Original Article

MicroRNA-1297 regulates hepatocellular carcinoma cell proliferation and apoptosis by targeting EZH2

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Abstract: In this study, we suggested the level of miR-1297 was downregulated in the human hepatocellular carcinoma compared to the normal cells. We demonstrate ectopic expression of miR-1297 could significantly suppress hepatocellular carcinoma cells proliferation and enhance the cell apoptosis. In vitro reporter assay suggested EZH2 is a direct target gene of miR-1297. Furthermore, knockdown of EZH2 have the same effect with miR-1297 overexpression in hepatocellular carcinoma cells. These findings provide evidence that miR-1297 plays a key role in inhibition of the hepatocellular carcinoma cells proliferation, and enhancing cell apoptosis through targeting EZH2, and strongly suggest that exogenous miR-1297 may have therapeutic value in treating hepatocellular carcinoma.

Keywords: miRNA, miR-1297, EZH2, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer worldwide [1], but the second leading cause of cancer-related death in men, the sixth leading cause of cancer-related death in women. The two main risk factors for HCC are a viral hepatitis infection (hepatitis B or C) and cirrhosis (alcoholism being the most common cause of hepatic cirrhosis). Surgical resection is the optimal treatment for HCC, but only 10 to 20% of HCC can be surgically excised. Therefore, the elucidation of the molecular mechanisms underlying the tumorigenicity, proliferation and apoptosis of HCC is critical for the development of novel treatments for this disease. In this several years, a class of novel non-coding RNAs called microRNAs (miRNAs) had been discovered in plants and animals. MicroRNAs (miRNAs) include 18-26 nucleotides, which post-transcriptionally regulate gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. In the process of tumor formation, the abnormal expression or the loss of the dynamic balance between oncogenes and tumor suppressor genes, leads tumorigenesis and development of cancer.

Histone-lysine N-methyltransferase (EZH2) is an enzyme that in humans encoded by the EZH2 gene, which is located at chromosome 7q35 and contains 20 exons and 19 introns [2, 3]. EZH2 encodes a member of the polycomb-group (PcG) family, which forms multimeric protein complexes and is involved in maintaining the transcriptional repressive state of genes over successive cell generations. A previous study suggested that the EZH2-exerted transcription repression involves a mechanism that directly controls DNA methylation [4]. With the exception of certain stem cell types, the expression of EZH2 is barely detectable or is suppressed in normal cells [5, 6]. On the other hand, the dysregulated expression of EZH2 has been observed in many types of cancer, including prostate cancer, lymphoma and hepatic cancer [7-9], suggesting a role in cancer malignancy and progression.

As noted above, the incidence of hepatocellular carcinoma involves a number of oncogenes and tumor suppressor gene expression changes. Using quantitative real-time PCR, we found that microRNA-1297 (miR-1297) was significantly down-regulated in human hepatocellular carcinoma tissue samples, while EZH2 was up-regu-
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lated in human hepatocellular carcinoma. In this study, we focused on the effects of miR-1297 on the phenotypes of hepatocellular carcinoma cells as well as the identification of its direct target genes EZH2, in order to illuminate the molecular mechanisms of miR-1297 in the initiation and progression of hepatocellular carcinoma.

Materials and methods

Cell culture, transfection

Hep3B and HepG2 cells was cultured in MEM (GIBCO) and 10% heat-inactivated fetal bovine serum, 100 IU penicillin/ml, 0.1 mg streptomycin/ml in a humidified 5% (v/v) atmosphere of CO2 at 37°C. Transfected with Lipofectamine 2000 Reagent (Invitrogen), followed the manufacturer's protocol.

Fluorescent reporter assay

The Luciferase vector pGL3/Luciferase was purchased in Guangzhou Zhi-You Bio-company. The 3’-untranslated region of EZH2 mRNA containing the miR-1297 binding site was synthesized and cloned into pGL3/Luciferase vector. The fragment of EZH2 3’UTR mutant was also cloned into pGL3/Luciferase vector at the same sites. Cells were transfected with miR-1297 or control in 96-well plates, and with the reporter vector EZH2 3’UTR-WT or EZH2 3’UTR-MUT. The intensities of luciferase were detected according to the manufacturer's protocol.

Quantitative RT-PCR

To detect the relative level of transcript, real-time RT-PCR was performed. Briefly, a cDNA library was generated through reverse transcription using M-MLV reverse transcriptase (Promega) with 2 μg of the large RNA extracted from the cells. The cDNA was used for the amplification of EZH2 gene and the β-actin gene was used as an endogenous control for the PCR reaction. PCR was performed under the following conditions: 94°C for 4 min followed by 40 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min. To detect the mature miRNA level, stem-loop RT-PCR assay was performed. Briefly, 2 μg of small RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega). PCR cycles were as follows: 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 40 s. SYBR Premix Ex Taq™ Kit (TaKaRa) was used following the manufacturer’s instructions, and the real-time PCR was performed and analyzed by 7300 Real-Time PCR system (ABI).

