

Inhibition of c-FLIP expression by miR-512-3p contributes to Taxol-induced apoptosis in hepatocellular carcinoma cells

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Abstract. Dysregulation of the antiapoptotic protein cellular FLICE-like inhibitory protein (c-FLIP) has been proven to be associated with tumorigenesis and progress of most human cancers. However, its aberrant expression is poorly elucidated. MicroRNAs (miRNAs) are small non-coding RNAs that are involved in tumorigenesis through negatively regulating gene expression. Our study disclosed that c-FLIP was overexpressed in HepG2 hepatocellular carcinoma cells and down-regulation of c-FLIP enhanced Taxol-induced apoptosis. Taxol induction significantly decreased the protein level of c-FLIP. While no decrease in c-FLIP mRNA level was observed, indicating Taxol decreased c-FLIP expression through a post-transcriptional mechanism. miR-512-3p was a predicted suppressor of c-FLIP and exhibited an opposite expression manner to c-FLIP before and after Taxol induction. Luciferase report assay demonstrated miR-512-3p negatively regulated c-FLIP expression via a conserved miRNA-binding site in 3' untranslated region (3'UTR) of c-FLIP. The decrease of c-FLIP protein due to transfection of miR-512-3p further validated the inhibitory effect of miR-512-3p on c-FLIP. Additional transfection of miR-512-3p remarkably promoted Taxol-induced apoptosis, confirming its involvement in apoptosis. In summary, our study disclosed a novel regulatory mechanism that down-regulation of c-FLIP by miR-512-3p contributed to Taxol-induced apoptosis. Importantly, the pivotal role of miR-512-3p in determining c-FLIP abundance helps to broaden the implications for cancer therapy by developing small molecules to directly target c-FLIP at mRNA level.

Introduction

Protection from death receptor (DR)-mediated apoptosis has been proposed as an important step in the development of malignancy. An important regulator of DR-induced death is the cellular FLICE-like inhibitory protein (c-FLIP). c-FLIP has homology to caspase-8 and -10 but lacks their protease activity and thereby prevents the activation of procaspase-8 and potentially inhibits apoptosis mediated by death receptors (1,2). As an important antiapoptotic protein, c-FLIP has been shown to be overexpressed in human tumor cells, including colorectal carcinoma, hepatocellular carcinoma, pancreatic carcinoma, and prostate carcinoma (3-7). Dysregulation of c-FLIP expression has been proven to be one of the major determinants of the resistance to death ligands such as FasL and TRAIL, which suggests that targeting c-FLIP is an appealing way to anticancer therapy (8-10). Cisplatin, doxorubicin, actinomycin D, camptothecin, and Trichostatin A have been known to lower c-FLIP expression at transcriptional level and to sensitize the resistant tumor cells to death receptor-mediated apoptosis (11-15). However, due to limited information about c-FLIP regulation, the agents directly targeting c-FLIP at mRNA or protein levels have not yet been developed.

Taxol, as a DNA-damaging agent, just like cisplatin and doxorubicin, is one of the most active cancer chemotherapeutic agents and is effective against several human tumors including ovarian, breast, non-small cell lung tumors, and head and neck carcinomas (16-18). It is evident that Taxol-induced apoptosis is primarily through caspase-8 and -10 activation, which indicated that Taxol could prevent c-FLIP expression to facilitate caspase-8 activation and apoptosis signaling (19,20). However, the regulation of Taxol on c-FLIP expression and its molecular mechanisms are still poorly understood.

In our study, we demonstrated that during the process of Taxol-induced apoptosis, the decrease of c-FLIP expression by Taxol was through a post-transcriptional mechanism. We verified that it was miR-512-3p that inhibited c-FLIP expression via directly binding to c-FLIP 3'UTR. The findings implied that inhibition of c-FLIP by miR-512-3p might contribute to Taxol-induced apoptosis in hepatocellular carcinoma cells. Our study suggested that, compared to those agents that prevented the transcription of c-FLIP by blocking

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the corresponding signaling pathways, developing small molecules that directly targeting c-FLIP at mRNA level is expected to be efficient and valuable for cancer therapy.

Materials and methods

Cell culture and reagents. HepG2 cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Taxol (paclitaxel, Sigma Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 µM as a stock.

