

# Next generation deep sequencing identified a novel lncRNA n375709 associated with paclitaxel resistance in nasopharyngeal carcinoma

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**Abstract.** Paclitaxel chemoresistance restricts the therapeutic efficacy and prognosis of patients with nasopharyngeal carcinoma (NPC). Accumulating evidence suggests that aberrant expression of long non-coding RNAs (lncRNAs) contributes to cancer progression. Therefore, we aimed to identify lncRNAs associated with paclitaxel resistance in NPC. First, paclitaxel-resistant CNE-2 cells (CNE-2-Pr) were successfully established and confirmed to be 33.26±8.70 times more resistant than parental CNE-2 cells. Then, differential expression profile of lncRNAs associated with NPC paclitaxel resistance, which contained a total of 2,670 known lncRNAs and 4,820 novel lncRNAs, was constructed via next generation sequencing technology. Our qRT-PCR confirmed that 7 of the top 8 lncRNAs were expressed with the same trend as the prediction, including 4 known lncRNAs (n375709, n377806, n369241 and n335785) and 3 novel lncRNAs (Unigene6646, Unigene6644 and Unigene1654). Our group initially focused on lncRNA n375709, which was the most significantly overexpressed lncRNA of the known lncRNAs. CCK-8 assays demonstrated that further inhibition of lncRNA n375709 increased the paclitaxel sensitivity in NPC 5-8F and 6-10B cells. In conclusion, the present study provided an overview of the expression profiles of lncRNAs correlated with paclitaxel resistance. lncRNA n375709 was identified to be involved in the regulation of NPC paclitaxel resistance.

## Introduction

Nasopharyngeal carcinoma (NPC), one of the most common head and neck malignant tumors, is prevalent in Southern China and Southeast Asia (1). For patients with advanced NPC, first-line radiotherapy is far from satisfactory. Chemotherapy is offered as the most efficacious auxiliary treatment strategy (2). However, similar to the obstacle of paclitaxel application in other solid tumors, the emergence of resistant cancer cells to paclitaxel limits its clinical efficiency in NPC, which eventually brings NPC recurrence and metastasis (3). Previous studies have focused on discovering diverse molecules including protein, miRNAs and mRNAs to serve as biomarkers and therapy targets of paclitaxel resistance (4,5). However, paclitaxel resistance still remains unclear, making it urgent to clarify paclitaxel resistance from a brand-new perspective.

lncRNAs are a family of transcripts with >200 nucleotides (nt) in length with no protein-coding potential, which are involved in numerous biological processes such as their protein-coding counterparts (6). This class of lncRNAs makes up the largest portion of the mammalian non-coding transcriptomes. Most of the currently known lncRNAs show their functional role by participating in the biological progressions at the epigenetic, transcriptional and post-transcriptional levels (7,8). Moreover, dysregulated expression of lncRNAs in cancers involves in a spectrum of disease progression, serving as an independent predictor for patient outcomes and associates with therapeutic efficacy (9,10). *In vitro* and *in vivo* functional analyses also indicate that lncRNAs can take part in multiple cancer malignant behaviors, such as unlimited proliferation, metastasis, radioresistance and cancer stem cell phenotype (11,12). In respect to the correlation between lncRNA and chemoresistance, limited lncRNAs including MEG3, MALAT-1, UCA1 and HOTAIR have been reported to regulate the chemoresistance of cisplatin and doxorubicin in several solid human tumor (13-15). Taken together, current research of lncRNAs in tumors shows the need to illuminate the mechanism of chemoresistance in NPC based on lncRNAs.

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Paclitaxel, one of the taxane families, is widely used in many solid human malignancies including NPC (16). The emergence of paclitaxel resistance greatly restricts its clinical efficiency (3). Therefore, the present study investigated lncRNAs association with paclitaxel resistance in NPC. Considering the widespread application of next generation sequencing (NGS) technology and its advantage of massively discovering existing and novel molecule candidates, it is now employed in our research to comprehensively and systematically screen lncRNA candidates associated with paclitaxel resistance (17).

