Metabolomics and transcriptomics joint analysis reveals altered amino acid metabolism in esophageal squamous cell carcinoma

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Research Article

Keywords: esophageal squamous cell carcinoma, metabolic pathway, joint-pathway analysis, amino acid metabolism, arginine and proline metabolism.

Posted Date: June 30th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3117927/v1

Additional Declarations: No competing interests reported.

Abstract

Introduction:

Metabolic reprogramming plays a crucial role in tumor development by modifying tumor cell metabolism, which was also found in esophageal squamous cell carcinoma (ESCC).

Objectives

This study aims to explore the altered metabolic pathways for ESCC through joint-pathway analysis of differentially expressed metabolites and genes.

Methods

Differentially expressed metabolites in ESCC were collected from published tissue-based metabolomics studies. Differentially expressed genes in ESCC were obtained using bioinformatic analysis of online ESCC transcriptome data. Then, joint-pathway analysis was performed to explore the altered metabolic pathways in ESCC. Immunohistochemistry (IHC) staining and arginine-deprivation experiments were conducted to verified the key enzymes in metabolic pathway and their potential function in ESCC.

Results

A total of 9 tissue-based metabolomics studies revealed 495 differentially expressed metabolites in ESCC. Enrichment analysis of the 69 high-frequency metabolites, defined as reported by over 2 studies, showed that the top enriched pathways were urea cycle, arginine and proline metabolism and ammonia recycling. Besides, bioinformatic analysis of a dataset (GSE53625) showed 2679 differentially expressed genes in ESCC. Joint-pathway analysis illustrated that the top 5 significantly altered metabolic pathways were glycerolipid metabolism, ascorbate and aldarate metabolism, histidine metabolism, arginine and proline metabolism, and linoleic acid metabolism. IHC staining and arginine-deprivation experiments revealed the up-regulating of arginine transporter (CAT1) and characteristic of arginine-dependent proliferation in ESCC.

Conclusions

This study revealed the altered amino acid metabolism, especially arginine and proline metabolism, as the most significant metabolic characteristic in ESCC. However, further functional study is needed.

1. Introduction

Esophageal cancer is an extremely dangerous form of cancer with an aggressive nature and high mortality rate. It is ranked 6th among the leading causes of cancer-related deaths worldwide and is the 8th most prevalent form of cancer globally. The 5-year survival rate is only around 15%-25%(Domper Arnal et al., 2015), and this highlights the challenging aspect of treating this disease. Esophageal cancer has two main pathological types: adenocarcinoma (EAC) and squamous cell carcinoma (ESCC), with the latter accounting for 80% of all esophageal cancer cases(Xi et al., 2022). ESCC has a discouraging prognosis and a high fatality rate mainly due to its difficult detection in the early stages. It is typically identified at later disease stages by enhanced thoracic computerized tomography (CT) and gastroscopy(Baba et al., 2018). Although surgical resection, radiotherapy, and chemotherapy are the primary clinical treatments for ESCC, their efficacy is limited, and they often have severe adverse effects(Yang et al., 2020). As a result, it is crucial to explore new therapeutic options and targets, particularly those focused on future research and development.

It is well-known that metabolic reprogramming is one of the hallmarks of cancer(Ward and Thompson, 2012), and emerging evidence has revealed that tumor cells undergo metabolic reprogramming to fuel their proliferation and differentiation(Sun et al., 2019). As a result, identifying therapeutic targets or biomarkers for cancer based on altered metabolome has emerged as a

promising strategy(Martinez-Outschoorn et al., 2017). Metabolomics is the study of small molecule metabolites, typically less than 1000, in a biological system such as cell, tissue, organ, or organism. There are three primary analytical platforms for metabolomics, including liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR), and each with their unique analytical range. Unlike other "omics" approaches, metabolomics can provide a snapshot of changes at the biochemical level, making it a highly sensitive tool for identifying pathological variants(Griffin and Shockcor, 2004)·(Schmidt et al., 2021)·(Nicholson et al., 2002).

While several metabolomics studies have investigated the metabolomic profile of ESCC(Xu et al., 2013; Zhang et al., 2012), the results reported by each study are limited by the analytical coverage of the platforms utilized, such as LC-MS, GC-MS, and NMR. As a consequence, the number of differentially expressed metabolites reported in each study is relatively small. Furthermore, the results of these studies have sometimes been contradictory, making it challenging to determine the precise changes in metabolite levels associated with ESCC. Thus, integrating different metabolomics studies, carefully analyzing and weighting the results from each, is critical for identifying the metabolic processes that are truly altered in ESCC. By doing so, researchers can provide effective targets for the treatment of ESCC and enhance the understanding of its pathogenesis.

A multi-molecule level approach that systematically combines genes and metabolites can provide a new direction for disease research, as studying biomolecular changes at a single level is insufficient for systems biology research(Hasin et al., 2017; Yan et al., 2018). Controversial results have emerged in recent years regarding multi-omics studies on ESCC, due to the lack of strict inclusion criteria such as sample size, sample type, clinical information, metabolomics testing methods, and other factors. Thus, it is essential to systematically review and select appropriate studies for multi-omics analysis of ESCC, in order to explore the molecular features and potential targets.

This study collected differentially expressed metabolites from published studies and obtained differentially expressed genes through bioinformatic analysis of online data. Joint-pathway analysis of gene and metabolite was utilized to investigate metabolic alterations and identify potential therapeutic targets in ESCC. Key enzymes in the feature metabolic pathway were validated by immunohistochemistry (IHC) staining. These findings may offer promising biomarkers and therapeutic strategies for ESCC.

2. Materials and Methods

2.1. Collection of differentially expressed metabolites and pathway analysis

To collect tissue-based metabolomics studies on ESCC, a literature search was conducted on PubMed (https://pubmed.ncbi.nlm.nih.gov/), using the following inclusion criteria: 1) based on metabolomics, 2) ESCC tissue samples, and 3) complete metabolomics results and patient clinical information. A total of 9 metabolomics studies met the criteria, and 495 unique differential metabolites were obtained after removing duplicates. High-frequency metabolites refers to metabolites that appeared in two or more studies with consistent or inconsistent trends.

Enrichment analysis was performed using online software MetaboAnalyst (https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml). This method utilizes SMPDB, which included 99 metabolite sets based on normal human metabolic pathways, as the metabolite set library for enrichment analysis.

