Global and Targeted Metabolomics of Esophageal Squamous Cell Carcinoma Discovers Potential Diagnostic and Therapeutic Biomarkers*

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Diagnostic and therapeutic biomarkers useful for esophageal squamous cell carcinoma (ESCC) have the ability to increase the long term survival of cancer patients. A metabolomics study, using plasma from four groups including ESCC patients before, during, and after chemoradiotherapy (CRT) and healthy controls, was originally carried out by LC-MS to determine global alterations in the metabolic profiles and find biomarkers potentially applicable to diagnosis and monitoring treatment effects. It is worth pointing out that a clear clustering and separation of metabolic data from the four groups was observed, which indicated that disease status and treatment intervention resulted in specific metabolic perturbations in the patients. A series of metabolites were found to be significantly altered in ESCC patients versus healthy controls and in pre- versus post-treatment patients based on multivariate statistical data analysis (MVDA). To further validate the reliability of these potential biomarkers, an independent validation was performed by using the selected reaction monitoring (SRM) based targeted approach. Finally, 18 most significantly altered plasma metabolites in ESCC patients, relative to healthy controls, were tentatively identified as lysophosphatidylcholines (lysoPCs), fatty acids, L-carnitine, acylcarnitines, organic acids, and a sterol metabolite. The classification performance of these metabolites were analyzed by receiver operating characteristic (ROC) analysis and a biomarker panel was generated. Together, biological significance of these metabolites was discussed. Comparison between pre- and post-treatment patients generated 11 metabolites as potential therapeutic biomarkers that were tentatively identified as amino acids, acylcarnitines, and lysophosphatidylcholines. Levels of three of these (octanoylcarnitine, lysoPC(16:1), and decanoylcarnitine) were closely correlated with treatment effect. Moreover, variation of these three potential biomarkers was investigated over the treatment course. The results suggest that these biomarkers may be useful in diagnosis, as well as in monitoring therapeutic responses and predicting outcomes of the ESCC. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.022830, 1306–1318, 2013.

Worldwide, esophageal cancer (EC) is the eighth most prevalent cancer, and it is also one of the most lethal, accounting for more than 300,000 deaths per year. There are two major histological types of EC, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), of which ESCC is dominant globally (1–3). Massive studies have revealed the prevalence of this disease in China (1, 2, 4). However, the current diagnostic, screening, and surveillance methods for EC such as upper gastrointestinal endoscopy, BMI, Body Mass Index; CRT, Chemoradiotherapy; EC, Esophageal Carcinoma; ESCC, Esophageal Squamous Cell Carcinoma; FA, Fumaric Acid; LysoPCs, Lysophosphatidylcholines; MVDA, Multivariate Statistical Data Analysis; NC, Normal Control; OR, Overall Responder; Non-OR, Nonoverall Responder; OPLS-DA, Orthogonal Partial Least Square Discriminant Analysis; PCA, Principal Component Analysis; PLS-DA, Partial Least Squares Discriminant Analysis; QC, Quality Control; RRLC, Rapid Resolution Liquid Chromatography; RRLC-(−)ESI-MS, RRLC-MS Analysis by ESI in Both Positive and Negative Ion Modes; RRLC-(+)ESI-MS, RRLC-MS Analysis by ESI in Positive Ion Mode; RRLC(−)ESI-MS, RRLC-MS Analysis by ESI in Negative Ion Mode; TIC, Total Ion Chromatogram; XIC, Extracted Ion Chromatogram; TNM, Tumor Nodes Metastasis; VIP, Variable Importance in Projection Values; SRM, Selected Reaction Monitoring; CE, Collision Energy; MS/MS, Tandem Mass Spectrometry; RRLC-MS/MS SRM, Rapid Resolution Liquid Chromatography-Tandem Mass Spectrometry in Selected Reaction Monitoring Mode.
barium swallows, and serology makers etc. (5), has specific limitations in their own way. In addition, chemoradiotherapy (CRT) is recognized as one of the most effective current treatments for EC (6–8). However, as with all other treatments, variability in clinical response to CRT is observed among individuals, and this has a major influence on clinical care outcomes. Hence, biomarkers indicating EC pathogenic processes or responses to therapeutic interventions are required to diagnose and facilitate early interventions, as well as for monitoring therapeutic responses and predicting outcomes.

Metabolomics, a growing field in systems biology (9–11), has been shown to be a powerful approach to quantitatively measure global changes in the metabolic profiles of individuals in response to disease or treatment via noninvasive analyses of biofluids (12–16). Hence, metabolomics represents an excellent developing prospect for the discovery of diagnostic and therapeutic biomarkers. Recently, Arun et al. reported that components of the sarcosine pathway may have potential as biomarkers for prostate cancer progression and serve as new avenues for therapeutic interventive using the metabolomics approach (15). Moreover, Xuan et al. used metabolomics to identify potential biomarkers associated with schizophrenia and drug treatment thereof (17). There is also a growing interest in identifying the molecular alterations associated with EC by GC-MS- and 1H NMR- based metabolomics approach (18, 19). However, the metabolite variations and the disturbances of metabolic pathways of ESCC by the metabolomics analyses are still far from complete.

In this study, a non-targeted metabolomics approach based on rapid resolution liquid chromatography-mass spectrometry (RRLC-MS) in conjunction with multivariate statistical data analyses (MVDA) was employed to determine global alterations in the metabolic profiles of healthy controls and ESCC patients before, during, and after cisplatin-based CRT. Metabolic alterations in response to pathological conditions or CRT treatment were evaluated to discover potential diagnostic and therapeutic biomarkers, respectively. Furthermore, a targeted metabolomics approach was carried out to validate the reliability of these potential biomarkers based on rapid resolution liquid chromatography-tandem mass spectrometry in selected reaction monitoring mode (RRLC-MS/MS SRM). Potential diagnostic biomarker metabolites were evaluated by receiver operating characteristic (ROC) analysis. In addition, this research focused in-depth on variations of potential therapeutic biomarkers in ESCC patients responding differently to CRT treatment, with the aim of allowing monitoring of treatment progress and outcome prediction. A flow chart illustrating the study design is shown in supplemental Fig. S1.

