1H-NMR based metabonomic profiling of human esophageal cancer tissue

Liang Wang†, Jie Chen†, Longqi Chen, Pengchi Deng, Qian bu, Pu Xiang, Manli Li, Wenjie Lu, Youzhi Xu, Hongjun Lin, Tianming Wu, Huijuan Wang, Jing Hu, Xiaoni Shao, Xiaobo Cen and Ying-Lan Zhao*

Abstract

Background: The biomarker identification of human esophageal cancer is critical for its early diagnosis and therapeutic approaches that will significantly improve patient survival. Specially, those that involves in progression of disease would be helpful to mechanism research.

Methods: In the present study, we investigated the distinguishing metabolites in human esophageal cancer tissues (n = 89) and normal esophageal mucosae (n = 26) using a 1H nuclear magnetic resonance (1H-NMR) based assay, which is a highly sensitive and non-destructive method for biomarker identification in biological systems. Principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were applied to analyse 1H-NMR profiling data to identify potential biomarkers.

Results: The constructed OPLS-DA model achieved an excellent separation of the esophageal cancer tissues and normal mucosae. Excellent separation was obtained between the different stages of esophageal cancer tissues (stage II = 28; stage III = 45 and stage IV = 16) and normal mucosae. A total of 45 metabolites were identified, and 12 of them were closely correlated with the stage of esophageal cancer. The downregulation of glucose, AMP and NAD, upregulation of formate indicated the large energy requirement due to accelerated cell proliferation in esophageal cancer. The increases in acetate, short-chain fatty acid and GABA in esophageal cancer tissue revealed the activation of fatty acids metabolism, which could satisfy the need for cellular membrane formation. Other modified metabolites were involved in choline metabolic pathway, including creatinine, creatine, DMG, DMA and TMA. These 12 metabolites, which are involved in energy, fatty acids and choline metabolism, may be associated with the progression of human esophageal cancer.

Conclusion: Our findings firstly identify the distinguishing metabolites in different stages of esophageal cancer tissues, indicating the attribution of metabolites disturbance to the progression of esophageal cancer. The potential biomarkers provide a promising molecular diagnostic approach for clinical diagnosis of human esophageal cancer and a new direction for the mechanism study.

Keywords: Metabonomic profiling, Human esophageal cancer, 1H-NMR

Background

Esophageal cancer is one of the most common newly diagnosed cancers and the fourth cause of digestive system cancer mortality in the United States in 2012 [1]. Esophagectomy is the mainstay of curative treatment for localized esophageal cancer [2]. However, the treatment outcome is far from satisfactory [3-5]. The patients with low-stage cancer have a 45% to 73% chance of survival, and the patients with high-stage tumors of larger size and higher metastatic potential have only a 18% chance of survival within 3 years [6]. The underlying reasons for this disappointingly low survival rate are multifold, including ineffective screening tools and guidelines; cancer detection at an advanced stage, with over 50% of patients with unresectable disease or distant metastasis at the time of presentation; unreliable noninvasive tools to measure complete response to chemoradiotherapy; and limited survival achieved with palliative chemotherapy alone for
patients with metastatic or unresectable disease. Therefore, early and accurate diagnosis of esophageal cancer is important for patient survival and improving therapeutic options for different stage of esophageal cancer.

Over the past decades the methods, such as on-endoscopy-based balloon cytology and upper gastrointestinal (GI) endoscopy, have been widely used to improve the diagnosis. However, they have certain limitations including the poor specificity and sensitivity, resulting in detection of the disease at an advanced stage [7]. At the molecular level, numerous studies reporting specific alterations in proteins and genes in esophageal cancer might be useful for the diagnosis, prognosis and treatment of esophageal cancer [8-10]. However, reliable markers, especially at an early and potentially curative stage, are still unknown.

Metabonomics is a systematic approach focusing on the profile of low molecular weight metabolites in cells, tissues, and biofluids [11,12]. It is a powerful tool for analyzing the chemical composition and providing important information on disease process, biochemical functions and drug toxicity [13]. Thus, it has been widely used in disease diagnosis [14,15], biomarker screening [16,17] and safety assessment of chemical [18,19]. Two most powerful and commonly used analytical methods for metabolic fingerprinting are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry [20,21]. NMR is a non-destructive and non-invasive technique that can provide complete structural analysis of a wide range of organic molecules in complex mixtures [22]. Although a growing number of NMR-based metabonomics aim at finding possible biomarkers of presence and/or grade of different cancers such as prostate cancer [23,24], colorectal cancer [25], brain cancer [26] and breast cancer [27,28], there are only few researches on esophageal cancer [29-31]. Only one report used NMR method to investigate the difference of metabolites in esophageal cancer tissue. Moreover, the number of cancer samples was only 20 ~ 35 in these studies, which may be difficult to provide accurate and comprehensive information of metabolites. Especially, none of these reports systematically investigated the discriminating metabolites involved in the different pathological stages of esophageal cancer.

Multivariate statistical analysis is commonly applied to metabonomic data including the unsupervised (principal component analysis, PCA) and supervised (partial least-squares-discriminant analysis, PLS-DA) methods [32]. In addition, to optimize the separation, thus improving the performance of subsequent multivariate pattern recognition analysis and enhancing the predictive power of the model in NMR-based metabonomics studies [33], orthogonal partial least-squares-discriminant analysis, OPLS-DA is carry out to visualize the metabolic alterations between the esophageal cancer tissues and normal esophageal mucosae. In the present study, we applied $^1$H-NMR to study metabonomic profiling of human esophageal cancer tissues. We identified a total of 45 distinguishing metabolites, 12 of which were modified along with the aggressive process of esophageal cancer. These metabolites are closely associated with the energy supplies, fatty acids and choline metabolic pathways. Our results provide the potential biomarkers for clinical diagnosis for different stages of human esophageal cancer and new insights for the mechanism research. Moreover, this study demonstrates that a NMR-based metabolomics approach is a reliable and sensitive method to study the biochemical mechanism underlying human esophageal cancer.

Results

Clinical population
We investigated a total of 115 samples, 89 of which were from primary esophageal cancer and 26 from normal esophageal mucosa. For 26 cases paired samples of cancer tissue and normal tissue were available from the same patient. The clinical information of patients was summarized in Table 1. As listed in Table 1, the patients aged 39–79 years old. The stage of all tissue specimens was determined according to the American Joint Committee on Cancer (AJCC) for esophageal tumors: stage II, 28 patients; stage III, 45 patients; stage IV, 16 patients. Though the tissue specimens of stage I patient was absent because of the limited specimens, it is still worth investigating. 85 cases of cancer samples were esophageal squamous cell carcinoma. All patients were subjected to surgical resection of the primary tumor and lymph nodes. Tumor size, location, lymph node numbers, differentiation status and lymphovascular invasion were also evaluated.

