Downregulation of miR-33a-5p in Hepatocellular Carcinoma: A Possible Mechanism for Chemotherapy Resistance

Authors' Contribution:
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Background: Multi-drug resistance is one of the major problems limiting the efficacy of cisplatin (CDDP) in treatment of hepatocellular carcinoma (HCC), and abnormal microRNA (miRNA) expression in drug-resistant cell lines plays an important role in liver cancer chemotherapy resistance.

Material/Methods: We established stable Hep3B and 97L HCC cell strains resistant to CDDP, both *in vitro* and *in vivo*. A combination of microRNA microarray and RT-qPCR experiments were used to screen differentially expressed miRNAs in HCC cell strains. A CCK-8 assay was carried out to detect and calculate the survival rates and relative inhibitory rates. Oligonucleotide transfection was used to confirm the regulatory function of the miRNA in HCC drug resistance.

Results: The IC50 of Hep3B/CDDP(v), 97L/CDDP(v), Hep3B/CDDP(s), and 97L/CDDP(s) were significantly higher than that of their parental cells. Moreover, the doubling time of drug-resistant cells increased compared with their parent cells. MiRCURYTM LNA Array (v 16.0) high-throughput tests of resistant cell models and their parent cells showed that there were 5 downregulated microRNAs in the 4 drug-resistant cell lines, and we chose hsa-miR-33a-5p as our target for further study. Oligonucleotide transfection showed that miR-33a-5p overexpression increased the cisplatin sensitivity of Hep3B/CDDP(v) and 97L/CDDP(v) drug-resistant cells and reduced their resistance. Additionally, inhibition of miR-33a-5p expression reduced cisplatin sensitivity in Hep3B and 97L and increased their drug resistance.

Conclusions: This study confirmed that the most downregulated microRNA, miR-33a-5p, can mediate the cisplatin resistance of HCC cells, providing a new and feasible direction for research into combatting liver cancer chemotherapy resistance.

MeSH Keywords: Carcinoma, Hepatocellular • Drug Resistance, Multiple • MicroRNAs

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Background

Primary hepatocellular carcinoma (HCC) is one of the most common malignant tumors, and ranks third in cancer mortality, worldwide [1]. It has a high degree of malignancy and a poor prognosis. Advanced HCC has low radical resection rate and high relapse rate, of which standardized treatment is sorafenib, and chemotherapy is also one of the treatments for it [2,3]. Transcatheter arterial chemoembolization with platinum-containing drugs or systemic chemotherapy are routinely used to control local tumor lesions and improve cancer-related symptoms in patients with intermediate- and advanced-stage HCC. In 2013, the EACH study showed that FOLFOX4 systemic chemotherapy using platinum-based drugs could induce local control and survival benefits in patients with advanced HCC [4,5]. Cisplatin is one of the most important anticancer drugs in TACE and systemic chemotherapy. Nevertheless, multidrug resistance significantly limits its efficacy. The underlying mechanisms are not yet fully understood [6].

MicroRNAs (miRNA) are a class of highly conserved non-coding small RNA. They are involved in a wide variety of physiological processes, such as cell proliferation, differentiation, apoptosis, and formation of tissues and organs. miRNA also plays an important role in tumorigenesis. In recent years, more and more studies have reported abnormal miRNA expression in drug-resistant cell lines such as cancers of the breast [7], ovary [8], and lung [9], as well as leukemia [10]. Through directly regulating key target genes, miRNAs can cause phenotypic alterations in tumor cells, thereby mediating tumor resistance to chemotherapy. For example, Climent found that breast cancer cells with a knocked-out 11q region containing miR-125 were more sensitive to anthracycline drugs, suggesting that miRNAs and cancer chemotherapy are significantly correlated [7]. Establishing a stable and reliable drug-resistant cell model is foundational to the study of the mechanisms of HCC cell resistance to chemotherapy. Most previous research has focused on *in vitro* induction methods to establish drug-resistant cell models. However, the *in vitro* induction of a drug-resistant HCC cell model through the application of subcutaneous xenografts in nude mice + intraperitoneal chemotherapy is considered to be an ideal modeling method, because it can more realistically simulate the true biological environment of chemotherapy resistance [9]. Until now, studies using a drug-resistant HCC cell model to investigate the mechanisms of HCC resistance to cisplatin *in vitro* and *in vivo* have not been reported.

An understanding of the molecular mechanisms of the miRNA imbalance in drug resistance must be obtained in order to overcome cisplatin resistance in future cancer treatment. Previous studies have shown that transcription disorders, mutations, DNA replication anomalies, and a faulty miRNA biogenesis pathway might be the main reasons for tumor miRNA

disorders [10]. For example, the enzyme Dicer, which activates miRNA, and the Argonaute2 protein were significantly downregulated in the Adriamycin-resistant breast cancer cell line MCF-7/DOX. Argonaute2 plays a key role in RNA silencing [11]. However, in recent years, studies have also shown that many miRNAs are influenced at the transcriptional level by DNA methylation, histone modifications, and other epigenetic mechanisms. Many studies have also shown that epigenetic drugs can alter miRNA expression by changing DNA methylation and chromatin remodeling patterns to re-induce miRNA expression [12,13]. Therefore, the exploration of the epigenetic regulation of miRNA involved in drug resistance is important for future clinical research.

This study aimed to provide new evidence for the mechanism of miRNA involvement in the cisplatin resistance of HCC cells. We established the first stable CDDP-resistant 97L and Hep3B HCC cell strains, both *in vitro* and *in vivo*. A combination of miRNA microarray and RT-qPCR experiments was used to screen differentially expressed microRNAs in the 4 drug-resistant HCC cell strains for further investigation. A CCK-8 assay was carried out to detect and calculate the survival rates and relative inhibitory rates.