2.2. Bioinformatics analysis of ESCC transcriptome

Based on sample size and clinical information integrity, microarray data, which was deposited in Gene Expression Omnibus (GEO) under accession number GSE53625 (Agilent-038314 CBC Homo sapiens lncRNA + mRNA microarray V2.0), were processed as described previously(Li et al., 2021). In briefly, the probe sets in GSE53625 were re-annotated by mapping all sequences provided in GPL18109 annotation file to human genome (hg38) using SeqMap(Qu et al., 2022). Probes that were mapped to protein-coding transcripts were remained. Average value was used for the genes with multiple probes. Differentially expression analysis between cancer and normal tissues was performed using R package *limma* (version 3.6.3). KEGG pathway enrichment analysis of differential genes was conducted using online tool "bioinformatics network analysis" from online software DAVID (https://david.ncifcrf.gov/).

2.3. Joint-pathway analysis of differential metabolite and gene

To gain a comprehensive understanding of metabolic reprogramming in esophageal squamous cell carcinoma (ESCC), we employed the joint-pathway analysis method from Metaboanalyst (https://www.metaboanalyst.ca/) to perform integrative analysis of differentially expressed metabolites and genes in ESCC. This method utilizes hypergeometric testing for enrichment analysis, degree centrality for topology measurement, and combine p values (unweighted) for integration of results.

2.4. Immunohistochemistry (IHC) staining

ESCC tissue samples were collected from 119 patients recruited after histopathologic confirmation of ESCC and radical resection at Zhejiang Cancer Hospital, China, from May 2010 to December 2012. The clinical stages of ESCC patients were determined based on the American Joint Committee on Cancer 8th edition staging system. This study was approved by the Institutional Ethical Review Board of Zhejiang Cancer Hospital and all patients were informed about and gave their consent for the study before surgery. Detailed clinical information is presented in the table **(Supplemental file3: Table S9)**.

The IHC staining procedure was processed as described previously(Zhu et al., 2020). In briefly, the tissue microarray slides were deparaffinized in xylene and gradient ethanol. Slides were immunohistochemically stained according to the manufacturer's instructions. Antibodies for identification of protein expression of CAT1 (Affinity Biosciences, Jiangsu, China; Cat#:DF13433) at a dilution of 1:200, ASS1 (Affinity Biosciences, Jiangsu, China; Cat#:BF0242) at a dilution of 1:500, and ODC1 (Affinity Biosciences, Jiangsu, China; Cat#:DF6712) at a dilution of 1:200 were used in this study. The IHC staining results were reviewed by a pathologist, and the staining score was defined as multiplying the percentage of positive cells by staining intensity.

2.5. Arginine-deprivation experiments

Human ESCC KYSE150 and KYSE30 cell lines were purchased from Nanjing Kebai Biotechnology Co., Ltd. (Nanjing, China) in 2016, and authenticated by a short tandem repeat (STR) report by Shanghai biowing biotechnology Co., Ltd (Nanjing, China) in 2019. The cells were cultured in RPMI 1640 (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/100 μg/mL streptomycin at 37°C under 5% CO_2.

CCK8 assay

Cells were seeded into 96-well plates at a density of 3000 cells per well and incubated at 37°C and 5% CO_2 with costumed (without arginine) RPMI 1640 Medium (Coolaber Technology Co., Ltd., Beijing, China) adding a series of concentrations (0, 0.25, 0.5, 1.0, 1.5, 2.0 mM) of arginine. CCK8 assay (APExBIO Technology LLC, USA) was performed according to the product's protocol at a series of time points (24, 48, 72, and 96 hours). Each experiment consisted of five replicates and was repeated at least three times.

Clone formation assay

Cells were seeded in 6-well plate at a density of 1000 cells per well, and incubated under the same conditions as described above. After 3 weeks, the cells were fixed with 4% polyformaldehyde for 20 minutes, and stained with 1% crystal violet, and the numbers of colonies containing more than 50 cells were counted microscopically. The experiment was performed in triplicate and repeated three times.

3. Results

3.1. Differentially expressed metabolites from 9 ESCC tissue-based metabolomics studies

With the keywords of "ESCC", "esophageal squamous cell carcinoma", and "metabolomics ", a total of 58 related articles retrieved. After systematically review, in which studies of non-ESCC tissue-based research or with incomplete information were excluded, a total of 9 eligible metabolomics studies(Chen et al., 2020; Chen et al., 2021; Sun et al., 2019; Tokunaga et al., 2018; Wang et al., 2013; Wu et al., 2009; Xu et al., 2022; Yang et al., 2022; Zang et al., 2021) was remained **(**Table 1**).**

The 9 studies included the three primary analytical platforms for metabolomics (NMR, GC-MS, LC-MS). Besides, mass spectrometry imaging (MSI), capillary electrophoresis-mass spectrometry (CE-MS) were also used **(**Fig. 1A**)**. After collecting differential metabolites from the 9 studies, there were 740 differential metabolites including duplicates **(Supplemental file1: Table S1)**. After

removing exogenous compounds and duplicate metabolites, 495 unique differential metabolites were remained, including 327 up-regulated and 168 down-regulated ones. In terms of high frequency metabolite, there was a total of 69 metabolites reported in over two studies, of which 11 had inconsistent change trends and 58 had consistent trend (Fig. 1B, Table 2, **Supplemental file1: Table S2)**.

Enrichment analysis of 495 differential metabolites revealed a total of 42 significant metabolic pathways (FDR < 0.05) (Fig. 1C, **Supplemental file1: Table S3)**, and the top 5 were apartate metabolism; glycine and serine metabolism; urea cycle; nicotinate and nicotinamide metabolism; glutamate metabolism. Enrichment analysis of 327 up-regulated metabolites revealed 18 significantly enriched metabolic pathways and the top 5 metabolic pathways were aspartate metabolism; glycine and serine metabolism; nicotinate and nicotinamide metabolism; purine metabolism; methionine metabolism (Fig. 1D, **Supplemental file1: Table S4)**. While pathway enrichment analysis of 168 down-regulated metabolites showed that there were 47 significantly enriched metabolic pathways and the top 5 included warburg effect; citric acid cycle; gluconeogenesis; galactose metabolism; purine metabolism (Fig. 1E, **Supplemental file1: Table S5)**. Furthermore, Enrichment analysis of 69 high-frequency metabolites, there were a total of 19 metabolic pathways were significantly enriched, and the top 5 pathways were urea cycle; arginine and proline metabolism; ammonia recycling; aspartate metabolism; glycine and serine metabolism (Fig. 1F, **Supplemental file1: Table S6)**.