Metabonomic profiling of samples
Normal mucosa and esophageal cancer tissue samples underwent extraction, and the aqueous fractions were investigated using NMR. The representative $^1$H-NMR spectra of aqueous phase extracts of normal mucosae and esophageal cancer tissues were showed in Figure 1. The spectra were processed and converted into 419 integral regions of 0.02 ppm width as described in Materials and Methods. The major metabolites in the integrate regions were identified by a comparison with literature data and spectra of standards acquired in Human Metabolome Database. As a result, a series of changes of endogenous metabolite levels were observed in esophageal cancer when compared with the normal mucosa (Figure 1A and 1B). The majority of metabolites were assigned to amino acid, lipid, carbohydrate, organic acid and nucleotide, which are known to be involved in multiple biochemical processes, especially in energy and lipid metabolism [34].
To optimize the separation of the two groups, we then utilized OPLS-DA to visualize the metabolic difference between the esophageal cancer tissues and normal esophageal mucosa. As the results shown, two groups achieved distinct separation in the scores plot of PC1 and PC2 of OPLS-DA analysis (Figure 2B). Moreover, the corresponding PLS-DA model parameters for the explained variation, $R^2 = 0.75$, and the predictive capability, $Q^2 = 0.64$, were significantly high, indicating that it is an excellent model suitable for data analysis (Figure 2C).

According to the chemical shifts between the two groups (VIP $> 1$ and $p < 0.05$), the significantly distinguishing metabolites were identified (Table 2). These metabolites are involved in key metabolic pathways including glycolysis, TCA cycle, urea cycle, pyrimidine metabolism, gut flora metabolism and fatty acids metabolism.

To study the predictive power of the model to unknown samples, we randomly selected 80% of samples (normal mucosae = 20, esophageal cancer = 71) as training set to construct OPLS-DA model, which was used to predict the class membership of the remaining 20% of samples (the ‘testing set’, normal mucosae = 5, esophageal cancer = 18). As shown in Figure 2D, normal mucosae of testing set were correctly located in the region of normal mucosae of training set, and the same results were obtained in esophageal cancers of testing set ($R^2_{X_{cum}} = 0.17$, $R^2_{Y_{cum}} = 0.531$, $Q^2_{Y_{cum}} = 0.322$). These results show that OPLS-DA model can not only distinguish normal mucosae from esophageal cancers, but also achieve excellent predictive power to the unknown samples.

---

**Table 1 Clinical information of esophageal cancer patients and normal mucosae**

| Clinical features      | Esophageal cancer | Normal control |
|------------------------|-------------------|----------------|
| Number                 | 89                | 26             |
| Sex                    |                   |                |
| Male                   | 82                | 22             |
| Female                 | 7                 | 4              |
| Age at diagnosis       |                   |                |
| Median(years)          | 61                | 59.5           |
| Range                  | 39-79             | 43-70          |
| Histology              |                   |                |
| ESCC                   | 85                |                |
| Adenocarcinoma         | 4                 |                |
| Pathologic grade       |                   |                |
| PD                     | 22                |                |
| MD                     | 62                |                |
| HD                     | 5                 |                |
| Disease stage          |                   |                |
| I                      | 0                 |                |
| II                     | 28                |                |
| III                    | 45                |                |
| IV                     | 16                |                |
| Lymph node metastasis  |                   |                |
| NO                     | 34                |                |
| N1                     | 55                |                |

MD: poorly differentiated; PD: moderately differentiated; HD: highly differentiated.

---

**Figure 1** 600.13 MHz CPMG $^1$H-NMR spectra of tissue specimens from esophageal cancer patients and normal mucosae. A: normal mucosae, B: esophageal cancer. The grey region represents the detailed spectra between 2 and 4.5 ppm of normal mucosae and esophageal cancer.
PR analysis of normal mucosae and different stages of esophageal cancer

The differences of metabolites profiling among various stages of esophageal cancer are important for biomarker identification for accurate diagnosis and therapy. The PR analysis showed the metabolites modified, and we then used the OPLS-DA model to investigate the metabolites differentially regulated. As shown in Figure 3A, the scores plot of PC1 and PC2 indicated that all stages (II, III and IV) of esophageal cancer could be clearly separated from normal mucosae. The statistical validations of the corresponding PLS-DA model by permutation analysis were shown in Figure 3B. The parameters for different stages were as follows: stage II: $R^2 = 0.92$, $Q^2 = 0.76$; stage III: $R^2 = 0.82$, $Q^2 = 0.71$ and stage IV: $R^2 = 0.88$, $Q^2 = 0.76$. The panel of 45 metabolites with VIP > 1 and $p < 0.05$ of these three groups were listed in Table 3. The majority metabolites were similar to those of metabolites between normal mucosae and esophagus cancers. Interestingly, we identified dimethylamine (DMA), dimethylglycine (DMG), polyunsaturated fatty acids and histidine, which are associated with the stage of esophageal cancer. DMG significantly decreased in stage III, and the changes of DMA and histidine were significant only in stage IV in comparison to normal mucosae. In addition, polyunsaturated fatty acids were altered significantly in stage II.

Finally, we randomly used 80% of samples to construct OPLS-DA model, and then detected the predictive power to the remaining 20% of samples. As shown in Figure 3C, the majority samples of testing set were correctly classified as esophageal cancer and normal mucosae. In the scores plot of stage II and normal mucosae ($R^2_{X\text{cum}} = 0.287$, $R^2_{Y\text{cum}} = 0.895$, $Q^2_{Y\text{cum}} = 0.725$), the green diamond which represented normal mucosae of testing set were correctly located in the region of normal mucosae, and most of the stage II samples of testing set located in the region of stage II. These results indicate that esophageal cancer of stage II are correctly discriminated
from normal mucosae. However, there were 2 samples of stage II located in the edge of the two training set clusters. One possible reason might be that the changes of the metabolites were not significant in the earlier stage. For example, DMA was only remarkably increased in stage IV.

