# Analysis of novel microRNA targets in drug-sensitive and -insensitive small cell lung cancer cell lines

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**Abstract.** Advances in chemotherapy have failed to improve the long-term survival rate of small cell lung cancer (SCLC) patients due to multidrug resistance (MDR). The mechanisms of MDR are complex involving multiple genes and a variety of mechanisms. MicroRNAs (miRNAs) are non-coding RNAs theoretically involved in gene regulation. The aim of the present study was to explore the role of miRNAs in SCLC occurrence and multidrug resistance. Expression levels of known miRNAs in SCLC cell line H446 and its multidrug-resistant cell line H446/CDDP were analyzed using the next generation high through-put Illumina Solexa sequencing technology, and expression of a group of specific miRNAs was validated by quantitative polymerase chain reaction (qPCR). Furthermore, novel miRNAs and their putative target genes in the two SCLC cell lines were predicted with the help of software developed by Beijing Genomics Institute and analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The results revealed a set of known miRNAs with altered expression in the H446 and H446/CDDP cells which may be associated with multidrug resistance of SCLC. Biological information analysis of the novel miRNAs and their putative target genes further elucidated the role of miRNAs in MDR. In addition, the pathway prediction by KEGG analysis may provide clues for further research on MDR of SCLC.

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## Introduction

Lung cancer is clinically classified into two major histological types: non-small cell lung cancer (NSCLC), which accounts for more than 80% of all lung cancer cases; and small cell lung cancer (SCLC), which accounts for 13-15% (1). Although SCLC incidence is relatively lower, its poor prognosis is a cause for concern. The standard treatment for SCLC has not dramatically changed during the past 30 years, and platinum-based chemotherapy (2,3), hyper-fractionated thoracic radiation (4), and prophylactic cranial irradiation (5,6) are still used therapeutically. However, multidrug resistance (MDR) following chemotherapy increases the relapse ratio and mortality. The median survival time is only 6-8 months and the 5-year survival rate is <15% (7). In the absence of any meaningful advances in chemotherapy or programs to improve the survival rates, MDR has become a major clinical obstacle in the treatment of SCLC (8), warranting investigation into the mechanisms underlying MDR.

Previous studies suggest complex MDR mechanisms involving multiple genes and a variety of mechanisms at the tissue, cell and molecular levels (8-10). As transcription regulating factors, microRNAs (miRNAs) have become the 'hot topic' in gene regulation (11,12). miRNAs, evolved from an RNA polymerase 2 transcript (pri-miRNA) with a strong secondary structure (stem-loop), are classified into diverse small RNA families. The miRNAs are 21-24 nucleotides (nt) in length, single-stranded and non-coding (13). The miRNAs may theoretically target any mRNA, and therefore, participate in a very broad functional spectrum including the cell cycle, cell growth, apoptosis, cell differentiation and stress response (14). It is no surprise that miRNAs are involved in human cancer development and tumor metastasis. However, only a few studies are available discussing the role of miRNAs in the multidrug resistance of SCLC.

The aim of the present study was to elucidate the role of miRNAs in SCLC. Using the next generation high throughput Solexa-Illumina sequencing technology, we determined the varied expression of known miRNAs as well as discovered novel miRNAs. Furthermore, the possible biological significance of putative target genes of novel miRNAs in H446 and H446/CDDP cells was determined using Gene Ontology (GO)

analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The findings should help understand the role of miRNAs in MDR of SCLC and provide ideas for future research.

## Materials and methods

*Cell lines and culture*. The SCLC cell line H446 and its multidrug-resistant cell line H446/CDDP were kindly gifted by Dr Guisheng Qian (Institute of Human Respiratory Disease, Xinqiao Hospital, The Third Military Medical University, China). More importantly, the H446 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and the H446/CDDP cell line was derived from H446 cells. These cells were maintained in RPMI-1640 medium with 10% fetal calf serum (Gibco-BRL, Grand Island, NY, USA) in a humidified atmosphere containing 5% $CO_2$ at 37˚C. In order to maintain the MDR phenotype, cisplatin (at a final concentration of 0.5 $\mu$g/ml) was added to the culture medium for the H446/CDDP cells.

*Validation of drug resistance*. Sensitivity of the H446 and H446/CDDP cells to cisplatin was evaluated by a microculture tetrazolium (MTT) assay. Briefly, the cells were seeded in 96-well plates at a density of $1x10^4$ cell/well. The cells were treated with 5 $\mu$g/ml cisplatin for 24, 48 or 72 h, respectively. The supernatant was discarded, and 20 $\mu$l MTT (5 mg/ml, dissolved in PBS and filtered through a 0.22-mm membrane) was added into each well, followed by incubation for 4 h at 37˚C. Finally, absorbance values were determined at 492 nm on an automated Bio-Rad 550 microtiter plate reader.

*Total RNA extraction*. Total RNA from H446 and H446/CDDP cell lines was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Total RNA samples were subjected to an RNA quality control process for Solexa sequencing.

*Data cleaning and length distribution*. Different small RNAs vary in length. For example, miRNAs are normally 21 or 22 nt, siRNAs are 24 nt, and piRNAs are 30 nt. Therefore, length distribution analysis was helpful to determine the composition of small RNA samples. In the present study, clean reads were obtained from raw reads after eliminating contaminant reads, including low quality reads, reads with 5' primer contaminants, reads without 3' primer, reads without the insert tag, reads with poly A and reads shorter than 18 nt. The length distribution of these clean reads was summarized using software developed by BGI (formerly known as Beijing Genomics Institute).

*Small RNA annotation*. Small RNA tags from the two libraries were aligned to associated repeat RNAs and annotated with rRNA, scRNA, snoRNA, snRNA, tRNA exon and intron alignment in order to eliminate matched tags. Furthermore, the small RNA tags were also annotated with sequences from Rfam and matched tags were also discarded. Above all, in order to map each unique sRNA to only one annotation, the following priority rule was strictly followed: rRNA (in which Genbank > Rfam) > known miRNA > repeat > exon > intron.

