Differential miRNA expression profiles in hepatocellular carcinoma cells and drug-resistant sublines

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Abstract. Altered expression of miRNAs may contribute to multidrug resistance (MDR) in human cancers. This study investigated the association between miRNAs and MDR in five different drug-resistant hepatocellular carcinoma (HCC) cell sublines. The HCC Huh-7 cell line was treated with adramycin (ADM), cisplatin (DDP), carboplatin (CBP), mitomycin C (MMC) or vincristine (VCR) at increasing concentrations to develop drug-resistant sublines. The cell viability MTT assay was used to detect drug resistance. Five different drug-resistant HCC sublines, Huh-7/ADM, Huh-7/CBP, Huh-7/DDP, Huh-7/MMC and Huh-7/VCR, were established. Cells that were resistant to one drug were also found to be resistant to the other drugs. miRNA microarrays were analyzed to identify differential miRNA expression profiles in these cell lines, and real-time PCR was used to validate miRNA microarray data. miRNA microarray analysis showed that there were 53 upregulated miRNAs in Huh-7/ADM, 56 in Huh-7/CBP, 58 in Huh-7/DDP, 58 in Huh-7/MMC and 49 in Huh-7/VCR, whereas there were 52 downregulated miRNAs in Huh-7/ADM, 50 in Huh-7/CBP, 41 in Huh-7/DDP, 55 in Huh-7/MMC and 56 in Huh-7/VCR. Moreover, 26 simultaneously upregulated and 25 simultaneously downregulated miRNAs were noted in the Huh-7/ADM, Huh-7/CBP, Huh-7/DDP and Huh-7/MMC sublines compared to the parental Huh-7 cell line. In contrast, among these 51 upregulated and downregulated miRNAs, 12 miRNAs were upregulated and 13 miRNAs were downregulated in Huh-7/VCR. Upregulation of miR-27b, miR-181a, miR-146b-5p, miR-181d and miR-146a expression was verified using real-time RT-PCR in the parental and the five drug-resistant cell lines. In conclusion, the present study demonstrates that the differentially expressed miRNA profiles in these five drug-resistant HCC sublines could be useful to further investigate the association of miRNA expression with drug resistance in HCC.

Introduction

Hepatocellular carcinoma (HCC) is a significant worldwide health problem accounting for more than 750,000 new cases and more than 690,000 cancer-related deaths in 2011 (1), in which half of these cases and deaths were estimated to have occurred in China alone. HCC incidence rates are increasing in many parts of the world, including the US and Central Europe. To date, surgery is still the primary treatment for HCC. However, there are not many patients who are eligible to receive surgery due to a variety of reasons, such as unresectable tumors, distant tumor metastasis, insufficient hepatic function and poor health conditions. Moreover, HCC has a high recurrence rate after resection. Thus, neoadjuvant therapy is usually used to treat HCC patients. For example, transarterial chemoembolization (TACE), which is an important neoadjuvant treatment against HCC, has been extensively used to delay HCC progression in the clinic and to improve the prognosis of HCC patients. Chemotherapeutical drugs, such as adriamycin (ADM), mitomycin C (MMC), vincristine (VCR), 5-fluorouracil (5-FU), cisplatin (DDP) and carboplatin (CBP), are frequently used in HCC treatment (2,3). Among them, ADM is an antibiotic that eliminates tumor cells by preventing RNA biosynthesis. MMC, also an antibiotic, is able to directly destroy DNA and prevent DNA replication. Both DDP and CBP confer antitumor effects through inhibition of DNA biosynthesis and replication. In contrast, VCR targets the microtubules in cells, interferes with protein metabolism and inhibits activity of RNA polymerase and synthesis of plasmalemma adipoid. In the clinic, two or three drugs are combined together with TACE. However, multidrug resistance (MDR) prevents successful long-term use of chemotherapy. MDR mechanisms are usually complicated and many factors, such as P-glycoprotein (P-gp), resistance-associated protein (MRP), lung resistance protein (LRP), glutathione S-transferase (GST), cyclooxygenase 2 (COX-2), nuclear factor-κB (NF-κB) (4-9), have been found to participate in MDR.