| Table 2 Summary of different metabolites between esophageal cancers and normal mucosae (Continued) |
|---------------------------------------------------------------|
| **Carbohydrate**                                               | **Esophageal cancer vs Normal control** |
| Glucose                                                       | VIP<sup>a</sup> | p-value<sup>b</sup> | FC<sup>c</sup> |
|                                                               | 3.25            | 0.026               | −1.16          |
|                                                               | 3.53            | 2.11                | <0.0001        | −1.97 |
| Glycoprotein                                                  | 2               | 2.08                | <0.0001        | 1.70  |
| Polyol                                                        |                |                     |                |
| Ethanol                                                       | 3.65            | 2.07                | <0.0001        | −1.97 |
| Acetone                                                       | 2.22            | 1.28                | <0.0001        | 1.56  |
| **Organic acid**                                              |                |                     |                |
| α-ketoglutaric acid oxime                                      | 2.44            | 1.53                | <0.0001        | −1.60 |
|                                                               | 2.48            | 1.93                | <0.0001        | −1.56 |
| Malonate                                                      | 3.16            | 1.34                | <0.0001        | 1.46  |
| Acetoacetic acid                                              | 2.27            | 1.27                | <0.0001        | 1.25  |
|                                                               | 3.43            | 1.02                | <0.0001        | 3.09  |
| Acetate                                                       | 1.92            | 1.23                | <0.0001        | 3.12  |
| Trimethylamine                                                | 2.88            | 1.19                | <0.0001        | 2.19  |
| Formate                                                       | 8.45            | 1.01                | 0.0005         | 8.79  |
| **Nucleotide**                                                |                |                     |                |
| Uracil                                                        | 5.81            | 1.82                | <0.0001        | 39.93 |
|                                                               | 7.54            | 1.26                | <0.0001        | 2.13  |
| AMP                                                           | 6.15            | 1.76                | <0.0001        | −2.05 |
|                                                               | 8.67            | 1.19                | <0.0001        | −3.10 |
| NAC1                                                          | 2.1             | 1.15                | <0.0001        | 1.49  |
| Adenine in ATP/ADP and NAD/NADH                              | 8.25            | 1.56                | <0.0001        | 1.41  |
| **Cofactors and vitamins**                                    |                |                     |                |
| NAD                                                           | 8.27            | 1.58                | <0.0001        | 1.75  |
|                                                               | 8.36            | 1.45                | <0.0001        | 1.97  |
| NAC2                                                          | 2.04            | 1.69                | <0.0001        | 1.68  |
| **Inorganic acid**                                            |                |                     |                |
| NAD                                                           | 8.83            | 1.65                | <0.0001        | −2.81 |
|                                                               | 9.15            | 1.49                | <0.0001        | −2.44 |
|                                                               | 9.35            | 1.53                | <0.0001        | −2.43 |
| Acetyl hydrazine                                              | 1.96            | 1.63                | <0.0001        | 2.14  |
| **Xenobiotics**                                               |                |                     |                |
| Hippurate                                                     | 7.55            | 1.00                | 0.0005         | 1.77  |
|                                                               |                |                     |                |
| **Variable importance in the projection (VIP > 1) was obtained from OPLS-DA analysis.** |
| **p-value determined from Student’s t-test.**                 |
| **FC: fold change between esophageal cancers and normal mucosae. Positive sign indicates a higher level in esophageal cancer and a negative value indicates a lower level.** |
The scores plot of stage III, IV (stage III: $R^2_{X\text{cum}} = 0.276$, $R^2_{Y\text{cum}} = 0.798$, $Q^2_{Y\text{cum}} = 0.671$; stage IV: $R^2_{X\text{cum}} = 0.296$, $R^2_{Y\text{cum}} = 0.912$, $Q^2_{Y\text{cum}} = 0.765$) were illustrated in Figure 3C. Almost all testing set were correctly located in the corresponding region except for one testing set of stage IV. These results indicate that metabonomics difference could be used for the grading of esophageal cancer.

**Trending markers**

To further study which biomarkers are mainly responsible for the pathological process of esophageal cancer disease, we used box-and-whisker plots to clarify the relative altered levels of those identified 45 metabolites, and obtained 12 representative metabolites among normal control mucosae and esophageal cancer at different stages (Figure 4). Interestingly, these metabolites included glucose, formate, AMP, NAD, creatine, creatinine, DMG, DMA, trimethylamine (TMA), short-chain fatty acids, acetate and GABA, which are mainly involved in energy, fatty acids and choline metabolic pathways.

Glucose, the main source of energy metabolism and precursors for biosynthesis of macromolecules in cells [35], was decreased along with the progression of esophageal cancers when compared with normal mucosae. Formate, the product of sugar utilization of Enterococcus casseliflavus [36], was significantly increased along with the progression; moreover, it upregulated 21.48 folds in stage IV compared to normal mucosae. The AMP, which can be transformed to ATP as energy donor, and NAD, an important coenzyme, were downregulated in esophageal cancer tissues, suggesting a great quantity of energy consumption due to accelerated
Table 3 Summary of different metabolites between each stage of esophageal cancers and normal mucosa

| Metabolite              | Chemical shift (ppm) | Normal vs II VIP<sup>a</sup> | p-value<sup>b</sup> | FC<sup>c</sup> | Normal vs III VIP<sup>a</sup> | p-value<sup>b</sup> | FC<sup>c</sup> | Normal vs IV VIP<sup>a</sup> | p-value<sup>b</sup> | FC<sup>c</sup> |
|-------------------------|---------------------|-------------------------------|---------------------|-------------|-------------------------------|---------------------|-------------|-------------------------------|---------------------|-------------|
| **Amino acid**          |                     |                               |                     |             |                               |                     |             |                               |                     |             |
| Creatine                | 3.04                | 1.97                          | <0.0001             | -1.71       | 1.97                          | <0.0001             | -1.70       | 1.89                          | <0.0001             | -1.91       |
| NAA                     | 2.6                 | 2.06                          | 0.001               | 1.59        | 1.25                          | <0.0001             | 1.84        | 1.17                          | 0.0003              | 1.58        |
| Glutamate               | 2.05                | 1.77                          | <0.0001             | 1.34        | 1.50                          | <0.0001             | 1.28        | 1.77                          | <0.0001             | 1.39        |
| Glutamine               | 2.36                | 1.38                          | <0.0001             | 1.27        | 1.11                          | <0.0001             | 1.19        | 1.29                          | <0.0001             | 1.26        |
| Glycine                 | 2.45                | 1.73                          | <0.0001             | -1.66       | 1.74                          | <0.0001             | -1.52       | 1.74                          | <0.0001             | -1.52       |
| Creatinine              | 3.56                | 1.58                          | 0.0003              | -1.48       | 1.97                          | <0.0001             | -1.66       | 1.66                          | <0.0001             | -1.71       |
| Leucine/Isoleucine      | 3.04                | 1.97                          | <0.0001             | -1.71       | 1.97                          | <0.0001             | -1.70       | 1.89                          | <0.0001             | -1.91       |
| L-tyrosine              | 4.05                | 1.56                          | 0.0005              | -1.45       | 1.85                          | <0.0001             | -1.55       | 1.74                          | <0.0001             | -1.69       |
| Glutamate γ-H          | 2.36                | 1.38                          | <0.0001             | 1.27        | 1.11                          | <0.0001             | 1.19        | 1.29                          | <0.0001             | 1.26        |
| Valine                  | 0.98                | 1.46                          | 0.0008              | 1.45        | 1.61                          | 0.0009              | 1.51        | 1.45                          | 0.0001              | 1.49        |
| Taurine                 | 3.43                |                               |                     |             |                               |                     |             |                               |                     |             |
| Leucine/Isoleucine      | 0.96                | 1.43                          | <0.0001             | 1.40        | 1.55                          | <0.0001             | 1.45        | 1.36                          | <0.0001             | 1.44        |
| L-tyrosine              | 6.93                | 1.22                          | 0.005               | 1.61        | 1.41                          | <0.0001             | 1.62        | 1.21                          | <0.0001             | 1.66        |
| Methionine              | 7.22                | 1.11                          | 0.013               | 1.49        | 1.27                          | <0.0001             | 1.49        | 1.11                          | <0.0001             | 1.54        |
| L-aspartate             | 3.92                |                               |                     |             |                               |                     |             |                               |                     |             |
| GABA                    | 1.91                | 1.58                          | <0.0003             | 2.74        | 1.21                          | <0.0001             | 2.88        | 1.50                          | <0.0001             | 3.65        |
| Methionine              | 3.01                | 0.049                         | 1.15                |             | 0.012                         | 1.19                | 1.11        | 0.0005                        | 1.29                |             |
| Phenylacetylglutamine   | 2.11                | 0.04                          | 1.26                | 1.11        | 0.0122                        | 1.22                | 1.06        | 0.001                         | 1.34                |             |
| Histidine               | 7.42                | 1.01                          | 0.024               | 1.38        | 1.16                          | 0.0006              | 1.43        | 1.12                          | 0.0005              | 1.52        |
| 4-HPPA                  | 7.09                | 1.03                          | 0.027               | -2.22       | 1.30                          | <0.0001             | -3.48       | 1.99                          | <0.0001             | -2.03       |
| Dimethylglycine         | 7.79                | 0.032                         | -2.82               | 0.038       | -2.01                         | 1.48                | 0.0003      | -3.93                         |                     |             |
| Pheny lacetylglutamine  | 2.45                | 1.16                          | 0.0116              | -1.61       | 1.37                          | <0.0001             | -1.65       | 1.01                          | 0.0026              | -1.53       |
| Pheny lacetylglutamine  | 3.72                |                               |                     |             |                               |                     |             |                               |                     |             |
| Cholesterol             | 7.42                | 1.01                          | 0.024               | 1.38        | 1.16                          | 0.0006              | 1.43        | 1.12                          | 0.0005              | 1.52        |
| Phosphocholine          | 7.35                | 1.3                            | 0.0381              | 1.28        | 1.28                          | 0.0038              | 1.2         | 1.23                          | 0.0024              | 1.35        |
| **Phospholipid**        |                     |                               |                     |             |                               |                     |             |                               |                     |             |
| Polyunsaturated fatty acids | 2.84                  | 0.0113                         | 1.25                | 1.02        | 0.0023                        | 1.29                | 1.03        | 0.033                         | 1.24                |             |
| Unsaturated lipids      | 2.27                | 1.04                          | 0.01                | 2.23        | 1.17                          | 0.0002              | 2.24        | 1.19                          | 0.0002              | 2.09        |
| Short-chain fatty acids | 1.04                | 1.39                          | 0.001               | 1.41        | 1.49                          | <0.0001             | 1.48        | 1.53                          | <0.0001             | 1.55        |
| Phosphocholine          | 3.22                | 1.56                          | 0.0002              | 1.29        | 1.07                          | 0.03                | 1.11        | 1.25                          | <0.0001             | 1.05        |
| Myo-inositol            | 3.55                | 1.81                          | <0.0001             | -1.76       | 2.10                          | <0.0001             | -2.01       | 1.71                          | <0.0001             | -2.04       |
| Choline                 | 3.63                | 1.71                          | <0.0001             | -1.62       | 2.01                          | <0.0001             | -1.81       | 1.68                          | <0.0001             | -1.86       |
| Cho                     | 4.07                | 1.56                          | 0.0005              | -1.45       | 1.85                          | <0.0001             | -1.55       | 1.74                          | <0.0001             | -1.69       |
| Choline                 | 3.51                | 1.47                          | 0.001               | -1.43       | 1.63                          | <0.0001             | -1.53       | 0.006                         | -1.34               |             |
| Glycine                 | 4.05                | 1.56                          | 0.0005              | -1.45       | 1.85                          | <0.0001             | -1.55       | 1.74                          | <0.0001             | -1.69       |
cell proliferation in esophageal cancer. The downregulation of creatine, creatinine and DMG, and the elevation of DMA and TMA along with the stages of esophageal cancer indicate the disturbance of choline metabolism. Acetate and GABA, two intermediates in the synthesis of fatty acids, were significantly elevated along with the progression, and both of them upregulated 3.65 folds in stage IV compared to normal mucosae. In addition, the upregulation of short-chain fatty acids, a kind of fatty acid materials, indicated a highly activated fatty acids metabolism in esophageal cancer.

