5-Fluorouracil in combination with cisplatin alters the microRNA expression profile in the CNE nasopharyngeal carcinoma cell line

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Abstract. Chemotherapy constitutes one of the chief supplementary methods in the treatment of nasopharyngeal carcinoma (NPC). 5-Fluorouracil (5-FU) and cisplatin are classic chemotherapeutic drugs that have been widely used for NPC treatment. Although the aberrant expression of protein-coding genes has been observed after chemotherapy, the regulatory mechanisms involved remain poorly understood. MicroRNAs (miRNAs) are a newly identified class of small regulatory RNAs involved in multiple biological processes and metabolic regulation, including the initiation, progression and metastasis of human cancers. In this study, using a label-free high-throughput microRNA array technology, the stacking-hybridized universal tag (SHUT) assay, we show that 5-FU in combination with cisplatin significantly alters the global expression profile of miRNAs in CNE cells. After 48 h of treatment with a low dose [10% inhibitory concentration (IC_{10})] of 5-FU and/or cisplatin, numerous key miRNAs were shown to be regulated. Compared to the 431 miRNAs detected in the control cells, 184 miRNAs were significantly expressed in the 5-FU-treated cells, while 336 miRNAs were expressed in the cisplatin-treated cells and 13 miRNAs in the cells treated with the combination of both drugs. The majority of these miRNAs are associated with cancer development, progression and metastasis. This is the first time that miRNA expression profiles in the CNE cell line are shown. Our findings elucidate a potential mechanism involved in the chemotherapy of NPC and provide new clues for the treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells that cover the surface and line the nasopharynx (1). NPC differs significantly from other cancers of the head and neck in its occurrence, causes, clinical behavior and treatment. It is vastly more common in certain regions of East Asia and Africa than elsewhere, with viral, dietary and genetic factors implicated in its causation (2,3). Although rare, NPC accounts for one third of childhood nasopharyngeal neoplasms in the USA (4).

Chemotherapy with anti-neoplastic (or cytotoxic) drugs is one of the most extensively adopted practices for managing cancers (5). Apart from radiation therapy and surgical resection, chemotherapy should be considered for patients with metastatic disease or local recurrence that is no longer amenable to surgery or radiation therapy (6-8). For NPC therapy, it is most common to use a combination of two or more chemotherapeutic drugs, known as combination chemotherapy. Using two or more drugs together is often more effective than using one drug.

Two of the main drugs used in the treatment of NPC are cisplatin and 5-fluorouracil (5-FU) (9,10). Cisplatin is a DNA-binding agent that is widely used to treat different types of cancer. At the centre of this drug is a platinum metal atom. The drug forms DNA crosslinks via the platinum which damages the cancer cells. 5-FU is one of the most commonly used drugs to treat cancer. It is part of a group of chemotherapeutic drugs known as the anti-metabolites. In cancer cells, 5-FU is converted intracellularly into three cytotoxic metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These metabolites inhibit the formation and repair of DNA in cancer cells (11,12).

Despite the widespread clinical use of cisplatin and 5-FU for over 40 years and the aberrant expression of protein-coding genes observed after chemotherapy (13-15), the regulatory mechanisms invovled remain poorly understood. We hypothesized that microRNAs (miRNAs), a class of post-transcriptional gene regulators, may play an important role in the cisplatin- and 5-FU-induced alteration of gene expression in cancer cells. miRNAs are a group of small (20-22 nt) endogenous non-protein-coding RNA molecules that negatively regulate gene expression (16,17). miRNAs are involved in multiple biological processes and metabolic regulation, including cell proliferation, differentiation and programmed cell death. As the dysregulation of these processes is a hallmark of cancer, miRNAs are viewed as major contributors to the pathogenesis

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of cancer, including the initiation and progression of cancer (18). A growing body of evidence has suggested the importance of miRNAs in managing the efficiency of chemotherapy in several human cancers (19,20).

Although a number of studies have shown that chemotherapy drugs alter miRNA expression in many cancer cells, there is no report on the effect of cisplatin or 5-FU on miRNAs in human NPC (21,22). In this study, we used a label-free high-throughput miRNA array technology, the stacking-hybridized universal tag (SHUT) assay (23), to investigate the effect of cisplatin and/or 5-FU exposure on the global expression profile of miRNAs in the CNE human NPC cell line.