Material and Methods

Cell culture and subculture

The human HCC cell lines Hep3B and 97L were seeded into 25-cm sterile culture flasks, then we added 5-ml DMEM high-glucose medium containing 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). This was cultured in 5% CO_2 at 37°C and the culture solution was changed every 1 to 2 days. When the cells were in the logarithmic growth phase, 0.5~1 ml of 0.25% trypsin was added and allowed to digest for 2 to 3 min, and then 4~5 ml DMEM medium containing 10% fetal bovine was added to terminate digestion, followed by a 1: 3 subculture.

Establishment of an Hep3B/CDDP(v), 97L/CDDP(v) drug-resistant cell model through intermittent *in vitro* induction with large doses of CDDP

Hep3B cells were dosed with 1 μg/ml and 97L cells with 4 μg/ml of the CDDP culture medium. After 24 h, the drug-containing culture medium was discarded and 0.25% trypsin was added for digestion. The medium was replaced every 1 to 2 days. When cell growth recovered, the medium was replaced with a low concentration of 0.1 μg/ml CDDP for continuous culture. After the cells immersed in the low-CDDP medium resumed exponential growth, 97L cells were again impacted using culture medium containing 4 μg/ml CDDP and Hep3B cells with

culture medium containing 1 µg/ml CDDP. Impacts were repeated 6 times.

Subcutaneous xenografts in nude mice + intraperitoneal chemotherapy to establish the Hep3B/CDDP(s)- and 97L/CDDP(s)-resistant cell models

We injected 1×10^5 Hep3B or 97L cells into the subcutaneous tissue on the right side of the backs of 4- to 6-week-old male nude mice. When the subcutaneous tumor reached a diameter of approximately 4 mm, the mice were given 1, 2, or 5 mg/kg CDDP intraperitoneal chemotherapy once every 4 days, 7 times total. After intraperitoneal chemotherapy, the tumor was aseptically removed for primary separation, and primary cells were purified by the successive differential adherence method. It took approximately 50 days to create the *in vivo* Hep3B/CDDP(s)- and 97L/CDDP(s)-resistant cell models.

Test CDDP resistance of HCC cells using the CCK-8 assay

HCC cells in the logarithmic growth phase were seeded into 96-well plates and cultured for 24 h. The culture medium was replaced with CDDP culture medium containing 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, or 16 µg/ml CDDP. Each well received 10 µl CCK-8 + 100 µl DMEM every 24, 48, or 72 h after the addition of CDDP, and cultured at 37°C in a chamber containing 5% CO_2 for 100 min. The 490-nm absorbance value (A) was detected using a microplate reader and the relative inhibitory rate. The IC50 and resistance index (RI) were also calculated.

MicroRNA chip

Total RNA was extracted using the TRIzol (Invitrogen) and miR-Neasy mini kits (QIAGEN). Samples were labeled with a label kit (miRCURY™ Hy3™/Hy5™ Power labeling kit) after detection with a visible spectrophotometer (NanoDrop 1000). They were then hybridized with the hybridization chip miRCURY™ LNA Array (v.16.0) and the chips were scanned (Axon GenePix 4000B microarray scanner). Grid alignment and data extraction of the images were performed with the GenePix Pro 6.0 image software (Axon). Repeated data was averaged and all samples with miRNA expression intensity ≥50 were selected as a normalization factor. MicroRNAs with significantly different expressions in drug-resistant cells and control cells were selected after normalization using a multiple differential screening method. The miRNA expressions of all differentially expressed samples were clearly displayed by a hierarchical clustering heat map.

Real-time quantitative PCR

Total RNA was extracted and reversely transcribed to cDNA. The specific primer was designed with Primer 5.0 software and synthesized by the Shanghai Kangcheng Biotechnology Company. Specific gene levels were detected with a real-time PCR reaction system and reaction parameters.

Oligonucleotide transfected cells

Cells in the logarithmic phase were made into single-cell suspension, seeded at 4×10^5 cells/well in 6-well plates, and cultured in antibiotic-free DMEM at 37°C and 5% CO_2. When the cell density reached 30-50%, hsa-miR-33a-5p mimic and a negative control were transfected into 97L/CDDP(v) with an hsa-miR-33a-5p inhibitor, while the negative control was transfected into Hep3B, using the LipofectamineTM2000 transfection kit.

Western blot analysis

Cells were lysed and total protein was extracted using a protein extraction kit (Lysates Lysis, protease inhibitor, phenylmethylsulfonyl fluoride PMSF, phosphatase inhibitor 2). The protein was quantified with a BCA protein quantitation kit and separated by electrophoresis using polyacrylamide gel. Next, protein transmembranes were blocked with 5% skim milk, incubated with antibody, and were exposed and developed. Images were taken and analyzed by computer.

Statistical Analysis

Experimental data were analyzed with SPSS 16.0 statistical software and are presented as mean ±SD. Homogeneity of variance among groups was analyzed with ANOVA, using the LSD statistical method for multiple comparisons and the Student's t-test for pairwise comparison. Either the paired or unpaired t-test was used, according to the situation. $P<0.05$ was considered statistically significant.

