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MicroRNA 506 regulates expression of PPAR alpha in hydroxycamptothecin-resistant human colon cancer cells

Jin Lu Tong [a], Chen Peng Zhang [b], Fang Nie [a], Xi Tao Xu [a], Ming Ming Zhu [a], Shu Dong Xiao [a], Zhi Hua Ran [a,*]

[a] Division of Gastroenterology and Hepatology, Shanghai Jiao-Tong University School of Medicine, Renji Hospital, Shanghai Institute of Digestive Disease; Key Laboratory of Gastroenterology & Hepatology, Ministry of Health (Shanghai Jiao-Tong University), 145 Middle Shandong Rd, Shanghai 200001, China
[b] Department of Nuclear Medicine, Renji Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai 200001, China

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ABSTRACT

Chemotherapeutic drug resistance remains a major obstacle to the successful treatment of colon cancer. Here, we show that 77 differentially expressed miRNAs were identified in SW1116/HCPT versus SW1116, and over-expressed miR-506 in SW1116/HCPT cells was validated. Then it was indicated that PPARα is a common target of miR-506 by using a luciferase reporter assay. Our results also demonstrated that cytotoxic ability of HCPT requires the concomitant presence of PPARα, and that loss of PPARα expression imparts resistance to HCPTs anti-tumor effects. All together, our studies indicate that miR-506 over-expression in established HCPT-resistant colon cancer cell line confers resistance to HCPT by inhibiting PPARα expression, then providing a rationale for the development of miRNA-based strategies for reversing resistance in HCPT-resistant colon cancer cells.

1. Introduction

Colon cancer is the third most common cancer for both men and women in the United States [1]. Now, there is a growing importance of chemotherapy for malignant colon tumors. However, resistance to anticancer drugs is still a major cause in the failure of chemotherapy in cancer patients.

Inhibitors of topoisomerase I have demonstrated potent antitumor activity in both preclinical and clinical trials [2–4]. Camptothecin (CPT) was first isolated from the bark of the Chinese/Tibetan ornamental tree, Camptotheca acuminata. The development of camptothecin-like compounds (topotecan and irinotecan) as inhibitors of topoisomerase I for the treatment of resistant tumors has generated clinical excitement in this new class of drugs. As anti-cancer drugs, the efficacy of camptothecin is limited by the onset of drug resistance. Reversing such resistance could allow the decrease of the dose necessary to eradicate the tumor, thus diminishing the side effects of the drug.

In previous work, we established a new hydroxycamptothecin-resistant cell line (SW1116/HCPT) from human colon cancer cell line SW1116 by treatment with step-wise increasing concentrations of hydroxycamptothecin (HCPT) [5]. MicroRNAs play important roles in the regulation of normal gene expression for developmental timing, cell proliferation, and apoptosis [6–8]. Despite strong evidence that miRNAs are associated with cancer and are potential biomarkers for outcome, little is known about how they affect the response of a tumor to cytotoxic treatment.

In this work, to study the involvement of miRNAs in drug resistance, we examined the differences in the miRNAs expression profiles of hydroxycamptothecin-sensitive and hydroxycamptothecin-resistant cells by miRNA microarray analyses, then exploring the potential role of specific miRNA in drug resistance to HCPT.

2. Materials and methods

2.1. Cell culture

Human colon cancer cell line SW1116 (obtained from Academy of Military Medical Science, Shanghai, China) and its hydroxycamptothecin-resistant variant SW1116/HCPT [5] (established and maintained in our laboratory) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified incubator at 37 °C.

2.2. RNA extraction and miRNA microarray

Total RNA was extracted from SW1116 and SW1116/HCPT with Trizol (Invitrogen). The isolated miRNAs from the two cell lines

* Corresponding author. Address: Department of Gastroenterology, Renji Hospital, Shanghai Institute of Digestive Disease, Shanghai 200001, PR China. Fax: +86 21 63266027.
E-mail addresses: ranzhihua1962@126.com, z-ran@online.sh.cn (Z.H. Ran).

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were then labeled with Hy3 using the miRCURYTM Array Labelling kit (Exiqon, Vedbaek, Denmark), and then hybridized respectively on a miRCURYTM LNA microRNA Array (v 8.1, Exiqon) as described [9]. Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Devices). Images were quantified by the GenePix Pro 6.0 (Axon Instruments). Raw data were further automatically processed in Microsoft Excel.

2.3. Real-time PCR analysis for miRNA

TaqMan MicroRNA Reverse Transcription kit and TaqMan MicroRNA Assay were used to detect and quantify mature microRNA-506, microRNA-452, and microRNA-373*. Normalization was performed with RNU6B. All primers were provided by Applied Biosystems. All reactions were performed according to manufacturer's protocols. Experiments were carried out in triplicate.

2.4. SYBR Green quantitative real-time reverse transcription-PCR assays

The isolated RNA was DNase treated and reverse-transcribed using First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. RTQ-PCR was performed using the SYBR Green method (Toyobo) and analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). GAPDH was employed as internal standard. Experiments were carried out in triplicate.

2.5. miRNA target predictions

The analysis of miRNA predicted targets was carried out using the algorithm TargetScanS [10]. The algorithm produced a list of hundreds of target genes for miR-506 by searching for the presence of conserved 8-mer and 7-mer sites matching the seed region of a miR.

2.6. Reporter constructs

Three putative miRNA506-recognition elements from the PPARα gene, two from RXR alpha and corresponding mutants were cloned into the 3′-untranslated region (UTR) of the luciferase gene in the pMIR-REPORT luciferase vector (Ambion). The sense and antisense strands of the oligonucleotide sequences (Table 1) were annealed by adding 2 μg of each oligonucleotide to 46 μL of 1×oligonucleotide hybridization solution (100 mM potassium acetate, 30 mM HEPES, pH 7.4, and 2 mM magnesium acetate) and incubated at 90 °C for 3 min and then at 37 °C for 1 h. The oligonucleotide sequences were designed to carry the HindIII and SpeI sites at their extremities facilitating ligation into the HindIII and SpeI sites of pMIR-REPORT vector. The oligonucleotides used in the studies were listed in Table.1. All constructs were confirmed by DNA sequence analysis.

