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Activation of choline kinase drives aberrant choline metabolism in esophageal squamous cell carcinomas

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is a major health threat worldwide. Research focused on molecular events associated with ESCC carcinogenesis for diagnosis, treatment and prevention is needed. Our goal is to discover novel biomarkers and investigate the underlying molecular mechanisms of ESCC progression by employing a global metabolomic approach. Sera from 34 ESCC patients and 32 age and sex matched healthy controls were profiled using two-dimensional liquid chromatography-mass spectrometry (2D LC–MS). We identified 120 differential metabolites in ESCC patient serums compared to healthy controls. Several amino acids, serine, arginine, lysine and histidine were significantly changed in ESCC patients. Most importantly, we found dysregulated lipid metabolism as an important characteristic in ESCC patients. Several free fat acids (FFA) and carnitines were found down-regulated in ESCC patients. Choline was significantly increased and phosphatidylcholines (PC) were significantly decreased in ESCC serum. The high expression of choline and low expression of total PC in patient serum were associated with the high expression of choline kinase (Chok) and activated Kennedy pathway in ESCC cells. Chok expression can serve as a significant biomarker for ESCC prognosis. In conclusion, metabolite profiles in the ESCC patient serum were significantly different from those in the healthy controls. Phosphatidylcholines and Chok, the key enzyme in the PC metabolism pathway, may serve as novel biomarkers for ESCC.

1. Introduction

Esophageal cancer is the eighth most common cancer worldwide with geographical and ethnic characteristics [1]. In China, esophageal cancer ranks the third most common and the fourth leading cause of cancer mortality [2]. Esophageal squamous cell carcinoma (ESCC) is the dominant type of esophageal malignancy in China. Although the early intervention, surgery, medical and radiation therapy have demonstrated improved patient outcomes, the five-year survival for patients with esophageal cancer is still low, ranging from 15% to 20% [3]. Moreover, it is estimated that esophageal cancer rates will increase in the next decades, while many other types of cancer will decrease in incidence. In summary, ESCC represents a worldwide health problem.

ESCC carcinogenesis is driven by multiple molecular events. The multistage process is characterized by morphological changes from normal esophagus to basal cell hyperplasia, dysplasia, carcinoma *in situ* and ESCC [4]. Strategies using genomics, transcriptomics, proteomics and metabolomics have been employed to investigate molecular mechanisms underlying the pathogenesis of esophageal cancer carcinogenesis and identify potential prevention and therapeutic targets for ESCC. Metabolic reprogramming is one of the

Abbreviations: ESCC, esophageal squamous cell carcinoma; 2D LC–MS, two-dimensional liquid chromatography-mass spectrometry; FFA, free fat acids; PC, phosphatidylcholines; Chok, choline kinase; ESI, electrospray ionization; PCA, principal component analysis; PLS-DA, partial least squares discriminate analysis; HCA, hierarchical cluster analysis; IHC, immunohistochemistry; TMAs, tissue microarrays; DAB, 3,3′-diaminobenzidine; QC, quality control; LPC, lysophosphatidylcholine; PC-MUFA, polyunsaturated phosphatidylcholines; PC-PUFA, monounsaturated phosphatidylcholines; PC-SFA, saturated phosphatidylcholines; PE, phosphatidylethanolamine; cPLA2, cytosolic phospholipase A2.

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critical characteristics of cancer. Metabolic reprograming includes changes in biosynthetic and bioenergetic pathways such as glycolysis, glutaminolysis, lipid metabolism, mitochondrial biogenesis, or the pentose phosphate pathway [5]. Moreover, metabolic reprograming plays a profound role in cancer cell gene expression, cellular differentiation, and changes in the microenvironment [6].

Metabolomic analysis is a strategy to systematically investigate the small molecular weight metabolites present within a biological system [7,8]. Nontargeted metabolomic approaches have been used in new biomarker development and investigations of carcinogenesis mechanisms in numerous diseases, including esophageal cancers [7,9,10]. Serum has an abundant pool of metabolites that can be used to reflect the systemic metabolic aberration in patients [7]. Metabolomic approaches have identified metabolites that are significantly changed in ESCC, with the potential to serve as biomarker for diagnosis, therapeutic response monitoring, prognosis or outcome prediction [11–14]. For example, significant differences in serum free amino acid profiles were found between ESCC patients and healthy controls [14]. Metabolic approaches also showed that phospholipid metabolism plays a role in ESCC carcinogenesis [9,12]. Metabolomics approaches identified the prognostic value of a combination of valine, γ-aminobutyric acid, and pyrrole-2-carboxylic acid as potential biomarkers for metastatic ESCC [10].

However, these differently accumulated metabolites have yet to uncover the key mechanisms that drive ESCC carcinogenesis. In this study, we attempted to discover novel biomarkers by profiling serum metabolites from ESCC patients along with age and gender matched controls using two dimensional liquid chromatography-mass spectrometry (2D LC–MS), which is valuable for serum samples by achieving comprehensive metabolomics and lipidomics analysis in a single injection [15]. We also aimed to identify the molecular events associated with these dysregulated metabolites to shed light on the pathogenesis of ESCC.