Results

Establishment of *in vivo* an *in vitro* drug-resistant HCC cell models

The Hep3B/CDDP(v) and 97L/CDDP(v) drug-resistant cell models were established using intermittent *in vitro* induction with large doses of CDDP. Hep3B/CDDP(s)- and 97L/CDDP(s)-resistant cell models were established using subcutaneous xenografts in nude mice + intraperitoneal chemotherapy. An optical microscope showed that the drug-resistant cells were significantly enlarged with irregular streaked shapes and tended to accumulate. Large numbers of granules and vacuoles were observed in the cytoplasm and the cell boundaries became blurred (Figure 1A, Supplementary Figure 1).
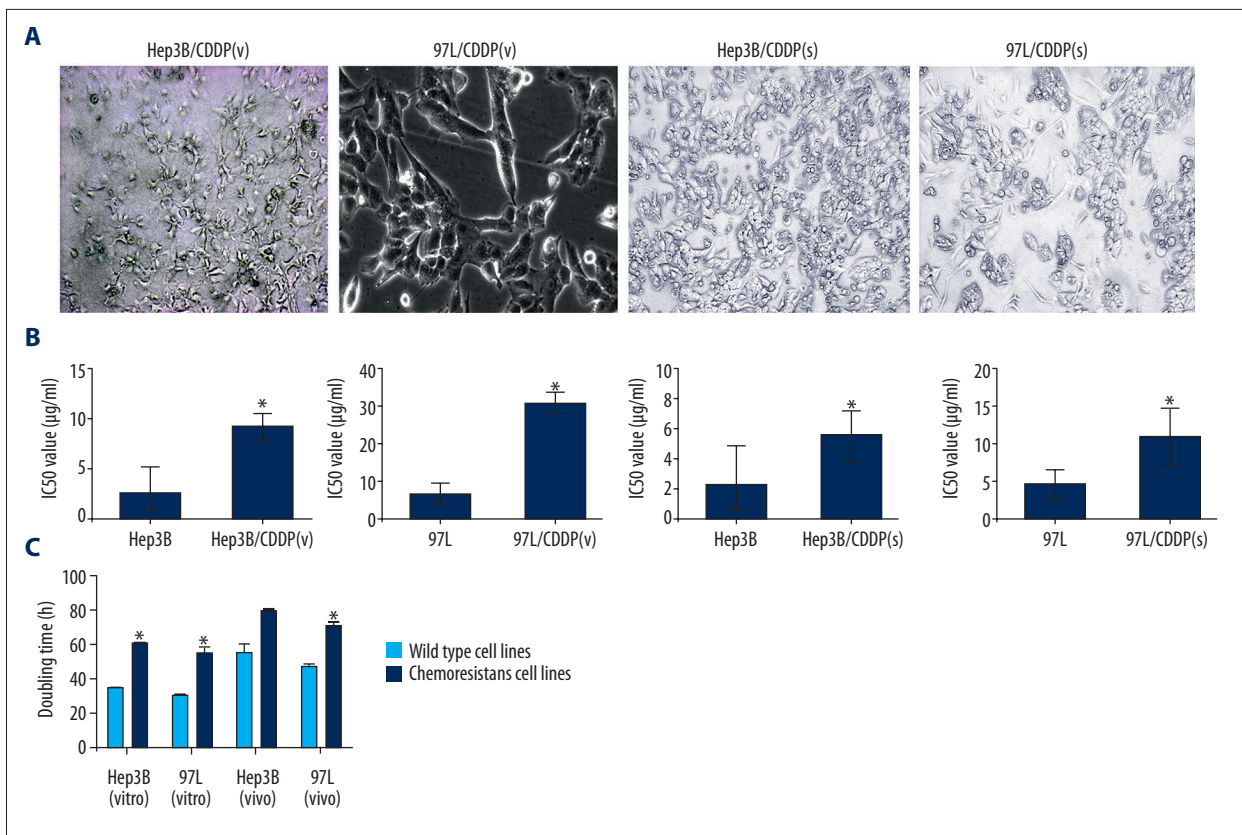
Figure 1. Establishment of *in vivo* and *in vitro* drug-resistant HCC cell models. (**A**) Morphological observation of a drug-resistant cell model; (**B**) Detection of the CDDP resistance capacity of the drug-resistant cell models; (**C**) Doubling time of drug-resistant cell models (* p<0.05).

Both the drug-resistant and the parent cells were processed for 24 h with stepped concentrations of CDDP. Survival rates were detected using CCK-8 and the relative inhibitory rates were calculated. Results showed that the IC50 of Hep3B/CDDP(v) and Hep3B were 9.24 and 2.63 μg/ml (p<0.05) and the IC50 of 97L/CDDP(v) and 97L were 30.77 and 6.71 μg/ml (p <0.05), respectively. The resistance indexes (RI) of Hep3B/CDDP(v) and 97L/CDDP(v) were 3.51 and 4.59. The IC50 of Hep3B/CDDP(s) and Hep3B(s) were 5.51 and 2.30 μg/ml (p <0.05) and the RI of Hep3B/CDDP(s) and 97L/CDDP(s) were 2.40 and 2.38. The IC50 of 97L/CDDP(s) and 97L(s) were 10.91 and 4.58 μg/ml (p<0.05), respectively (Figure 1B, Supplementary Figure 2). Therefore, compared with their parent cells, all drug-resistant cell models showed significant resistance to CDDP.

In addition, cell counts, growth curves, and doubling times were calculated 24, 48, 72, 96, 120, and 144 h after the seeding of drug-resistant cells and their parent cells. Results showed that the doubling time of Hep3B/CDDP(v) was 60.10±1.20 h and the doubling time of Hep3B was 34.51±1.16 h; thus, the doubling time of the drug-resistant cell population increased by 1.74 times. Likewise, the doubling time of 97L/CDDP(v) was 54.48±8.67 h and the doubling time of 97L was 29.92±3.04

h, showing that doubling time increased by 1.82 times in the drug-resistant cells. Hep3B/CDDP(s) and Hep3B(s) showed doubling times of 79.51±3.37 and 54.75±12.13 h, respectively. Thus, the doubling time of the drug-resistant cell population was extended by 1.45 times, compared with the control group. In addition, the doubling times of 97L/CDDP(s) and 97L(s) were 70.84±4.70 and 46.99±3.85 h, showing an increase in the doubling time of the drug-resistant cell population by 1.51 times, compared with the control group (Figure 1C). The growth of drug-resistant cells was slower than that of their parent cells (Supplementary Figure 3).

