

Prediction of Potential Prognostic Tumor Markers of Re-ascites After Chemotherapy for Ovarian Cancer Based on LC/MS Method

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Research

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Abstract

Background: Tumor cells are not only in a state of exuberant metabolism, but also inseparable from the surrounding microenvironment for the occurrence and development of tumors. By means of metabonomics technology, the metabolic profiles of re-ascites after chemotherapy in ovarian cancer ascites microenvironment were compared, and new tumor markers were screened for the treatment and prognosis of ovarian cancer.

Methods: Thirty-seven cases of ovarian cancer were selected, of which 12 cases had ovarian cancer tissues, ascites supernatant and precipitated cells. By using UHPLC-Q-TOF MS/MS and combined with HMDB database, differential metabolites are identified and then differential pathways are enriched.

Results: The results revealed that 10 differential metabolites were screened between chemotherapy and no-chemotherapy groups, and 9 differential metabolites were found in chemotherapy-sensitive and chemotherapy-resistant groups, in the ascites of ovarian cancer. Among the 19 differential metabolites, 9 metabolites combined with serum CA125 were analyzed by receiver operating characteristic analyses, which showed more the predictive value of diagnosis of malignancy. Among them, Two differential metabolites (2/9) were correlated with the expression of CA125, five differential metabolite (5/9) related enzyme genes were statistically significant in overall survival and progression free survival (PFS) analysis. Four differential metabolites (4/9) were correlated with the expression of uric acid, creatinine, γ -GGT and ALP in serum.

Conclusions: The results suggest that differential metabolites are mainly involved in PPAR signal pathway, steroid biosynthesis, fatty acid metabolism, secondary bile acid biosynthesis and so on. Lipid metabolites of ovarian cancer ascites cells may promote fatty acid oxidation by activating AMPK, promote lipid synthesis through mTOR/PPAR, provide energy for cancer cells, inhibit apoptosis and promote metastasis. Thus targeted lipid metabolism signal transduction axis can inhibit ovarian cancer metastasis and drug resistance.

Introduction

Ascites formation is a common complication of advanced ovarian cancer [1]. Some patients still produce ascites repeatedly after chemotherapy, indicating that they are resistant to chemotherapy. At present, there is a lack of clinical markers related to chemosensitivity or drug resistance, obtained from ascites. Therefore, it is very important to find potential tumor markers that can predict the efficacy and drug resistance of platinum chemotherapy for ovarian cancer.

Cancer is a metabolic disease characterized by metabolic reprogramming of cells, which is essential to maintain a high proliferation rate and resist changes in cell death signals [2, 3]. Metabonomics is a rapid and effective method to identify new cancer biomarkers, which has gradually become a supplementary technology of genomics and proteomics. It integrates high-throughput, high-resolution analysis technology with bioinformatics to conduct research on biology, and at the metabolic level. It provides a unique perspective for understanding biology, and its applications in tumor research are becoming increasingly mature and has become a hot spot in domestic and foreign research [4, 5]. It promotes the discovery of

biomarkers in disease and drug treatment targets. In addition, it can also provide valuable information about drug resistance and disease recurrence [6], The application of this technology can help discover new targets for the treatment of ascites for ovarian cancer.

In this study, ascites and cancer tissues were divided into no-chemotherapy and chemotherapy, chemotherapy-sensitive and chemotherapy-resistant groups. we performed Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF MS/MS) metabonomics to analyze the characteristics of metabolites between the two groups and the relationship between differential metabolites and platinum efficacy and to look forward to providing new clues and new hopes in guiding clinical diagnosis and treatment.

Materials And Methods

Collection of ascites and tissue samples from ovarian cancer

82 cases of high-grade serous ovarian cancer ascites from November 2014 to June 2019 from the Harbin Medical University Affiliated Cancer Hospital were collected. 37 ascites samples were selected and divided into non-chemotherapy group (No chemotherapy, N) and platinum chemotherapy group (Chemotherapy, C). The patients who underwent chemotherapy were further divided into the chemo-sensitive group (≥ 6 months for platinum sensitivity, CS) and the chemo-resistant group (< 6 months is platinum resistance, CR) [7]. We also acquired 22 fresh ovarian cancerous tissue samples of the 37 cases. Among them, 12 cancer tissues can correspond to the ascites supernatants and precipitated ascites cells to form self-pairing (Table S1). All cases had complete clinicopathological data and written informed consent.

processing of ascites and cancer tissue

The supernatants of cancerous ascites were collected into 1.5 mL centrifuge tubes. Then, moderate ACK lysis (Leagene Biotechnology, China) buffer was added to the precipitates, which were collected into 1.5 mL centrifuge tubes. The paraffin blocks of ovarian cancer tissues were cut into 20 μm and stored at -80°C . Experimental details about metabolite extraction and liquid chromatography-mass spectrometry (LC-MS) data acquisition are provided in the Supporting information.