Table I. Primer sequences of the genes used in this study.

| miRNA name | miRNA sequence |
|---|---|
| hsa-miR-323a-3p | 5'-cacauuacacggucgaccucu-3' |
| hsa-miR-411-5p | 5'-uaguagaccguauagcguacg-3' |
| hsa-miR-382-5p | 5'-gaaguuguucgugguggauucg-3' |
| hsa-miR-486-5p | 5'-uccuguacugagcugccccgag-3' |
| hsa-miR-323b-3p | 5'-cccaauacacggucgaccucuu-3' |
| hsa-miR-485-3p | 5'-gucauacacggcucuccucucu-3' |
| hsa-miR-876-5p | 5'-uggauuucuuugugaaucacca-3' |
| hsa-miR-379-5p | 5'-ugguagacuauggaacguagg-3' |
| hsa-miR-486-3p | 5'-cggggcagcucaguacaggau-3' |
| hsa-miR-487b | 5'-aaucguacagggucauccacuu-3' |
| hsa-miR-2277-5p | 5'-agcgcgggcugagcgcugccaguc-3' |
| hsa-miR-137 | 5'-uuauugcuuaagaauacgcguag-3' |
| hsa-miR-369-3p | 5'-aauaauacaugguugaucuuu-3' |
| hsa-miR-3911 | 5'-uguguggauccuggaggaggca-3' |
| hsa-miR-204-3p | 5'-gcugggaaggcaaagggacgu-3' |
| hsa-miR-432-5p | 5'-ucuuggaguaggucauugggugg-3' |
| hsa-miR-204-5p | 5'-uucccuuugucauccuaugccu-3' |
| hsa-miR-485-5p | 5'-agaggcuggccgugaugaauuc-3' |
| hsa-miR-127-3p | 5'-ucggauccgucugagcuuggcu-3' |
| hsa-miR-494 | 5'-ugaaacauacacgggaaaccuc-3' |
| hsa-miR-382-3p | 5'-aaucauucacggacaacacuu-3' |
| hsa-miR-409-3p | 5'-gaauguugcucggugaaccccu-3' |
| hsa-miR-584-5p | 5'-uuaugguuugccugggacugag-3' |

In addition, the total rRNA represented the quality of samples, usually <40% in animal samples of high quality.

*Expression analysis of known miRNAs*. The two small RNA libraries were aligned to the miRNAs in miRBase 21 in order to obtain the miRNA data in H446 and H446/CDDP cell lines. The expression of miRNAs in the two libraries was normalized to obtain the expression of transcript per million (TPM), using the normalization formula: Normalized expression = Actual miRNA count/total count of clean reads x 1,000,000. Subsequently, fold-change and p-value of the normalized expression were calculated. Finally, the scatter plot and fold-change table were generated to reveal the differential expression of known miRNAs in the H446 and H446/CDDP cells.

*Validation of known miRNA expression in the H446 and H446/CDDP cell lines*. The differential expression of miRNAs in the H446 and H446/CDDP cells was validated by qPCR. Briefly, total RNA was extracted from the H446 and H446/CDDP cells in the logarithmic phase with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was monitored using agarose electrophoresis and miRNA levels were expressed as the ratio to U6 level in the same RNA sample. PCR primers are listed in Table I. Amplification and detection of chosen miRNAs were carried out using iQ™ SYBR Green Supermix reagent and iQ™

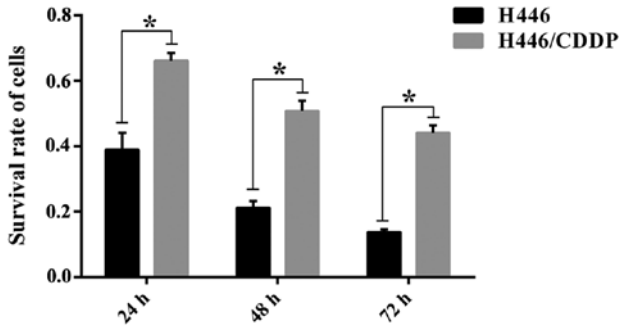


Figure 1. Sensitivity of H446 and H446/CDDP cells to cisplatin was determined by MTT assay. Cells were treated with 5 $\mu$g/ml cisplatin for 24, 48 and 72 h. Data are expressed as means ± SD of three independent experiments. Comparison was carried out between the survival rate of H446 and H446/CDDP cells, *P<0.01.

Single-Color Real-Time PCR detection system (both from Bio-Rad, USA).

*Novel miRNA prediction*. The characteristic hairpin structure of the miRNA precursor was used in the present study to predict novel miRNAs with the help of software, Mireap (http://sourceforge.net/projects/mireap/), developed by BGI. Novel miRNAs were predicted by exploring the secondary structure, the Dicer cleavage site and the minimum free energy of the non-annotated small RNA tags.

*Target gene prediction of the novel miRNAs*. Target genes of novel miRNAs released by Mireap were also predicted according to previous suggestions (15,16): i) no more than 4 mismatches between sRNA and target (G-U bases count as 0.5 mismatches); ii) no more than 2 adjacent mismatches in the miRNA/target duplex; iii) no adjacent mismatches in positions 2-12 of the miRNA/target duplex (5' of miRNA); iv) no mismatches in positions 10-11 of miRNA/target duplex; v) no more than 2.5 mismatches in positions 1-12 of the miRNA/target duplex (5' of miRNA); and vi) minimum free energy (MFE) of the miRNA/target duplex at least 75% of the MFE as the miRNA bound to its complement. The prediction process was carried out using software developed by BGI.

*Functional analysis of predicted target genes*. GO analysis is a popular standardized classification system for gene function, which provides a set of controlled vocabulary to comprehensively describe genes and gene products. The GO results revealed molecular function, cellular components and biological processes of novel miRNAs by mapping target genes to GO terms in the database (http://www.geneontology.org/), calculating gene numbers for each term, and determining significantly enriched GO terms in target gene candidates.

Pathway analysis facilitates the understanding of biological functions of genes. KEGG (http://www.genome.ad.jp/kegg/pathway.html) is a major public pathway-related database (17,18). KEGG pathway analysis identifies significantly enriched metabolic pathways or signal transduction pathways in target gene candidates compared with the whole reference gene background. Therefore, in the present study, KEGG pathway analysis was adopted to analyze the function of putative target genes.

Table II. Summary of reads and unique sequences in small RNA libraries from the H446 and H446/CDDP cells.

| Types of reads | H446 | H446-CDDP |
|---|---|---|
| Raw reads | 7,703,264 | 9,151,947 |
| Adaptor removed | 36,549 | 44,975 |
| Exon-/intron-antisense removed | 5,405 | 12,530 |
| Exon-/intron-sense removed | 28,987 | 69,948 |
| r-/sc-/sn-/sno-/srp-/t-RNA removed | 216,256 | 377,967 |
| Putative small RNA population | 7,096,132 | 8,157,598 |

*Statistical analysis*. Sensitivity of H446 and H446/CDDP cells to cisplatin was determined by MTT test and the differences among groups were compared by one-way classification ANOVA; the differential expression of known miRNAs in H446 and H446/CDDP cells was compared by t-test. Fold-change and p-value of normalized known miRNA expression were determined using software developed by BGI. GO analysis was accomplished with a hyper-geometric test to unravel significantly enriched GO terms in target gene candidates, and the formula is not shown in the manuscript.

## Results

*Evaluation of cisplatin sensitivity*. The sensitivity of H446 and H446/CDDP cells to cisplatin was evaluated to develop the basis for Solexa-Illumina sequencing. The MTT test results (Fig. 1) revealed a differential survival rate in the H446 and H446/CDDP cells challenged by cisplatin at each time-point (P<0.01). Furthermore, the survival rate in both the H446 and H446/CDDP cells decreased with prolonged stimulation, and the difference in survival rate was statistically significant.

