Overexpression of Acyl-CoA Ligase 4 (ACSL4) in Patients with Hepatocellular Carcinoma and its Prognosis

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Background: Recently, accumulating studies have found that ACSL4 dysregulation is related to a great number of malignant tumors. The purpose of the present study was to explore the relationship between ACSL4 expression level and clinical prognosis of hepatocellular carcinoma (HCC) patients.

Material/Methods: The Oncomine and TCGA databases were used to predict the expression of ACSL4 mRNA in HCC and its association with HCC prognosis. Further, immunohistochemistry was performed to verify the ACSL4 protein expression in 116 paired HCC and adjacent normal tissues. Kaplan-Meier and cox analysis were performed to validate the correlation between ACSL4 expression and HCC prognosis.

Results: We first used the Oncomine database to find that ACSL4 mRNA expression level was significantly higher in HCC tissues than that in normal tissues (p all <0.001). The results were consistent with those in the TCGA database. Then, immunohistochemical results demonstrated that the ACSL4 positive expression rate was 70.7% in HCC tissues. ACSL4 differential expression level was significantly related to Edmondson grade (p=0.010), AFP (p=0.001) and TNM stage (p=0.012). Survival analysis revealed that both overall survival (OS) and disease-free survival (DFS) time were remarkably reduced in HCC patients with ACSL4 high expression (p=0.001 and 0.000, respectively). Moreover, Cox multivariate analysis demonstrated that ACSL4 expression was the only independent prognostic factor for both OS and DFS (both p values=0.001).

Conclusions: Taken together, our study demonstrated that ACSL4 was overexpressed in HCC, and it will be a new potential therapeutic target for HCC as an independent adverse prognostic parameter.

MeSH Keywords: Acyl-CoA Oxidase • Carcinoma, Hepatocellular • Prognosis

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Background

Hepatocellular carcinoma (HCC) as one of the most common malignancies of the digestive tract, it is the third cause of cancer death worldwide [1] and it ranks second in China’s malignant tumor mortality incidence [2]. Most HCC patients miss the chance of receiving radical resection or liver transplantation due to their disease being diagnosed at a late stage [3]. Therefore, it is helpful to develop diagnostic and therapeutic targets through identification of genes which are differentially expressed in HCC.

Bioinformatics is a powerful tool for mining tumor differential genes. In early studies, it was found that the long chain fatty acyl-CoA ligase 4 (ACSL4) gene was highly expressed in HCC compared with normal tissues. ACSL4, also known as FAC1L4, was initially identified as its mutation in non-specific X-linked mental retardation in 2002 [4]. Maloberti et al. [5] found that ACSL4 possessed a substrate preference for eicosapentaenoic acid and arachidonic acid (AA). More interestingly, the level of intracellular ACSL4 protein can be altered in turn by the amounts of free AA [6]. So far, it has been proven that the ACSL4 dysregulation is related to a great number of diseases including diabetes [7], atherosclerosis [8], obesity [9], and malignant tumors [10–17]. In a previous study, Sung et al. [10,11] found that ACSL4 was overexpressed in HCC cell lines and tissues compared to normal cell lines. However, the relationship between ACSL4 expression level and clinical prognosis of HCC patients remains largely unclear.

Therefore, in the present study, immunohistochemical staining was done to examine the expressions of ACSL4 in 116 paired HCC and adjacent normal tissue samples. In addition, ACSL4 expression in HCC and its relationships with patients’ clinico-pathological factors and prognosis were investigated.

Material and Methods

Bioinformatics prediction

First, the Oncomine database (https://www.oncomine.org/resource/login.html) was used to predict the ACSL4 mRNA expression levels in HCC and normal tissues. Then the Cancer Genome Atlas (TCGA) database was used to predict the relationship between expression levels of ACSL4 mRNA and clinical prognosis of HCC patients.

Patients and samples

One hundred and sixteen cases of HCC patients who had received the curative operation at Anhui Provincial Hospital from January 2009 to June 2013 were selected. We used the tumor-node-metastasis (TNM) classification (sixth edition) of the Union for International Cancer Control (UICC) to evaluate tumor stage. The detailed information on ACSL4 protein expression and other clinico-pathological factors (such as gender and age) are listed in Table 1. The study was approved by the Ethics Committee of Anhui Provincial Hospital and all patients signed the written informed consent.