Materials and methods

Cell line and cell culture. All the cell culture reagents were purchased from Invitrogen, Inc. (Carlsbad, CA, USA). The CNE human NPC cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (CAS). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). They were maintained at 37˚C in a humidified incubator with 5% CO_2.

Drug treatment and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 5-FU and cisplatin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at 4˚C, protected from lighting and moisture. For the cell treatments, 5-FU and cisplatin were freshly prepared in DMEM complete medium in 5-fold series dilution concentrations, with final starting concentrations of 48.05 and 1.04 mM. The cells were seeded 10,000 cells/well (100 μl) in a 96-well plate (Corning Costar, #3599) and treated with the freshly prepared 5-FU or cisplatin in duplicate for 48 h. The half maximal inhibitory concentration (IC_{50}) (concentration of the two drugs to produce half maximal cell inhibition) values were determined by the MTT method and their 10% inhibitory concentration (IC_{10}) values were calculated. Briefly, after 48 h of drug incubation, 90 μl fresh medium and 10 μl MTT-Dulbecco's phosphate-buffered saline (DPBS) solution were added to each well, and the plate was incubated in the dark for 4 h at 37˚C. The medium was removed by inverting and tapping the plate, and 100 μl/well of dimethyl sulfoxide (DMSO) were added and shaken for 5 min to dissolve the formazan crystals. The absorbance at 570 nm was read on a microplate spectrophotometer (BioTek) and the results were expressed as a percentage of the control.

RNA isolation. Cells were seeded at 300,000 cells/well in a flat-bottom 6-well plate in 2 ml of complete medium. After 18 h, the medium was replaced with fresh medium. The cells were dived into four groups: control group (CNE-normal), IC_{10} of 5-FU (CNE-5-FU), IC_{10} of cisplatin (CNE-Cis), or both IC_{10} values of 5-FU and cisplatin (CNE-Cis + FU) in complete medium, respectively. After 48 h, the cells were collected and total RNA was prepared using TRIzol (Invitrogen, Inc.) following the manufacturer's instructions, except that the precipitation was allowed to proceed for 12 h at -20˚C, which efficiently recovers all RNA species, including miRNAs. RNA concentration was determined using a nanodrop spectrophotometer. RNA integrity was determined by TAE agarose gel electrophoresis and the relation of the 28S, 18S and 5S ribosomal RNA (rRNA) bands.

Microarray miRNA profiling. The SHUT assay, recently presented by Duan *et al* (23), is an efficient technique for high-throughput miRNA profiling using microarrays. Being a label-free approach, it eliminates the cost and potential biases of the fluorescent-labeling of miRNAs, while offering good specificity and sensitivity. Following the strategies presented by the authors, we designed a full-spectrum microarray targeting of each of the 1,733 mature human miRNAs listed in miRBase release 17. This customized microarray design was ordered from Agilent Technologies Inc. (Santa Clara, CA, USA) via its eArray online service. The final product is an *in situ* synthesized high-definition DNA microarray of the format 8x15 k (arrays per slide x probes per array; Agilent G2509F). For each array, 5.0 μg total RNA extracted from each cell line and 200 nM (final concentration) Cy3-labeled universal tag DNA oligonucleotides (UT, of the sequence AGGTCGCA; synthesized by Integrated DNA Technologies, Inc., Coralville, IA, USA) were dissolved in hybridization buffer (5X SSC with 0.2% SDS in nuclease-free water) to make a 40-μl sample solution. In a typical experiment, the four cell samples along with a blank buffer were applied respectively to one of the eight arrays on the same microarray slide, which was part of the SureHyb hybridization assembly (with G2534-60014 and G2534A; Agilent Technologies, Inc.). The assembly was placed in a hybridization oven (Agilent 2545A) at 42˚C for 22 h with a constant rotation speed of 20 rpm. After hybridization, the slides were washed in 5X SSC and 0.1% SDS at 30˚C for 6 min, and then washed for 3 min twice at room temperature in 0.2X SSC. The slides were immediately dried and scanned.

Image scanning and data analysis. Following hybridization and washing, the slides were scanned using a GenePix 4100A Microarray Scanner (Molecular Devices, Inc., USA) at constant power and photomultiplier (PMT) gain settings through a single-color channel (532 nm wavelength). The raw pixel intensities were extracted using the GenePix Pro 7.0 software (Molecular Devices, Inc.). Data processing (including filtration, background-correction, transformation and normalization) and subsequent statistical analysis were performed using the methods provided by Duan *et al* (23). Finally, the expression levels of all mature miRNAs were plotted on a heatmap.