Table 1
Nine eligible ESCC tissue-based metabolomics research articles

Studies	Journal	Year	Platform[a]	Paired[b]	Sample[c]	Cmpd[d]	DOI
Metabolomic study for diagnostic model of oesophageal cancer using gas chromatography/mass spectrometry	*J Chromatogr B Analyt Technol Biomed Life Sci.*	2009	GC-MS	Yes	Tissue (tumor = 20 and normal = 20)	20	DOI: 10.1016/j.jchromb.2009.07.039
1H-NMR based metabonomic profiling of human esophageal cancer tissue	*Mol Cancer.*• • • .	2013	NMR	No	Tissue (tumor = 89 and normal = 26)	42	DOI: 10.1186/1476-4598-12-25
Metabolome analysis of esophageal cancer tissues using capillary electrophoresis-time-of-flight mass spectrometry	*Int J Oncol.*	2018	CE-TOFMS	Yes	Tissue (tumor = 35 and normal = 35)	110	DOI: 10.3892/ijo.2018.4340
Spatially resolved metabolomics to discover tumor-associated metabolic alterations	*Proc Natl Acad Sci USA.*	2019	AFADESI-MSI	Yes	Tissue (tumor = 256 and normal = 256)	27	DOI: 10.1073/pnas.1808950116
Metabolomic Characterization Reveals ILF2 and ILF3 Affected Metabolic Adaptions in Esophageal Squamous Cell Carcinoma	*Front Mol Biosci.*	2021	CE-MS/LC-MS	Yes	Tissue (tumor = 28 and normal = 28)	112	DOI: 10.3389/fmolb.2021.721990
Tissue-based metabolomics reveals metabolic biomarkers and potential therapeutic targets for esophageal squamous cell carcinoma	*J Pharm Biomed Anal.*	2021	UPLC/MS	No	Tissue tumor = 141 and normal = 70)	41	DOI: 10.1016/j.jpba.2021.113937
Combined Metabolomic Analysis of Plasma and Tissue Reveals a Prognostic Risk Score System and Metabolic Dysregulation in Esophageal Squamous Cell Carcinoma	*Front Oncol.*	2020	LC-MS	Yes	Tissue (tumor = 23 and normal = 23)	26	DOI: 10.3389/fonc.2020.01545
Untargeted metabolomics analysis of esophageal squamous cell cancer progression	*J Transl Med.*	2022	LC-MS/MS	No	Tissue (tumor = 60 and normal = 15)	145	DOI: 10.1186/s12967-022-03311-z

Studies	Journal	Year	Platform[a]	Paired[b]	Sample[c]	Cmpd[d]	DOI
Metabolomics of Esophageal Squamous Cell Carcinoma Tissues: Potential Biomarkers for Diagnosis and Promising Targets for Therapy	*Biomed Res Int.* .	2022	HPLC-TOF-MS/MS	Yes	Tissue (tumor = 210 and normal = 210)	269	DOI: 10.1155/2022/7819235

[a]: Metabolomics detection platform used in the study: GC-MS(gas chromatography-mass spectrometry), NMR(1H nuclear magnetic resonance), CE-TOFMS(capillary electrophoresis time-of-flight mass spectrometry), AFADESI-MSI(airflow-assisted desorption electrospray ionization mass spectrometry imaging), CE-MS/LC-MS(capillary electrophoresis-mass spectrometry and liquid chromatography-mass spectrometry), UPLC-MS(ultra-high-performance liquid chromatography coupled with high resolution mass), LC-MS(liquid chromatography-mass spectrometry), LC-MS/MS(liquid chromatography with tandem mass spectrometry), HPLC-TOF-MS/MS(high-performance liquid chromatography time-of-flight mass spectrometry with tandem mass spectrometry).

[b]: Whether the tissues used in the study were paired, “yes” means paired, “no” means unpaired.

[c]: The number of ESCC tissue as well as normal esophageal tissue used in the study.

[d]: The result of the number of differential metabolites in the metabolomics studies.

Table 2

Summary of the high-frequency metabolites[a]

Metabolites identified by > 2studies	Studies[b]	Up/down regulation	Metabolites identified by > 2studies	Studies[b]	Up/down regulation
Beta-Alanine	2	Up	D-Phenyllactic acid	2	Up
Creatine	3	Down	Gluconic acid	4	Up
Deoxyguanosine	2	Up	Glutamine	3	Down
Glycerophosphocholine	3	Up	L-Kynurenine	4	Up
Citric acid	2	Down	L-Leucine	2	Up
GABA	2	Up	L-Methionine	3	Up
Glutathione	2	Up	4-Hydroxyproline	2	Up
Guanosine	2	Up	Myristic acid	3	Up
Fumaric acid	2	Down	N-Acetyl-L-aspartic acid	2	Up
Glutamate	4	Up	L-Valine	4	Up
Hypoxanthine	3	Up	Citrulline	2	Up
L-Tyrosine	5	Up	L-Tryptophan	4	Up
Phenylalanine	7	Up	S-Adenosylmethionine	2	Up
L-Proline	3	Up	N'-Formylkynurenine	2	Up
L-Threonine	3	Up	ADP	2	Down
L-Isoleucine	5	Up	N-Acetyl-glucosamine 1-phosphate	2	Up
L-Histidine	3	Up	Guanosine monophosphate	3	Up
L-Lysine	2	Up	Putrescine	2	Up
L-Serine	2	Up	CDP	2	Down
L-Lactic acid	3	Up	Phosphorylcholine	2	Up
L-Aspartic acid	3	Up	Palmitoylethanolamide	2	Up
Ornithine	2	Up	Hypogeic acid	2	Up
Palmitic acid	2	Up	N8-Acetylspermidine	2	Up
Palmitoylcarnitine	3	Up	Ophthalmic acid	2	Down
Pyruvic acid	2	Down	LysoPC(18:2(9Z,12Z)/0:0)	2	Up
Uracil	3	Up	gamma-Glutamylglutamic acid	2	Up
N-alpha-Acetyl-L-lysine	2	Up	N-Acetyl-L-methionine	3	Up
L-Arginine	2	Up	Ricinoleic acid	2	Up
Adenosine triphosphate	2	Down	Creatinine	2	Down
Aminoadipic acid	2	Up/down	Arachidonic acid	2	Up/down
	3	Up/down	Dihydroxyacetone phosphate	3	Up/down
Glucose	2	Up/down	Glycine	4	Up/down
l-Asparagine	5	Up/down	LysoPC(24:1(15Z)/0:0)	2	Up/down

Metabolites identified by > 2studies	Studies[b]	Up/down regulation	Metabolites identified by > 2studies	Studies[b]	Up/down regulation
Myo-inositol	3	Up/down	N-Acetylputrescine	3	Up/down
Phosphocreatine	3	Up/down			

[a]: High-frequency metabolites: metabolites identified by > 2 studies, with or without consistent trends.

[b]: Number of studies reporting this differential metabolite.