2.7. Luciferase activity assay

SW1116 cells were cultured in 24-well plates and each transfected with 200 ng of either Reporter constructs or corresponding mutants together with 200 ng of pMIR-REPORT β-gal Control Plasmid and 30 pmol of miR-506 precusor (Ambion) using Lipofectmine 2000 (Invitrogen). Twenty-four hours following transfection, luciferase and β-galactosidase activities were assayed with the Dual-Light Combined Reporter Gene Assay System (Applied Biosystems) and Promega Turner TD-20/20 Luminometer. Luciferase activity was normalized to β-galactosidase activity. All assays were performed in triplicate.

Table 1
Complementary pairs of oligonucleotides were designed to construct luciferase reporter vectors.

Putative miRNA-binding sites	Complementary pairs of oligonucleotides
Position 3547-3553 of PPARα 3′ UTR	3′-ATTCCGTGGTCGTCGGCATTCGA-5′ 5′CTAGTAAGGCACCAGCAGCCGTA-3′
Mutant of position 3547-3553	3′-A*TAGGCACG*TCGTCGGCATTCGA-5′ 5′-CTAGT*ATCCGTGC*AGCAGCCGTA-3′
Position 7930-7937 of PPARα 3′ UTR	3′-AATTCCGTGAGTGATCATCTTTCGA-5′ 5′CTAGTTAAGGCACTCACTAGTAGAA-3′
Mutant of position 7930-7937	3′-A*TAAGGCAG*AGTGATCATCTTTCGA-5′ 5′-CTAGT*ATTCCGTC*TCACTAGTAGAA-3′
Position 8335-8341 of PPARα 3′ UTR	3′-AATTCCGTTTTGACATGGGATTCGA-5′ 5′-CTAGTTAAGGCAAAACTGTACCCTA-3′
Mutant of position 8335-8341	3′A*AAAGGCT*TTTGACATGGGATTCGA-5′ 5′CTAGT*TTTCCGA*AAACTGTACCCTA-3′
Position 2093-2099 of RXRα 3′ UTR	3′-ATTCCGTGGTCCACTTCGA-5′ 5′-CTAGTAAGGCACCAGGTGA-3′
Mutant of position 2093-2099	3′-A*TAGGCA*GGTCCACTTCGA-5′ 5′-CTAGT*ATCCGT*CCAGGTGA-3′
Position 3381-3387 of RXRα 3′ UTR	3′-AATTCCGTAACGATTCCTTTCGA-5′ 5′-CTAGTTAAGGCATTGCTAAGGAA-3′
Mutant of position 3381-3387	3′-A*AAAGGCT*TTTGACATGGGATTCGA-5′ 5′-CTAGT*TTTCCGA*AAACTGTACCCTA-3′

2.8. Short hairpin RNA expression plasmids and stable transfection

We established a vector-based RNA interference expression system using pRNAT-U6.1/Neo (Genscript, Piscataway, NJ, USA) small interfering RNA (siRNA) expression vector. A shRNA expression vector against PPARα (pRNATU6.1-PPARAi) was prepared by cloning of double-stranded oligonucleotides into the BamHI and HindIII sites in the pRNAT-U6.1/Neo vector. The sequence of shRNA against PPARα was 5′ TGGGAAACATCCAAGAGAT 3′, a second shRNA 5′ CATTGAACATCGAATGTAGAA 3′, and a third shRNA 5′ CGATCAAGTGACATTGCTAAA 3′. All of the constructs were confirmed by DNA sequencing. The pRNATU6.1-PPARAi vector was then used to induce PPARα stable suppression in SW1116 cells using Lipofectmine 2000 (Invitrogen). Stable transfectants were selected by growing cells in 600 μg/ml G418; the cells (SW1116-PPARAi) that survived were then expanded in the absence of G418 for additional studies.

2.9. Cytotoxicity assay

SW1116, SW1116-pMIF-miR-506 and SW1116-PPARAi cells were seeded into 96-well plates at a density of 5000 cells/well in RPMI 1640 medium with 10% FBS. The cells were treated with several concentrations of HCPT for 72 h. After the incubation period, the medium was removed, and 20 μL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) were added to each well, and plates were placed at 37 °C for 4 h. At the end of the incubation period, the untransformed MTT was removed, and 100 μL dimethylsulfoxide was added to each well to lyse the cells. Absorbance was measured at 570 nm using a 96-well microplate reader (ELx-808, Cambrex, Baltimore, MD). The absorbance values were corrected for non-specific conversion of MTT by preparation of blank wells containing all additions apart from the cells. All of the experiments were performed in triplicate.

2.10. Apoptosis assay

Following 48 h of treatment, cells were harvested. Apoptotic and necrotic cells were assayed in each sample using the Vybrant

apoptosis assay kit #2 and the protocol supplied by the manufacturer (Invitrogen). Each sample was evaluated by flow cytometry with a Coulter Epics XL flow cytometer (Beckman-Coulter, Villepinte, France). Data were processed with Expo 32 cytometer software (Beckman-Coulter). Experiments were done three times in duplicate.

2.11. Western blot analysis

Western blots were done as previously described. The dilutions of the primary antibodies were anti-PPARα (ab-2779, Cambridge, MA) in 1:1000, and anti-GAPDH (Sc-32233, Santa Cruz Biotechnology, Inc) in 1:5000.

2.12. Statistical analysis

Unpaired Student's t test was performed for all experiments. All data, unless stated otherwise, are expressed as mean ± standard error of mean (SE). A P value of less than 0.05 was considered statistically significant. SPSS Software, version 11 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

3. Results

3.1. Comparison of microRNA profiles between SW1116/HCPT and SW1116

We analyzed miRNA expression profiles of SW1116 and its hydroxycamptothecin-resistant variant SW1116/HCPT using a miRNA microarray platform (Exiqon). Seventy-seven miRNAs emerged as differentially expressed in SW1116/HCPT with respect to SW1116. Specifically, 30 miRNAs were down-regulated and 47 miRNAs were up-regulated in SW1116/HCPT cells compared with SW1116 (Fig. 1A and B). MiR-548d was the highest up-regulated microRNA (124.55-fold), while miR-641 was the most highly down-regulated one (60.20-fold).

At the same time, the differential pattern of gene expression between SW1116 and SW1116/HCPT showed that PPARα was the highest down-regulated transcript in resistant variant (about 404.1-fold) (Table S1). Combining data from microRNA chip results, gene-chip results and the results of target prediction (Fig. 4A), two down-regulated miRNAs (hsa-miR-452 and hsa-miR-373*) and one up-regulated miRNA (hsa-miR-506) were selected for validation by the Taqman MicroRNA Assays. The expression level of hsa-miR-452 and hsa-miR-506 from real-time RT-PCR were consistent with results from miRNA array analysis (Fig. 1C). However, the expression of hsa-miR-373* was not detected by real-time RT-PCR.