Immunohistochemistry and analysis

Immunohistochemistry was performed according to the manufacture protocol. After the sections were treated with dewaxing, antigen repair, and serum sealing, ACSL4 antibody (1: 500, ab110007, Abcam, UK) was added at 4°C overnight. The next day, after rewarming for 45 minutes, PBS was used to wash the sections, and then the sections were incubated at room temperature. The staining results were observed by microscope and immunohistochemical scores were calculated as described in a previous report [18].

Statistical analysis

SPSS 19.0 software was used to do statistical analysis. Pearson chi-squared test or Fisher’s test was selected to analyze the correlation between ACSL4 expression and clinico-pathological factors. Kaplan-Meier and Cox regression model were employed to analyze the parameters associated to the disease-free survival (DFS) and overall survival (OS) of HCC patients. A p value <0.05 was regarded as statistical significance.

Results

ACSL4 high expression in HCC

We first used the Oncomine database to find that ACSL4 mRNA expression level was significantly higher in HCC tissues than that in normal tissues (Figure 1, p all <0.001). The results were consistent with those from the TCGA database (Figure 2A). Then, in order to verify the real expression level of ACSL4 in HCC, immunohistochemistry was selected to examine the
Figure 1. High expression levels of ACSL4 mRNA in HCC predicted by the Oncomine database. The Oncomine database mining analysis of ACSL4 mRNA levels in (A) Wurmbach liver (GEO: GSE 6764), (B) Roessler Liver2 (GEO: GSE 14520/GPL3921), (C) Roessler Liver (GEO: GSE 14520/GPL571), and (D) Mas liver (GEO: GSE 14323) grouped by HCC and normal liver.

Figure 2. Relationship between ACSL4 mRNA and the prognosis of HCC patients predicted by TCGA database. TCGA database mining analysis of (A) ACSL4 mRNA levels grouped by HCC and normal liver and (B) relationship between ACSL4 mRNA and the prognosis of HCC patients.
Table 2. Relationships among ACSL4 and clinicopathological parameters in HCC patients.

| Variables                | Total (n=116) | ACSL4 expression level | P     |
|--------------------------|---------------|------------------------|-------|
|                          | Low (n=34)    | High (n=82)            |       |
| Age                      |               |                        |       |
| <60                      | 76            | 23                     | 53    | 0.756 |
| ≥60                      | 40            | 11                     | 29    |       |
| Sex                      |               |                        |       |
| Male                     | 71            | 19                     | 52    | 0.449 |
| Female                   | 45            | 15                     | 30    |       |
| Tumor Size (cm)          |               |                        |       |
| >5                       | 38            | 10                     | 28    | 0.621 |
| ≤5                       | 78            | 24                     | 54    |       |
| Tumor Nodules            |               |                        |       |
| Single                   | 89            | 26                     | 63    | 0.967 |
| Multiple                 | 27            | 8                      | 19    |       |
| Tumor capsula            |               |                        |       |
| Complete                 | 90            | 24                     | 66    | 0.244 |
| None                     | 26            | 10                     | 16    |       |
| Edmondson grade          |               |                        |       |
| I–II                     | 75            | 28                     | 47    | 0.010*|
| III–IV                   | 41            | 6                      | 35    |       |
| HbsAg                    |               |                        |       |
| Positive                 | 97            | 27                     | 70    | 0.430 |
| Negative                 | 19            | 7                      | 12    |       |
| Cirrhosis                |               |                        |       |
| Yes                      | 106           | 30                     | 76    | 0.437 |
| No                       | 10            | 4                      | 6     |       |
| Child-Pugh grade         |               |                        |       |
| A                        | 110           | 33                     | 77    | 0.485 |
| B                        | 6             | 1                      | 5     |       |
| AFP (ng/ml)              |               |                        |       |
| >20                      | 79            | 14                     | 65    | 0.001*|
| ≤20                      | 37            | 18                     | 19    |       |
| TNM stage                |               |                        |       |
| I–II                     | 68            | 26                     | 42    | 0.012*|
| III–IV                   | 48            | 8                      | 40    |       |

Figure 3. Immunochemical staining of ACSL4 in paired HCC and adjacent normal tissues. (A) High expression of ACSL4 in HCC tissues; (B) Low expression of ACSL4 in HCC tissues; (C) Low expression of ACSL4 in adjacent normal tissues.
Table 3. Kaplan-Meir analysis of ACSL4 and other clinicopathological parameters in HCC patients.