Data Processing

Raw MS data (.d) files are preprocessed by XCMS online [8] and MetaboAnalyst 4.0 [9]. The generated data matrix consisted of the mass-to-charge ratio (m/z) value, retention time (RT), and peak abundance. Multivariate statistical analysis (PCA and PLS-DA) was carried out by SIMCA-P software package (14.0). The validity of the model is tested by replacement to avoid the overfitting. The variable important in the projection (VIP) value was calculated for each variable in the PLS-DA model. In addition, An independent t-test was used for comparison. The differential metabolites with p value less than 0.05 and VIP value greater than 1 were selected. The combination of accurate mass and experimental MS/MS spectra are matched with the

structural information of metabolites in human metabolite database (HMDB). Dysregulated metabolites were mapped into the KEGG database for pathway enrichment analysis using the hypergeometric test.

Statistical Analyses

Clinical data are represented as mean \pm sd. Statistical analyses were performed using SPSS 21.0 software. Spearman's test was used to evaluate the associations between differential metabolite and serum biochemical index. Comparisons between two or multiple parameters were performed using the chi-square test. A p value less than 0.05 was set as the significance level. Kaplan-Meier analysis is used to evaluate the prognosis of patients with ovarian cancer. Random forest analysis estimates the importance of each metabolite in MetaboAnalyst 3.0. The receiver operating characteristic (ROC) was used to evaluate the diagnostic performance of metabolite biomarkers.

Results

Metabonomics analysis of ovarian cancer tissue and ascites

The metabonomic data were visualized by unsupervised principal component analysis(PCA). In 37 cases of ascites supernatant, 23 cases of precipitated cells and 22 cases of cancer tissue samples, QC samples in PCA plots were clustered tightly (Fig. S1), which indicated that the data quality was very good. In a supervised model of PLS-DA, ascites and tissue samples were clearly separated between N and C, CS and CR, indicating that all the three groups had different metabolic profiles and there was no over-fitting (Fig. 1).

Select differential metabolites in ovarian cancer tissues and ascites

Metabolic characteristics of supernatant, precipitated cells and cancer tissues in ovarian cancer

A total of 7579, 10053 and 11053 metabolic peaks were detected in 37 cases of ascites supernatant, 23 cases of precipitated cells and 22 cases of ovarian cancer, respectively (Fig. 2a). Heatmap and correlation analysis showed that metabolites were well distinguished in no-chemotherapy, chemosensitivity and Chemotherapy resistance groups, and there was a certain correlation between them (Fig. 2b). It is suggested that there may be different metabolic patterns in N vs C and CS vs CR and the metabolites promote or inhibit each other.

Analysis of differential metabolites and common differential metabolites in each sample set

Differential metabolites were screened in N vs C and CS vs CR for each sample set(Table S2-3). The corresponding coexisting metabolites of differential metabolites in other samples of each group were 49, 31 and 9, respectively (Table 1). All the differential metabolites were combined and the differential metabolites with higher significance were screened by random forest analysis (n = 15, respectively (Fig. 2c). The coexistence metabolites were intersected with random forest results in N vs C and CS vs CR, respectively. Screening shared metabolites in co-existing metabolites and random forests, and further combining them (Fig. 2d). 10 and 9 differential metabolites were obtained in N vs C and CS vs CR, respectively.

Table 1

Results of different metabolites in ovarian cancer tissues, ascites supernatant and precipitated cells

	37 unpaired cases(n = 82)-I				12 paired cases (n = 44)-II				12 paired cases (n = 36)-III	
	different		coexisting		different		coexisting		different	coexisting
	N vs C	CS vs CR	N vs C	CS vs CR	N vs C	CS vs CR	N vs C	CS vs CR	N vs C	N vs C
supernatant	26	20	13	14	18	4	10	2	7	5
cells	6	10	2	2					3	1
tissues	14	16	10	8	14	16	10	9	7	3
Total	46	46	25	24	32	20	20	11	17	9
sum	92		49		52		31		17	9

Note: 37 cases of unmatched-I group, 22 cases of paired-II group, 12 cases of paired-III group; bold text means co-existing metabolites.

Correlation between differential metabolites and clinical pathological data

ROC analysis of differential metabolites combined with serum CA125

ROC analysis showed that the distinguishing ability of metabolites was less than that of CA125, but when combined with CA125, nine metabolites increased. They are carnosine, 20-COOH-leukotriene E4 (20-COOH-LTE4), sebacic acid in N vs C; 1,25-dihydroxyvitamin D3-26,23-lactone (1,25-Lactone), 20a,22b-dihydroxycholesterol, 3a,6a,7b-trihydroxy-5b-cholic acid (Muricholic acid), 3a,7a,12a,19-tetrahydroxy-5b-cholic acid (3,7,12,19-THCA), dihydrothymine, hexadecanoic acid in CS vs CR (Fig. 3a). The combination of metabolites and CA125 may improve the predictive value of ovarian cancer diagnosis.

Correlation between differential metabolites and clinical serum biochemical indexes in patients

The serum biochemical indexes of 82 patients with ovarian cancer showed that serum CA125, uric acid and α -hydroxybutyrate dehydrogenase (HBDH) were significantly decreased, γ -glutamyl transpeptidase (γ -GGT) was significantly increased in chemotherapy group, serum CA125, creatinine, lactate dehydrogenase (LDH), alkaline phosphatase (ALP) were increased, and total cholesterol was significantly decreased in Chemotherapy resistance group ($p < 0.05$, Fig. 3b). Correlation analysis showed that 20-COOH-LTE4 and hexadecanoic acid were positively correlated with CA125 ($p < 0.05$), 20-COOH-LTE4 was negatively correlated with creatinine ($p < 0.05$), carnosine was positively correlated with uric acid ($p < 0.05$), 20a-22b-dihydroxycholesterol was positively correlated with γ -GGT and ALP, hexadecanoic acid was positively correlated with γ -GGT and HBDH, and other metabolites were not significantly correlated with serum biochemical indexes (Fig. 3c).

