Anaphylatoxin C3a: A potential biomarker for esophageal cancer diagnosis

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Abstract. Esophageal carcinoma is a common malignancy worldwide, with a low 5-year survival rate. As the majority of cases are diagnosed at an advanced stage, there is an urgent need for an effective biomarker for early diagnosis of esophageal cancer patients. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) was applied to detect the serum protein expression in esophageal cancer patients using ProteinChip software, and the results were analyzed and screened using Biomarker Patterns and SPSS16.0 software. The ELISA method was conducted to determine the concentration of anaphylatoxin C3a, which is one of the complement proteins, in the serum of esophageal cancer patients and non-esophageal cancer participants. A total of 144 effective differential expression protein peaks in the window of 1-10 kDa were obtained ($P<0.05$). M/Z 8,926.478 ($P<10^{-6}$) protein peak was employed as the diagnostic biomarker for esophageal carcinoma. This established diagnostic biomarker has a sensitivity of 95% (19/20) and an accuracy of 100% (19/19) for positive prediction. The results suggested that anaphylatoxin C3a may be a promising biomarker in the diagnosis of esophageal carcinoma, and may play a key role in promoting esophageal carcinogenesis.

Introduction

Esophageal carcinoma is a common malignancy worldwide, with a 5-year survival rate of ~30%, even with chemotherapy, surgery and radiation therapy, due to tumor heterogeneity (1-9). It is well-known that early diagnosis and timely treatment may improve the survival rate in early-stage cancer patients, with a 5-year survival rate as high as 90% (10,11). Cancer Research UK (http://www.cancerresearchuk.org/about-cancer/cancer-symptoms/why-is-early-diagnosis-important) clearly reported the association of survival rate with stage at diagnosis for several cancers, such as breast, ovarian, lung and bowel cancer. However, the majority of esophageal cancer patients are often diagnosed at an advanced stage, as there are no obvious symptoms in the early stages of the disease. Therefore, it is crucial to identify an effective biomarker for early diagnosis. Recently, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MOLDI-TOF-MS) and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) techniques have been widely employed in the search for cancer biomarkers in brain cancer (12), oral squamous cell carcinoma (13), pancreatic cancer (14), lung cancer (15), esophageal squamous cell carcinoma (16) and breast cancer (17,18), among others. There have been several attempts to identify esophageal carcinoma biomarkers using these techniques (16,19-22). However, those studies failed to identify any single biomarker for the diagnosis of esophageal cancer, but rather indicated several proteins. In addition, the identified proteins differed among different research groups. This makes it difficult to establish a unified standard for rapid and accurate diagnosis of this type of cancer. In the present study, a single protein, anaphylatoxin C3a, which is one of the complement proteins, was investigated as a diagnostic biomarker to distinguish between esophageal cancer patients and healthy individuals.

Materials and methods

Research subjects. A total of 40 serum samples were included in this study, 20 of which were collected from esophageal carcinoma patients from Yancheng First People's Hospital (group A), whereas the others were collected from non-esophageal carcinoma participants recruited from the First Hospital of Nanjing Medical University (group B) between November 2014 and May 2015. The participants were aged 25-76 years, with a mean age of 55.7 years. In group A, there were 8 cases of patients who were undergoing therapy. All the blood collections were performed an overnight fast. Blood was collected in 5-ml blood collection tubes without anticoagulant and the serum samples were stored at -80˚C for further use.

Ethics statement. All patients signed an informed consent form and the study protocol was approved by the Institutional

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Key words: esophageal carcinoma, surface-enhanced laser desorption ionization time-of-flight mass spectrometry, anaphylatoxin C3a, diagnostic biomarker, tumor heterogeneity, immune escape

