Long noncoding RNA MACC1-AS1 promotes the stemness of hepatocellular carcinoma cells by antagonizing miR-145

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Abstract

Objectives: This work aimed to investigate the roles of long noncoding (lnc)RNA MACC1-AS1 (MACC1 antisense RNA 1) in progression of hepatocellular carcinoma (HCC).

Methods: Real-time quantitative PCR, western blot, spheroid formation, aldehyde dehydrogenase isoform 1 (ALDH1) activity analysis, luciferase reporter assay, and RNA pull-down analysis were used to examine MACC1-AS1–mediated effects on HCC cell stemness.

Results: MACC1-AS1 was highly expressed in HCC tissues and cells. MACC1-AS1 positively regulated the expression of stemness master regulators and inhibited spheroid-forming ability and ALDH1 activity. Furthermore, MACC1-AS1 promoted the stemness of HCC cells by antagonizing microRNA (miR)-145 activity. Overexpression of miR-145 also attenuated HCC cell stemness.

Conclusions: This work revealed a novel MACC1-AS1/miR-145 axis that regulates the stemness of HCC cells.

Keywords

LncRNA, MACC1-AS1, miR-145, hepatocellular carcinoma, stemness, cancer stem cell

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Introduction

Hepatocellular carcinoma (HCC) is one of the five most common cancers in the world.1 In China, HCC ranks second in
number of deaths caused by malignant tumors, after lung cancer, which has brought greater health threats and economic burden to China. In recent years, the concept of cancer stem cells (CSCs) has been proposed: CSCs are stem cell-like cells that exist in some cancer tissues and have the characteristics of infinite proliferation, metastasis, and strong drug resistance. CSCs are regarded as the root of cancer progression. So far, the treatment of HCC is still limited to partial hepatectomy, radiotherapy, and chemotherapy, and outcomes are less than ideal. Because of the high recurrence rate of HCC after surgery, it is speculated that HCC stem cells exist in HCC and play an important role in HCC progression. Understanding the role and mechanism of HCC stem cells in HCC progression might provide a new direction for the research, diagnosis, and treatment of HCC.

Long noncoding RNAs represents a group of RNA molecules whose transcript length is more than 200 nucleotides. LncRNAs encode only very short polypeptides or do not encode any protein. Only 2.94% of human genome transcripts encode proteins. Genome and transcriptome sequencing evidence suggests that complex organism functions may be regulated by a series of RNAs from the noncoding regions of the genome. Recent studies have shown that lncRNAs are involved in many important biological processes, such as X chromosome inactivation, stemness maintenance, transcriptional regulation, and epigenetic regulation, among others. Additionally, lncRNAs participate in the regulation of many diseases, being involved especially in tumorigenesis and development. With advances in high-throughput screening methods, an increasing number of lncRNA molecules have been identified, and they are expected to be confirmed as novel tumor diagnostic markers and targets for cancer treatment.

The lncRNA MACC1-AS1 was recently found to be highly expressed in gastric cancer tissues and to promote the metastasis and stemness of gastric cancer cells. Furthermore, recent work showed that MACC1-AS1 promotes pancreatic carcinoma metastasis through MACC-AS1/PAX8/Notch1 signaling. However, the roles of MACC1-AS1 have not yet been revealed in HCC progression.

Here, we focused on exploring the roles and related mechanisms of MACC1-AS1 in HCC cell stemness because CSCs are regarded as the root of cancer progression. We found that lncRNA MACC1-AS1 negatively regulated the stemness of HCC cells by antagonizing miR-145 activity.

