Circular RNA hsa_circ_0000437 may be used as a new indicator for the diagnosis and prognosis of hepatocellular carcinoma

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**ABSTRACT**
Circular RNAs (circRNAs) play an essential role in hepatocellular carcinoma (HCC); however, the precise role of circRNAs in the diagnosis and prognosis of HCC remains unclear. The circRNA circ_0000437 was identified in the microarray dataset GSE166678 and was detected in HCC and paired adjacent tissue and serum samples in both the HCC and control groups by reverse transcription quantitative PCR. The association between circ_0000437 expression and clinicopathological characteristics was investigated. Furthermore, the diagnostic and prognostic values of circ_0000437 were determined using receiver operating characteristic (ROC) and Kaplan-Meier curves. Circ_0000437 expression was markedly upregulated in the tumor group compared with the control group and was correlated with tumor node metastasis (TNM) classification, differentiation degree, tumor size, and Barcelona Clinic Liver Cancer (BCLC) stage ($P<0.05$) in both the tumor tissues and serum. Furthermore, poor overall survival (OS) was correlated with high circ_0000437 expression, and the area under the ROC curve (AUC) of circ_0000437 for the diagnosis of HCC was 0.9281 in the serum. Our findings suggest that circ_0000437 may be used as a novel biomarker for the diagnosis and prognosis of patients with HCC.

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Key policy insights

- Circ_0000437 expression was markedly upregulated in the HCC tumor group compared with the control group.
- Circ_0000437 was correlated with TNM classification, differentiation degree, tumor size, and BCLC stage ($P < 0.05$) in both the tumor tissues and serum.
- Circ_0000437 may be used as a novel biomarker for the diagnosis and prognosis of patients with HCC.

Introduction

Liver cancer usually begins with chronic hepatitis and progresses to fibrosis, cirrhosis, and eventually liver cancer [1]. Most patients with liver cancer are diagnosed with advanced liver cancer as soon as it is discovered [2]. Hepatocellular carcinoma (HCC) is the most common type of liver cancer [3]. The prognosis of early HCC is better than that of late HCC due to the continuous improvement of comprehensive treatment methods such as surgery; thus, early diagnosis is crucial for the timeliness of treatment and quality of life of patients with HCC [4,5].

Circular RNAs (circRNAs) are endogenous non-coding RNAs that are highly conserved and widely expressed in human cells [6]. In recent years, a large number of studies have detected the abnormal expression of circRNAs in malignant tumors through high-throughput sequencing or microarray analysis. Dysregulated circRNAs can affect the aggressiveness, angiogenesis, and drug resistance of HCC cells [7–9]. Furthermore, some circRNAs have been found to be associated with HCC clinicopathological characteristics and have the potential to be novel biomarkers of HCC [10,11]. Therefore, it is particularly important to establish a circRNA biomarker network for the diagnosis and prognosis of HCC.

In the current study, we screened for abnormal circRNAs in HCC using a microarray dataset and detected their expression in clinical samples. Abnormal circRNAs may be combined with clinicopathological information to facilitate the early diagnosis and prognosis of HCC.

Materials and methods

Identification of aberrantly expressed circRNAs in HCC

The microarray dataset GSE166678 generated using the GPL28148 Agilent-084217 CapitalBio Technology Human CircRNA Array platform from the Gene Express Omnibus (GEO) database was downloaded. This dataset included plasma samples from 3 patients with HCC and 3 healthy controls. The online tool GEO2R [12] (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to analyze circRNAs in the plasma samples of patients with HCC and healthy controls based on an adjusted $P$ value < 0.01 and $|\log_2$ fold change$| > 2$ (fold change indicates the presence or absence of multiple circRNAs).

Patients

A total of 100 patients with pathologically confirmed HCC admitted to The First Affiliated Hospital of Jinan University were selected and assigned to the tumor group. The clinicopathological characteristics of the patients are presented in Table 1. A total of 100 healthy volunteers were included in the control group. None of the subjects had a history of diabetes, hypertension, chronic obstructive pulmonary disease, autoimmune diseases, immunosuppressive drug use, liver infection, or AIDS. Pregnant and lactating women were excluded from this study. The study was approved by the ethics committee of The First Affiliated Hospital of Jinan University (Ethical number 20201020), and all subjects provided written informed consent. In addition, all patients in the tumor group were pathologically diagnosed with HCC and had complete clinical data without preoperative radiotherapy or chemotherapy. Radically resected or biopsy tissues and normal tissues adjacent to cancer tissues were collected from patients in the tumor group, and blood samples were collected from both groups (the supernatant was obtained after low-speed centrifugation at 4°C). All samples were stored at $-80^\circ$C for future use.
Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was used to determine the mRNA levels of circRNAs. Total RNA was isolated from the tissues and serum, and cDNA was synthesized using a reverse transcription kit. qPCR was subsequently performed using the Power SYBR™ Green RNA-to-C<sub>T</sub>™ 1-Step Kit. CircRNA expression was quantified using the 2<sup>−ΔΔCT</sup> method and normalized to GAPDH expression [8]. Primer sequences were listed as follows:

circ_0000437: forward 5'-CAGCAAGGAGCCCTCAGAGAG-3', reverse 5'-TGAACCCAGTGGTGAAGACA-3';
circ_0028071: forward 5'-ATGGGTTACATGCCCAGAGAG-3', reverse 5'-TGGCAATGACCTAGATGA-3';
circ_0036409: forward 5'-AAGAGGAAGACTGACACCAGATGGT-3', reverse 5'-GAAGACAATTGCTCCAGTGG-3'.

Statistical analysis

Data were analyzed using GraphPad 8.3 (GraphPad Software, USA) and expressed as the mean ± standard deviation (SD). Quantitative data were compared between the tumor and control groups by Student's t-test. The potential diagnostic value of circ_0000437 in HCC was determined by receiver operating characteristic (ROC) curve analysis. Logistic regression analysis of clinical parameters was carried out to clarify the factors affecting the prognosis of HCC patients. P< 0.05 was regarded as significant.

Results

Differentially expressed circRNAs identified in HCC

Firstly, we screened candidate circRNA in HCC through bioinformatics. A total of 466 abnormally expressed circRNAs in the microarray dataset GSE166678 were screened, including 324 upregulated and 142 downregulated circRNAs (Figure 1(a–b)). The levels of circ_0000437, circ_0097182, circ_0000437, circ_0028071, and circ_0036409 were

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Table 1. Correlation between hsa_circ_0000437 expression and clinical parameters in HCC.

| Clinicopathologic characteristics | Tissue | Serum |
|----------------------------------|--------|-------|
| Age (years)                      |        |       |
| < 55                             | 23     | 0.7444| 0.7970|
| ≥ 55                             | 76     | 0.3733| 0.7917|
| Sex                              |        |       |
| Male                             | 83     | 0.0215* | 0.0342* |
| Female                           | 17     | 0.0353* | 0.0348* |
| TNM stage                        |        |       |
| I+II                            | 52     | 0.0788 | 0.3690|
| III+IV                          | 48     | 0.0322* | 0.0195* |
| HCC history                      |        |       |
| Positive                         | 71     | 0.0322* | 0.0195* |
| Negative                         | 29     | 0.0353* | 0.0348* |
| Differentiation                  |        |       |
| High/moderate                    | 73     | 0.0353* | 0.0348* |
| Low                              | 27     | 0.0322* | 0.0195* |
| Tumor size                       |        |       |
| < 50 mm                          | 81     | 0.0371* | 0.0258* |
| ≥ 50 mm                          | 19     | 0.7679 | 0.1510|
| Tumor number                     |        |       |
| Single                           | 67     | 0.0215 | 0.0342|
| Multiple                         | 33     | 0.0353 | 0.0348|
| BCLC stage                       |        |       |
| A + B                            | 88     | 0.0322* | 0.0195* |
| C + D                            | 12     | 0.0353* | 0.0348* |
| HbsAg                            |        |       |
| Negative                         | 23     | 0.0353* | 0.0348* |
| Positive                         | 77     | 0.0353* | 0.0348* |

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significantly increased in HCC samples. We further measured the levels of these five circRNAs in the HCC and normal tissues obtained in this study, and the results demonstrated that circ_0000437 was the most markedly upregulated circRNA in HCC (Figure 1(c)).

**Upregulation of circ_0000437 expression in HCC tissues and serum**

Then the mRNA levels of circ_0000437 in the tumor tissue and serum samples of patients with HCC were quantified by PCR, and the results indicated that circ_0000437 expression was markedly increased in both the tissues and serum in the tumor group compared with the healthy control group (Figure 2(a,b)). As shown in Table 1, the circ_0000437 levels of 100 patients were divided into low and high levels according to the median expression in the tumor tissues and serum, and circ_0000437 expression was independent of age, sex, HCC history, tumor number, and HbsAg level ($P > 0.05$) but correlated with tumor-node-metastasis (TNM) classification, differentiation degree, tumor size, and Barcelona Clinic Liver Cancer (BCLC) stage ($P < 0.05$). Then, univariate analysis suggested that three parameters, including...
TNM classification, differentiation degree, tumor size, and elevated serum circ_0000437 expression in HCC, were the risk factors of HCC (Table 2).