Table 3 Summary of different metabolites between each stage of esophageal cancers and normal mucosae (Continued)

| Category                      | Carbohydrate | Glycoprotein | 2 | 2.00 | <0.0001 | 1.64 | 1.89 | <0.0001 | 1.66 | 1.95 | <0.0001 | 1.81 |
|-------------------------------|--------------|--------------|---|------|---------|------|------|---------|------|------|---------|------|
|                               | Glucose      |              | 3.25 | 1.16 | 0.0005 | -1.34 |
|                               |              |              | 3.53 | 1.81 | <0.0001 | -1.76 | 2.10 | <0.0001 | -2.01 | 1.71 | <0.0001 | -2.04 |
|                                | Acetol       | 2.22 | 1.47 | 0.0007 | 1.38 | 1.32 | <0.0001 | 1.49 | 1.47 | <0.0001 | 1.79 |
|                                | Ethanol      | 3.65 | 1.71 | <0.0001 | -1.62 | 2.01 | <0.0001 | -1.81 | 1.68 | <0.0001 | -1.86 |
|                                | Organic acid | 2.44 | 1.16 | 0.01 | -1.61 | 1.37 | <0.0001 | -1.65 | 1.01 | 0.002 | -1.53 |
|                                |              | 2.48 | 1.74 | <0.0001 | -1.52 | 1.74 | <0.0001 | -1.52 | 1.73 | <0.0001 | -1.66 |
|                                | Malonate     | 3.16 | 1.11 | 0.013 | 1.35 | 1.31 | <0.0001 | 1.45 | 1.38 | <0.0001 | 1.53 |
|                                | Acetate      | 1.92 | 1.58 | <0.0003 | 2.74 | 1.21 | <0.0001 | 2.88 | 1.50 | <0.0001 | 3.65 |
|                                | Acetoacetic acid | 2.27 | 1.04 | 0.01 | 1.22 | 1.17 | 0.0002 | 1.25 | 1.19 | 0.0002 | 1.28 |
|                                |              | 3.43 | 1.18 | 0.0005 | 1.39 | 1.39 | 0.03 | 1.24 |
|                                | Formate      | 8.45 | 1.44 | <0.0001 | 13.51 | 1.11 | <0.0001 | 14.84 | 1.31 | <0.0001 | 21.48 |
|                                | Trimethylamine | 2.88 | 1.23 | <0.0001 | 2.09 | 1.41 | <0.0001 | 2.24 | 1.28 | 0.005 | 2.23 |
|                                | Dimethylamine | 2.73 | 1.31 | 0.0057 | 1.67 |
|                                | Nucleotide   | 2.04 | 1.69 | <0.0001 | 1.35 | 1.50 | <0.0001 | 1.31 | 1.65 | <0.0001 | 1.44 |
|                                | NAC2         | 2.1  | 0.0495 | 1.26 | 1.11 | 0.0122 | 1.22 | 1.06 | 0.0011 | 1.34 |
|                                | NAC1         | 6.15 | 1.50 | <0.0001 | -1.76 | 1.72 | <0.0001 | -2.10 | 1.60 | <0.0001 | -2.16 |
|                                | AMP          | 8.67 | 1.20 | <0.0001 | -3.18 | 1.12 | 0.0007 | -3.84 |
|                                | Adenine in ATP/ADP and NAD/NADH | 8.36 | 1.33 | <0.0001 | 2.02 | 1.45 | <0.0001 | 2.00 | 1.14 | 0.011 | 1.78 |
|                                |              | 8.27 | 1.52 | <0.0001 | 1.44 | 1.51 | <0.0001 | 1.41 | 1.59 | <0.0002 | 1.38 |
|                                |              | 8.25 | 1.46 | <0.0001 | 1.44 | 1.51 | <0.0001 | 1.41 | 1.59 | <0.0002 | 1.38 |
|                                | Uracil       | 5.81 | 1.75 | <0.0001 | 4.30 | 1.83 | <0.0001 | 3.79 | 1.84 | <0.0001 | 41.03 |
|                                |              | 7.54 | 1.31 | 0.003 | 2.12 | 1.28 | <0.0001 | 2.06 | 1.26 | <0.0001 | 2.24 |
|                                | Cofactors and vitamins | NAD | 8.83 | 1.56 | 0.0004 | -2.28 | 1.53 | <0.0001 | -2.44 | 1.81 | <0.0001 | -4.55 |
|                                |              | 9.15 | 1.54 | 0.0005 | -2.09 | 1.41 | <0.0001 | -2.22 | 1.65 | <0.0001 | -3.28 |
|                                |              | 9.35 | 1.52 | 0.0005 | -2.12 | 1.39 | <0.0001 | -2.09 | 1.78 | <0.0001 | -3.65 |
|                                | Inorganic acid | Acetyl hydrazine | 1.96 | 1.99 | <0.0001 | 2.02 | 1.62 | <0.0001 | 2.03 | 1.75 | <0.0001 | 2.40 |
|                                |            | Hippurate | 7.55 | 1.08 | 0.0171 | 1.78 | 0.0027 | 1.75 | 1.13 | 0.0019 | 1.81 |
|                                |            | 7.64 | 1.21 | 0.0079 | 1.12 | 1.28 | 0.0351 | 1.38 | 1.03 | 0.0006 | 1.16 |

