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Original Article

Genome-wide analysis of microRNA and mRNA expression signatures in hydroxycamptothecin-resistant gastric cancer cells

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Aim: To investigate the involvement of microRNAs (miRNAs) in intrinsic drug resistance to hydroxycamptothecin (HCPT) of six gastric cancer cell lines (BGC-823, SGC-7901, MGC-803, HGC-27, NCI-N87, and AGS).
Methods: A sulforhodamine B (SRB) assay was used to analyze the sensitivity to HCPT of six gastric cancer cell lines. The miRNA and mRNA expression signatures in HCPT-resistant cell lines were then identified using DNA microarrays. Gene ontology and pathway analysis was conducted using GenMAPP2. A combined analysis was used to explore the relationship between the miRNAs and mRNAs.
Results: The sensitivity to HCPT was significantly different among the six cell lines. In the HCPT-resistant gastric cancer cells, the levels of 25 miRNAs were deregulated, including miR-196a, miR-200 family, miR-338, miR-126, miR-31, miR-98, let-7g, and miR-7. Their target genes were related to cancer development, progression and chemosensitivity. Moreover, 307 genes were differentially expressed in HCPT-resistant cell lines, including apoptosis-related genes (BAX, TIAL1), cell division-related genes (MCM2), cell adhesion- or migration-related genes (TIMP2, VSNL1) and checkpoint genes (RAD1). The combined analysis revealed 78 relation pairs between the miRNAs and mRNAs.
Conclusion: Hierarchical clustering showed that the miRNA and mRNA signatures in our results were informative for discriminating cell lines with different sensitivities to HCPT. However, there was slightly lower correlation between the expression patterns of the miRNA and those of the predicted target transcripts.

Keywords: hydroxycamptothecin (HCPT); gastric cancer; microRNA; signature; drug resistance; gene expression regulation; cluster analysis; messenger RNA

Introduction

Gastric cancer is the second leading cause of cancer death worldwide and accounts for approximately 10% of newly diagnosed cancers[1]. Camptothecins (CPTs) have been approved for the treatment of various malignancies, including gastric cancer. By binding to the covalent complex formed by Top1 and DNA, CPTs prevent the re-ligation of DNA, which leads to persistent DNA breaks, subsequently inducing cell apoptosis[2]. Clinical data show that the efficiency of CPTs varies between patients, and the resistance mechanism remains to be determined[3]. Previous studies indicated that the resistant phenotype may be the result of inadequate drug accumulation in cancer cells, alterations in the target Top1 and alterations in cellular drug responses including DNA repair and apoptosis[4–6]. Drug transporters that are ATP-dependent, such as P-glycoprotein, multidrug resistance-related protein (MRP) and breast cancer resistance protein (BCRP), were involved in the efflux of CPTs. UDP glucuronosyltransferases, such as UGT1A1, UGT1A3, UGT1A6, and UGT1A9, were associated with the glucuronidation of CPTs, which increased the efflux of this drug. Top1-binding protein nucleolin and small ubiquitin-like modifier (SUMO) could relocalize TOP1, the target of CPTs. The checkpoint genes *ATM*, *MEC1*, *MEC2*, *CHK1*, *RAD9*, *RAD17*, and *ATR* and the DNA repair genes *CSA*, *CSB36*, *RAD6*, *TDP*, *TRF4*, *MSM2*, *MSM3*, *XRCC*, and *PNK1* were related to the repair of DNA damage caused by CPTs. Bax, p53, NF-κB, and survivin participated in the regulation of cell apoptosis induced by CPTs[7–9]. All of these genes have

been reported to be related to the resistance to CPTs.

MiRNAs are a class of non-coding RNAs that are 21 to 25 nucleotides in length and function as regulators of gene expression. Recently, miRNAs have been identified as oncogenes or tumor suppressors[10, 11], and they have been regarded as potential drug targets[12] due to many of them being located at the fragile sites of chromosomes or cancer-associated genomic regions[13]. Bertino *et al* first introduced the idea of "miRNA pharmacogenomics," which is defined as the study of miRNAs and polymorphisms affecting miRNA function to predict drug response and improve drug efficiency[14]. MiRNA misexpressions or mutations result in a gain or loss of miRNA function and, therefore, a downregulation or upregulation of the target protein, which is involved in the drug response. In previous studies, miR-24, miR-21, let-7a, miR-214, miR-221, miR-222, miR-328, and miR-27a have been reported to be involved in the regulation of chemosensitivity[15]. However, there have been no reports regarding miRNAs and their involvement in the resistance to CPTs of gastric cancer.

To find the miRNAs that are involved in intrinsic resistance to 10-hydroxycamptothecin (HCPT), we conducted a miRNA pharmacogenomics study on HCPT by performing a genome-wide expression profiling of the miRNAs and mRNAs in six different gastric cancer cell lines. Our study provides a resource for integrated chemo-genomic studies that aimed to elucidate the molecular markers for personalized therapy and to understand the mechanisms of HCPT resistance.

Materials and methods
Cell culture
Six human gastric adenocarcinoma cell lines, BGC-823 (low-differentiated human gastric adenocarcinoma cell line), SGC-7901 (moderate-differentiated human gastric adenocarcinoma cell line), MGC-803 (low-differentiated human mucinous gastric carcinoma), HGC-27 (undifferentiated human mucinous gastric carcinoma), NCI-N87 (well-differentiated human carcinoma cell line) and AGS (low-differentiated human gastric adenocarcinoma cell line), were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China. The six cell lines were all derived from patients who had received no prior therapy. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY) at 37 °C in 5% CO_2.

SRB assay
Cells were seeded in 96-well plates with 100 µL (5000 to 10000 cells/well) for 24 h and then exposed to freshly prepared HCPT (HSFY Pharma, China) at a final concentration that was 0.01, 0.1, 1, 10, and 100 times the human peak plasma concentration for HCPT (1.0 µg/mL). Simultaneously, one plate of each cell line was fixed with TCA. The cells were then incubated for 48 h, and their viability was assessed using the Sulforhodamine B (SRB) assay (Sigma, St Louis, MO). The absorbance at 515 nm (OD_{515}) was read using the automated plate reader. Seven OD_{515} were measured: at time zero (Tz), the growth of control cells (C), and the growth in the presence

of the drug at the five concentration levels (Ti). The percentage growth inhibition was calculated as [(Ti–Tz)/(C–Tz)]×100, when Ti>/=Tz; [(Ti–Tz)/Tz]×100, when Ti<Tz. The concentration resulting in a 50% reduction in the net protein increase in control cells during the drug incubation (GI50) was calculated using the percentage growth inhibition curve. All of the experiments were individually performed in triplicate.

