Long non-coding RNA TRPM2-AS as a potential biomarker for hepatocellular carcinoma

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Abstract

Background There is increasing evidence that long non-coding RNAs are involved in hepatocellular carcinoma (HCC) tumorigenesis. The expression level of TRPM2-AS in HCC and its clinical association remain poorly defined.

Method qRT-PCR was performed to detect the expression of TRPM2-AS in 108 HCC patients. The correlations between TRPM2-AS expression and clinicopathological factors and prognosis were evaluated. The inference of TRPM2-AS to the proliferation and apoptosis of HCC cells was detected.

Aims The aim of our study was to explore the expression of TRPM2-AS in hepatocellular carcinoma (HCC) and the relation with prognosis and clinical features.

Results The expression of TRPM2-AS was higher in most HCC tissues and was significantly correlated with tumor size, AJCC stage, tumor differentiation, and the prognosis of HCC patients. Interfering TRPM2-AS expression using siRNA significantly inhibited cell proliferation and promoted cell apoptosis in two HCC cell lines.

Conclusion Long non-coding RNA TRPM2-AS is upregulated in HCC and represents a new biomarker for HCC and the

inhibition of TRPM2-AS promotes apoptosis in HCC cells in vitro.

Keywords Biomarker · Hepatocellular carcinoma · Long non-coding RNA · TRPM2-AS

Introduction

Hepatocellular carcinoma (HCC) consists of diverse, histologically distinct hepatic neoplasms and is the fifth most common cancer worldwide [1]. HCC is among the most lethal liver cancers and represents 70–90% of primary liver cancers [2]. In the early stage, HCC is usually asymptomatic and most patients are diagnosed at an advanced stage [3]. As most patients do not have the opportunity to undergo radical surgery, the overall patient outcome is poor. Therefore, novel, reliable, predictive biomarkers and treatments for HCC are urgently needed.

Long non-coding RNA (lncRNAs) is generally defined as RNA that lacks coding potential and has a transcript length of more than 200 nucleotides (nt) [4–6]. Using high-throughput technologies, thousands of lncRNAs have recently been identified, and a large number of studies have demonstrated that dysregulated lncRNAs are closely related to human cancers [7], such as HCC [8], lung cancer [9], breast cancer [10], and colorectal cancer [7]. The expression levels of certain lncRNAs are associated with the recurrence [11], metastasis [12], and prognosis of cancers. Therefore, the identification of cancer-associated lncRNAs will lead to a better understanding of the molecular biology of tumors and aid in developing novel therapeutic targets.

lncRNA TRPM2-AS, which is an antisense lncRNA of TRPM2, is 875 nt in length and located in the chr21q22.3 locus [13]. TRPM2-AS was first studied in prostate

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cancer [13] and then studied in the context of non-small cell lung cancer (NSCLC), where it was found to be related to the survival of NSCLC patients and chemoresistance in NSCLC [14, 15]. However, until now, the role of lncRNA TRPM2-AS in HCC has not been studied.

In the current study, we aimed to determine the expression of TRPM2-AS in HCC tissues and its relationship with HCC patient outcome and clinicopathological factors. Our findings provide new insights into HCC pathogenesis and suggest candidates for potential novel biomarkers for HCC.

Methods and materials

Study subjects

One hundred eight fresh HCC tissue and matched normal adjacent tissue samples were selected from patients with HCC at our hospital (Yuhuangding Hospital of Qingdao University Medical College) between 2010 and 2012. None of the patients had received preoperative therapy. The diagnosis of each specimen was confirmed histopathologically. Letters of consent were obtained from all patients, and the experimental protocols were approved by the local ethics committee. Patient charts were reviewed to obtain clinical data including age, gender, tumor size, AFP, HBsAg, vascular invasion, TNM stage (AJCC), tumor differentiation, and death or time of last follow-up. Patient survival was calculated from the day of surgery until death in months.

Cell lines and transfection

The HCC cell lines HCCLM3, Huh7, SMMC-7721, SK-Hep1, and HepG2 and one normal liver cell line (QSG-7701) were maintained at our institute. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA), and SK-Hep1, SMMC-7721, Huh7, HCCLM3, and QSG-7701 cells were cultured in Medium 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO, USA).

Si-NC and Si-TRPM2-AS were used to transfect the cells at a concentration of 20 nM. Oligonucleotide transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were collected 48 h after transfection. The siRNA sequences for TRPM2-AS1 were as follows: siRNA sense, 5'-GGGAAGAUGUCUCAGCAGACG-3', and antisense, 5'-UCUGCUGAGACAUCUCCCCU-3'.