Vectors, Stealth™ siRNAs and microRNA mimics. pGL3-FLIP-3'UTR reporter plasmid (designated as pGL3-F) was constructed as follows: the 3'UTR sequence of FLIP was amplified by RT-PCR from HepG2 cDNA using specific primers (forward: 5'-gaaaccaaaggctggcgct-3'; and reverse: 5'-cccaggttgtaagc-3'). This fragment was cloned into pGL3 vector downstream of the luciferase gene. We also constructed pGL3-FLIP-3'UTR mutant, in which two sites of the seed sequence was deleted and referred to as pGL3-FM. miR-512-3p and negative control mimics were synthesized from Dharmacon and dissolved in DEPC-treated H₂O at a concentration of 20 pmol/µl as a stock. Stealth siRNAs targeting FLIP were synthesized from Invitrogen and dissolved in DEPC-treated H₂O at a concentration of 20 pmol/µl as a stock.

Semi-quantitative RT-PCR. Total RNA was extracted from HepG2 cells using the TRIzol reagent (Invitrogen, CA, USA). The primers for amplification were: c-FLIP, forward 5'-ggga gaagtaagaacaaag-3' and reverse 5'-cgtaggcacaatcacgcat-3'; β-actin, forward 5'-gaaatctggcaccacacct-3' and reverse 5'-ggc cggactcgtcatactc-3'. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized under UV transilluminator.

Real-time PCR for miRNA. Total RNA was extracted from HepG2 cells using the TRIzol reagent (Invitrogen). Real-time PCR was performed with the TaqMans microRNA assays (Applied Biosystems) in the ABI PRISM® 7500 Real-time PCR system (Applied Biosystems). The mean Ct was determined from triplicate PCRs. miR-512-3p expression was calculated relative to 18S rRNA. The primer sequences were: miR-512-3p, forward 5'-uugcacuggcucagucuggc-3' and reverse 5'-cctcagtcattggacctcag-3'; 18S, forward 5'-ctacca ctccaaggaagcca-3' and reverse 5'-tttttcgtcactactccccg-3'.

Western blotting. Total protein from HepG2 cells was extracted using RIPA buffer (1 mM MgCl₂, 10 mM Tris-HCl pH 7.4, 1% Triton X-100, 0.1% SDS, 1% NP-40) and measured for total protein concentration using Bradford reagent (Bio-Rad, Hercules, CA). Protein extracts (20 µg) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Before staining, unspecific sites were blocked in 5% non-fat milk at room temperature for 1 h. The rabbit polyclonal c-FLIP antibody (1:1000; Santa Cruz Biotechnology) was used to evaluate c-FLIP expression. The secondary horseradish peroxidase-conjugated antibody was detected using ECL plus Western blotting detection reagents

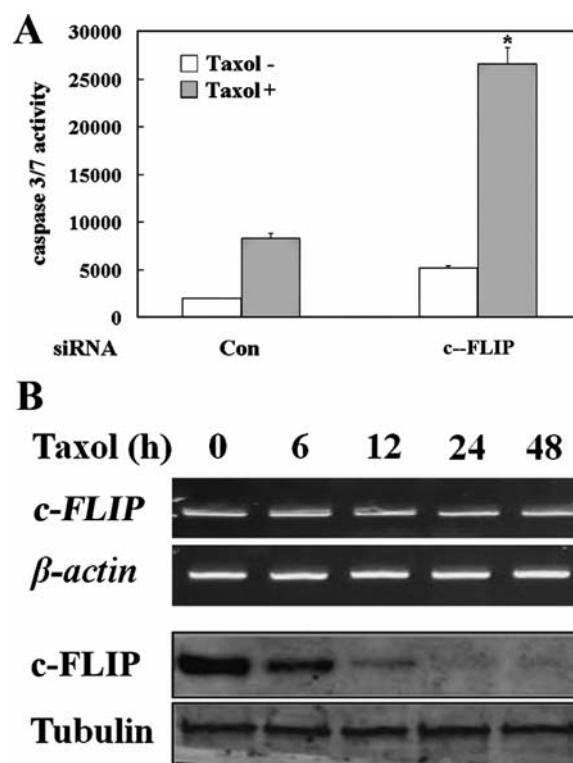


Figure 1. c-FLIP participated in Taxol-induced apoptosis. (A) HepG2 cells were seeded in 96-well plates and transfected with siRNAs specifically targeting FLIP, then treated with Taxol (100 nM) for 24 h. Caspase-3 activities were measured using a Caspase-Glo 3/7 assay kit. Error bars represent SD and are obtained from three independent experiments; *p<0.01. (B) HepG2 cells were plated in 6-well and incubated with Taxol. At the indicated time points (0, 6, 12, 24, and 48 h), total RNA and proteins were extracted. RT-PCR (upper panel) and Western blot (lower panel) were performed to detect the expression of c-FLIP. β-actin and Tubulin were used as internal controls. Results presented here are representative of three different experiments.

