

Gene expression analysis of the L-arginine pathway in renal cell carcinoma

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Abstract: Objective: To explore the key genes and their expression profiles in L-arginine biosynthesis and metabolic pathways in renal cell carcinoma (RCC) using bioinformatics methods. **Methods:** Differential expression analysis, prediction of upstream transcription factors, and survival analysis were carried out using gene expression data from RCC patients in the TCGA public database and clinical data. **Results:** Among 64 genes related to L-arginine anabolism, only the gene PYCR1 was up-regulated ($P < 0.01$); 31 genes were down-regulated ($P < 0.05$), including argininosuccinate synthase (ASS1), argininosuccinate lyase (ASL), and arginase 2 (ARG2). The transcriptional activators of these three genes, BRD2, EGR1, HNF4A, JUN, NFYA, NFYB, NFYC, SPI1, and TCF7L2, were down-regulated in cancer, whereas the transcriptional repressors c-Myc, ATF4, and ZNF263, were up-regulated. It is hypothesised that the down-regulation of these three genes is associated with changes in the expression of the above transcription factors. In addition, compared to control samples, the correlation between ASL and ASS1 in expression became weaker in clear cell renal cell carcinoma (ccRCC), papillary renal cell carcinoma (pRCC), and chromophobe renal cell carcinoma (chRCC), from 0.77, 0.86, and 0.85, respectively, to 0.36, 0.16, and 0.24. Respectively, high expression of ASL corresponded to longer ccRCC patients' overall survival (OS) ($P = 0.024$), which could be an independent prognostic factor for pRCC ($P = 0.04$). **Conclusions:** It is hypothesized that down-regulation of ASL, ASS1 and ARG2 expression leads to inhibition of the L-arginine-related pathway, which in turn correlates with RCC development, and that this down-regulation may be due to changes in transcription factor expression.

1 Preface

L-Arginine, as a core metabolic module, is directly or indirectly involved in a variety of biological phenomena, and it is a key pathway in the control of immune cell function. Increased levels of L-arginine can have multiple effects on T-cell activation, differentiation, and function^[1, 2], patients with higher blood arginine levels have a longer survival period^[3, 4]. Therefore, it is of great significance to explore the expression of genes related to L-arginine anabolism in RCC and to find potential specific molecular and biological targets for the diagnosis and treatment of RCC.

The intracellular metabolic pathway of L-arginine is shown in Figure 1: Arginine is converted to citrulline by urea cycle enzymes, then to arginine succinate by ASS1, then to arginine using ASL, and finally catabolized into urea and ornithine by ARG2. RCC is a malignant tumor originating from the epithelial system of the parenchymal urinary tubules of the kidney^[5] and the common types are ccRCC, pRCC and chRCC^[6]. The proximal tubular epithelial cells of the kidney are the main site of L-arginine synthesis, and through the expression of ASS1 and ASL, the kidney is able to utilize the citrulline produced by the small intestine as the main site of L-arginine source

synthesis, and ARG2 breaks down L-arginine and produces urea and ornithine, which have been correlated with T-cell survival and antitumor properties^[7, 8]. Currently, more studies have been conducted on ccRCC, and relevant literature indicates that downregulated expression of ASL, ASS1 and ARG2 can promote the proliferation of renal clear cell carcinoma^[9, 10]. The literature indicates that down-regulation of ASL, ASS1 and ARG2 can promote the proliferation of ccRCC. In this paper, we further explored the expression of L-arginine pathway-related genes in RCC.

