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*Original Article*

# Upregulation of circulating miR-21 is associated with poor prognosis of nasopharyngeal carcinoma

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**Abstract:** MicroRNAs (miRNAs) have been demonstrated to be implicated in the pathogenesis of a number of human diseases including cancer. The aim of current study was to reveal the potential clinical significance of serum miR-21 in nasopharyngeal carcinoma (NPC). CCK8 assay was used to evaluate the effects of miR-21 overexpression/downregulation on the chemoresistance of NPC cells. Real-time PCR was performed to detect the expression level of serum miR-21 in NPC patients and healthy volunteers. Then clinical significance of serum miR-21 was further investigated. Our results showed that miR-21 overexpression could enhance the chemoresistance of NPC cells to cisplatin, and *vice versa*. In addition, serum miR-21 was significantly upregulated in NPC patients and enhanced serum miR-21 level was associated with poor prognosis of NPC. Furthermore, its level was much higher in NPC patients resistant to cisplatin based chemotherapy and could discriminate the patients in the responding group from the non-responding group with high accuracy. The proportions of patients that resistant to chemotherapy were higher in the high serum miR-21 group. Finally, Kaplan-Meier survival analysis showed that enhanced serum miR-21 was a poor indicator of both overall and disease free survival among the patients who received cisplatin based chemotherapy. Taken together, serum miR-21 might be employed as a potential biomarker for predicting the clinical outcome and chemoresistance of NPC patients.

**Keywords:** Chemoresistance, serum miR-21, biomarker, prognosis

## Introduction

Nasopharyngeal carcinoma (NPC), arising from the nasopharynx epithelium, is one of the most common epithelium malignancies in the head and neck region [1]. The pathogenesis of NPC is associated with many risk factors including genetic, environmental, viral and dietary factors [2]. Although chemotherapy is currently an important therapeutic option for the treatment of NPC patients especially those with distal metastasis, the clinical outcome is modest due to the chemoresistance [3]. Therefore, it is of great clinical value to explore novel biomarkers that associated with NPC chemoresistance, which contributes to monitor therapeutic responses in real time.

MicroRNAs (miRNAs) are a class of small, highly conserved noncoding RNA molecules that act as key regulators of gene expression at the post-transcriptional level by reducing target mRNAs with partial complementarity in their 3'-untranslated regions (UTRs) [4]. Due to the fact that miRNAs involve in regulating a number of important biological processes, dysregulated miRNAs signature has been widely observed in many types of cancers including NPC [5, 6]. Downregulation of miR-21 suppressed the proliferation and migration capacity of nasopharyngeal carcinoma cells. In addition, B-cell lymphoma 2 was identified as a downstream target of miR-21 in NPC [7], suggesting reduced miR-21 expression might promote the carcinogeneiss of NPC. The expression level of miR-21 was significantly upregulated in NPC tissues compared with the adjacent normal tissues. In addition, higher miR-21 expression was correlated with advanced TNM stage, higher risk of positive lymph node metastasis as well as worse overall survival. Moreover, miR-21 was an independent risk factor for NPC [8].

**Table 1.** Association between serum miR-21 and clinicopathological parameters of NPC

| Variables | n | Serum miR-21 expression | | *P* |
|---|---|---|---|---|
| | | Low (n = 40) | High (n = 37) | |
| Age | | | | 0.9336 |
| <50 | 42 | 22 | 20 | |
| ≥50 | 35 | 18 | 17 | |
| Gender | | | | 0.6366 |
| Female | 23 | 11 | 12 | |
| Male | 54 | 29 | 25 | |
| T classification | | | | 0.0320 |
| T1-T2 | 47 | 29 | 18 | |
| T3-T4 | 30 | 11 | 19 | |
| N classification | | | | 0.0164 |
| N0-N1 | 38 | 25 | 13 | |
| N2-N3 | 39 | 15 | 24 | |
| Distant metastasis | | | | 0.9554 |
| Positive | 2 | 1 | 1 | |
| Negative | 75 | 39 | 36 | |
| Clinical stage | | | | 0.0052 |
| I-II | 29 | 21 | 8 | |
| III-IV | 48 | 19 | 29 | |

Although miR-21 has been demonstrated to play important roles in the development of NPC, the clinical value of serum miR-21 in NPC remains poorly known. Thus the main purpose of this study was to elucidate the potential clinical significance of serum miR-21 in NPC.