Material and methods

Reagents

Primary antibodies against octamer-binding transcription factor 4 (Oct4; Cat. No. 11263-1-AP, 1:1000), Nanog homeobox (Nanog; Cat. No. 14295-1-AP, 1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cat. No. 60004-1-Ig, 1:2000) were purchased from Proteintech (Wuhan, China). Antibodies to SRY-box transcription factor 9 (Sox9; ab185230, 1:3000) were purchased from Abcam (Cambridge, MA, USA). Lipofectamine 3000 reagent was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Clinical samples and cell culture

Twenty-three pairs of HCC and adjacent paraffin-embedded tissue samples were obtained from patients undergoing surgery at the First Affiliated Hospital of Nanchang University between March 2016 and January 2019. The experiments were undertaken with the understanding and written consent of each patient. Written informed
consent from all patients and approval of the hospital ethics review committees were obtained. The HCC cell lines SMMC7721, HepG2, Hep3B, Bel-7402, and Huh7, and normal hepatocellular cell line L02 were obtained from the Cell Bank of Chinese Academy of Medical Science (Shanghai, China). HepG2, Hep3B, and Bel-7402 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), and SMMC7721, Huh7, and L02 cells were cultured in RPMI 1640 medium. Fetal bovine serum (10%) was added to both types of medium, and cells were cultured in an incubator at 37°C and 5% CO2.

**Real-time quantitative PCR**

Total RNA was extracted by the Trizol method (ThermoFisher Scientific). According to Takara’s reverse transcription instructions (Cat. No. RR047A, Takara, Tokyo, Japan), RNA was reverse transcribed into complementary (c)DNA. The ABI7900 system (Applied Biosystems/ThermoFisher Scientific) was used to amplify the cDNA by PCR. The total RNA was 40 cycles at 95°C for 5 minutes, 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The relative levels of gene expression were evaluated by using the 2−ΔΔCT method.

**Western blot**

The detailed procedure was described previously. Cells were washed with PBS twice before radioimmunoprecipitation assay (RIPA) lysis solution (100 μL) was added, followed by 5× loading buffer; then, the solution was fully mixed and boiled for 5 minutes at 95°C, before being preserved at −20°C. Protein concentration was determined using the Protein Concentration Assay Kit (Keygene, Nanjing, China). In the assay, 30 μg of protein sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and sealed with 5% skimmed milk powder for 2 hours. After adding the primary antibody, the membranes were shaken overnight at 4°C. The membranes were washed three times with Tris-buffered saline-Tween (TBST), incubated with secondary antibodies for 2 hours at room temperature, washed three times with TBST, and then developed and exposed using enhanced chemiluminescence.

**Lentivirus package**

The knockdown and overexpression lentivirus of lncRNA MACC1-AS1 and control virus were packaged by GenePharma (Shanghai, China), and named len-MS1-kd and len-MS1-OE, respectively. The infection efficiency was examined by RT-qPCR assay.

**MiR-145 mimics, inhibitor, and negative control, and transfection**

The miR-145 mimics, inhibitor, and negative control (NC) were purchased from GenePharma, and the concentration of 50 nM was used for transfection using Lipofectamine 3000 reagent.

**Spheroid-forming analysis**

The detailed procedure was described previously. Cells were inoculated at a density of 1 × 104 cells/mL in serum-free DMEM/F12 containing 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF), and 2% B27 and cultured in ultra-low-adsorption plates. The production of suspended cell spheres was determined, and sphere size was measured under a microscope after 2 weeks of culture.
**ALDH1 activity assay**

ALDH1 activity was determined using ALDH Activity Assay Kit (Abcam) following the manufacturer’s recommendation.

**Luciferase reporter analysis**

The MACC1-AS1 sequences with wild-type (wt) or mutant (mut) binding sites of miR-145 were inserted into pMIR-Reporter vector (Addgene, Promega, Madison, WI, USA) and named MACC1-AS1-wt and MACC1-AS1-mut, respectively. HCC cells were transfected with MACC1-AS1-wt or MACC1-AS1-mut, Renilla luciferase plasmid, miR-145-5p mimics, inhibitor, or NC using Lipofectamine 3000. The cells were harvested after 72 hours, and luciferase activity was assessed using a dual-luciferase reporter assay system (Promega).

**RNA pull-down assay**

miR-145 probes coupled with biotin were synthesized by GenePharma. Cells were lysed in RNA immunoprecipitation lysis buffer and incubated with streptavidin-coated magnetic beads. Then, the lysates were incubated with probe-coated beads and washed using wash buffer. Real-time quantitative PCR was used to detect MACC1-AS1 expression in the RNA complex bound to the beads.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism software (version X; GraphPad Inc., La Jolla, CA, USA). The Student’s t-test was used to measure differences between two groups. *P* < 0.05 was regarded as statistically significant.