Total RNA extraction and quality assessment
The cells were cultured for 24 h and were then collected for total RNA extraction. Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Ambion, Austin, Texas) following the manufacturer's instructions. Then the RNA quantity was determined using Nanodrop1000. The RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California). All of the samples were assayed and qualified in triplicate using the miRNA microarray and DNA oligonucleotide microarray.

MiRNA microarray profiling
The input for Agilent's miRNA labeling system was 100 ng total RNA. After dephosphorylation and denaturation, the total RNA was labeled with cyanine 3-pCp and then hybridized to Agilent Human miRNA Microarray V2 using the miRNA Complete Labeling and Hyb Kit (Agilent, Santa Clara, California). After hybridization for 20 h, the slides were then washed using the Gene Expression Wash Buffer Kit (Agilent, Santa Clara, California) and scanned using an Agilent Scanner. The images were processed and analyzed with Agilent Feature Extraction Software.

The raw data were normalized using quantile normalization and then analyzed in GeneSpring GX (zcomSilicon Genetics, Redwood City, CA, USA). Statistical analysis using ANOVA was conducted to compare the differentially expressed miRNAs.

Whole genome gene expression microarray
Gene expression profiles were analyzed using the Affymetrix HG-U133+PM Array Plate (Affymetrix, Santa Clara, CA). Three biological replicates of each cell line were assayed. The probes were labeled, hybridized and scanned according to the manufacturer's instructions. The data were extracted through Expression Console (Affymetrix, Santa Clara, California). Data normalization and analysis were performed in GeneSpring GX software as mentioned above.

Quantitative real-time PCR (qRT-PCR)
For the miRNA, the reverse transcription of RNA was conducted using the miScript Reverse Transcription Kit. The cDNA serves as the template for real-time PCR analysis using a miScript Primer Assay in combination with the miScript SYBR Green PCR Kit (Qiagen, Germany). For the mRNA, the total RNA was reversed transcribed using the OneStep RT-PCR Kit. Then the cDNA served as the template for the real-time PCR analysis using the QuantiTect Primer Assay (Qiagen, Germany). The PCR was conducted using a 7900HT Sequence

Detection System (Applied Biosystems, Austin, TX, USA). All of the reactions were run in triplicate. The relative expression levels of the different miRNAs were calculated based on U6 RNA levels and multiplied by 10^2. The relative expression levels of the mRNAs were calculated based on GAPDH levels and multiplied by 10^2.

Statistical analysis

Each experiment was repeated at least three times. The continuous variables were expressed as the mean values±standard deviations (SD). The Student's *t*-test was used to analyze the different genes between cell lines with different drug sensitivities. A *P*<0.05 was considered statistically significant. All of the statistical analyses, including the normalization of data, choosing the differentially expressed genes and hierarchical clustering, were processed through GeneSpring GX Software.

MiRNAs targets prediction

The target genes of the miRNAs were predicted through miR-Gen, which incorporates algorithms including TargetScan, miRanda and PicTar (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen).

Gene ontology and pathway analysis

The gene ontology and pathway analysis was conducted through GenMAPP2 (http://www.genmapp.org/). The permutated *P*-value cutoff was set below 0.05, and the Z-score was set higher than 2.

Results

The growth inhibition effects of HCPT on human gastric cancer cells

Six human gastric cancer cell lines (BGC-823, SGC-7901, MGC-803, HGC-27, NCI-N87, and AGS) were used for the HCPT sensitivity analysis. The SRB assay showed a significant difference in sensitivities among these six cell lines at each concentration of HCPT. The concentration leading to 50% growth inhibition (GI50) was significantly different between the BGC-823 and AGS cell lines (Figure 1A). When exposed to HCPT at 1.0 µg/mL (the human peak plasma concentration), the AGS cells died, while the BGC-823, SGC-7901, MGC-803, HGC-27, and NCI-N87 cell lines displayed 24%, 36%, 65%, 72%, and 90% growth inhibition respectively, compared to the control (Figure 1B). In terms of percentage growth inhibition, there were significant differences (*P*<0.05) among the six cell lines, and they could be sorted according to their sensitivity to HCPT in ascending order: BGC-823, SGC-7901, MGC-803, HGC-27, NCI-N87, and AGS.

MiRNA expression profiling in HCPT-resistant versus HCPT-sensitive gastric cancer cell lines

To investigate the role of miRNAs in intrinsic HCPT resistance, we conducted a comprehensive miRNA expression profiling of the six gastric cancer cell lines using the Agilent Human miRNA Microarray V2.0. This microarray contains 15 744 probes representing 723 human miRNAs. After quan-
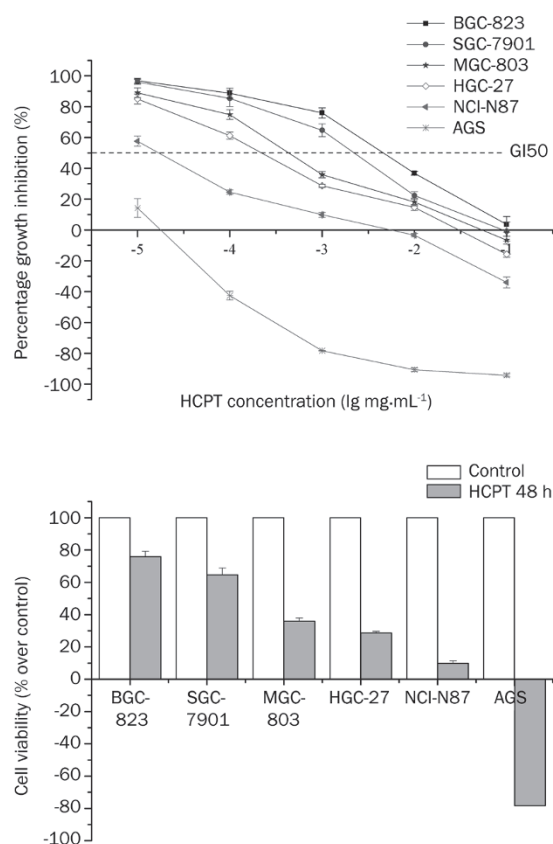