3.2. Differentially expressed genes in ESCC

Differentially expressed genes (DEG) were defined as adjusted P value less than 0.05 and $|\log_2 FC| > 1$. A total of 2679 differentially expressed genes, including 1080 up-regulated and 1599 down-regulated, were obtained (Fig. 2A, **Supplemental file2: Table S7**). Heatmap plotting showed that these DEGs could significantly distinguish between ESCC cancer tissues and normal tissues (Fig. 2B).

Pathway enrichment analysis of DEGs was performed using DAVID (https://david.ncifcrf.gov/), and a total of 17 pathways was significantly (FDR < 0.05) enriched. There was a total of 253 metabolic genes, suggesting a metabolic reprogramming in ESCC (Fig. 2C, **Supplemental file2: Table S8**).

Interestingly, the arginine and proline metabolism pathway was ranked high, with 16 differential genes and an enrichment ratio of 2.27 (Fig. 2D). This pathway was also enriched in the differential metabolite analysis, further supporting the importance of the arginine and proline metabolism pathway in ESCC.

3.3. Joint-pathway analysis of differentially expressed metabolites and genes

Joint-pathway analysis revealed that there were 12 significantly enriched metabolic pathways (FDR < 0.05) (Table 3), with the top 5 being: glycerolipid metabolism; ascorbate and aldarate metabolism; histidine metabolism; arginine and proline metabolism; linoleic acid metabolism. All the pathways, except for mucin type O-glycan biosynthesis, had hits from both metabolite and gene. Among the 12 pathways, there were 7 ones related to amino acid metabolism, indicating a potential role of amino acid metabolism in ESCC. Of them, altered arginine and proline metabolism had 12 metabolites hits, including L-arginine, creatine, 4-aminobutanoate, putrescine, S-adenosyl-L-methionine, spermidine, spermine, D-proline, hydroxyproline, L-glutamate, ornithine, pyruvate, while had 16 genes hits, including ARG1, NOS2, GATM, CKMT2, CKMT1A, ALDH2, ALDH9A1, ALDH3A2, ALDH7A1, MAOA, MAOB, L3HYPDH, PYCR1, P4HA3, P4HA1, ODC1.

Table 3
Joint-pathway analysis of genes and metabolites

Pathway name	Total[a]	Hits (cmpd)[b]	Hits (gene)[c]	FDR[d]	Impact
Glycerolipid metabolism	35	3	18	3.01E-05	1.18
Ascorbate and aldarate metabolism	13	1	6	2.29E-04	1.00
Histidine metabolism	32	6	10	9.22E-04	0.81
Arginine and proline metabolism	78	12	16	1.22E-03	0.90
Linoleic acid metabolism	17	3	8	2.76E-03	2.00
Valine, leucine and isoleucine biosynthesis	12	6	2	5.11E-03	1.55
Phenylalanine metabolism	21	4	7	6.25E-03	1.55
Mucin type O-glycan biosynthesis	22	0	8	7.79E-03	0.62
Glutathione metabolism	56	8	12	7.79E-03	0.85
Arginine biosynthesis	27	8	4	8.14E-03	1.23
beta-Alanine metabolism	44	7	9	1.82E-02	0.86
Nitrogen metabolism	10	2	4	2.15E-02	0.78

[a]: The number of total of genes and metabolites in the pathway.

[b]: The number of metabolite hits in the joint-pathway analysis.

[c]: The number of gene hits in the joint-pathway analysis.

[d]: False discovery rate.

3.4. Up-regulated arginine transporter CAT1 and down-regulated succinic acid synthetase1 (ASS1) in ESCC

To investigating the potential mechanism for the accumulation of arginine in ESCC, arginine transporter CAT1, which is responsible for uptake arginine extracellularly, as well as succinic acid synthetase1 (ASS1), which mediates biosynthesis of arginine from the urea cycle, were included in the study. Representative images of IHC staining of ESCC tissue microarray were shown in Fig. 3. CAT1 was significantly up-regulated in ESCC tissues compared to normal esophageal tissues (mean IHC score: 7.36 vs. 4.86, $p = 0.03$) (Fig. 3A); while ASS1 was shown significantly down-regulated in ESCC tissues compared to normal ones (mean IHC score, 8.00 vs. 4.53; $p = 0.01$) (Fig. 3B). These findings suggest that the elevated expression of the amino acid transporter CAT1 may be involved in the up-regulation of arginine in ESCC.

3.5. Arginine influences ESCC cell proliferation

The CCK8 assay revealed that the proliferative capacity of KYSE30 cells was relatively low in the control group (0 mM arginine), and showed a significant increase when arginine was added to the medium ($p < 0.01$). However, the proliferative ability of KYSE30 cells displayed growth that was independent of concentration (Fig. 4A). Similar trends were observed in KYSE150 cell (Fig. 4B).

The clone-formation assay results showed that there were no KYSE30 cell clones in the control group (0 mM arginine). When different concentrations of arginine were added to the medium, KYSE30 cell clones were formed. The clone formation was independent of concentration, which is consistent with that in CCK8 experiment (Fig. 4C). These findings suggest that within a certain range of concentrations, arginine can enhance the proliferation ability of ESCC cells, which provides valuable insights into the potential utility of manipulating arginine levels as a therapeutic strategy for treating ESCC.

4. Discussion

Integrative metabolomics and genomics become a popular strategy in cancer research, which facilitate in discovering biomarkers and understanding the molecular mechanisms of carcinogenesis. Since a single metabolomics study with a single analytical platform is hardly able to cover the whole metabolic profile of a disease, systemic reviewing of the published metabolic studies is a convenient way to collect the available differentially expressed metabolites and analyze the metabolism features for a disease. In fact, Li et al. performed similar study, in which seven metabolomics articles and six ESCC mRNA datasets were used for joint-pathway analysis for ESCC(Li et al., 2017). However, only two of the seven studies reviewed in their study were tissue-based metabolomics research article, and the others were from plasma/serum/other fluid -based metabolomics. Metabolite pool in plasma is obviously different from that in tumor. Therefore, the discover from their study can hardly represent the real metabolism features of ESCC. Recently, there is growing number of studies from ESCC tissue-based metabolomics studies available, and it is worthy to conducted a systemic review of ESCC tissue-based metabolomics study to investigate the big metabolic landscape of ESCC. Thus, we performed this study, and screened out 9 relevant articles between 2009 and 2022.

