COL14A1 promotes self-renewal of human liver cancer stem cells through activation of ERK signaling

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Abstract

Objective: Liver cancer stem cells (CSCs) are the culprits of hepatocellular carcinoma metastasis and recurrence. Only by eliminating tumor stem cells can malignant tumors be fundamentally cured. This study aimed to identify the role and underlying mechanism of aberrant Collagen Type XIV Alpha 1 Chain (COL14A1) overexpression in liver CSCs, and improve understanding of the molecular basis of hepatocellular carcinoma metastasis and recurrence.

Methods: First, quantitative real-time polymerase chain reaction was used to confirm aberrant high-expression of COL14A1 in liver CSCs. Next, interference experiments were performed to determine the key role of COL14A1. To explore the mechanism of COL14A1 overexpression in liver CSCs, putative microRNA (miRNAs) targeting COL14A1 were analyzed using the miRTarBase database. Next, quantitative real-time polymerase chain reaction, western blotting, and luciferase reporter assays were performed to verify the interaction between miR-7108-3p and COL14A1. Lastly, key target proteins of the COL14A1-extracellular-regulated signal kinase (ERK) signaling pathway were identified through western blotting analysis. This study was approved by the Ethics Committee of Shanghai Fourth People’s Hospital, Tongji University School of Medicine, China (approval No. 2019tjdx17) on February 21, 2019.

Results: COL14A1 is abnormally highly expressed in liver CSCs, which is necessary for liver CSCs to maintain their self-renewal capability. Mechanistically, COL14A1 is post-transcriptionally regulated by miR-7108-3p in a negative manner. Low expression of miR-7108-3p increased translation of COL14A1, which subsequently activated ERK signaling, ultimately maintaining the self-renewal and stem cell-like properties of liver CSCs.

Conclusion: COL14A1, which is negatively regulated by miR-7108-3p, was found to play a crucial role in maintaining the self-renewal and stem cell-like properties of liver CSCs through activation of ERK signaling.

Keywords: COL14A1, ERK signaling, hepatocellular carcinoma, liver cancer stem cells, miR-7108-3p

Introduction

Hepatocellular carcinoma (HCC), representing the major subtype of primary liver cancer, is one of the malignancies with the highest morbidity and mortality globally.¹ Due to insidious onset and the low effectiveness of screening methods, 60% to 70% of HCC patients are already in the advanced stage at the time of diagnosis, and their 5-year survival rate is less than 10%.² High recurrence and low survival, characteristic for HCC, are closely related to tumor heterogeneity.³ It is believed that a subset of tumor cells, called cancer stem cells (CSCs), construct a hierarchical tumor of heterogeneous cancer cells.⁴ CSCs share many characteristics with normal stem cells, including self-renewal ability and multi-directional differentiation potential. Importantly, CSCs play a crucial role in tumor deterioration and treatment resistance. Although many aberrantly expressed genes have been identified in liver CSCs in recent years,⁵ it is believed that a subset of tumor cells, called cancer stem cells (CSCs), construct a hierarchical tumor of heterogeneous cancer cells.⁴

Methods

Objective: Liver cancer stem cells (CSCs) are the culprits of hepatocellular carcinoma metastasis and recurrence. Only by eliminating tumor stem cells can malignant tumors be fundamentally cured. This study aimed to identify the role and underlying mechanism of aberrant Collagen Type XIV Alpha 1 Chain (COL14A1) overexpression in liver CSCs, and improve understanding of the molecular basis of hepatocellular carcinoma metastasis and recurrence.

Materials and methods

Oncomine platform

Oncomine (www.oncomine.org) is a large database of tumor gene chips with 715 datasets and 86,733 samples, integrating RNA and DNA-seq data from sources such as GEO, TCGA and
published literature. The transcript levels of COL14A1 in various cancers were analyzed and assessed relative to that in normal tissue with the Oncomine 4.5 database.

**Cell culture**

Primary HCC cells (1#, 2#, 3#, 4#) were prepared from tumor tissues of four male HCC patients (50–70 years old) using a Human Tumor Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Informed consent was obtained from volunteers undergoing surgical resection in the Eastern Hepatobiliary Surgery Hospital (Shanghai, China). Four HCC cell lines (Hep3B, Huh7, HepG2, SMMC7721) and HEK293T cells were obtained from the Chinese Academy of Sciences Cell Bank (Beijing, China). For adherent cell culture, cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Corning, Corning, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) at 37°C and 5% CO2 conditions. For suspension CSC culture or tumor spheroid formation, 1 × 10^5 tumor cells were trypsinized with Trypsin (0.25%; Gibco) as single-cell suspensions and seeded into 6-well plates treated with poly(2-hydroxyethyl methacrylate) (Poly-HEMA; Gibco) as single-cell suspensions and seeded into 6-well plates treated with poly(2-hydroxyethyl methacrylate) (Poly-HEMA; Sigma, St. Louis, MO, USA) in DMEM/F12 (1:1) (Hyclone, Logan, UT, USA) with 20ng/mL insulin growth factor (IGF; PeproTech), 1.0ng/mL basic fibroblast growth factor (bFGF; PeproTech), and 20ng/mL epidermal growth factor (EGF; PeproTech) under 37°C and 5% CO2 conditions for 1 week. This study was approved by the Ethics Committee of Shanghai Fourth People’s Hospital, Tongji University School of Medicine, China (approval No. 2019txjd17) on February 21, 2019.

