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Disturbed tryptophan metabolism correlating to progression and metastasis of esophageal squamous cell carcinoma

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is one of the most frequent malignancies worldwide. Lymph node metastasis is the leading cause of death in ESCC patients. To identify early diagnostic and prognostic biomarkers of ESCC and elucidate underlying pathogenesis of the disease, a targeted metabolomics strategy based on liquid chromatography combined with tandem mass spectrometry was applied to explore tryptophan metabolism between ESCC patients, metastatic ESCC patients (mESCC), and healthy controls. Statistical analysis on metabolite expression abundance and compound concentration ratio was conducted to discriminate patients from healthy controls. The concentration ratio of kynurenine, 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, 5-hydroxytryptamine to their precursor tryptophan were identified as potential biomarkers, presenting high diagnostic capacity for distinguishing ESCC and mESCC patients from healthy controls. Moreover, a prognostic prediction model was also built on these ratios to distinguish metastasis patients from non-metastasis patients successfully. The high performance of ESCC prediction models suggest that concentration ratios of compounds may be used as biomarkers for early diagnosis and prognosis of the disease. In addition, concentration ratios of compounds show a progressively increased trend from non-metastasis to metastasis patients compared with healthy controls, which is in accordance with process of malignant transformation of ESCC. This interested finding suggests that disturbed tryptophan metabolism is correlated to progression and metastasis of ESCC since concentration ratios of compounds reflect activity of enzymes involved in tryptophan metabolism. This study reveals the impact of tryptophan metabolism to tumorigenesis and metastasis of ESCC, which help biologists investigate mechanism of the disease.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one major form of esophageal cancer, which is a common malignant cancer worldwide and top 10 leading cause of death in cancer [1]. Most ESCC related deaths are results of systemic metastasis, mainly through lymphatic vessels [2]. The overall 5-year survival rate following surgery is about 40% for non-metastatic ESCC patients, whereas it is less than 10% for metastatic ESCC (mESCC) patients [3]. Thus, diagnosis of ESCC before lymph node metastasis is significant for clinical treatment. Previous studies have shown the prognostic significance of several clinic pathologic factors for ESCC, such as age of patients, tumor size and tumor primary site [4]. However, the prognosis of advanced ESCC patients still remains very poor because of few early symptoms and scarce inspection methods [5]. Therefore, it is crucial to identify prognostic factors and establish sensitive methods for monitoring the metastasis of ESCC, which can help surgeons select proper treatment and therapy for patients.

Metabolomics, a fast growing field of systems biology, aims to quantitatively measure small molecule metabolites in a given biological system through a targeted or non-targeted strategy generally. As for non-targeted metabolomics, a global analysis of metabolites within a biological system is conducted to give a

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holistic view of the system. While for targeted metabolomics, it focuses on detecting metabolites involved in a specific biological process or pathway. Targeted metabolomics provide a complementary means for non-targeted metabolomics in terms of its high specificity and sensitivity. It is known that a little change at gene and protein level usually results in a significant alteration in metabolite level; therefore, metabolomics is a highly sensitive and direct approach for disease inspection. Currently, metabolomics is widely applied to identify biomarkers for cancer diagnosis [6,7] as well as to investigate trigger factors of tumor progression and metastasis [8,9].

Recently, many clinic efforts have been made for ESCC based on metabolomics technology [10,11]. Most of these efforts are non-targeted metabolomics studies, which mainly focus on identifying biomarkers for ESCC diagnosis rather than ESCC metastasis prognosis [5]. A targeted metabolomics study reported that the response of cancer cells to immune escape has a close correlation to tumor formation, progression and migration [12]. It is worth noting that an imbalance of tryptophan metabolism is implicated in tumor immune escape [13]. Srikanth and his colleagues have investigated relations between the inflammation and tryptophan metabolism in colon and gastrointestinal cancer [14]. In this study, a targeted metabolomics approach is applied to quantify compounds participated in tryptophan metabolism, which presents variations of these compounds and their concentration ratios between ESCC, mESCC patients and healthy controls. Our study provides not only potential biomarkers for diagnosis and prognosis of ESCC but also metabolic profile of tryptophan metabolism about progression and migration of the disease, which helps understand the mechanism of tumorigenesis and metastasis of the disease.

2. Materials and methods

2.1. Chemicals

Acetonitrile and methanol of high-performance liquid chromatography grade were purchased from CNW Technologies GmbH (Düsseldorf, Germany). All standard compounds, benzoyl chloride, benzoyl chloride-d5, and sodium tetraborate were purchased from Sigma-Aldrich.

