Modifications of The Human Liver Cancer Cells through microRNA-145-Mediated Targeting of CDCA3

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Abstract

Objective: MicroRNA-143 (miR-143) has shown promising tumor-suppressive effects in different types of cancer and could potentially act as a tumor suppressor in liver cancer. To explore this further, the present study aims to investigate the protective function of miR-145 in human liver cancer.

Materials and Methods: In this experimental study, we analyzed miR-145 expression in human tissue samples and liver cancer cell lines using quantitative real time polymerase chain reaction (qRT-PCR). Cancer cell lines were transfected using Lipofectamine 2000. We assessed cell viability of HepG2 liver cancer cell line using the MTT assay and analyzed colony forming potential through clonogenic assay. Flow cytometry was employed to evaluate cell cycle phase distribution in cancer cell lines expressing either miR-145 inhibitor or miR-145 mimics. The motility of cancer cell lines was determined using the transwell chamber assay. Protein expression levels of Cyclin B1 and CDCA3, important for cell cycle progression and cell division, respectively, were measured using western blotting. Finally, we conducted a dual luciferase assay to investigate the interaction between miR-145 and CDCA3.

Results: Downregulation of miR-145 was observed in liver cancer cells. Overexpression of miR-145 through transfection inhibited cancer cell proliferation, while transfection of miR-145 inhibitor increased proliferation rate. MiR-145 overexpression led to cell cycle arrest at the G2/M phase by suppressing cyclin B1 protein expression. Moreover, miR-145 overexpression suppressed migration and invasion of cancer cells. CDCA3 gene was identified as the intracellular target of miR-145, and the inhibitory effects of miR-145 were mediated through the CDCA3 protein.

Conclusion: MiR-145 regulates CDCA3 in liver cancer, affecting its development. Decreased miR-145 allows CDCA3 accumulation, promoting liver cancer progression. MiR-145 targets CDCA3 to inhibit viability, migration, and invasion of liver cancer cells. Further research is needed to understand miR-145’s regulatory role and develop more effective strategies against liver cancer.

Keywords: Apoptosis, CDCA3, Cell Viability, Liver Cancer, miR-145


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Introduction

Liver cancer is a significant global health concern, ranking as the fifth most prevalent cancer and the third most deadly cancer. It carries a high fatality rate, ranking fourth among cancer-related deaths worldwide (1). Annually, liver cancer is responsible for over 780,000 deaths across the globe. The primary liver cancer will be diagnosed in 41,210 persons (27,980 men and 13,230 women) in the US in 2023. The prevalence of liver cancer has more than tripled since 1980. In China alone, liver cancer accounts for approximately 50% of all liver cancer cases (2). Major primary liver cancer includes hepatocellular hepatocarcinoma (HCC), and intrahepatic cholangiocarcinoma (ICC). The incidence trends vary greatly when compared between the various nations and also change over time. According to a recent prevalence survey that included information from more than 30 nations, Japan, China, and Singapore had the greatest frequency of primary liver tumors, but it was lowest in countries in northern Europe and southwestern countries (3, 4).

MicroRNAs (miRNAs) have gained considerable attention in cancer research, including liver cancer. Dysregulation of miRNAs has been implicated in the development and progression of liver cancer. A growing evidence points to the importance of miRNAs in the control of metabolic and developmental processes in the liver. As a result, changes in intrahepatic miRNA networks are one of the risk factors of a liver cancer emergence (5).

MiRNAs are a class of small non-coding RNA molecules that are expressed in the higher organisms (6). They mainly repress the mRNA expression level of specific target genes at the post-transcriptional phase. MicroRNAs are involved in the development of various human diseases, including different cancers (7, 8). Expression levels of specific microRNAs have been shown to change significantly during a cancer development, the subject
that proposes them as molecular markers of particular types of a cancer (9). Recent studies have highlighted the role of various protein-coding genes, and also non-coding genes such as microRNAs in the development and progression of the liver cancer (10-12). The miR-145 expression level was found to decrease significantly in the hepatic cancer cell lines and a cancerous liver tissue. The miR-145 expression restoration decreases migration and invasion abilities, and also inhibits proliferation of different cell lines, including HepG2, Hep3B and HCC.