| Variable                      | OS             | DFS            |
|-------------------------------|----------------|----------------|
|                               | HR  | 95% CI      | P    | HR  | 95% CI      | P    |
| ACSL4 expression              |     |             |      |     |             |      |
| Low                           | 0.377| 0.214–0.665 | 0.001* | 0.358| 0.203–0.632 | 0.000* |
| High                          |     |             |      |     |             |      |
| Age                           |     |             |      |     |             |      |
| <60                           | 0.744| 0.467–1.184 | 0.212 | 0.738| 0.465–1.171 | 0.197 |
| ≥60                           |     |             |      |     |             |      |
| Sex                           |     |             |      |     |             |      |
| Male                          | 1.279| 0.811–2.108 | 0.289 | 1.224| 0.777–1.927 | 0.383 |
| Female                        |     |             |      |     |             |      |
| Tumor size (cm)               |     |             |      |     |             |      |
| >5                            | 0.759| 0.464–1.241 | 0.272 | 23.268| 0.518–1.376 | 0.497 |
| ≤5                            |     |             |      |     |             |      |
| Tumor nodules                 |     |             |      |     |             |      |
| Single                        | 0.907| 0.540–1.526 | 0.714 | 0.931| 0.554–1.564 | 0.788 |
| Multiple                      |     |             |      |     |             |      |
| Tumor capsula                 |     |             |      |     |             |      |
| Complete                      | 0.779| 0.465–1.307 | 0.345 | 0.785| 0.472–1.307 | 0.352 |
| None                          |     |             |      |     |             |      |
| Edmondson grade               |     |             |      |     |             |      |
| I–II                          | 0.587| 0.371–0.928 | 0.022* | 0.566| 0.358–0.894 | 0.015* |
| III–IV                        |     |             |      |     |             |      |
| HbsAg                         |     |             |      |     |             |      |
| Positive                      | 1.154| 0.643–2.073 | 0.631 | 1.181| 0.658–2.121 | 0.577 |
| Negative                      |     |             |      |     |             |      |
| Cirrhosis                     |     |             |      |     |             |      |
| Yes                           | 0.911| 0.417–1.989 | 0.814 | 1.108| 0.508–2.417 | 0.797 |
| No                            |     |             |      |     |             |      |
| Child-Pugh grade              |     |             |      |     |             |      |
| A                             | 0.450| 0.180–1.124 | 0.087 | 0.601| 0.242–1.495 | 0.274 |
| B                             |     |             |      |     |             |      |
| AFP (ng/ml)                   |     |             |      |     |             |      |
| >20                           | 0.720| 0.445–1.167 | 0.183 | 0.784| 0.484–1.270 | 0.323 |
| ≤20                           |     |             |      |     |             |      |
| TNM stage                     |     |             |      |     |             |      |
| I–II                          | 0.624| 0.398–0.978 | 0.040* | 0.608| 0.388–0.951 | 0.029* |
| III–IV                        |     |             |      |     |             |      |
protein level of ACSL4 in 116 paired HCC and adjacent normal tissues (Figure 3). ACSL4 protein staining was mainly located in the cytoplasm (Figure 3A). ACSL4 positive expression rate was 70.7% (82/116) in HCC tissues (Table 1). The associations of ACSL4 expression with clinicopathological parameters are listed in Table 2. ACSL4 differential expression level was significantly related to Edmondson grade ($p=0.010$), AFP ($p=0.001$) and TNM stage ($p=0.012$).

**Table 4.** Cox regression analysis of ACSL4 and other clinicopathological parameters in HCC patients.

| Variable                          | OS HR 95% CI   | P     | DFS HR 95% CI   | P     |
|-----------------------------------|----------------|-------|-----------------|-------|
| ACSL4 expression (low vs. high)   | 0.359 0.191–0.674 | 0.001* | 0.347 0.185–0.650 | 0.001* |
| Age (<60 vs. ≥60)                 | 0.707 0.421–1.187 | 0.190 | 0.714 0.430–1.186 | 0.193 |
| Sex (Male vs. Female)             | 1.266 0.771–2.079 | 0.351 | 1.288 0.789–2.104 | 0.312 |
| Tumor size (≤5 vs. >5)            | 1.847 0.372–1.201 | 0.042* | 0.786 0.443–1.392 | 0.408 |
| Tumor nodule (single vs. multiple)| 0.941 0.526–1.682 | 0.836 | 0.919 0.514–1.641 | 0.774 |
| Edmondson grade (I–II vs. III–IV) | 0.564 0.332–0.957 | 0.034* | 0.589 0.344–1.008 | 0.053 |
| Tumor capsula (complete vs. none) | 0.584 0.325–1.051 | 0.073 | 0.586 0.325–1.058 | 0.076 |
| HbsAg (positive vs. negative)     | 2.221 0.986–5.002 | 0.054 | 1.714 0.782–3.757 | 0.178 |
| Cirrhosis (present vs. absent)    | 0.521 0.168–1.620 | 0.260 | 1.033 0.349–3.054 | 0.954 |
| Child-Pugh grade (A vs. B)        | 0.698 0.252–1.935 | 0.681 | 1.021 0.376–2.775 | 0.968 |
| AFP (>20 vs. ≤20)                 | 1.231 0.707–2.144 | 0.463 | 1.297 0.752–2.238 | 0.350 |
| TNM stage (I–II vs. III–IV)       | 0.898 0.539–1.497 | 0.681 | 0.818 0.491–1.364 | 0.441 |

