Identification of Expression Pattern and Clinical Significance of the Small Cajal Body-specific RNA SCARNA16 in Hepatocellular Carcinoma

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Abstract

Background and Aims: For high morbidity and mortality, hepatocellular carcinoma (HCC) becomes a major health issue worldwide. Nowadays, numerous non-coding RNAs (ncRNAs) are known to regulate the occurrence and pathogenesis of tumors. Some ncRNAs have also been developed as tumor biomarkers and therapeutic targets. However, the potential function of the small Cajal body-specific RNA (scRNA) SCARNA16, a newly identified ncRNA, remains to be explored in HCC. Methods: In both HCC cell lines and specimens from 120 enrolled patients, the expression values of SCARNA16 were detected. We divided patients into SCARNA16 high and low expression subgroups, and then analyzed the differences of various clinical characteristics and prognosis data between subgroups. Results: Compared to paired controls, SCARNA16 was significantly down-regulated in HCC cell lines and clinical specimens (p<0.01). Besides, HCC patients with lower SCARNA16 expression commonly presented with larger and more tumor lesions, more vesical carcinoma emboli, more capsular invasion and higher TNM stages (p<0.05). Moreover, SCARNA16 expression was negatively correlated with postoperative prognosis of HCC patients in 5-year follow-up, including tumor-free survival (TFS) (median time of low vs. high subgroups: 14 vs. 48 months, p=0.006) and overall survival (OS) (median time of low vs. high subgroups: 39 vs. 52 months, p=0.001). Besides, SCARNA16 acted as an independent prognostic biomarker in TFS (hazard ratio [HR]: 0.578, 95% CI: 0.345–0.969, p=0.038) and OS (HR: 0.366, 95% CI: 0.178–0.752, p=0.006). Conclusions: Low expression patterns of SCARNA16 remarkably associated with severe clinical status and poor survival of patients, suggesting that SCARNA16 possesses potency as a novel biomarker for HCC.

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Introduction

Nowadays, primary hepatic carcinoma has been ranked as the fifth most common cancer along with the fourth largest cause of cancer-related mortality. Indeed, the morbidity of liver cancer remains remarkably high around the world, especially in Eastern Asia. Among those cases, hepatocellular carcinoma (HCC) accounts for approximately 90% of the primary liver cancer cases. Although a lot of clinical therapies have been applied to treat HCC, the survival benefit to HCC patients is still limited for its high metastasis and relapse as well as fatality rates. In view of the severe malignancy and heterogeneity of HCC, an individualized treat-
ment strategy is deemed to improve the prognosis of these patients. Apparently, it is particularly necessary to determine some novel suitable biomarkers, which can be used in clinical assessment and prognostic analysis of HCC patients. In addition to the typically identified oncogenic and tumor suppressor genes, nowadays, more and more RNAs derived from noncoding regions within the genome (noncoding RNA, ncRNA) are ascertained to be extensively associated with tumorigenesis.\textsuperscript{5,6} As generally demonstrated by many researches, ncRNAs could directly participate in plentiful intracellular process by regulating transcription and translation of corresponding proteins.\textsuperscript{7} Among that, the small Cajal body-specific RNAs (scaRNAs), one special subset representing an ncRNA family derived from Cajal bodies, are commonly supposed to play vital roles in modifying other RNA family members, including mRNAs, tRNAs, rRNAs and snRNAs.\textsuperscript{8} However, it was demonstrated that dysregulation of the scaRNA SCARNA2 could promote tumor development and chemotherapy resistance of colorectal cancer via facilitating EGFR and Bcl-2 protein expression.\textsuperscript{9} Moreover, the expression pattern of sno/scaRNAs was successfully applied to characterize distinct molecular subtypes of multiple myeloma by Ronchetti's team,\textsuperscript{10} and Chu et al.\textsuperscript{11} also reported that the sno/scaRNAs expression profile could function as novel biomarkers and predict the clinical outcome of chronic lymphocytic leukemia patients. Thus, it is necessary to find the underlying tumor-related scaRNAs and explore its regulatory roles as well as clinical value in HCC.

