Original Article

Up-regulation of miR-877 induced by paclitaxel inhibits hepatocellular carcinoma cell proliferation though targeting FOXM1

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Abstract: Paclitaxel is an effective chemotherapeutic agent for treatment of cancer patients, and frequently, clinical outcome is influenced by paclitaxel sensitivity. Despite this, our understanding of the molecular basis of paclitaxel response is incomplete. Recently, it has been shown that microRNAs (miRNAs) influence messenger RNA (mRNA) transcriptional control and can contribute to human carcinogenesis. In the present study, our objective was to identify miR-877 associated with HCC cell lines response to paclitaxel and to evaluate these miRNAs as therapeutic targets to increase paclitaxel sensitivity. We measured the expression of miR-877 in paclitaxel-treated HCC cell lines. We verified that miR-877 was up-regulated in paclitaxel-induced HCC cells by real-time PCR. We further investigated the role and mechanisms of miR-877. Over-expression of miR-877 in HCC cells partially restores paclitaxel sensitivity. The proliferation activity and the colony formation activity of HCC cells were both inhibited after transfected with miR-877. MiRNA targets prediction algorithms imply FOXM1 serves as a target gene for miR-877. A fluorescent reporter assay confirmed that miR-877 binds specifically to the predicted site of the FOXM1 mRNA 3'-untranslated region (3'UTR). When miR-877 was overexpressed in HCC cells, the protein levels of FOXM1 was downregulated. These results indicate that miR-877 could influence the sensitivity of paclitaxel treatment in hepatocellular carcinoma cell lines by targeting FOXM1.

Keywords: Hepatocellular carcinoma, paclitaxel, miR-877, FOXM1

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, chemotherapeutic intervention and treatment is essential for achieving favorable prognosis [2]. The elucidation of the molecular mechanisms underlying the tumorigenicity, proliferation and apoptosis of HCC is critically important for the development of novel treatments for this disease. In recent years, improved knowledge on taxane, paclitaxel or docetaxel, has widened up its application to many cancers. The systemic chemotherapy or combination chemotherapy of paclitaxel is utilized for treating non-small cell lung cancers, ovarian, prostate, head and neck, bladder, esophageal cancers, prostate tumors, gastric carcinoma, adenocortical carcinoma, leukemia, human glioma and hepatocellular carcinoma [3-5]. Paclitaxel-induced cell death is mediated by multiple mechanisms. Paclitaxel's mechanism of action involves its stabilization of cellular microtubules by inhibiting their assembly and disassembly. This blocks progression of mitosis, and prolonged activation of the mitotic checkpoint triggers apoptosis or reversion to the G-phase of the cell cycle without cell division [6, 7]. Apoptosis occurs either during mitotic arrest or after slippage from the spindle checkpoint [8]. It has been also shown that paclitaxel-induced cell death is mediated through the phosphorylation of Bcl-2 that decreases its antiapoptotic activity [9]. Under-
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standing molecular mechanisms underlying paclitaxel-mediated cell death has contributed to the increase in treatment efficacy as well as selection for appropriate combination with other therapy that alone has a limited benefit. So, it is of great interest to find potential biomarkers for paclitaxel sensitivity evaluation to improve the therapy. Several genes or microRNAs (miRs) have been implicated in paclitaxel sensitivity or resistance of various cancers. For example, expression of β-tubulin isotypes [10], γ-actin [11] and LIMK2 [12], and the extracellular matrix protein transforming growth factor-β induced (TGFBI) [13] was correlated with paclitaxel sensitivity in different cancers. MicroRNAs (miRNAs), a class of novel non-coding RNAs had been discovered in plants and animals. MicroRNAs (miRNAs) include 18-26 nucleotides, which regulate gene expression by binding to specific mRNA targets and promoting their degradation and/or translational inhibition. Recently, accumulating evidence has indicated that the altered miRNA levels are related to the response to chemotherapeutic agents as well as oncogenesis. Paclitaxel sensitivity of various cancer cells was also associated to expression of miR-200c [14], miR-148a [15], miR-125b [16], miR-21 [17], miR-337-3p [18] and miR-34a [19]. However, there are few studies on biomarkers of HCC cells for paclitaxel. Of these, the mechanism by which microRNAs (miRNAs) regulate HCC development has recently become a focus of research in molecular biology. Increasing evidence indicates that miRNAs are expected to become new diagnostic markers and therapeutic targets of HCC.