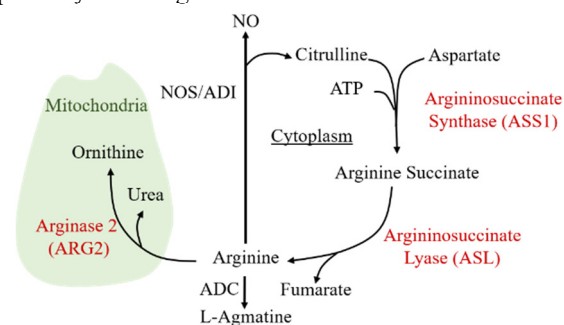


Fig 1. Metabolic flow chart based on common pathways of arginine metabolism

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2 Data and methods

2.1 Data

A collection of genes involved in the L-arginine-related pathway was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.kegg.jp/kegg/pathway.html>), and gene expression data from patients with RCC in The Cancer Genome Atlas (TCGA) database were selected for the study. We included data from a total of 516 ccRCC patients, 289 pRCC patients, and 66 chRCC patients. These specific subtypes were selected for study based on their prevalence in RCC and the reliability of the gene expression data in the TCGA library. ccRCC cohort, as the most common type of RCC, included 290 tumor samples and 32 normal control samples. pRCC cohort, as the second most common subtype, included 533 tumor samples and 72 normal control samples. Finally, the chRCC cohort, a rarer subtype, included 66 tumor samples and 25 normal control samples. This stratified approach allowed us to compare gene expression patterns in different RCC subtypes and their corresponding normal tissues. We also considered patient demographics and clinical characteristics. The median age of patients diagnosed with ccRCC was 59 years, and the group included a slightly greater percentage of male individuals, accounting for 58% of the cases; pRCC cohort had a median age of 62 years, with a similar proportion of males and females; and chRCC cohort had a median age of 65 years, with a higher proportion of male patients (62%). For a subset of patients, we were also able to obtain clinical outcome data including overall survival (OS) and disease progression, which allowed us to study the correlation between gene expression patterns and patient prognosis. ccRCC cohort had a median follow-up of 30 months and pRCC cohort had 36 months. By analysing this well-characterised and large patient cohort, we aimed to reveal the role of the L-arginine pathway in RCC and identify potential therapeutic targets and biomarkers. The robustness of our findings is emphasised by the diversity and size of the patient cohort, which enhances the general applicability of our results to the wider RCC patient population.

2.2 Methods

First, the differential expression of L-arginine-related pathway genes in cancer and normal tissues was analysed. Then, the genes were analysed via the hTFtarget website (<https://guolab.wehscu.cn/hTFtarget/#/>) and GeneCards website (<https://www.genecards.org/>) to predict potential upstream transcription factors of ASS1, ASL and ARG2 in renal tissues. Subsequently, Spearman correlation analysis was employed to assess the relationship between the expression levels of ASL and ASS1 in RCC. Moreover, the impact of ASL expression on the prognostic outcomes of patients with ccRCC was examined through Kaplan-Meier survival analysis. To identify the independent risk factors for patients with pRCC, univariate and

multivariate Cox proportional hazards regression analyses were conducted utilizing the clinical data of pRCC patients.

3 Results

3.1 Differential expression of L-arginine-related pathway genes

The L-arginine-related pathway genes were downloaded from KEGG pathway, containing two metabolic pathways: arginine biosynthesis and arginine and proline metabolism, as shown in Figure 2, totaling 64 genes. Then, the differential expression of L-arginine-related pathway genes in cancer and normal tissues was analyzed using the data of RCC samples downloaded from the TCGA database. In this analysis, 31 genes were simultaneously down-regulated in chRCC, pRCC, and ccRCC ($P < 0.05$). These included ABHD14A, ACY1, AGMAT, ALDH1B1, ALDH3A2, ALDH4A1, ALDH7A1, ALDH9A1, AMD1, ARG2, ASL, ASS1, CARN1, CKM, CNBP1, CPS1, DAO, GATM, GAMT, GLS, GLUD1, GLUD2, GPT, LAP3, MAOA, NAGS, NOS1, OAT, SAT1, SAT2, and SMS. Conversely, only one gene, PYCR1, showed an up-regulation in expression ($P < 0.01$). This suggests that the L-arginine-related pathway is generally inhibited, with key enzymes ASS1 and ASL, crucial for L-arginine synthesis, being down-regulated in RCC. As a result, arginine loses its intracellular synthesis source and instead relies on extracellular uptake. This is in agreement with the results of the literature^[10].