2.2. Sample collection

This study was approved by the ethics committee of Changhai Hospital (Shanghai, China). All subjects participated in this study provide written informed consents in accordance with institutional guidelines. All patients did not receive any radiotherapy or chemotherapy before blood collection. Fasting blood samples were collected from patients with clinically proven ESCC (n = 38) and mESCC (n = 38) at Changhai Hospital. Blood samples from healthy volunteers (n = 28) were obtained under the identical fasting conditions. Table 1 lists basic information of the patients and the healthy volunteers. There was no statistical difference in average age between patients and health subjects. Each blood sample was clotted for 45 min at room temperature prior to centrifugation at 3000*g* for 10 min. The serum was aliquoted into a separate vial and frozen at −80 °C before instrumental analysis.

Table 1
Clinical characteristics of patients and healthy subjects.

	Healthy controls	ESCC patients	mESCC patients
No. of subjects	28	38	38
Age(mean)	58	60	61
Gender(male/female)	20/8	27/11	36/2

2.3. Sample preparation

The protocol of sample preparation was revised from previous publication [14,15]. 20 μL of standard mixture (including all standard compounds detected in this study, each 5 μg/mL) was derivatized by sequentially adding 30 μL of 100 mM sodium tetraborate and 50 μL of 2% benzoyl chloride-d5 (BzD5) in acetonitrile (v/v). The resulting BzD5 labeled standard mixture was diluted to a final concentration of 2 ng/mL in 50% aqueous acetonitrile (v/v) as quantitative internal standard (BzD5-IS).

Thawed serum sample was vortexed for 5 s at room temperature, and 20 μL of serum was derivatized by orderly spiking 30 μL of 100 mM sodium tetraborate and 50 μL of 2% benzoyl chloride (Bz) in acetonitrile (v/v). The serum derivatives (100 μL) were mixed with equivoluminal BzD5-IS (2 ng/mL) prior to centrifugation at16000*g* for 10 min. The supernatant was applied to LC-MS/MS analysis.

2.4. UPLC-MS/MS analysis

An Ultra Performance Liquid Chromatography system (ACQUITY UPLC system; Waters) and a tandem mass spectrometry system (AB SCIEX Triple Quad™ 5500 System) were used to separate and detect the derivatives in this study. 2 μL of derivatized sample was injected and separated by a Poroshell 120 EC-C18 column (50 mm × 3 mm, 2.7 μm, Agilent). The column temperature was 40 °C. The derivatives were eluted from column at 0.4 mL/min using mobilephase consisted of 10 mM ammonium formate and 0.15% (v/v) formic acid in water (A) and acetonitrile (B). The program of elution was initially 5% B for 0.5 min, ramped to 25% B at 1.5 min, ramped to 70% B at 6 min, to 100% B at 6.5 min and held to 7.5 min, then back to 5% B for equilibration of column. The total analysis time was 10 min. Electrospray ionization (ESI) was used in positive mode for detection of the eluents. MS conditions were as follows: source temperature at 600 °C, curtain gas (CUR) at25 psi, ion source gas 1 (GS1) at 50 psi, ion source gas 2 (GS2) at50 psi, collision gas (CAD) at 8 psi, ion spray voltage (ISV) at 5500 V, entrance potential (EP) at 10 V, collision cell exit potential (CXP) at10 V. Scheduled multiple-reaction monitoring (S-MRM) was performed to qualitatively and quantitatively detect the nine metabolites, and the declustering potential (DP) and collision energy (CE) of their Bz- and BzD5-derivatives are listed in Table 2. The software Analyst® (version 1.5.2, AB Sciex) was applied to control LC-MS/MS instrument, as well as data collection and analysis.

2.5. Data preprocessing and statistical analysis

The raw data were preprocessed by the Analyst® (version1.5.2). Peak areas ratio of Bz- and BzD5-derivatives were calculated for relative quantization. In practice, abundance of each metabolite was first calculated for every sample. Then, statistical analysis on metabolite's abundance and the ratio of four metabolites to TRP was conducted among healthy controls, ESCC and mESCC patients by the R software. In this procedure, the ratios of metabolites/TRP were normalized into the 0–1 interval. Student's *t*-test (normal distribution) or Mann–Whitney *U* test (non-normal distribution) was performed to analyze the difference of metabolites/TRP among healthy controls, ESCC and mESCC patients. The differential ratios of metabolite/TRP (with p-value < 0.05 or p-value < 0.1) were kept as indicators, which were utilized to build prediction models for ESCC diagnosis and prognosis using the logistic regression method. After that, a five-fold cross validation test was carried out to inspect performance of these prediction models. Subsequently, the receiver