3.2. Overexpression of miR-506 confers resistance to hydroxycamptothecin in human colon cancer cell SW1116

Among above selected miRNAs, the most significant changes in the expression pattern were miR-506. Therefore, we further investigated the role of miR-506 in resistant variant SW1116/HCPT cells. To investigate the possible influence of miR-506 status on the sensitivity of colon cancer cells to HCPT, we firstly restored expression of miR-506 in SW1116 cells through stable transfection of miR-506 expression vector PMIRH-506, which was constructed from vector PMIRNA1 (System Biosciences, Mountain View, CA).. The expression of exogenous miR-506 in clone transfected with miR-506 was confirmed by Taqman MicroRNA Assays. As shown in Fig. 2A, the SW1116 cell line transfected with empty vector express low levels of miR-506, whereas individual miR-506-transfected clone express high levels of exogenous miR-506. Next, we did MTT assays to determine whether the expression of miR-506 would affect the sensitivity of SW1116 cells to HCPT. Dose-response curves for HCPT treatment were determined in SW1116 and transfected cells, and the IC_{50} was calculated. The IC_{50} concentrations of HCPT were 92.6 μM and 145.5 μM in SW1116 transfected with empty vector and SW1116-pMIF-miR-506 cells ($P < 0.05$) (Fig. 2B). Next, we examined the possible inhibitory effect of miR-506 on the ability of HCPT to induce tumor cell apoptosis. 100 μM and 150 μM concentrations of HCPT were used to treat both cell lines. At the same concentrations of HCPT, the flow cytometric analysis indicates that in SW1116-pMIF-miR-506 cells, the apoptotic rate induced by HCPT alone was significant lower than that of treatment in the parental SW1116 cells (150 μM: 18.2 ± 4.7% vs 33.6 ± 3.9%) ($P < 0.05$) (Fig. 2C). We also performed the same experiment in SW1116/HCPT by transfection with antisense (anti)-miR-506 (Shanghai GenePharma Co., Ltd). Real-time RT-PCR analysis confirmed 80.7 ± 19.3% reduction in miR-506 levels after introduction of anti-miR-506 compared with negative control group. The IC_{50} concentrations of HCPT were 812.3 μM and 393.8 μM in SW1116/HCPT transfected with negative control and anti-miR-506. A significant elevated level of apoptosis (37.9 ± 2.2)% was observed after 48 h transfection with anti-miR-506 compared with the negative control group (18.5 ± 2.1)% ($P < 0.05$) in SW1116/HCPT cell by treatment with HCPT (800 μM) (Fig. 3).

3.3. MicroRNA 506 regulates expression of PPARα in hydroxycamptothecin-resistant human colon cancer cells

The differential expression profiles observed in the microarray experiments (Table S1) were confirmed for two representative genes (PPARα and RXRα) using RTQ-PCR. As shown in Fig. 4B, differential expression of these genes was confirmed. Therefore, PPARα and RXRα became our research interest, because of the strikingly differential expression. Furthermore, the high expression level of PPARα was also reported in an adriamycin-resistant hepatocellular carcinoma cell line HepG2/ ADM [11].

Using bioinformatic tools (TargetScanS), we searched for potential mRNA targets of human miR-506. Among the candidates targeted, 3′-UTR of human PPARα contains three putative regions (nucleotides 3547-3553, 7930-7936, 8335-8341) that matches to the seed sequence of hsa-miR-506, two putative regions for RXRα (nucleotides 2093-2099, 3381-3387) (Fig. 4A). To investigate the influence of miR-506 on predicted mRNA targets, we transfected the pre-miR-506 (Ambion) into SW1116 cells (which expressed low level of miR-506) and searched for changes of PPARα protein levels. We found that introduction of miR-506 precursor decreased PPARα protein levels in a dose-dependent manner (Fig. 4C). Then we transfected the antisense (AS)-miR-506 (Shanghai GenePharma Co., Ltd) into SW1116/HCPT cells (which expressed high level of miR-506), we also found that introduction of AS-miR-506 increased PPARα protein levels in a dose-dependent manner, namely an inverse relationship between miR-506 and PPARα (Fig. 4C).

To examine whether PPARα and RXRα are indeed the targets of miR-506, luciferase constructs were made by ligating oligonucleotides containing the wild-type or mutant putative target site of the PPARα and RXRα 3′ UTR into the multi-cloning site of the pMIR Reporter Luciferase vector. Both the wild-type and the mutant reporters were introduced into SW1116 cells, pMIR-REPORT Beta-gal was used as a reference control. For 3 report vectors of PPARα, the changes of luciferase were about 2.68 ± 0.55 fold, 1.43 ± 0.22 fold and 1.34 ± 0.20 fold for position 7930-7937, position 3547-3553 and position 8335-8341, respectively. However, for 2 report vectors of RXRα, the changes of luciferase were all insignificant (Fig. 4D). These findings indicate that miR-506 negatively regulates the expression of PPARα through its three predicted binding sites, not for RXRα.