2. Materials and methods

2.1. Patients and samples

ESCC patients and healthy subjects were recruited at the First Affiliated Hospital of Zhengzhou University, whereas ESCC patients were diagnosed and received surgery or chemotherapy. Blood samples were collected on the date of diagnosis and prior to initial treatment. Blood samples were obtained from healthy individuals who underwent physical examination and showed no chronic diseases. Healthy individuals were chosen to match with ESCC patients on age and gender. Written informed consent was obtained from each subject before entering this study, and the Ethical Committee of the First Affiliated Hospital of Zhengzhou University approved the protocols outlined in the following text. Blood samples were allowed to clot for 45 min, centrifuged at 4 °C for 10 min at 1300 g, and the resulting sera were separated into 100 μl aliquots and frozen at −80 °C. All specimens were processed and frozen for long-term storage within 4 h post-sample preparation. Serum samples were delivered on dry ice to Dalian Institute of Chemical Physics and stored at −80 °C until use.

2.2. Metabolic profiling analysis

2.2.1. Serum pretreatment

50 μl of serum was added to 200 μl methanol (containing internal standards) and vortexed for 1 min 50 μl isopropanol was added to the sample, and the resulting solution was vortexed for 30 s. After centrifugation at 14,000 rpm, 4 °C, for 10 min, 250 μl of supernatant was transferred to a clean tube and lyophilized by freeze-drying. Prior to LC–MS analysis, 50 μl water/isopropanol (1:1, v:v) solution was added to the lyophilized sample for analysis. Eighteen internal standards are listed in Table A1.

Table A1
Internal standards and their concentrations.

ID	Concentration (μg/mL)
Carnitine C2:0-d3	0.16
Carnitine C10:0-d3	0.10
Carnitine C12:0-d3	0.15
CDCA-d4	1.45
CA-d4	1.85
Tryptophan-d5	4.25
Phenylalanine-d5	3.61
Leucine-d3	7.70
Alanine-d3	8.02
Valine-d8	4.00
Choline-d4	0.26
FFA C16:0-d3	2.50
FFA C18:0-d3	2.50
PC 38:0	1.25
PE 34:0	0.62
SM (d18:1/12:0)	0.31
TG 45:0	1.00
Cer (d18:1/17:0)	0.31

2.2.2. Instrumental analysis

The samples were run in random order. One quality-control sample and one blank vial were run after each of the 10 samples. A two-dimensional liquid chromatography (2D LC) system equipped with Shimadzu Prominence and Nexera modules (Shimadzu Corporation, Kyoto, Japan) coupled to a Q Exactive HF orbitrap mass spectrometer (ThermoFisher Scientific Corporation, Rockford, USA) equipped with electrospray ionization (ESI) source was used as the LC–MS platform to obtain comprehensive information of metabolome and lipidome. In addition, the times used in the sample preparation, reconstitution and column equilibrium can be saved to improve total analytical throughput. This 2D-LC strategy is significantly valuable for small amounts of samples such as stem cells, biopsy tissues, cerebrospinal fluid, serum etc. The specific procedures were reported previously [15]. Briefly, a BEH C8 column (2.1 × 5 mm, 1.7 mm, Waters Corporation, USA) was used in the first dimension to pre-separate the metabolome from lipidome. The metabolome and lipidome were on-line transferred to parallel LC separation systems. A BEH C18 column (2.1 × 50 mm, 1.7 mm, Waters Corporation, USA) was used for metabolome analysis. The mobile phases A1 and B1 were water and acetonitrile (containing 0.1% formic acid), respectively. The flow rate and column temperature were set at 0.35 ml/min and 55 °C. The initial percent of gradient elution was 5% B1, and maintained for 0.3 min. The percent of B1 was linearly increased to 100% in 13.8 min and maintained for another 0.5 min, and then reduced to 5% B1 for equilibrium. A BEH C8 column (2.1 × 50 mm, 1.7 mm, Waters Corporation, USA) was used for lipidome analysis. The mobile phases A2 and B2 were water/acetonitrile (4:6, v:v) and iso-propanol/acetonitrile (9:1, v/v), respectively, both containing 10 mM ammonium acetate. The flow rate and column temperature were set at 0.3 ml/min and 55 °C. The initial percent of gradient elution was 40% B2 and maintained for 15.3 min. The percent of B2 was linearly increased to 70% in 9.7 min and increased to 100% in 3 min. After 1 min, the ratio was reduced to 40% B2 for equilibrium. The injection volume was 5 μl.

Data were acquired in full scan mode. The m/z values ranging from 80 to 1000 were detected. Mass spectrometry parameters were as follows: spray voltage, 3.5 kV (ESI+) and 3 kV (ESI−); sheath gas flow rate, 45 arbitrary units; aux gas flow rate, 10 arbitrary units; capillary temperature, 300 °C; aux gas heater temperature, 350 °C; S-lens RF level, 50.