## Materials and methods

*Study population and sample preparation*

This study was approved by the China-Japan Union Hospital of Jilin University and written informed consent was obtained from each subject. Seventy-seven patients with NPC and thirty healthy volunteers were enrolled in this study. None of the patients received surgery, chemotherapy or radiotherapy before the collection of serum samples. The clinicopathological variables (summarized in **Table 1**) such as gender, age and TNM stage were analyzed. The diagnosis of NPC was confirmed by histological evaluation. All the blood samples were centrifuged at 3000 g for 10 min at 4°C and the supernatant were stored at -80°C until used.

*Cell culture and reagents*

The CNE2 and C666-1 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 100 U/ml penicillin-streptomycin. This cell line was maintained in 5% $CO_2$ at 37°C in a humidified chamber. Transfections of miR-21 or negative control (NC) mimics were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

*Cell viability assay*

The NPC cells were cultured with various concentrations of cisplatin (1, 2, 4, or 16 μM). After the indicated treatment and incubation period, the number of viable cells was determined with a CCK-8 kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). The signal was detected at 450 nm with a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

*Real-time PCR*

Total RNA was extracted from the 300 μL serum samples using a miRVana PARIS Kit (Ambion, Austin, TX, USA) according to the manufacturer's directions. The first strand cDNA was synthesized by TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then the cDNA was amplified by SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan) and real-time PCR was run on a 7500 Real-Time PCR system (Applied Biosystems). Synthetic miRNA-39 from Caenorhabditis elegans (cel-miRNA-39) was used as the internal control and 2-ΔCt method was employed to quantify the relative expression of miR-21 in the serum samples.

*Statistical analysis*

Statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL) or GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA). The $P<0.05$ was considered to indicate a statistically significant difference. The Mann-Whitney U test was used to evaluate the expression level of serum miR-21 between NPC patients and healthy controls as well as NPC patients with or without chemotherapy response. Chi-square test was performed to find out the potential association