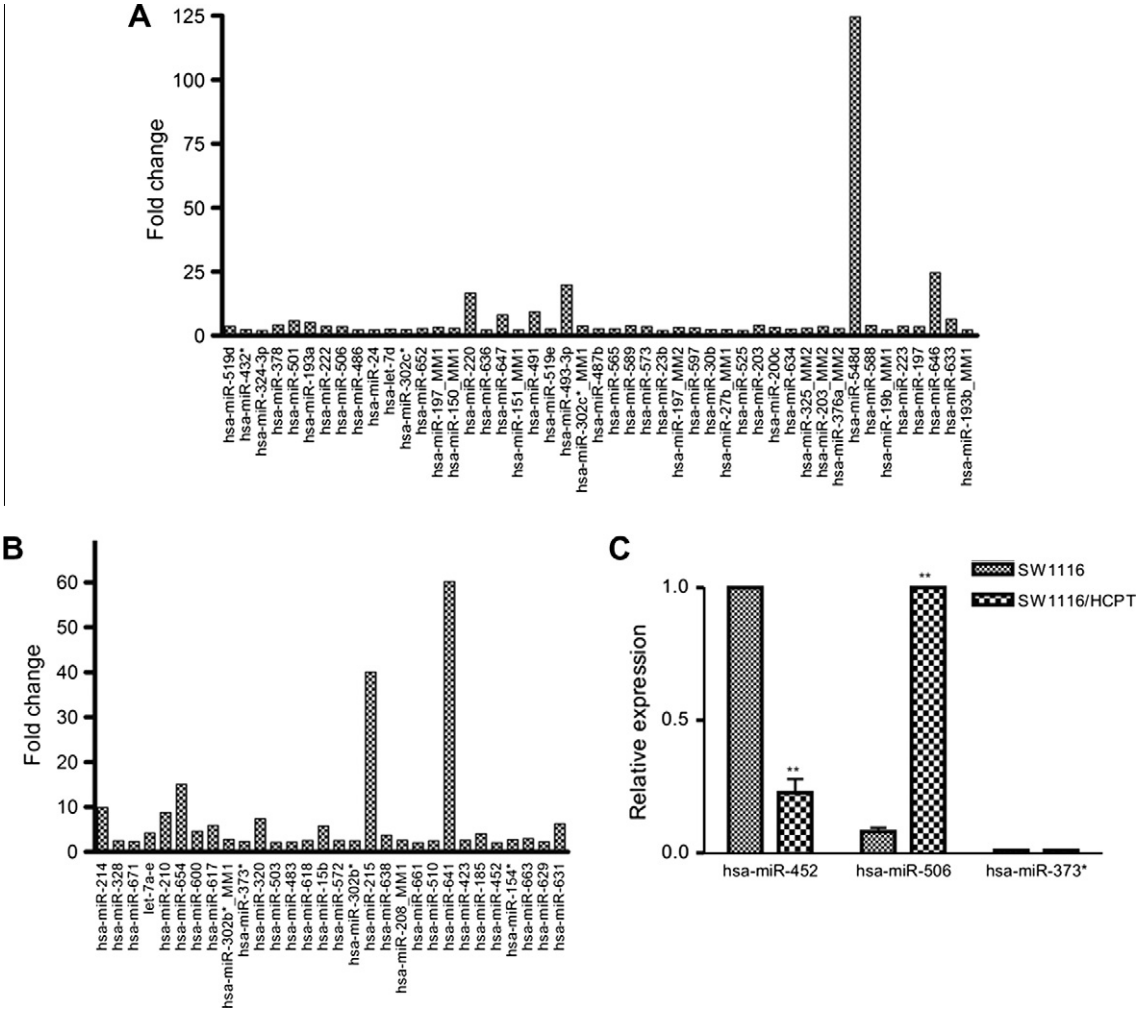

Fig. 1. Microarray analyses indicate that miRNAs are dysregulated in HCPT-resistant cells. (A) Up-regulated miRNAs from miRNA microarray of SW1116/HCPT versus SW1116 cells (fold change > 2). (B) Down-regulated miRNAs from miRNA microarray of SW1116/HCPT versus SW1116 cells (fold change > 2). (C) Changes in expression of hsa-miR-452, hsa-miR-506, hsa-miR-373* were confirmed by RTQ-PCR. Means from triplicate experiments in all cases; bars, SE. $^{**}P < 0.01$.

3.4. PPARα expression plays an important role in HCPT-induced resistance in human colon cancer cells

To evaluate the potential correlation between PPARα mRNA/protein expression and HCPT sensitivity in colon cancer cells, we first examined the expression of endogenous PPARα mRNA/protein in HCPT-sensitive and HCPT-resistant cell lines by real-time PCR (Fig. 4B) and Western blot analysis (Fig. 6B). A high level of PPARα mRNA/protein was detected in the colon cancer cell line SW1116 that was established from tumors sensitive to HCPT, but low level of PPARα mRNA/protein expression was detected in the HCPT-resistant SW1116/HCPT cell line, suggesting that PPARα is involved in HCPT-induced resistance in vitro.

To further assess the biological involvement of PPARα activity in HCPT-mediated cytotoxicity, we began by analyzing individual and combined effect of PPARα ligands and HCPT. We first investigated the sensitivity of SW1116/HCPT cells to a PPARα agonist wy-14643 (BIOMOL) as a single agent. As shown in Fig. 5A, wy-14643 only slightly inhibited the proliferation of SW1116/HCPT cells. Surprisingly, we found that wy-14643 strongly enhanced HCPT-induced inhibition of cell vitality in a synergistic manner. Furthermore, we showed that the above effect was in a dose-dependent manner. At the same time, wy-14643 also potentiates HCPT-induced apoptosis in a dose-dependent manner (Fig. 5B). Next, the effects of treatment with HCPT and PPARα antagonist MK 886 (SIGMA), alone or in combination were examined in SW1116 cells. We found that MK 886 alone could inhibit cell vitality and induce apoptosis in SW1116 cells, and pretreatment with MK 886 for 24 h before addition of HCPT enhanced the cytotoxicity of HCPT (Fig. 5C and D)

According to our hypothesis, inhibition of PPARα by treatment of MK 886 would augment resistance of SW1116 to cytotoxicity of HCPT. However, the above data did not support our initial idea. We supposed that whether there was non-specific effects of the antagonist MK 886 used at this concentration on cell vitality. To address this problem, we used RNAi vectors to inhibit PPARα expression in stably transfected SW1116 cells. After screening by culturing with G418, expression of PPARα mRNAs in the G418-selected cell pools was verified by semi-quantitative RT-PCR. Following identification of a correctly targeted transfectant, the expression level of protein was further immunobloted. As Fig. 6A shows, real-time RT-PCR analysis confirmed 94.1 ± 3.4% ($P < 0.05$) reduction in PPARα mRNA levels in the SW1116-PPARAi clones III relative to the vector control. Furthermore, the stably transfected clone expressing shRNA against PPARα displayed a 68.9 ± 8.3% reduction in protein level (Fig. 6B).