More recently, it has been shown that altered expression of miRNAs plays an important role in MDR (10). miRNAs

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are small non-coding RNA molecules of 20-23 nucleotides in length and regulate a variety of biological processes. miRNAs inhibit mRNA translation of target genes through imperfect base pairing with the 5'- or 3'-untranslated region of the target mRNAs. Previous studies have underlined the involvement of miRNAs in drug resistance (11-18). Different miRNAs have been shown to be important in the mediation of chemosensitivity or chemoresistance in different cancer types, which occurs through the regulation of MDR- or cell-growth-related protein expression. Expression of miR-326 was found to downregulate MRP-1 expression and sensitize tumor cells to VP-16 and doxorubicin (13). Overexpression of miR-122 modulated HCC sensitivity to chemotherapeutic drugs through downregulation of MDR-related genes MDR-1, GST-π, MRP, antiapoptotic gene Bcl-w and cell cycle-related gene cyclin-B1 (15). Expression of miR-34a can negatively regulate, at least in part, resistance of colorectal cancer DLD-1 cells to 5-FU through targeting Sirt1 and E2F3 genes (19). In contrast, stable transfection of miR-21 induced drug resistance in K562 cells, while suppression of miR-21 in K562/DNR cells enhanced DNR cytotoxicity (20). miR-214 induces cell survival and DDP resistance primarily through targeting the PTEN/Akt pathway (21). miR-328 targets ABCG2 3'-UTR and, consequently, controls ABCG2 protein expression and influences drug disposition in human breast cancer cells (22). However, upregulation of miR-138 reverses resistance of both P-gp-related and P-gp-non-related drugs in HL-60/VCR cells and promotes adriamycin-induced apoptosis (16). Although these studies provide insightful information regarding miRNA-mediated MDR in human cancers, a complete profile of miRNA-mediated MDR is needed to systematically understand the role of miRNAs in MDR in HCC. Therefore, in this study, we first established five chemotherapeutical drug-resistant HCC cell sublines, Huh-7/ADM, Huh-7/DDP, Huh-7/CBP, Huh-7/MMC and Huh-7/VCR. We then subsequently profiled altered miRNA expression in these sublines in comparison to the parental HCC cell line using miRCURY™ LNA array (v. 16.0).

Materials and methods

Cell line and culture. The human HCC Huh-7 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL; Invitrogen Life Technologies, Carlsbad, CA, USA) in a humidified incubator at 37˚C with 5% carbon dioxide.

Generation of drug-resistant HCC cell sublines. The different drug-resistant HCC cell sublines were established by exposing the parental Huh-7 cells to increased concentrations of various chemotherapeutic drugs. Briefly, Huh-7 cells were inoculated in a 10-ml cell culture flask and cultivated for 72 h in culture medium containing a low concentration of drugs (0.02 μg/ml ADM, 0.02 μg/ml CBP, 0.0375 μg/ml DDP, 0.0015 μg/ml MMC, or 0.01 μg/ml VCR). Subsequently, the cells were continuously cultured without drug exposure for ~2 weeks. When cell growth was in the logarithmic phase, the cells were collected and re-inoculated in a 10-ml culture flask in

culture medium containing the above-mentioned drugs at an elevated concentration (1.5- to 2-fold of the previous dose) or at a previous concentration. This procedure was repeated until the cells exhibited stable growth and proliferation in a culture medium with 4.0 μg/ml ADM, 0.4 μg/ml CBP, 0.6 μg/ml DDP, 0.1 μg/ml MMC or 4.0 μg/ml VCR. A period of ~10-15 months was required to establish these drug-resistant HCC cell sublines. The level of drug resistance was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell viability MTT assay. Exponentially growing cells were seeded at 6,000-10,000 cells/well in 96-well plates with 100 μl of culture medium/well and incubated for 6 h. The cells were then exposed to different concentrations of the drugs for 65 h. At the end of the experiments, 20 μl of MTT (5 mg/ml in PBS) was added in each well, and the cells were cultured for an additional 4 h for formation of formazan crystals. Subsequently, 150 μl of DMSO was added to each well to dissolve the crystals. The values of the optical density at 570 nm were then measured using a microplate ELISA reader. Each experiment was performed in triplicate and repeated trice. Resistance factors (RF) were calculated by dividing the IC$_{50}$ value (drug concentration resulting in 50% reduction in absorbance compared with the control) of the drug-resistant cells with that of the parental control cells.

miRNA microarray analysis. Total cellular RNA was isolated using TRIzol (Invitrogen Life Technologies) and then cleaned using the miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA quality and quantity were measured using a Nanodrop spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE, USA), and RNA integrity was determined using gel electrophoresis. Subsequently, the miRCURY™ Hy3/Hy5 Power labeling kit (Exiqon, Vedbaek, Denmark) was used to label the RNA as probes according to the manufacturer's guideline. Next, the Hy3TM-labeled RNA samples were hybridized on miRCURY™ LNA array (v. 16.0) (Exiqon) slides according to the array manual. The slides were then washed three times with wash buffer (Exiqon) and dried using centrifugation for 5 min at 400 rpm. Finally, the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA).