*Length distribution of small RNAs in the SCLC cell lines*. Differential sensitivity to cisplatin indicated that H446/CDDP cells were tolerant to cisplatin. We, therefore, explored whether the drug resistance was related to miRNA expression. In order to identify miRNAs in the H446 and H446/CDDP cell lines, two small RNA libraries from the two cell lines were constructed and sequenced independently. The sequencing results (Table II) exhibited 7,703,264 and 9,151,947 raw reads from the H446 and H446/CDDP cells, respectively. The raw reads were filtered according to several criteria. After removing low quality reads, 3' adaptor sequence, 5' adaptor contaminants and reads smaller than 18 nt, 7,346,780 and 8,618,043 clean reads for the H446 and H446/CDDP cells were obtained, and the length distribution was analyzed to characterize the small RNAs in SCLC. As shown in Fig. 2, the majority of reads, 79.86% for H446 (Fig. 2A) and 80.58% for H446/CDDP (Fig. 2B), were in the range of 21 to 24 nt in length, with 22 nt being the most abundant group of small RNAs. Read number and unique read number were the highest in this range, which indicated the abundance of these regulatory RNAs in SCLC. The 22 nt length was predominant, consistent with previous reports involving different species (19). We also found the

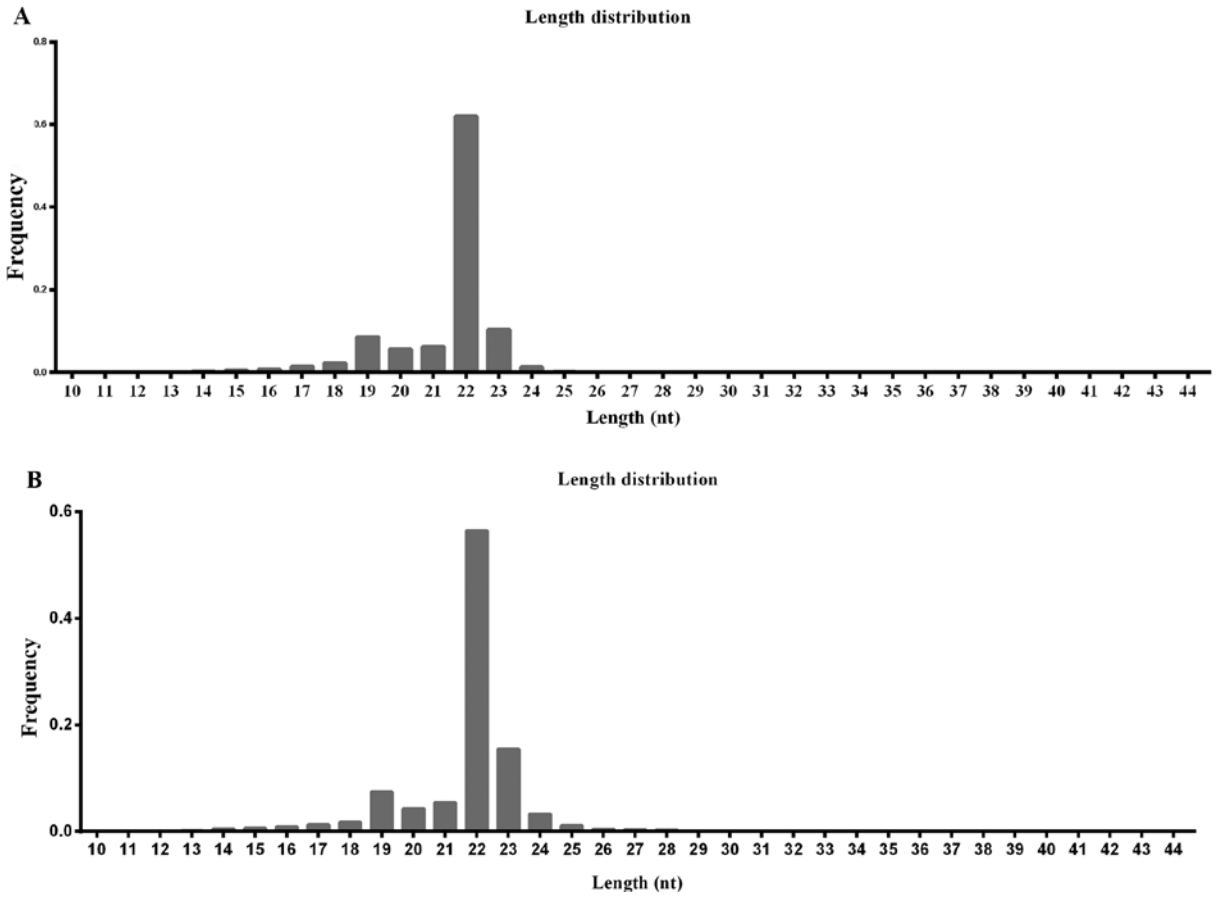


Figure 2. Length distribution of small RNAs identified in the H446 and H446/CDDP cells. (A) Length distribution of sRNAs from the H446 cells. (B) Length distribution of sRNAs from the H446/CDDP cells.