Survival analysis of genes related to differential metabolites

Progression free survival analysis (PFS) showed that the high expression of enzyme gene had a poor prognosis, which were CNDP1 (carnosine), LTC4S (20-COOH-LTE4), CYP11A1 (20a,22b-dihydroxy cholesterol), CYP7A1 (W-Muricholic acid), CYP27A1 (3,7,12,19-THCA) ($p < 0.05$, Fig. 4a). Low expression has a poor prognosis, which are ACADL (sebacic acid), DHCR24 (1,25-lactone), AGXT2 (dihydrothymine), ACSL1 (hexadecanoic acid) ($p < 0.05$, Fig. 4b). Elevated carnosine in no-chemotherapy group is also higher in patients with poor progression free survival. Carnosine that is increased in no chemotherapy group is also higher in patients with poor disease-free survival. Similarly, among drug-resistant patients, increased 20a, 22b-dihydroxycholesterol is also higher in patients with poor progression-free survival, and decreased 1,25-lactone, dihydrothymine and hexadecanoic acid are also lower in patients with poor progression-free survival. In addition, LTC4S, CYP11A1, CYP27A1, ACSL1, and AGXT2 all showed significance in overall survival and progression-free survival analysis. It suggests that 20a, 22b-dihydroxycholesterol, 1,25-lactone, dihydrothymine, and hexadecanoic acid may become the most promising signs for judging whether ovarian cancer develops drug resistance Things.

Pathway analysis of differential metabolites in ovarian cancer tissue and ascites

Metabolite pathway analysis showed that 25 and 12 pathways were enriched in N vs C, CS vs CR (Table S4-5), and there were 6 common pathways (Fig. 5a-b). Differential metabolites and clinical serological indicators also participate in the same pathway, including arachidonic acid, purine, arginine, folate metabolism and steroid synthesis, etc. The change trend of metabolic pathways is consistent. This may explain that changes in metabolites in cancer tissue not only lead to changes in ascites metabolites, but also cause serological changes.

The potential mechanism of differential metabolites in the progression of ovarian cancer

For 51 genes of 9 metabolite-related enzymes, STRING protein interaction network analysis (Fig. 5c) was performed, which has significant correlation ($p < 0.001$). KEGG enrichment analysis shows that it is mainly involved in the PPAR signaling pathway, steroid biosynthesis, fatty acid metabolism, secondary bile acid biosynthesis, et al. Molecular pathways that may be involved in ovarian cancer are constructed (Fig. 5d). Metabolites and related enzymes may play a role in the progression of ovarian cancer and the development of drug resistance, including malignant proliferation, invasion, apoptosis, et al. (Table 2).

Table 2
Biological function of potential biomarkers and related enzyme genes in ovarian cancer

Metabolites	Log FC	CA125 + AUC	Gene	KM p-value	poor survival	pathway	function
Carnosine	0.65	0.884	CNDP1	< 0.001	high	Histidine metabolism	Apoptosis
20-COOH-LTE4	-0.28	0.899	LTC4S	0.012	high	Arachidonic acid metabolism	Angiogenesis
Sebacic acid	0.2	0.879	ACADL	< 0.001	low	Fatty acid metabolism	Inhibit metastasis
Calcitriol lactone	-0.66	0.963	DHCR24	0.011	low	Steroid biosynthesis	Inhibit proliferation
20a,22b-Dihydroxycholesterol	0.3	0.88	CYP11A1	0.046	high	Steroid hormone biosynthesis	Proliferation
Muricholic acid	-0.28	0.944	CYP7A1	0.032	high	bile acid biosynthesis	Proliferation drug resistance
3,7,12,19-THCA	-0.47	0.935	CYP27A1	0.01	high	bile acid biosynthesis	Proliferation drug resistance
Dihydrothymine	-0.48	0.88	AGXT2	0.034	low	Pyrimidine metabolism	Anti-apoptosis drug resistance
Hexacosanoic acid	-0.14	0.926	ACSL1	0.018	low	Fatty acid metabolism	Proliferation drug

Discussion

At present, metabonomics is applied in a variety of tumors [10–13]. Biological samples, including urine, plasma and tumor tissue, have been applied to metabolism-based ovarian cancer research [14–17]. It is often used to distinguish between healthy and ovarian cancer patients, ovarian malignant tumors and borderline tumors, and can also be used to detect recurrent tumors. Compared with urine and plasma, ascites is highly selective in filtering and concentrating plasma metabolites through the serosa. Therefore, ascites may become an ideal fluid for the study of metabonomics for the discovery of biomarkers [18, 19]. Recurrent ascites and rapid resistance to platinum therapy are common in advanced ovarian cancer. The five-year survival rate of EOC has been around 30% [20]. Metabolomic profiling of human malignant effusion remain a field poorly investigated. At present, there are few studies on predicting chemotherapy resistance. In this study, metabonomic analysis was carried out on platinum sensitive and drug resistant cancer tissues and ascites in order to discover the new biomarkers to monitor disease progression and drug resistance for precise treatment.

