

# Diagnostic and prognostic value of plasma microRNA deregulation in nasopharyngeal carcinoma

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**Keywords:** nasopharyngeal carcinoma, NPC, miRNA, plasma, biomarker, diagnosis, prognosis

**Abbreviations:** CLL, chronic lymphatic leukemia; CT, computed tomography; Ct, cycle threshold; EBV, Epstein–Barr virus; ECT, emission computed tomography; HIV, human immunodeficiency virus; IMRT, intensity-modulated radiation therapy; miRNA, microRNA; MRI, magnetic resonance imaging; NCCN, National Comprehensive Cancer Network; NIH, National Institutes of Health; NPC, nasopharyngeal carcinoma; NPI, nasopharyngitis; PCR, polymerase chain reaction; RT-qPCR, quantitative real-time PCR

Nasopharyngeal carcinoma (NPC) is uncommon worldwide but often highly invasive in late stages. Due to its special location and lack of specific symptoms, NPC is hardly detected in regular medical examination at the beginning. Development of sensitive and specific biomarkers should help to save lives against this type of disease. In the present report, we investigated the value of plasma miRNAs for diagnosis and prognosis of NPC. Using candidate approach, we selected 21 miRNAs from literature to compare their expression levels in the plasma of NPC patients and controls. As a result, 5 miRNAs showed diagnostic potentials ( $P < 0.01$ ). Among them, miR-16, -21, -24, and -155 had increased levels in NPC patients, whereas the level of miR-378 was decreased. There was a negative correlation between plasma miRNA expression and cancer progression, where miR-21 was statistically significant in T and N staging and miR-16 and 24 were significant in N staging only. Combination of miR-16, -21, -24, -155, and -378 gives 87.7% of sensitivity and 82.0% of specificity for NPC diagnosis. Without miR-16, combination of the rest 4 miRNAs gives the same sensitivity but a slightly reduced specificity. After treatment, all 5 miRNAs were somewhat back to normal levels in patients without cancer recurrence but the prognostic value was not statistically significant. In conclusion, plasma miRNA expression is a useful biomarker for NPC diagnosis but not for its prognosis. More importantly, it is simple, effective, and non-invasive. Combination of several plasma miRNAs can increase both NPC diagnostic sensitivity and specificity.

## Introduction

Cancer can grow from any tissue of human body. Nasopharyngeal carcinoma (NPC) is not a common type of cancer that we often see in clinic worldwide. Instead, it is characterized by its confined geographical prevalence. In some area like the southern part of mainland China, as well as Hong Kong, Taiwan, and some southeast Asian countries, NPC is on the top list of deadly cancerous diseases.<sup>1</sup> Although many studies have shown that NPC has very close association with Epstein–Barr virus (EBV) infection, the cause of this type of cancer is still not very well understood.<sup>2,3</sup> The clinical symptoms of NPC are usually nonspecific, but its location is somewhat special and examination of nasopharynx area requires expertise, which render early

detection of NPC very difficult. To date, it remains a challenge to find efficient biomarkers for early detection/diagnosis and prognosis of this type of malignant disease.

MicroRNAs (miRNAs) are a class of non-translated small nuclear RNAs (snRNAs), which play important roles in cell growth, differentiation and specific functions.<sup>4–6</sup> Currently, close to 2000 mature miRNAs derived from over 1500 gene loci have been identified in human cells, and maybe more await discovery.<sup>7</sup> Expression of miRNAs can be tissue-specific, and cancer cells often contain dysregulated levels of miRNAs.<sup>8,9</sup> Studies have shown that miRNA profiling can be useful biomarkers for diagnosis, prognosis, and predicting a patient's response to cancer treatment.<sup>10</sup> Meanwhile, it has been known for a long time that the circulating bloodstream carries not only cells and chemicals vital