Figure 1. Sensitivity of six gastric cancer cell lines to HCPT. (A) Gastric cancer cells were incubated with HCPT (five different concentrations: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} mg/mL) for 48 h, and the viability was evaluated as described in the Materials and Methods section. The experiments were performed in triplicate. The six cell lines showed different sensitivities at each HCPT concentration. The concentrations that resulted in 50% growth inhibition (GI50) were significantly different between the cell lines. (B) At the human peak plasma concentration (1.0 µg/mL), the AGS cells experienced cell death, while the other cells proliferated at different rates.

tile normalization and filtering on flags (present or marginal), 279 miRNAs were selected for further analysis. ANOVA analysis revealed that 137 miRNAs were differentially expressed between the BGC-823 (HCPT-resistant) and AGS (HCPT-sensitive) cell lines with a more than 1.5-fold change (*P*<0.05) (Figure 2A). Further exploration of the other cell lines that had intermediate HCPT sensitivity (SGC-7901, MGC-823, HGC-27, and NCI-N87) revealed 25 miRNAs whose expression levels were also intermediate. Compared with their sensitive counterparts, 12 miRNAs were downregulated and the rest were upregulated in HCPT-resistant gastric cancer cells (Table 1). These results indicated that these 25 miRNAs might play important roles in intrinsic HCPT resistance. Hierarchical cluster analysis based on the expression patterns of these 25 miRNAs accurately separated the HCPT-sensitive cell line from the HCPT-resistant cell lines (Figure 2B).

To validate the miRNA microarray results, 12 deregulated miRNAs were randomly chosen for analysis by qRT-PCR. These miRNAs were miR-196a, miR-365, miR-424, miR-99b,
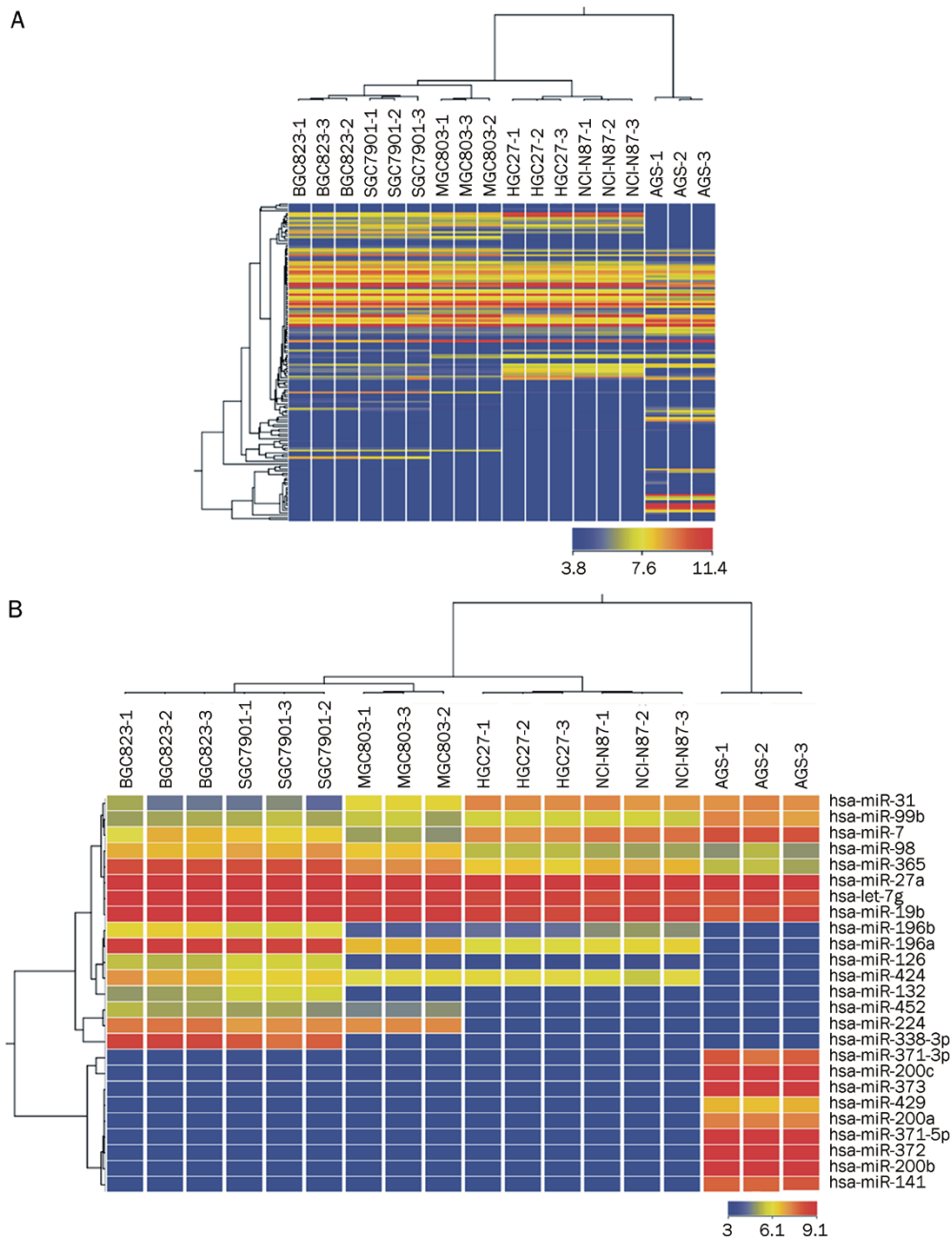
A

B

Figure 2. The miRNA expression signature in HCPT-resistant versus HCPT-sensitive gastric cancer cells. The tree was constructed using hierarchical clustering based on the $\log_2$ transformation of the normalized probe signal intensity. (A) The heatmap was based on 137 miRNAs that were differentially expressed between the most resistant cell line BGC-823 and the most sensitive cell line AGS. (B) The heatmap was based on 25 differentially expressed miRNAs, the expressions of which correlated with HCPT sensitivity.

miR-98, miR-224, miR-338-3p, miR-141, miR-200a, miR-200b, miR-372, and miR-373. The qRT-PCR results confirmed the miRNA microarray results (Figure 3). MiR-196a, miR-365, miR-424, miR-99b, and miR-98 were expressed in both the HCPT-sensitive cell lines and the HCPT-resistant cell lines. Cells with higher expression levels of miR-196a, miR-365, miR-424, and miR-98 or lower levels of miR-99b were less sensitive to HCPT. MiR-224 and miR-338-3p were only expressed in HCPT-resistant cells, and miR-141, miR-200a, miR-200b, miR-372, and miR-373 were only expressed in HCPT-sensitive cells. These results indicated that the sensitivity to HCPT of human gastric cancer cell lines may be interfered by the intrinsic expression levels of miRNAs.

