Original Research

snoRNA23 enhances the progression of hepatocellular carcinoma via regulation of the Wnt/β-catenin pathway

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Abstract: Small nucleolar non-coding RNA(snoRA)23 is upregulated in human pancreatic ductal adenocarcinoma. However, to the best of our knowledge, the role of snoRA23 in hepatocellular carcinoma progression has not been determined. MTT and colony formation assays were used to assess the cell viability and proliferation of HCC cells with snoRA23 knocked down, respectively, and a lymphatic vessel formation assay was used to determine tube formation ability of Human dermal lymphatic endothelial cells treated with conditioned media from HCC cell cultures. The results showed that snoRA23 knockdown attenuated cell viability, colony formation,and lymphatic vessel formation in HCC cells. snoRA23 was correlated with the prolonged overall survival of patients with HCC. Additionally, snoRA23 knockdown downregulated the Wnt/β-catenin signaling pathway by decreasing Wnt3a expression and β-catenin levels.α-methylacyl-CoA racemase (AMACR) levels were notably decreased by snoRA23 depletion. Finally, it was confirmed that AMACR overexpression partially rescued snoRA23-modulated HCC tumorigenesis. The results of the present study provide further insight into the role of non-coding RNAs in the development and progression of HCC.

Key words: Hepatocellular carcinoma; Tumorigenesis; Small nucleolar non-coding RNA23; Wnt/β-catenin pathway; α-methylacyl-CoA racemase.

Introduction

Hepatocellular carcinoma (HCC) is the most main common type of cancer worldwide (1-5). Nearly 80% of HCC patients were caused by chronic hepatitis B and C(6, 7). Till now, only sorafenib, lenvatinib, regorafenib are well-established therapies for HCC(8-13). Thus, there is an urgent need for improved treatments and an increased understanding of the pathogenesis of HCC(14-17).

Small nucleolar RNAs (snoRNAs) are defined as non-coding RNAs60-300 nucleotides in length that are primarily located in the nucleoli(18). snoRNA can be classified into two groups: C/D box snoRNAs and H/ACA box snoRAs (19). Functionally, snoRAs are involved in post-transcriptional modification of ribosomal RNAs and other RNAs(20). It has been reported that snoRAs participate in several biological processes, including cancer cell proliferation, invasion, and metastasis(21). For example, Zhang et al showed that snoR-NA71A promoted colorectal cancer (CRC) progression(22). snoRA23 is upregulated in human pancreatic ductal adenocarcinoma(23); however, the specific role of snoRA23 in HCC remains unknown.

The Wnt signaling pathway consists of two modes: A canonical and non-canonical signaling pathway. The canonical pathway is β-catenin-dependent, whereas the non-canonical pathway is β-catenin-independent(24). Previous studies have shown that the Wnt signaling pathway is involved in hepatocellular carcinoma progression as well as other cancers(25, 26) , and is a key oncogenic driver(26-28). Pharmacological intervention of the Wnt signaling pathway significantly inhibits xenograft tumor formation in animals inoculated with cholangiocarcinoma cells by decreasing Wnt and β-catenin expression(29).

α-methyl-acyl-CoA racemase (AMACR) is an enzyme located in the mitochondria and peroxisome(30, 31), where it serves a crucial role in β-oxidation of branched-chain fatty acids and derivatives(32). AMACR has been demonstrated to be overexpressed in various types of tumors, including 45-83% of cases of CRC, prostate (11, 33-37). Although AMACR serves as a prognostic marker for HCC recurrence, the detailed biological function of AMACR in HCC remains unknown(38).

The present study aimed to determine the function of snoRA23 in the initiation and progression of HCC. The data provided herein may provide a novel insight into the development and progression of HCC.

Materials and Methods

Cell culture

Human HCC cells (HepG2 and Huh-7), as well as human embryonic kidney cells 293T were purchased from the American Type Culture Collection. According to the manufacturer's instructions, cells were cultured in DMEM (Hyclone) supplemented with 10% FBS (Gibco)and 1% penicillin/streptomycin (Thermofisher) with 5% CO_2 at 37°C. Human dermal lymphatic endothelial cells (HDLECs) (PromoCell GmbH) were cultured in an endothelial cell growth medium with 5% CO_2 at 37°C. After 72 h, the conditioned medium (CM) ofHepG2 and Huh-7 cells were collected and used in

subsequent experiments.