Quantitative real-time PCR

Total RNA was extracted from tissues using TRIzol® Reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol, and cDNA was synthesized (Bio-Rad, Hercules, CA, USA). QPCR (7500Fast; ABI, Foster City, CA, USA) was performed to compare the mRNA expression levels of TRPM2-AS1 in 108 paired tumor and peritumor tissues from HCC patients. The primer sequences for TRPM2-AS1 were as follows: sense, 5'-CGTGACCAGGTTCA GACACA-3', and antisense, 5'-TGGGCAGTTTGGTT CTGGTT-3'. The primer sequences for the house-keeping gene β -actin were as follows: sense, 5'-TGTCCACCTTCCAG CAGATG-3', and antisense, 5'-TGTCACCTTCACCG TTCCAG-3'. Bax, Bcl-2, caspase-3, cyclin D1, cyclin E1, cyclin B1, and cyclin A2 expression was measured using the SYBR green qPCR assay (Takara, Dalian, China) and the primer sequences were shown in Table 1.

Cell viability assay

Cell viability assays were performed using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Cells were cultured in 96-well plates at a cell density of 1000 cells per well for 24, 48, 72, and 96 h. The absorbance was read at 450 nm to determine the cell viability in each well. All experiments were independently repeated three times.

Apoptosis assay

Cells were plated in 6-well plates, and 48 h after transfection, the cells were collected and resuspended in staining solution containing Annexin V-FITC and propidium iodide (PI; Tianjin Sungene Biotech Co. Ltd., Tianjin, China). The stained cells (1×10^5) were analyzed using a flow cytometer.

Cell cycle assay

Forty-eight hours after transfection, the cells were collected and fixed in 75% ethanol overnight at 4 °C and then incubated with 1 mg/mL RNase A for 30 min at 37 °C. Subsequently, cells were stained with propidium iodide (PI; Becton Dickinson, San Jose, CA, USA) in PBS according to the manufacturer's instructions. The stained cells (1×10^5) were analyzed by flow cytometry.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS), Version 18 (SPSS, Chicago, IL, USA). Pearson's chi-squared test was used to analyze the relationship between TRPM2-AS1 expression and clinicopathological

Table 1 Primers for qRT-PCR

	Upstream primer (5′ – 3′)	Downstream primer (5′ – 3′)	Size (bp)
Bax	CCCGAGAGGTCTTTTCCGAG	CCAGCCCATGATGGTTCTGAT	155
Bcl-2	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTACTCAGTCATCC	89
Caspase-3	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA	139
cyclin D1	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA	135
cyclin E1	GCCAGCCTTGGGACAATAATG	CTTGACGTTGAGTTTGGGT	104
cyclin A2	CGCTGGCGGTACTGAAGTC	GAGGAACGGTGACATGCTCAT	120
cyclin B1	AATAAGGCGAAGATCAACATGGC	TTTGTTACCAATGTCCCCAAGAG	111

characteristics. Kaplan-Meier plots were constructed to visualize survival outcomes, which were compared using a log-rank test. All experiments were performed in triplicate. The differences between the groups were analyzed using Student's *t* test, and $p < 0.05$ was considered statistically significant in all cases.

Results

Expression of TRPM2-AS in HCC tissue samples

We first detect the expression of TRPM2-AS in samples from 108 HCC patients using quantitative reverse transcription (qRT)-PCR. The expression level of TRPM2-AS in cancer tissues from patients with HCC was significantly higher than that in adjacent normal tissues ($p < 0.001$; Fig. 1a). Based on the relative expression levels in tumor and adjacent normal tissues, all 108 patients with HCC were divided into two subgroups. As shown in Fig. 1b, 32 patients (low-expression group) had a low expression level of TRPM2-AS in carcinoma tissues compared with that of adjacent normal liver tissues, and 76 patients (high-expression group) had a high expression level of TRPM2-AS.

Clinicopathological significance of TRPM2-AS expression in HCC

We then determined whether the expression level of TRPM2-AS was associated with patient characteristics, such as gender, age, tumor size, tumor differentiation, and TNM stage. TRPM2-AS expression was significantly correlated with tumor size, AJCC stage, and tumor differentiation ($p = 0.005$, 0.014, and 0.021, respectively) (Table 2). However, there was no association between TRPM2-AS expression and age, sex, AFP, vascular invasion, or liver cirrhosis.

Association between TRPM2-AS and patient prognosis

We then performed a survival analysis of the patients with HCC. Patients in the TRPM2-AS high-expression group had

a worse overall survival than those in the low-expression group (Fig. 2). There was not much difference in the survival rate between the two groups after the first year. However, the 3-year and 5-year survival rates were worse for the TRPM2-AS high-expression group (32.9 and 28.7%, respectively) than those of the TRPM2-AS low-expression group (58.6 and 48.8%, respectively) ($p = 0.021$) (Table 3). Thus, high expression levels of TRPM2-AS in HCC predict a poor prognosis.