(Amersham Biosciences). As a loading control, the β-tubulin expression level was measured using rabbit polyclonal anti-β-tubulin antibody (Santa Cruz Biotechnology).

Luciferase gene assay. The HepG2 cells were cultured in 24-well plates for 24 h, then transfected with 400 ng of pGL3-F (or pGL3-FM), and different dose of miR-512-3p mimics 20, 40, 80 pmol) using DharmaFECT® Duo Transfection Reagent (Dharmacon). pBIND plasmids (50 ng) were cotransfected to normalize for transfection efficiency. At 48 h post-transfection, the firefly and Renilla luciferase activities were measured using the dual-luciferase reagent assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

Cell apoptosis assay. HepG2 cells were plated in 6-well plates, then transfected with siRNAs targeting c-FLIP for 24 h and treated with Taxol. After 24 h, cells were incubated with propidium iodide exclusion and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry.

Caspase activity assay. HepG2 cells were plated in replicates of 6 in 96-well plates at a density of 5x10³ cells/well for 24 h,

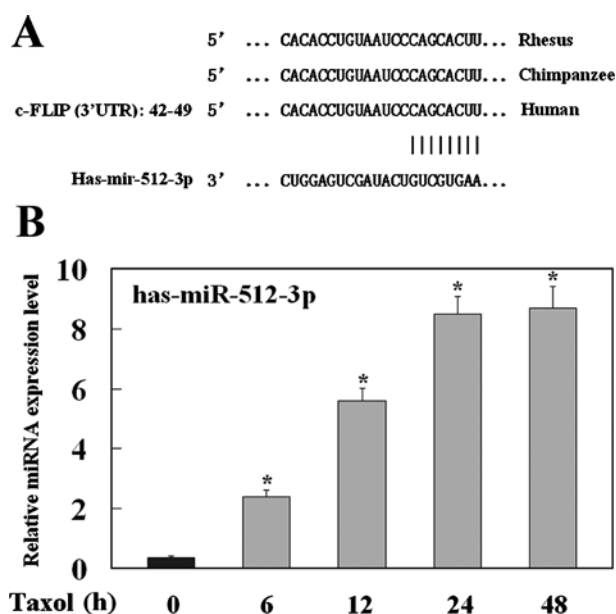


Figure 2. miR-512-3p as a putative inhibitor of c-FLIP by bioinformatics analyses. (A) A schematic representation illustrating the c-FLIP mRNA 3'UTR putative sites targeted by miR-512-3p predicted by Pictar and TargetScanS database. (B) HepG2 cells were plated in 6-well plates and treated with 100 nM of Taxol. At the indicated time points (0, 6, 12, 24, 48 h), total RNAs were extracted and real-time PCR was performed to examine the miR-512-3p level. Samples from three independent experiments are analyzed and the data are expressed as means \pm SD; * $p < 0.01$.

then transfected with siRNAs targeting c-FLIP for 24 h and treated with Taxol. Annexin V-FITC positive cells were determined by flow cytometry. For apoptotic effects of miR-512-3p, cells were transfected with miR-512-3p and control mimics for 24 h and subsequently treated with Taxol. After indicated treatment, 50 μ l of Caspase-Glo 3/7 Reagent (Promega) were added into each well and incubated for 1 h. The luminescence of each well was measured in the GENios Pro Multifunctional Reader from Tecan.

Results

Knockdown of c-FLIP enhanced Taxol-induced apoptosis. It has been recently reported that Taxol-induced apoptosis is FADD dependent, combined with the inhibitory role of c-FLIP in death receptors-induced apoptosis, we wonder whether knockdown of c-FLIP sensitized hepatocellular carcinoma cells to Taxol induction. As shown in Fig. 1A, Taxol induction caused a significant increase of caspase-3/7 activity by 3.3-fold in siFLIP cells, compared to its effects in siCon cells, which demonstrated that c-FLIP was involved in Taxol-induced apoptosis.

