LncRNA DLGAP1-AS2 SiRNA Silencing Inhibits Hepatocellular Carcinoma Cell Migration and Invasion by Up-regulating MiR-154-5p Through Methylation

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Abstract

Background:DLGAP1-AS2 has been characterized as an oncogenic lncRNA in glioma. This study was performed to explore the role of DLGAP1-AS2 in hepatocellular carcinoma (HCC).

Methods:Expression of DLGAP1-AS2 and miR-154-5p in paired HCC and non-tumor tissues from 62 HCC patients was determined by RT-qPCR. The 62 HCC patients were followed up for 5 years to analyze the prognostic value of DLGAP1-AS2 for HCC. DLGAP1-AS2 siRNA silencing and miR-154-5p overexpression was achieved in HCC cells to study the relationship between them. Methylation of miR-154-5p was analyzed by methylation-specific PCR. Cell proliferation was analyzed by CCK-8 assay.

Results: DLGAP1-AS2 was upregulated in HCC and predicted poor survival. MiR-154-5p was downregulated in HCC and inversely correlated with DLGAP1-AS2. In HCC cells, DLGAP1-AS2 siRNA silencing resulted in the upregulation of miR-154-5p and decreased methylation of miR-154-5p gene. Transwell assay showed that, DLGAP1-AS2 siRNA silencing and miR-154-5p overexpression inhibited cell invasion and migration, and the combination of LGAP1-AS2 siRNA silencing and miR-154-5p overexpression showed stronger effects.

Conclusion: DLGAP1-AS2 siRNA silencing may inhibit HCC cell migration and invasion by up-regulating miR-154-5p through methylation.

Introduction

Liver cancer is a common malignancy for both incidence and mortality (Siegel et al. 2020). It is reported that liver cancer in 2018 caused a total of 781,631 deaths, which were the 8.2% of all cancer deaths, and affected a total of 841,080, accounting for 4.7% of all new cancer cases (Bray et al. 2018). Hepatocellular carcinoma (HCC) is the major subtype of liver cancer (McGlynn et al. 2020). It is reported that the median survival time of HCC patients is only 6 to 20 months, and only less than 10% of HCC patients can survive for more than 5 years after the initial diagnosis (Wallace et al. 2019). Therefore, novel treatments are needed to improve the survival of HCC patients. Hepatitis B or Hepatitis C infections, alcohol consumptions and obesity are the main risk factors for HCC (Caldwell et al. 2004; Morgan et al. 2004; Rongrui et al. 2014). However, molecular pathogenesis of HCC is still unclear. Therefore, the development of novel anti-HCC approaches is limited.

It has been well established that the development and progression of HCC requires the involvement of molecular alterations (Ho et al. 2016; Tornesello et al. 2016). Functional characterization of these molecular factors facilitates the development of novel therapies, such as targeted therapy that can be applied to suppress cancers by regulation cancer-related gene expression network (Llovet et al. 2008). Despite of the lacking of protein-coding capacity, non-coding RNAs (ncRNAs), such as miRNAs and lncRNAs, regulate cancer progression by affecting gene expression (Farazi et al. 2011; Yang et al. 2014). Therefore, ncRNAs are promising targets for cancer treatment and function analysis of the roles of ncRNAs is required. DLGAP1-AS2 is a recent study has been characterized as an oncogenic lncRNA in

glioma (Miao et al. 2020), while its role in other cancers is unknown. Our deep sequencing analysis revealed the alteration of DLGAP1-AS2 in HCC and its inverse correlation with miR-154-5p (data not shown), which is also a critical player in cancer biology (Wang et al. 2017). This study was therefore carried out to analyze the interactions between DLGAP1-AS2 and miR-154-5p in HCC.

Materials And Methods

HCC patients

At Affiliated Hospital of Chengde Medical University, hospital, we enrolled 62 patients (36 males and 26 females) from May 2012 to January 2015. Age of the patients ranged from 42 years to 68 years (54.8 ± 5.8 years). Ethics Committee of the aforementioned hospital approved this study. All these HCC patients were confirmed by histopathological exam. No recurrent HCC patients were included, and all the 62 HCC patients were newly diagnosed cases. It is known that gene expression may be affected by therapies and other clinical disorders, this study excluded patients complicated with other clinical disorders and the ones with initiated therapy. Based on medical record, the 62 patients included 27 cases of HBV positive, 26 cases of HCV positive, and 9 cases of negative for both. All patients signed informed consent.