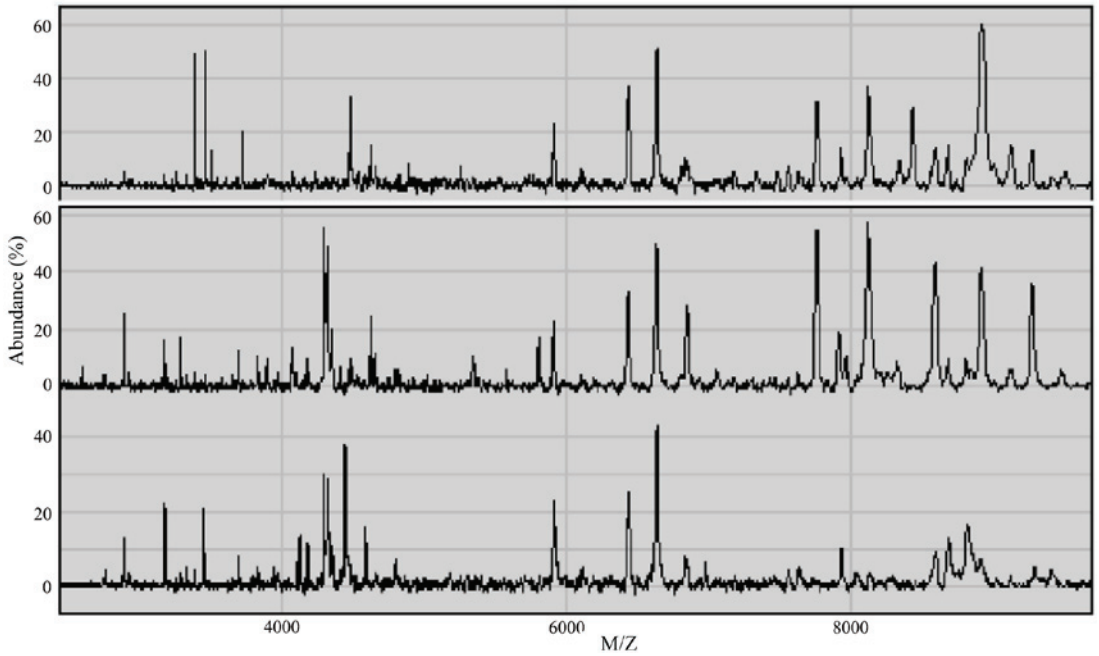

Figure 1. Protein mass spectrum peaks in the sera of esophageal cancer patients prior to treatment (upper) and after treatment (middle), and of non-esophageal carcinoma participants (bottom).

Review Board of the First People's Hospital of Yancheng (Yancheng, China). The study was reviewed and approved by Yancheng Medical Ethics Committee.

SELDI-TOF-MS assay. SELDI-TOF-MS (Ciphergen Biosystems, Fremont, CA, USA) was applied to collect the raw mass spectrometry data. Biomarker Wizard software (Ciphergen Biosystems, Fremont, CA, USA) was used to export the raw data into a digital format, according to the standard Excel format. A t-test was conducted with the Excel data using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

Enzyme-linked immunoabsorbent assay (ELISA). The concentrations of C3a in the serum samples were quantified by ELISA according to the manufacturer's protocol (cat. no. ab133037; Abcam, Cambridge, MA, USA). Briefly, 50 μl standard samples and 50 μl serum samples were diluted 5 times with phosphate-buffered saline, placed into a 96-well plate and cultured at 37˚C for 30 min. The plate was washed 5 times, after which time 50 μl reagent A was added into each well, followed by 50 μl reagent B. The samples were mixed well and cultured for a further 15 min at 37˚C in the dark; stop solution was then added into each well. An ELISA plate reader (Synergy HXT; BioTek Instruments Inc., Winooski, VT, USA) was used at a wavelength of 450 nm; the inter-assay and intra-assay coefficients of variation of the ELISA kits for C3a were <10%.

Statistical analysis. The SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA) was used for data analysis, and the data are expressed as means ± standard deviation. The comparison between the groups was performed using one-way analysis of variance. P<0.05 indicates that the difference was statistically significant.

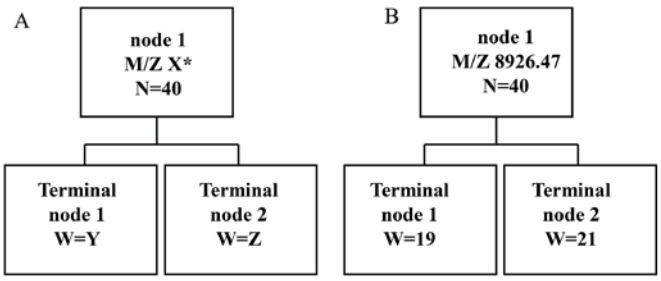

Figure 2. (A) The classical tree model; (B) the predicted results of M/Z 8,926.47.