**Short hairpin RNA (shRNA) plasmid construction**

Sense and antisense sequences of 21-mer targets of the COL14A1 gene (Table 1; shCOL14A1#1 and shCOL14A1#2; Tsingke, Beijing, China) were inserted into oligos compatible with pLKO.1-puro empty vector (Sigma). A corresponding scrambled sequence (See Table 1, scramble; Tsingke) was used to construct the control plasmid. Next, annealed oligos were ligated into a linearized pLKO.1 vector (digested with AgeI and EcoRI). A screen for successfully ligated plasmids was performed using Sanger sequencing.[11]

**Cell transfection**

Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) was used to transiently transfet plasmids and mRNA inhibitors or inhibitors of miR-7108-3p and negative control (GenePharma, Shanghai, China) into Huh7 and HEK293T cells at 60% to 80% confluency. In addition, lentivirus was used to establish cell lines with stable expression as previously described.[5] Cells were incubated with infectious lentiviruses for about 12hours, and with stable expression as previously described.[5] Cells were incubated with infectious lentiviruses for about 12hours, and with stable expression as previously described.[5] Cells were incubated with infectious lentiviruses for about 12hours, and with stable expression as previously described.[5] Cells were incubated with infectious lentiviruses for about 12hours, and with stable expression as previously described.[5] Cells were incubated with infectious lentiviruses for about 12hours, and with stable expression as previously described.[5] Cells were incubated with infectious lentiviruses for about 12hours, and with stable expression as previously described.[5] Cells were incubated with infectious lentiviruses for about 12hours, and with stable expression as previously described.[5] Cells were incubated 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**Cell apoptosis detection**

Hep3B and HuH7 cells at 80% to 90% confluency were trypsinized with Trypsin (0.25%; Gibco, Gaithersburg, MD, USA) into single cells to detect apoptotic cells with an Annexin V-FTTC/PI Apoptosis Detection Kit (Elabscience, Wuhan, China). Fluorescence of annexin V bound to apoptotic cells was quantitatively determined by flow cytometry (BD FACSCanto™ II; BD Biosciences, San Jose, CA, USA).

**Cell proliferation assay**

A total of 4 × 10^4 Hep3B or HuH7 cells were seeded into 24-well plates in advance. When Hep3B or HuH7 cells reached about 60% confluency, a Click-i™ EdU Cell Proliferation Assay Kit (Invitrogen) was used to detect proliferation according to the manufacturer’s recommended protocol. Fluorescent images were obtained using OLYMPUS IX73 and acquisition software (Olympus, Tokyo, Japan).

**Luciferase reporter assay**

The 3′ untranslated region (3′UTR) of COL14A1 with a wild-type (WT) or mutant target site of miR-7108-3p was cloned into the pmirGLO vector (Tsingke) to generate a WT or mutated pmirGLO-COL14A1-3′UTR reporter. The sequence (position 1450-1749) of wild-type COL14A1-3′UTR was synthesized as follows: TTTCTGTCCCTTCTCAATAATCTCCCTGATCTTTGGAGATGAAGATCTCCTTATCTTCATCTGAAGGGATTCTCAAGAAGTCTCATTCCGAGGCTTTGAAAGGGCGGAGGAGGAGGGAGGTTCAGTTTGAAAGGGAATTCTTATTATCTGGACTCACATCTGGTCTTGGTCACCTGAGGAAATTGAAAGGATAATAGACGATAATACGAGCGCATTTCTCTATATGTTATTTTTGGTCAGTGACGAGATAGAAGAAGAAGAAGAAATACTGGTCTTGGTTTACATATTATCTGGTCTTGGTCACCTCGCTGAGAAGCAGGATTTATGGGAAAGAAACATTGGTCT. Mutated COL14A1-3′UTR was cloned with substitution of miR-7108-3p target site (position 1597-1603). The reporter and pRL-TK vector were subsequently co-transfected into HEK293T cells with miR-7108-3p mimic or inhibitor using Lipofectamine 3000 (Invitrogen). At 48 hours post-transfection, cells were collected for luciferase activity detection using a Dual Luciferase Reporter Gene Assay Kit (KeyGen Biotech, Nanjing, China). Mimic and inhibitor of miR-7108-3p or negative control (Table 1) were synthesized by GenePharma (Shanghai, China).