As noted above, miRNAs constitute a newly regulatory layer of gene expression and suggest that miRNAs may influence cancer cell response to chemotherapy. In present study, to systematically understand the roles of genes or miRs in paclitaxel sensitivity, hepatocellular carcinoma cells, treated with different dose of paclitaxel for 24 h, we investigated that miR-877 was differentially expressed in paclitaxel treated human hepatocellular carcinoma cell lines HepG2 and QGY-7703. However, the contribution of miR-877 in the tumorigenesis and progression of HCC has not been fully clarified. We also hypothesized that FOXM1 could be a target gene of miR-877. We also studied the effect of miR-877 on the malignant genotypes of HCC cells in vitro. In addition, we studied the relationship between miR-877 and FOXM1 in HCC.

Materials and methods

Cell culture and transfection

Human hepatocellular carcinoma cell line HepG2 and QGY-7703 were maintained in DMEM and RPMI 1640 (GIBCO), respectively, with 10% heat-inactivated fetal bovine serum, 100 IU penicillin/ml, and 0.1 mg streptomycin/ml in a humidified 5% (v/v) atmosphere of CO2 at 37°C.

Fluorescent reporter assay

Cells were transfected with miR-877 or control vector pcDNA3 in 96-well plates with the reporter vector pcDNA3/EGFP-FOXM1 3'UTR or pcDNA3/EGFP-FOX1 3'UTR-mut. The vector pDsRed2-N1 (Clontech, Mountain View, CA) expressing RFP was spiked in and used for normalization. The intensities of EGFP and RFP fluorescence were detected with Fluorescence Spectrophotometer F-4500 (Hitachi, Tokyo, Japan).

Quantitative RT-PCR

To detect the relative levels of the transcripts, real-time RT-PCR was performed. Briefly, a cDNA library was generated through reverse transcription using M-MLV reverse transcriptase (Promega, Madison, WI) with 2 μg of the large RNA extracted from the cells. The cDNA was used for the amplification of FOXM1 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, and 72°C for 1 min. PCR primers were as follows: FOXM1-3'UTR-S: 5’-…-3’; FOXM1-3'UTR-A: 5’-…-3'. GAPDH-S: 5’-CGTGACATTAAGGAGAAGCTG-3'; GAPDH-AS: 5’-CTAGAGCATTTGCGGTGGAC-3'. To detect the expression level of miR-877, an RT-PCR assay was performed. Briefly, 2 μg of small RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega) with the following primers: miR-877-RT primer: 5’-…-3'; U6-RT primer: 5’-GTCGTATCCAGTGCAAGGCTGAGGTATTCGCACTGGATACGAAATATGGAGACGCTTGACATTTGCGGTGGAC-3'.
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The following PCR primers were used: miR-877-Fwd: 5'-TGGCATAGCAGGCTCCTGGCAGC-3'; U6-Fwd: 5'-TGCGGGTGCTCGCTTGTGGCCAGC-3'; Reverse: 5'-CCAGTGCAGGGTCCAGGTAGGT-3'.

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, and 72°C for 40 s. SYBR Premix Ex Taq™ Kit (TaKara, Madison, WI) was used following the manufacturer's instructions, and real-time PCR was performed and analyzed by the 7300 Real-Time PCR system (ABI). All primers were purchased from AuGCT Inc.

MTT assay

HepG2 and QGY-7703 cells were seeded in a 96-well plate at 6,000, 7,000, and 8,000 cells per well one day prior to transfection, respectively. The cells were transfected with miR-877 mimics or control vector, 0.15 μg per well. The MTT assay was used to determine cell viability 24 h, 48 h and 72 h after transfection. The absorbance (A) at 570 nm was measured using a μQuant Universal Microplate Spectrophotometer (BioTek, Winooski, VT).

Colony formation assay

After transfection, HepG2 cells and QGY-7703 cells were counted and seeded in 12-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. The rate of colony formation was calculated with the equation: colony formation rate = (number of colonies/number of seeded cells) × 100%.