Generation of stably transfected cells

A total of 2 μg vectors [1 μg pLKO.1-short hairpin (sh)RNA, 0.5 μg VSVG and 0.5 μg PAX2]was used to transfect 293T cells (~2×10^6 cells/1.5 mL medium) using Lipofectamine® 3000reagent at 37 °C. The volume of transfection reagents and vectors was 100ul. Lentiviruses were collected 36-48 h after transfection. Virus stock was mixed with fresh media at a ratio of 1:4 and then added toHepG2 and Huh-7 cells. After a further 24 h,1 μg/ml puromycin was utilized to select resistant cells. The sequences of the shRNAs used were: sh-NC,AUCAGUCAUCAUAACCUCGAAGG; sh-snoRA23-1, GCUGCUGUAAUGUGUGCAU; and sh-snoRA23-2,GUGCAUAGGUUUCAUCUGU.

Transfection

AMACR Cdna was amplified and subcloned into a pcDNA3.1vector. The recombinant construct,pcDNA3.1-AMACRwas transfected into HepG2 and Huh-7 cellsusingLipofectamine® 3000 reagents. After 24-36 h of transfection, the cells were used for subsequent experiments.

Soft agar assay

Soft agar assay was performed as described previously (39)). In total, ~2×10^3HepG2 or Huh-7cells/well (6-well plate) were plated. Cell clones that had formed after 28 days were counted and imaged.

MTT assay

HepG2 and Huh-7 cells were suspended and seeded into 96-well plates (~5×10^3cells). MTT (Sigma-Aldrich; Merck KGaA) was added to the well and incubated for 4 h with 5% CO_2 at 37 °C. Signals were measured after 0-72 h, following the addition of 150 μl DMSO to each well for 10 min. The absorbance was measured at 490 nm.

Western blotting

Proteins (~50 μg)were loaded on a 10% SDS-gel, resolved using SDS-PAGE, and transferred to a PVDF membrane. The membranes were blocked using 5% non-fat milk to reduce non-specific binding. Primary antibodies were used to probe the indicated proteins overnight at 4 °C. After rinsing, the membranes were incubated with secondary antibodies for 0.5-1 h at room temperature. The antibodies used were: Wnt3a antibody (cat. no. 2391; 1:1,000;Cell Signaling Technologies, Inc.), anti-β-catenin antibody (cat. no. 9562; 1:1,000; Cell Signaling Technologies, Inc.), anti-AMACR antibody (cat. no. MA5-14793; 1:1,000; Thermo Fisher Scientific, Inc.), anti-GAPDH antibody (cat. no. 10094-R004; 1:1,000;SinoBiological), anti-mouse HRP-conjugated IgG(cat. no 7076; 1:3,000; Cell Signaling Technologies, Inc.), or anti-rabbit HRP-conjugated IgG(cat. no. 7074; 1:3,000; Cell Signaling Technologies, Inc.). The statistics using ImageJ software.

Lymphatic vessel formation assay

Matrigel was diluted with PBS (1:5 ratio), used to coat the wells of a 24-well plate (0.2 ml/well), and incubated at 37°C for 5 h. HDLECs were seeded in Growth Factor Reduced (GFR) Matrigel and cultured in the CM. Cells (~5×10^3 cells/well) were seeded. An inverted microscope was used to observe the tubes.

The Cancer Genome Atlas (TCGA) analysis

We downloaded the colon adenocarcinoma TCGA dataset (275tumors and 349 normal tissues). The relative expression was analyzed using a two-tailed unpaired Student's t-test.