In conclusion, Hep3B/CDDP(v), 97L/CDDP(v), Hep3B/CDDP(s), and 97L/CDDP(s) are ideal models for further investigation of the mechanism of drug resistance in HCC cells.

miR-33a-5p regulating HCC drug resistance

MiRCURYTM LNA Array (v. 16.0) high-throughput tests of the resistant cell models and their parent cells showed that, compared with Hep3B, there were 190 upregulated microRNAs and 143 downregulated microRNAs in Hep3B/CDDP(v), compared with 97L, which showed 26 upregulated and 60 downregulated

microRNAs in 97L/CDDP(v). There were 22 upregulated and 45 downregulated microRNAs in Hep3B/CDDP(s), compared with 97L(s), in which there were 283 upregulated and 135 downregulated microRNAs (Figure 2A). Mutual intersection data from downregulated microRNAs in the 4 drug-resistant cell lines showed that there were 5 downregulated microRNAs in the 4 drug-resistant cell lines: hsa-miR-33a-5p, hsa-miR-4301, hsa-miR-455-3p, hsa-miR-483-3p, and hsa-miR-483-5p (Figure 2B). Recent studies have shown that hsa-miR-33a-5p is involved in tumor development, metastasis, and prognosis. Therefore, we chose hsa-miR-33a-5p as our research target. Real-time quantitative PCR was then performed to confirm its expression in drug-resistant cells, and the results showed that it was downregulated in the 4 cell lines, in accordance with the results from the microRNA array analysis (Figure 2C).

To verify whether hsa-miR-33a-5p regulates the drug resistance of HCC cells, we transfected Hep3B/CDDP(v) and 97L/CDDP(v) using an oligonucleotide pre-miR-33a-5p (miR-33a-5p mimic), and Hep3B and 97L with an oligonucleotide anti-miR-33a-5p (miR-33a-5p inhibitor) (Supplementary Figure 4). Transfected cells and their parent cells were treated with stepped concentrations of CDDP for 24 h, whereupon their survival rates and relative inhibitory rates were detected and calculated with the CCK-8 method. The results showed that the IC50 of the Hep3B/CDDP(v) transfection group and the negative control group were 8.03 and 12.86 ($p<0.05$), respectively, and the IC50 of the 97L/CDDP(v) transfection group and the negative control were 21.54 and 31.49 ($p<0.05$) (Figure 2D, 2E). This suggests that the overexpression of miR-33a-5p increased the sensitivity of Hep3B/CDDP(v) and 97L/CDDP(v) drug-resistant cells to cisplatin and reduced their drug resistance. The IC50 of the Hep3B transfection group and the negative control group were 4.17 and 2.18 ($p<0.05$), respectively, and the IC50 of the 97L transfection group and the negative control group were 14.06 and 9.89 ($p<0.05$), respectively (Figure 2F, 2G, Supplementary Figure 5), suggesting that inhibition of miR-33a-5p expression reduced the cisplatin sensitivity of both Hep3B and 97L and increased their drug resistance. In general, the expression of miR-33a-5p directly affected the cisplatin resistance of Hep3B and 97L HCC cells.

Discussion

Multi-drug resistance is one of the key factors limiting the efficacy of cisplatin in the comprehensive treatment of HCC [14,15]. In the present study, we established the first *in vitro* Hep3B/CDDP(v) and 97L/CDDP(v) drug-resistant cell models and the first *in vivo* drug-resistant Hep3B/CDDP(s) and 97L/CDDP(s) cell models. We found that, compared to the respective parent cells, cell line proliferation slowed significantly and the population doubling time increased. We postulate that the change in doubling time was related to the cell cycle and the various

cell cycle arrest periods. Delayed cell cycle progression could enable cells to escape cytotoxic injury from the cycle-specific chemotherapy drug cisplatin, resulting in drug resistance. Our results showed the consistent performance of the 4 different drug-resistant cell lines. They can be used to reflect a clinical cisplatin-resistant environment, for the purpose of establishing a foundation for the exploration of the drug-resistant mechanisms discussed in the present study.

In an attempt to elucidate the correlation between miRNAs and cisplatin, various studies have reported that miR-214 might induce cisplatin resistance in ovarian cancer cells by negatively regulating PTEN [6] and that the high expression level of miR-33a-5p could reduce cisplatin sensitivity via SOCS3 [16]. In the present study, we screened the differential expression map of miRNAs in drug-resistant cells, based on *in vitro* and *in vivo* drug-resistant cell models, and found that miR-33a-5p was downregulated in all 4 drug-resistant cell strains, confirming that its expression directly influences cisplatin resistance in HCC cells. MiR-33a-5P is mainly produced by mature miR-33a; the latter has been shown to have significantly decreased expression in lymphoma, colon cancer, and other cancers. The downregulation of miR-33a-mediated oncogenic kinase PIM-1 is an important strategy for the treatment of malignant tumors [17,18]. Therefore, miR-33a-5p might alter the phenotype of tumor cells through regulating the direct key target genes, thus mediating tumor resistance to chemotherapy. Our study verified that HSPA8 was the direct downstream target gene for miR-33a-mediated drug resistance (data not shown). In addition, studies have confirmed that epigenetic drugs can alter miRNA expression by changing DNA methylation and chromatin remodeling to re-induce miRNA expression [12,13], which might contribute to the way in which miR-33a-5p mediates cisplatin resistance of HCC cells at a transcriptional level. Interestingly, Zhou et al. observed that miR-33a expression was significantly higher in cisplatin-resistant osteosarcoma cells and could enhance its cisplatin resistance by downregulating TWIST [19]. Compared with the present study, we could confirm, on the one hand, that miR-33a-5p is highly correlated with cisplatin resistance and, on the other hand, that its expression in various types of drug-resistant cancer cells is significantly different.