Next, we tested the expression of c-FLIP in HepG2 cells following Taxol treatment. As shown in Fig. 1B, the protein level of c-FLIP was down-regulated dramatically by Taxol in a time-dependent manner, especially at 12 h of Taxol incubation; while, its mRNA level showed slight alteration, indicating that Taxol controls c-FLIP expression through a post-transcriptional mechanism.

miR-512-3p is a potent suppressor of c-FLIP predicted by bioinformatics analyses. MicroRNAs regulate a variety of

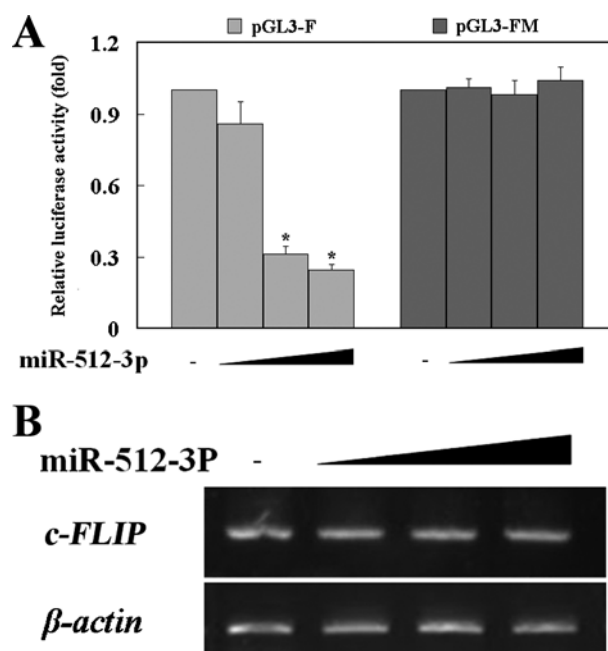


Figure 3. Negative regulation of miR-21 on c-FLIP expression. (A) HepG2 cells were plated in 24-well plates, and cotransfected with pGL3-FLIP-3'UTR (wild-type or mutant, designated as pGL3-F and pGL3-FM) and different dose of miR-512-3p mimic (20, 40, and 80 pmol). Luciferase activity was determined 48 h after transfection. Activity of the firefly luciferase was normalized to that of the renilla luciferase and expressed as relative luciferase unit (RLU). Error bars represented SD and were obtained from three independent experiments. Student's t-test was applied for analysis of significant difference; * $p < 0.05$. (B) HepG2 cells were plated in 6-well plates and transfected with different dose of miR-512-3p mimics (20, 40, and 80 pmol) and control mimic. At 48-h post-transfection, the c-FLIP RNA and protein levels were assessed by RT-PCR and Western blotting.

cellular activities through regulation of the expression of multiple target genes via repressing translation or promoting mRNA decay (21-23). To examine the involvement of microRNAs in c-FLIP expression during the process of Taxol incubation, we focused on the identification of putative microRNAs by Pictar and TargetScan software. There were 15 miRNAs that were predicted to bind to the 3'UTR of human c-FLIP by the two softwares. Based on published studies, majority of predicted targets function as anti-apoptotic proteins, for example miR-150, miR-17-5p, miR-93 and miR-221. Among them, miR-512-3p is the unique candidate because it has been verified as a pro-apoptotic tumor suppressor in variety of carcinomas (24). Fig. 2A shows that the conserved target sites for miR-512-3p in the 3'UTR of c-FLIP is at nucleotides 1937-1942, conservation of which was also observed in other species. To characterize miR-512-3p was a putative suppressor of c-FLIP, we examined its expression in Taxol-treated cells. The expression level of miR-512-3p was nearly undetectable in HepG2 cells. While, its expression elevated rapidly at 6 h of Taxol incubation and nearly reached the maximum 24 h later (Fig. 2B), hinting that miR-512-3p expression was triggered by Taxol.

Negative regulation of miR-512-3p on c-FLIP. We next examined the effect of miR-512-3p on endogenous c-FLIP expression. To test this, we subcloned the c-FLIP 3'UTR (designated as pGL3-F) downstream of luciferase gene.