Reverse transcription-quantitative (RT-q)PCR

Briefly, total RNAs were extracted from HCC-cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 1 μg of total RNA was used to generate cDNAs using a PrimeScript RT reagent kit (Takara Bio, Inc.). AnSYBR Green I MasterMix kit was used for qPCR. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$method(40). The sequences of the primers used were: snoRA23 forward,ATGGCTGCTGTAATGTGTGCand reverse,GTGTGGGAATTTGGAGGCTG; WNT3A forward, GGACAAAGCTACCAGGGAGTand reverse, ACCATCCCACCAAACTCGAT; β-catenin forward, CTTACACCCACCATCCCACTand reverse, CCTCCACAAATTGCTGCTGT; AMACR forward,AGGGTCAGGTCATTGATGCAand reverse,GTCCTTTGATCAGCAGCTCG; and β-actin forward,GGGAAATCGTGCGTGACATTand reverse,AGGTAGTTTCGTGGATGCCA

Statistical analysis

Data were analyzed using GraphPad Prism version 8.0 (GraphPad Software, Inc.). Most of the experiments were performed independently in three replicates unless western blot result. Data were presented as mean ± SD (n=3). Comparisons between two groups were compared using an unpaired Student's t-test. Comparisons between multiple groups were compared using a one-way ANOVA with a post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

snoRA23 is associated with the prognosis of human patients with HCC

To assess the clinical significance of snoRA23, we analyzed colon adenocarcinoma TCGA dataset to determine whether snoRA23 was associated with HCC progression in human tissues. Gene expression analysis showed that snoRA23 expression was upregulated in HCC tumors compared with normal tissues (Figure 1A). Low levels of snoRA23 were associated with longer survival times in patients with HCC(Figure 1B). Additionally, higher stage (Stage III and IV)HCC tumors were associated with increased snoRA23 expression (Figure 1C). These data suggest that snoRA23may potentially act as an oncogene in human clinical tissues.

snoRA23 knockdown suppresses tumorigenesis of HCC cells

To investigate the role of snoRA23 in HCC, shRNAs against snoRA23 were designed and used to transfect HepG2 and Huh-7 cells. The expression levels of
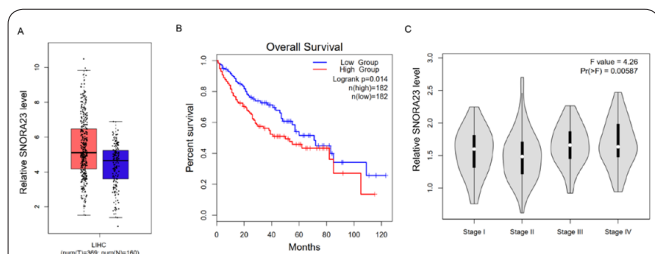
Figure 1. snoRA23 expression is associated with the prognosis of patients with HCC. (A) snoRA23 levels in clinical HCC tumors. *P<0.05. T indicated tumor samples, N indicated normal samples. (B) Overall survival of HCC patients with high or low snoRA23 levels was analyzed using Kaplan-Meier analysis. n (high)=135, n (low)=134. P=0.011. (C) Violin plot showing snoRA23 levels in different stages of HCC. ***P<0.001 (stage III+IV vs I+II). HCC, hepatocellular carcinoma; snoRA23, small nucleolar non-coding RNA 23. Data were presented as mean ± SD (n=3).
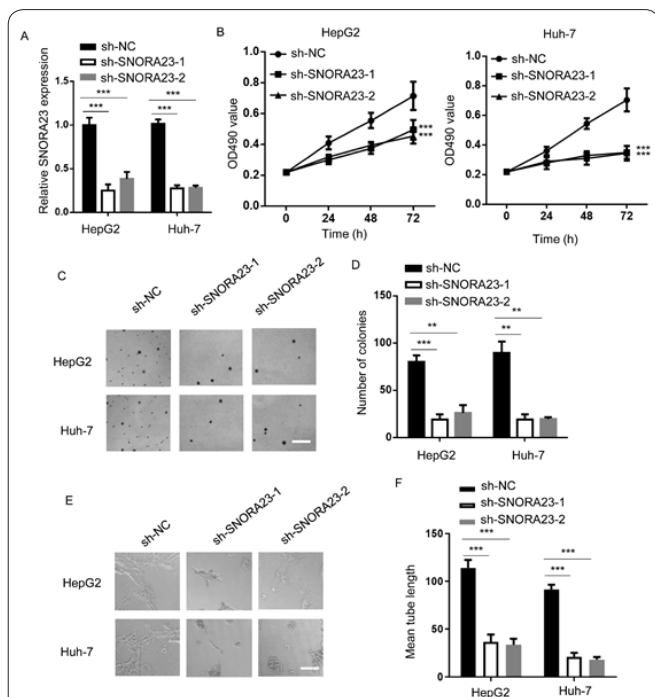