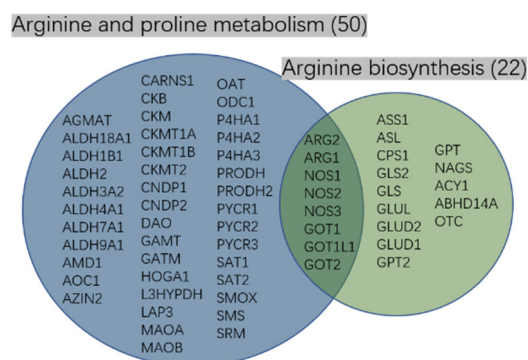


Fig 2. L-arginine-related pathway genes

3.2 Differential expression of ASL, ASS1 and ARG2 genes

The aim of this study was to explore the role of three genes, ASL, ASS1 and ARG2, in the L-arginine pathway in RCC. Differential expression of ASL, ASS1 and ARG2 in cancer and normal tissues was analysed based on data from RCC samples downloaded from the TCGA database. As shown in Figure 3, the distribution of gene expression levels was plotted using a box-and-line plot, and significance was calculated using the Wilcoxon test. As can be seen in Figure 3, the expression of ASL, ASS1 and ARG2 in chRCC, pRCC and ccRCC tumor tissues was consistently down-regulated and significantly lower than the corresponding normal tissues.

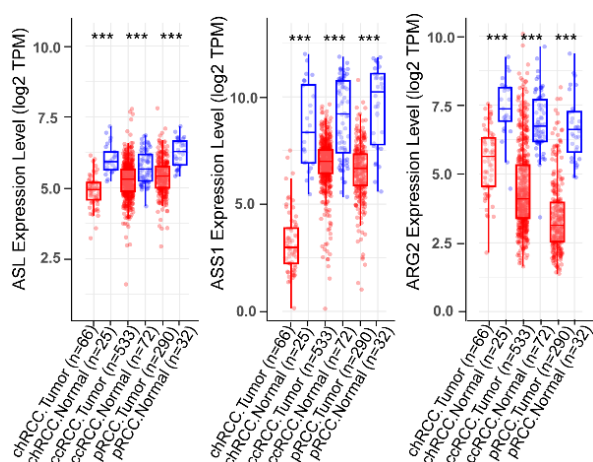


Fig 3. Box line plots of expression of ARG2, ASS1 and ASL genes in RCC subtypes
Note: *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Expression of ASS1 and ASL makes the kidney a major site of L-arginine source synthesis through absorption of citrulline produced in the small intestine. The down-regulation of ASS1 and ASL leads to restricted L-arginine synthesis, which in turn has multiple effects on T cell activation, differentiation, and function^[11-13]. Meanwhile, Rabinovich et al. demonstrated reduced expression of ASL in several tumor types. This downregulation of ASL is attributed to promoter hypermethylation. Concurrently, the primary metabolic consequence of ASS1 downregulation is an increased availability of its substrate, aspartic acid, which promotes the activation of CAD. This activation, in turn, fosters the synthesis of pyrimidines, ultimately supporting the proliferation of tumor cells^[14, 15]. In addition, ASS1 downregulation is also associated with high tumor proliferation rates, increased metastasis and poorer prognosis.

The arginase ARG2 is predominantly distributed in mitochondria and is more widely expressed in the kidney. The catalytic activity of specific ARG2 proteins on mitochondria is essential for oxidative phosphorylation, maintaining cellular energy supply and metabolic homeostasis. There are two key mechanisms for down-regulation of ARG2, namely avoidance of depletion of pyridoxal, an important biosynthetic cofactor, and avoidance of toxic accumulation of polyamines to promote tumor growth.