Results

Serum proteomic profiles. SELDI-TOF-MS was applied to examine the serum samples within a window of 1-10 kDa, and 253 protein peaks were detected in the sera of esophageal cancer patients. There were 144 statistically significant difference peaks (P<0.05): 56 peaks had higher protein expression and 88 had lower protein expression in esophageal cancer patients. The mass spectra (MS) of the serum proteins of esophageal cancer patients and non-esophageal cancer participants are shown in Fig. 1.

Establishment of diagnostic biomarker. In order to establish the diagnostic biomarker, 6 MS peaks, i.e., M/Z 2,748.87 ($P=2.38\times10^{-7}$), M/Z 4,119.31 ($P=3.18\times10^{-7}$), M/Z 4,425.94 ($P=2.38\times10^{-7}$), M/Z 4,798.62 ($P=3.67\times10^{-7}$), M/Z 9,136.76 ($P=6.45\times10^{-7}$) and M/Z 8,926.47 ($P=7.33\times10^{-8}$), exhibiting statistically significant differences, were further analyzed. The classical tree model was employed to examine the predictor variables (Fig. 2A). The protein M/Z 8,926.47 had the best prediction result (Fig. 2B). The sensitivity and specificity of

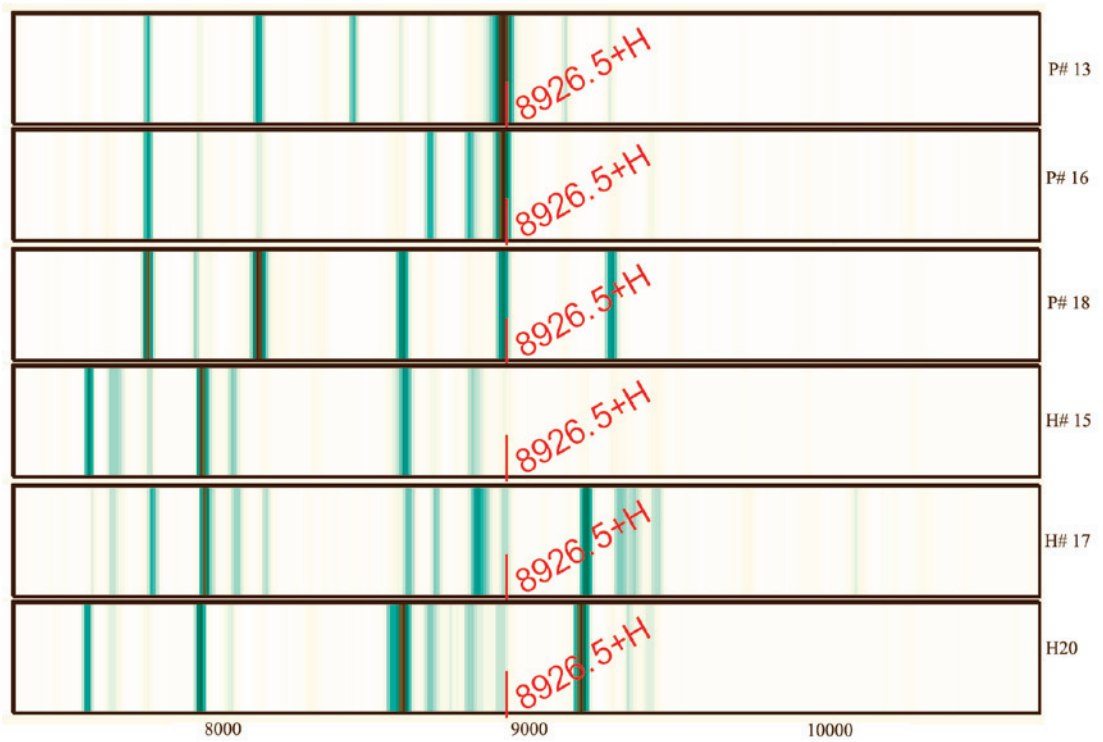

Figure 3. Serum protein mass spectra. Upper three, esophageal carcinoma patients (P); bottom three, non-esophageal carcinoma participants (healthy; H).