Treatment and follow-up

The 62 patients were classified into AJCC stage I and II ($n=28$), and III and IV ($n=34$). Treatment approaches were determined based on AJCC stages and the health conditions of patients. The 62 patients were followed up for 5 years through telephone in a monthly manner. Patients' survival conditions were recorded. In survival analysis, we excluded the patients died of causes unrelated to HCC.

HCC tissues and cells

Fine needle aspiration was performed to obtain HCC and paired non-tumor tissues from the 62 HCC patients. After histopathological confirmation, all tissue samples were immediately subjected to RNA isolation.

HCC cell line SNU-398 (ATCC, USA) was included. RPMI 1640 (90%) was mixed with FBS (10%) to serve as culture medium for SNU-398 cells. In a 5% CO₂ and 95% humidity incubator cells were cultivated at 37 °C.

Transient transfections

DLGAP1-AS2 siRNA and negative control (NC) siRNA, as well as NC miRNA and mimic of miR-154-5p were all purchased from Invitrogen (Shanghai, China). Through lipofectamine 2000 (Invitrogen)-mediated transient transfections, SNU-398 cells were transfected with 40 nM siRNA or miRNA. To perform NC experiments, cells were transfected with either NC siRNA or NC miRNA. Incubation with transfection mixture was performed for 6h, followed by cell culture in fresh medium for further 48h. To perform Control (C) experiments, untransfected cells were cultivated in fresh medium for 54h.

RNA isolation and process

SNU-398 cells and paired tissue samples were subjected to RNA isolations, which were performed using RNAzol (Invitrogen). Incubation with DNase I (Invitrogen) was performed for 100 min at 37°C to achieve complete genomic DNA removal. Ratios of OD260/280 were determined to check RNA purity.

RT-qPCR

Reverse transcriptions (RTs) were performed using RNA samples with a ratio of OD260/280 with SSRT IV system (Invitrogen) to prepare cDNA samples. All cDNAs were subjected to qPCRs using GeneRead qPCR SYBR Green Mastermix (QIAGEN) to determine the expression of DLGAP1-AS2. Internal control of DLGAP1-AS2 was 18S rRNA. To determine the expression of miR-154-5p, mature miRNAs were added with poly (A), followed by miRNA RTs and qPCRs. U6 was used as internal control of miR-154-5p. Each experiment was performed in three technical replicates and method of $2^{-\Delta\Delta C_T}$ was used for Ct value normalizations.

Methylation-specific PCR (MSP)

Genomic DNAs were extracted from SNU-398 cells with DLGAP1-AS2 siRNA silencing. Genomic DNA Extraction Kit (ab156900, Abcam) was used. DNA samples were converted using EZ DNA Methylation Lighting Kit (ZYMO research, followed by PCRs to determine the methylation of miR-154-5p.

Transwell assays

Invasion and migrations of SNU-398 cells were determined using Transwell filters (8 µm, Dojindo). To perform invasion assay, Matrigel (Dojindo) was used to coat membranes for 12h at 37 °C to mimic in vivo invasion conditions. In contrast, uncoated membranes were used in migration assay. Cells were transferred to upper Transwell chamber with 5000 cells in 0.1 ml serum-free medium, and the lower chamber was filled with medium containing 20% FBS to induce cell migration and invasion. At 37 °C cells were cultivated for 12h and the upper surface of membranes was cleaned with a cotton swab. Crystal violet (0.1%) was used to stain the lower surface of membranes for 20 min. A light microscope was used to observe cells.

Statistical analysis

Gene expression levels in paired tissues were expressed as average values of 3 technical replicates and were compared by paired t test. Data of multiple transfection groups were expressed as mean +/- SD values of 3 biological replicates and were compared by ANOVA Tukey's test. Correlations were analyzed by linear regression. With median level of DLGAP1-AS2 expression in HCC tissues as a cutoff value, the 62 patients were divided into high and low DLGAP1-AS2 level groups (n=31). Survival curves were plotted and compared by log-rank test. $P < 0.05$ was deemed statistically significant.