Identification of mRNA expression profiles in HCPT-resistant versus HCPT-sensitive gastric cancer cell lines

To explore the impact of gene expression on HCPT sensitivity, we further studied the mRNA expression patterns of these human gastric cancer cell lines using the Affymetrix HG-U133+PM Array Plate. In total, 307 genes were identified as differentially expressed between the HCPT-resistant cell lines and the HCPT-sensitive cell lines. The six cell lines clustered into different groups based on the expression profiles of the 307 genes (Figure 4A). Among these genes, 33 had been reported to be involved in cancer development, cell progression and/or chemoresponse (Figure 4B). Some of the genes are apoptosis-related genes (BAX, TIAL1, TPD52L1, BAG, SP3,

Table 1. miRNAs correlated to intrinsic HCPT resistance in gastric cancer cells.

miRNA	Regulation in resistant cells	Chromosome location
miR-132	Up	17p13.3
miR-224	Up	Xq28
miR-338-3p	Up	17q25.3
miR-452	Up	Xq28
miR-141	Down	12p13.31
miR-200a	Down	1p36.33
miR-200b	Down	1p36.33
miR-200c	Down	12p13.31
miR-371-3p	Down	19q13.41
miR-371-5p	Down	19q13.41
miR-372	Down	19q13.41
miR-373	Down	19q13.41
miR-429	Down	1p36.33
let-7g	Up	3p21.1
miR-126	Up	9q34.3
miR-196a	Up	17q21.32 12q13.13
miR-196b	Up	7p15.2
miR-19b	Up	13q31.3 Xq26.2
miR-27a	Up	19p13.12
miR-31	Down	9p21.3
miR-365	Up	16p13.12 17q11.2
miR-424	Up	Xq26.3
miR-7	Down	9q21.32 15q26.1 19p13.3
miR-98	Up	Xp11.22
miR-99b	Down	19q13.33

Table 2. The miRNA-mRNA regulation pairs.

Regulation in resistant cells	miRNA	Target genes
miRNA up-target down	let-7g	BTG2, VSNL1, MAP4K4, SLC35D2, CHD7, FRAS1, EPHA4, PGM2L1
	has-mir-19b	ADD3, EFNB2, STRN3, GAD1, SPHK2, VPS37B, TP53INP1, PGM2L1, BMPR2
	has-mir-132	BTG2, SOX2
	has-mir-224	SOCS2, JAG1, AMIGO2
	has-mir-338	HIATL1
	has-mir-365	ADD3, KALRN
	has-mir-424	ACTR1A, PSME3, BTG2, EFNB2, PDLIM5, PTPN3, SLC12A2, EPHA1, PIK3R1, ATF7IP2, SEH1L, AXIN2, WDR22, KALRN, PGM2L1
	has-mir-452	EIF2S1
	has-mir-98	BTG2, VSNL1, MAP4K4, SLC35D2, CHD7, FRAS1, EPHA4,PGM2L1
miRNA down-target up	has-mir-200a	OLFM1
	has-mir-200b	MITF, GABARAPL2, SLC38A2, FBXL16, NOG
	has-mir-200c	MITF, GABARAPL2, SLC38A2, FBXL16, NOG
	has-mir-141	OLFM1, GLRX, GABARAPL2, HOXA11, BCOR
	has-mir-429	MITF, PCTK1, GABARAPL2, SLC38A2, FBXL16, NOG, SP3
	has-mir-7	SLC38A2
	has-mir-31	OSBP2
	has-mir-372	SMAD2, SUV39H1
	has-mir-373	SMAD2, SUV39H1

RPS27L, PLSCR3, TP53INP1, *etc*), cell division-related genes (MCM2, *etc*), cell adhesion/migration-related genes (TIMP2, VSNL1, FUT8, TBX3, *etc*) and checkpoint genes (RAD1, *etc*).

To validate the microarray data, nine differentially expressed genes were randomly chosen for qRT-PCR. The results showed that these genes were differentially expressed between different cell lines (Figure 5). Gene ontology and pathway enrichment analysis of these 307 genes demonstrated that two significant pathways ($P<0.05$) were involved: the irinotecan pathway and the IL-3 pathway. The irinotecan pathway is very important for the biotransformation of CPTs, and the IL-3 pathway is related to the proliferation and differentiation of hematopoietic cells. In addition, we examined the focal adhesion pathway and the cell cycle pathway and achieved a P-value of 0.1.

Integrated analysis of deregulated miRNAs and mRNAs

MiRNAs modulate gene expression through inducing mRNA degradation or translational repression, or both. Therefore, we further performed an integrated analysis of miRNA and mRNA expression patterns in the six gastric cancer cell lines. The target genes of the 25 deregulated miRNAs were predicted using the intersections of PicTar and TargetScanS in miRgen (http://www.diana.pcbi.upenn.edu/cgi-bin/