It was observed that there were different expression profiles in patients no-chemotherapy, chemo-sensitive and chemo-resistant. Cancer cells exhibited increased lipid dependency during growth and proliferation. The high free fatty acid content in ascitic fluid provides a huge energy source and may force ovarian cancer cells to undergo metabolic reprogramming from aerobic glycolysis to fatty acid β -oxidation to produce energy for tumor growth [21]. Compared with chemotherapy samples (ascites and tissues), the levels of saturated/unsaturated fatty acids (sebacic acid, 20-COOH-LTE4), glycerol phospholipids (PC, PGP), steroids (7-Dehydrocholesterol, Squalene, Ergosterol) and amino acid (2-Aminomuconic acid, L-Tryptophan, Formiminoglutamic acid) metabolites were down-regulated in untreated samples. Consistent with Anna, et al. [18]. It was confirmed that the decrease of lipids and essential amino acids was directly related to tumor metabolism and promoted cell proliferation and growth. According to the analysis of coexisting metabolites in ascites and cancer tissues, nine metabolites were selected as possible biomarkers of ovarian cancer. The AUC values of nine metabolites combined with CA125 were greater than 0.875, which showed high diagnostic ability.

20-COOH-LTE4, a class of inflammatory liposomes synthesized by arachidonic acid, is often used to evaluate mast cell activation [22]. Recently, it has been found that [22, 23] leukotriene E4 (LTE4) can also induce prostaglandin D2 (PGD2) production in human mast cells by activating peroxisome proliferator receptor (PRPP) and inhibit the growth of cancer cells. It is an independent indicator of good prognosis. Sebacic acid is a saturated, straight-chain naturally occurring dicarboxylic acid. deficiency of medium chain acyl-CoA dehydrogenase (ACADM) results in the inhibition of β -oxidation and indirectly leads to the increase of sebacic acid. This study found that sebacic acid increased in chemotherapy ascites, which may be related to the inhibition of ACADM activity. Abnormal expression of ACADM was also found in hepatocellular carcinoma [24]. It promotes cell proliferation, migration and invasion by regulating fatty acid metabolism. Therefore, it is suggested that ACADM is expected to be a therapeutic target.

Compared with the sensitive group, the recurrent group also showed significant changes in fatty acid metabolism, in which hexadecanoic acid increased significantly in drug resistant ascites. Hexadecanoic acid is a very long-chain fatty acid, which was once considered as a sign of peroxisome metabolism disorder. However, Adriana et al. [25] proposed that cetanoic acid is the product of fatty acid prolongation in tumor tissue. Fatty acid prolonging enzyme overexpression [26] promotes peroxisome β oxidation, relatively increases fatty acid uptake and utilization, and promotes drug resistance. It is consistent with the plasma study of recurrent ovarian cancer by Ke. et al. [27].

In addition, the four metabolites in the drug resistant group were all involved in the metabolism of 7 dehydrocholesterol. String protein interaction analysis also showed a significant correlation. Emerging evidence has suggested [28] that a high-fat diet and maladjusted WNT signals synergistically increased intestinal secondary bile acid levels in colorectal cancer. It also can activate proteasome to degrade p53 and promote the production of drug resistance. This study found that 20a-22b-dihydroxycholesterol was increased in the resistant group, and CYP11A1 can convert it into pregnenolone indirectly. The latest research shows that CYP11A1 exerts its anti-tumor activity through vitamin D substitution [29]. At the same time, this study also found that 1, 25-lactone was down-regulated in the drug-resistant group. This consistency can confirm the beneficial role of vitamin D in cancer prevention and treatment. These results suggest that

cholesterol, as a precursor of bile acid and steroid synthesis, promotes cancer cell proliferation, invasion and inhibition of apoptosis [30]. In a word, dynamic lipid metabolism changes occur in advanced ovarian cancer cells to adapt to the changes of nutritional microenvironment.

Studies have shown that [31] diabetes is a risk factor for many cancers, especially ovarian cancer. In this study, the incidence of diabetes was higher in the drug resistant group. Long-term hyperglycemia can promote the growth of tumor cells. In turn, it can promote the expression of glycolysis-related enzymes such as lactate dehydrogenase (LDH) [32]. In this study, it was also found that LDH, ALP and γ -GGT were up-regulated in drug-resistant group, and HBDH was up-regulated in non-chemotherapy group. These metabolic enzymes that promote malignant transformation are expected to become therapeutic targets.

Ovarian cancer cells utilize lipid metabolism in the ascites or omental microenvironment through AMPK/ACC/FASN-mediated lipogenesis and mTOR /TAK1 /NF- κ B signaling cascades, which in turn, enhanced ovarian cancer metastasis and aggressiveness [21]. Cholesterol can promote angiogenesis of tumor cells through mTOR signal [33]. The signal transduction network in which metabolites may be involved needs further study.

Conclusions

In this study, the metabolic changes of paired ascites and tissues during chemotherapy and relapse resistance were analyzed for the first time, and the potential differential metabolites in ascites were screened to reflect the efficacy of chemotherapy and monitor drug-resistant recurrence. The biomarkers of diagnosis and prognosis of ovarian cancer were screened by metabolomics technology to provide new ideas and new hope for judging the prognosis of ovarian cancer ascites and revising the treatment plan in time.