**Results**

**LncRNA MACC1-AS1 expression is significantly upregulated in HCC tissues and cells**

We found that MACC1-AS1 expression was significantly upregulated in HCC tissues and cells (*P* < 0.01; Figure 1a and b). MACC1-AS1 expression was highest and lowest in Huh7 and SMMC7721 cells, respectively. Thus, in a subsequent experiment, MACC1-AS1 was knocked down in

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**Figure 1.** LncRNA MACC1-AS1 expression was significantly upregulated in HCC tissues and cells. (a) RT-qPCR assay on MACC1-AS1 expression was constructed in clinical samples (HCC and adjacent tissues). (b) MACC1-AS1 level was detected in different HCC cells (HepG2, SMMC7721, Bel-7402, Hep3B, and Huh7) and normal hepatic cells (L02). **P** < 0.01. LncRNA, long noncoding RNA; MACC1-AS1, MACC1 antisense RNA 1; RT-qPCR, real-time quantitative PCR; HCC, hepatocellular carcinoma.
Huh7 cells and overexpressed in SMMC7721 cells to evaluate its effects.

**LncRNA MACC1-AS1 increases the stemness in HCC cells**

As CSCs play important roles in and are considered the root of tumor progression, we assessed whether MACC1-AS1 could regulate the stemness of HCC cells. We found that knockdown of MACC1-AS1 downregulated the mRNA levels of the stemness master regulators Nanog, Oct4, and Sox9, whereas overexpression of MACC1-AS1 upregulated the mRNA levels of the stemness master regulators (Figure 2a). Protein levels of the stemness master regulators showed consistent results (Figure 2b). The infection efficiency was confirmed by RT-qPCR (Figure 2c).

**LncRNA MACC1-AS1 binds directly to and downregulates miR-145**

Because a previous study indicated that MACC1-AS1 could antagonize miR-145 function, we assumed that this MACC1-AS1/miR-145 axis existed in HCC cells. As expected, MACC1-AS1 negatively regulated miR-145 level in HCC cells by RT-qPCR assay ($P < 0.01$; Figure 3a). Conversely, miR-145 exerted the same effect on MACC1-AS1 expression (Figure 3b). Additionally, MACC1-AS1 was significantly enriched in RNA complex pulled down by miR-145 probes (Figure 3c). Transfection of miR-145 mimics decreased MACC1-AS1-wt activity, and transfection of miR-145 inhibitor increased MACC1-AS1-wt activity (Figure 3d). These findings demonstrated that lncRNA MACC1-AS1 could directly

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**Figure 2.** LncRNA MACC1-AS1 promotes the stemness of HCC cells. (a) RT-qPCR analysis on the expression of stemness master regulators (Nanog, Oct4, and Sox9) was performed in HCC cells with MACC1-AS1 overexpression (SMCC7721) or knockdown (Huh7). (b) Protein levels of the stemness master regulators were examined in the cells depicted in (a). (c) The infection efficiency of len-MS1-kd (knockdown) and len-MS1-OE (overexpression) was confirmed by RT-qPCR assay. (d and e) The size and number of spheres were evaluated in HCC cells treated as indicated. (f) ALDH1 activity was determined in the cells described in (a). **$P < 0.01$. LncRNA, long noncoding RNA; MACC1-AS1, MACC1 antisense RNA 1; HCC, hepatocellular carcinoma; RT-qPCR, real-time quantitative PCR; ALDH1, aldehyde dehydrogenase isoform 1.
bind to miR-145 and downregulate it in HCC cells.

**miR-145 overexpression reduces the stemness of HCC cells**

We then determined whether miR-145 had the opposite effect on HCC cell stemness. As shown in Figure 4a–4e (P < 0.01), miR-145 overexpression negatively regulated the expression of stemness master regulators, spheroid formation ability, and ALDH1 activity in clinical samples (Figure 4f) and cells (Figure 4g) in the same way that MACC1-AS1 did, and miR-145 expression was negatively correlated with MACC1-AS1 expression in clinical samples (Figure 4h).