**Statistical analysis**

Statistical analysis was performed by GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as mean±standard deviation. Differences between groups were evaluated using two-tailed Student’s t-tests. P < 0.05 was considered statistically significant.

**Results**

**COL14A1 was overexpressed in liver CSCs**

For cancer patients, the biggest fear is that malignant tumor cells continue to metastasize and spread. The reason metastasis occurs is rooted in the tumor seed, that is, CSCs left in the body following removal of primary lesions.[12] In view of the vital role of CSCs in various cancers, we have performed a lot of work in this area,[5,13,14] and identified many aberrantly expressed molecules in CSCs. COL14A1, a molecule overexpressed in a variety of cancers (Fig. 1A), especially HCC tumors (Fig. 1B), was notable among them. Through suspension culture,[14] we confirmed that COL14A1 was significantly upregulated in CSCs of both HCC primary cells (Fig. 1C, top panel) and HCC cell lines (Fig. 1C, bottom panel).

**COL14A1 was required for liver CSCs to maintain their self-renewal capability**

To explore the function of COL14A1 in liver CSCs, we constructed two shRNAs to silence COL14A1. Both shRNAs achieved good knockdown efficiency in HCC cell lines Hep3B and HuH7 (Fig. 2A). Notably, both mRNA and protein expression of the pluripotency transcription factor NANOG was significantly attenuated with COL14A1 depletion (Fig. 2B). Additionally, COL14A1 knockdown remarkably increased cell apoptosis (Fig. 2C) and impaired cell proliferation (Fig. 2D) of Hep3B and HuH7 cells. More importantly, tumor sphere formation, the most direct and powerful assay to evaluate the self-renewal capability of CSCs, was greatly inhibited by knockdown of COL14A1 expression (Fig. 2E). Collectively, these results clearly indicate that COL14A1 was required for the maintenance self-renewal in liver CSCs.

**miR-7108-3p regulated COL14A1 expression in liver CSCs**

To elucidate the mechanism of COL14A1 overexpression in liver CSCs, we analyzed miRNAs targeting COL14A1 mRNA. Twelve miRNAs experimentally validated to have interactions with COL14A1 mRNA have been reported in the miRTarBase database. We tested expression of these twelve miRNAs in liver CSCs, and found that miR-7108-3p was significantly down-regulated in both HCC primary cells (Fig. 3A, top panel) and the HuH7 cell line (Fig. 3A, bottom panel). Next, we performed miRNA overexpression and inhibition experiments to verify that regulation occurs. COL14A1 expression was effectively inhibited by miR-7108-3p mimic (Fig. 3B) and remarkably enhanced by its inhibitor (Fig. 3C). To further confirm the interaction, we constructed two luciferase reporter plasmids containing the 3′UTR of COL14A1 mRNA and either the wild-type (pmirGLO-COL14A1-3′UTR-WT) or mutated (pmirGLO-COL14A1-3′UTR-Mut) putative miR-7108-3p binding site, respectively. As a result, a significant change was only observed in the luciferase activity of pmirGLO-COL14A1-3′UTR-WT, not the mutated pmirGLO-COL14A1-3′UTR-Mut (Fig. 3D). Collectively, these results suggest that lower expression of miR-7108-3p allowed more COL14A1 to be expressed in liver CSCs.

**COL14A1 activated ERK signaling in liver CSCs**

Analysis using GeneCards (https://pathcards.genecards.org/) revealed five potential superpathways for the COL14A1 gene. Notably, ERK signaling, a critical pathway in normal cells and for stemness of certain cancer cells,[15] was among them. We found that ERK signaling was extremely activated in liver CSCs (Fig. 4A). To verify the interaction between COL14A1 and ERK signaling, we examined protein levels of total ERK1/2 (ERK1/2) and phosphorylated extracellular signal-regulated kinase (p-ERK1/2). We observed a significant change in the percentage of p-ERK1/2 to total ERK1/2 in COL14A1-silenced Hep3B and HuH7 cells compared with control cells (Fig. 4B). This finding strongly indicates that COL14A1 maintained the self-renewal of CSCs in HCC through ERK signaling.
Discussion

In many solid tumors, only the population of CSCs (a minority among all cancer cells) could maintain a tumor, while the majorities were terminally differentiated and lost clonogenic potential.[16,17] Nevertheless, research directly confirming the existence of CSCs in human cancer was recently reported by Melenhorst et al,[18] who identified a shocking clinical case of leukemic relapse after chimeric antigen receptor (CAR) T-cell immunotherapy. Strikingly, the cancer cells that caused the patient’s death all came from a single edited leukemic B cell, which was unintentionally introduced by the CAR gene. Therefore, CSCs are critical for tumor metastasis and recurrence.