Abbreviations

ALP alkaline phosphatase

AUC Area under the ROC curve

CHOL Cholesterol

GLU Glucose

CREA creatinine

HBDH α -hydroxybutyrate dehydrogenase

HMDB Human metabolome database

KEGG Kyoto encyclopedia of genes and genomes

LDH lactate dehydrogenase

MS/MS Tandem mass spectroscopy

PFS Progression free survival

QC Quality control

QTOF Quadrupole time-of-flight

RF Random forest

ROC Receiver operating characteristic curve

TRIG Triglyceride

UA uric acid

UPLC Ultra-performance liquid chromatography

VIP Variable importance in the projection

γ -GGT γ -glutamyl transpeptidase

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of The Third Affiliated Hospital of Harbin Medical University, and written informed consent was obtained from all patients. This study was approved by the ethics committee of Harbin Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

X.-M. Jin and D. Kong designed the research study and interpreted the data. D. Kong, S. Jin, Y.-F. Li, M.-J. Weng and H. Feng collected samples and clinical-pathological data. M. Liu, Y. Liu, Y.-X. Jing, Y.-H. Yang, S.-J. Yang and N. Zhang performed the experiments. M. Liu, Y.-X. Jing, Y.-H. Yang and S. Jin analyzed Metabonomic data. X.-M. Jin, D. Kong, M. Liu and Y. Liu wrote the manuscript. All authors have read and approved the final manuscript.

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Figures

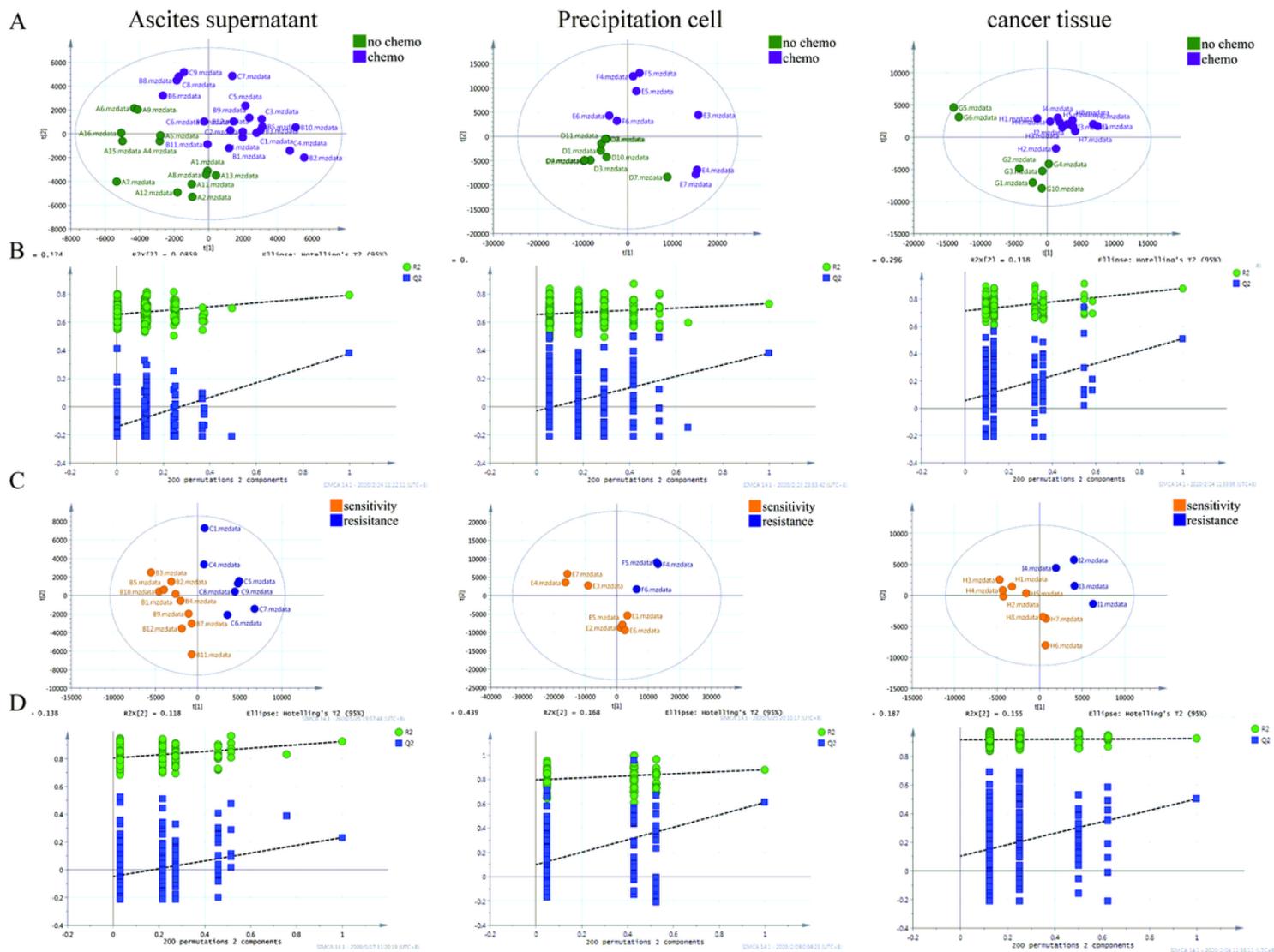


Figure 1

Results of supervised partial least squares discriminant analysis (PLS-DA) and permutation test. a-b. No chemotherapy and chemotherapy groups. c-d. chemosensitivity and chemotherapy resistance groups. Ovarian cancer ascites supernatant, precipitated cells and tissue samples can be separated. The permutation test indicates that the PLS-DA model of tissue data set is not over-fitting.