*aVariable importance in the projection (VIP > 1) was obtained from OPLS-DA analysis.

*bP-value determined from Student’s t-test.

*FC: fold change between esophageal cancers and normal mucosae. Positive sign indicates a higher level in esophageal cancer and a negative value indicates a lower level.
Discussion

In the present study, we discriminated 89 esophageal cancer tissues from 26 normal mucosae using an OPLS-DA model, and analyzed the metabolites difference between each stage of esophageal cancer and normal mucosae to identify the potential biomarkers involved in the development of esophageal cancer. Forty-five of distinguishing metabolites were identified and 12 of them, including glucose, formate, AMP, NAD, creatine, creatinine, DMG, DMA, TMA, short-chain fatty acids, acetate and GABA, were significantly changed along with the progression of esophageal cancer. Though there are several reports showing the metabolic profiling of esophageal cancer, to the best of our knowledge, the present study is the first to show that some specific metabolites are modified along with the stage of esophageal cancer. Remarkably, the highly activated fatty acids metabolism, increased energy supplies and disturbance of choline metabolism are mainly responsible for the process of pathological development of esophageal cancer.

Identifying the related metabolic pathways of distinguishing metabolites is very important for understanding the biochemical alterations during neoplastic occurrence and development. In order to enhance the information obtained from global metabonomic profiling of esophageal cancer, the human metabolome database and the Kyoto encyclopedia of genes and genomes (KEGG) were utilized to map the marker metabolites with regards to the human metabolic pathways. The findings about the key sets of marker metabolites, related metabolic pathways are summarized in Figure 5. These distinguishing metabolites are involved in detailed metabolic pathway, including fatty acids metabolism (polyunsaturated lipids, short-chain fatty acids, phospholipid, NAA and acetate, GABA), choline metabolism (choline, DMA, DMG, TMA creatine and creatinine), amino acid metabolism (glycine, L-aspartate, glutamine, valine, leucine/isoleucine, methionine and tyrosine), glycolysis (glucose), glutaminolysis (glutamine and glutamate) and tricarboxylic acid cycle (2-oxoglutarate). These results indicate that several specific metabolic pathways are disturbed in esophageal cancer tissue and, particularly, fatty acids metabolism, energy supplies and choline metabolic pathways are involved in the progression of esophageal cancer.

Figure 4 Box-and-whisker plots illustrated progressive changes of the metabolites among different stages of esophageal cancer relative to normal mucosae. Horizontal line in the middle portion of the box, median; bottom and top boundaries of boxes, lower and upper quartile; whiskers, 5th and 95th percentiles; open circles, outliers.
In the process of cancer development, cancer cells increase and alter the metabolism of major nutrients, glucose via glycolysis to meet the high-energy demand under hypoxic conditions [37,38]. In 1920s, Otto Warburg first discovered that cancer cells prefer to metabolize glucose through glycolysis to generate ATP instead of oxidative phosphorylation even in presence of ample oxygen [39]. One molecule of glucose only generates 2 molecules of ATP through glucolysis. This process is a less efficient pathway compared with oxidative phosphorylation which generated ~36 molecules of ATP [40]. Therefore, plenty of glucose was consumed, resulting in glucose reduction. Our results showed that the level of glucose was decreased in esophageal cancer tissue, which is similar to the previous studies that decreased glucose is detected in human colorectal cancer, cervical cancer and hepatoma [41-43]. Moreover, formate, the product of glucose utilization, was significantly increased along with the progression. Remarkably, it reached to the 21 fold in the stage IV in comparison to normal mucosae. Thus, it should be considered as a potential biomarker involved in the progression of human esophageal cancer. Besides glycolysis, increased glutaminolysis is recognized as a vital metabolism pathway of cancer cells to meet the high-energy demand under hypoxic conditions. In cancer cells, glutamine is converted to glutamate by mitochondrial glutaminase. Glutamate is an important energy source via anaerobic input into the tricarboxylic acid (TCA) cycle after conversion to α-ketoglutarate. The decrease of glutamine observed in esophageal cancer could satisfy the production of energy. After conversion to glutamate and ketoglutarate, the final major fate of glutamine is the oxidation of its carbon backbone in the mitochondria, leading to energy production. Glutaminolysis contributes to production of mitochondrial NADH, which is used to support ATP production by oxidative phosphorylation. In the present study, the decreased AMP in esophageal cancer tissue suggests a rapid energy transformation due to a great demand of ATP synthesis.

Besides supporting ATP production, glutamine also contributes to the biosynthesis of lipids and nucleic acids, and regulation of redox homeostasis [44,45]. The catabolism of glutamine is initiated by glutaminolysis [46]. In glutaminolysis, malate is converted into pyruvate or carboxylation is reduced to produce acetyl-CoA. Both catabolism could be useful for maintaining lipogenesis [47,48]. In proliferating cancer cells, the lipogenesis is especially needed for the formation of cellular membranes [39,49]. Therefore, another reasonable explanation for the decreased glutamine observed in present study might be that glutamine is used for maintaining the formation of cellular membranes in esophageal cancer tissues. In the cancer cell proliferation, besides glutamine, the fatty acids are required for the membrane lipids synthesis due to accelerated cell proliferation. In present study, the family of fatty acid materials, short-chain fatty acids, phospholipid, and polyunsaturated fatty acids were upregulated, indicating the activation of fatty acids metabolism. In addition, previous studies reported that cancer tissue can utilize GABA to produce propylene glycol, a precursor of pyruvate derived from glycine [50]. Therefore, GABA upregulation in esophageal cancer tissue