Western blotting

Cultured cells were lysed by RIPA (0.1% SDS, 1% Triton X-100, 1 mM MgCl2, 10 mM Tris-HCl (pH 7.4) in 4°C for 25 min. Collected the lysates and cleared by centrifugation, and protein concentration was determined. Total cell lysates (50 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes. Nonspecific binding sites of membranes were saturated with 5% skim milk in TBST solution (100 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) and incubated for 2 hours with antibodies at room temperature. The following antibody was used: anti-EZH2 and anti-β-actin. After 4 washes with TBST, the filters were incubated with goat anti-mouse peroxidase-conjugated secondary antibody (Sigma) in 5% skim milk in TBST solution for 1 hour at room temperature; reactions were developed using enhanced chemiluminescence (Perkin Elmer, USA).

Cell proliferation assay

HepG2 and Hep3B cells were seeded in 96-well plate at 6,000, 7,000 and 8,000 cells per well the day before transfection. The cells were transfected with miR-1297 or control. MTT assay was used to measure the viable, proliferating cells at 48 h after transfection. The absorbance at 570 nm was measured using a μQuant Universal Microplate Spectrophotometer (Biotek Instruments). Cell proliferation inhibition rate (%) = (1-A of experimental group/A of control group) × 100%.

Colony formation assay

After transfection, HepG2 and Hep3B cells were counted and seeded in 6-well plates (in triplicate) at 50, 60 and 75 cells per well. Fresh culture medium was replaced every 3 days. Colonies were counted only if they contained more than 50 cells, and the number of colonies was counted from the 6th day after seeding and then the cells were stained using crystal violet.
Flow cytometry analysis

After 48 hours transfection as earlier described, the cells were harvested and washed twice with PBS. Washed cells were resuspended in 0.6 mL PBS, and fixed by the addition of 1.4 mL 100% ethanol at 4°C overnight. The fixed cells were rinsed twice with PBS, and resuspended in propidium iodine (PI) solution, including 50 mg/mL PI and 50 mg/mL RNaseA (Sigma) in PBS without calcium and magnesium, and incubated at 37°C for 30 minutes in the dark. Stained cells were passed through a nylon-mesh sieve to remove cell clumps and analyzed by a FACScan flow cytometer and Cell Quest analysis software (Becton Dickinson, San Jose, CA, USA). Flow cytometry analysis was repeated 3 times.

SiRNA transfection

EZH2 and control siRNAs were synthesized by Shanghai GenePharma (Shanghai, China). Cells were seeded in a 6-well plate and grown in serum- and antibiotic-free medium. Transfection was performed when the cells reached 60% confluence using Oligofectamine 2000.

Statistical analysis

Data are expressed as means ± standard deviation (SD), and P < 0.05 is considered as statistically significant by Students-Newman-Keuls test.

Results

MiR-1297 and EZH2 expression level in human hepatocellular carcinoma

We use quantitative Real-time PCR to detect miR-1297 differential expression level in 35 pairs of human hepatocellular carcinoma and corresponding adjacent normal tissue. The result showed that, miR-1297 in hepatocellular carcinoma was significantly lower than their corresponding adjacent normal tissues (Figure 1B). We used bioinformatics methods to pre-
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Figure 2. EZH2 is a directly target gene of miR-1297. (A) The predicted binding sites of miR-1297 on EZH2 mRNA are shown. The mutant UTR with a 7-base pair for site-directed mutagenesis in the complementary seed sequences (Figure 1A). We also use quantitative Real-time PCR to detect EZH2 mRNA differential expression level in these 35 pairs of human hepatocellular carcinoma and corresponding adjacent normal tissues. The results showed that, EZH2 mRNA in hepatocellular carcinoma was significantly higher than their corresponding adjacent normal tissues (Figure 1C).

MiR-1297 directly inhibits expression of EZH2 via its 3’UTR

We used bioinformatics methods to predict miR-1297 potential target genes. The 3’UTR region of EZH2 mRNA, contains miR-1297 complementary binding sites (Figure 1A). We also use quantitative Real-time PCR to detect EZH2 mRNA differential expression level in these 35 pairs of human hepatocellular carcinoma and corresponding adjacent normal tissues. The results showed that, EZH2 mRNA in hepatocellular carcinoma was significantly higher than their corresponding adjacent normal tissues (Figure 1C).