The cell division cycle-associated 3 (CDCA3), an F-box protein, is considered a cell cycle trigger and mediates cell cycle entry. This happens throughout targeting mitotic inhibitory kinases via Skp1-Cul1-F-box (SCF) protein, an E3 ligase (9). Recently, it has been reported that the miR-145 regulates the expression level of CDCA3 protein in the colorectal cancer (10). The collection of large-scale research data on large panels of tumor cell lines is an interesting subject of cancer research. Therefore, the present study was designed to study the role of miR-145 and its possible regulation on CDCA3 expression in the progression of liver cancer.

**Materials and Methods**

Ethical approval for the use of human tissue samples (liver cancer tissues and normal tissues) was granted by the Guangdong Second Provincial General hospital, Guangzhou (XTC/20200619). Prior to surgery, written informed consent was obtained from all patients (total number of patients were 87), granting permission to utilize their liver tissues for research purposes. Our study prospectively included patients who underwent liver partial resection surgery in the Second Department of General Surgery, Guangdong Second Provincial General Hospital, located in Guangzhou, Guangdong, China.

**Human tissues, cell culture and transfection**

Our patients were histologically confirmed to have HCC. Specimens of liver tissues were collected from these HCC patients, including the carcinoma liver tissues and the adjacent paracancerous liver tissues, which were obtained at a distance of 2 cm away from the carcinoma liver tissue.

Liver cancer cell lines SNU-182 (CRL-2235™), SNU-423 (CRL-2238) and HepG2 (HB-8065) and normal liver epithelial cells, THLE-2 (CRL-2706) were used. The cells were cultured, using RPMI-1640 medium (11875085, ThermoFisher scientific, USA) supplemented with 10% heat inactivated FBS (F2442, Sigma-Aldrich, USA). The cell lines were incubated at 37°C with 5% CO2 concentration in a humidified CO2 incubator. Transfection of cancer cell lines was performed using Lipofectamine 2000 (11668019, ThermoFischer scientific, USA). 3'- U6 -nc, miR-145 mimics and miR-145 inhibitor constructs were ordered from RiboBio (Guangzhou, China). The sequence of miRNA-145 mimics was (sense): 5’-GUCAUUUCCAGGAUCCCU-3’, and antisense 3’-CAGGUCAAAAGGGCUUUAGGA-5’). The miR-145 inhibitor sequence was (5’-AGGGAUUC-CUGGAAAACUGGAC-3’). The sequence of the miR-NC was (Sense 5’-CGCUGGAGAAAAACUGAU-UCUTT-3’, and antisense 5’-AGAAUAGCUUUCUC-CAGCGTT-3’).

The pcDNA3.1 (23252, Addgene, USA) was employed to facilitate the overexpression of CDCA3.

**Quantitative real time polymerase chain reaction analysis**

Totally, 1.0 mL of the TriZOL, (15596026, ThermoFisher scientific, USA) was used to isolate the total RNA from human liver tissues and cell lines. The cDNA was synthesized from this extracted RNA using Revert Aid First Strand cDNA Synthesis Kit (K1621, ThermoFisher scientific, USA) followed by the manufacturer’s protocol. Quanta Studio 3.0 Real-Time PCR System (A28567, Applied Biosystems, USA) was used for real time PCR analysis with SYBR Green (4309155, ThermoFisher scientific, USA). The PCR reaction conditions were 10 minutes at 95°C followed by 40 cycles of 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 15 seconds. The internal control for expression analysis was human GAPDH protein. The primer sequences used for expression study were:

**CDCA3**

- F: 5’-TGGTATTTGCACGGACACCTA-3’
- R: 5’-TGTTTCACCAGTGGGCTTG-3’

**GAPDH**

- F: 5’-AGGTCTGGTGTAACGGATTTG-3’
- R: 5’-GGGCTGTGTTGATGGCAACA-3’