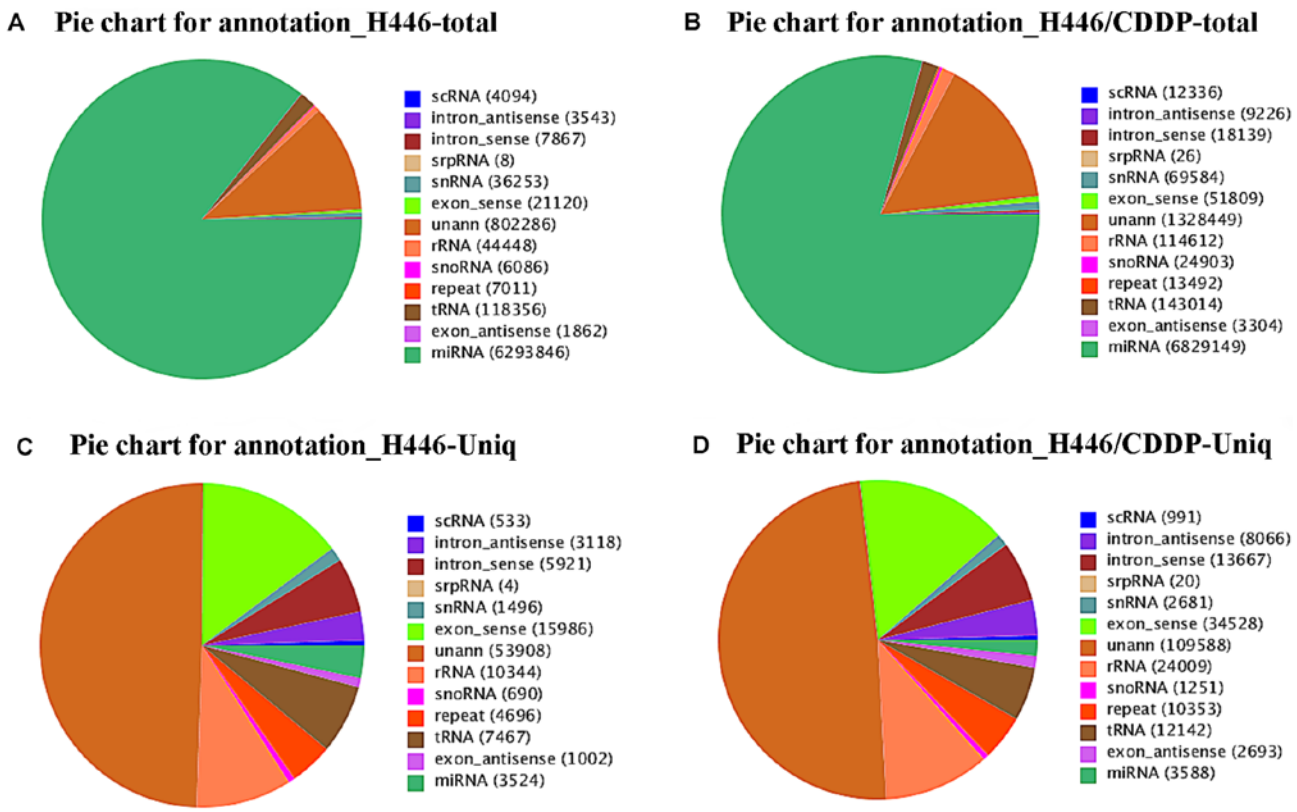


Figure 3. Annotation of small RNAs in the H446 and H446/CDDP cells. (A and B) Classification of total small RNAs in the H446 and H446/CDDP cells. (C and D) Classification of unique small RNAs in the H446 and H446/CDDP cells.

Table III. Top 10 most abundant miRNAs in the H446 cells.

| miR name | H446 expressed | H446/CDDP expressed | H446-std | H446/CDDP-std | Fold-change (log2 H446-CDDP/H446) | P-value |
|---|---|---|---|---|---|---|
| hsa-miR-452-5p | 9,604 | 2,667 | 1,307.2394 | 309.467 | -2.07866589 | 0 |
| hsa-miR-21-5p | 127,238 | 63,455 | 17,318.8798 | 7,363.0405 | -1.23397208 | 0 |
| hsa-miR-29a-3p | 14,327 | 7,210 | 1,950.106 | 836.6169 | -1.2209135 | 0 |
| hsa-miR-222-3p | 74,587 | 38,395 | 10,152.3388 | 4,455.1878 | -1.18825397 | 0 |
| hsa-miR-23a-3p | 7,412 | 3,941 | 1,008.8774 | 457.2964 | -1.1415494 | 0 |
| hsa-miR-224-5p | 3,457 | 1,543 | 470.5463 | 179.043 | -1.39403057 | 8.89E-239 |
| hsa-miR-100-5p | 3,275 | 1,788 | 445.7735 | 207.4717 | -1.10339629 | 5.24E-157 |
| hsa-miR-181a-2-3p | 2,205 | 1,131 | 300.1315 | 131.2363 | -1.19342792 | 1.96E-120 |
| hsa-miR-125b-5p | 2,301 | 1,273 | 313.1984 | 147.7133 | -1.08427711 | 7.75E-108 |
| hsa-miR-27b-5p | 1,403 | 608 | 190.968 | 70.5497 | -1.43661906 | 1.36E-102 |

Table IV. Top 10 most abundant miRNAs in the H446/CDDP cells.

| miR name | H446 expressed | H446/CDDP expressed | H446-std | H446/CDDP-std | Fold-change (log2 H446-CDDP/H446) | P-value |
|---|---|---|---|---|---|---|
| hsa-miR-1307-3p | 696 | 5,475 | 94.7354 | 635.295 | 2.74545114 | 0 |
| hsa-miR-10a-5p | 1,243 | 7,504 | 169.1898 | 870.7313 | 2.36358499 | 0 |
| hsa-miR-423-5p | 27,054 | 104,330 | 3,682.4296 | 12,105.9967 | 1.71699201 | 0 |
| hsa-miR-320a | 136,101 | 385,405 | 18,525.259 | 44,720.7098 | 1.2714494 | 0 |
| hsa-miR-320b | 10,816 | 29,499 | 1,472.2096 | 3,422.9349 | 1.21725077 | 0 |
| hsa-miR-92b-5p | 547 | 2,557 | 74.4544 | 296.7031 | 1.99459099 | 1.47E-243 |
| hsa-miR-92a-1-5p | 592 | 2,508 | 80.5795 | 291.0173 | 1.85262016 | 8.82E-217 |
| hsa-miR-204-5p | 5 | 474 | 0.6806 | 55.0009 | 6.33650426 | 5.69E-118 |
| hsa-miR-7-5p | 166 | 682 | 22.5949 | 79.1363 | 1.80834244 | 2.23E-58 |
| hsa-miR-204-3p | 2 | 225 | 0.2722 | 26.108 | 6.58368107 | 3.40E-57 |

second highest diversity of 23-nt small RNAs compared with the total read number in the H446/CDDP cells (Fig. 2B). Similar small RNA length distribution was discovered in the H446 cells with a limited significance for 23-nt small RNAs (Fig. 2A).

*Annotation of small RNAs*. In order to explore the components of the two libraries, small RNAs from the H446 and H446/CDDP cells were annotated. Pie charts (Fig. 3) revealed the percentage of each type of small RNA, and the numbers in the figure labels in parentheses indicate the different types of small RNAs expressed. As shown in Fig. 3A and B, most small RNAs from both H446 and H446/CDDP cells were characterized as miRNAs. The rRNA proportion in the two libraries was <40%, which indicated that the quality of our samples was adequate for Solexa-Illumina sequencing. Furthermore, we also observed a relatively smaller percentage of miRNAs expressed in the H446 and H446/CDDP cells, respectively (Fig. 3C and D).

Therefore, small RNA annotation together with length distribution, exhibited the complexity and diversity of the small RNAs in SCLC, and provide clues for further analysis of miRNAs in the two cell lines. Several types of small RNAs that could be annotated were discarded, such as rRNAS, tRNAs, snRNAs, snoRNAs, known miRNAs, exon intron and non-annotated sRNAs. Finally putative small RNA populations including 7,096,132 H446 cells and 8,157,598 H446/CDDP cells were obtained (Table II).