SCARNA16, a newly identified scaRNA located in the Cajal body, has been demonstrated to directly participate in RNA processing.\textsuperscript{8} However, its potential association with biological or clinical characteristics of malignant tumors remains unknown. In the present research, we aimed to explore the expression pattern of SCARNA16 in HCC cells as well as tissues. Then, we investigated its potential value in clinical assessment and prognostic analysis for HCC patients.

Methods

Cell culture

The human HCC cell lines, including Hep3B, PLC/PRF/5, SK-HEP-1 and Huh-7, and normal hepatocyte cell line QSG-7701 were used in this study. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Waltham, MA, USA) with 10% fetal bovine serum (Gibco) and 1% streptomycin/penicillin (Gibco) under an atmosphere of 5% CO\textsubscript{2} at 37°C.

Patient enrollment and specimen collection

This retrospective research study enrolled 120 consecutive HCC patients from January 2013 to December 2014, who underwent hepatectomy and were diagnosed with HCC according to histological tests. Any patient with history of pre-operative radiotherapy, chemotherapy and immunotherapy before hepatectomy was excluded. Informed consent was obtained from all enrolled patients. The clinical characteristic and basic information of patients were collected from the hospital case system. HCC and adjacent liver specimens were acquired from resected liver tissues and quickly stored in −80°C refrigerator for future examination.

Postoperative follow-up

Within 5 years after hepatectomy, the valid survival and tumor recurrence information was obtained from enrolled HCC patients by telephone or periodic review in hospital. The time interval of tumor-free survival was recorded from the date of hepatectomy to tumor recurrence, distant metastasis, or individual death. The measurement end point of overall survival (OS) was individual death or the last follow-up date. All patients and their family members were blinded to the grouping of this research, and the experimenters responsible for postoperative follow-up were blind to clinical data of the enrolled patients.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

With use of Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions, RNA extraction was performed for collected cells and tissues. Then, total RNA was assessed by Nanodrop 2000 spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA) and then it was reverse transcribed to cDNA using a Reverse Transcriptase kit (Vazyme, Nanjing, China). To calculate the relative expression values of SCARNA16, cDNA samples mixed with SYBR Green Master ROX (Roche, Basel, Switzerland) were subjected to qRT-PCR according to the manufacturer’s instructions. GAPDH was tested as endogenous control, and quantification of SCARNA16 expression was calculated by the 2\textsuperscript{−ΔΔCt} formula. All samples were tested in triplicate. The primer sequences included: SCARNA16: 5′-GGGAAAGGCTCCTGTGTTG-3′ (forward), 5′-CTTTAGGTCAGGGTTGGGC-3′ (reverse); GAPDH: 5′-CAGGAGGCATTGCTGATGAT-3′ (forward), 5′-GAAGGCTGGGGCTCAAGG-3′ (reverse); GAPDH: 5′-GAAGGCTGGGGCTCAAGG-3′ (reverse).

Statistical analysis

Statistical software package SPSS19.0 (IBM Corp., Armonk, NY, USA) was used to accomplish all statistical analysis. Quantitative variables were assessed by Student’s t-test. Chi-square test and Fisher’s exact test were applied to analyze the correlations between SCARNA16 expression and clinical characteristics. Tumor-free survival (TFS) and OS data were evaluated by the Kaplan-Meier method and Log-rank test. Cox proportional hazards regression models were used to assess the independent influence of each index, and the index with p of less than 0.1 in the univariate Cox model was entered into the multivariate Cox model. A p value of less than 0.05 was considered to be statistically significant.

Ethical statement

This study was conducted according to the ethical guidelines of the Helsinki Declaration as revised in 2013. All participants signed the written informed consent form. Ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. All data were analyzed anonymously and identified prior to analysis.