### Experimental Procedures

**Chemicals**—ACN (HPLC grade) and formic acid (FA, HPLC grade) were purchased from Merck (Darmstadt, Germany). Standard compounds, including valine, phenylalanine, L-carnitine, lactic acid, uric acid, cholic acid, linoleic acid, 1-palmitoyl-sn-glycero-3-phosphocholine, and 1-stearoyl-sn-glycero-3-phosphocholine were purchased from Sigma-Aldrich (St. Louis, MO). Citric acid, trioxymethylen-thraquinone, 2-hydroxybenzoic acid, levofloxacin, hesperidin, and rhein were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

**Sample Collection**—All of the fasting samples were recruited from the Cancer Institute and Hospital of the Chinese Academy of Medical Sciences (Beijing, China). The cancer hospital had the standard sample collection protocols. The current patients selecting standards were as follows: patients signed the informed consent of research; diagnosed with ESCC by cytology or histology; not suitable for surgical operation; age between 18—80; in generally, ECOG performance status ≤2; weight loss <10% in recent 6 months; had CT image in last 4 weeks and had completed staging examination; sufficient heart, lung, liver, kidney, and hematopoietic function. All patients had not received any other therapy including medications except CRT during the sampling period. Cancer stage was established according to the 2002 Tumor Nodes Metastasis (TNM) staging system. Controls were recruited in the department of cancer prevention of the same hospital when they did the health examination and signed the informed consent. The study was approved by the hospital ethics committee and with the approval of corresponding regulatory agencies.

**Pre-treatment Samples**—44 males diagnosed with ESCC (ESCC group, 64.4 ± 10.8 years old, BMI: 23.0 ± 3.9). Mid- and post-treatment samples, were collected after the first course of cisplatin-based CRT (ESCC-M group) and upon completion of CRT (ESCC-P group) from 32 ESCC patients with different responses to treatment (overall responders, OR group, n = 21; nonoverall responders, non-OR group, n = 11). The control group (NC group) consisted of samples from 24 male, age- and BMI-matched, healthy Chinese volunteers (detailed information in Table I). RRLC-MS/MS based validation test: ESCC group, n = 80; ESCC-M and ESCC-P group, n = 40, respectively (including 28 ORs and 12 non-ORs); NC group, n = 80. Fasting blood samples were collected into K2 EDTA vacutainer tubes and cooled down in freezer (4 °C) at once. Then they were centrifuged within 2 h at 3000 × g for 10 min at 4 °C. Supernatants (plasma) were separated and transferred into new vials, and immediately stored frozen (−80 °C) until preparation.

As part of the system conditioning and quality control (QC) process, a pooled QC (20) sample was prepared by mixing equal volumes (10 μl) of 132 samples.

**Sample Preparation**—A method of plasma preparation has been established in our previous study, and was applied here (21). The dry residue was reconstituted in 80 μl of acetonitrile (brevf vortext), followed by 20 μl of high purity grade water (brevf vortext), and then centrifuged again at 3000 × g for 10 min at 4 °C. Supernatant was removed and transferred into new vials ready for RRLC-MS analysis. For RRLC-MS/MS based validation test, plasma samples containing internal standards (levofloxacin, hesperidin, and rhein, 5 μg/ml) were prepared as described here.

**RRLC-MS Analysis**—Chromatographic separation was performed on a Zorbas Aq-C18 column (1.8 μm, 10 cm × 2.1 mm; Agilent, Santa Clara, CA), using an Agilent 1200 Series rapid resolution liquid chromatography system (1200 RRLC system; Agilent technologies, Waldbronn, Germany). The column was maintained at 60 °C. The injected sample volume was 5 μl for each run. Gradient conditions were as follows: 0–20 min linear gradient 0–100% B, and then solvent composition was maintained at 100% B for 3 min, followed by return to the starting conditions and re-equilibration of the column for 8 min. Solvents A and B were 0.1% FA-water and ACN, respectively, and the flow rate was 250 μl/min. Samples from healthy volunteers and ESCC patients were alternated in random order in the analysis batch. To visually evaluate chromatographic reproducibility, the QC sample was...
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Table I

| Characteristics                        | ESCC patients | ESCC-M and ESCC-P | Healthy controls |
|----------------------------------------|---------------|-------------------|------------------|
| Gender                                 | Male          | Male              | Male             |
| Race                                    | Chinese       | Chinese           | Chinese          |
| Total No. of subjects                  | 44            | 32                | 24               |
| Age (mean ± S.D., range)               | 64.4 ± 10.8, 45–79 | 62.2 ± 10.6, 46–70 | 60.3 ± 11.2, 42–74 |
| Cancer stage                           |               |                   |                  |
| T1 No. of subjects                     | 2             |                   |                  |
| Age (mean ± S.D., range)               | 62 ± 12.7, 53–71 |                   |                  |
| Smokers/non-Smokers                    | 2/0           |                   |                  |
| Alcohol/non-Alcohol                    | 2/0           |                   |                  |
| T2 No. of subjects                     | 6             |                   |                  |
| Age (mean ± S.D., range)               | 72 ± 7.4, 62–78 |                   |                  |
| Smokers/non-Smokers                    | 5/1           |                   |                  |
| Alcohol/non-Alcohol                    | 5/1           |                   |                  |
| T3 No. of subjects                     | 22            | 21                |                  |
| Age (mean ± S.D., range)               | 66 ± 9.7, 51–79 | 63 ± 9.3, 51–70 |                  |
| Smokers/non-Smokers                    | 20/2          | 20/1              |                  |
| Alcohol/non-Alcohol                    | 19/3          | 18/3              |                  |
| T4 No. of subjects                     | 14            | 11                |                  |
| Age (mean ± S.D., range)               | 60 ± 9.5, 45–74 | 58 ± 8.7, 46–69 |                  |
| Smokers/non-Smokers                    | 10/4          | 9/2               |                  |
| Alcohol/non-Alcohol                    | 11/3          | 9/2               |                  |
| Medicine of CRT                        |               |                   |                  |
| Cisplatin                              | 6             |                   |                  |
| 5-Fu/Cisplatin                         | 15            |                   |                  |
| Paclitaxel/Cisplatin                   | 11            |                   |                  |

analyzed repeatedly within the analytical run after every ten plasma samples.