![Figure 5 Altered metabolic pathways for the most relevant distinguishing metabolites between esophageal cancers and normal mucosae. Blue boxes indicated metabolites that were up-regulated in esophageal cancers, while red boxes indicated metabolites that were down-regulated.](http://www.molecular-cancer.com/content/12/1/25)
tissue could be used as building blocks for biosynthesis of cellular membranes. Importantly, GABA increased dramatically along with the progression of esophageal cancer. Therefore, GABA might play a role in the deterioration or matatasis of esophageal cancer. NAA, a free amino acid, synthesized from L-asparate and acetyl-CoA, serves as a source of acetate for lipid and myelin synthesis [51]. We found that both NAA and acetate were significantly increased in esophageal cancer tissue, but L-asparate was decreased. These results are consistent with other findings in ovarian cancer [52]. However, in the previous metabonomics research on esophageal cancer, none of the paper reported the identification of NAA and acetate. The possible reason is that NMR spectroscopy is a method that no selection to metabolites and thus all possible variables could be detected in which no prior information about metabolites is known or the higher sensitivity compared to $^1$H MAS-NMR [53].

Choline and its derivatives represent important constituents in phospholipid metabolism of cell membranes and have been previously identified as markers of cellular proliferation. Choline is degraded through two pathways. The one is to form creatinine via DMG, and the other is converted to methylamine [54]. In the present study, the levels of creatinine, creatine, glycerine, DMG and choline significantly decreased in esophageal cancer tissues. Meanwhile, DMA and TMA, the products of choline metabolism, remarkable increased [55]. Though these two products are widely regarded as nontoxic substance, their potential to form the carcinogen NDMA attaches great clinical interest [56]. Our results indicate the disturbance of choline metabolism in esophageal cancer tissue, which is supported by previous findings that choline and its metabolites allow a distinct differentiation in human oral cancer [32]. The elevation of choline metabolites in esophageal cancer may be resulted from the metabolism of membrane phospholipids due to accelerated cell proliferation. The levels of DMG, creatinine, creatine, DMA and TMA were altered along with development of esophageal cancer, indicating that they might be the potential biomarkers for diagnosis of esophageal cancer.

Conclusions
Overall, our findings confirm a distinct tissue metabolic profile of esophageal cancer patients characterized by altered levels of 45 metabolites mainly involved in fatty acids metabolism, energy supplies and choline metabolism. Particularly, a panel of 12 metabolite biomarkers was changed along with the development of esophageal cancer and might be related to the occurrence and aggression of this cancer. Our study highlights the significance of the distinct tissue metabolic profile of esophageal cancer. These 12 gradient biomarkers provide not only a new insight for the establishment of improved clinical biomarkers for esophageal cancer detection, but also potential information for mechanism study of esophageal cancer progression. Further investigation is needed to validate these initial findings in much larger samples and the related mechanism underlying the progression of esophageal cancer.

Methods
Chemicals
Deuterium oxide (99.8% D) was purchased from NORELL (Landisville, USA). Trimethylsilylpropionic acid-d4 sodium salt (TSP) was purchased from Sigma Aldrich (St. Louis, MO). HPLC-grade methanol and chloroform were purchased from Fisher Scientific (Fairlawn, NJ, USA). Deionized water was obtained from an EASYpure II UV water purification system (Barnstead International, Dubque, IA). All of the chemicals employed in this study were of analytic pure and culture grade.

Sample collection
The protocol of the present study was approved by the Ethics Committee of West China Hospital of Sichuan University. The informed consents were obtained from all patients.

In total, 115 case of surgical specimen came from eighty-nine esophageal cancer patients treated during 2010 to 2011 at West China Hospital of Sichuan University. Among them, 52 cases belonged to the matched tumor and normal mucosas, which were taken at least 5–10 cm away from the edges of a tumor from the same patient (n = 26). The patients enrolled in this research did not receive any neoadjuvant chemotherapy or radiation therapy prior to esophagectomy. Fresh tumor tissues or corresponding normal esophageal mucosas were immediately frozen in liquid nitrogen after dissection, then stored at −80 °C until processing. Tumor specimens were carefully microdissected to ensure at least 90% of the analyzed tissue contained cancer cells. The clinical diagnosis, tumor stage, histology differentiation and resection margin were determined by routine histopathological examination of H & E stained specimens by a blinded pathologist.

Sample Preparation
The 200–500 mg of frozen tissue samples were weighed and suspended in bidistilled water containing methanol (4 ml per gram of tissue). The samples were homogenized with 20 strokes at 800 rpm, and 2 ml/g chloroform was added and homogenization was repeated. Then, the suspension was mixed with 2 ml/g chloroform and 2 ml/g bidistilled water, and leaved on ice for 30 min, followed by centrifugation at 4000 g for 30 min. This procedure separated suspension to three phases, including a
water phase containing methanol at the top, a denatured proteins phase in the middle, and a lipid phase at the bottom. The upper phase (aqueous phase) of each sample were collected and evaporated to dryness under a nitrogen gas stream. The residue was reconstituted with 580 μl of D₂O containing 0.01 mg/ml sodium (3-trimethylsilyl)-2,2,3, 3-tetradeuteriopropionate (TSP) and 30 μmol/L phosphate buffer solution (PBS, pH = 7.4). The D₂O and TSP provided the deuterium lock signal for the NMR spectrometer and the chemical shift reference (80.0), respectively. After centrifuged at 12,000 g for 5 min, the supernatant was transferred into a 5-mm NMR tube for NMR spectroscopy [53].

1H-NMR Measurements
All samples were detected by 1H-NMR spectroscopy at 600.13 MHz using a Bruker Avance II 600 spectrometer operating (Bruker Biospin, Germany) at 300 K. A one-dimensional spectrum was acquired using a standard (1D) Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence to suppress the water signal with a relaxation delay of 5 sec. Sixty-four free induction decays (FIDs) were collected into 64 K data points with a spectral width of 12,335.5-Hz spectral, an acquisition time of 2.66 sec, and a total pulse recycle delay of 7.66 sec. The FIDs were weighted by a Gaussian function with line-broadening factor 20.3 Hz, Gaussian maximum position 0.1, prior to Fourier transformation [57].

1H-NMR spectral data processing
The raw NMR data (FIDs) was been manually Fourier transformed for obtaining NMR spectroscopy in MestRe-c2.3 software to reduce the complexity of the NMR data and facilitate the pattern recognition (http://mestre-c-lite.findmysoft.com/download/). After phase adjustment and baseline correction, the spectrum was divided into 419 segments ranging from 9.5 to 0.5 ppm, with equal width of each region (0.02 ppm). The region 5.2–4.6 ppm was removed for excluding the effect of imperfect water suppression. Moreover, the integrated data were normalized before pattern recognition analysis to eliminate the dilution or bulk mass differences among samples due to the different weight of tissue.