*Differentially expressed known miRNAs in H446 and H446/CDDP cells*. Putative small RNA populations from the two cell lines were aligned to the precursor/mature miRNAs in miRBase 21 and known miRNA data in H446 and H446/CDDP cells were collected. The analysis revealed more than 400 miRNAs, which were differentially expressed in the H446 and H446-CDDP cells (Fig. 4). More importantly, there were 72 de-regulated and 55 upregulated miRNAs in the H446/CDDP cells; the difference was statistically significant (P<0.01) compared with that of the H446 cells. We also identified the most markedly changed 10 miRNAs from the H446 and H446/CDDP cells (Tables III and IV). The counts of different miRNAs in the H446 cells changed from 1 (hsa-miR-3131, hsa-miR-3124-5p, hsa-miR-4647, hsa-miR-216b, hsa-miR-769-3p and hsa-miR-4750) to 2,368,107 (hsa-let-7a-5p). The expression changes of known miRNAs in the H446/CDDP cells ranged from 1 (hsa-miR-3148,

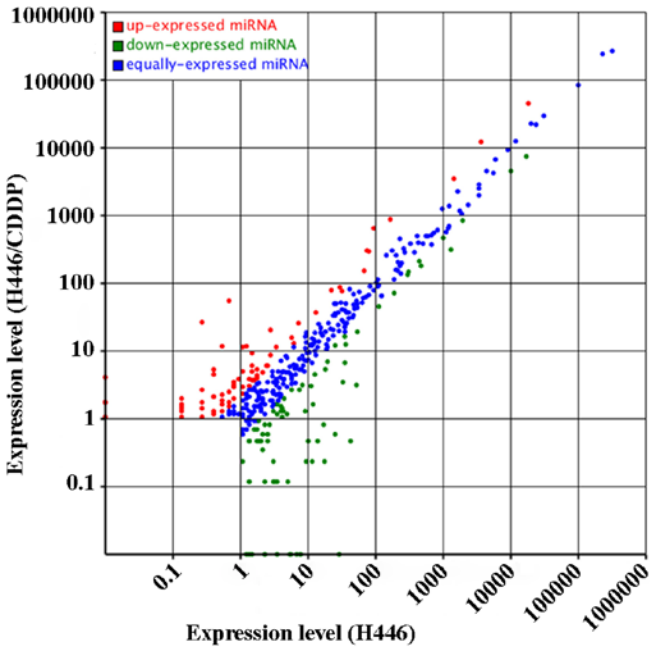


Figure 4. Digitalized expression levels of miRNAs in the H446 and H446/CDDP cells. Each point represents an miRNA; the x-axis and y-axis show expression levels. Point-color represents the relative expression level of miRNAs. Ratio = normalized expression in H446/CDDP cells/normalized expression in the H446 cells. A red point represents a ratio >2, a blue point represents a ratio ≥1/2 but ≤2; and a green point represents a ratio <1/2.

Table V. Novel miRNAs predicted in the H446 cells.

| Name | Sequence (5'-3') | Length (nt) | Arm (5'/3') | Chromosome location | Precursor length (nt) | MEF (kcal/mol) | Count |
|---|---|---|---|---|---|---|---|
| H446-m0001 | AGGGAGGGGTGGCAGGGATT | 20 | 5' | chr11:66638660:66638740:- | 81 | -30.7 | 6 |
| H446-m0002 | TACTTACCTGTCCCCTACCCCAC | 23 | 3' | chr12:53292676:53292766:- | 91 | -34.1 | 5 |
| H446-m0003 | GCTGGGGATGGAAGCTGAAGCC | 22 | 3' | chr13:111102941:111103018:+ | 78 | -25.7 | 23 |
| H446-m0004 | TTGGCCTGTAGCCCGGTCCGGT | 22 | 3' | chr17:61850673:61850755:- | 83 | -43.1 | 5 |
| H446-m0005 | CAAAATGATGAGGTACCTGATA | 22 | 5' | chr20:3194751:3194835:+ | 85 | -20.4 | 33 |
| H446-m0006 | CTGGGAGTGGAGGGGAGGGTA | 21 | 3' | chr20:60944769:60944849:+ | 81 | -32.9 | 8 |
| H446-m0007 | GGAGGAACCTTGGAGCTTCGGCA | 23 | 3' | chr22:31556037:31556127:- | 91 | -45.3 | 45 |
| H446-m0008 | TGGGGAGGTGTGGAGTCAGCAT | 22 | 5' | chr3:127294107:127294179:- | 73 | -46.3 | 14 |
| H446-m0009 | TCTGTTTGTCGTAGGCAGATGG | 22 | 3' | chr5:99385174:99385256:+ | 83 | -18 | 9 |
| H446-m0010 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6:28918820:28918902:+ | 83 | -21.5 | 3,647 |
| H446-m0011 | GCAAATGATGTGAGAGATTC | 20 | 3' | chr6:168343859:168343952:+ | 94 | -20.2 | 9 |
| H446-m0012 | CGCGCCTGCAGGAACTGGTAGA | 22 | 3' | chr6:1390549:1390646:- | 98 | -39.62 | 14 |
| H446-m0013 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_cox_hap2:437556:437638:+ | 83 | -21.5 | 3,647 |
| H446-m0014 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_mann_hap4:222225:222307:+ | 83 | -21.5 | 3,647 |
| H446-m0015 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_mcf_hap5:222373:222455:+ | 83 | -21.5 | 3,647 |
| H446-m0016 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_qbl_hap6:222220:222302:+ | 83 | -21.5 | 3,647 |
| H446-m0017 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_ssto_hap7:259727:259809:+ | 83 | -21.5 | 3,647 |
| H446-m0018 | CCGGGCGGGCGAGGAGCGGG | 20 | 5' | chr7:1200062:1200125:+ | 64 | -31.2 | 6 |
| H446-m0019 | TGGGCGGCCACTTGACATCCTC | 22 | 5' | chr7:128587281:128587362:- | 82 | -34.8 | 6 |
| H446-m0020 | AACTGGGCATAGCTGTACTTTT | 22 | 3' | chr8:141538823:141538913:- | 91 | -19.1 | 9 |
| H446-m0021 | AGGGCCGAAGGGTGGAAGCT | 20 | 5' | chr9:135927378:135927447:+ | 70 | -35 | 13 |

M, miRNA; MFE, minimum free energy.

hsa-miR-671-3p,hsa-miR-495,hsa-miR-2682-5p,hsa-miR-433, hsa-miR-493-5p, hsa-miR-584-5p and hsa-miR-494) to 2,278,377 (hsa-let-7a-5p) suggesting drastic variation in the expression of miRNAs in SCLC.