3.3 Transcription factor expression changes in ASS1, ASL and ARG2

Potential upstream transcription factors in kidney tissues were predicted by hTFtarget and GeneCards predicted potential upstream transcription factors for ASS1, ASL and ARG2 in renal tissues, and bubble plots show fold change and corrected P-values by bubble colour and size. Rows are genomic symbols and columns are selected cancer types. Bubble colours range from purple to red representing fold change between tumor and normal samples. Dot size is positively correlated with the significance of the P value. The transcriptional activators

BRD2, EGR1, HNF4A, JUN, NFYA, NFYB, NFYC, SPI1, and TCF7L2 were down-regulated in cancers ($P < 0.05$) (Figure 4). In contrast, the transcriptional repressors c-Myc, ATF4, and ZNF263 were upregulated ($P < 0.05$). They acted synergistically as negative regulators of ASS1, ASL, and ARG2 expression by binding to each other and to the promoters of ASS1, ASL, and ARG2. In the literature, over-activation of the gene MYC in adaptive immune cell subpopulations reduces tumor killing mainly through inhibition of effector T cells, and overexpression of c-Myc reduces the expression levels of ASS1 and ASL, thereby reducing arginine synthesis, which is consistent with the results of this paper.

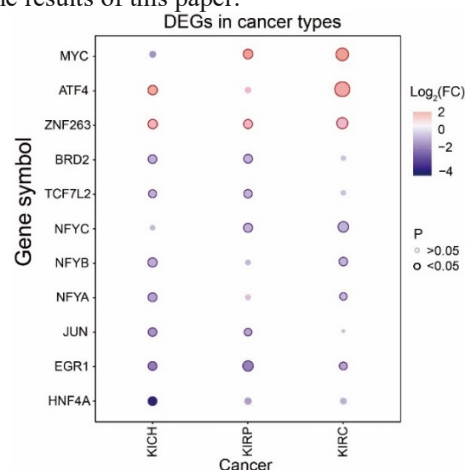


Fig 4. Potential upstream transcription factors for ASS1, ASL and ARG2 in renal tissues

3.4 Reduced correlation between ASL and ASS1 in expression in cancer

The correlation coefficients of ASL and ASS1 in RCC are presented in Figure 5, which illustrates the relationship between these genes upstream of L-arginine in the three renal cancer subtypes: chRCC, pRCC, and ccRCC. As shown in the figure, the Spearman correlation coefficients for ASL and ASS1 were 0.77, 0.86, and 0.85 in the respective control samples, indicating a strong correlation. However, in the cancer samples, these coefficients dropped to 0.36, 0.16, and 0.24, reflecting a weaker correlation. This suggests that in normal tissues, the expression of ASL and ASS1 is synergistically regulated, whereas in cancer tissues, this regulatory pattern is disrupted.

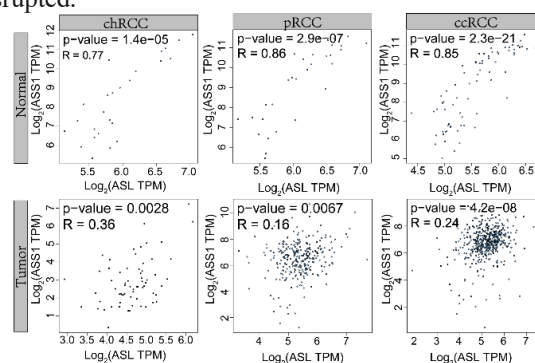


Fig 5. Spearman's correlation coefficient between ASL and ASS1 in renal cancer and normal samples

3.5 Expression of ASL indicates survival

By employing the median ASL expression level as a threshold, 516 ccRCC cases were segmented into high and low expression categories using the TCGA survival dataset. The relationship between ASL expression and the prognosis of ccRCC patients was examined through the Kaplan-Meier survival analysis (Figure 6). The analysis indicated that elevated ASL expression was correlated with an improved overall survival (OS) prognosis in ccRCC patients ($P = 0.024$). Inhibition of ASL activity was linked to a decrease in the production of arginine and nitric oxide, which can modulate the immune microenvironment and facilitate tumor growth in a context-dependent fashion. ASS1 and ASL are proposed to act as metabolic tumor suppressors in ccRCC, and their absence is associated with the regulation of cellular aspartate levels, nucleotide synthesis, and nitric oxide production, thereby enhancing ccRCC cell proliferation.