To test the sensitivity of SW1116-PPARAi clones to HCPT, SW1116-PPARAi clones and vector-transfected cells were exposed

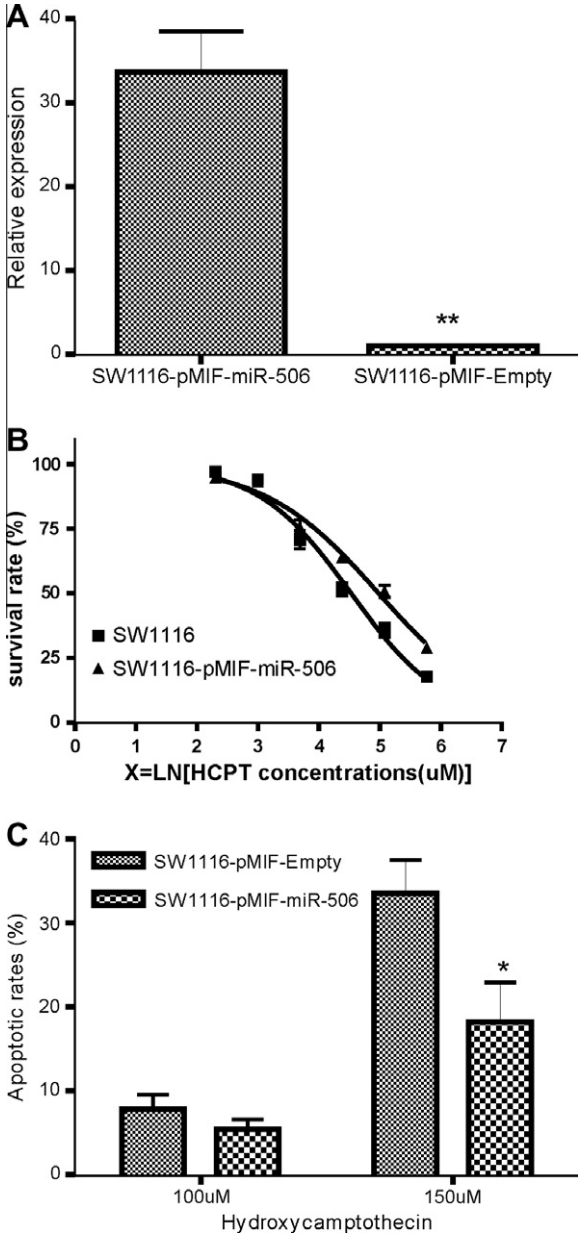

Fig. 2. Ectopic expression of miR-506 induces resistance to HCPT-induced apoptosis in SW1116 cells. (A) Ectopic expression of miR-506. SW1116 cells which express low levels of endogenous miR-506 were transfected with PMIRH-506 or empty vector (PMIRNA1). After 15 d under G418 selection, the expression level of mir-506 was determined by quantitative RT-PCR in stable cells versus cells transfected with control vector. Columns, mean; bars, SE. (B) MTT assay. The SW1116-pMIF-miR-506 cells or SW1116 transfected with empty vector were treated with different concentrations of HCPT for 3 days and examined for cell viability. (C) Flow cytometry. The vector-transfected and miR-506–transfected cells were treated with 100 μM and 150 μM HCPT, after 48 h of the treatment, cells were labeled with Annexin V and analyzed by flow cytometry. $^{*}P < 0.05$, $^{**}P < 0.01$.

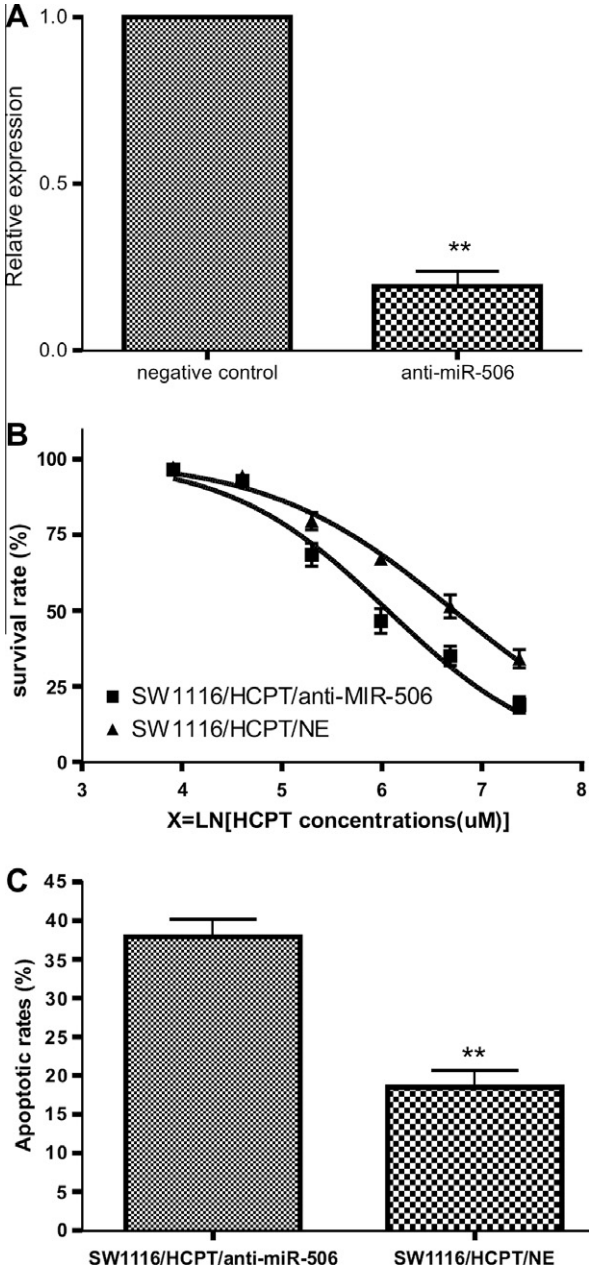

Fig. 3. Inhibition of miR-506 expression decrease resistance to HCPT-induced apoptosis in SW1116/HCPT cells. (A) Inhibition of miR-506 expression. SW1116/HCPT cells which express high levels of endogenous miR-506 were transfected with anti-miR-506 or negative control (NE) alone. After 48 h, the cells were harvested for the miR-506 quantitative analysis. Columns, mean; bars, SE. (B) MTT assay. The SW1116/HCPT cells transfected with NE and anti-miR-506 were treated with different concentrations of HCPT for 3 days and examined for cell viability. (C) Flow cytometry. The Anti-miR-506-transfected and NE–transfected cells were treated with 800 μM HCPT, after 48 h of the treatment, cells were labeled with Annexin V and analyzed by flow cytometry. $^{*}P < 0.05$, $^{**}P < 0.01$.

to various concentrations of HCPT and examined for cytotoxicity by MTT assay. As seen figure, the IC_{50} concentrations of HCPT were 87.0 μM and 225.9 μM in SW1116 and transfected cells ($P < 0.05$) (Fig. 6C). Next, we examined the possible inhibitory effect of PPARα on the ability of HCPT to induce tumor cell apoptosis. 150 μM and 300 μM concentrations of HCPT were used to treat both cell line. At the same concentrations of HCPT, the flow cytometric analysis indicates that in SW1116-PPARAi cell line, the apoptotic rate (Fig. 6D) induced by HCPT alone was significant lower than that of treatment in the parental SW1116 cells.