The scanned images were imported into GenePix Pro 6.0 software (Axon Instruments) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities >50 in all the samples were chosen to calculate a normalization factor. Expressed data were then normalized using the median normalization. Differentially expressed miRNAs were then identified according to the fold changes and intensities, and hierarchical clustering was performed using MEV software (v. 4.6, TIGR).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total cellular RNA samples from the cells were reversely transcribed into cDNA using a Universal cDNA synthesis kit (Exiqon) according to the manufacturer's instructions. Real-time PCR amplification was performed on an ABI 7500 Real-Time PCR system with the DNA-binding

Table I. IC_{50} values and resistance factors (RF) in the HCC Huh-7 cell line and the five drug-resistant sublines.

Drug	Huh-7 IC_{50}	Huh-7/ADM IC_{50} (RF)	Huh-7/CBP IC_{50} (RF)	Huh-7/DDP IC_{50} (RF)	Huh-7/MMC IC_{50} (RF)	Huh-7/VCR IC_{50} (RF)
ADM	1.6±0.44	117.24±22.96[a] (73.28)	74.66±0.55[b] (46.66)	11.87±0.81[b] (7.42)	28.44±6.75[a] (17.78)	148.73±13.52[b] (92.96)
CBP	19.79±1.86	27.43±1.31[b] (1.39)	214.55±36.57[a] (10.84)	56.87±1.03[b] (2.87)	74.71±3.93[b] (3.78)	103.11±20.08[a] (5.21)
DDP	3.47±0.71	14.36±6.47 (4.14)	15.87±1.57[b] (4.57)	13.66±0.41[b] (3.94)	4.37±0.71 (1.26)	12.33±1.64[b] (3.55)
MMC	1.98±0.12	6.9±0.84[b] (3.49)	2.7±0.1[b] (1.36)	1.72±0.394 (0.87)	12.76±0.44[b] (6.44)	9.77±0.84[b] (4.93)
VCR	4.88±0.7	217.22±49.84[a] (44.5)	69.91±16.38[a] (14.33)	46.52±4.43[b] (9.53)	100.57±13.72[b] (20.61)	243.45±60.23[a] (49.89)

The IC_{50} values are shown as means ± SD (n=3). [a]P<0.05, [b]P<0.01. Statistical analyses were performed with the independent samples t-test in SPSS 16.0.
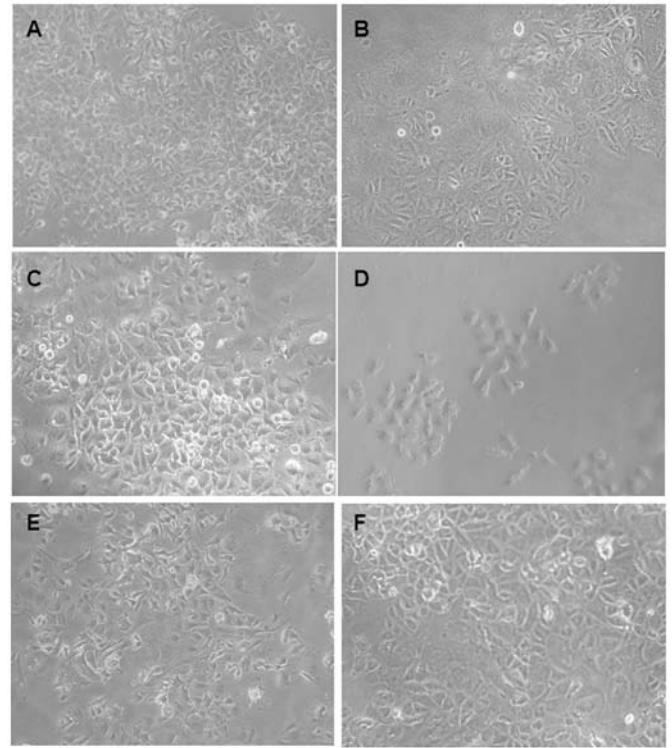