To investigate whether EZH2 can be directly targeted by miR-1297, we performed luciferase reporter assay, engineering luciferase reporter, that have either the wild-type 3’UTR of EZH2, or the mutant UTR with a 7-base pair for site-directed mutagenesis in the complementary seed sequence (Figure 2A). First, Hep3B and HepG2 cells were transfected with EZH2-wt, miR-1297 and control mimics. The results showed that, compared with the control group, co-transfected with miR-1297, the fluorescent EGFP expression were significantly lower (Figure 2B), indicating that overexpression of miR-1297 enhanced miR-1297 binding to its target gene EZH2 mRNA 3’UTR, so that luciferase activities were decreased. In contrast, mutant reporters were not repressed by miR-1297 (Figure 2B). These all results suggested that, miR-1297 could combine with the specific EZH2 mRNA 3’UTR binding sites and play a role in inhibiting the expression of EZH2 gene.
MiR-1297 plays a negative regulatory role at EZH2 post-transcriptional level

MiRNAs regulate the target genes at the post-transcriptional level by binding their target genes 3'UTR to silence the gene function. We transfected Hep3B and HepG2 cells with miR-1297, in order to examine whether miR-1297 depress endogenous EZH2 through translational repression, the expression of EZH2 protein was examined by Western blot. The results showed that overexpression of miR-1297 made the expression level of EZH2 protein decreased (Figure 2C), suggesting that miR-1297 negatively regulates endogenous EZH2 protein expression through translational repression mechanism. Meanwhile, high expression level of miR-1297 in Hep3B and HepG2 cells could also decrease the endogenous EZH2 mRNA level (Figure 2D). Furthermore, in the pairs of human hepatocellular carcinoma tissues, we found the expression level of EZH2 had a negative correlation with miR-1297 expression (Figure 1B). All these data suggest that miR-
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1297 negatively regulates the expression of EZH2 through mRNA cleavage mechanism at the post-transcriptional level.

Overexpression of miR-1297 suppresses cell proliferation and enhances cell apoptosis in hepatocellular carcinoma

To study the effects of miR-1297 on hepatocellular carcinoma cells proliferation, we respectively transfected miR-1297 into Hep3B and HepG2. After transfection of Hep3B and HepG2 cells, we test the validity of miR-1297 ectopic expression by quantitative Real-time PCR. The results revealed that miR-1297 expression level was significantly higher than the control group (Figure 3A). To test the effects of miR-1297 overexpression on hepatocellular carcinoma cells proliferation, we investigated cell growth by MTT assay and found that miR-1297 could reduce Hep3B and HepG2 cells growth (Figure 3B). The high dose of miR-1297 transfection showed a significantly lower survival than the low dose group (Figure 3B). We performed colony formation assay to further confirm the effects of miR-1297 on cell proliferation. The colony formation rate of Hep3B and HepG2 cells transfected with miR-1297 was significantly lower than the control group (Figure 3C, 3D). These two experiments sug-
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gested that miR-1297 played a role in inhibiting cell growth and proliferation in hepatocellular carcinoma cells. Upregulating miR-1297, cell viability and proliferation was significantly inhibited. To validate whether miR-1297 is able to influence apoptosis, Flow cytometry assay was performed (Figure 3E). The results indicated that the significant increase in the apoptosis was observed in the Hep3B and HepG2 cells transfected with miR-1297 (Figure 3E). These results strongly suggested that introduction of miR-1297 could inhibit human hepatocellular carcinoma cells growth by promoting apoptosis of cancer cells.

Knockdown of EZH2 inhibits hepatocellular carcinoma cell proliferation and growth in vitro

Previous studies show that EZH2 plays an important role in promoting tumor cell proliferation, and functions as an oncogene. Accordingly, we detected whether EZH2 affects Hep3B and HepG2 cell growth. We used si-EZH2 to knockdown the expression level of EZH2 and then identified the expression of EZH2 protein by Western blot. The si-EZH2 was confirmed effectively suppress the EZH2 protein expression level (Figure 4A). The effect of EZH2 knockdown on cell growth was evaluated by MTT assay. As a result, interference with EZH2 decreased cell growth activity. At 48 h after transfection, we observed that cells transfected with the high dose of si-EZH2 grew more slowly than the low dose group (Figure 4B). Also, the colony formation rate of the cells transfected with si-EZH2 was obviously lower than the control group (Figure 4C). These data, consistent with the previous findings, provided further evidence that EZH2 is an oncogene. The oncogenic role of EZH2 in hepatocellular carcinoma can also explain why overexpression of miR-1297 can inhibit hepatocellular carcinoma cell growth.

EZH2 siRNA-treated Hep3B and HepG2 cells undergo apoptosis

To investigate whether the increased apoptosis accounted for the inhibition of cell growth observed in the EZH2 siRNA-treated Hep3B and HepG2 cells, the apoptotic cells were probed using dual staining with PI and Annexin V. Increased numbers of apoptotic cells were detected in the EZH2 siRNA-treated cells (Figure 4D).