## Materials and methods

**Cell culture and construction of paclitaxel-resistant CNE-2 cells.** Poorly differentiated NPC cell line CNE-2, 5-8F and 6-10B were provided by the Cell Center of Central South University and were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS) and 1% antibiotics (both from Gibco-BRL, Gaithersburg, MD, USA). Paclitaxel-resistant CNE-2 cell line (termed CNE-2-Pr) was established by exposure to the cell culture medium with gradually increased concentrations of paclitaxel (Sigma-Aldrich, St. Louis, MO, USA). The initial and final concentrations of Paclitaxel were 0.2 and 2 nM. The above cells were propagated in an incubator at 37°C with saturated humidity and 5% CO<sub>2</sub>. CNE-2-Pr was cultured in the RPMI-1640 medium with 1 nM paclitaxel to keep its chemoresistance. The CNE-2-Pr cells were sub-cultured <20 passages and used for the following experiments.

**Paclitaxel cytotoxicity assays by CCK-8 methods.** Cells were suspended in essential medium, plated into a 96-well plate (5x10<sup>3</sup> cells/well) and incubated for 24 h at 37°C. Paclitaxel was separately added to each well at different concentrations and the plate was incubated for another 48 h before further analyses. Triplicate wells were used for each drug treatment. Cell Counting Kit-8 (CCK-8; Beyotime, Co., Guangzhou, China) was used according to its instructions. The absorbance of each well was then detected (18,19).

**RNA extraction.** Total RNA was extracted from paclitaxel resistant CNE-2-Pr and parental CNE-2 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The qualities and concentrations of RNA samples were monitored by gel electrophoresis and at absorbance ratios of A260/A280 using a NanoDrop ND-1000 spectrophotometer. RNAs of appropriate quality were stored at -80°C for lncRNA detection and subsequent experiments.

**Construction of an RNA library for sequencing.** Total RNA was extracted from CNE-2-Pr and CNE-2 cells. After removal of rRNA, the remaining RNA was cut randomly into short fragments. Using random hexamers, first-strand cDNA was synthesized based on these fragments; second-strand cDNA was also synthesized by mixing first-strand cDNA with buffer, dNTPs, RNase H and DNA polymerase I. This cDNA was then purified by a QIAquick PCR kit (Qiagen, Valencia, CA, USA) and subsequently degraded by uracil-N-glycosylase. RNA fragments were separated by agarose gel electrophoresis, and

fragments were then expanded with polymerase chain reaction (PCR). The PCR products were sequenced using an Illumina HiSeq™ 2000 instrument (Illumina, Inc., San Diego, CA, USA), and the original image data were converted into '.fq' files by base calling software. The relative data were submitted to NCBI under BioProject accession no. PRJNA 254709.

**Transcript identification and establishment of differential expression profiles.** Sequence data (.fq files) was filtered to remove rRNA sequences. We then used TopHat2 and Cufflinks strategies to reconstruct transcripts in samples. The transcripts were BLASTed against the NONCODE v3.0 database (<http://www.noncode.org/NONCODERv3/>) to identify already known non-coding RNAs (identity >0.9 and coverage >0.8 as the selection criteria). Transcripts without annotations in the ncRNA library were then compared to protein databases, and the mapped transcripts were considered as mRNA (identity >0.9 and coverage >0.8). The remaining transcripts, which were not aligned with the protein library, were then entered into the Coding Potential Calculator program to distinguish coding and non-coding sequences (20). To obtain the differential expression profiles, read counts and reads per kilobase per million read (RPKM) values were calculated for each gene, and a likelihood ratio test was used to assess the significance of expression differences as described in our previous study (21). lncRNAs were considered to be differentially expressed when the fold-change was >2 (log<sub>2</sub> ratio ≥1) and the false discovery rate (FDR) was 0.01 or less.

**Quantitative real-time reverse transcription PCR (qRT-PCR).** Briefly, cDNA was synthesized from total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Shiga, Japan). Primers for lncRNAs were designed and synthesized. Then, qPCR assays were performed using a Bio-Rad iQ5 Multicolor Real-Time qRT-PCR Detection system (Bio-Rad, Hercules, CA, USA). The expression levels of lncRNAs were detected as previously described (21). Human β-actin was used as a housekeeping gene for normalization. The expression levels of lncRNAs were measured in terms of the cycle threshold (CT) and were then normalized to β-actin expression using the 2<sup>-ΔΔCt</sup> method (21).