Figure 1. Changes in cell morphology of the five drug-resistant HCC sublines. (A) Huh-7; (B) Huh-7/ADM; (C) Huh-7/CBP; (D) Huh-7/DDP; (E) Huh-7/MMC; (F) Huh-7/VCR. The cell morphology of the drug-resistant cells reveals larger and more cellular pleomorphism than that of the parental Huh-7 cells, although their shapes are polygon and similar to the Huh-7 cells. In contrast, some cells have long pseudopodia (magnification, x200).

dye technique (SYBR Green) according to the manufacturer's instructions. The primers for the different miRNAs and PCR reagents were purchased from Exiqon. U6 snRNA was used as the reference gene, and $2^{-\Delta\Delta ct}$ was used to calculate the expression levels of miRNAs in the drug-resistant HCC cell sublines compared to the parental Huh-7 cell line.

Results

Establishment of different chemotherapeutical drug-resistant HCC cell sublines. In this study, we first established drug-resistant HCC cell sublines by treating HCC Huh-7 cells with increasing doses of ADM, DDP, CBP, MMC and VCR for ~10-15 months. The drug-resistant concentration of ADM was 4.0 μg/ml, CBP was 0.4 μg/ml, DDP was 0.6 μg/ml, MMC was 0.1 μg/ml and VCR was 4.0 μg/ml. The HCC cell sublines were named Huh-7/ADM, Huh-7/DDP, Huh-7/CBP, Huh-7/MMC and Huh-7/VCR, respectively. The IC_{50} values and RF, which are shown in Table I, indicated that these drug-resistant sublines were not only resistant to the treatment drug, but also resistant to the other drugs. Under an inverted microscope, although the drug-resistant cells were similar to the parental Huh-7 cells, the drug-resistant cells grew slowly as colonies and most of the cell lines had an enlarged cell body (Fig. 1).

Differential expression of miRNAs in the drug-resistant HCC cell sublines. To profile differential expression of various miRNAs in the drug-resistant HCC sublines, we performed
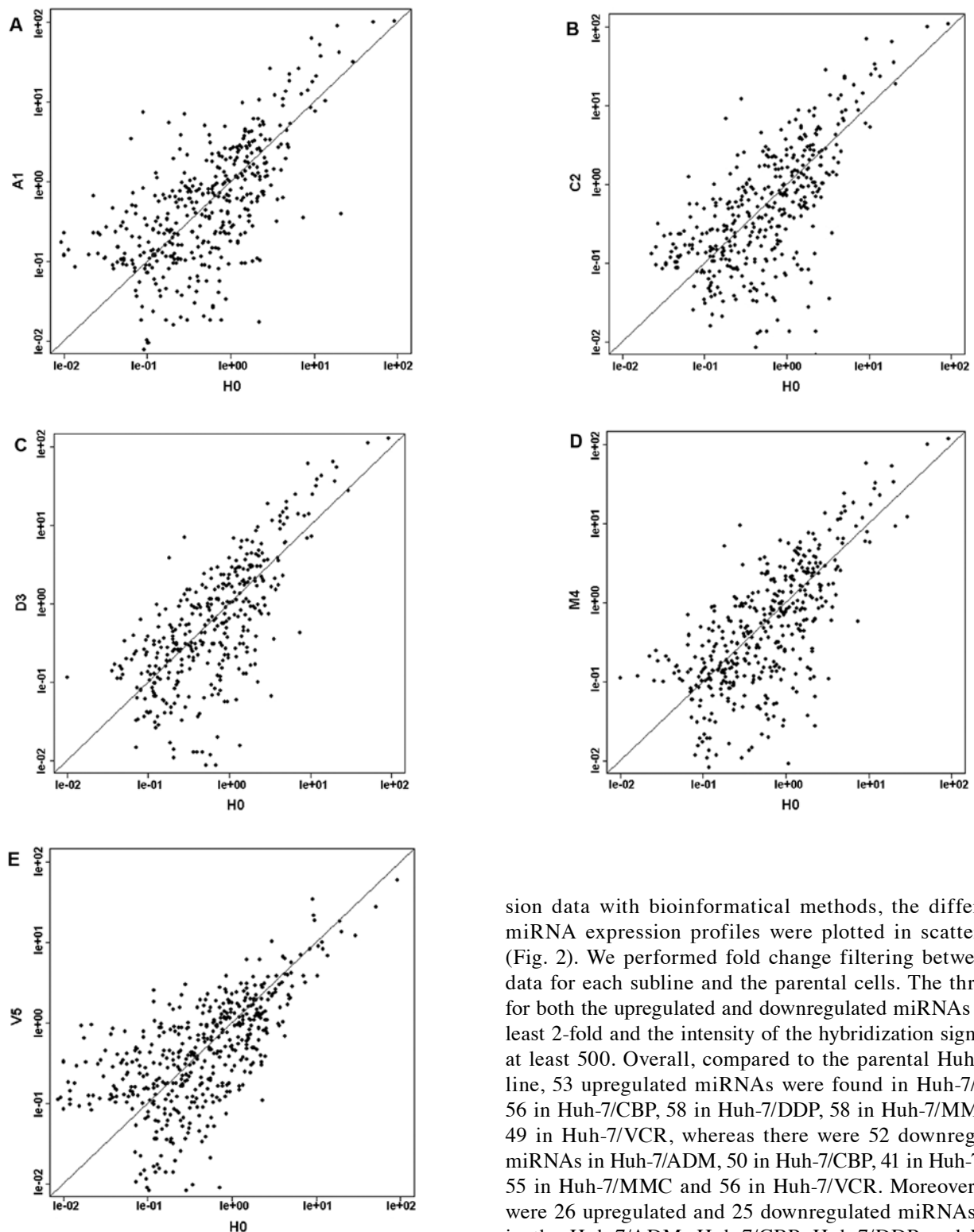
Figure 2. miRNA expression profiles between the five drug-resistant cell sublines and their parental Huh-7 cell line. The axes of the scatter-plot are the normalized signal values (ratio scale). The variation in miRNA expression levels in the drug-resistant sublines compared to Huh-7 cells were similar for Huh-7/ADM, Huh-7/CBP, Huh-7/DDP and Huh-7/MMC. H0, Huh-7; A1, Huh-7/ADM; C2, Huh-7/CBP; D3, Huh-7/DDP; M4, Huh-7/MMC; V5, Huh-7/VCR. (A) $R_{A1-H0}=0.8277$, (B) $R_{C2-H0}=0.8451$, (C) $R_{D3-H0}=0.9093$, (D) $R_{M4-H0}=0.8964$, (E) $R_{V5-H0}=0.8647$.