Results

SCARNA16 expression patterns in HCC

To explore the potential functional role of SCARNA16 in HCC, the SCARNA16 expression values were initially detected in HCC cell lines and the normal hepatocyte cell line QSG-7701. As shown in Figure 1, SCARNA16 expression...
values of several HCC cells were significantly lower than that of normal hepatocytes ($p<0.05$; Fig. 1). Recognizing QSG-7701 as the normal control, the relative expression levels of SCARNA16 in different HCC cell lines were 0.845 (Hep3B), 0.511 (PLC/PRF/5, $p<0.01$), 0.485 (SK-HEP-1, $p<0.001$) and 0.171 (Huh-7, $p<0.001$), respectively.

To further verify the abnormal expression patterns of SCARNA16 were detected in HCC, HCC and related adjacent liver specimens from HCC patients. In Figure 2A, the results revealed that SCARNA16 expression of HCC tissues was remarkably lower than that of adjacent liver tissues ($p=0.002$; Fig. 2A). Among that, the down-regulated expression features of SCARNA16 were identified in 82.9% paired clinical HCC specimens (Fig. 2B). By reference to the verification results of both cell lines and tissues, it was demonstrated that SCARNA16 universally presented with decreased expression patterns in HCC and suggested it might function as a tumor biomarker in tumorigenesis.

**Correlation between SCARNA16 expression and clinicopathological characteristics of HCC patients**

According to the clinical values of SCARNA16 expression in...
As shown in Figure 3, the results of Kaplan-Meier analysis of survival time of low vs. high expression subgroups: 14 vs. 48 months, demonstrated that the high SCARNA16 expression subgroup showed larger tumor lesions (p=0.001), higher incidences of vessel carcinoma emboli (p=0.006) and capsular invasion (p=0.023), and more severe TNM stage (p<0.001), revealing that low SCARNA16 expression was strongly associated with advanced clinical status of HCC. However, there was no distinct relevance found between SCARNA16 expression and α-fetoprotein value, HBV infection, liver cirrhosis or other basic liver function indexes (p>0.05; Table 1). Meanwhile, there was no significant difference in SCARNA16 expression between HCC patients with or without specific liver disease background, including HBV infection (p=0.664; Supplementary Fig. 1) or liver cirrhosis (p=0.313; Supplementary Fig. 2).

**Association between SCARNA16 and OS in HCC patients**

As shown in Figure 3, the results of Kaplan-Meier analysis demonstrated that the high SCARNA16 expression group showed a more favorable OS curve (median survival time of low vs. high expression subgroups: 14 vs. 48 months, p=0.006), indicating that the HCC patients with low SCARNA16 expression had relatively higher risk of tumor relapse. Furthermore, the independent risk impact of each characteristic on OS was assessed using multivariate Cox proportional hazards regression analysis. As shown in Table 2, besides TNM stage, SCARNA16 expression also behaved as another independent risk factor for OS of HCC patients (hazard ratio [HR]: 0.578, 95% confidence interval [CI]=0.345–0.969, p=0.036). Meanwhile, no impact of sex, age, HBV infection, liver cirrhosis or tumor size was found on the OS of HCC patients.