**siRNA construction and transfection.** To estimate inhibition of lncRNA n375709, the specific lncRNA n375709 siRNA, universal negative control and the transfection agent (riboFECT™ CP Buffer) were designed and purchased from RiboBio Co. Ltd. (Guangzhou, China). NPC cells (1x10<sup>5</sup>) were grown in triplicate in 6-well plates. Twenty-four hours later, 100 nM of lncRNA n375709 siRNA, the transfection agent and negative control which were used as controls were separately transfected into NPC cells according to the manufacturer's instructions. Forty-eight hours later, the initial transfection medium was changed for fresh medium and NPC cells were harvested for following experiments.

**Paclitaxel cytotoxicity assays.** Cells (2x10<sup>3</sup>) were separately cultured into 96-well plates in triplicate and were treated with varying concentrations of paclitaxel for 48 h. A CCK-8 was applied to determine the growth curves for both cell lines.

Inhibition fraction was used to compare the sensitivity of paclitaxel in each group. Each experiment was performed in triplicate. Inhibition fraction =  $1 - A_{450}$  value (paclitaxel group) /  $A_{450}$  value (parental group).

**Statistical analysis.** The results of the quantitative data in the present study are expressed as the mean  $\pm$  standard deviation. The statistical significance of the differences between two groups were analyzed using a two-sided, unpaired Student's t-tests (for equal variances) or Welch's corrected t-tests (unequal variances) performed in SPSS 18.0 software. Differences with P-values of  $<0.05$  were considered to indicate a statistically significant result.

## Results

**Generation of paclitaxel-resistant NPC cells.** To obtain the lncRNAs correlated with paclitaxel resistance, CNE-2 cells were exposed to gradually increased concentration of paclitaxel in complete medium for 10 months, and the remaining CNE-2 cells were abbreviated as CNE-2-Pr and its resistance to paclitaxel was then validated by CCK-8 assays. As shown in Fig. 1, the half maximal inhibitory concentration ( $IC_{50}$ ) of paclitaxel for CNE-2-Pr and parental CNE-2 cells were  $1098.66 \pm 31.95$  and  $32.76 \pm 1.67$  nM, respectively, which indicates that paclitaxel resistance of CNE-2-Pr cells was  $33.26 \pm 8.70$  times higher than that of its parental CNE-2 cells. Therefore, we considered the remaining CNE-2-Pr cells post-paclitaxel exposure as paclitaxel resistant cells. CNE-2-Pr cells kept the paclitaxel resistant characteristics after  $>20$  passages under the continuous exposure to 1 nM paclitaxel (data not shown), which were used for our further experiments to screen lncRNAs correlated with paclitaxel resistance.

**Establishment of lncRNA differential expression profile associated with paclitaxel resistance in NPC.** To obtain lncRNAs associated with paclitaxel resistance in NPC, cDNA libraries were initially constructed via our previously established CNE-2-Pr and parental CNE-2 cells. As depicted in Fig. 2, a total of 65,925,308 and 63,933,890 clean reads were detected in CNE-2-Pr and CNE-2 cells, respectively. After eliminating reads mapped to rRNA, TopHat2 and Cufflinks were used to reconstruct transcripts in both samples. Then, these reconstructed transcripts were BLASTed against the NONCODE v3.0 database. Transcripts (8,121) in CNE-2-Pr and 9,635 transcripts in CNE-2 cells were annotated as known lncRNAs. Additionally, following another elimination of transcripts mapped to mRNA and coding sequences, 7,018 (CNE-2-Pr) and 8,511 (CNE-2) transcripts were separately identified as novel lncRNAs. The unique mapped reads for each lncRNA were then counted, and the RPKM value for each lncRNA was calculated. Based on the criteria of an absolute fold-change  $>2.0$  and an FDR  $<0.001$ , 2,670 known lncRNAs and 4,820 novel lncRNA candidates were finally obtained, which constituted the differential lncRNA expression profiles associated with NPC paclitaxel resistance.