Fig. 1 Upregulated expression of the long non-coding RNA TRPM2-AS in HCC. **a** The expression level of TRPM2-AS in HCC tissues was significantly higher than that in non-tumorous tissues ($n = 108$) ($p < 0.001$). **b** The TRPM2-AS expression levels in HCC tissues were increased (70%) compared to those of the adjacent normal tissues. Bars represent the ratio between the expression in HCC and that in adjacent normal tissues

Table 2 Correlation of TRPM2-AS expression and clinicopathological characteristics

Variable In general	Low	High	<i>p</i> value
	32	76	
Sex			
Male	28	68	0.5
Female	4	8	
Age (years)			
≤ 50	9	15	0.238
> 50	23	61	
Tumor size (cm)			
≤ 5	18	18	0.005
> 5	14	48	
AFP (ng/mL)			
≤ 400	18	36	0.264
> 400	14	40	
HBsAg			
Positive	28	70	0.336
Negative	4	6	
Anti-HCV			
Positive	2	4	0.577
Negative	30	72	
Vascular invasion			
Yes	6	18	0.386
No	26	58	
AJCC stage			
I–II	19	26	0.014
III–IV	13	50	
Tumor differentiation			
I–II	11	44	0.021
III–IV	21	32	
Liver cirrhosis			
Yes	16	46	0.212
No	16	30	

Italic numbers: $P < 0.05$

Downregulation of TRPM2-AS inhibited HCC proliferation

To further ascertain the function of lncRNA TRPM2-AS in HCC, we first examined the TRPM2-AS expression level in HCC cell lines using qRT-PCR. The expression of TRPM2-AS in five HCC cell lines, HCCLM3, Huh7, SMMC-7721, SK-Hep1, and HepG2, was significantly higher compared to that in the normal liver cell line QSG-7701 (Fig. 3a). We used SMMC-7721 and SK-Hep1 cells, which had the highest TRPM2-AS levels of these cell lines, to determine if Si-TRPM2-AS could effectively inhibit the expression of TRPM2-AS (Fig. 3b). The influence of TRPM2-AS on HCC cell proliferation was investigated using the CCK-8

assay. The inhibition of TRPM2-AS significantly inhibited SMMC-7721 and SK-Hep1 cell growth compared with that of the control, respectively (Fig. 3c, d) (Table 3).

Downregulation of TRPM2-AS causes apoptosis of HCC cells

To evaluate the apoptotic effects of TRPM2-AS in HCC cells, the SMMC-7721 and SK-Hep1 cell lines were treated with Si-TRPM2-AS. The inhibition of TRPM2-AS significantly induces SMMC-7721 and SK-Hep1 cell apoptosis compared with that of the control. The downregulation of TRPM2-AS increased the apoptosis rate from 8.5 to 27.7% in SMMC-7721 cells and from 7.6 to 18.5% in SK-Hep1 cells (Fig. 4a, b).

We examined the effect of TRPM2-AS on apoptosis mediators, in order to elucidate the molecular mechanism of TRPM2-AS-induced apoptosis. As shown in Fig. 4c, Si-TRPM2-AS significantly increased the mRNA levels of caspase-3 and Bax, but not the expression of Bcl-2.

Downregulation of TRPM2-AS causes cell cycle arrest of HCC cells

Then, we analyzed cell cycle alterations in HCC cells by flow cytometry. In the Si-TRPM2-AS group, cells in the G0/G1 phase increased significantly for both cell lines, accompanied by a significant decrease in cells at in G2/M phase (Fig. 5a, b). However, no statistically significant differences were observed for cells in the S phase. The percentage of SK-Hep-1 cells in the G0/G1 phase was found to have increased from 33.69 to 47.11%, and those in G2/M phase decreased from 31.25 to 12.19%. This tendency was also observed in SMMC-7721 cells.

To understand these cell cycle alterations, we analyzed the expression of various cyclins, which are important regulators of the cell cycle. Inhibition of TRPM2-AS caused significant decrease in the mRNA level of cyclin D1, cyclin E1, and cyclin B1, but not cyclin A2 (Fig. 5c).