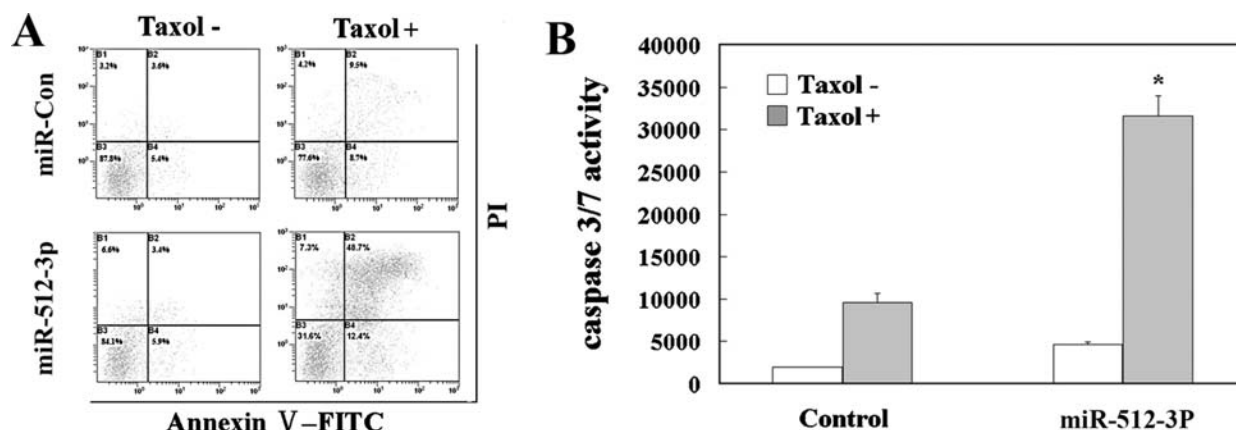


Figure 4. miR-512-3p enhances Taxol-induced apoptosis. (A) HepG2 cells were plated in 6-well plates and transfected with miR-512-3p and control mimics for 24 h and subsequently treated with Taxol. After 24 h of incubation, the apoptotic cells were measured by PI and Annexin V-FITC staining and analyzed by flow cytometry. Percentages in the figures represent the mean from three independent experiments. (B) HepG2 cells were plated in 96-well plates and treated as described above. Caspase-3 activities were measured using a Caspase-Glo 3/7 assay kit (Promega). Error bars represent SD and were obtained from three independent experiments; * $p < 0.01$.

Cotransfection of cells with pGL3-F plus the miR-512-3p decreased the luciferase activity in a dose-dependent manner. To further determine the suppression of miR-512-3p on c-FLIP by binding to c-FLIP 3'UTR, the seed region of c-FLIP 3'UTR was deleted and subcloned downstream of luciferase gene (designated as pGL3-FM). As we expected, the luciferase activity in pGL3-FM was unchanged by overexpression of miR-512-3p (Fig. 3A). Our data demonstrated that negative post-transcriptional regulation of miR-512-3p on c-FLIP was dependent on miR-512-3p target sites in c-FLIP 3'UTR. To check if miR-512-3p actually affected c-FLIP expression in cellular environment, we analyzed the consequences of the ectopic expression of miR-512-3p. We found that the protein level of c-FLIP was increased in a dose-dependent manner in the miR-512-3p-transduced cells, while no significant difference in c-FLIP mRNA level was observed between miR-512-3p-transduced cells and control cells (Fig. 3B). These data suggested that miR-512-3p specifically down-regulated c-FLIP expression at the post-transcriptional level.

miR-512-3p restoration promotes Taxol-induced apoptosis. Considering the negative regulation of miR-512-3p on c-FLIP expression, combined with its increased expression after Taxol induction, we next evaluated the role of miR-512-3p in the process of Taxol-induced apoptosis. In the presence of Taxol, 61.1% of cells transfected with miR-512-3p mimics were Annexin V positive, compared with 18.2% Annexin V positivity in Taxol-treated negative control cells (Fig. 4A, right panels).

MiR-512-3p overexpression in combination with 24 h of Taxol treatment resulted in increased activation of caspase-3 compared to control cells (Fig. 4B, $p < 0.01$). These findings suggested that miR-512-3p may function as a pro-apoptotic activator of cell death.