**Characteristics of known and novel lncRNAs.** Based on the above-mentioned lncRNA profiles, the features of lncRNAs were further analyzed. Our data revealed that the most known

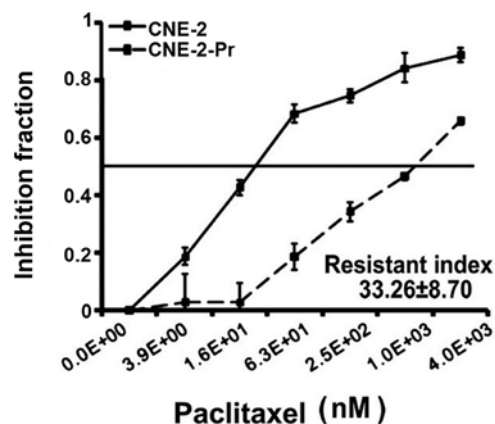


Figure 1. CCK-8 assays confirmed that CNE-2-Pr cells were significantly more resistant to paclitaxel than the CNE-2.

lncRNAs were abundant in 200 b-3 kb in length, and novel candidates mainly distributed in the region of 200 b-2 kb. Among these 2,670 known lncRNAs, 2,413 lncRNAs were expressed in both cell lines, while 255 and 2 were present only in CNE-2-Pr or CNE-2 cells, respectively. Compared with parental CNE-2 cells, 25 lncRNAs were upregulated and 2,645 lncRNAs were downregulated in paclitaxel-resistant CNE-2-Pr cells. Similarly, in the 4,820 novel lncRNA candidates, 3,518 lncRNAs were expressed in both cells, while 1,264 and 38 existed only in CNE-2-Pr or CNE-2. Of the lncRNAs, 193 were elevated and 4,627 were decreased in paclitaxel-resistant CNE-2-Pr cells.

**Validation of the lncRNA differential expression profile via qRT-PCR.** To confirm the lncRNA expression profile findings, the expression level of top 8 upregulated and downregulated in both known and novel lncRNAs were detected by qRT-PCR. Compared with the deep sequencing prediction findings, qRT-PCR verified that the same significant differential expression trends were presented in 7 of 8 lncRNAs, which included all the known lncRNAs (n375709, n377806, n369241 and n335785) (Fig. 3A) and 3 novel lncRNAs (Unigene6646, Unigene6644 and Unigene1654) (Fig. 3B). Our validation showed a high consistency with the lncRNAs predicted by the expression profile established via next generation deep sequencing.

**Inhibition of lncRNA n375709 in NPC cells increases their sensitivity to paclitaxel.** The above abundant lncRNAs provide us a solid basis to investigate paclitaxel resistance of NPC. Our group initially focused on lncRNA n375709, which was the most significantly overexpressed lncRNA in known lncRNAs. Thus, the role of lncRNA n375709 in paclitaxel resistance of NPC was further explored *in vitro*. siRNA interference was employed to inhibit the expression of lncRNA n375709 in both 5-8F and 6-10B cells. To make the results more credible, two different siRNAs targeting the different sites of lncRNA n375709 were used. qRT-PCR assays revealed that lncRNA n375709 was successfully downregulated by two siRNA in NPC 5-8F and 6-10B cell (Fig. 4A-a and -b, and B-a and -b). Further paclitaxel cytotoxic assays demonstrated that inhibition of lncRNA n375709 increased the paclitaxel sensitivity