The cDNA was synthesized from RNA using TaqMan MicroRNA reverse transcription kit (4366596, ThermoFisher scientific, USA) following the manufacturer’s protocol. The miRNA RT-PCR primers for miR-145 and endogenous control RNU6B were purchased from Applied Biosystems, Germany). A Quant Studio 3.0 Real-Time PCR System (A28567, Applied Biosystems, USA) was used for real time PCR analysis with TaqMan method. The PCR reaction conditions were 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. Normalization was performed with respect to RNU6B gene (internal control). The primer sequences used for miR expression study were:

**miR-145**

- F: 5’-GGATCGAGAGAGAGACTCCA-3’
- R: 5’-CTTCATCCTGTGAGCCACG-3’

**U6**

- F: 5’-CTCCTCCGGCCAGCAC-3’
- R: 5’-AAGGCTTCAGAATTTGCGT-3’

The experiments were repeated thrice.

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**MTT assay**

In order to assess cell viability, the 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test is employed to quantify cellular metabolic activity. A yellow tetrazolium salt is converted into purple formazan crystals in this colorimetric experiment by metabolically active cells. Briefly, about 0.6×10⁶ cells were plated in each well of a 96-well plate and the plate was incubated for 24 hours at 37°C. Then, 10 µl of 0.5% MTT reagent was added to each well and incubated for 4 hours at 37°C. Following incubation, 100 µl of dimethyl sulfoxide (DMSO, 100 µl) (D8418, Sigma Aldrich, USA) was added to each well to solubilize the formazan crystals. Using a microplate reader (ELx808™, Agilent, USA), optical density (OD) was recorded at 570 nm and values obtained were used for plotting the cell proliferation curve. The experiment was repeated thrice.

**Clonogenic assay**

Using a hemocytometer, cell suspension containing about 200 transfected HepG2 cancer cells was added to each well of a 6-well plate and incubated for 6 days at 37°C with 5% CO₂ to form colonies. Then, the colonies were fixed using fixation buffer (1:7 acetic acid: methanol) for 10 minutes methanol and subsequently stained with crystal violet stain (0.5%) (548-62-9, Sigma Aldrich, USA) for 2 hours. Then, pictures were taken and colonies were counted and comparison of colonies was done in terms of percent values. The experiment was repeated thrice.

**Flow cytometry for cell cycle phase dissemination**

The transfected HepG2 cancer cell lines at a cell density of 2×10⁶ cells/well were added to the wells of a 6-well plate and incubated at 37°C for 24 hours. The cells were then centrifuged to obtain a cell pellet, which was washed thoroughly with PBS buffer (P3813, Sigma Aldrich, USA). The cells were fixed with 70% ethanol (64-17-5, Sigma Aldrich, USA) and incubated overnight at 4°C. Next, the cells were resuspended in a mixture of 40 µg/mL propidium iodide (PI, P4170-10MG, Sigma Aldrich, USA), 0.1% Triton X-100 (Sigma Aldrich, USA), and 0.1 mg/mL RNase (10109134001, Sigma Aldrich, USA) in a dark room and incubated for 30 minutes at 37°C. The cells were then subjected to cell cycle analysis using flow cytometry (BD FACSMelody™ 4-Way Cell Sorter, BD, Franklin Lakes, NJ, USA). The experiment was repeated thrice.

**Analysis of migration and invasion of cancer cells**

The ability of cancer cells to directionally respond to different drugs/agents can be examined using the transwell migration and invasion assays. The HepG2 cancer cell line was transfected for 24 hours with miR-NC, miR-145 mimics, and miR-inhibitor. Cell migration was determined using a 24-well Tranwell chamber assay. In brief, 100µl of the HepG2 cell suspension containing about (10⁵) cells was added to the upper well of the Tranwell chamber, and only RPMI-1640 (11875085, ThermoFisher scientific, USA) with 10% FBS (F2442, Sigma-Aldrich, USA) was added to the lower well. The cells were allowed to grow for 24 hours at 37°C in a humidified CO₂ incubator. After 24 hours, the cells that had migrated into the lower well were fixed with 4% paraformaldehyde (158127-5G, Sigma-Aldrich, USA) for 20 minutes, stained with crystal violet (548-62-9, Sigma Aldrich, USA), and then visualized microscopically (CKX53, Olympus, USA)

Cell invasion was also estimated using the same procedure, but the chamber was coated with matrigel (CB-40234, ThermoFisher scientific, USA) instead. The migration and invasion of HepG2 cells transfected with different constructs were compared in terms of percentage values, using miR-NC transfected cells as the standard. The experiments were repeated thrice.