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for life, but also small amount of genetic materials, i.e., nucleic acids such as various types of DNAs and RNAs.<sup>11</sup> The possible source of genetic materials found in blood varies from naturally dead cells, physiological secretion, and viral infections. Under normal conditions in a healthy human body, miRNAs are also present in circulating peripheral blood, and predicted to regulate differentiation of primary blood cells, maintain biological homeostasis, and control metabolic pathways.<sup>12,13</sup> In cancer patients, elevated levels of miRNA in their serum have been found.<sup>14</sup> Notably, much of these miRNAs are protected in microvesicles or in exosomes, of which the latter are a type of small membrane vesicles of endocytic origin that is often released from cancer cells into peripheral blood circulation,<sup>15</sup> resulting in remarkable change in the miRNA profile in blood.<sup>16,17</sup> These extra miRNAs in a cancer patient's plasma may function in signaling events that affect the development of cancer cell's tolerance, tumorigenesis, and organ/tissue-specific metastasis.<sup>18-21</sup> Therefore, there are several reasons for plasma miRNA to become very promising cancer biomarkers: (1) miRNA expression is often dysregulated in cancer cells and seems to be tissue-specific; (2) miRNAs appear to be very stable in serum and/or plasma; (3) blood samples are easy to obtain and its method is non-invasive compared with tissue biopsy; (4) change of miRNA levels in the plasma are supposed to be earlier than any of their downstream effect on cancer growth. In fact, in the past few years studies on plasma miRNA profiling have been reported on breast, liver, lung, prostate, colorectal, pancreatic, gastric, head, and neck cancers.<sup>16,22,23</sup> Although not all studies had reached satisfactory results, they opened up an exciting prospective for cancer treatment.

MicroRNA plays an important role in the development of nasopharyngeal carcinoma (NPC). In recent years, miRNA expression in NPC has been studied in small or large scales. Most of them were focused on NPC-associated EBV viral miRNAs,<sup>24</sup> or cellular miRNAs isolated from cancer biopsy specimen.<sup>25,26</sup> There were also several attempts to evaluate miRNA as diagnostic and prognostic biomarkers for NPC.<sup>27-29</sup> However, studies on plasma miRNA in NPC patients have not been reported yet. In the present report, we examined expression of 21 miRNAs in the plasma of NPC patients with proper control. We performed statistical analysis for association studies on miRNA expression and NPC clinical staging, and also compared the levels of miRNA before and after treatment. Our findings indicate that plasma miRNA profiling is a simple, reliable and non-invasive biomarker for early detection of NPC and therefore will be helpful to treat this type of cancer in human.

## Results

### Patient characteristics

Among 217 NPC patients, there were 149 males and 68 females with age ranging from 17 to 77 y old on average of  $45.93 \pm 11.78$ , whereas in the NPI control group there were 50 males and 23 females with age ranging from 18 to 68 y old on average of  $40.08 \pm 10.79$ . The male to female ratio was about 2.19:1 among NPC patients, which was similar to that of control group. The distribution of NPC patients and NPI control

individuals among age groups and between sexes was shown in **Figure 1**. Most incidences of NPC were first diagnosed on patients between 35 to 55 y old.

NPC patients were classified using two different systems: (1) the TNM cancer staging system, which was created by the American Joint Committee on Cancer (AJCC); (2) the Chinese NPC 2008 staging system, which was created and recommended by the Chinese Committee for Clinical Staging of Nasopharyngeal Carcinoma. In the latter, the T, N, and M categories were combined and further divided into 4 groups designated as I–IV. Compared with its previous version, the newly established Chinese NPC 2008 staging system was largely based on MRI analysis, and proved to be more accurate and practical.<sup>30,31</sup> In the present study, the number of patients in stages I, II, III and IV was 10, 52, 69, and 86, respectively.