Different analytical tool platforms, including NMR, GC-MS, LC-MS and CE-MS, were used in metabolomics for ESCC. Each platform has its own advantages and limitations, and the reliability of the results obtained from different platforms might have inconsistent result. NMR is non-invasive, rapid, and can detect metabolites in vivo, but has lower sensitivity and limited dynamic range. LC-MS and GC-MS offer improved sensitivity and resolution, while CE-MS is often used for metabolite profiling due to its high sensitivity(Liu and Zhong, 2019). Combinations of multiple analytical techniques, including GC-MS, NMR, and LC-MS, have been widely used to improve the sensitivity, specificity, and selectivity of metabolite detection in recent years(Gao and Xu, 2015). The 9 studies reviewed in this study covered NMR, LC-MS, GC-MS and CE-MS, the combination of multiple analytical techniques provided a comprehensive overview of the differentially expressed metabolites in ESCC. This study revealed that there was a total of 495 unique differential metabolites, 58 high-frequency metabolites with consistent trends.

Based on the results of collected differential metabolites, especially high-frequency metabolites, and the pathway enrichment analysis, dysregulated amino acid metabolism was the most significant metabolic feature in ESCC. The high-frequency metabolite table showed 19 amino acids was reported to altered in ESCC. Most of amino acids, such as L-arginine, glutamate, L-proline, L-aspartic acid, were significantly accumulated in ESCC tissue compared to normal tissue, which indicating an increased uptake of amino acids in ESCC. Glutamine was the only down-regulated amino acid reported in ESCC, and it might be caused by the factor that consumption of arginine was much higher than its absorption from extracellular environment. While glycine and asparagine had inconsistent change trends in different studies. Additionally, the abnormal amino acid metabolism observed in ESCC may serve as a potential biomarker for diagnosis and monitoring of the disease. Targeting amino acid metabolism pathways could be a promising therapeutic strategy, as inhibitors of enzymes involved in amino acid metabolism have shown promising results in inhibiting tumor growth and improving survival. Our previous study(Chen et al., 2021)[22] revealed a significant alteration in amino acid metabolism, such as tryptophan metabolism, was significantly up-regulated in ESCC, and its corresponding amino acid transporters, such as SLC7A5, SLC1A5 and SLC16A10, were evidently over-expressed in ESCC. Some studies have shown that, amino acid transporters, such as SLC7A5 and SLC1A5, are over-expressed in several tumors and essential for cancer cell growth(Wang and Zou, 2020). And the pharmacologic inhibition and knockdown/knockout of these transporters can significantly suppress the proliferation of cancer cells(Kanai, 2022). Therefore, nutritional interventions that target specific amino acids, such as arginine or glutamine, may also be beneficial for patients with ESCC.

Based on the results from enrichment analysis of high-frequency metabolites and joint-pathway analysis, arginine and proline metabolism was illustrated to be a significantly dysregulated pathway in ESCC. Among the top 5 significantly enriched pathways of the high-frequency metabolites, the urea cycle, which was found deregulation in several cancers to maximize the body nitrogen incorporation into tumor growth(Keshet et al., 2018), is included in arginine and proline metabolism. The ammonia recycling exists down stream of arginine and proline metabolism to recover ammonia and keep the balance of nitrogen metabolism in the body, which performs a similar function to the urea cycle. Both of the aspartate metabolism and glycine and serine metabolism have more or less intersection with arginine and proline metabolism through transamination. All of these results hint at the importance of altered arginine and proline metabolism in ESCC.

Arginine, as an important amino acid that plays a critical role in cellular metabolism and immune function(Szefel et al., 2019), was found up-regulated in ESCC in this study. Existing literature reports, altered arginine and proline metabolism has been observed in

various tumors, particularly those with chemo resistance and poor prognosis. Arginine is obtained by cells through two pathways under normal physiological conditions: production via the ornithine cycle by ASS1(Szlosarek, 2014) and transport into cells via the CAT1(Satriano, 2004)(Fig. 5). In this study, IHC staining was performed and the result verified the up-regulating expression of CAT1 and the down-regulating expression of ASS1 in ESCC, which implied that CAT1 might be the main cause of increased arginine uptake in ESCC, and targeting this transporter might be a potential therapeutic strategy for this type of cancer. Other reference reported that circulating arginine promotes tumor growth(Poillet-Perez et al., 2018). And this study further supported the concept with CCK8 and clone-formation assays demonstrating that arginine enhances the capacity of proliferation in ESCC cell lines. Therefore, blocking arginine uptake through CAT1 or other means could be a viable therapeutic strategy for ESCC. However, more research is needed to fully understand the role of arginine in ESCC growth and to determine the best approach for targeting arginine and proline metabolism in cancer therapy.

The ornithine is first synthesized from glutamine via glutaminase (GLS), pyrroline-5-carboxylate synthase (P5CS) and ornithine aminotransferase (OAT), or be generated from proline via proline oxidase (PO). Ornithine then enters the urea cycle (shown in the blue area). The enzyme ornithine carbamoyltransferase (OCT) converts the ornithine to citrulline and the argininosuccinate synthetase1 (ASS1) combines citrulline with aspartate to generate argininosuccinate. After that, the enzyme argininosuccinate lyase (ASL) will remove fumaric acid from argininosuccinate to generate arginine. Extracellular arginine can be transported into cells by the cationic amino acid transporters (CAT1) as well. Arginine then converted into ornithine and urea by arginase I/II (ARGI/II). Subsequently, ornithine can be recycled back into arginine through the urea cycle or further converted to polyamines in spermidine and spermine metabolism through ornithine decarboxylase (ODC1) (showed yellow area). Among the above metabolites, up-regulated differentially expressed metabolites show red, down-regulated differentially expressed metabolites show blue and non-differentially expressed metabolites show grey.

Notably, spermidine and spermine biosynthesis, as a downstream metabolic reaction to arginine and proline metabolism, also shown significant alterations in the results of differential metabolite enrichment analysis, 8 out of 18 metabolites in this pathway were differential metabolites, 7 of which showed up-regulation, including ornithine, pyrophosphate, S-adenosylmethionine, spermine, spermidine, putrescine, and 5 were high-frequency metabolites, including adenosine triphosphate, ornithine, S-adenosylmethionine, spermidine, putrescine. In this study, polyamines, including spermidine, spermine, and putrescine, are up-regulated metabolites and IHC staining results indicated the up-regulation of ODC1, a key enzyme involved in polyamine synthesis (Fig. 3A, Fig. 3B). Studies reported that, polyamines are essential for normal cell growth and their depletion results in cytostasis. Dysregulation of polyamine metabolism is common in many cancers, such as prostate cancer, colorectal cancer and ovarian cancer(Du and Han, 2021; Holbert et al., 2022). Elevated polyamine levels are necessary for transformation and tumor progression and targeting polyamine metabolism with inhibitors such as difluoromethylornithine (DFMO), inhibitor of ODC1(Casero et al., 2018), has shown promising results in phase I trials for various cancers. These findings highlight the metabolic specificity of polyamine synthesis in ESCC and suggest the potential feasibility of polyamine metabolic inhibitors in ESCC treatment, which should be further explored.