**miR-145 target prediction and luciferase reporter assay**

TargetScan (http://www.targetscan.org) online bioinformatics software was used for predicting the target of miR-145. Dual-Luciferase® Reporter (DLR™) Assay System (E1910, Promega Corporation, USA) by following manufacturer’s guidelines was used to perform the interaction analysis of miR-145 with 3’-UTR of CDCA3 mRNA.

**Western blotting**

Concisely, the cancer cell lysate were obtained by digesting the cells with Radio-Immunoprecipitation Assay buffer (R0278-50ML, Sigma Aldrich, USA). The total protein concentration of cell lysates was calculated using Brad Ford assay. While the Lysate containing about 45µg of proteins, it was loaded and ran on the 12% SDS-PAGE and it is transferred to the PVDF membrane (HVL02500, Sigma Aldrich, USA) using the semi-dry method. The transferred membrane was probed using primary antibody, anti-Cyclin B1 antibody, (1:800 dilution, ab32053, Abcam, USA) or anti-CDCA3 antibody (1:1000 dilution, HPA026587, Sigma-Aldrich, USA) and β-actin (1:500 dilution, ab115777, Abcam, USA) was used as a housekeeping control. Following washing step, a horse radish peroxidase (HRP) conjugated anti-rabbit, IgG secondary antibody (1:800 dilution, CB-40234, ThermoFisher scientific, USA) instead. The procedure, but the chamber was coated with matrigel (CB-40234, ThermoFisher scientific, USA) instead. The migration and invasion of HepG2 cells transfected with different constructs were compared in terms of percentage values, using miR-NC transfected cells as the standard. The experiments were repeated thrice.

**Validation of statistical data**

All the experiments were performed in triplicates, and values were presented as a mean ± standard deviation (SD). The t test was performed using GraphPad Prism7.0 software to calculate the P value. A P<0.05 was P<0.05 was considered significant.
Results

miR-145 was downregulated in the liver cancer tissues and cell lines

The miR-145 expression levels were about three folds lower in the cancerous tissues (P=0.041) than normal liver tissue (Fig. 1A).

Similar results were obtained when miR-145 expression level was compared between liver cancer cell lines, including SNU-182, SNU-423 and HepG2 and normal liver epithelial cell line (Fig.1B).

This indicates that miR-145 is significantly down regulated during liver cancer development and progression. In this study cancer cell lines, the miR-145 expression level was lower in the HepG2 cell line and thus it was selected for further characterization of miR-145.

MiR-145 inhibited cancer cell proliferation

The HepG2 cancer cells were transfected with miR-NC, miR-145 mimics and miR-145 inhibitor constructs to generate negative control, miR-145 over expressing and miR-145 inhibitor lines, respectively (Fig.1C).

At different time intervals of cell transfection, the proliferation of cancer cells was estimated using MTT assay and it was observed that the proliferation rates of miR-145 over expressing HepG2 cancer cells were significantly lower in comparison with the negative control cells. The highest proliferation rate was observed at miR-145 inhibitor transfected cells (Fig.1D). The result suggests that miR-145 has growth inhibitory role in the liver cancer.

The statement was further supported by the observations of clonogenic assay where a liver cancer cell line (HepG2) transfected with miR-145 mimics was observed to form the least number of colonies in comparison with negative control, miR-145 over expressing and miR-145 inhibitor cancer cell lines (Fig.1E). Colony number was about 100% in the case of cells with miR-145 inhibitor while it was only 55% of cells over expressing miR-145.