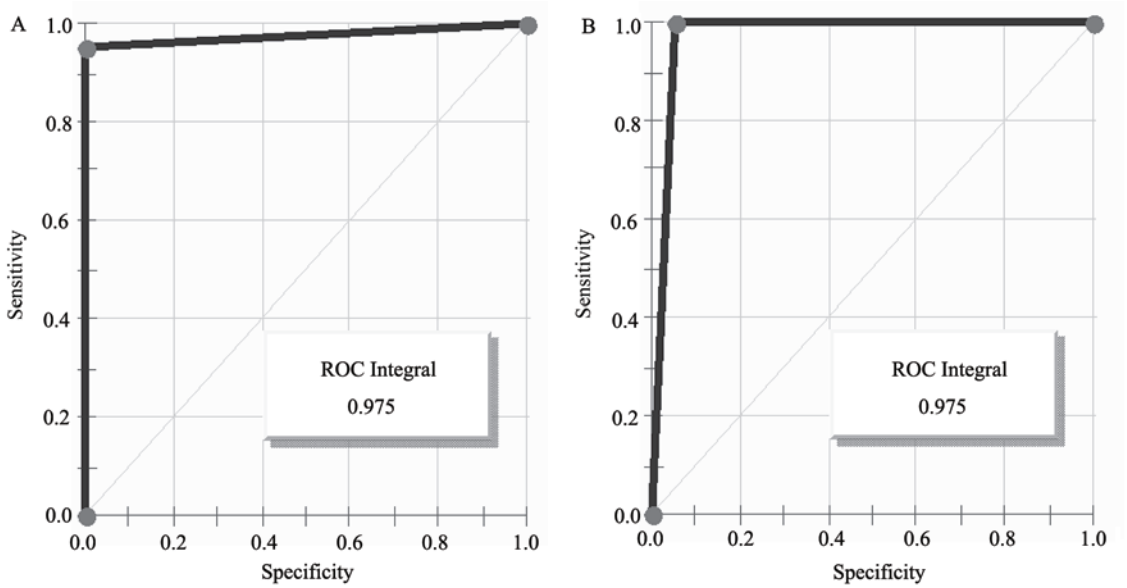

Figure 4. Receiver operating characteristics (ROC) curves of diagnostic biomarker. (A) ROC curve of diagnostic biomarker of patients; (B) ROC curve of diagnostic biomarker of non-esophageal carcinoma participants.

the biomarker were 95% (19/20) and 97.5% (39/40), respectively. The positive predictive accuracy was 100% (19/19) and the negative predictive accuracy was 95.2% (20/21). Thus, the protein at M/Z 8,926.47 may be an optimal biomarker for distinguishing the esophageal cancer and non-esophageal cancer sera with high accuracy and high specificity.

Further study indicated that the abundance of M/Z 8,926.47 was reduced from 60.69 prior to therapy to 43.39 after therapy (the mean abundance of this peak was 6.81 for non-esophageal carcinoma participants). Three of the serum protein MS of esophageal cancer patients and non-esophageal carcinoma participants are shown in Fig. 3. The intensities of M/Z 8,926.47 in the non-esophageal carcinoma participants were distinctly lower compared with those in esophageal carcinoma patients. The areas under the receiver operating characteristic curves of the diagnostic biomarker were 97.5% (Fig. 4).

Determination of the concentration of C3a via ELISA. The human C3a ELISA kit was used to determine the concentration of anaphylatoxin C3a using the obtained serum samples.