Pattern recognition (PR) analysis
For pattern recognition, the reduced and normalized NMR spectral data were imported into SIMCA-P (version 11, Umetrics AB) for analysis. PCA, the unsupervised PR method, was initially applied to analyze the NMR spectral data to separate the tumor samples from the normal samples. PLS-DA and OPLS-DA, the supervised PR method, were subsequently used to improve the separation and the data filtering method. The PLS-DA models were validated by a permutation analysis (200 times). The default 7-round cross-validation was applied with 1/seventh of the samples being excluded from the mathematical model in each round, in order to guard against overfitting. The variable importance in the projection (VIP) values of all peaks from OPLS-DA models was taken as a coefficient for peak selection, and these variables with VIP > 1 was considered relevant for group discrimination [58]. In addition to the multivariate statistical analysis method, unpaired Student’s t-test (p < 0.05) to the chemical shifts was also used to the significance of each metabolite. Only both VIP > 1 of multivariate and p < 0.05 of univariate statistical significance were identified distinguishing metabolites. Metabolites of corresponding chemical shift were identified according to the previous literatures and the Human Metabolome Database (http://www.hmdb.ca/), a web-based bioinformatic/cheminformatic resource with detailed information about metabolites and metabolic enzymes.

Abbreviations
1H-NMR: 1H nuclear magnetic resonance; PCA: Principal component analysis; PLS-DA: Partial least squares-discriminant analysis; OPLS-DA: Orthogonal partial least-squares-discriminant analysis; MS: Mass spectrometry; PC: Principal components; VIP: Variable importance; DMA: Dimethylamine; DMG: Dimethylglycine; TMA: Trimethylamine; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; NAD: Nicotinamide adenine dinucleotide; GABA: Gamma-aminobutyrate; TCA: Tricarboxylic acid; NAA: N-acetylaspargate; NDMA: N-nitrosodimethylamine.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
LW, XC-Draft the paper, processed, analyzed the 1H-NMR data, performed the multivariate analysis and also classification of the data. LC, PX, ML, WL-were involved in the collection and verification of tissue samples and clinical details. PD-mainly took part in the detection of samples. YZ, HJ-assisted the writing of the paper and the interpretation of its results. TW, HW, JH, XS-were mainly responsible for the extraction of the metabolites and its dryness. YZ-conceived and designed the study and assisted in the interpretation of results. All authors have read and approved the contents.

Acknowledgments
This work was supported by National S&T Major project (2011ZX09102-001-013 and 2012ZX09501001-003), Program for New Century Excellent Talents in University (NCET-11-0360) and Project of the National Natural Sciences Foundation of China (81272459). In addition, many thanks to Analytical & Testing Center, Sichuan University for assisting analyzed the 1H-NMR data.

Author details
1State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China.
2Department of thoracic surgery, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China.
3Analytical & Testing Center, Sichuan University, Chengdu 610041, China.

Received: 14 November 2012 Accepted: 17 March 2013

Published: 4 April 2013

References
1. Siegel R, Naishadham D, Jemal A: Cancer statistics, 2012. CA Cancer J Clin 2012, 62:10–29.
2. Hirst J, Smithers BM, Gostey OC, Thomas J, Barbera A: Defining cure for esophageal cancer: Analysis of actual 5-year survivors following esophagectomy. Ann Surg Oncol 2011, 18:1766–1774.

3. Allum WH, Stenning SP, Clark PI, Langley RE: Long-term results of a randomized trial of surgery with or without preoperative chemoradiotherapy in esophageal cancer. J Clin Oncol 2009, 27:5062–5067.

4. Kelsen DP, Winter KA, Gunderson LL, Martini J, Estes NC, Hacker DG, Ajan A, Kocha W, Minsky BD, Roth JA, et al: Long-term results of RTOG trial 8911 (USA Intergroup 113): a random assignment trial comparison of chemotherapy followed by surgery compared with surgery alone for esophageal cancer. J Clin Oncol 2007, 25:3719–3725.

5. Ajan A: Carcinoma of the esophagus: is biology screaming in my deaf ears? J Clin Oncol 2005, 23:4256–4323.

6. Hsu WH, Hsu PK, Hsieh CC, Huang CS, Wu YC: The metastatic lymph node number and ratio are independent prognostic factors in esophageal cancer. J Gastrointest Surg 2009, 13:1913–1920.

7. Tomizawa Y, Wang XK: Screening, surveillance, and prevention for esophageal cancer. Gastroenterol Clin N Am 2009, 38:59–73.

8. Tew WP, Kelsen DP, Hixon DH: Targeted therapies for esophageal cancer. Oncologist 2005, 10:650–661.

9. Wang DH, Souza RF: Biology of Barrett's esophagus and esophageal adenocarcinoma. Gastroenterol Endosc Clin N Am 2011, 21:25–38.

10. Ekman S, Bergqvist M, Heldin CH, Lennartsson J: Activation of growth factor receptors in esophageal cancer—implications for therapy. Oncologist 2007, 12:1165–1177.

11. Nicholas JK, Lindon JC, Holmes E: Metabonomics: understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999, 29:181–1189.

12. Finhn D: Metabolomics—the link between genotypes and phenotypes. Plant Mol Biol 2002, 48:155–171.

13. Nicholson JK, Connelly J, Lindon JC, Holmes E: Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov 2002, 1:153–162.

14. Claudino WM, Quattrone A, Biganzoli L, Pestrin M, Bertini I, Di Leo A: Metabolomics: available results, current research projects in breast cancer, and future applications. J Clin Oncol 2007, 25:2840–2846.

15. Yang J, Xu G, Hong Q, Liebich HM, Lutz K, Schmülling RM, Wahl HG: Discrimination of Type 2 diabetic patients from healthy controls by using metabolomics method based on their serum fatty acid profiles. J Chromatogr B 2008, 813:53–58.

16. Xue R, Lin Z, Deng C, Dong L, Liu T, Wang J, Shen XA: Serum metabolomic investigation on hepatocellular carcinoma patients by chemical derivatization followed by gas chromatography/mass spectrometry. Rapid Commun Mass Sp 2008, 22:3001–3008.

17. Bogdanov M, Matzon WR, Wang J, Matzon T, Saunders-Pullman R, Bressman SS, Beal MF: Metabolomic profiling to develop blood biomarkers for Parkinson’s disease. Brain 2008, 131:389–396.

18. Shi C, Wu C, Cao A, Sheng HZ, Yan X, Liao M: Metabolomic profiling detects field effects in nondysplastic tissue from esophageal cancer patients. Cancer Res 2010, 70:9129–9136.

19. Zhang J, Bowers J, Liu L, Wei S, Gowda GA, Hammond Z, Rafferty D: Esophageal Cancer Metabolite Biomarkers Detected by LC-MS and NMR Methods. PLoS One 2012, 7:e30181.

20. Wu H, Xue R, Tang Z, Deng C, Liu T, Zeng H, Sun Y, Shen X: Metabolomic Investigation of gastric cancer tissue using gas chromatography/mass spectrometry. Anal Bioanal Chem 2010, 396:1385–1395.

21. Tzian R, Lopes V, Günther U: Early stage diagnosis of oral cancer using 1H NMR-based metabolomics. Neoplasia 2009, 11:269–275.

22. Nogah VH, Wilson C, Nelson J, Nicholson J: Physiological variation in metabolic phenotyping and functional genomic studies: use of orthogonal signal correction and PLS-DA. FEBS Lett 2002, 530:191–196.

23. Postic C, Dentin R, Girard J: Role of the liver in the control of carbohydrate and lipid homeostasis. Diabetes metab 2004, 30:398–408.