Mass spectrometry experiments were performed on a Q-TOF (QSTAR Elite, Applied Biosystem/MDS Sciex, Foster City, CA) equipped with ESI sources. Data were acquired in both positive and negative ion modes. The measurement conditions were as follows: ESI: source voltage 5.5 kV or −4.5 kV, vaporizer temperature: 375 °C, turbo gas: 75 psi, nebulizer gas: 65 psi, curtain gas: 40 psi, and declustering potential: 50/-50 V. The scan range was m/z 65–850. Nitrogen gas was used for both nebulizing and drying. Data acquisition and processing were performed using Analyst QS 2.0 (QSTAR Elite, Applied Biosystem/MDS Sciex).

RRLC-MS/MS Analysis—For these experiments, information dependent acquisition (IDA) mode was applied, and the signals were detected in a TOF survey scan, followed by two product ion scans on the most intense parent ions as a data dependent MS/MS experiment. Three MS/MS experiments were triggered by each survey scan. For 60 s, compounds with identical mass-to-charge ratios were automatically excluded. Metabolites of interest were included in the MS/MS table list. Collision energy (CE) was set to 40/20 or −35/−20 eV. All other parameters were as RRLC-MS analysis. Data acquired in positive ion mode were auto-calibrated with background ions (phthalates: m/z 149.0233 and m/z 391.2843). In the negative ion mode, signals were also acquired with auto-calibration, using standard solutions of 2-hydroxybenzoic acid and trioxymethylanthraquinone, which were introduced by post-column mixing at concentrations of 2 ng/μl and a flow rate of 10 μl/min, generating [M-H]− ions of m/z 137.0244 and m/z 269.0455.

Data Processing—Novel data preprocessing approaches were required to correlate specific metabolites to their biological origin. Open-source software XCMS (22) (http://massspec.scripps.edu/xcms/xcms.php), operated within R statistical software (Version 2.10.0), was used in our study. RRLC-MS raw data files acquired from plasma analysis were initially converted to mzData format using Wiff to mzData translator software (version 1.0.0.4, Applied Biosystems/MDS Sciex) with the threshold set at 1%. Nonlinear retention time alignment, peak discrimination, filtering, alignment, matching, and identification were subsequently conducted using XCMS. The parameters were optimized step-by-step until aligned peak lists agreed with the visualization of data across multiple samples. Peak finding options were set as follows: proton method, binlin; method, centWave; ppm, 60; peakwidth, 8–30 scans; mass traces were only retained if they contained at least three peaks with intensity >100 counts (prefilter, c[3,100]); noise threshold, 20; mzdiff, 0.05 amu, and other parameters for detailed data preprocessing are available in the Supporting Information. Then, the resultant two-dimensional matrices, including observations (sample names) in columns, variables (m/z-retention time pairs) in rows, and peak areas were further imported into the SIMCA-P 12.0 software package (Umetrics AB, Umeå, Sweden) for MVDA. Mean-centering and pareto-scaling were applied to all data prior to multivariate statistical analysis to reduce noise and artifacts in the models. Principal component analysis (PCA) was used to visualize general clustering, trends, or outliers among the observations. To test the validity of the model against overfitting, the cross-validation parameter Q2 was computed as in partial least squares discriminant analysis (PLS-DA) by permutation testing using 100 random permutations. Discriminating variables were selected according to variable importance in projection values (VIP > 1), S-plot, jack-knifed-based confidence intervals, and raw data plot in orthogonal partial least square discriminant analysis (OPLS-DA) model. Furthermore, an independent t test (p < 0.05) (Microsoft Office Excel 2007) was used to determine if different biomarker candidates obtained from OPLS-DA
modeling were statistically significant between groups. Partial correlation coefficients between discriminating metabolites with high confidence level ($\alpha = 0.001$) conditioning on one variable were evaluated by software ParCorA, downloaded from: http://www.comp-sys-bio.org/tiki-index.php. After a tight relationship of variables was judged from a series of correlation analysis (Pearson correlation coefficients $>0.8$) (23), R-package CAMERA (http://www.biocductor.org/packages/release/bioc/) and corresponding extracted ion chromatograms (XICs), the fragment, isotope, and adduct ions were manually removed.