Results

High level of DLGAP1-AS2 in HCC predicted poor survival

Expression of DLGAP1-AS2 in paired HCC and non-tumor tissues was determined by RT-qPCR. Compared to non-tumor tissues, HCC tissues exhibited significantly higher levels of DLGAP1-AS2 expression (Fig.1A, $p < 0.001$). Survival curve analysis showed that patients in high DLGAP1-AS2 level group exhibited significantly higher mortality rate in comparison to patients in low DLGAP1-AS2 level group (Fig.1B). Therefore, DLGAP1-AS2 is overexpressed in HCC and its high expression levels in HCC predicted poor survival. It is worth noting that HBV and HCV infections failed to significantly affect the expression of DLGAP1-AS2 (data not shown).

MiR-154-5p is downregulated in HCC and was inversely correlated with DLGAP1-AS2

Expression of miR-154-5p in paired HCC and non-tumor tissues was determined by RT-qPCR. Compared to non-tumor tissues, HCC tissues exhibited significantly lower levels of miR-154-5p expression (Fig.2A, $p < 0.001$). Correlation analysis showed that miR-154-5p was inversely correlated with DLGAP1-AS2 across HCC tissues (Fig.2B), but not non-tumor tissues (Fig.2C). Therefore, DLGAP1-AS2 and miR-154-5p may interact with each other in HCC.

DLGAP1-AS2 siRNA silencing upregulated miR-154-5p through methylation

To explore the interaction between DLGAP1-AS2 and miR-154-5p, SNU-398 cells were transfected with DLGAP1-AS2 siRNA or miR-154-5p mimic, followed by the confirmation of transfections by RT-qPCR (Fig.3A, $p < 0.05$). It was observed that cells with DLGAP1-AS2 siRNA showed significantly higher levels of miR-154-5p expression (Fig.3B, $p < 0.05$). In contrast, cells with miR-154-5p overexpression showed no significant alterations in DLGAP1-AS2 expression (Fig.3C). MSP was performed to analyze the methylation of miR-154-5p. Compared to cells transfected with NC siRNA, cells transfected with DLGAP1-AS2 siRNA silencing showed decreased methylation of miR-154-5p gene (Fig.3D).

DLGAP1-AS2 siRNA silencing and miR-154-5p overexpression inhibited cell invasion and migration

Transwell assay showed that, DLGAP1-AS2 siRNA silencing and miR-154-5p overexpression inhibited cell invasion (Fig.4A) and migration (Fig.4B), and the combination of LGAP1-AS2 siRNA silencing and miR-154-5p overexpression showed stronger effects ($p < 0.05$).

Discussion

This study analyzed the roles of DLGAP1-AS2 and miR-154-5p in HCC and explored the interactions between them. We found that DLGAP1-AS2 was upregulated in HCC and could regulate the methylation of miR-154-5p gene to regulate the invasion and migration of HCC cells.

The functionality of DLGAP1-AS2 has only been characterized in glioma (Miao et al. 2020). DLGAP1-AS2 is overexpressed in glioma and may target Yes Associated Protein 1 to suppress cancer cell apoptosis, but promote cell proliferation and migration (Miao et al. 2020). We in this study observed the

upregulation of DLGAP1-AS2 in HCC and decreased invasion and migration of HCC cells after DLGAP1-AS2 siRNA silencing. Therefore, DLGAP1-AS2 is likely an oncogenic lncRNA in HCC and DLGAP1-AS2 siRNA silencing may be a potential target for the treatment of HCC. Hepatitis B and Hepatitis C infections are the major cause of HCC (Rongrui et al. 2014). However, in this study we found that the expression of DLGAP1-AS2 was not significantly affected by Hepatitis B and Hepatitis C infections. Therefore, DLGAP1-AS2 may participate in HCC through a different pathway.

Most HCC patients were diagnosed at late stages, leading to poor prognosis (Stefaniuk et al. 2010). Effective tumor markers for the early diagnosis of HCC remain lacking, as the consequence, early diagnosis of HCC is unlikely to be improved in near future (Fujiyama et al. 2002). In this study we found that high levels of DLGAP1-AS2 expression in HCC tissues were correlated with the poor survival of HCC patients. Therefore, DLGAP1-AS2 may be used as a tumor marker to assist the prognosis of HCC patients, thereby guiding the determination of treatments and improving patients' survival.