miRGen) to reduce the chance of false positives. For each deregulated miRNA, 13 to 600 target genes were predicted. As was expected, many known chemoresistant targets were found among the candidates, including CDKN1B (p27), ANXA1 (p35), PDCD4, UGT1A1, TOP1, CYP3A4, ABCG2 (BCRP), CHEK1, TDP1, BCL-2, and SUMO1. The pathways of these predicted target genes were enriched using GenMAPP 2. We also identified 47 biological pathways (permuted P-value<0.05), including classical cancer-related pathways (EGFR1, Wnt signaling, MAPK signaling, C-kit receptor, focal adhesion pathways, *etc*); drug metabolism-associated pathways (cytochrome P450, *etc*) and cell apoptosis-related pathways (S1P signaling pathway, *etc*). Fifty percent of these 47 biological pathways had been reported to be implicated in carcinogenesis or chemoresponse (Figure 6A). Moreover, the gene ontology terms of these target genes included cell cycle, cell proliferation, G1/S transition of the mitotic cell cycle, negative regulation of programmed cell death, cell adhesion, positive regulation of cell motility, cell-matrix adhesion and regulation of DNA repair (Figure 6B). These results indicated that the cells were not sensitive to HCPT partially due to their
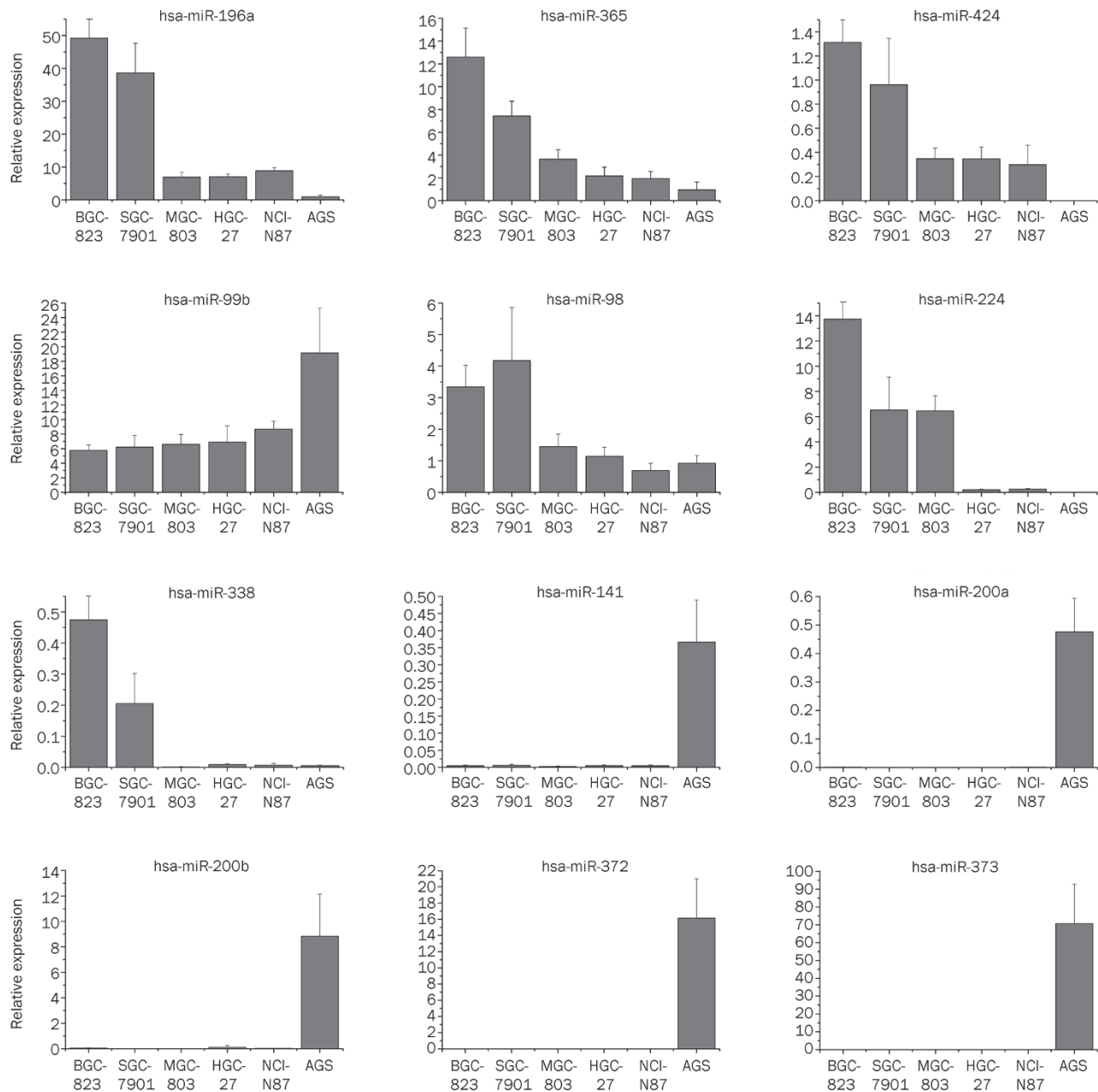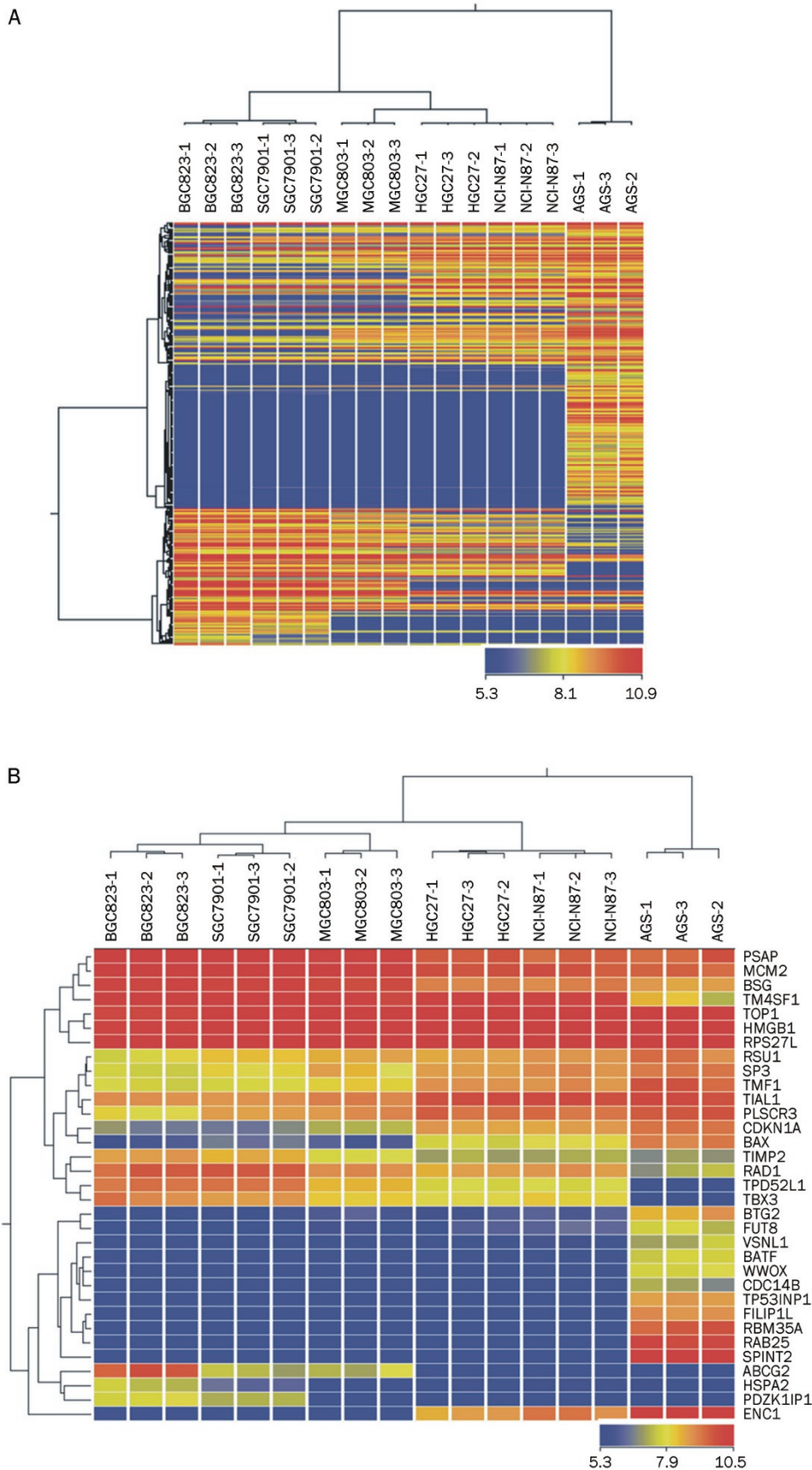
Figure 3. Validation of the differentially expressed miRNAs by real-time PCR. As described in the Materials and Methods, real-time PCR was performed in triplicate, and the relative expression levels of different miRNAs were calculated based on U6 RNA levels and multiplied by 10^2. MiR-196a, -365, -424, -98, -338, and -224 were markedly upregulated in the resistant cells, but not in the sensitive cells, while miR-99b, -141, -200a, -200b, -372, and -373 were markedly downregulated.

