.694
Location of the esophageal cancer Upper Middle Lower	 1.136±1.159 1.870±1.906 1.232±1.635	 0.812 (Upper vs. Middle) 0.727 (Upper vs. Lower) 0.037 (Middle vs. Lower)
Tumour size < 5cm > 5cm	 1,583±1,888 1,519±1,489	 0.649
T-stage T1 T2 T3 T4	 1.010±1.799 1.157±1.066 1.707±1.992 1.640±1.811	 0.589(T1vs.T2) 0.395(T2vs.T4) 0.963(T3vs.T4) 0.395(T2vs.T3)
N-stage N0 N1	 0.998±1.114 1.733±1.875	 <0.0389
M-stage M0 M1	 1.332±1.558 2.139±2.135	 <0.0388
TNM-stage I II III IV	 1.437±1,642 0.958±0.859 1.483±1.762 2.362±2.286	 0.611(Ivs.II) 0.281 (IIvs.III) <0.034(IIvs.IV) <0.041(IIIvs.IV)
Differentiation G1 G2 G3	 0.814±0.874 1.595±1.626 1.692±2.011	 <0.027(G1vs.G2) 0.112(G1vs.G3) 0.515(G2vs.G3)
Tobacco (+) (-)	 1.651±2.249 0.651±0.902	 0.068
Alcohol (+) (-)	 1.290±1.727 1.683±1.810	 0.318

tissues and their lymph node metastases in patients with squamous cell esophageal cancer, and cell death was detected in tumor infiltrating lymphocytes [4,18,19]. The biologic significance of circulating sFasL in esophageal cancer is currently unknown. High levels of sFasL in serum have been generally detected in several haematological diseases including NK-cell leukaemia, NK cell lymphoma [20], but there is little published data relating to patients with non-hematopoietic malignancies [12,23,26,27], and none

concerning the role of sFasL in esophageal carcinoma. We investigated the role of serum sFasL concentration in the biology of squamous cell carcinoma. We demonstrated that the level of serum sFasL in patients with esophageal cancer was significantly higher than in healthy volunteers, a finding that is in line with the data reported by Lim [27], Hara *et al.* [28] and Mizutani *et al.* [22] in different kinds of tumors. Furthermore, the serum levels of sFasL in stage III to IV esophageal cancer patients were significantly higher than they were in patients with stage II disease. These results suggest that the increase in serum sFasL in patients with squamous cell esophageal cancer might be reflective of their tumor burden insofar as the tumor may release sFasL.

Ohbu *et al.* [29] reported that the rate of apoptosis was higher in well-differentiated esophageal tumors than in poorly differentiated tumors. Our date demonstrate that the level of serum sFasL in patients with well-differentiated esophageal cancer was significantly lower than in patients with moderate differentiated. Therefore, it would be possible that the survival advantage seen in low level serum sFasL cases is due to the occurrence of frequent apoptosis in well differentiated esophageal squamous cell cancers (data not shown).

The metastatic cascade is a complex series of processes including angiogenesis, intravasation of tumor cells, transport by circulation, adhesive interaction with the endothelial cells, and extravasation. Our results show circulating serum sFasL concentration are significantly higher in patients with regional lymph node metastases and/or distant metastases than in patients without metastases. These findings suggest that elevated levels of serum sFasL may induce the apoptosis of activated lymphocytes and thus facilitate tumor cell progression as a result of immunosurveillance escape [30]. An evasion of the host immune surveillance may be important for metastasis when cancer cells enter into circulation. Our data supports the hypothesis of others [4,6,19] who have recently proposed a "counterattack model" suggesting that tumor cells use FasL and sFasL as cytolytic effectors to kill Fas-expressing activated lymphocytes, thus indicating that both may offer subsequent tumor survival advantage and regional lymph node and/or distant metastasis.

The precise cellular origins of sFasL have not been elucidated. The present study has shown that high serum levels of sFasL were mostly observed in advanced stage of disease and moderate and poorly differentiated tumors. We speculate that sFasL may be derived from cancer cells which revealed high overexpression [31]. It has been reported that sFasL was detected in culture supernatant of several cancer cells [20,21,30].

High serum sFasL levels in esophageal cancer patients may represent a further mechanism of immune escape as a result of an attack against immune cytotoxicity cells [23], but their clinical significance has not been clarified and their molecular mechanisms are complex [33]. In future sFasL may serve as an anti-cancer therapeutic target, and it is important to block sFasL to improve clinical outcome.

Our results showed a lack of correlation between sFasL levels and patients survival. It is in line with results by Shibakita *et al.* [32] who studied the expression of FasL in esophageal carcinomas.

In conclusion, the data presented in this paper demonstrated that elevated serum soluble Fas ligand were associated with advanced stage of disease and poorly differentiated tumors of squamous cell esophageal cancer.

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