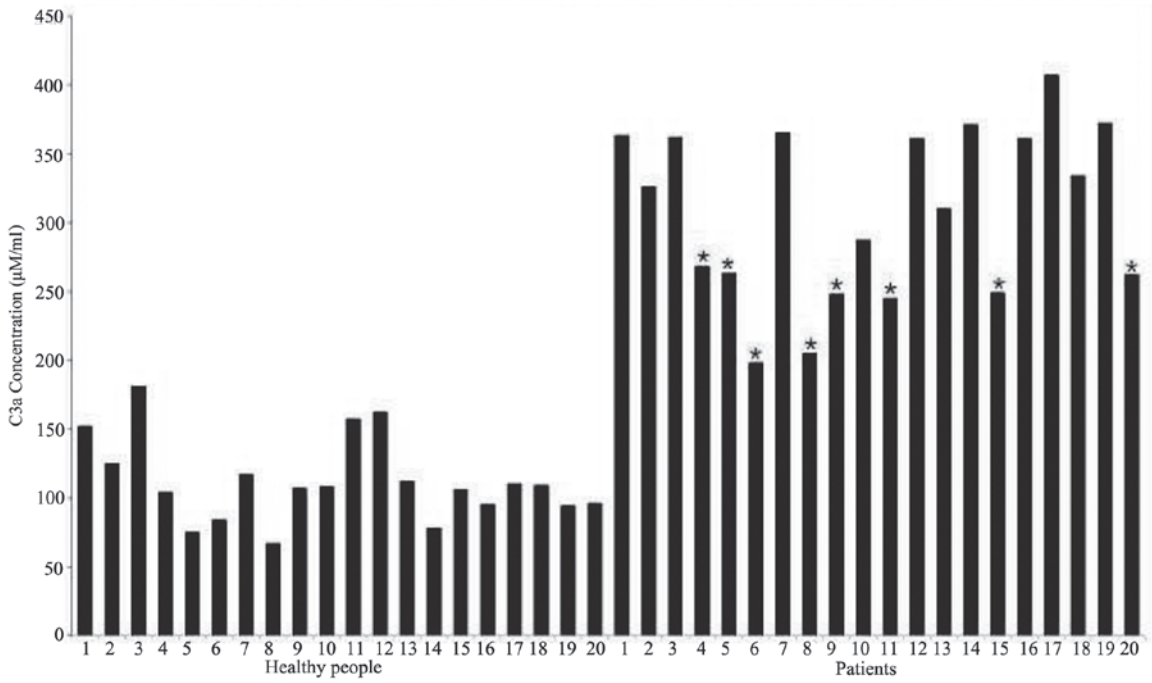

Figure 5. Concentrations of C3a in different serum samples determined by ELISA. The first 20 bars represent the C3a concentrations in healthy participants, whereas the remaining bars represent those in esophageal cancer patients (P<0.01). *sera from esophageal carcinoma patients undergoing chemotherapy and surgery.

The ELISA experimental results (Fig. 5) revealed that the concentration of anaphylatoxin C3a in the sera of esophageal cancer patients (mean, 308±60 ng/ml) were significantly higher compared with those in healthy participants (mean, 112±30 ng/ml). Among esophageal carcinoma patients, the mean concentration of C3a in the sera of those without therapy and those undergoing therapy was 352±31 and 242±25 ng/ml, respectively.

Discussion

Regarding the SELDI-TOF MS results, significant differences were observed in the serum samples between the esophageal carcinoma patients and healthy participants for 6 protein peaks ($P<10^{-6}$). Among those, the abundance of the peak M/Z 8,926.47 ($P=7.33x10^{-8}$) markedly increased from 6.81 (non-esophageal carcinoma) to 60.69 (esophageal carcinoma), suggesting that the expression of M/Z 8,926.47 was significantly increased in the plasma of esophageal carcinoma patients. This particular peak distinguished between the sera of esophageal carcinoma patients and non-esophageal carcinoma participants with high accuracy (100%) and high efficiency (97.5%), indicating that M/Z 8,926.47 may be a promising biomarker for early diagnosis. Based on our experience, this peak is likely to be complementary to the C3a protein.

The ELISA results demonstrated that the concentrations were statistically significantly different (P<0.01) and the mean concentration of anaphylatoxin C3a in esophageal carcinoma and non-esophageal carcinoma samples was 308±60 and 112±30 ng/ml, respectively, which was in agreement with the MS peak abundance of 53.98 and 6.81. Furthermore, among esophageal cancer samples, the C3a concentration was 352±31 and 242±25 ng/ml for samples before and after treatment, respectively, which was also in agreement with the MS peak abundances of 60.69 and 43.39. This finding indicates that, after treatment, the concentration of C3a in the plasma was markedly decreased. According to the ELISA results, it may be concluded that, when the serum C3a concentration is >120 ng/ml, the risk of esophageal cancer is increased.

C3a is a serum protein first discovered in 1896, which plays a key role in either antitumor immune response (23-27), or in promoting tumor growth and progression (28,29). However, the role of C3a in esophageal cancer has not been determined to date. According to the MS and ELISA results, the anaphylatoxin C3a concentrations in the sera of treated patients are significantly lower compared with those without treatment. It may be hypothesized that C3a plays a key role in promoting esophageal tumorigenesis. As previously reported, anaphylatoxin C3a may contribute to cancer cell immune escape via promoting local immunosuppression (30,31). However, more studies should be conducted to elucidate the mechanisms through which C3a promotes esophageal tumorigenesis.

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