Table VI. Novel miRNAs predicted in the H446/CDDP cells.

| Name | Sequence (5'-3') | Length (nt) | Arm (5'/3') | Chromosome location | Precursor length (nt) | MEF (kcal/mol) | Count |
|---|---|---|---|---|---|---|---|
| H446-CDDP-m0001 | AGCTGGGGATGGAAGCTGAAGCC | 23 | 3' | chr13:111102941:111103018:+ | 78 | -25.7 | 9 |
| H446-CDDP-m0002 | AGCCGCCTCCACCAAGCCTGGA | 22 | 3' | chr14:70449623:70449708:+ | 86 | -35.1 | 6 |
| H446-CDDP-m0003 | GGAGGGAGGGGGACGAGCGCGCG | 23 | 5' | chr14:32545963:32546039:- | 77 | -45.2 | 7 |
| H446-CDDP-m0004 | TGAGTGCGGGCTGGGCACAAGT | 22 | 5' | chr17:6347800:6347869:+ | 70 | -31.7 | 15 |
| H446-CDDP-m0005 | CAGGGCTGGGAGGGAGTGGGA | 21 | 3' | chr1:153585537:153585627:- | 91 | -34.1 | 8 |
| H446-CDDP-m0006 | CGGCGGCTCCCCGCTCCCCGGA | 22 | 3' | chr1:214454448:214454532:- | 85 | -55.1 | 6 |
| H446-CDDP-m0007 | GCAAAATGATGAGGTACCTGATA | 23 | 5' | chr20:3194750:3194836:+ | 87 | -20.4 | 23 |
| H446-CDDP-m0008 | TGCGGGTGAGGATGAGGGTGC | 21 | 3' | chr20:61276739:61276835:+ | 97 | -43.5 | 15 |
| H446-CDDP-m0009 | GGAGGAACCTTGGAGCTTCGGCA | 23 | 3' | chr22:31556037:31556127:- | 91 | -45.3 | 56 |
| H446-CDDP-m0010 | TGGGGAGGTGTGGAGTCAGCAT | 22 | 5' | chr3:127294107:127294179:- | 73 | -46.3 | 48 |
| H446-CDDP-m0011 | TGGGGGACGGTGGGCACCGAG | 21 | 5' | chr4:184329416:184329499:- | 84 | -49.6 | 15 |
| H446-CDDP-m0012 | TCAGACACAGGTATGGCTGGCT | 22 | 5' | chr5:171831829:171831908:- | 80 | -30.6 | 14 |
| H446-CDDP-m0013 | TAGCTCTGATGATGGTGGTTTCT | 23 | 3' | chr5:176878879:176878970:- | 92 | -23.2 | 7 |
| H446-CDDP-m0014 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6:28918820:28918902:+ | 83 | -21.5 | 7,600 |
| H446-CDDP-m0015 | AGCAAATGATGTGAGAGATTC | 21 | 3' | chr6:168343859:168343952:+ | 94 | -20.2 | 17 |
| H446-CDDP-m0016 | CGCGCCTGCAGGAACTGGTAGA | 22 | 3' | chr6:1390549:1390646:- | 98 | -39.62 | 22 |
| H446-CDDP-m0017 | TGAGATCAAGACCAGGGAAAAT | 22 | 3' | chr6:134609915:134609990:- | 76 | -30.3 | 5 |
| H446-CDDP-m0018 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_cox_hap2:437556:437638:+ | 83 | -21.5 | 7,600 |
| H446-CDDP-m0019 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_mann_hap4:222225:222307:+ | 83 | -21.5 | 7,600 |
| H446-CDDP-m0020 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_mcf_hap5:222373:222455:+ | 83 | -21.5 | 7,600 |
| H446-CDDP-m0021 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_qbl_hap6:222220:222302:+ | 83 | -21.5 | 7,600 |
| H446-CDDP-m0022 | TCGGGCGGGAGTGGTGGCTTT | 21 | 3' | chr6_ssto_hap7:259727:259809:+ | 83 | -21.5 | 7,600 |
| H446-CDDP-m0023 | TGAGTGTGTGTGTGTGAGTGTGA | 23 | 3' | chr8:79679467:79679541:+ | 75 | -20.3 | 21 |
| H446-CDDP-m0024 | AGGGCCGAAGGGTGGAAGCT | 20 | 5' | chr9:135927378:135927447:+ | 70 | -35 | 19 |
| H446-CDDP-m0025 | CCCTGGGGTTCTGAGGACATG | 21 | 5' | chr9:35710651:35710743:- | 93 | -39 | 7 |

M, miRNA; MFE, minimum free energy.

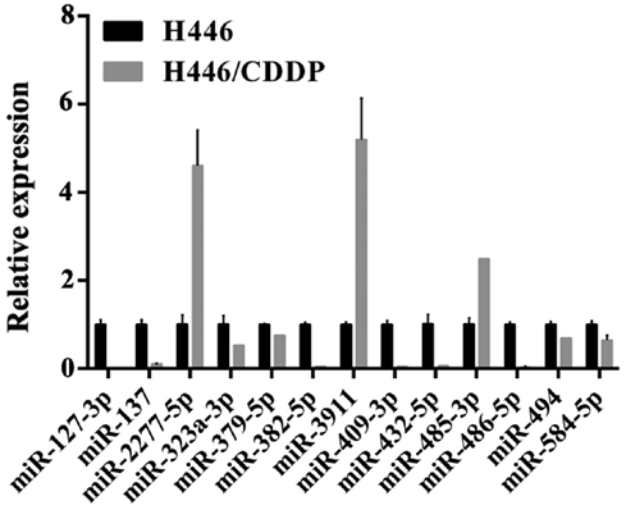


Figure 5. Expression levels of randomly selected known miRNAs in the H446 and H446/CDDP cells.

*Validation of miRNA expression in the H446 and H446/CDDP cells*. Among the differentially expressed miRNAs, 23 miRNAs were randomly selected and validated by qPCR. As shown in Fig. 5, expression of 15 miRNAs was significantly different including five miRNAs (miR-485-3p, miR-2277-5p, miR-3911, miR-204-3p and miR-204-5p) elevated >5-fold in the H446/CDDP cells. Markedly, the miR-204-3p expression was elevated 16 times and miR-204-5p was increased >300-fold. By contrast, the expression of 6 miRNAs (miR-127-3p, miR-137, miR-382-5p, miR-3911, miR-409-3p, miR-432-5p, miR-485-3p, miR-486-5p and miR-494) in the H446/CDDP cells was decreased >5-fold. In addition, the miR-127-3p expression was decreased ~50 times. Above all, the expression trends of the selected miRNAs in the H446 and H446/CDDP were similar to the Solexa sequencing results.

*Prediction of novel miRNAs in the H446 and H446/CDDP cells*. The stem-loop hairpin secondary structure is an important feature, which can be utilized to distinguish miRNAs from each other. Furthermore, Solexa sequencing enables identification of novel transcripts. Therefore, our next objective was to predict novel miRNAs in the H446 and H446/CDDP cell lines by investigating the stem-loop hairpin structure using the Solexa sequencing method. We introduced 21 to 25 novel miRNAs into the H446 and H446/CDDP cells (Tables V and VI), and found the read number of the novel miRNAs was much lower than that of the known miRNAs,

Table VII. Distribution of putative target genes in cell component: Gene Ontology - cellular component term.

| Gene Ontology term component | Cluster frequency (%) | Corrected p-value |
|---|---|---|
| H446 cells | | |
| Cell | 96.90 | 0.00158 |
| Cell part | 96.90 | 0.00158 |
| Intracellular | 74.00 | 0.01422 |
| Intracellular part | 73.60 | 0.02138 |
| Cell fraction | 10.40 | 0.00015 |
| Insoluble fraction | 7.60 | 0.00047 |
| Neuron projection | 3.60 | 0.01808 |
| H446/CDDP cells | | |
| Cell | 96.90 | 5.89E-05 |
| Cell part | 96.90 | 5.89E-05 |
| Integral to membrane | 9.90 | 0.00278 |

indicating that novel miRNAs were usually expressed at lower levels.