sion data with bioinformatical methods, the differential miRNA expression profiles were plotted in scatter-plots (Fig. 2). We performed fold change filtering between the data for each subline and the parental cells. The threshold for both the upregulated and downregulated miRNAs was at least 2-fold and the intensity of the hybridization signal was at least 500. Overall, compared to the parental Huh-7 cell line, 53 upregulated miRNAs were found in Huh-7/ADM, 56 in Huh-7/CBP, 58 in Huh-7/DDP, 58 in Huh-7/MMC and 49 in Huh-7/VCR, whereas there were 52 downregulated miRNAs in Huh-7/ADM, 50 in Huh-7/CBP, 41 in Huh-7/DDP, 55 in Huh-7/MMC and 56 in Huh-7/VCR. Moreover, there were 26 upregulated and 25 downregulated miRNAs noted in the Huh-7/ADM, Huh-7/CBP, Huh-7/DDP and Huh-7/ MMC sublines. However, among these 51 upregulated and downregulated miRNAs, 12 miRNAs were upregulated and 13 miRNAs were downregulated in the Huh-7/VCR subline. We then performed a hierarchical clustering analysis for these miRNAs (Fig. 3). Fig. 4 shows the normalized value of these differentially expressed miRNAs between Huh-7 cells and the Huh-7/ADM, Huh-7/CBP, Huh-7/DDP, Huh-7/MMC and Huh-7/VCR sublines.

miRNA microarray analysis in these sublines, as well as in the parental HCC Huh-7 cells. After normalizing the expres-