Our study has several limitations. First, we selected miR-33a-5p, the most downregulated miRNA in the 4 drug-resistant HCC strains, as our target, but did not explore the other downregulated miRNAs, including miR-4301, miR-455-3p, and miR-483. Second, the use of ChIP-seq technology for the detection and modification of the whole genome of drug-resistant strains would be more helpful for the elucidation of the mechanisms of miR-33a disorders in drug-resistant HCC cells. Finally, we plan to explore whether other transcription factors or autophagy are involved in the process of HCC drug resistance mediated by HSPA8.
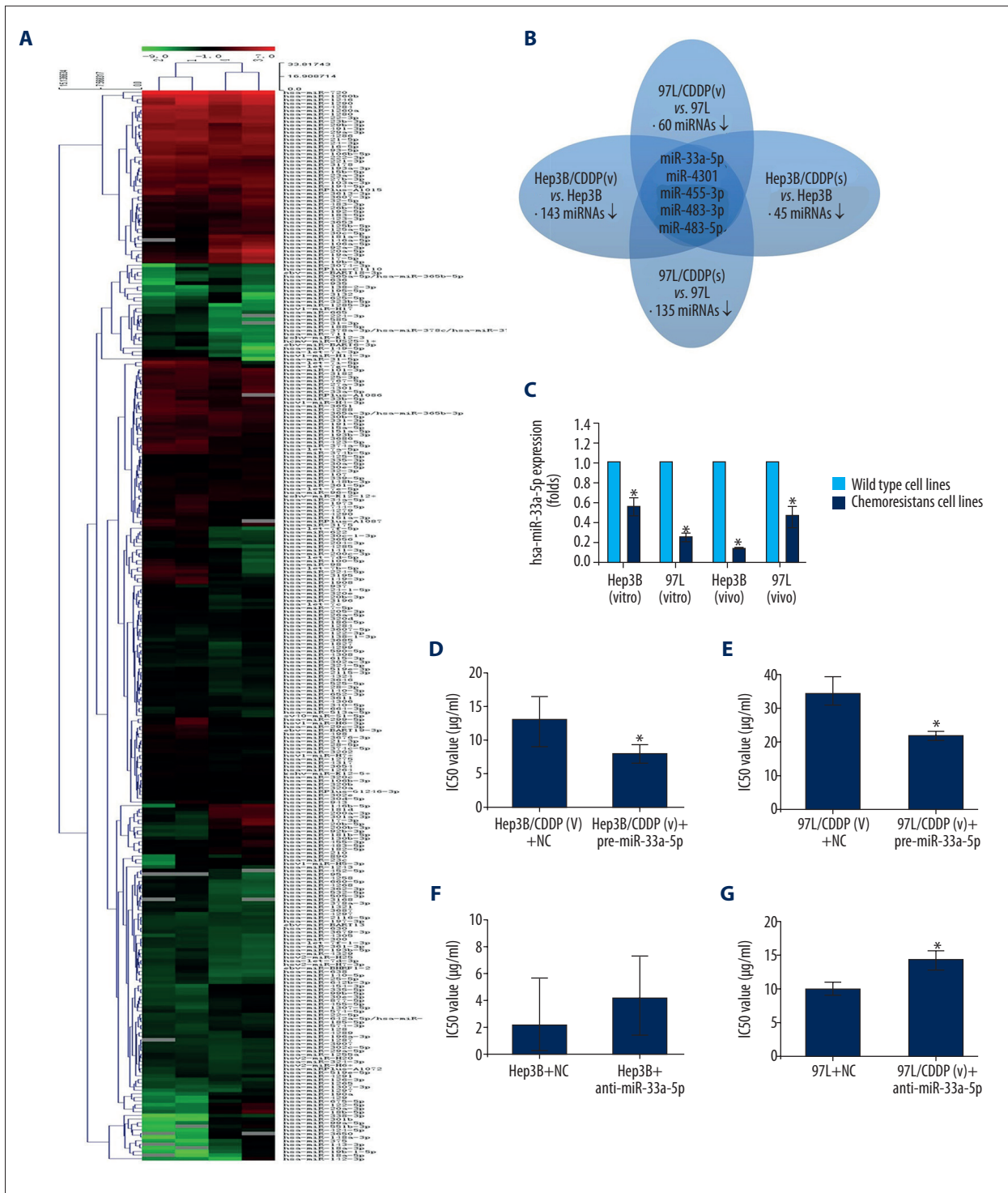
Figure 2. miR-33a-5p regulation of the drug resistance in HCC cells. (**A**) Hierarchical clustering heat map of differentially expressed microRNA standardized data; (**B**) Downregulated microRNA in drug-resistant cells compared with parent cells; (**C**) hsa-miR-33a-5p expression confirmed by real-time PCR; (**D**) Testing the drug resistance of Hep3B/CDDP(v) transfected with pre-miR-33a-5p; (**E**) Testing the drug resistance of 97L/CDDP(v) transfected with pre-miR-33a-5p; (**F**) Testing the drug resistance of Hep3B transfected with anti-miR-33a-5p; (**G**) Testing the drug resistance of 97L/CDDP(v) transfected with anti-miR-33a-5p (* p<0.05).

Conclusions

In summary, the present study established *in vitro* and *in vivo* drug-resistant HCC cell models and confirmed that the most downregulated miRNA, miR-33a-5p, was able to mediate the cisplatin resistance of HCC cells. We provided not only a new theory for the molecular mechanisms of cisplatin resistance in HCC cells, but also a new feasible direction for the reversal of liver cancer chemotherapy resistance.