In vivo tumorigenesis

Human hepatocellular carcinoma cell line HepG2 (106 cells/mouse) which transfected with miR-877 or mimics control were suspended in DMEM, injected subcutaneously into the right flank of nude mice (male; 4-8 weeks old) obtained from the ? ? , and allowed to grow for 50 days or until the tumor reached a volume of ? ? mm³ (tumor weight, ? ? mg). Animals (n = ? ? per group) were monitored daily and tumors were measured with a digital caliper rule twice a week. Tumor volume was estimated using the formula: Volume = (minor diameter × major diameter)/2.

Flow cytometry-based apoptosis and cell cycle analysis

Forty eight hours after transfection, cells were harvested and approximately 0.5 × 106 cells were suspended in 500 μl of propidium iodide (PI) solution [20 μg/ml PI, 50 μl/ml RNaseA, 0.02% NP40 in PBS] at 4°C for 30 min. DNA content analysis was performed by a FACS Calibur instrument (Becton-Dickinson, Mountain View, CA) and CellQuest software (Becton-Dickinson). Cells were synchronized at G1/S transition by serum deprivation for 12 h and 2 mM of hydroxyurea (HU) present for 16-18 h. Apoptotic cells were detected by FITC Annexin V apoptosis detection kit (BD Pharmingen) according to the protocols recommended by the manufacturer.

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 30 min. Lysates were collected and cleared by centrifugation, and the protein concentration was determined. Total cell lysates (50 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes. Nonspecific binding sites of membranes were saturated with 5% skim milk in TBST solution (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and incubated for 2 hours with antibodies at room temperature. The following antibodies were used: anti-FOXM1 and anti-GAPDH. After washing with TBST 4 times, the membranes were incubated with goat anti-rabbit peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) in 5% skim milk in TBST solution for 1 hour at room temperature; reactions were developed using enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA, USA).

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

Paclitaxel efficacy is correlated with the miR-877 expression level

Paclitaxel is a mainstay of treatment for many solid tumors, and frequently, clinical outcome
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is influenced by paclitaxel sensitivity. Despite this, our understanding of the molecular basis of paclitaxel response is incomplete. In order to identify miR-877 associated with liver cancer cell line response to paclitaxel and to evaluate miR-877 as therapeutic targets to increase paclitaxel sensitivity. We measured the expression level of miR-877 in hepatocellular carcinoma cell lines HepG2 and QGY-7703. We found that miR-877 was significant up regulated after paclitaxel treated HCC cell lines (Figure 1A, 1B). Furthermore, the expression level of miR-877 was associated with the dose of paclitaxel treated to HCC cell lines (Figure 1C, 1D).

Overexpression of miR-877 suppresses HCC cells proliferation in vitro

In order to study the effects of miR-877 on HCC cells proliferation, we constructed an overexpression vector: pcDNA3/pri-miR-877. To test the effect of miR-877 overexpression on HCC cell proliferation, we investigated cell growth by MTT assay and found that miR-877 could dramatic reduce HepG2 and QGY-7703 cells growth, which led to 30% and 35% inhibition of cell growth at 96h (Figure 2A, 2B). We also performed colony formation assay to further confirm the effect of miR-877 on cell proliferation. The colony formation rate of HepG2 and QGY-7703 cells transfected with miR-877 mimics was significantly lower than the control group (Figure 2C, 2D). These two experiments show that miR-877 plays a role in inhibiting cell growth and proliferation in HCC cells. Upregulating of miR-877, cell viability and proliferation was significantly inhibited.