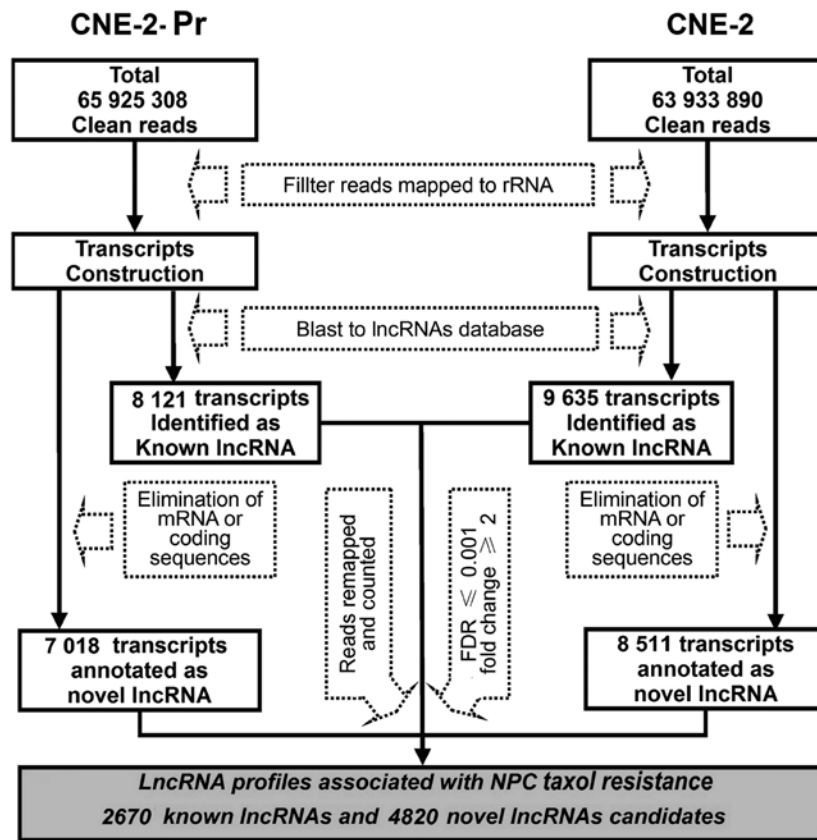


Figure 2. Discovery pipeline of lncRNAs associated with paclitaxel resistance in NPC.

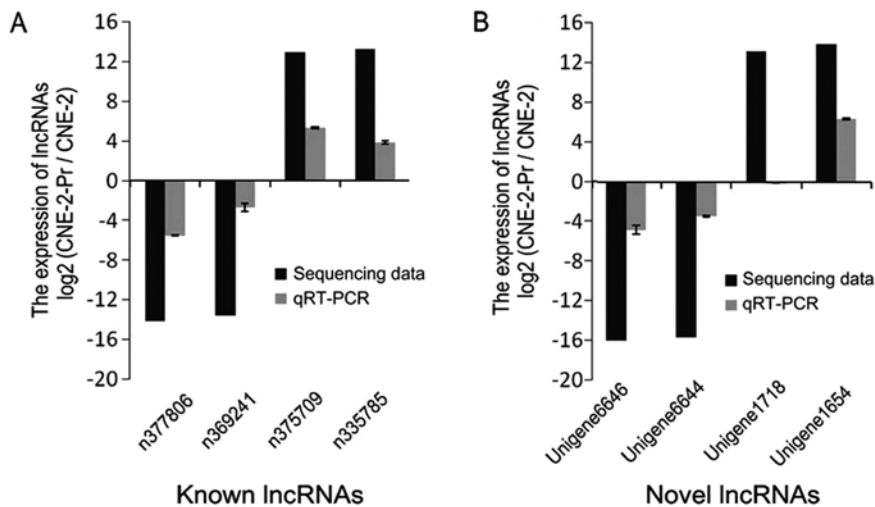


Figure 3. qRT-PCR validation of the top 8 differential expressed lncRNAs.

of NPC cells *in vitro*, which was indicated by the increased inhibition fraction of both cell lines (Fig. 4A-c and -d, and B-c and -d;  $p < 0.05$ ). Together, these data clearly showed that lncRNA n375709 could regulate the paclitaxel resistance of NPC cells.

**Discussion**

Paclitaxel resistance restricts the clinical response and prognosis of diverse human cancers including NPC (3),

therefore it is urgent to find biomarkers and therapeutic targets associated with paclitaxel resistance. In the present study, next generation sequencing technology was used to establish differential expression profile of lncRNAs associated with paclitaxel resistance in NPC, which embraced both known and novel lncRNAs. This profile provided us numerous potential lncRNAs that may function in NPC paclitaxel resistance, furthermore indicating that much more effort should be made to validate the exact function of these known and novel lncRNAs in paclitaxel resistance.