**Association between SCARNA16 and TFS in HCC patients**

Furthermore, to determine the impact of SCARNA16 expression on TFS, the OS curves based on 5-year follow-up of HCC patients were plotted (Fig. 4). The results showed that HCC patients with relatively high SCARNA16 expression had favorable OS (median survival time of low vs. high expression subgroups: 39 vs. 52 months, p=0.001). Following, multivariate Cox proportional hazards regression analysis demonstrated that expression of SCARNA16 functioned as a risk factor for long-term survival of HCC patients after hepatectomy (HR: 0.366, 95% CI=0.178–0.752, p=0.006; Table 3), which was independent of HBV infection, liver cirrhosis and other liver function-related indexes.
| Characteristics                  | Total | **SCARNA16 expression** | **p**  |
|---------------------------------|-------|-------------------------|--------|
|                                 |       | Low, *n*=60 | High, *n*=60 |
|                                 |       | (proportion) | (proportion) |
| Age (years)                     |       |              |              |
| <60                             | 73    | 34 (0.57)    | 39 (0.65)    |
| ≥60                             | 47    | 26 (0.43)    | 21 (0.35)    |
| Sex                             |       |              |              |
| Female                          | 24    | 8 (0.15)     | 16 (0.27)    |
| Male                            | 96    | 52 (0.85)    | 44 (0.73)    |
| HBV                             |       |              |              |
| Positive                        | 102   | 51 (0.85)    | 51 (0.85)    |
| Negative                        | 18    | 9 (0.15)     | 9 (0.15)     |
| Cirrhosis                       |       |              |              |
| Present                         | 73    | 38 (0.63)    | 35 (0.58)    |
| Absent                          | 47    | 22 (0.37)    | 25 (0.42)    |
| ALT (U/L)                       |       |              |              |
| ≤40                             | 90    | 48 (0.80)    | 42 (0.70)    |
| >40                             | 30    | 12 (0.20)    | 18 (0.30)    |
| Serum TBil (µmol/L)             |       |              |              |
| ≤17                             | 88    | 46 (0.77)    | 42 (0.70)    |
| >17                             | 32    | 14 (0.23)    | 18 (0.30)    |
| Serum albumin (g/L)             |       |              |              |
| ≥35                             | 106   | 51 (0.85)    | 55 (0.92)    |
| <35                             | 14    | 9 (0.15)     | 5 (0.08)     |
| AFP (ng/mL)                     |       |              |              |
| <200                            | 62    | 30 (0.50)    | 32 (0.53)    |
| ≥200                            | 58    | 30 (0.50)    | 28 (0.47)    |
| Tumor diameter (cm)             |       |              |              |
| <5                              | 35    | 9 (0.16)     | 26 (0.43)    |
| ≥5                              | 85    | 51 (0.84)    | 34 (0.57)    |
| Multiple lesions                |       |              |              |
| Absent                          | 97    | 45 (0.74)    | 52 (0.87)    |
| Present                         | 23    | 15 (0.26)    | 8 (0.13)     |
| Vessel carcinoma embolus        |       |              |              |
| Absent                          | 89    | 38 (0.63)    | 51 (0.85)    |
| Present                         | 31    | 22 (0.37)    | 9 (0.15)     |
| Microvascular invasion          |       |              |              |
| Absent                          | 112   | 56 (0.92)    | 56 (0.93)    |
| Present                         | 8     | 4 (0.08)     | 4 (0.07)     |
| Capsular invasion               |       |              |              |
| Absent                          | 74    | 31 (0.52)    | 43 (0.72)    |
| Present                         | 46    | 29 (0.48)    | 17 (0.28)    |
| Differentiation                 |       |              |              |
| Low                             | 65    | 34 (0.57)    | 31 (0.52)    |
| High/moderate                   | 55    | 26 (0.43)    | 29 (0.48)    |
| TNM stage                       |       |              |              |
| I–II                            | 74    | 33 (0.55)    | 41 (0.85)    |
| III–IV                          | 36    | 27 (0.45)    | 9 (0.15)     |

*p*<0.05. Values are presented as *n* (proportion). AFP, α-fetoprotein; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; TBil, total bilirubin; TNM, tumor-node-metastasis.
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To occur significantly more in hepatitis virus-associated HCC cell lines (PLC/PRF/5; HBV-related HCC cells; Huh7: hepatitis C virus [HCV]-related HCC cells). Meanwhile, considering that hepatitis virus infection and liver cirrhosis are another recognized pathogenic factor of HCC, we considered whether the SCARNA16 expression level was affected by these liver disease backgrounds. Since none of enrolled patients was infected by HCV, we subdivided them according to their medical history of HBV infection or liver cirrhosis, and then demonstrated that SCARNA16 expression showed no remarkable difference among these subgroups, which supported that SCARNA16 expression was not remarkably influenced by the liver disease background of enrolled patients. Besides, down-regulation of SCARNA16 was found to be independently associated with both high postoperative recurrence rate and poor survival of HCC patients. To sum up, low SCARNA16 expression was shown to be an independent risk factor for tumor relapse as well as unfavorable survival outcomes in HCC patients. What’s more, further investigations remain needed to illuminate the specific mechanism of SCARNA16 in HCC tumorigenesis. And, now, this finding about SCARNA16 and HCC patients is still a proof of concept, which needs more multicentric clinical data to be verified in following research studies.