Discussion

Transformation process of malignant tumors is regulated by the synergy of multiple genes, including overexpression of oncogenes and low expression or even loss of function of tumor suppressor genes. Recent research uncovered that the regulation of oncogenes and tumor suppressor genes was not only in the transcriptional level, but also in the post-transcriptinal level, which was more important and accurate. MiRNAs, together with partner proteins, bind to the 3'UTR region of their specific target mRNA to regulate target genes by degradation of target mRNAs or inhibition of gene expression [12]. Many studies have shown differences in miRNA expression between tumors and normal tissues. In the present study, we tried to identify a novel miRNA which regulates the expression of EZH2, and evaluate its effects on cell phenotype using hepatocellular carcinoma cells. Initially, we used real-time PCR to find that miR-1297 was significantly down-regulated in human hepatocellular carcinoma tissues, compared with the normal ones. The results suggested that alterations of miR-1297 could be involved in hepatocellular carcinoma progression. Therefore, we hypothesized that miR-1297 was a negative factor of carcinogenesis in human hepatocellular carcinoma cells due to the low expression levels in human hepatocellular carcinoma. We calculated the cell growth viability through the MTT and colony formation assay to detect the relationship between miR-1297 and the growth capacity of hepatocellular carcinoma cell line Hep3B and HepG2. The cell growth viability of hepatocellular carcinoma cells transfected with the miR-1297 was significantly decreased when compared to control group. We further showed that overexpressed miR-1297 in hepatocellular carcinoma cells promoted cell apoptosis. Secondly, we predicted the miR-1297 which could possibly targeting EZH2 by using microRNA analysis software online. A plasmid, containing the EGFP reporter gene followed by the 3'-UTR of EZH2, was constructed to analyze the interaction between EZH2 and miR-1297. Our results showed that miR-1297 could downregulate the EGFP expression compared with NC-treated cells. On the other hand, the endogenous EZH2 protein expression was decreased in hepatocellular carcinoma cells transfected with miR-1297. These results suggested that miR-1297 regu-
lated EZH2 protein expression at the post transcription level. Several previous studies showed that EZH2 acted as an oncogene and increasing EZH2 expression led to tumorigenesis [13]. The oncogenic role of EZH2 was further confirmed in our study, which showed increased levels of EZH2 in human hepatocellular carcinoma tissue. We also showed that inhibition of EZH2 expression could induce tumor cell apoptosis.

Enhancer of zeste homolog 2 (EZH2) is a member of the Polycomb Group (PcG) of proteins. PcG proteins are important epigenetic regulators which can function as transcriptional repressors that silence specific sets of genes through chromatin modification. PcG proteins are grouped in polycomb repressive complexes (PRC). PRC2 includes enhancer of zeste 2 (EZH2), suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED). EZH2 is the catalytically active component of the PRC2 complex that participates in transcriptional repression of specific genes by trimethylation of lysine 27 and, to a lesser extent, lysine 9 of histone H3. Recently, an increasing number of studies linked various oncogenic properties to EZH2. Overexpression of EZH2 is found in various types of solid tumors such as melanoma, breast cancer, cervical cancer, gastric cancer, and prostate cancer [9, 14-17]. High levels of EZH2 are linked to tumor growth, metastasis, and poor prognosis for cancer patients. EZH2 suppresses apoptosis in a variety of cancers, including gastric cancer, bladder cancer, leukemia, and prostate cancer [18-21].

These recent years, the research about miRNAs has been more in-depth. MiRNA-mediated post-transcriptional gene silencing (PTGS) and the relevance with tumor formation have become the focus of attention. Zhang and colleagues [22] reported that demonstrated that C/EBPα, as the downstream factor of TRIB2, was up-regulated after miR-511 (or miR-1297) treatment, and that miR-511 (or miR-1297) acts as a tumor suppressor genes to induce A549 cell apoptosis by targeting the oncogene TRIB2. Chen P [23] et al, found miR-1297 to be down regulated in both CRC-derived cell lines and clinical CRC samples, when compared with normal tissues. Furthermore, miR-1297 could inhibit human colorectal cancer LOVO and HCT116 cell proliferation, migration, and invasion in vitro and tumorigenesis in vivo by target-

ing Cox-2. Yang NQ [24] et al, also found that miR-1297 as a potential oncogene could induce cell proliferation by targeting PTEN in NCCIT cells.

In summary, we demonstrated that miR-1297 played an important role in the regulation of EZH2 gene expression. The effect of miRNAs on hepatocellular carcinoma cells expression occurred at both mRNA and transcription levels, and at least in part through targeting EZH2. However, we emphasize that miR-1297 may be capable of controlling tumor-specific gene(s), consequently favoring cell apoptosis. Therefore, our study suggests that down-regulation of miR-1297 may provide a better strategy to block tumor proliferation and promote cell apoptosis.

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Disclosure of conflict of interest

None.

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