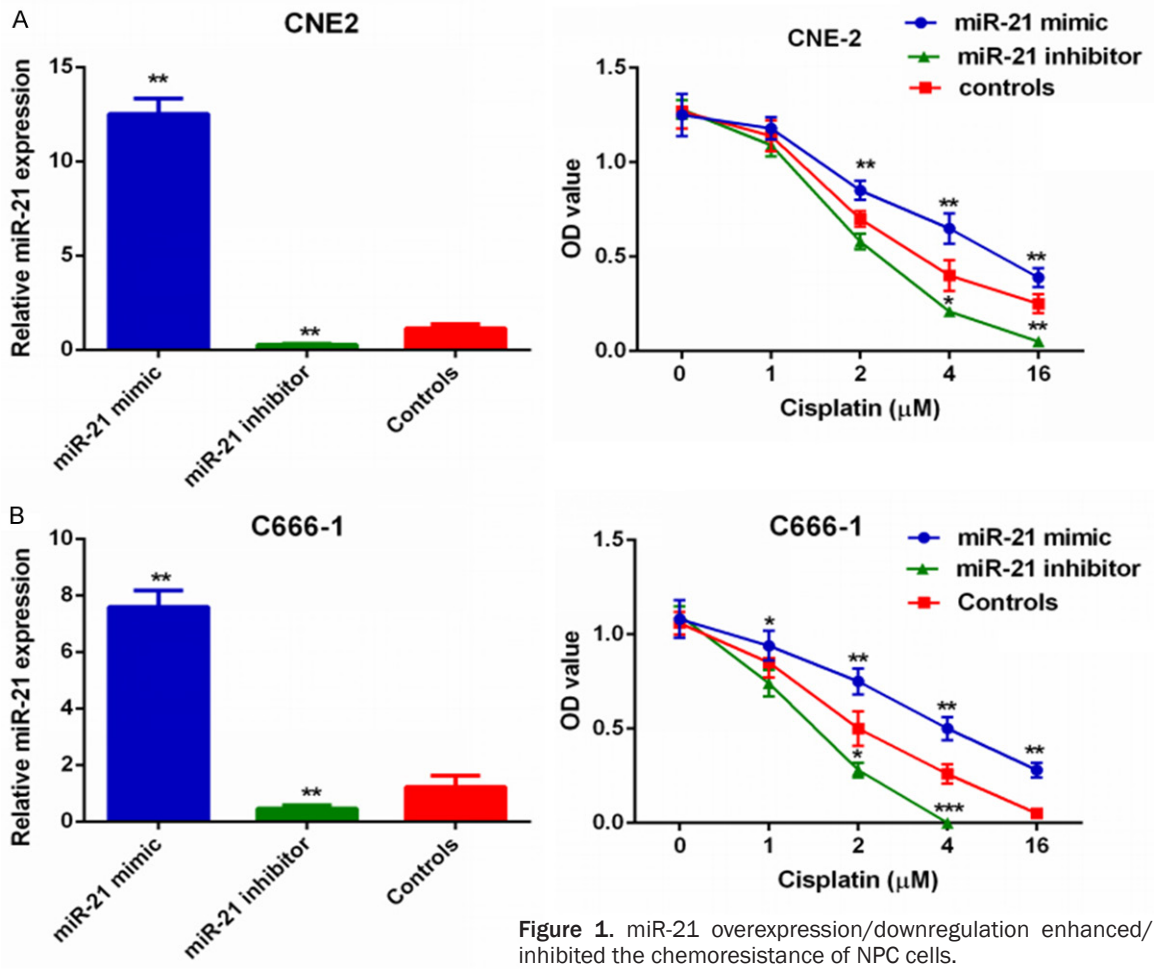


**Figure 1.** miR-21 overexpression/downregulation enhanced/inhibited the chemoresistance of NPC cells.

between serum miR-21 expression level and clinical parameters in NPC. For the survival analysis, a Kaplan-Meier survival curve was generated following a log-rank test.

## Results

*miR-21 overexpression/downregulation enhanced/inhibited the chemoresistance of NPC cells*

The NPC cells (CNE2 and C666-1 cells) successfully transfected with miR-21 mimic, miR-21 inhibitor or control were treated with increasing concentrations of cisplatin, and cell viability was measured using the CCK-8 assay. The results showed that the OD values was lowest in the cancer cells that treated with miR-21 inhibitor, while highest in the miR-21 mimic treated cells, indicating that miR-21 upregulation/downregulation promoted/suppressed the chemoresistance of NPC cells ($P<0.05$, $P<0.01$) (**Figure 1**).

*Expression level of serum miR-21 and its diagnostic value in NPC*

The real-time PCR results showed that the expression level of serum miR-21 was significantly increased in NPC patients compared to the healthy controls ($P<0.01$) (**Figure 2A**). In addition, serum miR-21 level could discriminate the NPC patients from healthy controls with an AUC value of 0.845 (sensitivity = 0.74, specificity = 0.82) (**Figure 2B**).