Fig.1: miR-145 is down regulated in liver cancer tissues and cell lines. A. miR-145 expression in the normal and liver cancer tissues. B. qRT-PCR analysis of miR-145 in liver cancer cell lines, including SNU-182, SNU-423 and HepG2, in comparison with the normal liver epithelial cell line. C. Expression analysis of transfected HepG2 cells with miR-145 mimics, miR-145 inhibitor and miR-NC. D. In vitro growth analysis of transfected HepG2 cells with miR-145 mimics, miR-145 inhibitor and miR-NC. E. Colony formation of transfected HepG2 cells with miR-145 mimics, miR-145 inhibitor and miR-NC. *; Denotes statistical significance at P<0.05 in comparison with the miR-NC control group, #; P<0.05 between the miR-145 mimics group and other groups, and qRT-PCR; Quantitative real time polymerase chain reaction.
MiR-145 induced cell cycle arrest in the liver cancer cell line (HepG2)

To infer the proliferation decrease of cancer cells under the miR-145 overexpression was due to the mitotic cell divisions arresting, transfected cancer cells were processed for flow cytometry analysis. The observations revealed that the cell number at G2/M phase was highest (70.34%) under a miR-145 overexpression while it was only 25.94% from the miR-145 inhibitor cell line and 27.62% of negative control cancer cells (Fig.2A). The percentage values indicate that cell cycle was halted at the G2/M stage.

The miR-145 overexpression induces cell cycle arrest was confirmed by western blotting of cyclin B1 which is considered as the mitotic entry protein. The cyclin B protein concentration was reduced significantly when the miR-145 was overexpressed (Fig.2B).

miR-145 restricted this study cancer cell lines metastasis

Wound healing assay, the present study, cancer cell lines migration rate (40%) was significantly reduced under miR-145 overexpression in comparison with the negative control cell line. However, the migration rate was about 140% under the miR-inhibitor transfection (Fig.3).

Similarly, the invasion rate of cancer cell lines was also significantly inhibited under miR-145 overexpression (Fig.4). Therefore, it can be concluded that miR-145 overexpression is effective in inhibiting the motility of these liver cancer cell lines and has potential to restrict a cancer metastasis.

MiR-145 targeted CDCA3 mRNA

Bioinformatics analysis (http://www.targetscan.org) was performed to predict the intracellular target of miR-145 and the target was identified as CDCA3 mRNA. When the miRNA is coupled to the target mRNA, it interacts with its target sites in the 3′UTR of the mRNA to reduce its expression. The miR-145 was predicted to bind the 3′-untranslated region (3′-UTR) of CDCA3 mRNA in a sequence specific manner (Fig.5A).

To further validate this interaction, a dual luciferase assay was performed using wild type (WT) and mutated (MUT) 3′-UTR constructs of CDCA3 cDNA. It was observed that the luciferase activity was comparatively higher in the cancer cell lines co-transfected with miR-145 mimics and CDCA-3′-UTR-WT constructs compared to cells transfected with miR-NC. It therefore indicates that CDCA3 is the target of miR-145 (Fig.5B).

Our results were also supported by analysis of CDCA3 protein expression. We observed that CDCA3 protein is highly over expressed in the cancerous tissues and cell lines (Fig.5C, D). The expression level of the CDCA3 protein was elevated in cancer tissues, which can be attributed to the repressor miR-145 being down-regulated.

Western blotting of CDCA3 protein also supported the finding that CDCA3 mRNA is targeted by the miR-145, intracellularly. We observed CDCA3 protein repression under the miR-145 over expression in the liver cancer cell lines (Fig.5E).
CDCA3 modulated the inhibitory effects of miR-145 in the liver cancer cell line (HepG2)

To investigate whether miR-145 exerts its inhibitory effects by repressing the CDCA3 protein, we used a RNA interference to silence CDCA3 mRNA in cancer cells (Fig.5F). The results showed that a decrease in the proliferation rate of cancer cells, same as the miR-145 overexpression consequences (Fig.5G). Furthermore, when CDCA3 mRNA was overexpressed in cancer cells that already overexpressed the miR-145, the cells exhibited a proliferation rate similar to the control cells, exhibited no loss of proliferation (Fig.5H). These results suggest that miR-145 growth inhibitory effects are mediated through the CDCA3 mRNA.
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Discussion

In the present study, we investigated the microRNA-145 (miR-145) role in the liver cancer development. Our results showed that miR-145 plays an inhibitory role in the liver cancer progression. It was observed that overexpression of miR-145 in liver cancer cells led to a decline in the cell proliferation and the cell cycle is arrested by decreasing the concentration of cyclin B1 in the HepG2 cancer cell line. Also, we observed that the miR-145 overexpression inhibited the cancer cell metastasis occurrence. The CDCA3 mRNA was found to be the intracellular target of miR-145.