Conflict of interest

These authors have no conflicts of interest to declare.
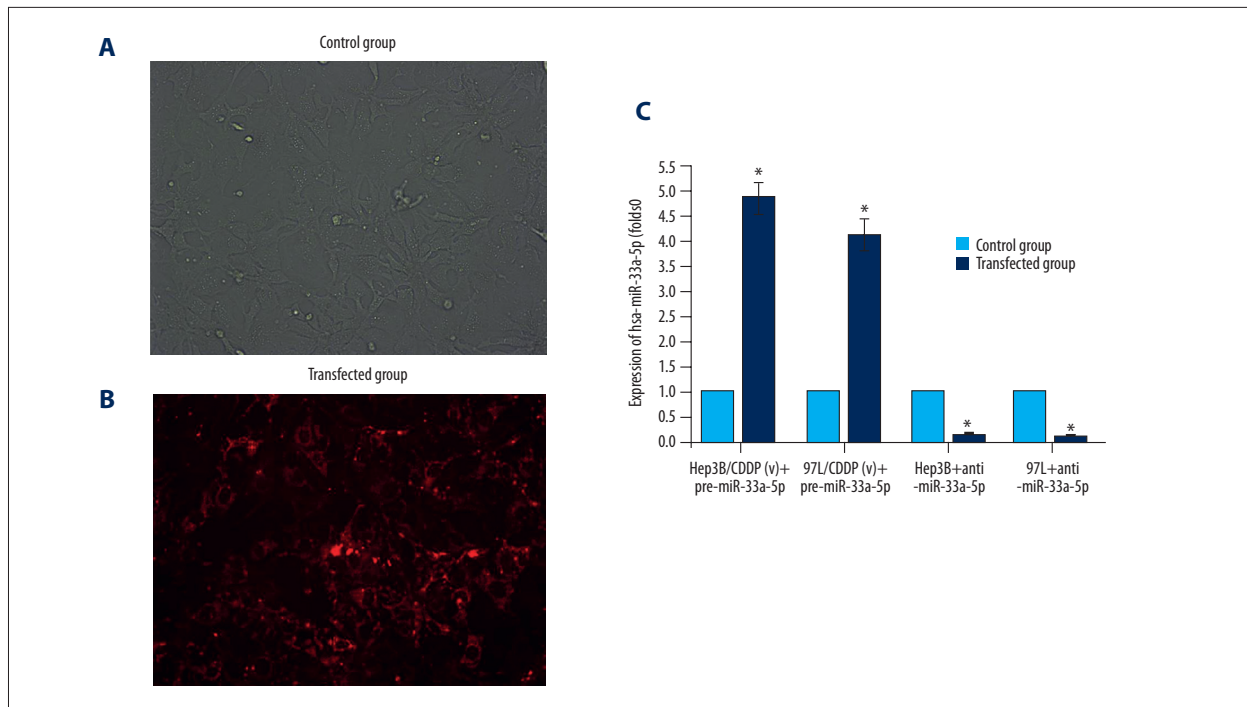
Supplementary Figures
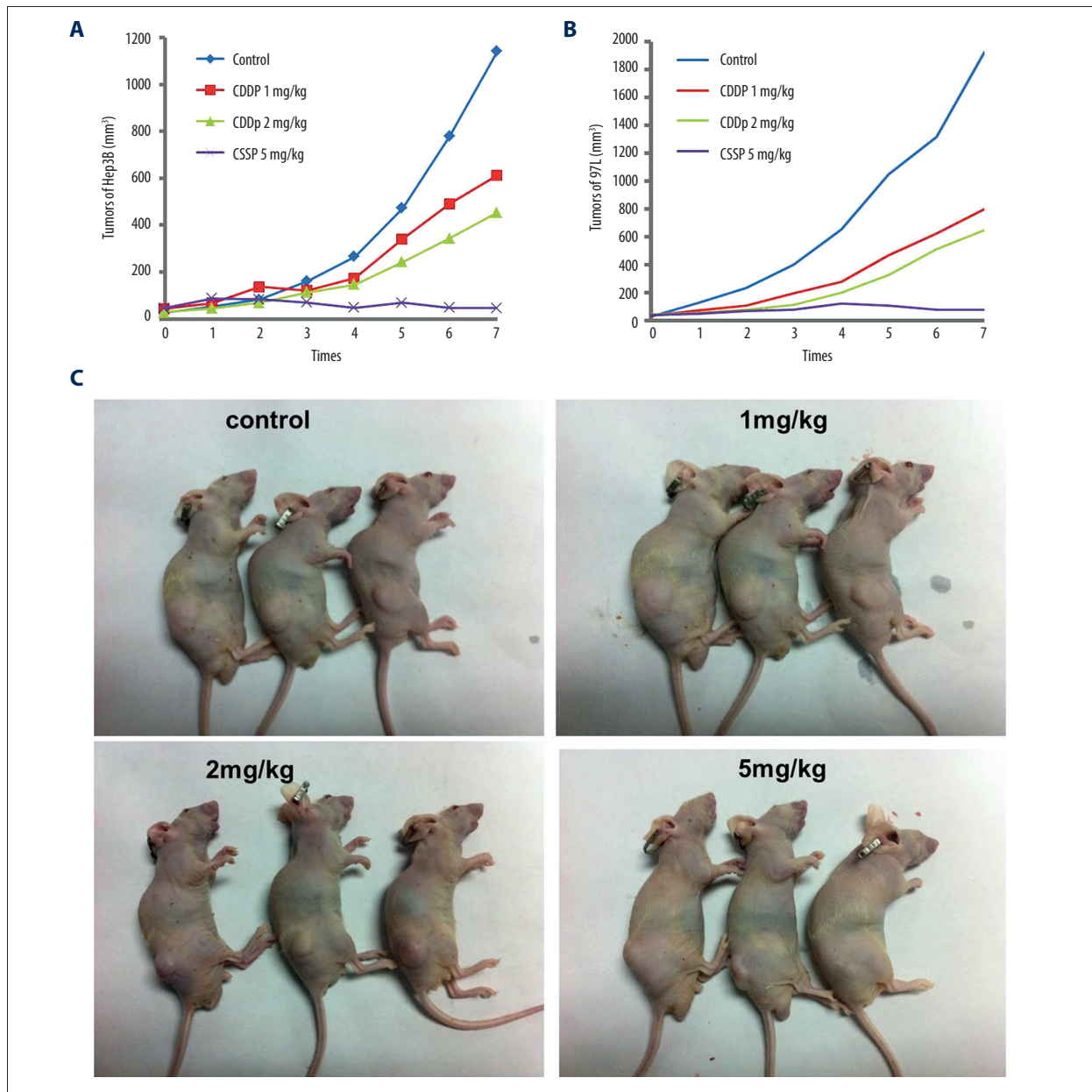
Supplementary Figure 1. Subcutaneous xenografts in nude mice + intraperitoneal chemotherapy to establish 97L/CDDP(s)- and Hep3B/CDDP(s)-resistant cell models; (**A**) Different concentrations of CDDP intraperitoneal chemotherapy on Hep3B subcutaneous xenografts; (**B**) Different concentrations CDDP intraperitoneal chemotherapy