**RRLC-MS/MS Based Validation Test**—The gradient conditions were adjusted as follows: The gradient started with 98% A and linear decreased to 40% in 10 min, and then maintained 40% A over 10–14 min. The gradient was then decreased to 0% A in 16 min and maintained for 6 min, followed by return to the initial conditions and re-equilibration of the column for 8 min. Solvents A and B were 0.1% FA-water and ACN, respectively, and the flow rate was 250 µl/min. The eluent was introduced into a triple quadrupole-trap mass spectrometer equipped with electrospray ionization source (Qtrap 5500, Applied Biosystem/MDS Sciex). All of the potential diagnostic and therapeutic biomarkers were detected by RRLC-MS/MS SRM method. The respective SRMs are as follows: positive ion mode including 110$\rightarrow$69, 116$\rightarrow$70, 118$\rightarrow$72, 132$\rightarrow$44, 140$\rightarrow$96, 162$\rightarrow$102, 166$\rightarrow$120, 177$\rightarrow$80, 182$\rightarrow$91, 185$\rightarrow$98, 189$\rightarrow$119, 203$\rightarrow$175, 205$\rightarrow$188, 209$\rightarrow$192, 218$\rightarrow$88, 246$\rightarrow$70, 262$\rightarrow$88, 274$\rightarrow$88, 288$\rightarrow$85, 290$\rightarrow$228, 302$\rightarrow$85, 314$\rightarrow$85, 316$\rightarrow$85, 318$\rightarrow$88, 330$\rightarrow$85, 334$\rightarrow$57, 338$\rightarrow$83, 344$\rightarrow$85, 346$\rightarrow$86, 358$\rightarrow$88, 362$\rightarrow$88, 368$\rightarrow$85, 370$\rightarrow$85, 374$\rightarrow$88, 400$\rightarrow$88, 412$\rightarrow$85, 415$\rightarrow$107, 468$\rightarrow$184, 494$\rightarrow$184, 496$\rightarrow$184, 510$\rightarrow$184, 516$\rightarrow$459, 520$\rightarrow$184, 522$\rightarrow$504, 524$\rightarrow$506, 546$\rightarrow$184, 583$\rightarrow$299, 585$\rightarrow$299, 617$\rightarrow$219. IS1 (rhein): 293$\rightarrow$196, 556$\rightarrow$196, 85, 204$\rightarrow$142, 213$\rightarrow$59, 215$\rightarrow$59, 239$\rightarrow$151, 241$\rightarrow$59, 243$\rightarrow$183, 263$\rightarrow$145, 267$\rightarrow$59, 267$\rightarrow$223, 277$\rightarrow$233, 279$\rightarrow$261, 281$\rightarrow$210, 303$\rightarrow$259, 307$\rightarrow$59, 335$\rightarrow$124, 367$\rightarrow$97, 389$\rightarrow$97, 398$\rightarrow$97, 397$\rightarrow$97, 407$\rightarrow$325, 428$\rightarrow$74, 464$\rightarrow$74, 471$\rightarrow$97, 476$\rightarrow$279, 480$\rightarrow$225, 485$\rightarrow$469, 500$\rightarrow$303, 507$\rightarrow$123, 512$\rightarrow$432, 524$\rightarrow$196, 556$\rightarrow$271. IS1 (rhein): 293$\rightarrow$221; IS2 (hesperidin): 609$\rightarrow$301. Analyst 1.5.1 was used for data processing.  

**Metabolite Identification**—Identification of metabolites was carried out following established methods (24, 25) and by searching the free databases METLIN (http://metlin.scripps.edu), Massbank (http://massbank.imm.ac.cn/MassBank) and HMDB (http://hmdb.ca/) using exact molecular weights. Subsequently, high resolution RRLC-MS/MS spectra were used for further identification. Commercially available standards were adopted to confirm the structure of some metabolites.

**Characterization of Potential Diagnostic Biomarkers**—Each potential biomarker, or the potential biomarker panel, was further evaluated for diagnostic power using the area under the receiver operating characteristic curve (AUC) (26). SPSS (version 17.0) was used to plot ROC curves for this purpose. The discriminatory capability of each marker metabolite was ranked and visualized by heat maps (27).

**RESULTS**

**Data Quality Assessment**—At the heart of any successful metabolomics study is a high quality data set that produces a biochemical snapshot, reflecting the temporal state of an organism through its endogenous small molecules or “metabolites” (28). To obtain reliable data, technical errors originating from sample collection, sample preparation, and RRLC-MS analysis must be minimized to avoid confounding multivariate data analysis. In our study, samples from each group were alternated in random order in each analysis batch. Moreover, a QC sample was analyzed in parallel with the actual samples to monitor the stability of the system (20).

Multivariate analysis results of the QC sample demonstrated that the peak areas deviation was <2SD, indicating that the data from the RRLC-MS were statistically acceptable (supplemental Fig. S2). In addition, the retention time deviation profiles (R-software) of all plasma samples generated values of less than ± 40 s and ± 20 s for the majority of RRLC-MS analysis by ESI in positive ion mode (RRLC-(+)ESI-MS) and in negative ion mode (RRLC-(−)ESI-MS) analyses, respectively, indicating high reproducibility (supplemental Fig. S3). These results confirm that the significant differences observed between groups by multivariate statistical analysis were more likely to be a result of genuine subtle changes in metabolites, rather than products of artifacts arising from technical errors. The typical total ion chromatograms (TICs) obtained from plasma samples in different groups by RRLC-(+)ESI-MS are shown in supplemental Fig. S4.

**Global Metabolomic Profiling of ESCC, ESCC-M, ESCC-P, and NC**—For obtaining useful metabolomics results, data analysis strategy is as important as the analytical technique employed. In this study, the RRLC-MS analysis by ESI in both positive and negative ion modes (RRLC-(±)ESI-MS) raw data were converted into mzData format. XCMS was then used to carry out peak discrimination, filtering, and retention time alignment, yielding 1012 peaks of positive ions and 1942 peaks of negative ions between retention times of 0.9–21 min (XCMS program used available in Supporting Information). Missing values caused by peaks that were not present in the sample/ chromatogram were reduced using the 80% rule (29). The resulting data were imported into SIMCA-P, and centered and pareto scaled to reduce the impact of noise and artifacts in the models.