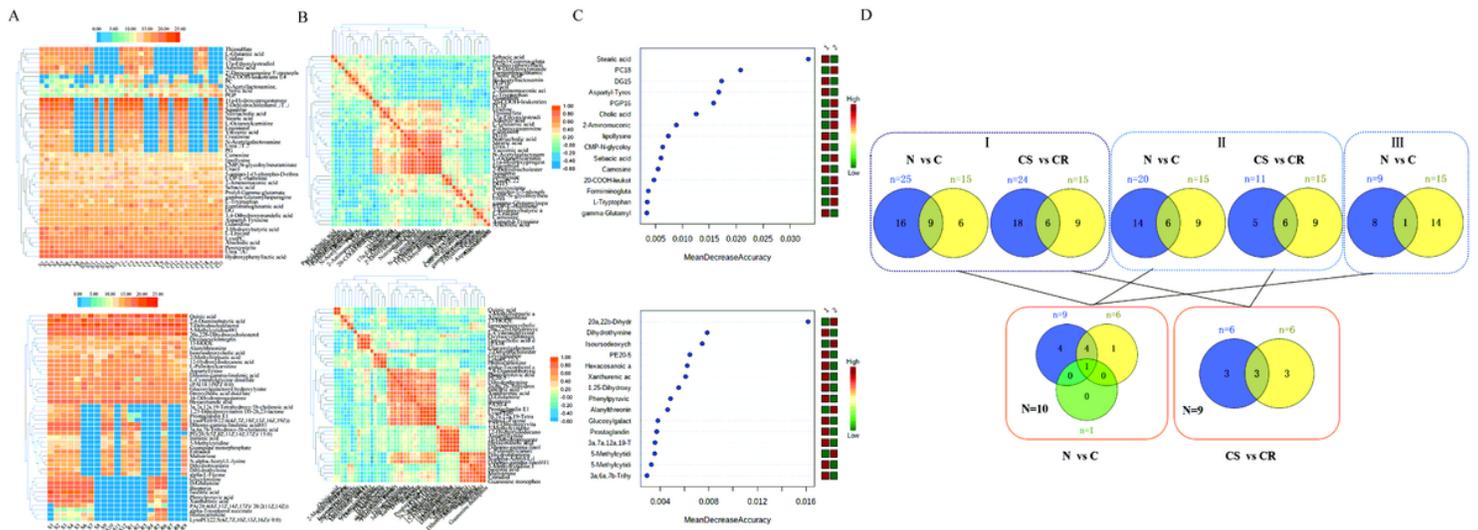


Figure 2

Metabolites Characteristics of ascites supernatant, precipitated cells and tissues in ovarian cancer. a. Results of hierarchical cluster analysis of metabolites of ascites supernatant (nasty 37), precipitated cells (nasty 23) and cancer tissue (naphtha 22) in ovarian cancer. Each row represents a metabolite, and each column represents a different group (N vs C and CS vs CR). b. Correlation heatmap of differential metabolites. c. Random forests show important differential metabolites in N vs C (up) and CS vs CR (down). d. The analysis flow of screening differential metabolites.