**MACC1-AS1 regulates the stemness of HCC cells dependent on miR-145 expression**

Finally, we determined whether the regulation of stemness of HCC cells by

![Figure 3. LncRNA MACC1-AS1 binds directly to miR-145 and downregulates its level. (a) The level of miR-145 was determined in HCC cells treated as indicated. (b) RT-qPCR assay on miR-145 and MACC1-AS1 levels were performed in HCC cells treated as indicated. (c) MACC1-AS1 level was detected in RNA complex pulled down by miR-145 probe or control probe in HCC cells. (d) Luciferase activity of MACC1-AS1-wt and MACC1-AS1-mut was determined in HCC cells with miR-145 overexpression (OE) or knockdown (kd). ***P < 0.01. LncRNA, long noncoding RNA; MACC1-AS1, MACC1 antisense RNA 1; miR-145, microRNA-145; HCC, hepatocellular carcinoma; RT-qPCR, real-time quantitative PCR; wt, wild-type; mut, mutant.]
MACC1-AS1 was dependent on miR-145 expression. miR-145 was overexpressed in SMMC7721 cells with MACC1-AS1 overexpression, and knocked down in Huh7 cells with MACC1-AS1 knockdown. Transfection efficiency was confirmed by RT-qPCR analysis (P < 0.01; Figure 5a). We found that miR-145 ectopic expression rescued the regulation of HCC cell stemness by MACC1-AS1 (Figure 5b–5f). Collectively, these results indicated that lncRNA MACC1-AS1 promoted the stemness of HCC cells by regulating miR-145 activity.

Discussion

HCC is the sixth most common malignant tumor in the world, and most patients undergo relapse, metastasis, and drug resistance after treatment. Therefore, there is an urgent need to find new diagnostic markers and therapeutic targets for HCC.

There are HCC stem cells in HCC, which participate in the occurrence, development, and relapse of HCC, as well as drug resistance. However, the regulatory mechanism of HCC stem cells is not clear. Recently, evidence has shown that many lncRNAs are not only highly expressed in CSCs, but also play an important role in regulating the biological functions of CSCs. Similar to other tumors, many lncRNAs show abnormal expression in HCC, including REIH, MALAT1, HULC, and HOTAIR. Therefore, the study of HCC-related lncRNAs may help identify early therapeutic targets of HCC. In this study, we found that expression of
lncRNA MACC1-AS1 was increased in HCC tissues and cells. The regulation of CSCs is a complex process, involving a variety of signaling pathways, such as the Wnt/β-catenin, Notch, transforming growth factor (TGF)-β, and interleukin (IL)-6/STAT3 signaling pathways. Previous studies have shown that miR-145 can enhance the chemosensitivity of glioma stem cells to demethoxycurcumin and regulate the epithelial–mesenchymal transition (EMT) process in osteosarcoma. Additionally, lncRNA Linc-DYNC2H1-4 promotes CSC phenotypes by acting as a sponge of miR-145 in pancreatic cancer cells. Moreover, evidence has shown that miR-145 regulates the normal stem cell progression in various tissues. These results suggest that miR-145 plays critical roles in CSC progression. Here, we revealed that miR-145 could suppress the stemness of HCC cells. Moreover, miR-145 is necessary for MACC1-AS1–mediated regulation of HCC cell stemness. However, the downstream effectors of the MACC1-AS1/miR-145 axis are still unclear in HCC stem cell progression. Notably, a recent study showed that miR-145 can target Smad2, and the miR-145/Smad2 axis is necessary for MACC1-AS1–mediated promotion of nasopharyngeal carcinoma cell stemness. This mechanism might exist in HCC and should be explored in the future.

In this study, we found that lncRNA MACC1-AS1 is highly expressed in HCC tissues and cells and positively regulates the stemness of HCC cells by binding to miR-145. Our findings provide a new theory for the mechanism underlying the
regulation of HCC stem cells and a new target for diagnosis and treatment of HCC.

**Declaration of conflicting interest**

The authors declare that there is no conflict of interest.

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