*Target prediction of novel miRNAs*. Target genes were predicted according to the aforementioned rules and principles discussed above. The results indicated the presence of 130,566 candidate target genes for 21 novel miRNAs in the H446 cell line and 153,092 putative target genes for the 25 novel miRNAs from the H446/CDDP cell line. We also annotated the biological processes, molecular functions and cellular components of the target genes using GO analysis (20,21). GO analysis revealed that in the cellular component category (Table VII), most target genes in the H446 cell line were localized in insoluble cell fraction, intracellular areas and dendrites ($P<0.05$), while target genes in the H446/CDDP cells were only restricted to the membrane ($P<0.05$). In the category of molecular function, the majority of targets in the H446 cells were significantly involved ($P<0.05$) (Table VIII) in transcriptional regulation, transferase and binding activities involving nucleosides and nucleotides (purine), adenyl nucleotides and ribonucleotides, cations, metal ions, sequence-specific DNA binding and enzymes. The target gene functions in the H446/CDDP cells were limited to transition metal ion binding and DNA binding ($P<0.05$) (Table VIII). The target genes in the H446 cell line participated in more biological processes (statistically significant processes are listed in Table IX) than those of putative target genes in the H446/CDDP cells (all listed in Table IX).

*Pathway annotation of target genes*. We were also interested in the signaling pathways of the target genes. KEGG analysis was adopted to determine the biological pathway of the novel miRNAs from the H446 and H446/CDDP cell lines. Results revealed that target genes of miRNAs from the H446 cell line participated in greater number of biological pathways than those of miRNAs from the H446/CDDP cells. A few pathways were shared by target genes of novel miRNAs from both the cell lines (Table X). The target genes of novel miRNAs from the H446 cells mediate a few pathways closely related to cancer, such as the ErbB signaling pathway, mTOR signaling pathway and Notch signaling pathway in chronic myeloid leukemia colorectal cancer, melanoma, pancreatic cancer, prostate cancer and thyroid cancer as well as non-small cell lung cancer. However, only the Wnt signaling pathway was closely related to cancer in the pathway list of target genes mediated by miRNAs from the H446/CDDP cells (Table X).

Table VIII. Top 10 most frequently enriched molecular functions by putative target genes of novel miRNAs in the H446 and H446/CDDP cells: Gene ontology - molecular function term.

| Gene Ontology term function | Cluster frequency (%) | Corrected p-value |
|---|---|---|
| H446 cells | | |
| Binding | 84.90 | 2.37E-05 |
| Ion binding | 26.00 | 0.00803 |
| Cation binding | 25.70 | 0.00676 |
| Metal ion binding | 20.40 | 0.01386 |
| Purine nucleotide binding | 13.50 | 0.02562 |
| Transferase activity | 12.70 | 0.04526 |
| Nucleoside binding | 11.50 | 0.0012 |
| Purine nucleoside binding | 11.50 | 0.00144 |
| Adenyl nucleotide binding | 11.20 | 0.00296 |
| Adenyl ribonucleotide binding | 10.60 | 0.01851 |
| Transcription regulator activity | 7.70 | 0.00271 |
| Enzyme binding | 5.00 | 0.03061 |
| Sequence-specific DNA binding | 1.80 | 0.02915 |
| H446/CDDP cells | | |
| Binding | 84.90 | 3.53E-05 |
| Transition metal ion binding | 14.10 | 0.00056 |
| DNA binding | 5.80 | 0.00135 |

## Discussion

Small RNAs (sRNAs) from the next generation high throughput Solexa sequencing cover almost every type of sRNAs, including miRNAs, siRNAs, piRNAs, rRNAs, tRNAs, snRNAs, snoRNAs, repeat associated sRNAs and degraded tags of exons or introns. The sRNAs were annotated into different categories by comparing the collected tags with those in databases. In the present study, digitalized sRNAs were obtained and initially subjected to data cleaning including elimination of low-quality tags and other contaminants. The length distribution was summarized to explore the possible composition of sRNA libraries from the H446 and H446/CDDP cells. Subsequently, standard bioinformatic analysis was carried out to annotate the clean tags into different categories in order to identify known miRNAs and predict novel miRNAs expressed in the H446 and H446/CDDP cell lines. On the one hand, the expression level of known miRNAs in the two cell lines was summarized and compared to identify the varied expression of the miRNAs. On the other hand, biological information

Table IX. Distribution of putative target genes in biological processes: Gene ontology - biological process term.

| Gene Ontology term-process | Cluster frequency (%) | Corrected p-value |
|---|---|---|
| H446 cells | | |
| Regulation of metabolic process | 22.50 | 5.27E-08 |
| Regulation of macromolecule metabolic process | 18.10 | 2.55E-07 |
| Regulation of macromolecule metabolic process | 17.90 | 5.30E-07 |
| Biopolymer modification | 14.60 | 8.55E-07 |
| Developmental process | 30.80 | 1.38E-06 |
| Intracellular signaling cascade | 10.20 | 2.06E-06 |
| Anatomical structure development | 23.30 | 2.78E-06 |
| Protein modification process | 13.70 | 3.35E-06 |
| Multicellular organismal development | 22.80 | 5.67E-06 |
| Regulation of gene expression | 16.30 | 3.30E-05 |
| H446/CDDP cells | | |
| Anatomical structure development | 23.20 | 2.80E-06 |
| System development | 20.80 | 1.39E-05 |
| Multicellular organismal development | 22.70 | 5.19E-05 |
| RNA metabolic process | 18.20 | 6.46E-05 |
| Cellular developmental process | 15.60 | 8.70E-05 |
| Organ development | 14.80 | 9.03E-05 |
| Transcription | 15.80 | 9.66E-05 |
| Nitrogen compound metabolic process | 29.70 | 0.00014 |
| Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 27.20 | 0.00019 |
| Cellular process | 82.90 | 0.00096 |

of novel miRNAs from the H446 and H446/CDDP cells was analyzed by GO and KEGG analysis. The results revealed differences in target gene expression, functions, processes and pathways, which may be linked to MDR of H446/CDDP cells.