In conclusion, the joint-pathway analysis of differential genes and differential metabolites explored a relatively wide metabolic landscape for ESCC, and revealed the amino acid metabolism pathways, such as arginine and proline metabolism pathway and polyamine metabolism, as the potential targets for ESCC. However, further functional studies are needed for investigating the potential clinical significance of these metabolic targets in ESCC.

Declarations

This research was supported by grants from the National Natural Science Foundation of China (No. 81672315,81302840), from the Medical and the Health Science Project of Zhejiang Province (2022KY622), from the Zhejiang Provincial Natural Science Foundation of China (LY23H010002), Key R&D Program Projects in Zhejiang Province (2018C04009), from the Medical and the Health Science Project of Zhejiang Province (2020KY487).

5.Data availability statement

The datasets generated for this study can be found in the GEO/GSE53625/ https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53625.

6.Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Zhejiang Cancer Hospital, China. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

7.Author contributions

Yang Chen, Zhongjian Chen, and Weimin Mao conceived, designed the study, interpreted the data, wrote the first draft of the manuscript, and contributed to the final version of the manuscript. Yang Chen, Huan Yang, Xiancong Huang and Ruting Wang performed the experiment, conducted the bioinformatics analysis. All authors approved the submitted version of this manuscript.

8.Funding

This research was supported by grants from the National Natural Science Foundation of China (No. 81672315,81302840), from the Medical and the Health Science Project of Zhejiang Province (2022KY622), from the Zhejiang Provincial Natural Science Foundation of China (LY23H010002), Key R&D Program Projects in Zhejiang Province (2018C04009), from the Medical and the Health Science Project of Zhejiang Province (2020KY487).

9.Acknowledgements

We thank Biobank in Zhejiang Cancer Hospital for providing all the samples in the study.

10. Conflict of interest statement

The authors declare that there is no conflict of interests, we do not have any possible conflicts of interest.

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Figures

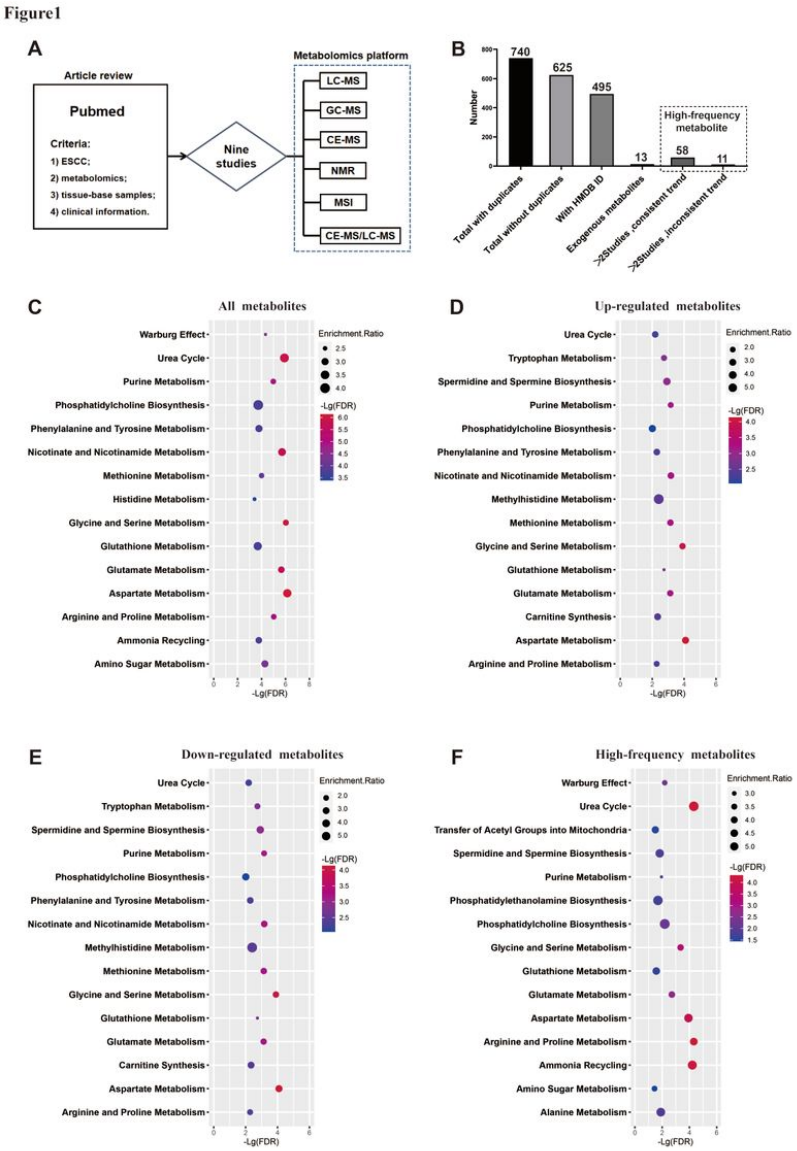

Figure 1

Differentially expressed metabolites from 9 ESCC tissue-based metabolomics studies

(A) Flow chart of the article reviewing for ESCC tissue-based metabolomics. **(B)** Summary of the differentially expressed metabolites from 9 ESCC tissue-based metabolomics studies. Enrichment analysis of the total of 495 differential metabolites (**C**), of 327 up-regulated differential metabolites (D), of 168 down-regulated differential metabolites (**E**), and of 69 high frequency differential metabolites (**F**).