increased proliferation, anti-apoptosis and/or migration abilities.

Finally, we deciphered the relationship between the deregulated miRNAs and the mRNAs. Among the 307 differentially expressed mRNAs, 50 genes are the predicted targets of the 25 differential miRNAs, forming 78 relation pairs (Table 2). These 50 genes included tumor suppressors, protein kinases, helicases, histone methyltransferases, transcription factors and proteasome-related proteins. The tumor suppressor gene BTG2, which inhibits proliferation and promotes the apoptosis

of gastric cancer cells, was targeted by the highly-expressed miRNAs let-7g, miR-98, and miR-132 in HCPT-resistant cells. Another tumor suppressor gene VSNL1, which inhibits cell migration, was also targeted by let-7g and miR-98. TP53INP1, which promotes TP53 phosphorylation on "Ser-46" and subsequent apoptosis, was predicted to be a target of miR-19b, an upregulated miRNA in HCPT-resistant cells. In addition, we found the components of cancer-related pathways such as SMAD2 and PIK3R1 in the relation pairs. Our systematic analysis revealed that approximately 1.3% of the predicted
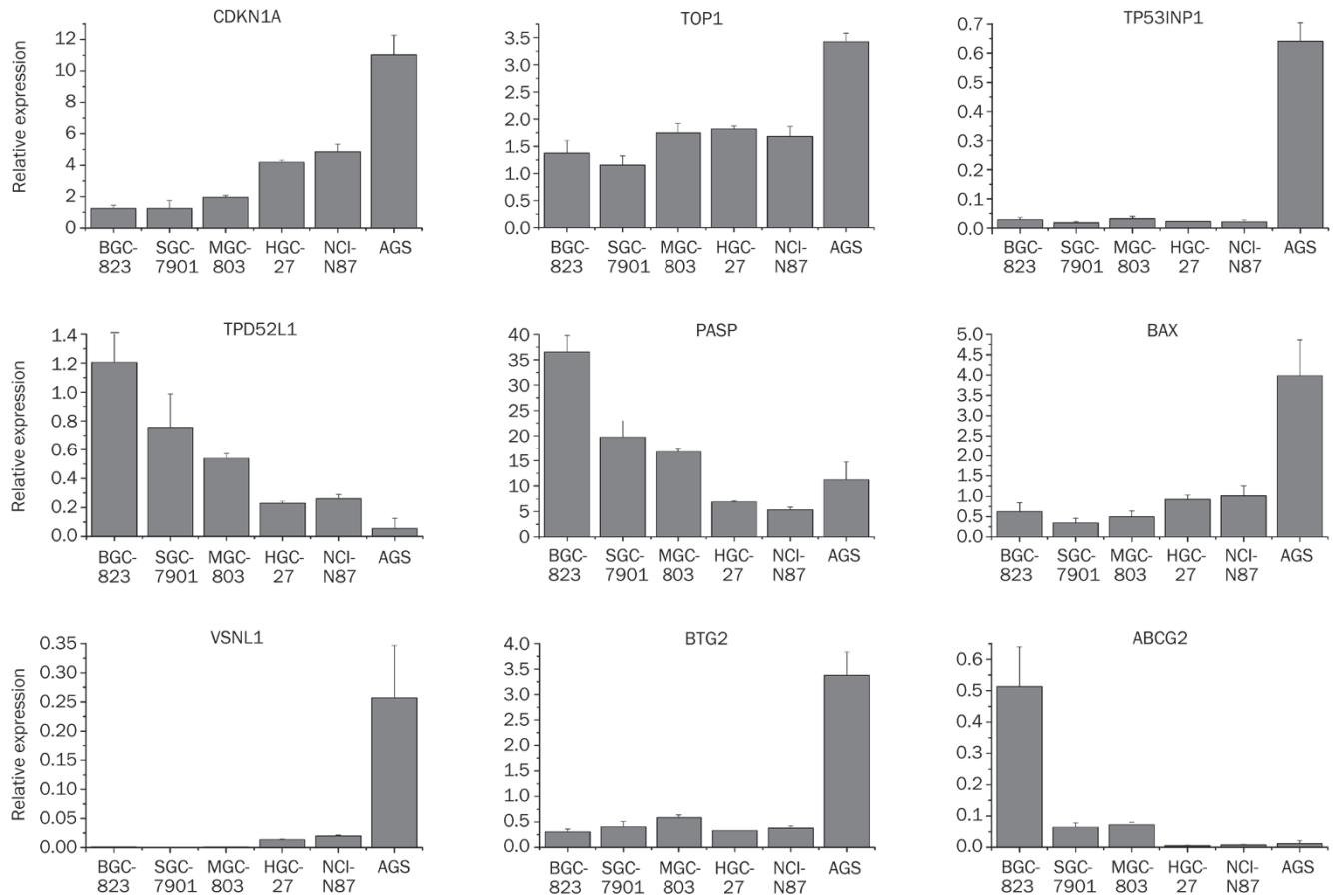
A