To investigate global metabolic alterations, all observations acquired in both ion modes were integrated and coanalyzed using PCA. However, the ESCC, ESCC-M, ESCC-P, and NC groups were not well distinguished in the PCA score plot. This is likely because human plasma samples are extremely complex, and the unsupervised PCA data analysis technique separates samples based on random, ESCC-irrelevant variation of metabolites (supplemental Fig. S5 A and B). To maximize the separation of sample classes, a further supervised analysis using OPLS-DA was performed. As presented in Fig. 1, clear separation was observed among four groups (ESCC, ESCC-M, ESCC-P, and NC groups) for RRLC-(+)ESI-MS analysis, suggesting that metabolic perturbations were evident in the patients, dependent on pathological condition and treatment intervention. Moreover, the results also showed that, combined with the response to treatment, the difference between ORs and non-ORs increased over time, with clear statistically significant differences between them in the ESCC-P group. This indicated that treatment progress could be reflected in the levels of some metabolites. For
negative data, an obvious separation trend was observed among ESCC, ESCC-T (ESCC-treated; including ESCC-M and ESCC-P), and NC groups, but minimal separation between the ESCC-M and ESCC-P groups was observed (supplemental Fig. S5C).

A series of OPLS-DA analyses were conducted to further investigate the metabolomic alterations that characterize ESCC diagnosis and treatment intervention. For further discovery of potential diagnostic biomarkers, an OPLS-DA model was constructed using data from pre-treatment ESCC patients and healthy controls. To discover potential therapeutic biomarkers, another OPLS-DA was constructed using data from pre- and post-treatment ESCC patients, combined with the therapeutic effect (responders and non-responders). Metabolites significantly altered only in ORs are more likely to be associated with therapeutic effects and could potentially be used as biomarkers for therapeutic response monitoring.

Comparison of Metabolic Profiles between Pre-treatment ESCC Patients and Controls—

Multivariate Statistical Analysis—To explore potential biomarker candidates better, a subset of ten samples (including five ESCC patients and five healthy controls) was randomly selected to form an independent test set, and the resulting samples formed the training set. For the training set, OPLS-DA was applied as a stoichiometric analysis method for the exploration of the difference between pre-treatment ESCC patients and controls. Score scatter plots for OPLS-DA models from both positive and negative ion modes showed clear differentiation of ESCC and NC groups (Fig. 2A and 2B). For RRLC-(±)ESI-MS data, the classification of ESCC and NC groups resulted in one predictive (t_y) and two orthogonal (t_o) (1 + 2) components with a cross-validated predictive ability, Q²(cum), of 54.7%. In addition, 58.5% of the variance in X [R²(X)] accounted for 83.2% of the variance of Y [R²(Y)].

To further guard against model overfitting, a default of seven rounds of cross-validation across three components was applied, with 1/7th of the samples being excluded from the model in each round. Validation with 100 random permutation tests generated intercepts of R² = 0.287 and Q² = −0.178 (Fig. 3A). For the negative model data R²(X) = 60.5%, R²(Y) = 89.6%, and Q²(cum) = 53.4% across one predictive and two orthogonal components, with validation intercepts of R² = 0.275 and Q² = −0.186 (Fig. 3B). These results indicate that OPLS-DA models derived from the RRLC-(±)ESI-MS data had good predictive ability and were reliable (30).

Moreover, to further evaluate the predictive ability of the models, an independent test set was used. None of those samples had been previously included in the supervised analysis, which therefore allowed for the estimation of true predictive accuracy. As shown in Fig. 3C and 3D, the OPLS-DA model correctly predicts all ESCC patients and healthy controls showing 100% sensitivity and specificity. This external validation study confirms the feasibility of the LC-MS based plasma metabolomics study as a potential diagnostic tool for ESCC.

Discovery of Potential Diagnostic Biomarker Candidates—To discover potential biomarkers among thousands of variables, the S-plot showing the covariance and correlation between the metabolite variables and the model was primarily used. Selection of metabolites with a high covariance combined with a high correlation was preferred (31). The VIP value reflects the influence of every variable on the classification. Variables with a VIP value of > 1.0 had an above average influence on the explanation of the Y matrix (30) and were,
therefore, highlighted. Furthermore, jack-knifed-based confidence interval and raw data plots were subsequently used to eliminate variables with low reliability. In parallel, an independent t test ($p < 0.05$) was used to validate the significance of the difference in concentration between variables identified by these methods in tested groups.

Pearson correlation coefficients, combined with the R-package tool, CAMERA, were applied to reduce redundant variables originating from the same compound, such as fragments, isotopes, and adduct ions. XICs of these ions were also similar, confirming that they originated from the same metabolite. The remaining 52 biomarker candidates were then selected for further validation by RRLC-MS/MS based targeted metabolomics.

Potential Biomarkers Validation by RRLC-MS/MS Based Targeted Metabolomics—To further validate the reliability of the screened potential biomarkers, and independent validation test based on RRLC-MS/MS targeted metabolomics was performed. Levofloxacin, hesperidin, and rhein were selected as the internal standards to monitor the stability of analysis. The CEs and precursor/fragment ion pairs were pre-optimized for each analyte of interest to give the best signal. The typical XICs of the RRLC-MS/MS SRM analysis in positive ion mode are shown in Fig. 4. According to the results of independent t test ($p < 0.05$) between ESCC and NC group, seventeen metabolites of the initial 52 biomarker candidates were screened out. The resulted 35 metabolites were picked out after the validation test, and regarded as the more reliable potential diagnostic biomarkers.