Figure 2. snoRA23 knockdown suppresses tumorigenesis of HCC cells. (A) snoRA23 expression levels in HepG2 or Huh-7 cells transfected with sh-NC, sh-snoRA23-1 or sh-snoRA23-2 were examined using reverse transcription-quantitative PCR. (B) Cell viability of HepG2 or Huh-7 cells transfected with sh-NC, sh-snoRA23-1 or sh-snoRA23-2 were examined using an MTT assay. (C and D) Colony formation assay of HepG2 or Huh-7 cells transfected with sh-NC, sh-snoRA23-1 or sh-snoRA23-2. Scale bar = 5μm. (E and F) Tube lengths of HDLECs treated with conditioned medium from the HepG2 or Huh-7 cells transfected with sh-NC, sh-snoRA23-1 or sh-snoRA23-2. Scale bar = 5μm. **P<0.01, ***P<0.001. HCC, hepatocellular carcinoma; snoRA23, small nucleolar non-coding RNA 23; siRNA, small interfering; NC, negative control; sh, short hairpin; HDLEC, Human dermal lymphatic endothelial cell. Data were presented as mean ± SD (n=3).

snoRA23 were significantly decreased following transfection with sh-snoRA23-1 and sh-snoRA23-2 compared with the sh-NC transfected cells (Figure2A). The viability of HepG2 and Huh-7 cells stably transfected with sh-snoRA23 was assessed. The viability of cells transfected with sh-snoRA23-1 and sh-snoRA23-2 was markedly reduced compared with the sh-NC transfected cells (Figure 2B). The colony formation assay demons-
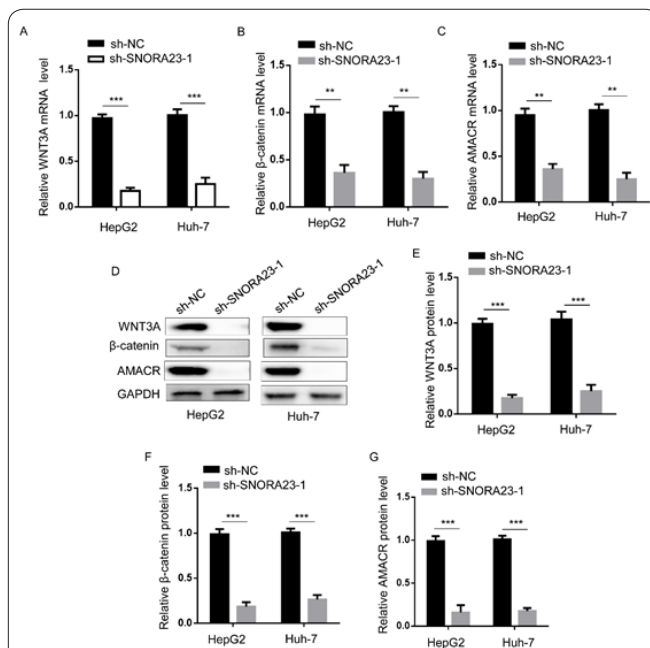

Figure 3. snoRA23 activates the Wnt/β-catenin pathway and upregulates AMACR expression. (A-C) Wnt3a, β-catenin and AMACR mRNA levels were detected using reverse transcription-quantitative PCR in HepG2 or Huh-7 cells transfected with sh-NC or sh-snoRA23-1. (D-G) Wnt3a, β-catenin and AMACR protein expression levels were detected by western blotting in HepG2 or Huh-7 cells transfected with sh-NC or sh-snoRA23-1. **P<0.01, ***P<0.001. HCC, hepatocellular carcinoma; snoRA23, small nucleolar non-coding RNA 23; siRNA, small interfering; NC, negative control; sh, short hairpin; AMACR, α-methylacyl-CoA racemase. Data were presented as mean ± SD (n=3). Statistical analysis of figure E-G of western blotting were based on numbers from three times of image J analysis of one set of samples.