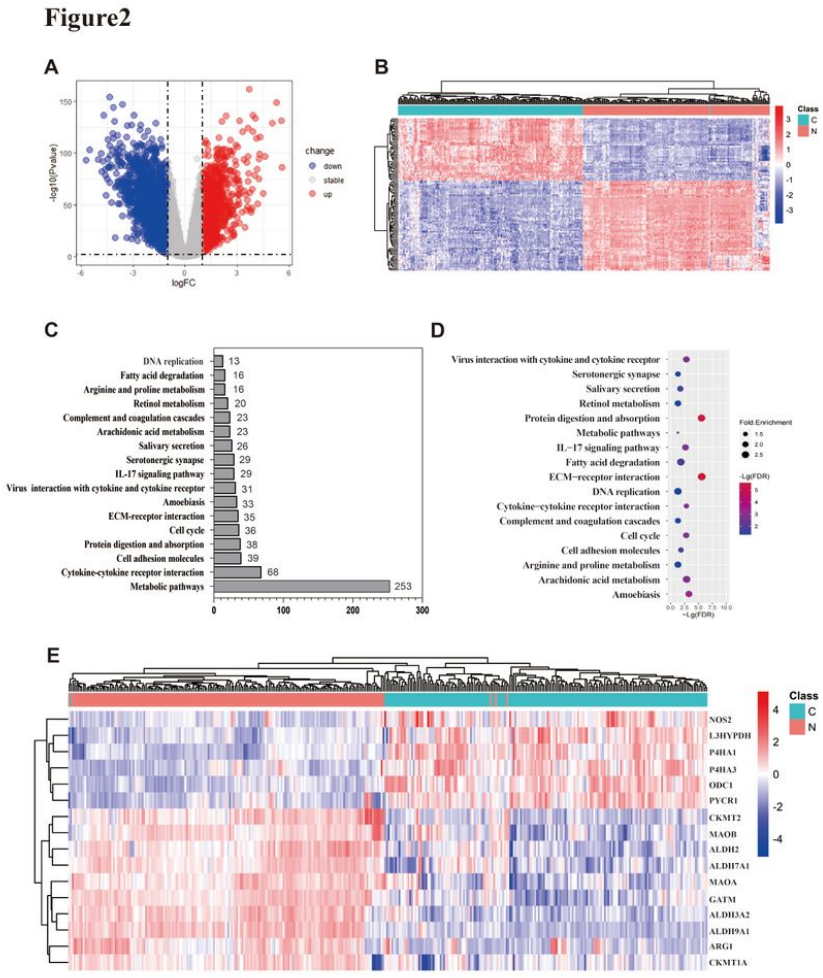

Figure 2

Differentially expressed genes in ESCC from GSE53625 dataset

(**A**) Volcano plot for differential genes. (**B**) Heatmap plotting with the differential genes, C: cancer, N: normal. (**C**) Pathway enrichment result. (**D**) Pathway analysis result. (**E**) Heatmap with differential metabolic genes belong to arginine and proline metabolism, C: cancer, N: normal.

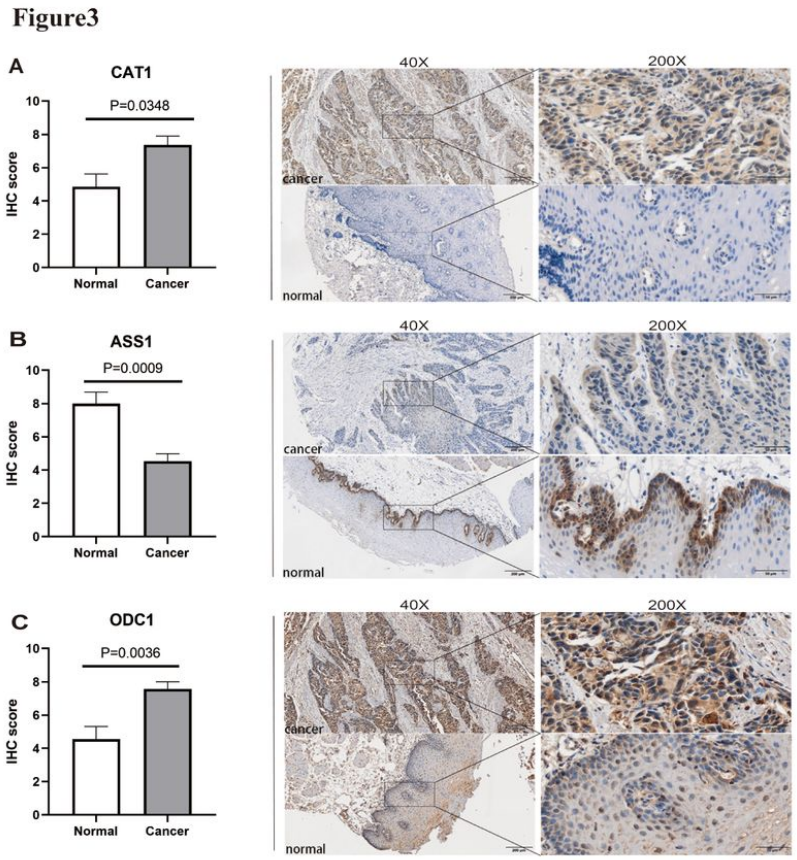

Figure 3

IHC staining showed the up-regulated arginine transporter (CAT1), down-regulated succinic acid synthetase1 (ASS1) in ESCC, and up-regulated ornithine decarboxylase (ODC1) in ESCC

(A) The bar graph of the IHC score of the CAT1 (**A**), ASS1 (**B**), and ODC1 (**C**) between cancer and normal tissue, and representative images of IHC staining at a magnification of 40x and 200x.