Discussion

c-FLIP is a recently identified intracellular inhibitor of caspase-8 activation, which inhibits the apoptosis signaling

mediated by the death receptors (2,25,26). Since c-FLIP is recognized more recently and the detailed molecular mechanisms on its regulation are deficient, thus developing small molecular inhibitors that aim at c-FLIP seems to be left behind in comparison to antiapoptotic proteins Bcl-2, IAP and MDM2, functions and molecular mechanisms of which are well-documented (27-29). Considering the characterized modulators regulate the expression of these antiapoptotic proteins at transcriptional, post-transcriptional, and translational levels, many small molecule inhibitors are designed to target Bcl-2, IAP, and MDM2 at either mRNA or protein levels are now in preclinical and early clinical trials (27-29). Cisplatin, doxorubicin, actinomycin D, and camptothecin have been proven to sensitize tumor cells to death receptor-mediated apoptosis by inhibiting the transcription of c-FLIP (11-15). However, small molecules, such as antisense oligonucleotides or miRNAs, which could directly aim at the mRNA of the targets, are seldom reported due to lack of valuable data on post-transcriptional regulation of c-FLIP.

Interestingly, our study observed that in hepatocellular carcinoma cells, Taxol induction significantly decreased the protein level of c-FLIP. While no decrease of c-FLIP mRNA level were observed, which indicated that Taxol decreased c-FLIP expression via a post-transcriptional mechanism. Our data are consistent with several published results. For example, PPAR γ ligands sensitized tumors to TRAIL-induced apoptosis by reducing c-FLIP through inducing ubiquitination and proteasome-dependent degradation of c-FLIP (30). Poukkula *et al* reported that c-FLIP could be down-regulated by a transcription-independent mechanism involving protein ubiquitylation (31). It has been reported that Taxol triggered caspase-8- and caspase-10-dependent apoptosis in human lymphoblastic leukemia cell line by inhibiting protein level of c-FLIP rather than mRNA level (32). Our data, combined with others, suggested that post-transcriptional regulation may be a common mechanism of c-FLIP expression.

MicroRNAs are a group of non-coding, single-stranded RNAs that negatively modulate gene expression at the

post-transcriptional level through association with the 3'UTR of protein coding genes and subsequent induction of translation inhibition or transcript destabilization (22,23). Accumulating evidence demonstrate that microRNAs contribute to cancer development by acting on oncogenes or tumor suppressor genes (33-36). For example, BCL2 are frequently highly expressed in chronic lymphocytic leukemia. Targeting BCL2 by miR-15a and miR-16 significantly enhance chemotherapy-induced apoptosis (37). Compared to oncogenes, p27 acts as a tumor suppressor and its expression is often disrupted in human cancers. High activity of miR-221 and miR-222 is required to maintain oncogenic state in cancer cells by inhibiting p27 expression (38).

Considering the inverse correlation between miR-512-3p and c-FLIP by Taxol induction and miRNAs as potent regulators of gene expression, we hypothesized that down-regulation of c-FLIP might be mediated by microRNAs. By using PicTar and TargetScan databases, we identified 15 putative targets. Among them, miR-512-3p was the only one that was able to induce apoptosis, which is contrary to c-FLIP function. Further data validated the association between miR-512-3p and the 3'UTR of c-FLIP mRNA and also demonstrated that the seed sequence strongly contributed to the miRNA-mRNA interaction which mediated the post-transcriptional inhibition on c-FLIP expression. Furthermore, we found that miR-512-3p over-expression inhibited c-FLIP mRNA level rather than protein level, which indicated that miR-512-3p controlled c-FLIP translation, but not mRNA stability.

Saito *et al* recently reported that miR-512-3p was silenced in gastric cancer cells (24). Epigenetic activation of silenced miR-512-3p resulted in apoptosis of gastric cancer cells by inhibiting the oncogene Mcl-1, which for the first time indicated that miR-512-3p could promote apoptosis. In agreement with this, our study verified that overexpression of miR-512-3p enhanced Taxol-induced apoptosis by inhibiting c-FLIP expression, which suggests that miR-512-3p may function as a novel tumor suppressor. Moreover, the inverse correlation between miR-512-3p and c-FLIP suggests that lower expression of miR-512-3p resulting in dysregulation of c-FLIP expression may lead to tumorigenesis of hepatocellular carcinoma.

In conclusion, our study disclosed that microRNA-512 functioned as a proapoptotic protein and inhibition of c-FLIP by miR-512-3p contributed to Taxol-induced apoptosis in hepatocellular carcinoma cells. The present study not only helps us to understand the molecular mechanism of tumorigenesis, but also provides a potent strategy for the treatment of carcinomas by developing small molecule inhibitors directly targeting c-FLIP at the levels of mRNA and protein.

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