Identification of the Potential Diagnostic Biomarkers—In the metabolomics analysis, biomarker identification, and further structure confirmation are considered to be the most challenging steps. A molecular formula was determined according
to the exact mass and the isotope pattern. Furthermore, RRLC-MS/MS experiments were carried out to identify potential biomarkers. The identification process is briefly outlined below, taking \( m/z \) 162.1131/1.18 min in ESI positive ion mode as an example (Fig. 5). Firstly, the corresponding quasi-molecular ion peak was located according to its retention time in the XIC of \( m/z \) 162.1 (Fig. 5A). The exact mass of the ion was found to be \( m/z \) 162.1131. Second, the element composition of the peak was calculated using software incorporated into Analyst QS 2.0 (Applied Biosystem/MDS Sciex). According to the exact mass and isotope pattern, three possible element compositions were calculated. Third, the elemental compositions were compared with those registered in public databases, and C7H16NO3 was found as the most likely match. Finally, a mass fragmentation experiment was conducted (Fig. 5B) and the standard MS/MS spectrum of possible compounds was searched. As a result, the biomarker was initially identified as L-carnitine and was finally confirmed by comparison with RRLC-MS/MS analysis of L-carnitine standard (Fig. 5C and 5D). Combined biomarkers were first analyzed by binary logistic regression, followed by ROC curve determination. The results (Fig. 6B) showed that a panel of potential biomarkers (decanoylcarnitine, octanoylcarnitine, lysoPC(18:0), lysoPC(16:1), lysoPC(16:0), linoleic acid, and uric acid) provided an AUC of 0.961. Sensitivity and specificity, which were calculated at best cut-off points, reached 90.2% and 96.0%, respectively. The results demonstrated the dependable capability of combined biomarkers to provide discrimination between ESCC patients and healthy controls. Future

Of these, eight potential biomarkers were confirmed using standard compounds (Table II).

Characterization of Potential Diagnostic Biomarkers and Biomarker Panel—To characterize these putative biomarkers, in-depth ROC analysis was performed. The 18 identified potential biomarkers were divided into two groups, 8 up-regulated in ESCC patients and 10 down-regulated (Table II). Heat maps were created to illustrate the discriminatory power of each potential biomarker (Fig. 6A), and each was ranked in order of AUC values. All potential biomarkers had AUC values within the range of 0.66–0.93. Because complex diseases involve systematic deregulation of biochemical pathways, a panel of multiple biomarkers, rather than a single biomarker, will have more power to discriminate and provide clinically useful information. Therefore, metabolites with AUC > 0.85 were used to construct a clinically valuable biomarker panel. Combined biomarkers were first analyzed by binary logistic regression, followed by ROC curve determination. The results (Fig. 6B) showed that a panel of potential biomarkers (decanoylcarnitine, octanoylcarnitine, lysoPC(18:0), lysoPC(16:1), lysoPC(16:0), linoleic acid, and uric acid) provided an AUC of 0.961. Sensitivity and specificity, which were calculated at best cut-off points, reached 90.2% and 96.0%, respectively. The results demonstrated the dependable capability of combined biomarkers to provide discrimination between ESCC patients and healthy controls. Future
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Tentatively identified metabolites accountable for the discrimination between ESCC patients and healthy controls. RT values in italics are potential biomarkers detected in negative ESI mode and those in non-italics detected in positive ESI mode; *Metabolites confirmed by standard compounds. Metabolites provisionally identified by database searches and MS fragmentation. MS/MS fragments was obtained with CE 40/-35eV, respectively. dp value of independent t-test. eFC was calculated from the ratio of the mean values of ESCC group and NC group. FC value >1 indicates a relative high concentration present in ESCC patients while <1 means a relative low concentration compared to the healthy control. VIP is variable importance in the projection obtained from OPLS-DA with a threshold of 1.0.

| No. | RT(min) | m/z   | Elemental composition | Metabolite identification | MS/MS fragments | p value | FC  | VIP |
|-----|---------|-------|-----------------------|---------------------------|-----------------|---------|-----|-----|
| 1   | 1.18    | 162.131 | C_{12}H_{14}NO_{3}    | L-Carnitine               | 102, 85, 60, 58, 57, 43 | 0.012   | 2.42 | 2.69 |
| 2   | 9.52    | 288.2168 | C_{12}H_{14}NO_{3}    | Octanoylcarnitine         | 229, 127, 85    | 0.035   | 0.35 | 3.79 |
| 3   | 10.12   | 302.2330 | C_{12}H_{14}NO_{3}    | Nonanoylcarnitine         | 243, 141, 85, 60 | 0.015   | 0.54 | 1.46 |
| 4   | 10.43   | 316.2483 | C_{12}H_{14}NO_{3}    | Decanoylcarnitine         | 257, 155, 85, 60 | 0.024   | 0.32 | 2.76 |
| 5   | 11.70   | 330.2650 | C_{12}H_{14}NO_{3}    | Undecanoylcarnitine       | 271, 249, 85    | 0.019   | 0.16 | 1.45 |
| 6   | 13.18   | 468.3083 | C_{20}H_{36}NO_{3}    | LysoPC(14:0)              | 184, 125, 104, 86 | 7.04E-06 | 0.36 | 1.75 |
| 7   | 13.61   | 494.3326 | C_{20}H_{36}NO_{3}    | LysoPC(16:1)              | 184, 125, 104, 86 | 0.035   | 0.41 | 1.98 |
| 8   | 14.30   | 496.3390 | C_{20}H_{36}NO_{3}    | LysoPC(16:0)              | 184, 125, 104, 86 | 0.011   | 0.42 | 1.45 |
| 9   | 15.43   | 524.3703 | C_{20}H_{36}NO_{3}    | LysoPC(18:0)              | 506, 184, 125, 104, 86 | 0.044   | 0.60 | 3.49 |
| 10  | 14.53   | 548.3537 | C_{20}H_{36}NO_{3}    | LysoPC(20:3)              | 184, 125, 104, 86 | 3.35E-04 | 0.49 | 2.41 |
| 11  | 7.49    | 89.0240  | C_{3}H_{3}NO_{2}      | Lactic acid               | 71, 43, 45, 41   | 2.39E-09 | 1.73 | 4.43 |
| 12  | 2.29    | 167.0216  | C_{3}H_{3}NO_{2}      | Oleic acid                | 124, 96, 69     | 0.044   | 4.50 | 1.44 |
| 13  | 7.47    | 191.0202  | C_{3}H_{3}NO_{2}      | Citric acid               | 111, 87, 85, 67  | 0.002   | 2.18 | 1.44 |
| 14  | 16.32   | 277.2186  | C_{18}H_{32}O_{2}     | Linolenic acid            | 259, 233, 205, 191, 178, 147, 103 | 0.038   | 1.34 | 1.27 |
| 15  | 16.82   | 279.2322  | C_{18}H_{32}O_{2}     | Linoleic acid             | 261, 71, 59      | 0.036   | 1.49 | 2.54 |
| 16  | 17.42   | 281.2485  | C_{18}H_{32}O_{2}     | Oleic acid                | 238, 210, 66     | 0.028   | 1.83 | 1.47 |
| 17  | 16.82   | 303.2331  | C_{18}H_{32}O_{2}     | Arachidonic acid          | 285, 259, 231    | 0.019   | 1.91 | 2.64 |
| 18  | 12.55   | 407.2795  | C_{18}H_{32}O_{2}     | Cholic acid               | 389, 345, 325, 251 | 0.003   | 0.39 | 1.10 |