MiR-154-5p plays opposite roles in different types of cancers (Wang et al. 2017; Lin et al. 2018). MiR-154-5p is downregulated in glioma and targets PIWIL1 to suppress tumor growth and metastasis (Wang et al. 2017). In contrast, miR-154-5p is overexpressed in renal cell carcinoma and promotes cancer cell migration and proliferation (Lin et al. 2018). In this study we first reported the upregulation of miR-154-5p in HCC and inhibitory effects on cancer cell invasion and migration. Therefore, miR-154-5p may serve as a tumor suppressor in HCC. The upstream regulator of miR-154-5p in cancer biology has not been reported by previous studies. In this study we showed that DLGAP1-AS2 may regulate the methylation of miR-154-5p to affects its expression, while the mechanism is unknown. Interestingly, miR-154-5p and DLGAP1-AS2 were only correlated across HCC tissues, not non-tumor tissues. Therefore, certain pathological factors may mediate the interaction between them in HCC.

Conclusions

In conclusion, upregulation of DLGAP1-AS2 in HCC predicts the poor survival of HCC patients. In addition, DLGAP1-AS2 may regulate the methylation of miR-154-5p gene to regulate the invasion and migration of HCC cells.

Abbreviations

HCC: Hepatocellular carcinoma;ncRNAs: non-coding RNAs

Declarations

Authors' contributions

Kai Chen conceived of and designed the project. Kai Chen and Zhuqing Zhang performed the experiments, analysed the data. Aijun Yu and Jian Lidone statistical analysis and interpreted the data. Jinlong Liu and Xuejun Zhang helped in histopathological findings for clinical samples. N.S., G.S. and

A.M. wrote the manuscript. Kai Chen correspond the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of the Affiliated Hospital of Chengde Medical University. All patients signed written informed consent before sample collection at the Affiliated Hospital of Chengde Medical University.

Consent for publication

All authors read and agreed to the content of the final manuscript, and consented to publish this material.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

High level of DLGAP1-AS2 in HCC predicted poor survival. Expression of DLGAP1-AS2 in paired HCC and non-tumor tissues was determined by RT-qPCR. Gene expression levels in paired tissues were expressed as average values of 3 technical replicates (A). ***, $p < 0.001$. With median level of DLGAP1-AS2 expression in HCC tissues as a cutoff value, the 62 patients were divided into high and low DLGAP1-AS2 level groups ($n=31$). Survival curves were plotted and compared by log-rank test (B).

Figure 2

MiR-154-5p is downregulated in HCC and was inversely correlated with DLGAP1-AS2. Expression of miR-154-5p in paired HCC and non-tumor tissues was determined by RT-qPCR. Gene expression levels in paired tissues were expressed as average values of 3 technical replicates (A). ***, $p < 0.001$. Linear regression was performed to analyze the correlations between miR-154-5p and DLGAP1-AS2 across HCC tissues (B), but not non-tumor tissues (C).

Figure 3

DLGAP1-AS2 siRNA silencing upregulated miR-154-5p through methylation To explore the interaction between DLGAP1-AS2 and miR-154-5p, SNU-398 cells were transfected with DLGAP1-AS2 siRNA or miR-154-5p mimic, followed by the confirmation of transfections by RT-qPCR (A). The effects of DLGAP1-AS2 siRNA silencing on miR-154-5p (B), or the effects of miR-154-5p overexpression on DLGAP1-AS2 (C) were also analyzed by RT-qPCR. MSP was performed to analyze the methylation of miR-154-5p in cells with DLGAP1-AS2 siRNA or NC siRNA transfection (D). Data of multiple transfection groups were expressed as mean $\pm$ SD values of 3 biological replicates. M, methylation; U, un-methylation. *, $p < 0.05$.

Figure 4

DLGAP1-AS2 siRNA silencing and miR-154-5p overexpression inhibited cell invasion and migration. Transwell assays were performed to analyze the effects of DLGAP1-AS2 siRNA silencing and miR-154-5p overexpression on the cell invasion (A) and migration (B) of SNU-398 cells. Data of multiple transfection groups were expressed as mean $\pm$ SD values of 3 biological replicates. *, $p < 0.05$.