24. Fan TW, Lane AN, Higa RH, Yan J: Stable isotope resolved metabolomics of lung cancer in a SCID mouse model. Metabolomics 2011, 7:267–269.

25. Williamson G, Day A, Plumb G, Couteau D: Human metabolic pathways of dietary flavonoids and cinamates. Biochem Soc Trans 2000, 28:16–22.

26. Zhang S, Nagawa Gowda GA, Assoa V, Shannah A, Barbas C, Rafferty D: Correlative and quantitative 1H NMR-based metabolomics reveals specific metabolic pathway disturbances in diabetic rats. Anal Biochem 2008, 383:76–84.

27. Gatenby RA, Gillies Rj: Why do cancers have high aerobic glycolysis? Nature Rev Cancer 2004, 4:891–899.

28. Warburg O: On the origin of cancer cells. Science 1956, 123:309–314.

29. Eng CH, Abraham RT: Glutaminase. Methods Enzymol 2005, 383:2846.

30. Piotto M, Mousallieh FM, Dillmann B, Imperiale A, Neuville A, Brigand C, Keun HC: Metabolic profiling detects field effects in nondysplastic tissue from esophageal cancer patients. Cancer Res 2010, 70:9129–9136.

31. Astrakas LG, Zurakowski D, Tzika A, Zarifi MK, Anthony DC, De Grolami U, Tarbell NJ, Black PM: Noninvasive magnetic resonance spectroscopic imaging biomarkers to predict the clinical grade of pediatric brain tumors. Clin Cancer Res 2004, 10:8220–8228.

32. Whitehead TL, Keiber-Emmons T: Applying in vitro NMR spectroscopy and 1H NMR metabolomics to breast cancer characterization and detection. Prog Clin Pharmac Res 2005, 47:165–174.

33. Postic C, Dentin R, Girard J: Role of the liver in the control of carbohydrate and lipid homeostasis. Diabetes Metab 2004, 30:398–408.

34. Fan TW, Lane AN, Higa RH, Yan J: Stable isotope resolved metabolomics of lung cancer in a SCID mouse model. Metabolomics 2011, 7:267–269.

35. Williamson G, Day A, Plumb G, Couteau D: Human metabolic pathways of dietary flavonoids and cinamates. Biochem Soc Trans 2000, 28:16–22.

36. Zhang S, Nagawa Gowda GA, Assoa V, Shannah A, Barbas C, Rafferty D: Correlative and quantitative 1H NMR-based metabolomics reveals specific metabolic pathway disturbances in diabetic rats. Anal Biochem 2008, 383:76–84.

37. Gatenby RA, Gillies Rj: Why do cancers have high aerobic glycolysis? Nature Rev Cancer 2004, 4:891–899.

38. Warburg O: On the origin of cancer cells. Science 1956, 123:309–314.

39. Eng CH, Abraham RT: Glutaminase. Methods Enzymol 2005, 383:2846.

40. Piotto M, Mousallieh FM, Dillmann B, Imperiale A, Neuville A, Brigand C, Keun HC: Metabolic profiling detects field effects in nondysplastic tissue from esophageal cancer patients. Cancer Res 2010, 70:9129–9136.

41. Astrakas LG, Zurakowski D, Tzika A, Zarifi MK, Anthony DC, De Grolami U, Tarbell NJ, Black PM: Noninvasive magnetic resonance spectroscopic imaging biomarkers to predict the clinical grade of pediatric brain tumors. Clin Cancer Res 2004, 10:8220–8228.

42. Whitehead TL, Keiber-Emmons T: Applying in vitro NMR spectroscopy and 1H NMR metabolomics to breast cancer characterization and detection. Prog Clin Pharmac Res 2005, 47:165–174.

43. Beckonert O, Monnerjahn J, Borik HL, Leifert D: Visualizing metabolic changes in breast cancer tissue using 1H-NMR spectroscopy and self-organizing maps. NMR Biomed 2003, 161–11.

44. Yokub D, Keun HC, Goldin R, Hanna GB: Metabolic profiling detects field effects in nondysplastic tissue from esophageal cancer patients. Cancer Res 2010, 70:9129–9136.

45. Zhang J, Bowers J, Liu L, Wei S, Gowda GA, Hammond Z, Rafferty D: Esophageal Cancer Metabolite Biomarkers Detected by LC-MS and NMR Methods. PLoS One 2012, 7:e30181.

46. Williamson G, Day A, Plumb G, Couteau D: Human metabolic pathways of dietary flavonoids and cinamates. Biochem Soc Trans 2000, 28:16–22.

47. Zhang S, Nagawa Gowda GA, Assoa V, Shannah A, Barbas C, Rafferty D: Correlative and quantitative 1H NMR-based metabolomics reveals specific metabolic pathway disturbances in diabetic rats. Anal Biochem 2008, 383:76–84.

48. Gatenby RA, Gillies Rj: Why do cancers have high aerobic glycolysis? Nature Rev Cancer 2004, 4:891–899.
dehydrogenase diverts glycolytic flux and contributes to oncogenesis.

50. Ippolito JE, Merritt ME, Bäckhed F, Moulder KL, Mennerick S, Manchester JK, Gammon ST, Pinnica-Worms D, Gordon JI. Linkage between cellular communications, energy utilization, and proliferation in metastatic neuroendocrine cancers. *Proc Natl Acad Sci U S A* 2006, 103:12505–12510.

51. Namboodiri A, Peethambaran A, Mathew R, Sambhu PA, Hershfeld J, Moffett JR, Madhavarao CN. Canavan disease and the role of N-acetylaspartate in myelin synthesis. *Mol Cell Endocrinol* 2006, 252:216–223.

52. Fong MY, McDunn J, Kakar SS. Identification of metabolites in the normal ovary and their transformation in primary and metastatic ovarian cancer. *PLoS One* 2011, 6:e19963.

53. Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, Nicholson JK. Metabolic profiling, metabolomic and metabolonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc* 2007, 2:2692–2703.

54. Dumas ME, Barton RH, Toye A, Cloarec O, Blacher C, Rothwell A, Fearnside J, Tatoud R, Blanc V, Lindon JC, et al. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. *Proc Natl Acad Sci USA* 2006, 103:12511–12516.

55. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, DuGar B, Feldstein AE, Britt EB, Fu X, Chung YM, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 2011, 472:57–63.

56. Cross AJ, Pollock RA, Bingham SA. Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Res* 2003, 63:2358–2360.

57. Hu Z, Deng Y, Hu C, Deng P, Bu Q, Yan G, Zhou J, Shao X, Zhao J, Li Y, et al. 1H NMR-based metabolomic analysis of brain in rats of morphine dependence and withdrawal intervention. *Behav Brain Res* 2012, 231:1–19.

58. Jansson J, Willing B, Lucio M, Fekete A, Dickove J, Halfvarson J, Tyk C, Schmitt-Kopplin P. Metabolomics reveals metabolic biomarkers of Crohn’s disease. *PLoS One* 2009, 4:e6386.

Cite this article as: Wang et al.: 1H-NMR based metabolomic profiling of human esophageal cancer tissue. *Molecular Cancer* 2013, 12:25.