Our data showed differential expression of known miRNAs in the H446 and H446/CDDP cells. Further sequence tag analysis revealed that the expression of 72 miRNAs was markedly decreased and that of 55 significantly increased in the H446/CDDP cells compared with that of the H446 cells (Fig. 4), respectively. These datasets showed that down-regulation of miRNAs was more common than upregulation in MDR cells of SCLC, which was consistent with previously published miRNA profiling studies involving bladder tumor tissues (22,23). Validation of the differentially expressed miRNAs, in the present study by real-time PCR, yielded results consistent with those obtained with Solexa sequencing. Our previous studies demonstrated that miRNA-137 was downregulated in H446/CDDP cells, which participated in the MDR of this cell line (24). Bier *et al* (25) demonstrated that decreased expression of miRNA-137 enhanced the stem-cell features of glioblastoma cells. Furthermore, deregulated miRNA-494 was related to the oncogenesis of pancreatic ductal adenocarcinoma and decreased miRNA-494 was considered as a therapeutic or prognostic marker for this cancer (26), which was also consistent with our results. Furthermore, a few miRNAs discussed in our study were similarly deregulated in other cancers: absence of miR-329 enhanced proliferation of cancer cells in neuroblastoma and glioma (27,28); renal childhood neoplasm was associated with de-regulated miR-215 (29) and its upregulation may inhibit the growth of osteosarcoma and colon cancer cells (30). Indeed, the varied expression of miRNAs provided reliable data to establish the role of miRNAs in the MDR mechanisms of SCLC.

After analyzing the expression of known miRNAs and exploring their possible relationship to MDR, we explored novel miRNAs in the H446 and H446/CDDP cells, which may also be associated with MDR. As described above, clean reads of digitalized sRNAs were annotated into different categories, such as rRNAS, tRNAs, snRNAs, snoRNAs, known miRNAs, exons/introns and unannotated sRNAs. Novel miRNAs were predicted from tags that were not annotated to any category. We discovered 21 and 25 novel miRNAs in the H446 and H446/CDDP cells, respectively. Although the expression of a majority of the novel miRNAs from both H446 and H446/CDDP cell lines was low, target genes of the novel miRNAs exhibited differences in distribution and pathophysiology.

Putative target genes of novel miRNAs from the H446 and H446/CDDP cells were analyzed by GO analysis (31). The results showed that a majority of novel miRNA target genes in the H446 cells were involved in regulation of a broad range of metabolic and physiological processes including metabolism of macromolecules, biopolymer modification, developmental processes, intracellular signaling cascades, anatomical structure, protein modification, tissue development, and regulation of gene expression. Compared with targets of novel miRNAs in the H446/CDDP cells, novel miRNA targets in the H446 cells participated in a greater number of processes, most of

Table X. Pathways mediated by putative target genes of novel miRNAs from the H446 and H446/CDDP cell lines.

| Pathways mediated by target genes |
|---|
| H446 cells only |
| Acute myeloid leukemia |
| Amoebiasis |
| Axon guidance |
| β-alanine metabolism |
| Chagas disease (American trypanosomiasis) |
| Chronic myeloid leukemia |
| Colorectal cancer |
| ECM-receptor interaction |
| Epithelial cell signaling in *Helicobacter pylori* infection |
| ErbB signaling pathway |
| Fc γ R-mediated phagocytosis |
| Glycerolipid metabolism |
| Inositol phosphate metabolism |
| MAPK signaling pathway - yeast |
| Measles |
| Melanoma |
| mTOR signaling pathway |
| Non-small cell lung cancer |
| Notch signaling pathway |
| Pancreatic cancer |
| Phosphatidylinositol signaling system |
| Prostate cancer |
| Thyroid cancer |
| Tryptophan metabolism |
| Ubiquinone and other terpenoid-quinone biosynthesis |
| H446/CDDP cells only |
| Cardiac muscle contraction |
| Hematopoietic cell lineage |
| Natural killer cell mediated cytotoxicity |
| Neurotrophin signaling pathway |
| NOD-like receptor signaling pathway |
| Osteoclast differentiation |
| Type II diabetes mellitus |
| Wnt signaling pathway |
| Common pathways |
| Aldosterone-regulated sodium reabsorption |
| African trypanosomiasis |
| Bile secretion |
| Biosynthesis of secondary metabolites |
| Carbohydrate digestion and absorption |
| Cysteine and methionine metabolism |
| Endocytosis |
| Gastric acid secretion |
| MAPK signaling pathway |
| Metabolic pathways |
| Pancreatic secretion |
| Pathways in cancer |
| Protein digestion and absorption |
| Proximal tubule bicarbonate reclamation |
| Purine metabolism |

which were related to transcription. A few novel miRNAs in the H446 cells were predicted to be involved in programmed cell death, cell differentiation, cell proliferation and cell adhesion. Those transcription factors were known to be targeted by conserved miRNAs and play a major role in various aspects of cancer cell growth and tumor metastasis (32,33). By contrast, target genes of novel miRNAs in the H446/CDDP cells were not involved in these regulation processes. Thus, in addition to the different expression levels of known miRNAs, the limited functional role of novel miRNAs in the H446/CDDP cells also highlights their MDR characteristics. Further studies are essential to explain the functional significance of the novel miRNAs and their targets in MDR of SCLC.

Based on the knowledge of putative target genes in regards to distribution range, pathophysiologic process and function type, we further analyzed possible pathways mediated by the novel miRNAs. The KEGG pathway analysis was adopted to identify significantly enriched metabolic pathways and signal transduction pathways. The results indicated that target genes of novel miRNAs from both the H446 and H446/CDDP cells participated in hundreds of pathways, but most target genes were limited to significantly enriched in metabolic pathways. Novel miRNAs from the H446/CDDP cells concentrated in fewer pathways than that of the H446 cells, and most of those pathways were shared with target genes of novel miRNAs from the H446 cells. Among the shared pathways, endocytosis, MAPK signaling pathway, metabolic pathways, pathways in cancer and purine metabolism pathways are believed to be associated with cancer metastasis (34,35), especially pathways in cancer. According to a map of pathways in cancer (which was not shown) most of the molecules were partially regulated by predicted genes, and most of them were involved in classic signal transduction pathways regulating the occurrence and metastasis of cancers (36-40). A few pathways were modulated only by target genes of novel miRNAs from H446 cells, such as the ErbB signaling pathway, mTOR signaling pathway, Notch signaling pathway, and pathways mediating acute myeloid leukemia, chronic myeloid leukemia, colorectal cancer, melanoma, non-small cell lung cancer, pancreatic cancer and prostate cancer. Interestingly, all those pathways were not mediated by target genes of novel miRNAs from the H446/CDDP cells. We believe that the differential regulation of the pathways may be associated with MDR of H446/CDDP cells. On the other hand, a few pathways were regulated only by target genes of novel miRNAs from the H446/CDDP cells, including neurotrophin signaling pathway, NOD-like receptor signaling pathway, Wnt signaling pathway and natural killer cell-mediated cytotoxicity. However, only the Wnt signaling pathway has been associated with carcinogenesis (41,42). In brief, KEGG analysis revealed pathways mediated by target genes of novel miRNAs from H446 and H446/CDDP cells, and the differential regulation of cancer-related pathways may be associated with MDR mechanisms in SCLC.

We analyzed the differential expression of known miRNAs in H446 and H446/CDDP cells and discovered a set of miRNAs with markedly altered expression in H446/CDDP cells. Furthermore, we predicted novel miRNAs in the two cell lines and analyzed their biological roles. The altered expression of known miRNAs and different functions of novel miRNAs may be related to MDR of SCLC.

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