However, there are some limitations in the present study that should be acknowledged. First, the postoperative follow-up of enrolled patients should be improved, to reduce random error from censored data. Besides, even though there was no significant difference in sex ratio between the SCARNA16 low and high expression groups, the sex ratio was almost 1:4 (female vs. male) in this study. Similarly, 90% of the enrolled HCC patients were infected by HBV, while no significant difference was found between HCC patients with or without HBV infection history. Indeed, HCC was largely attributable to chronic HBV infection in China, and almost 80% primary HCC patients had HBV infection history. Thus, to reduce selection bias caused by differ-

Table 2. Univariate and multivariate analysis of TFS in HCC patients

| Clinicopathological parameters | Univariate analysis | Multivariate analysis |
|--------------------------------|---------------------|----------------------|
|                                | HR (95% CI) p       | HR (95% CI) p        |
| Age (<60 vs. ≥60 years)        | 0.788 (0.479–1.298) | 0.350                |
| Sex (Female vs. Male)          | 1.478 (0.773–2.824) | 0.237                |
| Hepatitis B (Negative vs. Positive) | 1.254 (0.639–2.460) | 0.510                |
| AFP (<200 vs. ≥200 ng/mL)      | 1.087 (0.670–1.764) | 0.734                |
| Cirrhosis (Absent vs. Present) | 1.283 (0.777–2.120) | 0.331                |
| ALT (<40 vs. >40 U/L)          | 0.718 (0.397–1.296) | 0.271                |
| Serum TBil (≤17 vs. >17 µmol/L) | 1.050 (0.615–1.792) | 0.859                |
| Serum albumin (≥35 vs. <35 g/L) | 1.425 (0.726–2.795) | 0.303                |
| Tumor diameter (<5 vs. ≥5cm)   | 1.468 (0.861–2.505) | 0.159                |
| Microvascular invasion (Absent vs. Present) | 1.677 (0.759–3.703) | 0.201                |
| Differentiation (High/moderate vs. Low) | 1.137 (0.700–1.847) | 0.605                |
| Multiple lesions (Absent vs. Present) | 1.814 (1.031–3.190) | 0.039                |
| Capsular invasion (Absent vs. Present) | 1.632 (1.000–2.662) | 0.050                |
| TNM stage (I–II vs. III–IV)    | 2.057 (1.237–3.421) | 0.005                |
| Vessel carcinoma embolus (Absent vs. Present) | 1.789 (1.048–3.055) | 0.033                |
| SCARNA16 expression (Low vs. High) | 0.505 (0.308–0.829) | 0.007                |

*p<0.05. AFP, α-fetoprotein; ALT, alanine aminotransferase; CI, concordance index; HCC, hepatocellular carcinoma; HR, hazard ratio; TBil, total bilirubin; TFS, tumor-free survival; TNM, tumor-node-metastasis.
ent races, regions, liver disease background and medical technology levels, multiple medical centers should be united to perform the following research. More female and non-HBV-related HCC patients should also be enrolled into the research in the future. Besides, liver tissues collected from healthy people would help to determine the true baseline expression of SCARNA16 and verify its related carcinogenic mechanisms. Even so, these limitations did not compromise the integrity and scientificity of the present research.

In conclusion, the scaRNA SCARNA16 showed decreased expression pattern in both HCC cell lines and clinical specimens. Besides, we found significant association between low SCARNA16 level and several malignant characteristics, including large tumor size, vessel carcinoma embolus, capsular invasion, and severe TNM stages of HCC patients. During postoperative follow-up, HCC patients with low SCARNA16 expression generally manifested higher tumor recurrence rate and shorter long-term survival time. These findings suggested that SCARNA16 possesses potency as a novel biomarker for HCC.