Results

Effects of 5-FU and cisplatin on the CNE human NPC cell line. To determine the sensitivity of CNE cells to 5-FU or cisplatin, MTT assay was performed and dose-response curves were generated to determine the IC_{50} values. As shown in Fig. 1, the IC_{50} of 5-FU and cisplatin was approximately 1,058 and 5.237 μM, respectively. Using a formula from GraphPad $\{EC_F = IC_{50} \times [F/(100-F)]^{1/H}\}$, the IC_{10} values of these two drugs were calculated to be approximately 22.91 and 2.67 μM, respectively. To avoid genetic changes as much as possible, which are more sensitive as compared to cellular changes, low concentrations (IC_{10}) of these two drugs were selected to determine their

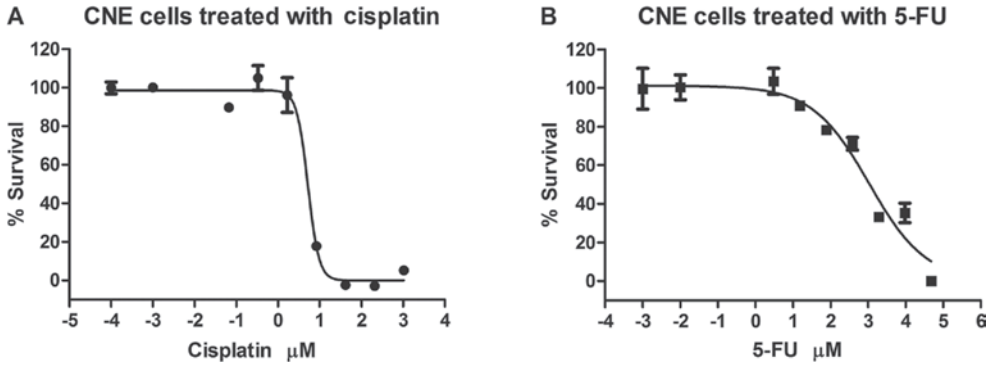

Figure 1. The half maximal inhibitory concentration (IC_{50}) determination of 5-fluorouracil (5-FU) and cisplatin against the CNE cell line, using a sigmoidal equation. (A) IC_{50} of cisplatin (5,237 μM). (B) IC_{50} of 5-FU (1,058 μM).

Table I. Comparison of miRNA expression profile between the four groups.

miRNA category	No.	miRNAs
Expressed significantly only in the CNE-normal, CNE-Cis and CNE-5-FU group	24	let-7f, miR-1302, miR-200c, miR-23c, miR-30b, miR-3145-5p, miR-3156-5p, miR-374a, miR-374b, miR-411, miR-4263, miR-4277, miR-4438, miR-4491, miR-466, miR-4698, miR-4753-5p, miR-4803, miR-508-3p, miR-515-5p, miR-625, miR-196a*, miR-3138, miR-761
Expressed significantly only in the CNE-normal, CNE-Cis and CNE-Cis + FU group	3	let-7d*, miR-19a, miR-372
Expressed significantly only in the CNE-normal, CNE-5-FU and CNE-Cis + FU group	1	miR-2116*
Expressed significantly only in the CNE-Cis, CNE-5-FU and CNE-Cis + FU group	2	miR-29c, miR-29a
Expressed in all the four groups	23	miR-100, miR-106a, miR-125b, miR-16, miR-17, miR-19b, miR-20a, miR-20b, miR-21, miR-22, miR-23a, miR-23b, miR-27a, miR-27b, miR-29b, miR-30a, miR-365, miR-4422, miR-4448, miR-24, miR-4436b-5p, miR-4454, miR-4734

Cis, cisplatin; 5-FU, 5-fluorouracil.

effects on the global expression levels of miRNAs in the CNE cells. Furthermore, as the combination of these two drugs is used as a popular first-line therapy (22.91 μM of 5-FU and 2.67 μM of cisplatin), we also investigated their combined effect on the global miRNA expression levels.

Alteration of miRNA expression profiles in CNE cells after drug treatment. Based on the SHUT assay, the miRNA expression profiles in CNE cells with different treatments were obtained and the heatmap of part of the results is shown in Fig. 2. Of the 1,733 human miRNAs analyzed, a total of 701 were detected to be expressed significantly with or without low-dose drug treatment, among which 23 miRNAs were detected in all the four groups (Table I and Fig. 2). Apart from the 23 miRNAs commonly expressed, 431 miRNAs were detected to be significantly expressed in the control cells, while 184 miRNAs were detected in the 5-FU-treated cells, 336 miRNAs in the cisplatin-treated cells and 13 miRNAs in the cells treated with the combination of both drugs. The above results showed that all the treated cells expressed a lower number of miRNAs as compared to the control cells.