trated that snoRA23 knockdown reduced the number of colonies formed compared with the sh-NC transfected HepG2 and Huh-7 cells (Figure 2C and D). Additionally, lymphatic vessel formation assays showed that the tube length of HDLECswas significantly reduced when incubated with CM from the snoRA23 knockdown HepG2 and Huh-7 cells (Figure 2E and F). Taken together, these results showed that snoRA23 regulated viability, colony formation and lymphatic vessel formation of HCC cells.

snoRA23 knockdown downregulates the Wnt/β-catenin pathway and AMACR expression

To determine the signaling pathway underlying snoRA23-mediated HCC progression, RT-qPCR was used. The results showed that snoRA23 knockdown resulted in lower expression levels of Wnt3a and β-catenin. Interestingly, AMACR expression was also downregulated (Figure 3A-C). The results of western blotting were consistent with that of the RT-qPCR analysis (Figure 3D-G). Taken together, these results suggest that snoRA23 depletion attenuates the Wnt/β-catenin signaling pathway and decreased AMACR expression.

AMACR is involved in snoRA23-modulated tumorigenesis of HCC cells

Finally, the role of AMACR in snoRA23-modulated tumorigenesis of HCC cells was assessed. AMACR was ectopically overexpressed in HepG2 and Huh-7 cells

(Figure 4A). Cell viability analysis demonstrated that AMACR overexpression markedly increased the viability of the HCC cells reduced by snoRA23 knockdown (Figure 4B). Similarly, colony formation assays showed that AMACR overexpression resulted in increased colony formation in the HepG2 and Huh-7 cells (Figure4C and D). Finally, the sh-snoRA23-inhibited-lymphatic vessel formation ability of the HepG2 and Huh-7 cells, and was restored by AMACRoverexpression (Figure 4E and F). To summarize, the data suggests AMACR is one of the factors involved in snoRA23-mediated HCC tumorigenesis.

Discussion

In the present study, it was confirmed that snoRA23 knockdown reduced the activity of the Wnt/β-catenin signaling pathway and regulated AMACR expression levels. snoRA23 promoted HCC tumorigenesis. Additionally, snoRA23 expression was shown to be upregulated in patients with HCC, and its expression was associated with survival.

Similar to the vast majority of cancer types, HCC may be caused by environmental and genetic factors(41-44). Despite improvements in surgical methods and the therapeutics available, the survival rate of patients with HCC remains unsatisfactory(45). Based on the results of the present study, snoRA23 may serve as an effective biomarker.

Increasing evidence has shown that non-coding RNAs (such as long non-coding RNAs, microRNAs, piRNAs, snoRAsand circRNAs) are implicated in HCC progression(46). snoRAs are generally encoded from the introns of genes. Several studies have shown that ncRNAs regulate gene expression at multiple levels. For example, snoRA27 affected alternative splicing of pre-mRNAs(47), and snoRA50A inhibited 3'-processing of mRNA via disruption of the Fib1-polyA site interaction(48). In the present study, snoRA23 was confirmed to modulate Wnt3a and β-catenin protein expression levels. However, the detailed regulatory mechanism underlying snoRA23regulation of the Wnt/β-catenin pathway requires further study.

AMACR performs the β-oxidation of branched-chain fatty acids. It converts (2R)-methyl-branched chain fatty acyl-CoAs to their (S)-stereoisomers(49). Although AMACR has been shown to serve as a biomarker for prostate cancer and CRC, the underlying mechanism remains unclear. In agreement with previous studies, AMACR was demonstrated to function as a downstream effector of snoRA23 in the tumorigenesis of HCC. However, the specific mechanism underlying this regulation requires further study.

In summary, the present study highlights a novel molecular mechanism involved in the tumorigenesis of HCC and suggests that snoRA23 may serve as a biomarker and/or therapeutic/target for the management of snoRA23.