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Figure 4. The mRNA expression signatures in HCPT-resistant versus HCPT-sensitive gastric cancer cells. The tree was based on the $\log_2$ transformation of the normalized probe signal intensity using hierarchical clustering. (A) The hierarchical clustering was based on 307 differentially expressed mRNAs whose expression levels were deregulated in HCPT-resistant cell lines. (B) The hierarchical clustering was based on 33 differentially expressed genes that were reported to be involved in cancer progression or chemoresponses.

Figure 5. Validation of nine differentially expressed mRNAs. We performed quantitative real-time PCR in triplicate, as described in the Materials and methods, and the relative expression levels of the different mRNAs were calculated based on GAPDH levels and multiplied by 10^2.

miRNA targets formed the reciprocal "up-down" or "down-up" expression relationships with miRNAs. On the one hand, miRNAs modulate gene expression through both mRNA degradation and translational repression mechanisms, and on the other hand, miRNA-mRNA regulatory networks are highly complex.

Discussion

Cancer drug resistance can be classified into acquired and intrinsic resistance. Acquired drug resistance is induced during the process of drug treatment, while intrinsic resistance is the inherent characterization of the original cells before drug treatment. For studying acquired resistance, drug-induced resistant cell lines are used as a model, and the acquired resistance mechanism is determined by comparing the characterization before and after drug treatment. However, for studying intrinsic resistance, untreated original cells are usually used for drug sensitivity assays, and these results indicate the original cell's drug response and give indications for clinical drug treatment. Previous studies of cancer drug resistance have mainly focused on DNA, mRNA and protein levels, including mutations, copy number variations, epigenetic changes at the DNA level and deregulation at the mRNA and protein

levels[16]. Different cells appear to achieve the same end result by modulating a definitive number of genes through different mechanisms[17]. Among these mechanisms, miRNA is a newly identified mechanism, and this field is potentially promising. The NCI-60s, a panel of 60 diverse human cancer cell lines established by the National Cancer Institute, have been profiled for their miRNA expression[18]. Using the expression data of the NCI-60 and their corresponding drug sensitivities, miRNA profiles were developed to represent the sensitivities to individual chemotherapeutic agents[19]. However, gastric cancer cell lines were not included in the NCI-60. Therefore, in this study, we examined the role of miRNAs in the intrinsic drug resistance of gastric cancer cell lines. Our results will benefit both the understanding of drug resistance mechanisms and the prediction of clinical drug responses.

First, to explore this new field, we examined the miRNA expression profiles of six gastric cancer cell lines. We identified 25 deregulated miRNAs, and the results were confirmed by qRT-PCR. The miRNA expression pattern was efficient to separate the six cell lines in terms of HCPT sensitivity. Interestingly, some of the deregulated miRNAs were from the same family, and they may produce synergistic effects in mediating the resistant phenotype. Among the 12 downregulated miR-
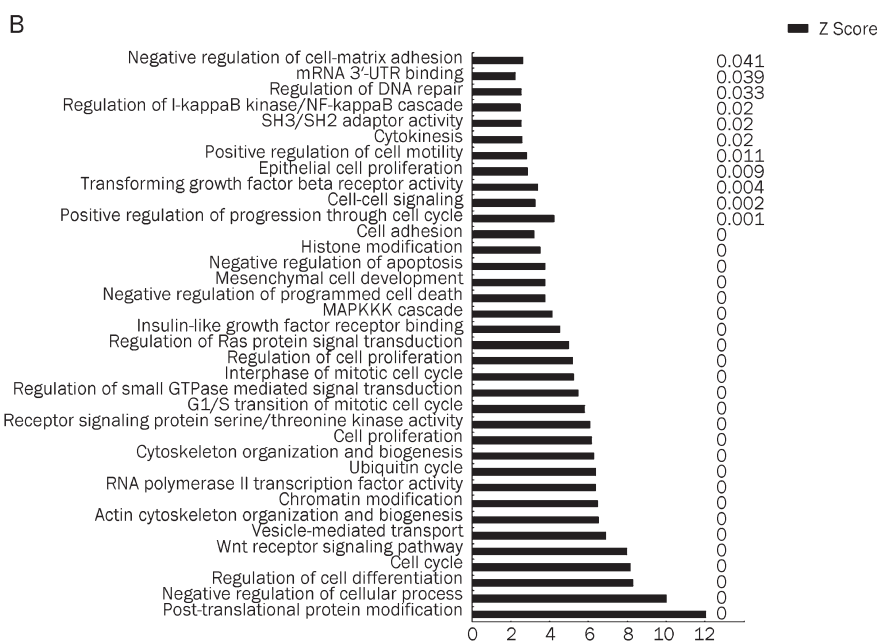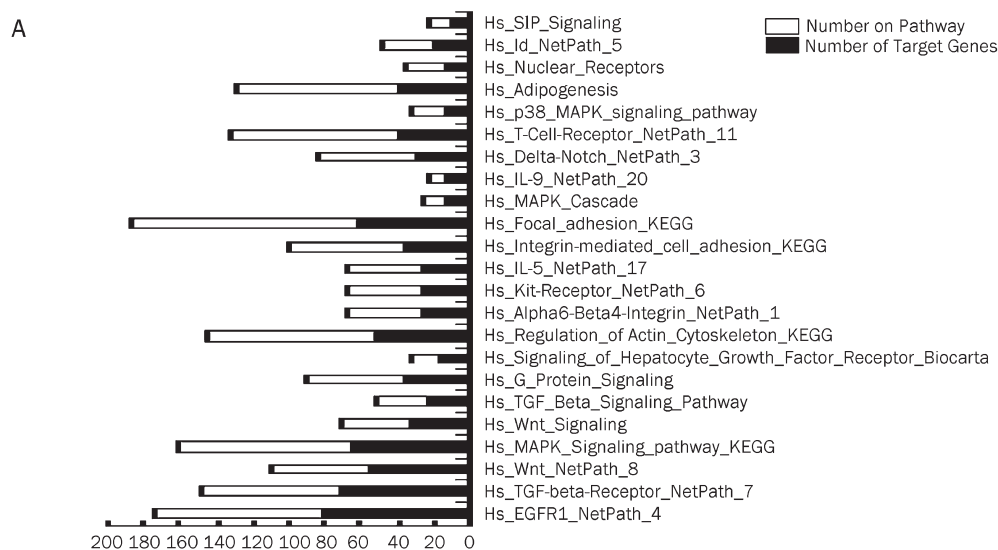
Figure 6. Gene ontology and pathway enrichment of predicted miRNA targets. (A) Significant pathways were enriched by GenMAPP2. The listed pathways are related to cell apoptosis or proliferation and have a *P*-value less than 0.05. The black column represents the number of target genes located in the pathway, while the white column represents the number of total genes located in the pathway. (B) Gene ontology was also enriched by GenMAPP2. The results indicated that these differential miRNAs may regulate genes involved in cell adhesion, cell proliferation and programmed cell death. The Z-score represents the enrichment extent, and the number listed beside each gene ontology term is the *P*-value.