When compared by group, the control cells collectively expressed 185, 80 and 28 miRNAs significantly with the cisplatin-, the 5-FU- and the combination-treated cells, respectively (Table II and Fig. 2). Furthermore, only 137 miRNAs were found in the control and the cisplatin-treated cells, 30 miRNAs in the control and the 5-FU-treated cells and only one miRNA, hsa-miR-374c, was found in the control and the combination-treated cells. The cisplatin-treated and 5-FU-treated cells exclusively expressed 44 miRNAs. As indicated in Table II and Fig. 2, there were only two miRNAs, hsa-miR-29c and hsa-miR-29a, expressed in the drug-treated groups, but not in the control. Three miRNAs, hsa-let-7d*, hsa-miR-19a and hsa-miR-372, were found to be expressed in all groups, except the cisplatin-treated group. One miRNA, hsa-miR-2116*, was expressed in all the groups, except for the 5-FU-treated group.

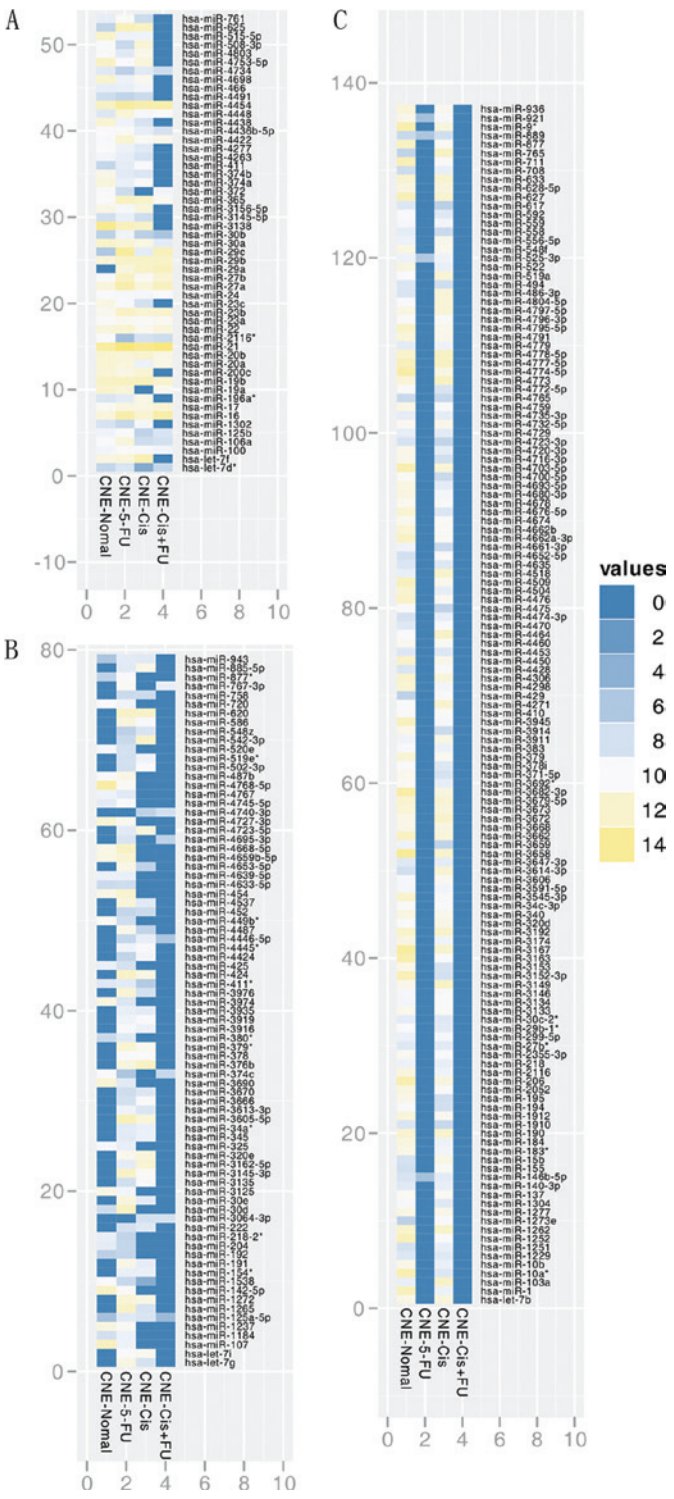

Figure 2. Heatmap of the miRNA expression profile in the CNE cell line. (A) Heatmap of the miRNA expression profile of the four groups (as listed in Table I). (B and C) Heatmap of the miRNA expression profile of miRNAs significantly expressed in two of the four groups (as listed in Table II). (C) Heatmap of the miRNA expression profile of miRNAs significantly expressed in the CNE-normal and CNE-Cis groups. Color represents log2-transformed expression data. The names of the miRNAs are in accordance with miRBase Release 16. The color key indicates the signal intensities based on their log2 values. Cis, cisplatin; 5-FU, 5-fluorouracil.

Notably, a total of 235 miRNAs were exclusively expressed in the control cells, while 116 miRNAs were expressed in the cisplatin-treated cells, 109 miRNAs in the 5-FU-treated group and only two miRNAs (hsa-miR-195* and hsa-miR-3714) in the combination-treated group. Therefore, it can be concluded that 5-FU, cisplatin and their combination treatment significantly altered the miRNA expression profile in the CNE human NPC cell line.

Discussion

miRNAs are an important class of gene regulators for a variety of human cancers. Each miRNA has the ability to regulate the expression of hundreds of target genes, including oncogenes and tumor suppressor genes (16,17). Extensive research is currently being focused on identifying miRNAs that may play an important role in cancer therapy. Thus, a study of the possible effects of chemotherapeutic drugs on the expression profile of miRNAs is of prime importance for cancer therapy and resistance.