*Clinical significance of serum miR-21 in NPC*

The Chi-square analysis showed that serum miR-21 expression level was significantly associated with various clinical features including T classification ($P = 0.0320$), N classification ($P = 0.0164$) and clinical stage ($P = 0.0052$). However, it was not correlated with age, gender and distant metastasis (**Table 1**). In addition, the median value of serum miR-21 was used to

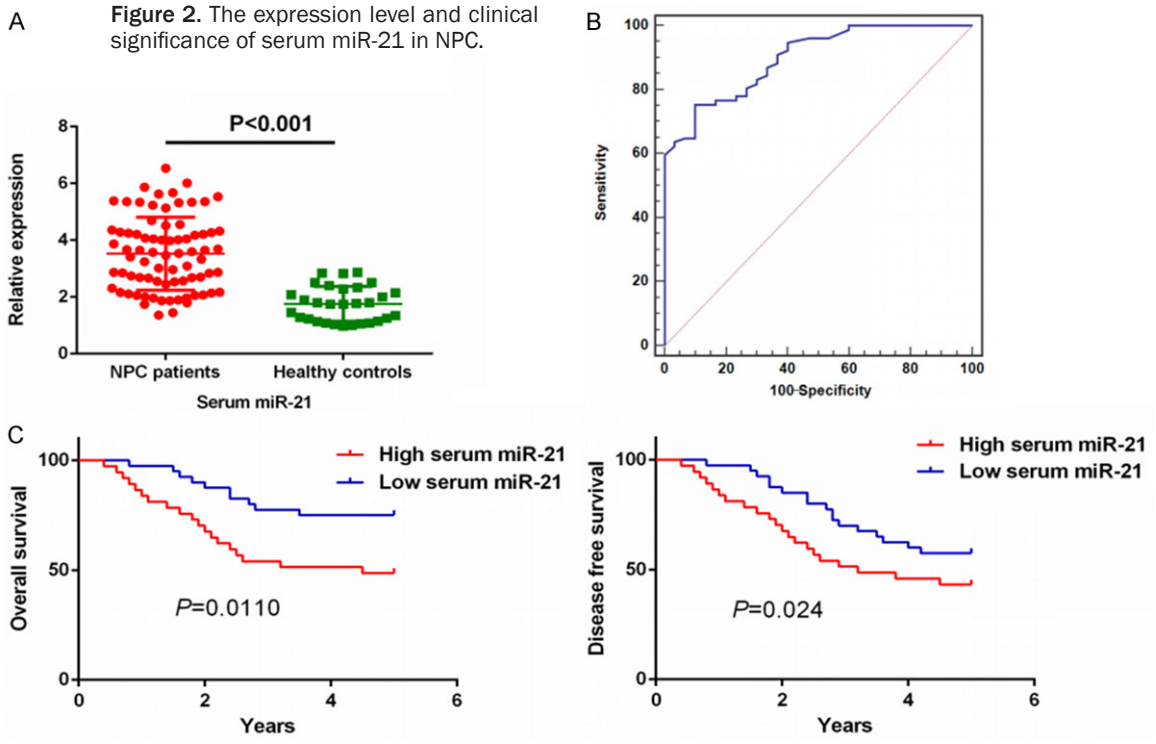


**Figure 2.** The expression level and clinical significance of serum miR-21 in NPC.

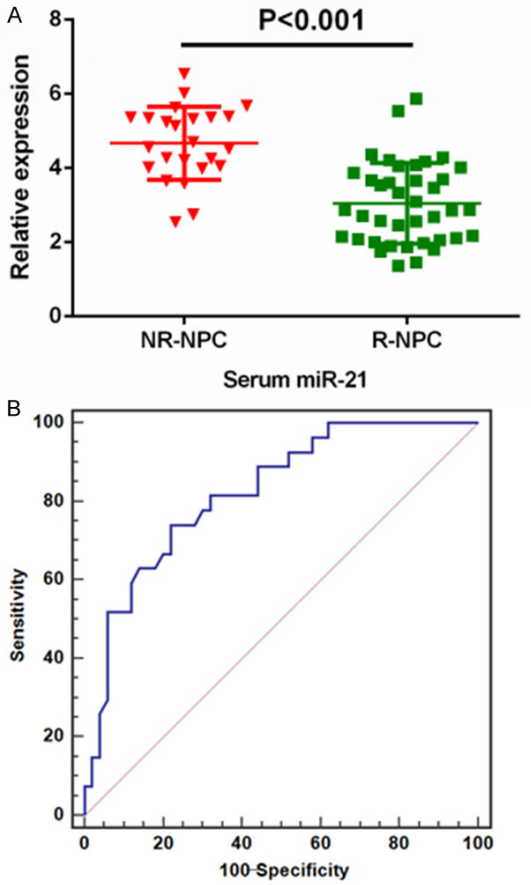


**Figure 3.** Serum miR-21 was upregulated in NPC patients resistant to chemotherapy NR = non-responsive; R = responsive.

divide the NPC patients into high serum miR-21 group and low serum miR-21 group. The survival analysis showed that the NPC patients in the high serum miR-21 group had significantly poorer 5 year overall ($P$ = 0.0110) and disease free survival ($P$ = 0.0240) than those in the low serum miR-21 group (**Figure 2C**).

*Association between serum miR-21 level and chemoresistance in NPC*

The NPC patients who were resistant to cisplatin based chemotherapy had a higher serum miR-21 expression than those who were response to chemotherapy ($P$<0.01) (**Figure 3A**). In addition, serum miR-21 level was able to distinguish the NPC patient resistant to cisplatin based chemotherapy from those patients response to chemotherapy with high accuracy (AUC = 0.812, sensitivity = 0.71, specificity = 0.75) (**Figure 3B**). These data not only demonstrated serum miR-21 was significantly associated with chemoresistance in NPC, but also further corroborated our above findings that miR-21 was positively correlated with chemoresistance in NPC cells.

We further compared the percentage of patients with chemoresistance in the high and lower serum miR-21 group. The proportion of patients not responding to chemotherapy was