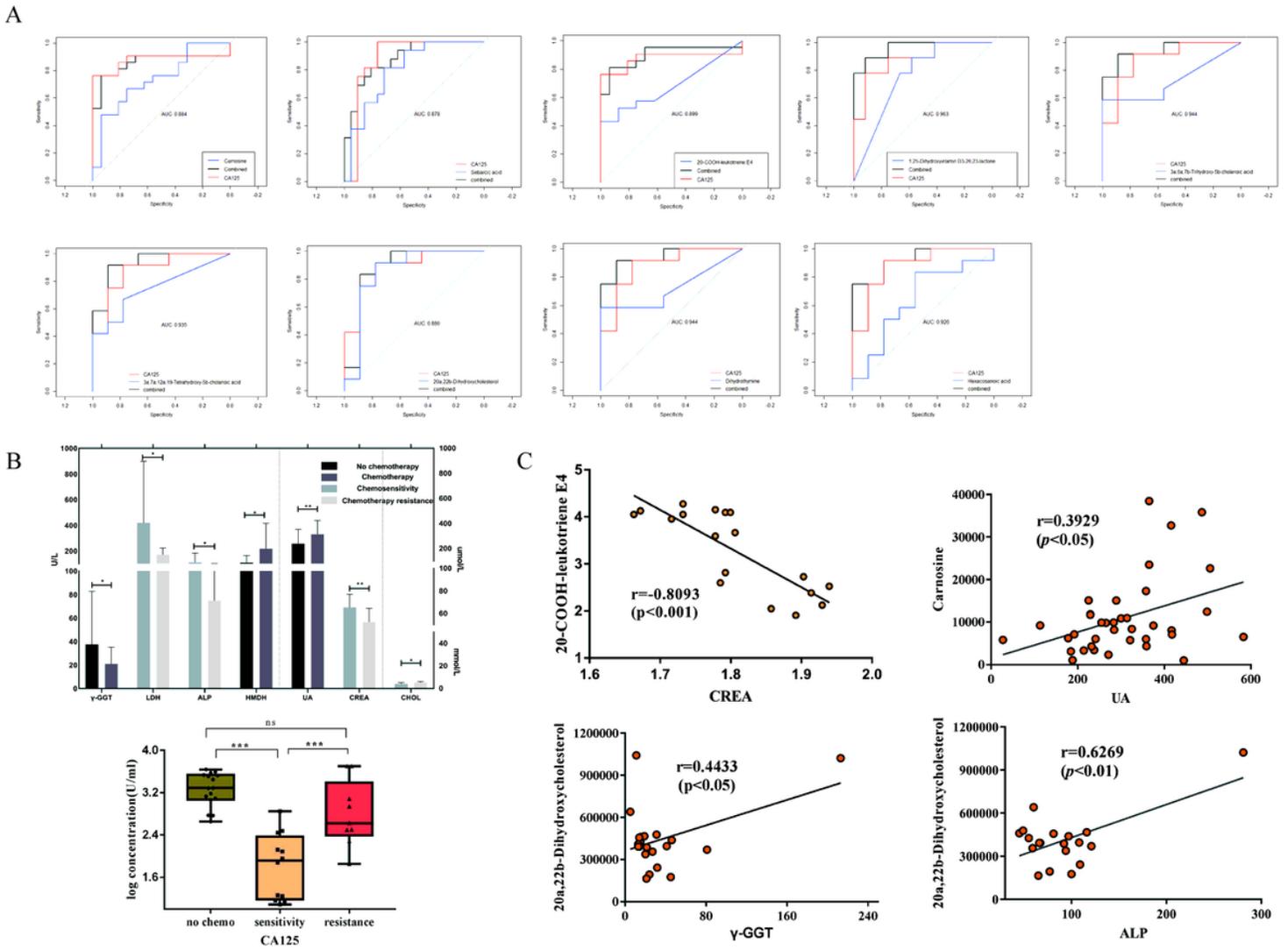


Figure 3

Analysis of differential metabolites and clinical data of patients. a. ROC curves of 9 metabolites combined with CA125 respectively. b. The expression of serological markers in N vs C and CS vs CR ($p < 0.05$). The ordinate is the concentration (log). c. Correlation analysis scatter plot for 20-COOH-LTE4 and creatinine, carnosine and uric acid, 20a,22 b-dihydroxycholesterol and γ -GGT, ALP ($p < 0.05$).

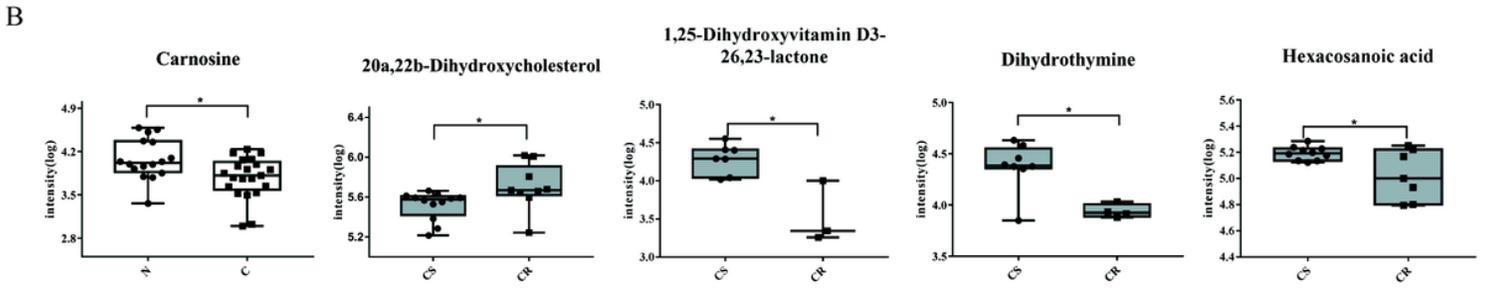
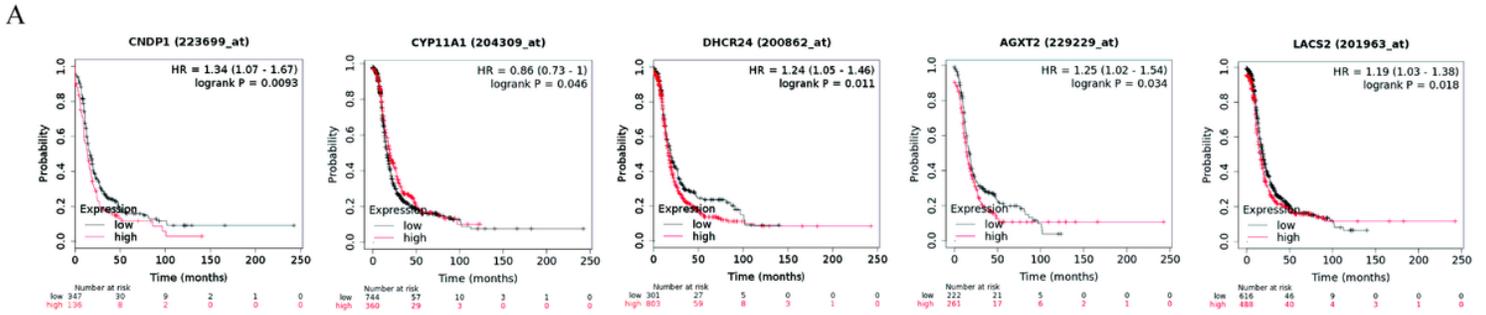