Figure4

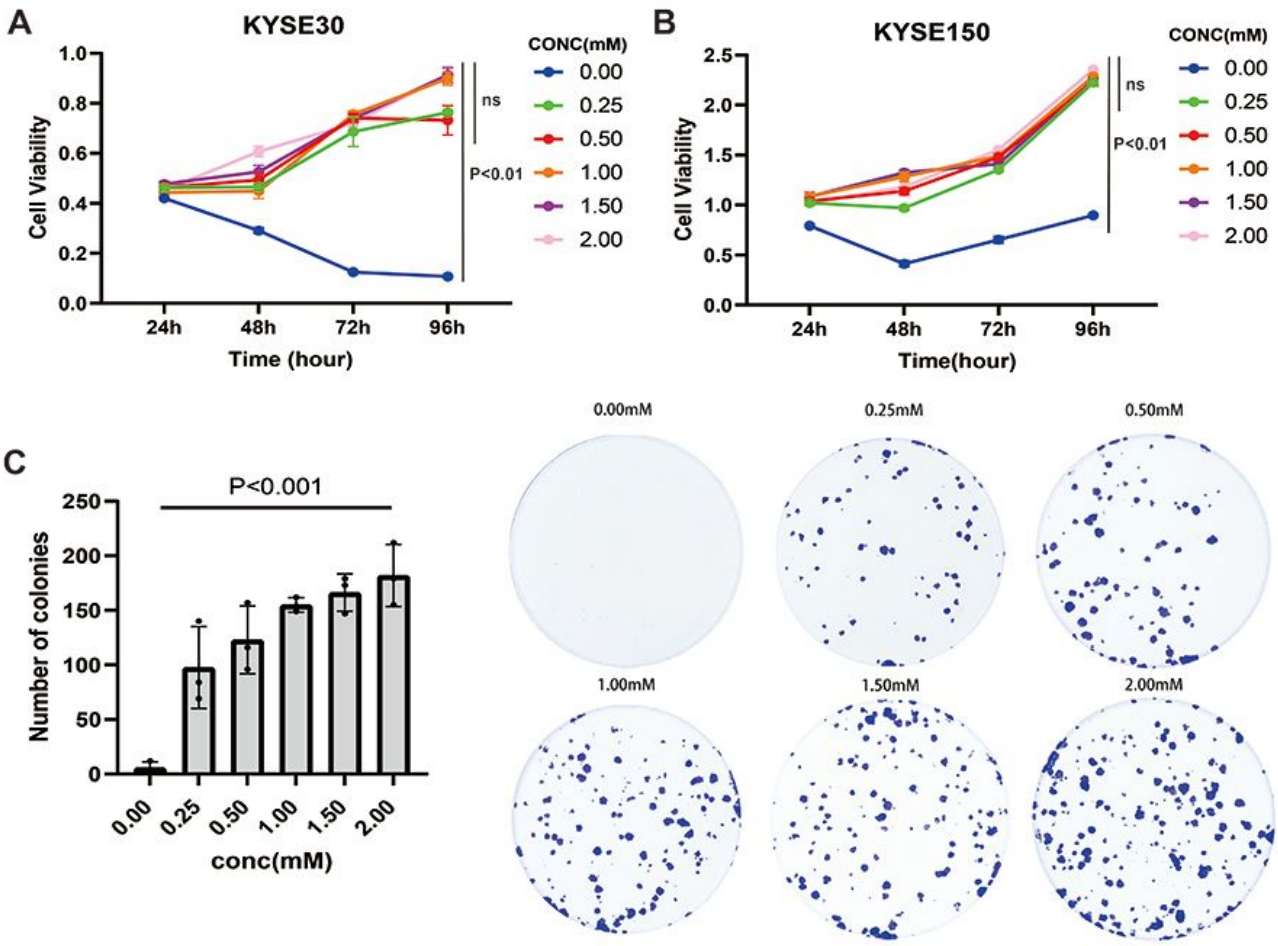

Figure 4

Arginine influences ESCC cell proliferation

Cells of KYSE30 **(A)** and KYSE150 **(B)** were cultured in media containing different concentrations of arginine (including 0.00, 0.25, 0.50, 1.00, 1.50, 2.00mM) with 6 technical replicates, and CCK8 assay were performed at 24h, 28h, 72h and 96h. **(C)** KYSE30 cells were used for clone formation assay, different concentrations of arginine (including 0.00, 0.25, 0.50, 1.00, 1.50, 2.00mM) with 3 technical replicates were used.

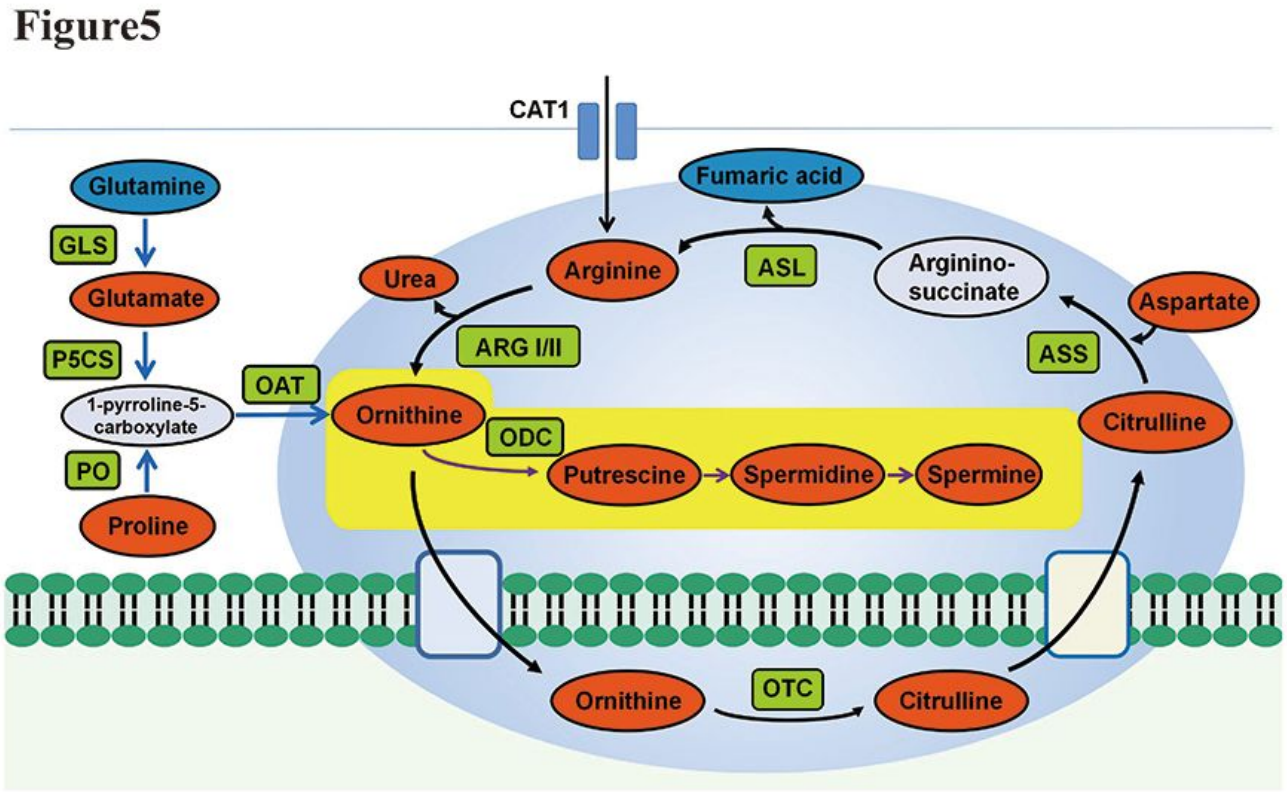

Figure 5

Schematic diagram for arginine and proline metabolic pathway in ESCC

The ornithine is first synthesized from glutamine via glutaminase (GLS), pyrroline-5-carboxylate synthase (P5CS) and ornithine aminotransferase (OAT), or be generated from proline via proline oxidase (PO). Ornithine then enters the urea cycle (shown in the blue area). The enzyme ornithine carbamoyltransferase (OCT) converts the ornithine to citrulline and the argininosuccinate synthetase1 (ASS1) combines citrulline with aspartate to generate argininosuccinate. After that, the enzyme argininosuccinate lyase (ASL) will remove fumaric acid from argininosuccinate to generate arginine. Extracellular arginine can be transported into cells by the cationic amino acid transporters (CAT1) as well. Arginine then converted into ornithine and urea by arginase I/II (ARGI/II). Subsequently, ornithine can be recycled back into arginine through the urea cycle or further converted to polyamines in spermidine and spermine metabolism through ornithine decarboxylase (ODC1) (showed yellow area). Among the above metabolites, up-regulated differentially expressed metabolites show red, down-regulated differentially expressed metabolites show blue and non-differentially expressed metabolites show grey.

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