NPC is a special type of squamous cell carcinoma of the head and neck. Clinically, poorly differentiated squamous cell carcinoma accounts for 98% of NPC cases in Southern China, where this disease is particularly prevalent (3). For NPC therapy, cisplatin and 5-FU are two of the main drugs that are widely used (9,10). The present study focused on the alteration of miRNA expression in the CNE cell line, with or without cisplatin and/or 5-FU treatment. We applied a label-free high-throughput microRNA array technology, the SHUT assay, to study the miRNA profiling between untreated and treated CNE cells.

The results from our study revealed that all the treated cells expressed a lower number of miRNAs as compared to the control cells. In total, 454 miRNAs were detected to be expressed in the control cells, over 200 in the 5-FU- or cisplatin-treated cells, while only 36 miRNAs were detected in the 5-FU and cisplatin combination-treated cells. Such a sharp decrease indicated that most of the miRNAs related to the regulation of cell growth and proliferation were suppressed by the combination treatment, which may be the main reason for the improved therapeutic effectiveness of the combination treatment. Furthermore, many of the miRNAs that were significantly detected only in the cisplatin-treated cells (109 miRNAs) or 5-FU-treated cells (116 miRNAs), as shown by our microarray analysis, have been shown to have tumor suppressive and anti-migration/anti-metastatic roles in cancer. Two miRNAs, hsa-miR-29c and hsa-miR-29a, were detected to be signigicantly expressed in the drug-treated groups, but not in the control group, and have been shown to induce apoptosis by targeting p85α and CDC42, both of which negatively regulate p53 (24). Hsa-let-7d*, hsa-miR-19a and hsa-miR-372 were found to be significantly expressed in all the groups, except for the cisplatin-treated group. Hsa-miR-2116* was significantly expressed in all the groups, except for the 5-FU-treated group. The regulation of hsa-let-7d* and hsa-miR-19a has been reported to be related to inflammation (25,26). Hsa-miR-372 has been reported to take part in tumorigenesis (27). However, the function of hsa-miR-2116* has not yet been reported. Also, several miRNAs with unknown function were detected significantly in some of the four groups. Hsa-miR-4695-3p and hsa-miR-767-3p were significantly expressed in both the cisplatin- and the combination-treated cells. Hsa-miR-3064-3p and hsa-miR-4740-3p were significantly expressed in both the

Table II. Comparison of miRNAs expressed significantly in only two of the four groups.