Validation of several differentially expressed miRNAs in HCC cells. We validated the microarray data using real-time RT-PCR
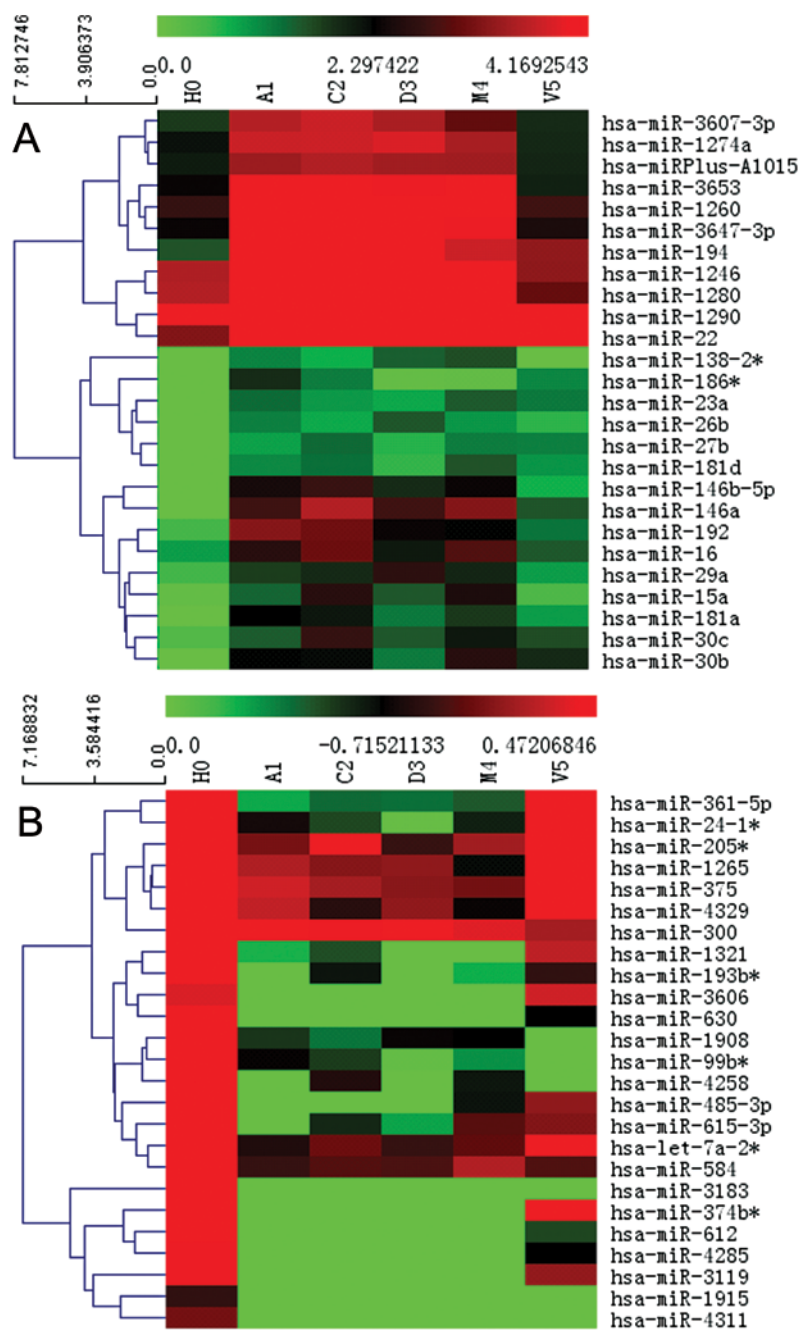
Figure 3. Hierarchical clustering analysis of the miRNA microarray data. (A) Analyses of the 26 upregulated miRNAs in Huh-7/ADM, Huh-7/CBP, Huh-7/DDP and Huh-7/MMC. (B) Analyses of the 25 downregulated miRNAs in Huh-7/ADM, Huh-7/CBP, Huh-7/DDP and Huh-7/MMC. The heat map diagram shows the results of the two-way hierarchical clustering analysis of miRNA expression levels and the cell lines. Each row represents an miRNA and each column represents a cell line. The miRNA-clustering tree is shown on the left and the cell line-clustering tree is at the top. The color scale shown at the top illustrates the relative expression level of an miRNA in a certain slide. A red color represents a high relative expression level and a green color represents a low relative expression level. H0, Huh-7; A1, Huh-7/ADM; C2, Huh-7/CBP; D3, Huh-7/DDP; M4, Huh-7/MMC; V5, Huh-7/VCR.

analysis. We chose the five most significantly expressed miRNAs (miR-27b, miR-181a, miR-146b-5p, miR-181d and miR-146a) to be verified in Huh-7, Huh-7/ADM, Huh-7/CBP, Huh-7/DDP, Huh-7/MMC and Huh-7/VCR cells. Our qRT-PCR data confirmed the microarray data (Table II). Table II also lists potential target genes of these miRNAs.