4. Discussion

Recently, accumulating evidence has indicated that altered miRNA level resulted from mutation or aberrant expression is correlated with various human cancers [12,13]. The role of miRNAs in multi-drug resistance was also reported in some studies. Xia et al. suggest that miR-15b and miR-16 could play a vital role in the development of MDR in gastric cancer cells at least in part by mod-

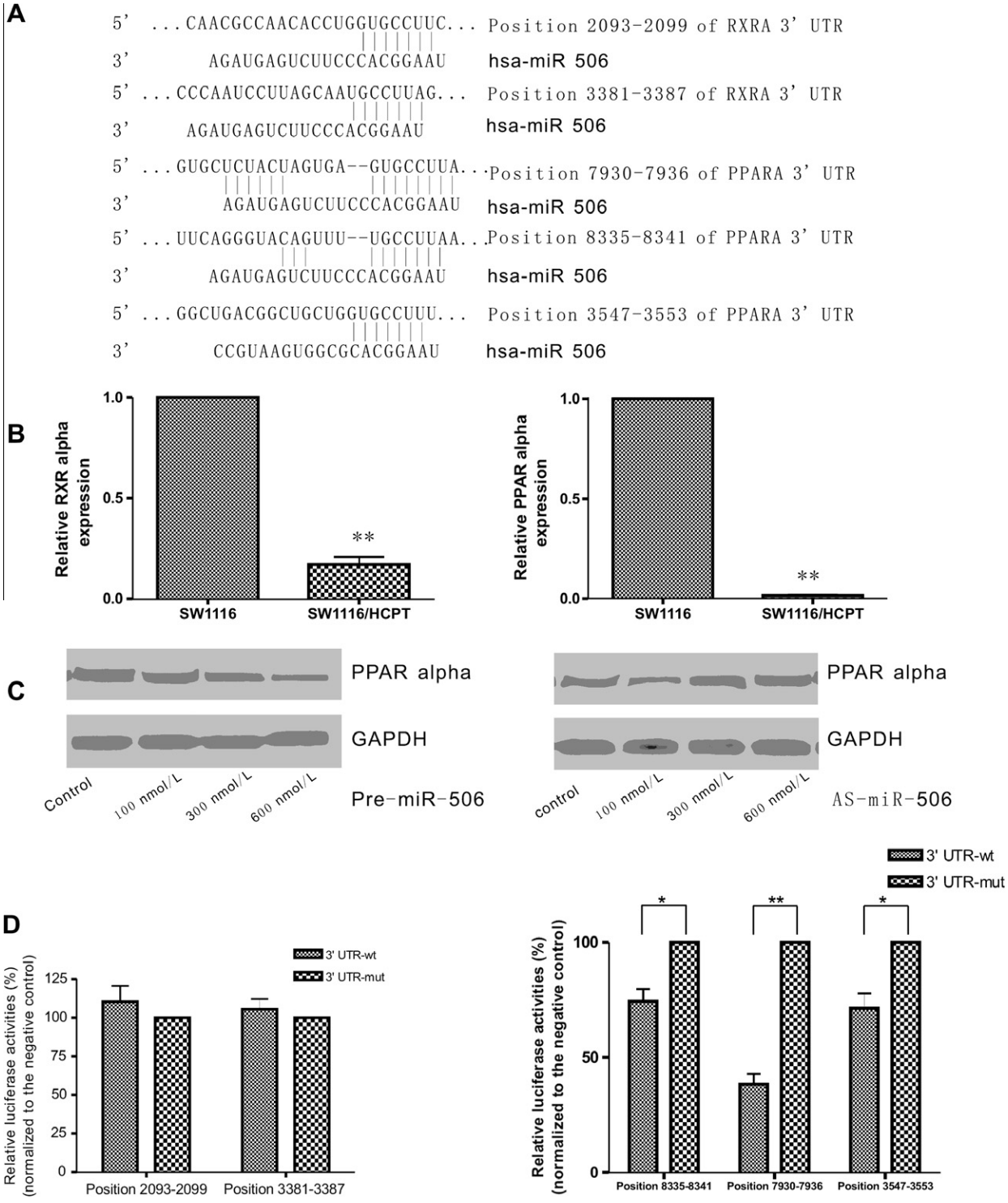

Fig. 4. MiR-506 directly targets PPAR alpha. (A) The predicted sites of miR-506 (Bottom) hybridization to the PPAR alpha and RXRA 3′-UTR region (top) as detected by TargetScan. (B) PPAR alpha and RXRA mRNA levels are significantly different in resistant cell line versus its parent cell line as determined by RT-PCR. Left, RXRA; right, PPAR alpha. (C) Effects of pre-miR-506 and AS-miR-506 on PPAR alpha protein expression. SW1116 cells were transfected with pre-miR-506 and SW1116/HCPT cells were transfected with AS-miR-506 both at a final concentration of 100, 300 and 600 nmol/L. PPAR alpha protein level were determined by western blot analysis as described in Section 2. Columns, means for three replicate determinations for each treatment group; bars, SE. The control treatment groups were transfected with 100 nmol/L of scrambled oligonucleotide and served as controls for the experiments. (D) miR-506 inhibits wild-type but not mutated ppar alpha-3′-UTR reporter activity. Expression of luciferase with the putative miR-506 target sites in wild-type (wt) or mutated 3′-UTR from PPAR alpha (right) and RXRA (left) were measured in a luminometer and shown as a fraction of control. Relative luciferase activity was normalized to the scrambled oligonucleotide control. Columns, mean; bars, SE. $^{*}P < 0.05$, $^{**}P < 0.01$.

ulation of apoptosis via targeting BCL2 [14]. Zhu et al. demonstrate the roles of microRNAs in the regulation of drug resistance mediated by MDR1/P-glycoprotein, and suggest the potential for targeting miR-27a and miR-451 as a therapeutic strategy for modulating MDR in cancer cells [15].