NAs, the miR-200 family was only expressed in the HCPT-sensitive cell line AGS. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration through the direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2[20]. In addition, the overexpression of miR-141 in the gastric cancer cell lines was correlated with the inhibition of cell proliferation[21]. MiR-7 inhibits the epidermal growth factor receptor and the Akt pathway in cancer cell lines (lung, breast, and glioblastoma), inducing cell cycle arrest and cell death[22, 23]. MiR-31 inhibits breast cancer metastasis and was shown to be downregulated in gastric cancer tissues[24]. We predicted that the downregulation of these miRNAs could promote cancer cell migration and cell proliferation, thus impairing the HCPT-induced responses. Among the 13 upregulated miRNAs, miR-338 and miR-126 were identified as the signature miRNAs for predicting the survival of gastric cancer patients[25]. MiR-98 and let-7g may play roles in both the apoptotic and cell-proliferation pathways[26], and tumor-suppressor gene FUS1 is a target of miR-98[27]. MiR-196a promotes the oncogenic phenotype of colorectal cancer cells by activating the AKT signaling pathway and promoting cancer cell detachment, migration and invasion[28]. MiR-27a functions as an oncogene in gastric adenocarcinoma by targeting an anti-proliferative protein prohibitin[29], and downregulation of miR-27a could reverse the multidrug resistance of esophageal cancer[30]. The upregulation of these miRNAs may strengthen cell proliferation or reduce apoptosis and therefore render cells insensitive to HCPT. Although no related reports have indicated the involvement of the other deregulated miRNAs in carcinogenesis and chemoresponses, we believe a detailed

study on these miRNAs is warranted.

Gene ontology and pathway enrichment analysis based on the 307 differentially expressed mRNAs revealed only two significant pathways with *P*-values<0.05, including the irinotecan pathway and the IL-3 pathway. In the irinotecan pathway, we found that ABCG2 was downregulated in the HCPT-resistant cell lines; this result was in agreement with a previous report that the ABCG2 protein mediated drug efflux from resistant cells and was directly involved in cellular resistance to SN38 (the active metabolite of irinotecan)[31]. We also found that UGT1A10 and UGT1A6, which catalyze the glucuronidation of SN-38, were only expressed in HCPT-sensitive cell lines; this result disagreed with the putative view that SN-38 glucuronidation could lead to cellular resistance to irinotecans. Therefore, these results indicated that the sensitivity of cells to HCPT was affected by multiple factors that contribute simultaneously.

To study the involvement of miRNA-mediated regulation of gene expression in HCPT resistance, we analyzed whether the 307 genes were the predicted targets of the 25 deregulated miRNAs, and finally, we found 50 genes and 78 relation pairs between them. However, we could not examine the enriched pathways of these 50 genes to analyze them individually. Some of these genes are tumor suppressor genes involved in regulating cancer cell proliferation or apoptosis. Because miRNAs are predicted to target multiple unrelated genes that are not coexpressed and one target gene is predicted to be targeted by multiple miRNAs, it is not surprising that miRNA expression levels do not tend to be strongly correlated with particular target transcripts. This phenomenon was also presented in Blower's study on the NCI-60[18].

This study was based on six gastric cancer cell lines, and we gained some information for intrinsic drug resistance, but more cell lines and clinical samples will need to be analyzed in a future study.

Conclusions

Our study demonstrated that the intracellular levels of miRNAs may interfere the chemoresponses of gastric cancer cells partially due to the regulation of cancer cell proliferation, migration and cell death pathways. Hierarchical clustering based on the expression patterns of 25 miRNAs showed that cell groupings were generally consistent with HCPT sensitivity. The mRNA signature we observed was also informative for discriminating cell lines with different sensitivities to HCPT. In addition, we found a slightly lower correlation between the expression patterns of miRNAs and those of their predicted target transcripts. Our study provides a resource for integrated chemo-genomic studies aimed to elucidate the molecular markers for personalized therapy and to understand the mechanisms of HCPT resistance.

Author contribution
Xue-mei WU designed the study, performed the SRB assay, microarray experiments and real-time PCR experiments, analyzed the data and wrote the manuscript. Li ZHU, Xian-xin MENG, and Xiao-na ZHANG performed the microarray experiments and generated the microarray data. Xiang-qiang SHAO, Shi-xu LIU, and Jian LIN performed the miRNA prediction and analysis. Hua-sheng XIAO coordinated the study and reviewed the manuscript. All of the authors have read and approved the final manuscript.

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