**Table 2. Relationship between the expression of hsa_circ_0000437 in serum and clinical characteristics of enrolled HCC patients.**

| Factors                                | Regression coefficient β | OR (95% CI)         | p value |
|----------------------------------------|--------------------------|---------------------|---------|
| Age (years, < 55 vs. ≥ 55)             | −0.03                    | 0.971 (0.546, 1.727) | 0.919   |
| Gender (Male vs. Female)               | −0.261                   | 0.777 (0.401, 1.478) | 0.423   |
| TNM stage (I-II vs. III+ IV)           | 1.788                    | 5.976 (2.701, 12.894)| <0.0001*|
| HCC history (Positive vs. Negative)    | −0.355                   | 0.701 (0.401, 1.226) | 0.213   |
| Differentiation (High/moderate vs. Low) | 0.964                    | 2.623 (1.39, 4.95)  | 0.003*  |
| Tumor size (<50 mm vs. ≥ 50 mm)        | 0.761                    | 2.141 (1.096, 4.185) | 0.026*  |
| Tumor number (Single vs. Multiple)     | 0.388                    | 1.474 (0.867, 2.505) | 0.152   |
| BCLC stage (A + B vs. C + D)           | 0.75                     | 2.118 (0.953, 4.705) | 0.065   |
| HbsAg (Negative vs. Positive)          | 0.437                    | 1.547 (0.853, 2.806) | 0.151   |

**Function of circ_0000437 as an indicator for HCC diagnosis and prognosis**

Afterward, the Kaplan-Meier survival curve revealed that high circ_0000437 expression in serum was correlated with poor overall survival (OS) (P= 0.0379, Figure 3(a)). The area under the ROC curve (AUC) of circ_0000437 in the serum was 0.9281, indicating the potential diagnostic value of circ_0000437 in HCC (P< 0.0001, Figure 3(b)).

**Discussion**

HCC is one of the most common cancers worldwide, and the prognosis of HCC is still not ideal despite continuous advances in treatment techniques in recent years. Due to the lack of promising and reliable biomarkers for the early diagnosis of HCC, most patients with HCC are diagnosed at an advanced stage.

CircRNAs have been reported to be involved in the progression of various diseases and are closely associated with prognosis [13,14]. With the development of high-throughput sequencing and bioinformatics, several circRNAs have been found to be abnormally expressed in HCC, which can be used as HCC biomarkers considering their involvement in the development of HCC [15,16]. For instance, Zhang et al. used a circRNA microarray to examine the circRNA expression profile of HCC tumors and normal controls. Bioinformatics analysis revealed that circ_0091579 was abnormally upregulated in HCC tissues and was correlated with the prognosis of patients with HCC. In addition, the ROC curve demonstrated the excellent specificity and sensitivity of circ_0091579 [17].

In the current study, circ_00004378 expression was significantly higher in HCC tissues than in normal tissues according to bioinformatics analysis. Furthermore, circ_00004378 was highly

![Figure 3. OS and ROC curves of circ_0000437 in HCC.](image-url)
expressed in the serum. The upregulation of circ_00004378 in HCC was associated with TNM stage, degree of differentiation, tumor size, and BCLC stage. In most studies, a higher TNM stage and a larger tumor size resulted in a decrease in the 5-year survival and an increase in recurrence and metastasis [18,19], which are in agreement with our results. Our findings suggest that patients with high levels of circ_00004378 had significantly shorter OS than that of patients with low levels. In a study by Geng et al., a high albumin-bilirubin (ALBI) grade was associated with poor OS among patients with HCC after liver resection, and the ALBI grade may be a predictive biomarker for prognosis in HCC [20]. Similarly, circ_00004378 may serve as an indicator for HCC prognosis. The results of ROC curve analysis demonstrated that circ_00004378 may be a novel biomarker for differentiating HCC tissues from adjacent noncancerous tissues with favorable sensitivity and specificity.

Our study has the following limitations: Only Han Chinese patients were included in this study, so it is necessary to further expand the sample size and enrich ethnic types in future studies. Besides, it will be the focus of future work to enrich the circRNA network related to HCC diagnosis and prognosis.

**Conclusion**

In this study, we identified circ_00004378 as a HCC-associated circRNA through a microarray dataset, and further emphasized its potential clinical application, which provides a new direction for developing HCC diagnostics and therapies, and raises the possibility of considering it as a potential target for cancer therapy. Furthermore, the detectability and specificity of circ_00004378 in serum make it possible to use it as a convenient clinical indicator. However, circ_00004378 contributing to HCC initiation and progression is not fully understood. It is highly anticipated that circ_00004378-based diagnostic and therapeutic interventions for HCC will emerge in the near future.

**Disclosure statement**

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**Data availability statement**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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