In this study, we identified that miR-506 was over-expressed in established HCPT-resistant colon cancer cell line SW1116/HCPT. Through bioinformatic prediction using targetScanS software, 3 sites in PPARα 3′ UTR region and 2 sites in RXRα 3′ UTR region become our interests. Moreover, PPARs all bind as heterodimers with

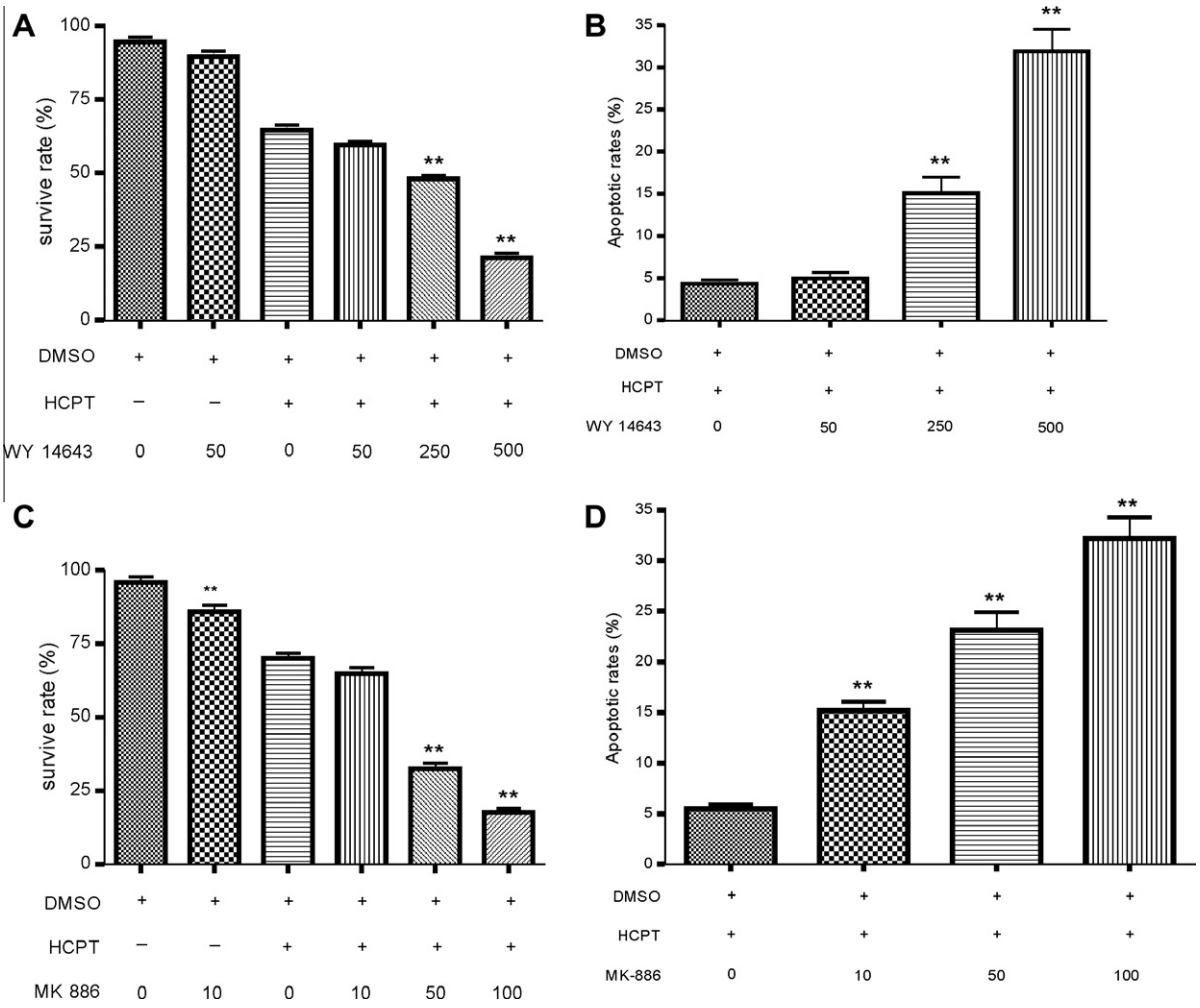

Fig. 5. Antiproliferative actions of hydroxycamptothecin in combination with WY 14643 or MK886 in human colon cancer cell line SW1116 and its HCPT-resistant variant SW1116/HCPT. (A) Effect of WY 14643 and HCPT treatment on cell viability of SW1116/HCPT cells. Cells were treated with either HCPT (2.4 mg/L) alone, or treated simultaneously with WY 14643 (0, 50, 250, 500 μmol/L) for 48 h and then analyzed by MTT assay. Columns, mean; bars, SE. (B) Sensitization of SW1116/HCPT cells to HCPT–induced and WY 14643-induced apoptosis as determined by flow cytometry analysis after 48 h treatment with HCPT (2.4 mg/L) alone, or combination of HCPT and WY 14643. Increased apoptotic response was evident in combination treatment group relative to untreated control or single-agent treated group. (C) Drug effects of MK886 or combination effects of MK886 plus HCPT on the colon cancer cell line SW1116. (D) MK886 potentiates apoptosis of SW1116 cells in response to HCPT in a dose-dependent manner. flow cytometry analysis cell apoptosis after 48 h treatment with HCPT (150 μg/L) alone, or the combination of HCPT and MK886. $^{**}P < 0.01$.

another nuclear receptor partner, the retinoid X receptor (RXR), to peroxisome proliferator response elements (PPRE) consisting of a direct repeat of the hormone receptor response element half-site spaced by one nucleotide in target genes [16]. Using luciferase assays, we documented a direct link between miR-506 and the putative target PPARα.

To date, the role of miR-506 in tumor cell is not clear. Zhao et al. found that expression of miR-506 was reduced in 16HBE-T transformed malignant human bronchial epithelial cells compared with 16HBE normal human bronchial epithelial cells and revealed that miR-506 acts as an anti-oncogenic miRNA (anti-oncomir) in malignantly transformed cells [17]. However, we show that miR-506 over-expression in established HCPT-resistant colon cancer cell line SW1116/HCPT confers resistance to HCPT by inhibiting PPARα expression. Peroxisome proliferator-activated receptors (PPARs) are steroid nuclear receptors that regulate diverse biological processes. Activation of PPARα has been reported to improve levels of triglycerides, HDL, and the overall atherogenic plasma lipid profile, while also potentially modulating inflammation as well insulin resistance itself [18,19]. Recently, it has been found that PPARs may also regulate the expression of some MDR proteins [20]. One of the major changes in the phenotype of MDR cells is a decreased accumulation of cytotoxic drugs, mainly due to P-glycoprotein overexpression, which uses ATP to extrude chemotherapeutic agents from the cells [21]. Such an energy-dependent process may require a high energy demand with an enhancement of energy-yielding pathways [22]. These pathways are strongly associated with ER-dependent signaling and significantly predict response to tamoxifen treatment, as measured by disease-free and overall survival [23]. Furthermore, some MDR proteins were regulated by PPARα [24,25]. Therefore, we hypothesized that PPARα play an important role in resistance of colon cancer cell to HCPT.