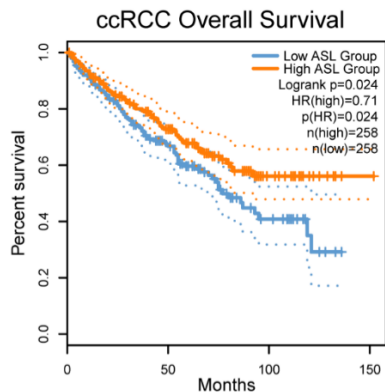


Fig 6. KM analysis of ASL and OS correlation in ccRCC

Independent risk factors for pRCC were identified by univariate and multivariate Cox regression analyses. The relationship between gene expression levels in the arginine pathway and overall survival (OS) was investigated using univariate Cox regression analyses with a 289-patient survival dataset from the TCGA database of pRCC patients. This analysis involved screening for genes with significant risk ratios at a threshold of $P < 0.05$, which included ABHD14A, AGMAT, ALDH3A2, ALDH4A1, ASL, CKMT1B, CKMT2, CNDP1, CPS1, GATM, GLS, GLUD1, GOT1, GOT2, GPT, MAOB, NAGS, P4HA2, PYCR2, SAT1, SAT2, and SMS. These genes were then included in the reference standard for multivariate Cox regression analysis. Based on this, prognostic models were evaluated with arginine prognostic factors of other genes in the pathway, as depicted in Figure 7. The results indicated that ASL could be an independent prognostic factor for pRCC, with a statistically significant P-value of 0.04.

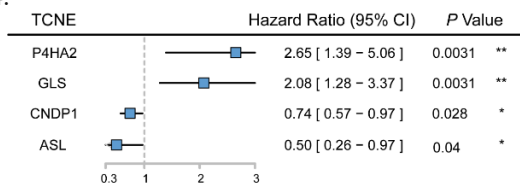


Fig 7. Multifactorial Cox forest plot of ASL-OS correlation in pRCC

4 Discussion

The study conducted an in-depth analysis of the expression patterns of crucial genes involved in the L-arginine pathway in RCC. It was observed that genes such as ASS1, ASL, and ARG2 exhibited significantly reduced expression in cancerous tissue compared to normal tissue. These findings not only offer potential biomarkers for the early detection of RCC but also highlight these genes as novel therapeutic targets. Specifically in ccRCC, higher expression levels of ASL were positively correlated with extended overall survival in patients. This suggests that manipulating the activity or expression of these genes could potentially inhibit the growth and spread of cancer cells, thereby providing more effective treatment options for patients with RCC.

In addition, the differences in the expression of L-arginine pathway-related genes in different RCC subtypes provide a basis for individualised medical treatment, which helps doctors to develop personalised treatment plans based on patients' gene expression profiles. Meanwhile, these findings also point to the direction of drug development, which may be possible in the future by developing activators or inhibitors targeting these genes to regulate L-arginine levels and affect the tumor growth environment.

In the field of immunotherapy, studies of the L-arginine pathway have also provided clues for the development of new therapeutic strategies. By regulating L-arginine levels in the tumor microenvironment, it may enhance the anti-tumor activity of immune cells and provide an adjunct to immunotherapy. In conclusion, this study not only provides a new perspective for the diagnosis and treatment of RCC, but also lays the foundation for the development of precision medicine and the improvement of patient outcomes. Future studies should further validate the efficacy and safety of these genes in clinical applications and explore their specific mechanisms of action in the development of RCC.

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