Discussion

In the present study, we first established five different chemo-therapeutic drug-resistant HCC cell sublines and then profiled the altered miRNA expression in these sublines in comparison to the parental HCC cell line using miRCURY™ LNA array (v. 16.0). We found that cells that were resistant to one drug were also resistant to the other drugs. miRNA microarray data revealed 53 upregulated miRNAs in Huh-7/ADM, 56 in Huh-7/CBP, 58 in Huh-7/DDP, 58 in Huh-7/MMC and 49 in the Huh-7/VCR subline. In contrast, there were 52 downregulated miRNAs in Huh-7/ADM, 50 in Huh-7/CBP, 41 in Huh-7/DDP, 55 in Huh-7/MMC and 56 in Huh-7/VCR. In total, there were 26 simultaneously upregulated and 25 simultaneously down-regulated miRNAs in Huh-7/ADM, Huh-7/CBP, Huh-7/DDP
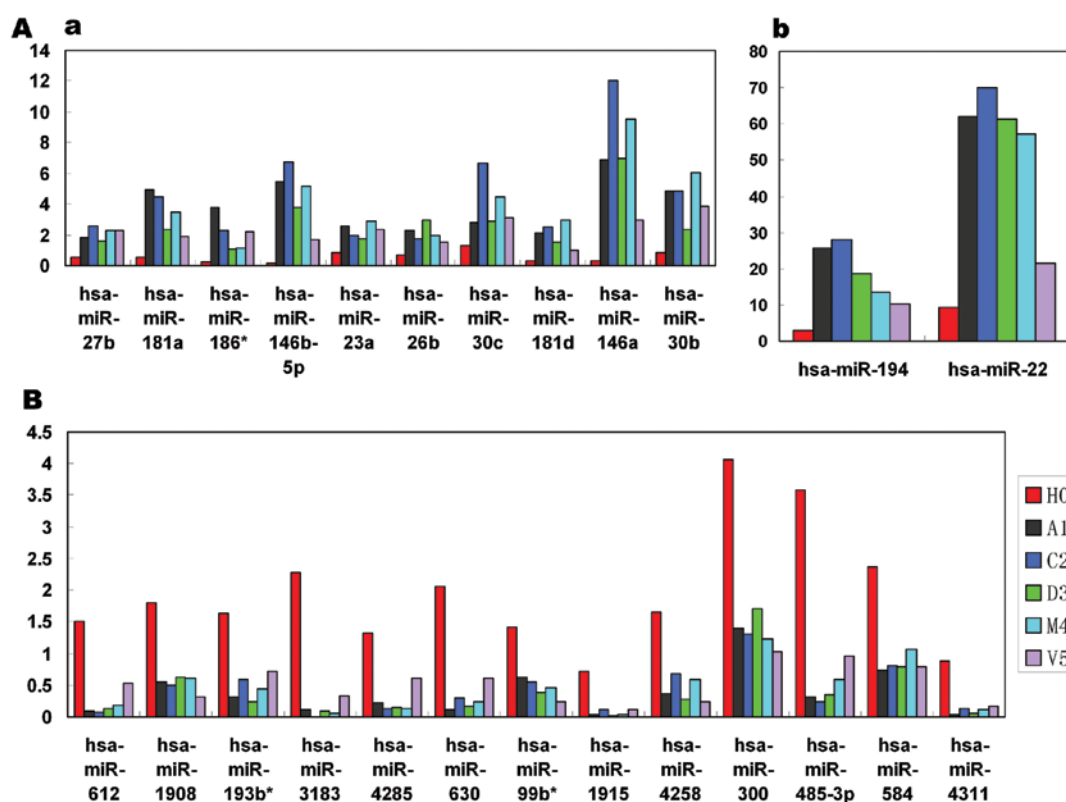
Figure 4. Analyses of the miRNAs exhibiting differential expression in the Huh-7 cell line and the drug-resistant sublines. (Aa and b) Normalized values of the simultaneously upregulated miRNAs in the five drug-resistant sublines. (B) Normalized values of the simultaneously downregulated miRNAs in the five drug-resistant cell sublines. H0, Huh-7; A1, Huh-7/ADM; C2, Huh-7/CBP; D3, Huh-7/DDP; M4, Huh-7/MMC; V5, Huh-7/VCR.

Table II. Confirmation of miRNA expression levels in the five drug-resistant sublines.