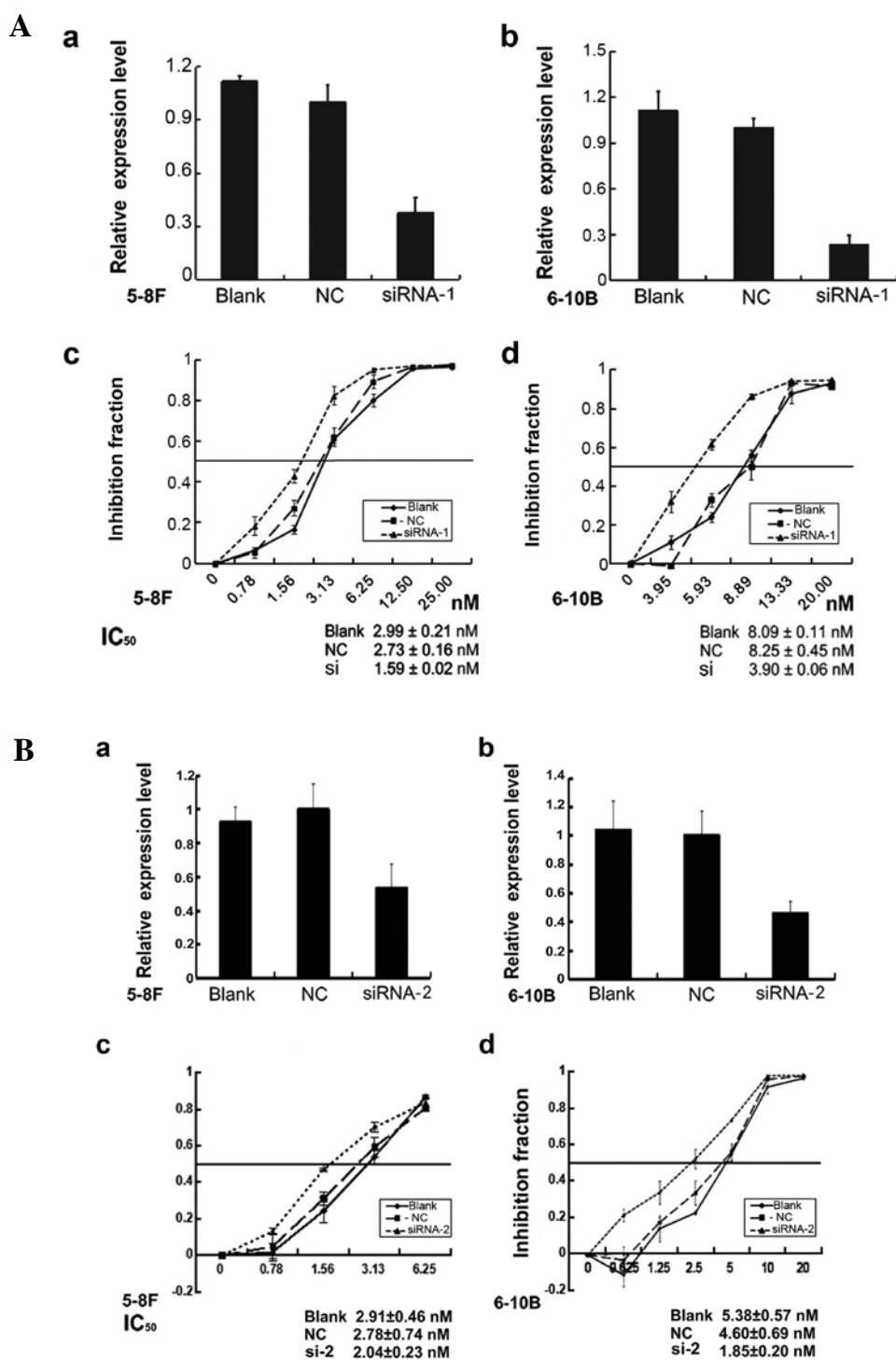


Figure 4. (A) (a-d) The downregulation of lncRNA n375709 interfered by siRNA-1 increased the paclitaxel sensitivity of NPC cells *in vitro*. (B) (a-d) The downregulation of lncRNA n375709 interfered by siRNA-2 increased the paclitaxel sensitivity of NPC cells *in vitro*.