miRNA category	No.	miRNAs
Expressed significantly only in the CNE-normal and CNE-Cis group	30	miR-142-5p, miR-192, miR-204, miR-30d, miR-3125, miR-325, miR-380*, miR-3974, miR-411*, miR-4633-5p, miR-4639-5p, miR-4659b-5p, miR-4668-5p, miR-4727-3p, miR-4768-5p, miR-487b, miR-520e, miR-720, miR-107, miR-1184, miR-1237, miR-1538, miR-218-2*, miR-3690, miR-425, miR-449b*, miR-4745-5p, miR-4767, miR-877*
Expressed significantly only in the CNE-normal and CNE-5-FU group	137	let-7b, miR-1, miR-10a*, miR-10b, miR-1252, miR-1262, miR-1277, miR-1304, miR-137, miR-146b-5p, miR-155, miR-15b,miR-183*, miR-184, miR-190, miR-194, miR-195, miR-2052, miR-206, miR-2116, miR-218, miR-2355-3p, miR-27b*, miR-299-5p, miR-29b-1*, miR-30c-2*, miR-3133, miR-3134, miR-3146, miR-3149, miR-3152-3p, miR-3163, miR-3167, miR-3174, miR-320d, miR-340, miR-3545-3p, miR-3591-5p, miR-3606, miR-3614-3p, miR-3647-3p, miR-3658, miR-3659, miR-3662, miR-3668, miR-3672, miR-3673, miR-3682-3p, miR-371-5p, miR-378i, miR-379, miR-383, miR-3914, miR-3945, miR-410, miR-4271, miR-429, miR-4306, miR-4450, miR-4453, miR-4460, miR-4464, miR-4475, miR-4476, miR-4504, miR-4509, miR-4635, miR-4652-5p, miR-4661-3p, miR-4662a-3p, miR-4662b, miR-4678, miR-4680-3p, miR-4693-5p, miR-4703-5p, miR-4720-3p, miR-4729, miR-4735-3p, miR-4759, miR-4765, miR-4772-5p, miR-4773, miR-4774-5p, miR-4777-5p, miR-4778-5p, miR-4779, miR-4791, miR-4795-5p, miR-4796-3p, miR-4797-5p, miR-4804-5p, miR-494, miR-519a, miR-522, miR-548f, miR-556-5p, miR-558, miR-559, miR-592, miR-617, miR-627, miR-628-5p, miR-633, miR-708, miR-765, miR-877, miR-889, miR-9*, miR-936, miR-103a, miR-1229, miR-1251, miR-1273e, miR-140-3p, miR-1910, miR-1912, miR-3153, miR-3192, miR-34c-3p, miR-3679-5p, miR-3692*, miR-3911, miR-4298, miR-4428, miR-4470, miR-4474-3p, miR-4518, miR-4674, miR-4676-5p, miR-4700-5p, miR-4716-3p, miR-4723-3p, miR-4732-5p, miR-486-3p, miR-525-3p, miR-711, miR-921
Expressed significantly only in the CNE-normal and CNE-Cis + FU group	1	miR-374c
Expressed significantly only in the CNE-Cis and CNE-5-FU group	44	let-7g, let-7i, miR-1265, miR-154*, miR-222, miR-30e, miR-3145-3p, miR-320e, miR-34a*, miR-3613-3p, miR-3666, miR-376b, miR-378, miR-379*, miR-3919, miR-3976, miR-424, miR-4424, miR-4445*, miR-4487, miR-452, miR-4653-5p, miR-519e*, miR-542-3p, miR-548z, miR-586, miR-620, miR-758, miR-885-5p, miR-125a-5p, miR-1272, miR-191, miR-3135, miR-3162-5p, miR-345, miR-3605-5p, miR-3670, miR-3916, miR-3935, miR-4446-5p, miR-4537, miR-4723-5p, miR-502-3p, miR-943
Expressed significantly only in the CNE-Cis and CNE-Cis + FU group	2	miR-4695-3p, miR-767-3p
Expressed significantly only in the CNE-5-FU and CNE-Cis + FU group	2	miR-3064-3p, miR-4740-3p

Cis, cisplatin; 5-FU, 5-fluorouracil.

5-FU- and the combination-treated cells. Hsa-miR-195* and hsa-miR-3714 were significantly detected only in the combination-treated cells. The function of these miRNAs was not fully elucidated and we believe that these miRNAs may also play a primary role in cancer.

In conclusion, in the present study, we performed a comparative miRNA expression profile analysis of the two drugs, cisplatin and 5-FU, and their combination, across the CNE cell line panel. A number of miRNAs were uncovered, which may form a basis for the rational clinical use of these drugs as anticancer agents and may correlate drug response with genomic characteristics. Our data provide insights into the cytotoxic mechanisms of anticancer drugs, as well as new clues for the treatment of NPC.

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