	Fold changes (microarray, real-time PCR[a])								
	A1/H0		C2/H0		D3/H0		M4/H0		
miRNA	Array	qPCR	Array	qPCR	Array	qPCR	Array	qPCR	Target genes
miR-27b	3.54	20.13	5.02	7.48	3.04	1.72	4.45	4.56	BAX, P53, FoxO1, KRAS
miR-181a	9.98	22.32	8.99	12.41	4.77	2.17	7.02	6.45	PTEN, KRAS, RB1
miR-146b-5p	29.84	35.47	36.96	10.14	20.75	4.28	28.15	3.85	TRAF6, PTGS2
miR-181d	7.08	6.97	8.20	3.86	4.97	2.02	9.80	3.19	PTEN, KRAS, RB1
miR-146a	24.44	441.13	42.60	96.45	24.57	12.95	33.69	50.66	TRAF6, PTGS2

H0, Huh-7; A1, Huh-7/ADM; C2, Huh-7/CBP; D3, Huh-7/DDP; M4, Huh-7/MMC; V5, Huh-7/VCR. [a]$P<0.001$ for all differences. Information regarding the miRNA target genes was obtained from http://www.microrna.org/.

and Huh-7/MMC. Among these 51 altered miRNAs, 12 were upregulated and 13 miRNAs were downregulated in the Huh-7/VCR subline. We chose miR-27b, miR-181a, miR-146b-5p, miR-181d and miR-146a to verify the results with real-time RT-PCR. This study profiled altered miRNA expression in the five most commonly used drug-induced HCC resistant sublines, which provides useful information for further investigation of miRNA-mediated drug resistance in HCC.

Generally, to study the mechanisms responsible for tumor multidrug resistance, three methods are usually used to establish a multidrug-resistant tumor cell line. These methods include the induction of drug resistant cell lines *in vitro*, MDR gene transfection, or the induction of drug resistance with a nude mouse implanted tumor model. To induce tumor cell MDR *in vitro*, two different cell culture methods are used: the drug concentration incremental gradient method and the high-concentration intermittent drug-induced method (23). In the present study, we established drug-resistant cell sublines using the drug concentration incremental gradient method. The drug-resistant cell sublines were resistant not only to the drug

used to induce the subline, but also to the other drugs, which is characteristic of MDR. These data provide the foundation to pursue subsequent research concerning the altered expression of miRNAs in these sublines.

A large number of miRNAs with altered expression were noted in these sublines compared to the parental HCC cell line. Several of the miRNAs, such as the miR-15/16 family (14), miR-30c (24,25), the miR-181 family (18,26,27), miR-23a (28), miR-192 (29), miR-27b (12), miR-194 (12,29), miR-22 (12), miR-29a (12,30), miR-146a (29) and the let-7 family (31,32), have been previously reported to be related to chemoresistance in different types of cancers. Specifically, miR-15b and miR-16 play a role in the development of MDR in gastric cancer through modulation of apoptosis by targeting Bcl-2 expression (14), while miR-30c was found to be downregulated in various chemoresistant tumor cell lines (23). Moreover, miR-181d was upregulated in two docetaxel-induced head and neck squamous cell cancer MDR cell lines (27), and miR-23a was shown to be an upstream regulator of TOP2B that mediated cisplatin chemoresistance in tongue squamous cell carcinoma cell lines (28). Furthermore, miR-192 was found to target dihydrofolate reductase, a key enzyme in folate metabolism that influences sensitivity in colorectal cancer cell lines when treated with 5-FU (29). In addition, breast cancer MCF-7/DOX cells resistant to DOX treatment were associated with the increased expression of miR-22, miR-29a, miR-194 and miR-132 (12). miR-let-7a was downregulated in the NCI-60 cell line, which is sensitive to cyclophosphamide (31). Our current data revealed 12 simultaneously upregulated and 13 downregulated miRNAs in the five HCC drug-resistant cell sublines. Further investigation of these 25 miRNAs may help elucidate HCC cell MDR to these five drugs.

In addition, we used a SYBR-Green miRNA real-time qPCR analysis to validate the expression of miR-27b, miR-181a, miR-146b-5p, miR-181d and miR-146a in the drug-resistant HCC cell sublines. miR-27b, which is localized at chromosome q22.32, has been reported to play a role in cell growth, tumor progression and the inflammatory response (33-36). miRNA-146a/b has been shown to be involved in the modulation of inflammatory responses (37), immune responses (38) and suppression of tumor metastasis (39-41). miR-181a was reported to be associated with patient prognosis (42,43). Finally, miR-181a and mir-181d (44) have been shown to have antitumor activity (43). However, their role in HCC MDR will be further investigated in our next study.

Acknowledgements

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