**Figure 4.** Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) curves of HCC patients based on ACSL4 expression as high- or low-expression. (A) OS curve of HCC patients based on ACSL4 expression; (B) DFS curve of HCC patients based on ACSL4 expression.
Correlation between ACSL4 expression and survival of HCC patients

Through mining the TCGA database, we found HCC patients with ACSL4 mRNA high expression had lower survival time than those with ACSL4 mRNA low expression (Figure 2B). Then, our own immunohistochemical results were consistent with the predictive findings. Survival analysis revealed that ACSL4 expression levels were significantly associated with the survival time of HCC patients. Moreover, compared to those with ACSL4 protein low expression levels, both OS and DFS time were remarkably reduced in HCC patients with ACSL4 high expression levels ($p=0.001$, Figure 4A and $p=0.000$, Figure 4B, respectively).

Prognostic value of ACSL4 expression in HCC patients

Initially, we used univariate analysis to reveal that ACSL4 expression, Edmondson grade and TNM stage had statistically prognostic influences on both OS and DFS (Table 3). In addition, Cox multivariate analysis demonstrated that ACSL4 expression was the only independent prognostic factor for both OS and DFS (both $p$ values=0.001, Table 4).

Discussion

There has been accumulating evidence from studies demonstrating that ACSL4 is overexpressed in parts of malignant tumors such as liver [10,11], prostate [12,13] and breast cancer [13–16]. In these findings, ACSL4 is reported to perform an oncogene role in promoting tumorigenesis and metastasis. While on the other hand, Ye et al. [17] recently found that ACSL4 may serve as a tumor suppressor gene in gastric cancer possibly involving FAK and P21 signaling. As for HCC, in order to verify the exact role of ACSL4 and explore the relationship between its expression and the prognosis of HCC patients, the present study was designed and completed.

We first used the Oncomine and TCGA databases to reveal that ACSL4 mRNA expression level was significantly higher in HCC tissues than that in normal tissues. Then, in order to validate this phenomenon, 116 cases of paired HCC and normal tissues were selected. Immunohistochemical results showed that ACSL4 positive expression rate was 70.7% (82/116) in HCC tissues. Compared to those in the adjacent normal tissues, ACSL4 protein expression levels were remarkably higher in HCC tissues. These findings were consistent with the bioinformatics analysis and a report by Sung et al. [10,11]. Moreover, ACSL4 differential expression level was significantly related to Edmondson grade ($p=0.010$), AFP ($p=0.001$) and TNM stage ($p=0.012$). Therefore, the above results suggest a key role for ACSL4 in HCC progression and development.

Though Sung et al. [10,11] reported that ACSL4 was overexpressed in HCC tissues and cell lines, whether it has prognostic significance in HCC has remained unclear. So next, we used the TCGA database to predict that HCC patients with ACSL4 mRNA high expression level had worse OS than those with ACSL4 mRNA low expression level. This finding was validated by our own experimental data. Kaplan-Meir analysis showed that HCC patients with ACSL4 protein high expression had significantly reduced OS and DFS than those with ACSL4 protein low expression. Moreover, both univariate and multivariate analyses demonstrated that ACSL4 was the only independent unfavorable predictor of OS and DFS in HCC patients.

There were several limitations to our study that should be noted. First, this was a retrospective study, possibly resulting in a selective bias. Second, only immunohistochemistry (a semiquantitative method) was used to detect the ACSL4 protein expression. Finally, we did not explore the exactly underlying molecular mechanisms in this study, which will be elucidated in future studies.

Conclusions

Collectively, our present study demonstrated that ACSL4 was overexpressed in HCC and patients with high expression level of ACSL4 had unfavorable prognosis. ACSL4, as an independent adverse prognostic parameter, will be a new potential therapeutic target for HCC.

Conflicts of interest

None.

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