in 97L subcutaneous xenografts; (**C**) Growth situation of the subcutaneous xenografts in nude mice.
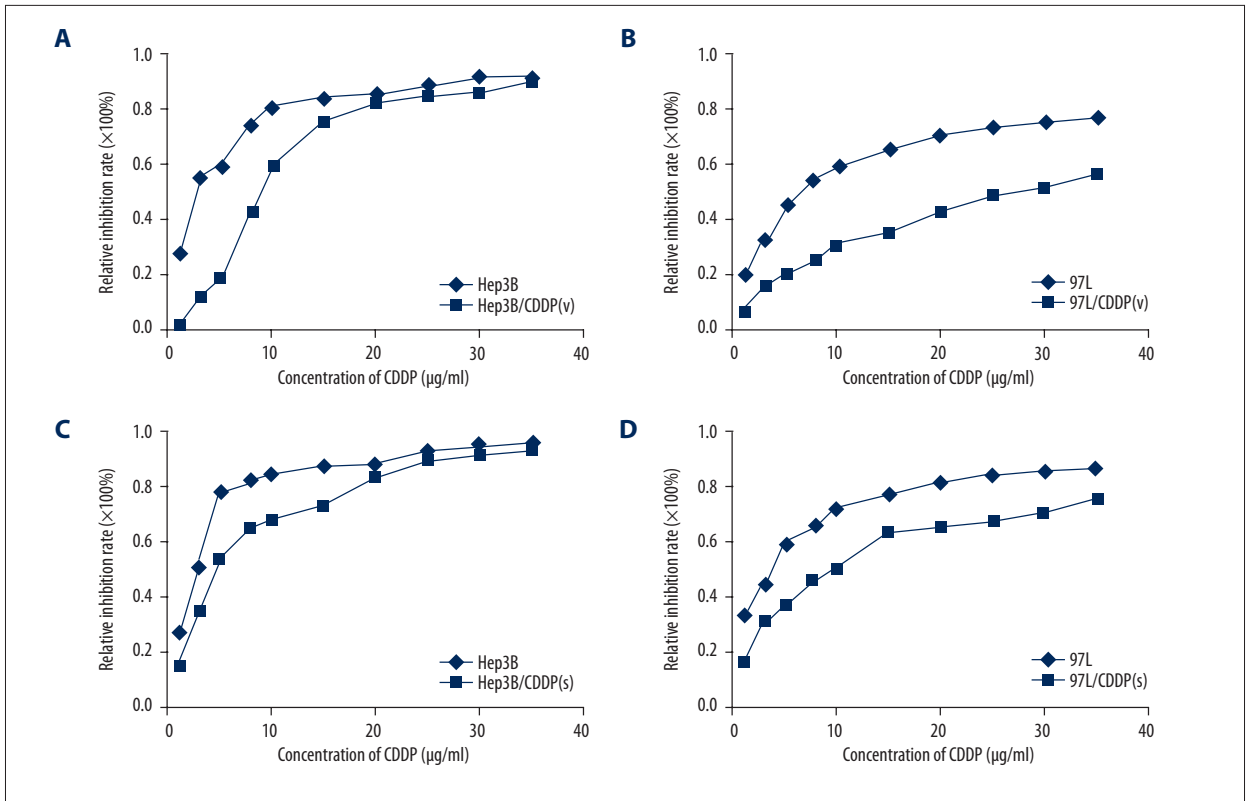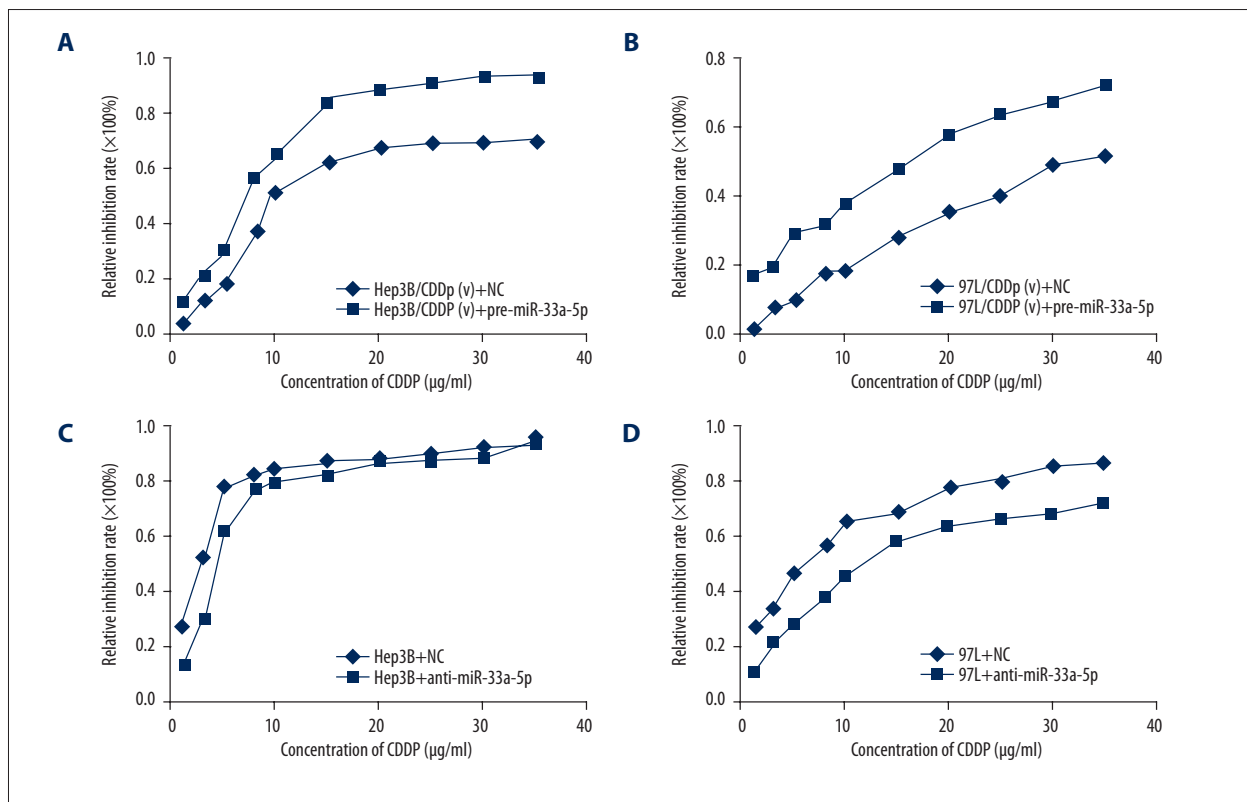
Supplementary Figure 2. Resistance capacity of HCC drug-resistant cell models to CDDP. (**A**) Hep3B/CDDP(v); (**B**) 97L/CDDP(v); (**C**) Hep3B/CDDP(s) D97L/CDDP(s).
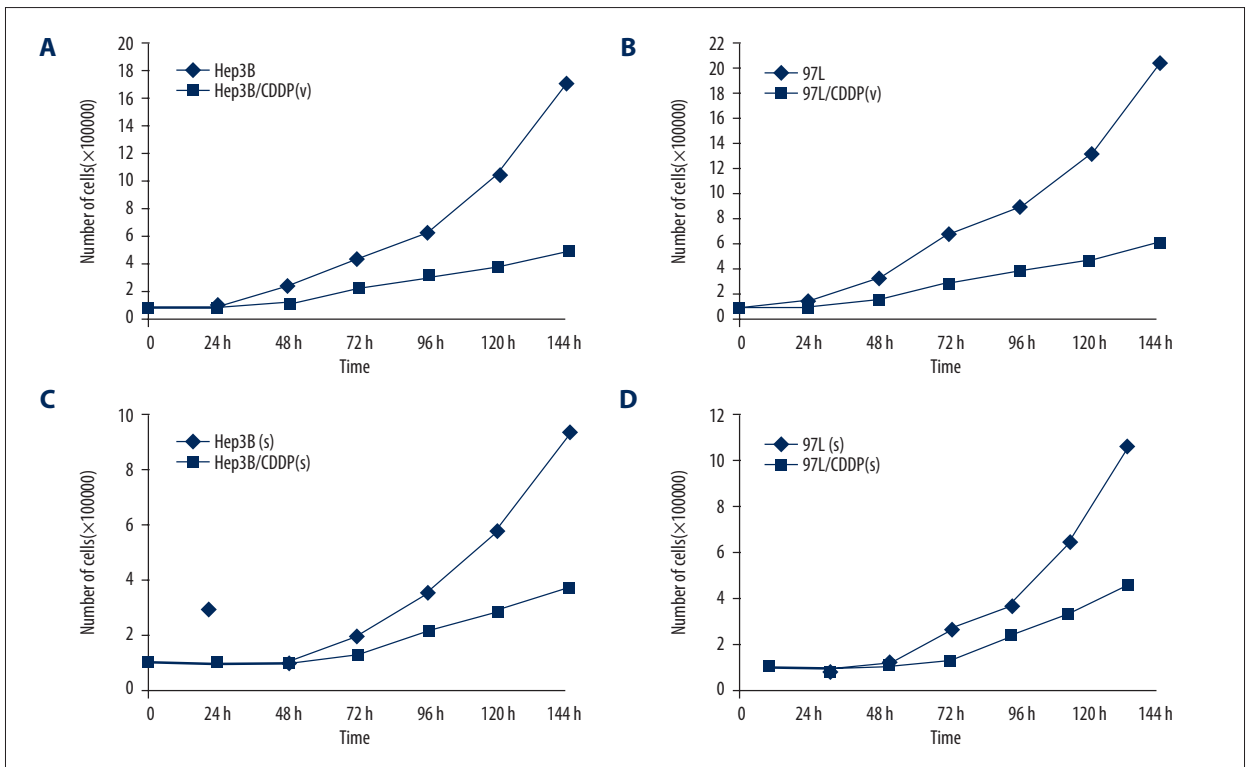
Supplementary Figure 3. Growth curve of drug-resistant HCC cell and parent cells.

Supplementary Figure 4. Oligonucleotide transfection efficiency. (**A, B**) Transfected cell (**A** before and **B** after); (**C**) Real-time PCR was used to detect transfection efficiency. (* p<0.05).

Supplementary Figure 5. Regulation effects of miR-33a-5p on HCC drug resistance. Detection of drug resistance after transfection with pre-miR-33a-5p. (**A**) Hep3B/CDDP(v); (**B**) 97L/CDDP(v). Detection of drug resistance after transfection with anti-miR-33a-5p. (**C**) Hep3B; D. 97L.

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