Discussion

In this study, we tested the expression of the novel lncRNA TRPM2-AS in HCC tissues and their surrounding non-tumorous tissues. We found that the upregulation of TRPM2-AS in HCC primary tumors was associated with a poor prognosis. When analyzing the clinicopathological factors, we found a significant association between TRPM2-AS expression and tumor size, AJCC stage, and tumor differentiation. We also identified the function of TRPM2-AS in HCC cells using a loss-of-function approach. The inhibition of TRPM2-AS inhibited HCC cell proliferation. Therefore, these results indicated that TRPM2-AS may function as a tumor oncogene and may explain the significant association between TRPM2-AS expression and

Fig. 2 Kaplan-Meier curves for the analysis of overall survival. The high expression of TRPM2-AS predicts a poor prognosis in HCC patients ($p = 0.021$)

Table 3 Relationship between TRPM2-AS expression and survival rate

Survival measurement	TRPM2-AS expression		<i>p</i> value
	Low TRPM2-AS	High TRPM2-AS	
1-year overall survival (%)	78.1 ± 7.30	72.4 ± 5.1	0.021
3-year overall survival (%)	58.6 ± 8.8	32.9 ± 5.4	
5-year overall survival (%)	48.8 ± 11.6	28.7 ± 5.2	

tumor size in HCC, which leads to a poorer prognosis in HCC patients with high TRPM2-AS expression levels.

lncRNAs were first regarded as spurious transcriptional noise [16, 17]. As thousands of lncRNAs were discovered,

Fig. 3 The expression level of TRPM2-AS in HCC cells. **a** TRPM2-AS expression levels in HCC cell lines (HCCLM3, Huh7, SMMC-7721, SK-Hep1, and HepG2) compared with those in a normal human liver cell line (QSG-7701). **b** TRPM2-AS expression level following treatment with Si-NC or Si-TRPM2-AS in SMMC-7721 and SK-Hep1 cells; **c, d** The inhibition of TRPM2-AS inhibited the proliferation of SMMC-7721 and SK-Hep1 cells in CCK-8 assays. * $p < 0.01$

Fig. 4 The effect of TRPM2-AS on HCC cell apoptosis in vitro. **a, b** Cells were treated with Si-NC or Si-TRPM2-AS, and apoptosis was detected using flow cytometry. Inhibition of TRPM2-AS promoted cell

apoptosis in SMMC-7721 and SK-Hep1 cells; **c** qRT-PCR analysis the mRNA levels of apoptosis associated regulators, caspase-3, Bax, and Bcl-2; * $p < 0.01$

however, they were found to affect a diverse range of biological processes, including the cell cycle, cell differentiation, and cell metabolism through various mechanisms at transcriptional, post-transcriptional, and epigenetic regulatory levels [17, 18]. Moreover, after the dysregulation of many lncRNAs was found to be associated with a variety of diseases [19],

including cancer, lncRNAs were also considered to be oncogenic or tumor-suppressor genes as well as protein-coding genes.

Although lncRNAs have been shown to play important biological roles and are dysregulated in many human diseases, the functional mechanism of lncRNAs is poorly understood

Fig. 5 The effect of TRPM2-AS on HCC cell cycle arrest in vitro. **a, b** Cells were treated with Si-NC or Si-TRPM2-AS, and cell cycle was detected using flow cytometry. Inhibition of TRPM2-AS cause cell cycle

arrest in SMMC-7721 and SK-Hep1 cells; **c** qRT-PCR analysis the mRNA levels of cell cycle associated regulators, cyclin D1, cyclin E1, cyclin B1, and cyclin A2; * $p < 0.01$

and represents a difficult aspect of lncRNA research [20]. Their mechanism of action mainly includes (1) chromatin remodeling by binding to the promoter regions or changing histone

markers and the chromatin state [21], (2) acting as scaffold molecules through the direct interaction with proteins or protein complexes as scaffolds or allosteric activators/inhibitors

[22], and (3) competing with endogenous RNAs by acting as competing endogenous RNAs that bind miRNAs and reduce their inhibitory effect on their natural targets [23]. These functions are likely not mutually exclusive and an individual lncRNA may act through several of them [20].

Orfanelli et al. [13] first investigated the function of TRPM2-AS in prostate cancer, demonstrating that high TRPM2-AS expression was associated with a poor clinical outcome and that, in vitro, TRPM2-AS knockdown led to prostate cancer cell apoptosis. TRPM2-AS was then shown to be related to resistance to anti-tumor drugs in carcinoma cells via the p53-p66 pathway in NSCLC. Here, we drew a similar conclusion that high TRPM2-AS expression was associated with a poor clinical outcome and that the inhibition of TRPM2-AS resulted in apoptosis in HCC cells, and this is the first report to demonstrate the function of TRPM2-AS in HCC.

Although we confirmed the role of TRPM2-AS in inhibiting proliferation in HCC cells, we did not clarify the mechanism of inhibition. Further studies are needed to determine whether TRPM2-AS retains this inhibitory effect in vivo and to identify the downstream molecules affected by TRPM2-AS.

In conclusion, we demonstrated that the upregulation of TRPM2-AS is associated with the patient overall survival rate after hepatic resection. TRPM2-AS knockdown was also found to effectively suppress the tumorigenicity of HCC cell lines. We suggest that controlling proliferation by inhibiting TRPM2-AS may represent a novel therapeutic approach for HCC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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