In efforts to explore the role of PPARα *in vitro*, most of the recent studies employed pharmacological approaches. WY 14643 is a potent exogenous PPARα agonist and MK886 is a PPARα antagonist. Here, we found that WY 14643 was able to induce apoptosis in a synergistic manner with HCPT, thereby enhancing the antitumor effect of HCPT in SW1116/HCPT cells. However, in SW1116 cells, synergistic cytotoxicity was also observed when MK 886 was administered with HCPT. The "off-target" effects of PPARα antagonist MK 886 necessitated the use of other approaches to evaluate the consequences of PPARα inactivation. Using RNAi techniques

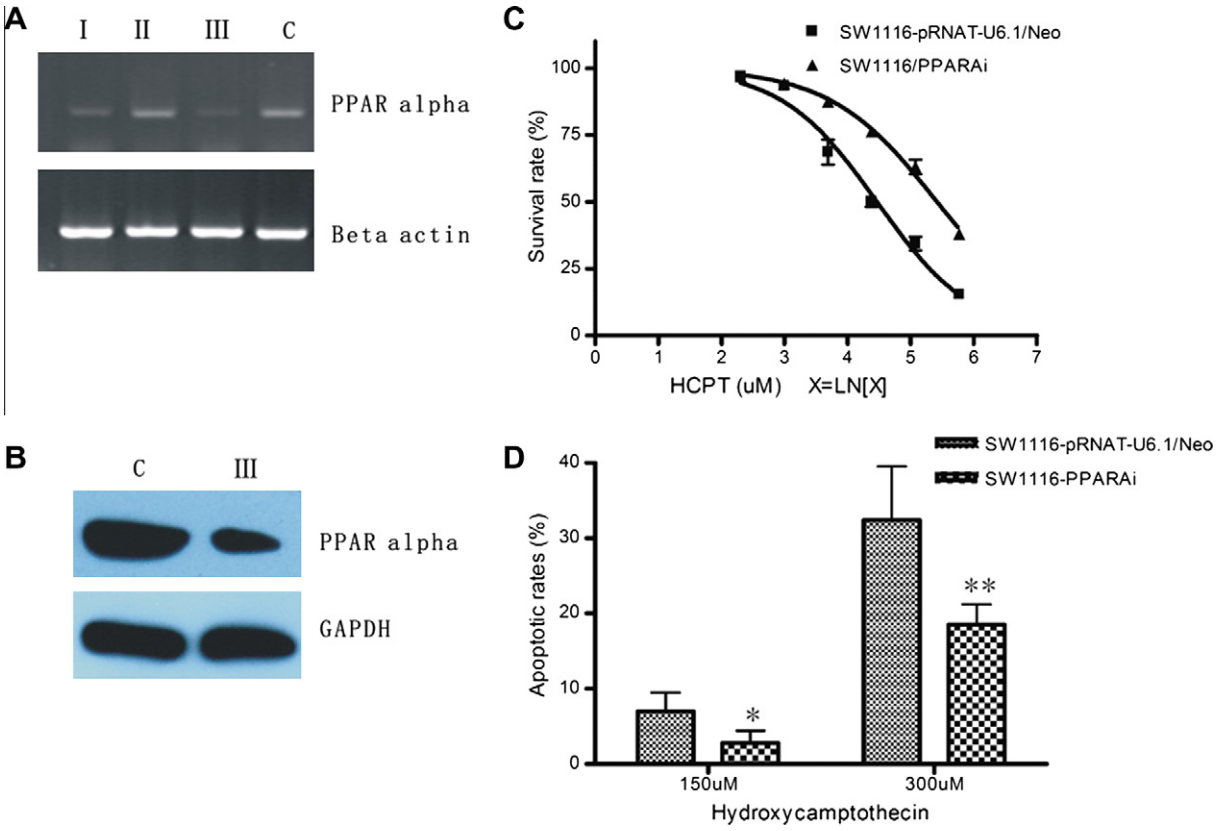

Fig. 6. PPARα down-regulation reduces sensitivity to HCPT in SW1116 cells. (A) SW1116 cells were stably transfected with either empty shRNA vector (SW1116-pRNAT-U6.1/Neo) (C) or PPARα shRNA-expressing shRNA vector (SW1116-PPARAi). PPARα expression in these clones (I, II, III) was analyzed by using semi-quantitative RT-PCR. (B) Expression of PPARα protein in SW1116-pRNAT-U6.1/Neo clone and SW1116-PPARAi clone III. Whole-cell lysates were used to detect the protein level of PPARα by immunoblot analysis (top) with GADPH as loading control (bottom). (C) SW1116-pRNAT-U6.1/Neo clone and SW1116-PPARAi clone were treated with concentrations of HCPT range 0 μmol/L from 320 μmol/L, and cell viability was determined 72 h later using the MTT assay. The IC_{50} values were calculated. Each assay was repeated at least thrice. (D) Analysis of Annexin V and propidium iodide staining in SW1116-pRNAT-U6.1/Neo clone and SW1116-PPARAi clone by flow cytometry after treatment with 150 or 300 μmol/L HCPT for 48 h. $^{*}P < 0.05$, $^{**}P < 0.01$.

to inhibit PPARα expression, we demonstrated that down-regulation of PPARα leads to a decrease in cellular proliferation and a simultaneous increase in HCPT-induced apoptosis in SW1116 cells. Interestingly, direct siRNA-mediated repression of PPARalpha or overexpression of miR-506 had similar effects on cellular proliferation and apoptosis upon HCPT treatment. This further suggests that PPARα is a true target gene of miR-506.

In summary, we show that miR-506 over-expression in established HCPT-resistant colon cancer cell line SW1116/HCPT confers resistance to HCPT by inhibiting PPARα expression.

Conflict of Interest

No conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.10.021.

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