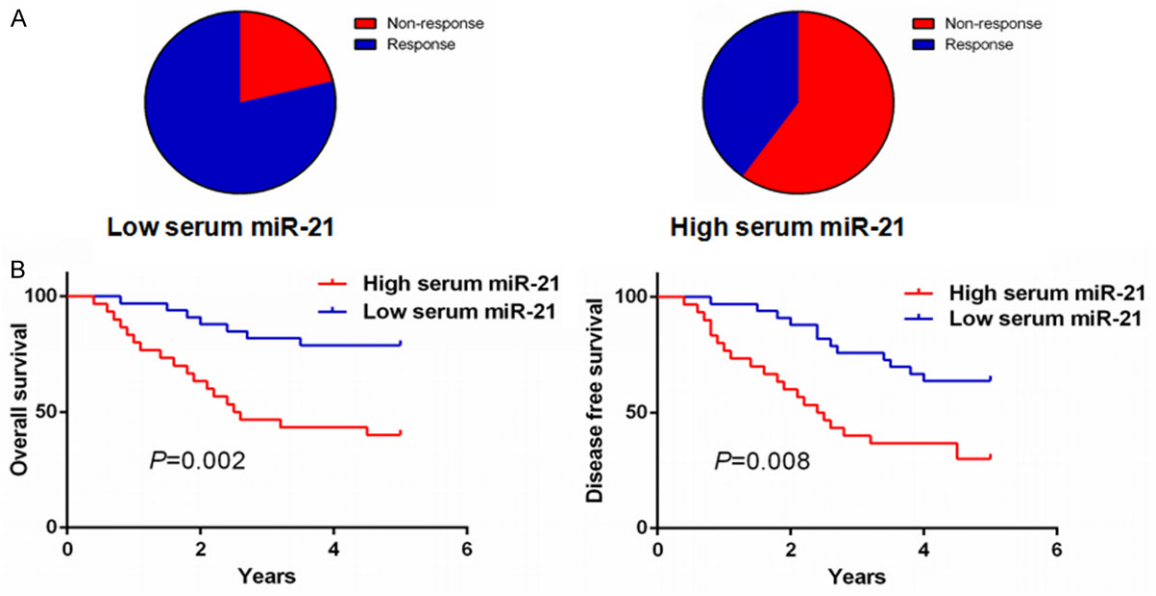


**Figure 4.** Association between serum miR-21 level and chemoresistance in NPC.

significantly higher in the high serum miR-21 group (18/30) than in the low group (7/33) (P<0.01) (**Figure 4A**). Patients with high miR-21 expression suffered remarkably shorter 5 year overall (*P* = 0.002)/disease free survival (*P* = 0.008) among NPC patients receiving cisplatin based chemotherapy (**Figure 4B**).

## Discussion

NPC is still a significant public health issue in China and chemoresistance is a major obstacle to the effective treatment of NPC patients especially those in the advanced stage [9]. miRNAs have been demonstrated to play critical roles in the initiation and development of NPC [10-12]. In addition, this class of biological molecules is highly stable in the serum samples [13, 14]. Therefore, detecting changes in miRNA expression in the serum sample might provide useful guidance for the treatment of NPC.

Our results demonstrated that ectopic expression of miR-21 could enhance the chemoresistance of NPC cells, and opposite results were observed when miR-21 was suppressed. In addition, the expression level of serum miR-21 was significantly upregulated in NPC patients and significantly associated with T classification, N classification, clinical stage and survival. Also its level was much higher in NPC patients resistant to chemotherapy response and could discriminate the patients in the responding group from the non-responding group with high accuracy. The proportions of patients that resistant to chemotherapy were higher in the high serum miR-21 group. Finally, enhanced serum miR-21 was a poor indicator of both overall and disease free survival among the patients who received cisplatin based chemotherapy. Therefore, our data suggested that serum miR-21 level might be of great value for monitoring prognosis and chemotherapeutic responses in NPC. Further studies are needed to perform to reveal the molecular mechanisms accounting for the chemoresistance promotion capacity of miR-21 in NPC.

Consistent with our findings, the expression level of serum miR-21 was remarkably increased in patients with NPC. In addition, its expression level was correlated with T and N staging [15]. Yang et al showed that the Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) enhanced the chemoresistant capacity of NPC cells by increasing the miR-21 expression level. Downregulation of miR-21 suppressed the resistance of the NPC cells to cisplatin treatment. In addition, programmed cell death 4 (PDCD4) and Fas ligand were identified as downstream targets of miR-21 associated with chemoresistance, indicating miR-21 plays a central role in maintaining the chemoresistant capacity of NPC cells [16]. The cor-

relation between miR-21 and chemoresistance has been also reported in many types of cancers. miR-21 was overexpressed in the resistant ovarian cancer cell line. miR-21 inhibition enhanced the chemosensitivity of cancer cells through upregulating PDCD4 and downregulating c-IAP2 [17]. Similarly, miR-21 overexpression protected the pancreatic cancer cells from gemcitabine-induced apoptosis, and opposite findings were observed when miR-21 was inhibited. In addition, higher serum miR-21 was significantly associated with poorer clinical outcome of pancreatic cancer [18]. The expression level of pretreatment plasma miR-21 was significantly higher in esophageal squamous cell carcinoma (ESCC) patients with a low histopathological response than in those with a high histopathological response. In addition, miR-21 was also an independent risk factor associated with chemoresistance in ESCC. Moreover, overexpression of miR-21 significantly suppressed the inhibitory effects of 5-FU or cisplatin on ESCC cells [19].

In conclusion, upregulation of miR-21 is correlated with clinical outcome and chemoresistance in NPC. Therefore, serum miR-21 level could be a useful biomarker for predicting the prognosis in patients with NPC.

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## Disclosure of conflict of interest

None.

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