**Fig. 6. Visualization of the discriminatory powers of individual and combined potential diagnostic biomarkers.** A. Heat map showing the discriminatory capacity of each metabolite estimated by AUC. Colors correspond to AUC values; red and blue represent high and low values, respectively. The metabolites with * represented which were confirmed by standard compounds. B. ROC curve of the combined potential biomarkers. 1 means a relative low concentration compared to the healthy control. VIF is variable importance in the projection obtained from OPLS-DA with a threshold of 1.0.

Biomarkers—To evaluate physiological responses to treatment, OPLS-DA was performed to discriminate between the pre- and post-treatment metabolic profiles of ESCC patients. For RRLC-(-)ESI-MS data, a clear separation was observed in the OPLS-DA model (Fig. 7A), indicating a significant impact of CRT treatment on global metabolism. R^2(Y), Q^2(Y), and validation results (Fig. 7A and 7B) indicated that the OPLS-DA model derived from the positive data was robust and valid. Metabolic alterations in ORs and non-ORs were also evaluated, respectively. Those metabolites only significantly altered in ORs are more likely to be associated with therapeutic effects, and could potentially be used as biomarkers for therapeutic response monitoring. Pre- and post-treatment groups in both ORs and non-ORs were readily discriminated. In addition, PLS-DA was carried out to validate the reliability of the model (supplemental Fig. S6). RRLC-(-)ESI-MS data were similarly analyzed (supplemental Fig. S7, S8). The results showed the clear distinction between pre- and post-treatment samples of all patients, ORs, and non-ORs.

Using similar selection and RRLC-MS/MS based validated processes as those for diagnostic biomarkers, 48 metabolites accountable for the discrimination between post-treatment and pre-treatment metabolic profiles were initially selected. Furthermore, eleven of the resulting 28 differential metabolites after validation test were tentatively identified as potential therapeutic biomarkers: two amino acids, five acylcarnitines, and four lysoPCs (summarized in Table III).

Variations of Potential Therapeutic Biomarkers during CRT Treatment—In comparison of post- with pre-treatment patients, levels of the amino acids were decreased, while those
of acylcarnitines and lysoPCs were increased (Table III). Furthermore, metabolic alteration trends between pre- and post-treatment in ORs and non-ORs could be divided into four categories: five metabolites had similar changes after treatment in ORs and non-ORs, one demonstrated opposite changes after treatment in ORs and non-ORs, two metabolites were only found to be significantly changed in non-ORs, and three were only significantly changed in ORs (Table III).

The three metabolites (lysoPC(16:1), octanoylcarnitine and decanoylcarnitine) that significantly changed only in ORs were chosen as biomarkers related to therapeutic effect and the variations of these were subjected to in-depth investigation over the treatment course. As illustrated in Fig. 8, levels of all three showed a tendency toward gradual recovery to the healthy state in ORs. In non-ORs, no obvious changes in the levels of these metabolites were observed over the treatment course.

**DISCUSSION**

The present study develops the feasibility and effectiveness of utilizing RRLC-MS-based metabolomics combined with global and targeted approach to capture metabolic markers...
representing biochemical changes in ESCC and CRT treatment along with therapeutic effects. The global metabolomics offers the widest chemical coverage, but does not provide the same data quality for all of the analytes covered (32), whereas the RRLC-MS/MS based targeted metabolomics are often used to determine precisely and accurately relative abundances and concentrations of a limited number of preknown and expected metabolites (33). Therefore, to discover more reliable biomarkers, it is necessary to integrate the global and targeted metabolomics. However, what we should point out is that non-identical analogs (levofloxacin, hesperidin and rhein), instead of heavy isotope labeled internal standards, were used in our targeted approach to monitor the stability of analysis, which might lead to some deviation of the results. In addition, the validity of our approach has been demonstrated by verifying part of potential biomarkers with standards. However, there remains some uncertainty regarding the verification of several metabolites we identified in this research because their commercially standards were unavailable (Table II and III). Understanding the biological significance of these potential biomarkers could provide further insight into the mechanisms underlying the pathophysiology of ESCC and treatment intervention, and facilitate the discovery of biomarkers for diagnosis, treatment monitoring, and response prediction in ESCC.

Compared with healthy controls, ESCC patients showed significantly higher plasma levels of l-carnitine, while levels of acylcarnitines (octanoylcarnitine, nonanoylcarnitine, decanoylcarnitine, and undecanoylcarnitine, Table II) were decreased. Carnitine and acylcarnitines are essential for the transport of long chain fatty acids across the mitochondrial membrane for degradation and energy production, and that they have the ability to shuttle short chain fatty acids from the inside of the mitochondria to the cytosol (34). During this process, carnitine is esterified to form acylcarnitine derivatives catalyzed by acetyl-CoA (35). Therefore, the lower levels of acylcarnitines might be a reflection of deficiency, or reduced activity, of acetyl-CoA dehydrogenase in ESCC patients, and also reflected the disturbed ability of β-oxidation of long-chain fatty acids. Numerous disorders have been described that lead to disturbances in energy production and intermediary metabolism, which are characterized by the abnormal levels of acylcarnitines (36–40). For example, four identical acylcarnitines (carnitine C8:1, C9:1, C9:0 and C10:1) have been found as potential biomarkers in liver cancer patients (37). This also implies a single metabolite will have limited use for an accurate diagnosis of ESCC, however, the biomarker panel would be more specificity.