CSCs, like normal pluripotent stem cells, have the ability to self-renew and differentiate into various types of cells.[19] CSCs maintain their stemness through multiple mechanisms, including activation of stem-cell promoting signaling[20] and inactivation of pathways inhibiting stem-cell characteristics.[21] Here, we showed that COL14A1 (negatively regulated by miR-7108-3p) could promote the self-renewal of liver CSCs through the ERK signaling pathway, which has been found to regulate NANOG expression and enhance CSC-like phenotypes (Fig. 5). Based on this, therapy targeting COL14A1-ERK signaling could contribute to the elimination of liver CSCs, thus inhibiting tumor recurrence to improve the survival rate of HCC.

CSC characteristics are regulated by transcription factors,[23] epigenetic regulators,[24] and extrinsic and intrinsic cell signaling factors.[25] It has been reported that collagens are aberrantly expressed in the tumoral extracellular matrix,[26] whereby they actively drive tumor growth and invasiveness.[27] In gastric cancer, as a component of extracellular matrix, COL14A1 exhibited high prevalence of nonsynonymous mutations.[28] Furthermore, an association between altered expression of COL14A1 and hepatic fibrosis has been confirmed.[29] However, the mode by which this extracellular collagen participated in HCC development and self-renewal of liver CSCs remained unknown. Here, we showed that COL14A1, which is upregulated in liver CSCs, is required to maintain their self-renewal
property through activation of ERK signaling by increasing p-ERK1/2. Our results suggest that extracellular collagens represent an important layer for regulation of oncogenesis. However, how COL14A1 increases levels of p-ERK1/2 in liver CSCs remains to be further investigated to uncover this regulation.

However, this study had some limitations. Our observations of the biological function of COL14A1 were based on ex vivo cell culture, which may not be able to express the complexity of in vivo situation. Therefore, the potential of COL14A1 as a therapeutic target for HCC treatment needs to be well evaluated in animal models and clinical trials.
In conclusion, our results show that COL14A1 plays a crucial regulatory role in liver CSCs. Interfering with COL14A1 expression had a striking effect on self-renewal of liver CSCs. Further research demonstrated that COL14A1 enhances phosphorylation of ERK protein to activate ERK signaling, which elevates NANOG expression and promotes CSC self-renewal. Our research provides a new target and theoretical basis for liver CSC elimination, as well as curbing HCC metastasis and recurrence.

**Figure 3.** miR-7108-3p regulated COL14A1 expression in liver CSCs. (A) miR-7108-3p, but not the other eleven miRNAs examined, was remarkably downregulated in CSCs of both HCC primary cells and Huh7 cell line compared with the matched cells. Cells were HCC cells in adherent culture, while CSCs were matched CSCs obtained by suspension culture. (B) miR-7108-3p mimic remarkably decreased COL14A1 RNA and protein expression when compared with control mimic (*P < 0.05, **P < 0.01). (C) miR-7108-3p inhibitor remarkably increased COL14A1 RNA and protein expression when compared with control inhibitor (*P < 0.05, **P < 0.01). (D) Luciferase activities of pmirGLO-COL14A1-3’UTR-WT and pmirGLO-COL14A1-3’UTR-Mut were analyzed when co-transfected with miR-7108-3p mimic (middle panel) or miR-7108-3p inhibitor (right panel), compared with the matched control mimic and control inhibitor, respectively. U6 snRNA and β-actin served as the control gene for miRNA and mRNA, respectively. Data are presented as mean ± SD, and derived from three independent experiments. *P < 0.05, **P < 0.01 (two-tailed Student’s t-test). COL14A1 = Collagen Type XIV Alpha 1 Chain, CSC = cancer stem cell, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, HCC = hepatocellular carcinoma, snRNA = small nuclear RNA.
Acknowledgement

None.

Author contributions

SL supervised the study. SL and RK designed the study and wrote the manuscript. RK, HL, YS and QM performed the experiments and analyzed the data. All authors edited the manuscript and approved the final version of the manuscript.

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Institutional review board statement

This study was approved by the Ethics Committee of Shanghai Fourth People’s Hospital, Tongji University School of Medicine, China (approval No. 2019tjdx17) on February 21, 2019.

Conflicts of interest

The authors declare that they have no conflicts of interest.

References