MiR-877 suppresses tumorigenicity of hepatoma cells in vivo

To further determine whether miR-877 is involved in tumorigenesis of hepatocellular carcinoma, nude mouse xenograft model was
Figure 2. Overexpression of miR-877 suppresses HCC cells proliferation. A. HepG2 cells were transfected with miR-877 or control vector. Cell growth activity was determined at 24 h, 48 h and 72 h post-transfection by MTT assay. Values are means ± SD of three duplications and the relative cell growth activity is shown (*P < 0.05). B. QGY-7703 cells were transfected with miR-877 or control vector. Cell growth activity was determined at 24 h, 48 h and 72 h post-transfection by MTT assay. Values are means ± SD of three duplications and the relative cell growth activity is shown (*P < 0.05). C. The cell independent growth activity in vitro was assessed by colony formation assay. HepG2 cells were transfected with miR-877 or control vector, and then seeded in 6-well plates. Colonies were counted only if they contained more than 50 cells, and the number of colonies was counted from the 6th day after seeding. The number of colonies was counted from the 6th day after seeding. The colony formation rate was calculated and was shown (*P < 0.05). D. QGY-7703 cells were transfected with miR-877 or control vector, and then seeded in 6-well plates. Colonies were counted only if they contained more than 50 cells, and the number of colonies was counted from the 6th day after seeding. The number of colonies was counted from the 6th day after seeding. The colony formation rate was calculated and was shown (*P < 0.05). E. MiR-877 suppresses tumorigenicity of hepatoma cells in vivo (*P < 0.05).
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used. Because we found that miR-877 played a more significant role in promoting HepG2 cells apoptosis than QGY-7703 cells in vitro, we explore the role of miR-877 in tumorigenesis of hepatocellular carcinoma using HepG2 cells. miR-control- and miR-877-tranfected HepG2 cells were injected subcutaneously into either posterior flank of the same nude mice. The mice were followed for observation of xenograft growth for 4 weeks. It was found that introduction of miR-877 into HepG2 cells led to a significant reduction in the size of tumor volume (Figure 2E), and tumors derived from miR-877 treated HepG2 cells grew lighter compared with the control group (Figure 2E). When tumors were harvested, the average volume of tumors derived from the miR-877 mimics group was only 2% of that in the miR-control group (Figure 2E).

MiR-877 mediates cell cycle arrest and potentiates apoptosis in HCC cells

To validate whether miR-877 is able to influence apoptosis, Flow cytometry assay was performed. The results indicated that the significant increase in the apoptosis was observed in the Hep2 and QGY-7703 cells transfected with the upregulation of miR-877. These results strongly suggested that introduction of miR-877 could inhibit hepatocellular carcinoma growth by promoting apoptosis of cancer cells. To confirm that the expression of miR-877 can cause G1 arrest, HepG2 and QGY-7703 cells transfected with miR-877 mimics were synchronized at the G1/S transition by serum starvation and Hydroxyurea (HU). DNA content was examined from the time of HU release. The results showed that all cells transfected with miR-877 mimics began to arrest at G1 phase and inhibited the transfection from G1 phase to S phase (Figure 3B).

MiR-877 directly inhibits expression of FOXM1 via its 3’UTR

We used bioinformatics methods to predict miR-877 potential target genes. The 3’UTR region of FOXM1 mRNA, contains miR-877 complementary binding sites (Figure 4A). Luciferase reporter assay has been widely used in verification of miRNA target genes [20, 21]. To investigate whether FOXM1 can be directly targeted by miR-877, we performed luciferase reporter assay, engineering lucifer-
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Figure 4. FOXM1 is a direct target of miR-877. A. The predicted binding sites of miR-877 on FOXM1 mRNA are shown. The mutant UTR contains 4 bases in the complementary seed sequence (Figure 4A). First, HepG2 and QGY-7703 cells were transfected with pGL3/EGFP-FOXM1 3’UTR, miR-877 and control mimics. The results showed that, compared with the control group, transfected with miR-877, the fluorescent EGFP expression were significantly lower (Figure 4B), indicating that overexpression of miR-877 enhanced miR-877 binding to its target gene mRNA 3’UTR, so that luciferase activities were decreased. In contrast, mutant reporters were not repressed by miR-877 (Figure 4B). These results suggested that, miR-877 could combine with the specific FOXM1 mRNA 3’UTR binding sites and play a role in inhibiting the expression of FOXM1 gene.

Discussion

Evidence is accumulating to support a role for miRNAs in the development and progression of human cancer [22-24]. Changes in miRNA expression have been found in various malignancies after exposure to cytotoxic stimuli such as radiotherapy or chemotherapy, by mechanisms that may be both cancer cell-type and drug specific. Previous studies have shown that paclitaxel sensitivity may be associated with the expression of miR-200c in both ovarian [25] and gastric cancer [26], miR-148 in prostate cancer cells [27], miR-337, miR-34 and miR-135a in lung cancer [28-30], miR-22 in...
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colon cancer [31], and miR-125b and miR-21 in breast cancer [32, 33].