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Conflict of interest
The authors have no conflict of interests related to this publication.

Author contributions
Conceived of and designed the study (WW, SY, SZ), conducted the study (SZ, YD, ZS, YG, YC), contributed to the acquisition of data (QX, LZ, HX, YB), analyzed the data (ZS, SZ, CX, YL), interpreted the data (WW, ZS, XH, HD), and reviewed and edited the manuscript (WW, SY, SZ, YD, YG). All authors read and approved the manuscript.

![Fig. 4. Kaplan-Meier analysis in OS of HCC patients with low and high SCARNA16 expression.](image)

**Table 3.** Univariate and multivariate analyses of OS in HCC patients

| Clinicopathological parameters | Univariate analysis | Multivariate analysis |
|-------------------------------|---------------------|----------------------|
|                               | HR (95% CI)         | p                    | HR (95% CI)         | p                  |
| Age (<60 vs. ≥60 years)       | 0.864 (0.448–1.667) | 0.663                | 1.219 (0.627–2.370) | 0.559              |
| Sex (Female vs. Male)         | 0.885 (0.417–1.876) | 0.750                | 0.780 (0.368–1.656) | 0.518              |
| Hepatitis B (Negative vs. Positive) | 1.059 (0.465–2.412) | 0.892                | 0.954 (0.293–3.110) | 0.938              |
| AFP (<200 vs. ≥200 ng/mL)     | 1.369 (0.710–2.641) | 0.349                | 0.611 (0.268–1.392) | 0.241              |
| Cirrhosis (Absent vs. Present) | 1.219 (0.627–2.370) | 0.559                | 1.219 (0.627–2.370) | 0.559              |
| ALT (≤40 vs. >40 U/L)         | 0.850 (0.368–1.656) | 0.518                | 1.789 (0.817–3.917) | 0.146              |
| Serum TBil (≤17 vs. >17 µmol/L) | 0.954 (0.293–3.110) | 0.938                | 1.359 (0.641–3.695) | 0.335              |
| Serum albumin (≥35 vs. <35 g/L) | 1.789 (0.817–3.917) | 0.146                | 0.691 (0.345–1.384) | 0.297              |
| Tumor diameter (<5 vs. ≥5cm)  | 0.954 (0.293–3.110) | 0.938                | 2.866 (1.449–5.669) | 0.002              |
| Microvascular invasion (Absent vs. Present) | 0.954 (0.293–3.110) | 0.938                | 1.900 (0.994–3.629) | 0.052              |
| Differentiation (High/moderate vs. Low) | 0.954 (0.293–3.110) | 0.938                | 2.906 (1.520–5.556) | 0.001              |
| Vessel carcinoma embolus (Absent vs. Present) | 0.954 (0.293–3.110) | 0.938                | 2.450 (1.221–4.915) | 0.012              |
| Capsular invasion (Absent vs. Present) | 1.900 (0.994–3.629) | 0.052                | 1.472 (0.755–2.871) | 0.257              |
| TNM stage (I-II vs. III-IV)   | 2.906 (1.520–5.556) | 0.001                | 1.696 (0.799–3.596) | 0.169              |
| Multiple lesions (Absent vs. Present) | 2.906 (1.520–5.556) | 0.001                | 2.450 (1.221–4.915) | 0.012              |
| SCARNA16 expression (Low vs. High) | 0.329 (0.162–0.669) | 0.002                | 0.329 (0.162–0.669) | 0.002              |

*p<0.05. AFP, α-fetoprotein; ALT, alanine aminotransferase; CI, concordance index; HCC, hepatocellular carcinoma; HR, hazard ratio; OS, overall survival; TBil, total bilirubin; TNM, tumor-node-metastasis.
Data sharing statement

The datasets used in support of the findings of this study are available from the corresponding author at wam@zju.edu.cn upon request.

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