While the regulation of eukaryotic gene transcription primarily occurs at the transcriptional level, the significance of regulatory mechanisms at the post-transcriptional level should not be overlooked (11). MicroRNAs, belonging to the post-transcriptional category of gene regulators, play a significant role in maintaining the homeostasis of the human body. Fluctuations in microRNA levels can disrupt basic cellular metabolism, paving the way for the onset of various disease conditions, including cancer (12). Recent studies have focused on the role of microRNAs in development and progression of human cancers (13, 14). There are a fine number of reports about the regulatory role of microRNAs in the human liver cancer (15, 16). The MiR-145 has been shown to regulate the development of different human cancers such as breast, lung, colon and cervical cancers (17, 18). In the present study, we found that the miR-145 is down regulated in the liver cancer cell lines. This finding suggests the miR-145 regulatory role in the liver cancer cell lines. While, we over expressed the miR-145 in these cell lines, we observed these cancer cell lines proliferation inhibition. Such results were also obtained for miR-145 against liver cancer previously where it was seen to target ROCKI mRNA (19). By targeting CDCA3 mRNA, miR-145-5p has been reported to reduce the growth, metastasis, and epithelial to mesenchymal transition (EMT) of colorectal cancer cell lines (10). Qiu et al. (20) revealed that the over expressing of miR-145 is associated with induction of mitotic arrest in liver cancer cell line (HepG2) and

Fig.5: miR-145 targets CDCA3 mRNA in liver cancer cell lines. A. In silico analysis predicted a specific miR-145 binding site in the 3´-UTR of CDCA3 gene. B. Luciferase activity of transfected HepG2 cells with miR-145 mimics, CDCA3-WT, miR-145 mimics and CDCA3-MUT. C. CDCA3 expression in normal and liver cancer tissues. D. CDCA3 protein expression in the liver cancer cell lines, including SNU-182, SNU-423 and HepG2, in comparison with the normal liver epithelial cell line. E. CDCA3 protein expression in the transfected HepG2 cells with miR-145 mimic, and miR-NC. F. CDCA3 protein expression in the HepG2 cells transfected with either siRNA-CDCA3 or si-NC. G. In vitro growth analysis of transfected HepG2 cells with siRNA-CDCA3 and si-NC. H. In vitro growth analysis of transfected HepG2 cells transfected with miR-145 mimics, pcDNA- CDCA3 and miR-NC. *; denotes statistical significance at P<0.05 compared to the miR-NC group. The experiments were repeated thrice.
a cell proliferation inhibition. Previous reports have shown that miR-145 is activated by restricting the cancer cell motility that prevents the metastasis rate (21). Consistent with that, in our study, we reported that in liver cancer cell lines, CDCA3 promotes cancer development by enhancing the cell proliferation through prevention of G1 cell cycle arrest (22). In summary, miR-145 regulates the control of liver cancer by targeting intracellular CDCA3 mRNA. When miR-145 expression levels fall beyond a threshold point, the repression of CDCA3 is alleviated and CDCA3 protein accumulates and adds to the development of liver cancer.

Conclusion

Our study demonstrates that miR-145 plays a critical role in targeting CDCA3 mRNA that leads to inhibiting proliferation and migration of liver cancer cell lines. It seems that miR-145 may serve as a potential target of the liver cancer therapy. Further investigations, in vivo and in vitro, is needed to better understanding of mechanisms of the liver cancer.

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Authors’ Contributions

Y.L., J.L.; Conceptualization, Methodology, and Software. X. H.; Data curation, Writing- Original draft preparation. X. Z.; Visualization and Investigation. P.G.; Writing- Reviewing, Editing and Supervision. All authors read and approved the final manuscript.

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