Marked abnormalities in the levels of a series of fatty acids and lysoPCs were also observed in ESCC patients. LysoPCs can hydrolyse into fatty acids in a reaction catalyzed by lyso-phospholipase A1, and subsequently disintegrate in the mitochondria to produce energy through β-oxidation (41). The abnormal levels of the fatty acids (linolenic acid, linoleic acid, oleic acid, and arachidonic acid) and lysoPCs (lysoPC (14:0), lysoPC(16:0), lysoPC (16:1), lysoPC (18:0) and lysoPC (20:3)) (Table II) in ESCC patients are indicative of disturbed lipid and energy metabolism. LysoPCs can also be transformed into lysophosphaticid acid (LPA) in a reaction catalyzed by lyso-phospholipase D (lyso-PLD). Thus, we speculate that the significant diminishment of five lysoPCs might be correlated with the overexpression of lyso-PLD, as the overexpression of lyso-PLD has been observed in several other malignancies, including glioblastoma, thyroid carcinomas, and breast cancer (42–45). Furthermore, LPA, the product of lysoPCs, is considered to be closely involved in the development of cancers because of its role in the promotion of cell proliferation (46, 47). In addition, lysoPCs themselves have been recognized as vital cell-signaling molecules acting through lysophospholipid receptors (LPL-R) (46). Thus, the down-regulation of lysoPCs may indicate an abnormality in this cell-signaling pathway in cancer patients.

We also observed a dramatic decrease in levels of cholic acid, the main metabolite of cholesterol in the liver, in ESCC patients. Its metabolic role is to affect the absorption of lipids from food and influence sterol hormone levels (48). Uric acid, as the final oxidation product of purine metabolism, was...
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found to be up-regulated in plasma of ESCC patients (Table II). The studies about human hepatocellular carcinoma also showed uric acid was elevated in urine sample of patients but not obvious variation in serum sample (14).

The abnormal levels of lactic acid and citric acid observed also indicate a disturbance of glycolysis and energy metabolism. Lactic acid is a product of glucose metabolism under conditions of oxygen deficiency and plays a key role in several biochemical processes. A number of studies have demonstrated that malignant transformation is associated with an increase in glycolytic flux, and anaerobic and aerobic cellular lactate excretion (49, 50). Citric acid is an important intermediate product in the TCA cycle (51) and is also a precursor in metabolic pathways, including the biosynthesis of fatty acids (52).

Although some of these metabolites were also discovered in other studies on cancer metabolomics (36–38) and other diseases (39), change tendencies of some metabolites in different diseases were consistent whereas some were not. For example, the carnitine was demonstrated up-regulated in colorectal cancer (36) and liver cancer (37), whereas the carnitine 9:0 (m/z 302) was down-regulated in liver cancer (37). However, the opposite change has been observed in patients with inflammatory disorders: nonesterified carnitine was extremely decreased, while acylcarnitines were significantly increased in patient plasma (39).

At the same time, because of the cancers involvement in the systematic deregulation of biochemical pathways, the single biomarker would be of limited use for diagnosis. So we conducted a ROC analysis on a generated logistic regression model to perform the diagnostic value of the biomarker panel. The diagnostic utility in combining measurement of the up-regulated (decanoylcarnitine, lysoPC(16:0), lysoPC(18:0), lysoPC(16:1) and octanoylcarnitine) with the down-regulated (linoleic acid and uric acid) was relatively high, with a sensitivity and specificity up to 90.2% and 96.0%, respectively. For example, the carnitine was demonstrated up-regulated in colorectal cancer (36) and liver cancer (37), whereas the carnitine 9:0 (m/z 302) was down-regulated in liver cancer (37).

The variations of three metabolites (lysoPC(16:1), octanoylcarnitine and decanoylcarnitine) with the down-regulated (linoleic acid and uric acid) was relatively high, with a sensitivity and specificity up to 90.2% and 96.0%, respectively. For further clinical applications, much more rigorous validation will be required. Larger and more diverse population including various cancer patients, non-cancer patients, and healthy controls, will be enrolled in the future to further verify the specificity and sensitivity of this newly discovered biomarker panel.

The variations of three metabolites (lysoPC(16:1), octanoylcarnitine and decanoylcarnitine) before, during, and after CRT treatment (Fig. 8) are suggestive of partial normalization in β-oxidation, glycolysis and energy metabolism in ESCC patients as a result of treatment. A few studies described the modulation of the carnitine system by anticancer therapy (38, 53, 54). However, our study drew the conclusion through a distinct aspect. Furthermore, variations of these three metabolites between ORs and non-ORs over the same treatment course became more pronounced over time (Fig. 8). Thus, we propose that these three metabolites might not only be useful in diagnosis, but also in monitoring treatment progress and outcome prediction.

In summary, the present study revealed that metabolomics combined global with targeted approach is able to discover biomarkers applicable to both diagnostic and therapeutic purposes. The results showed the global metabolic alterations of healthy controls and ESCCC patients before, during and after CRT treatment by RRLC-MS based metabolomics approach, which were potentially valuable both in aiding diagnosis and monitoring the therapeutic effect, and in providing novel insights regarding metabolism and systemic effects in ESCC. In the future, analysis of additional large-scale samples should be explored to further validate the clinical utility of biomarkers described in this study.

* This study has been supported by National Instrumentation Program (2011YQ170067) and the National High Technology Research and Development Program of China (863 Program) (No. 2012AA02A503).

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This article contains supplemental Figs. S1 to S8.

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