Chemotherapy is the most common treatment option for various cancers. The selection of the right chemotherapeutic agent for individual cancer patients remains a challenge. Chemotherapy-associated morbidity is a major concern, and strategies to limit therapies used to those that are most likely to be effective have the potential to change the current paradigm of cancer treatment. A recently study confirmed that the expression of miR-508-5p, miR-1260, miR-183*, miR-1274a, miR-21 and miR-877 was upregulated and the expression of miR-1290 and miR-1246 was downregulated in HepG2 cells treated with paclitaxel [34]. Molecular markers, such as miRNAs, might provide a promising new approach to investigate the pharmacological effects of paclitaxel. According to the published literature, We firstly identified miR-877 that is upregulated expressed following paclitaxel treatment of HCC cells. MiR-877 was selected for further examination based on associations between paclitaxel sensitivity and miR-877 expression. We also found that the expression level of miR-877 had a positive correlation with the dose of paclitaxel-induced in HCC cell lines. Therefore, we hypothesized that miR-877 is an inhibitory factor in HCC cells. We calculated the HCC cell proliferation through the MTT assay and colony formation assay to detect the relationship between miR-877 and the growth inhibition capacity of HCC cell lines. Overexpression of miR-877 did influence cell growth inhibition positively when compared to the control group. We further showed that overexpressed miR-877 in HCC cells induced G1 arrest, suppressed cell proliferation, and induced apoptosis. These data indicate that miR-877 may act as a tumor suppressor to inhibit cell proliferation by blocking the G1/S transition of HCC cells. In other words, reduced miR-877 expression in HCC cells and tissues may promote cell proliferation by activating the cell cycle. Secondly, bioinformatics analyses predicted a miR-877 binding site on the FOXM1 transcript. Experimental evidence indicated that FOXM1 was indeed a target of miR-877. On one hand, the ability of miR-877 to regulate FOXM1 expression was likely direct because it bound the 3'UTR of FOXM1 mRNA complementarily to the miR-877 seed region. The EGFP fluorescence intensity of EGFP-FOXM1-3'UTR was specifically responsive to miR-877 overexpression (Figure 3B). Furthermore, mutation of the miR-877 binding site abolished the effect of miR-877 on the regulation of EGFP fluorescence intensity. On the other hand, the endogenous FOXM1 protein expression was decreased in HCC cells transfected with miR-877. These results suggested that miR-522 regulated ABCB5 protein expression at the post transcription level.

Forkhead box M1 (FOXM1), which is characterized by the forkhead box domain, is a proliferation-associated transcription factor that has important roles in cellular proliferation, cell cycle progression, tissue repair and carcinogenesis [35, 36]. Genome-wide gene expression profiling of cancers has independently identified FOXM1 as one of the genes whose expression is most commonly up-regulated in human solid tumors, such as liver, prostate, brain, breast, lung, colon, and pancreatic tumors [37]. In addition, subsequent studies also demonstrated that high expression of FOXM1 predicted the poor prognosis of several malignancies, such as gastric cancer, lung cancer, and liver cancer [38-40]. FOXM1 aids cancer cell to obtain tumorigenic features and it plays a critical role in promoting their oncogenic phenotype. FOXM1 has been implicated in tumor initiation, expansion, and progression. A growing body of evidence also suggests that FOXM1 actively participates in drug resistance and evasion of cell death.

Cancer cells can survive and expand not only by the means of proliferation but also by resisting cell death signals [41]. Apoptosis, perhaps the most common form of cell elimination, is a strictly regulated stepwise process.

In conclusion, the development of liver cancer is characterized by multi-factorial and multi-stage process. Our studies implicated that miR-877 could unregulated by paclitaxel-treated along with the down regulation of FOXM1 expression, and investigate the potential role of miR-877 in tumorigenesis by regulating cell proliferation, apoptosis and cell cycle. The reported chemotherapy sensitivity signatures can be used effectively in the prediction of response, while other predictors of oncogenic pathway activation and even tumor microenvironment should add information that further improves our understanding of cancer biology while also leading to alternative therapeutics for predicted non-responders. Importantly, rel-
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event molecular or pathologic information may aid our understanding of chemotherapy resistance and sensitivity in HCC.

Disclosure of conflict of interest

None.

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