Figure 4

Expression of differential metabolites and correlation between related enzyme genes and progression-free survival of patients. a. Progression-free survival curve of differential metabolites ($p < 0.05$). b. Box plots of corresponding metabolite and biomarker in N vs C (blank) and CS vs CR (gray) samples ($p < 0.05$).

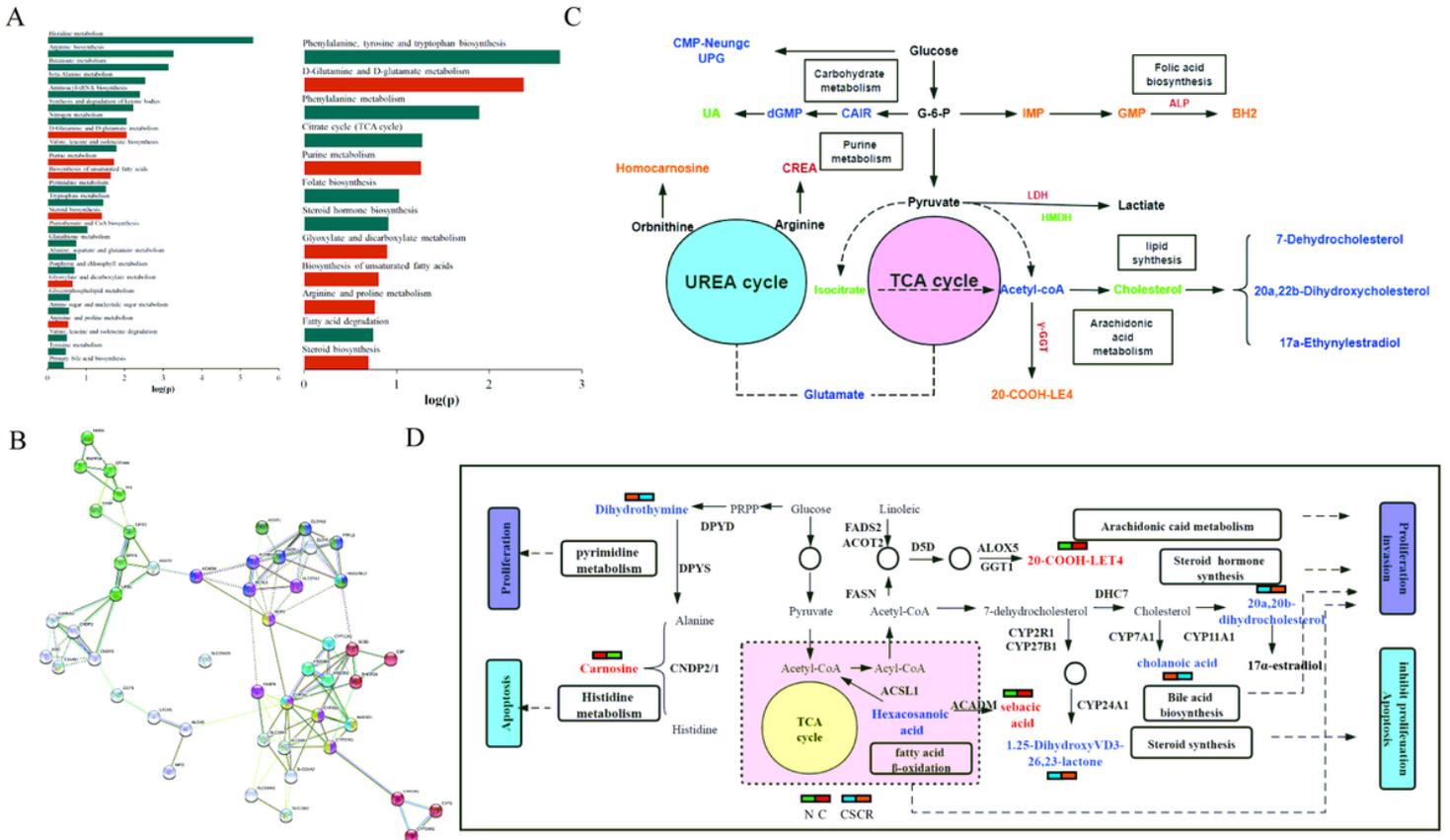


Figure 5

Pathway analysis of differential metabolites between ovarian cancer tissue and ascites. a. Enrichment pathway bar chart of differential metabolites (orange is shared pathway) in N vs C (left), CS vs CR (right) groups. b. The shared pathways involved in differential metabolites and serological indicators. c. Network view of STRING analysis of genes related to differential metabolites ($p < 0.001$). d. Proposed metabolic mechanisms associated with ovarian cancer. Red font represents the N vs C group and blue font represents the CS vs CR group. and denote down-regulation/up-regulation.

Supplementary Files

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