Acknowledgements
Not applicable.

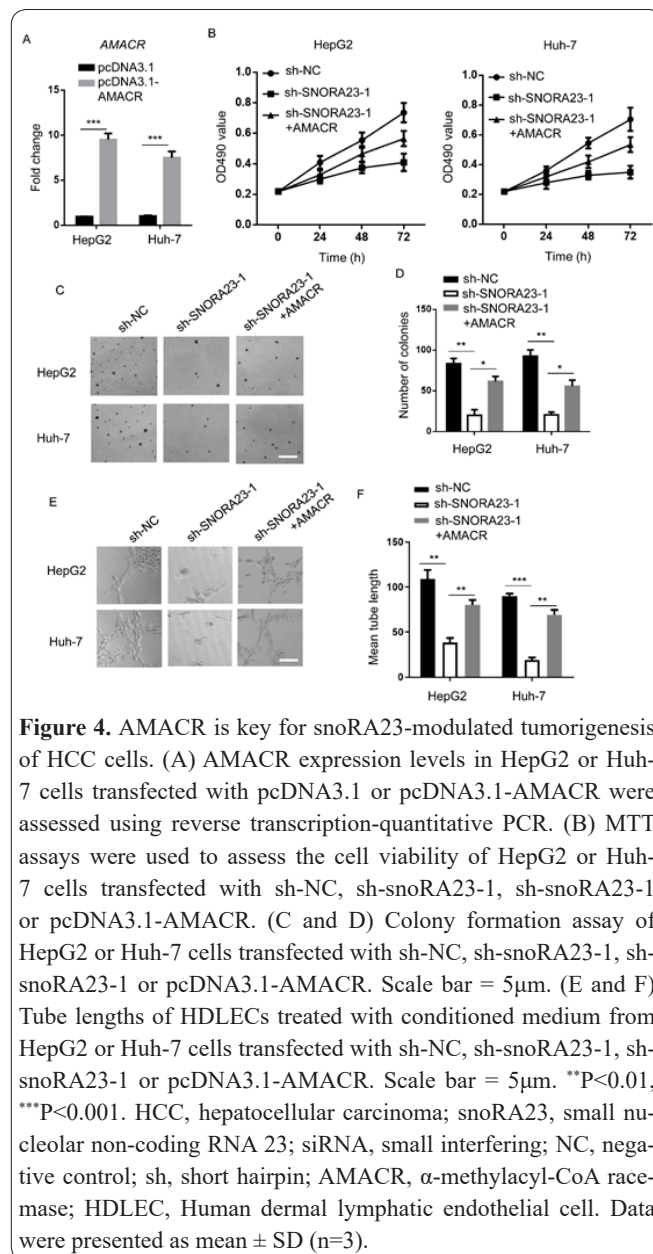## Interest conflict
We declare no conflict of interest.

Figure 4. AMACR is key for snoRA23-modulated tumorigenesis of HCC cells. (A) AMACR expression levels in HepG2 or Huh-7 cells transfected with pcDNA3.1 or pcDNA3.1-AMACR were assessed using reverse transcription-quantitative PCR. (B) MTT assays were used to assess the cell viability of HepG2 or Huh-7 cells transfected with sh-NC, sh-snoRA23-1, sh-snoRA23-1 or pcDNA3.1-AMACR. (C and D) Colony formation assay of HepG2 or Huh-7 cells transfected with sh-NC, sh-snoRA23-1, sh-snoRA23-1 or pcDNA3.1-AMACR. Scale bar = 5μm. (E and F) Tube lengths of HDLECs treated with conditioned medium from HepG2 or Huh-7 cells transfected with sh-NC, sh-snoRA23-1, sh-snoRA23-1 or pcDNA3.1-AMACR. Scale bar = 5μm. **P<0.01, ***P<0.001. HCC, hepatocellular carcinoma; snoRA23, small nucleolar non-coding RNA 23; siRNA, small interfering; NC, negative control; sh, short hairpin; AMACR, α-methylacyl-CoA racemase; HDLEC, Human dermal lymphatic endothelial cell. Data were presented as mean ± SD (n=3).

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