Currently, it is undeniable that available chemoresistant tissues are the best choice for screening biomarkers and targets correlated with paclitaxel resistance, particularly under the application of NGS technology (22). However, NPC is not suitable for surgery and shows high response to radiotherapy, which is the reason why these specific NPC tissues are hard to obtain in clinical settings. Therefore, we established paclitaxel resistant NPC CNE-2 cells named CNE-2-Pr, which were then used in NGS and bioinformatics analyses. The construction

of paclitaxel resistant NPC cells was by exposure of NPC cells to gradually increasing doses of paclitaxel. Compared with methods reported by other groups, such as high-dose paclitaxel impulse, high-dose paclitaxel impulse followed with low-dose paclitaxel maintenance, our method avoided lethal cell damage and unrepeatably dosage used in high-dose paclitaxel impulse (23-25).

lncRNAs have been demonstrated to be involved in the development of different human diseases, such as diabetes and

obesity (26), cardiovascular diseases (27), neurodegenerative disorders (28) and muscular dystrophies (29). Indeed, strong links between lncRNAs and cancers are also abundant. The most studied lncRNAs, such as HOTAIR and MALAT1, are abnormally expressed in a wide range of solid tumors and function in cancer malignant behavior (10,30-32). As to NPC, lncRNA profiles associated with metastasis and recurrence except to paclitaxel resistance has been recently reported (33,34). These groups used the microarray to screen differential lncRNAs, which is different to the NGS technology we used in the present study. NGS not only allowed massive parallel analyses of genome-wide expression as microarrays, but also had the advantages of calculating the absolute abundance of the transcripts, identifying variations in lncRNA sequences and discovering novel lncRNAs. According to the CPC website and the cited literature (20), a true protein-coding transcript is more likely to have a long and high-quality open reading frame (ORF) compared with a non-coding transcript. In the present study, we considered the following 6 features, such as log-odds score, coverage of the predicted ORF, integrity of the predicted ORF, number of HITs, hit score and frame score. When the transcripts matched the above criterion, we identified the transcripts as a candidate of protein-coding transcript. Accordingly, in addition to 2,670 known lncRNAs, we also obtained 4,820 novel lncRNA candidates that were associated with paclitaxel resistance. These lncRNAs may greatly enrich the human lncRNA pool.

Aberrant expression and dysfunction of lncRNAs in cancers, along with its tissue specific expression pattern, indicates specific-lncRNAs may be used as promising targets for the development of novel anticancer therapy (30-32). So far, multiple strategies have been proposed to restore the homeostatic levels of lncRNAs, in which siRNA is one of the most frequently used approaches to inhibit the upregulation of oncogenic lncRNAs (35,36). These siRNAs are complementary to target lncRNAs and induce degradation in RISC (RNA-induced silencing) complex, and consequently decline the levels of lncRNA transcripts (37). Although numerous lncRNAs were found in the present study, we initially focused on the most increased lncRNA n375709. As expected, siRNA inhibition of lncRNA n375709 led to enhanced paclitaxel sensitivity in two NPC cell lines. These data indicate that we successfully identified lncRNA n375709 as a potential target to regulate paclitaxel resistance in this differential expression profile. As a novel biomarker, there is scarce research of lncRNA n375709 in the field of tumor biology. lncRNA n375709 is a ~0.5 kb long non-coding RNA which non-coded gene ID is NONHSAG021653. However, we have to strengthen two points: firstly, we just confirmed the phenotype of lncRNA n375709 in regulating paclitaxel resistance. Many experiments are necessary to illuminate how it functions in the process; secondly, apart from lncRNA n375709, the remaining lncRNAs are also waiting to be evaluated and functionally analyzed.

In summary, we established an expression profile of lncRNAs associated with NPC paclitaxel resistance in human NPC cells and found that lncRNA expression differed in the chemoresistant cells, suggesting that these unique non-coding transcripts may contribute to the acquisition of chemoresistance in NPC. Although additional *in vivo* studies and clinical

trials are needed to verify the actual value of the lncRNAs mentioned above, the present study provides important insights into novel potential treatment strategies or prognostic indicators for patients with NPC.

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