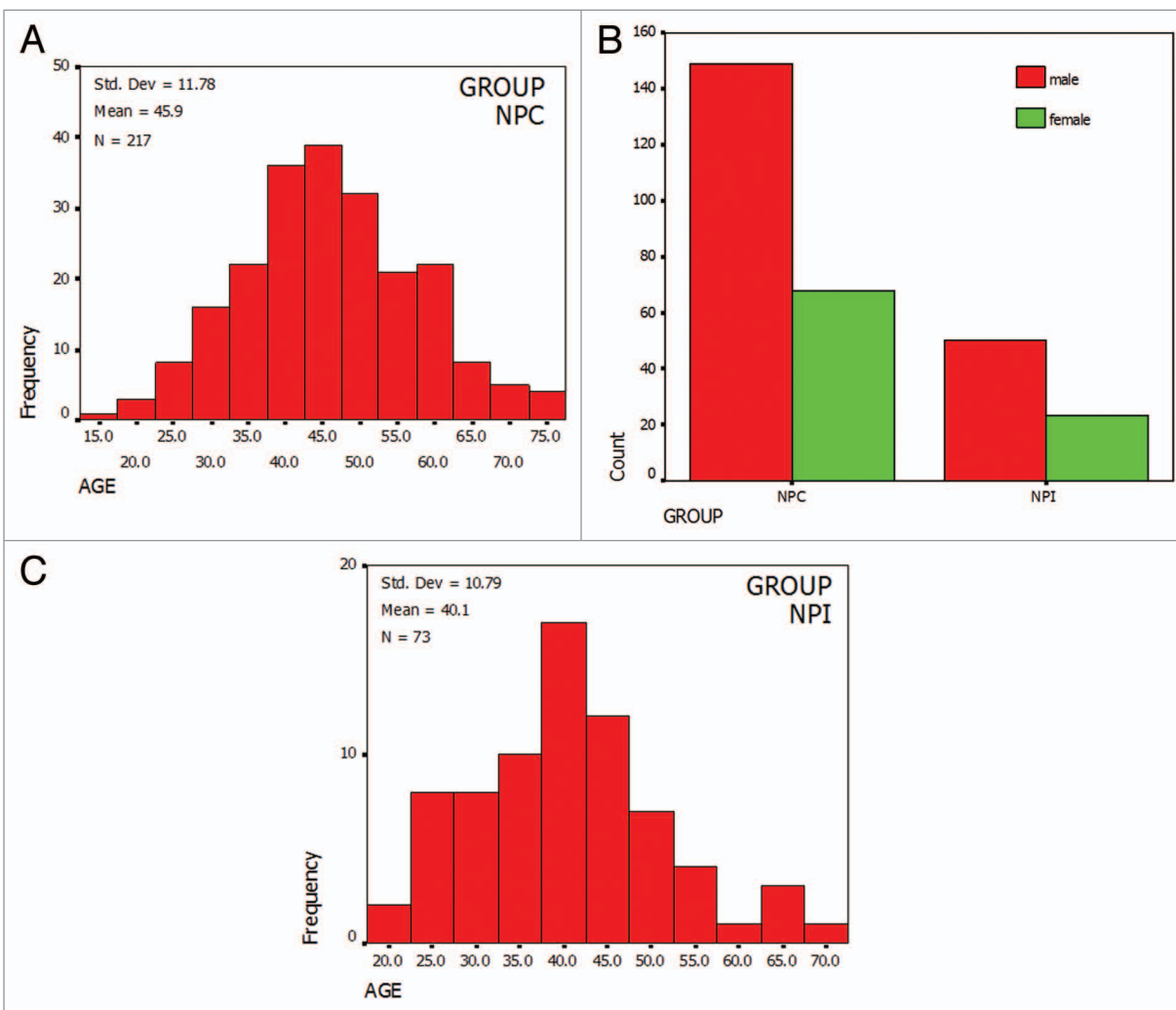
### Pilot study

A pilot study was performed to validate 21 pre-selected miRNA candidates (**Table 1**) in a small group (10%) of patients, which consisted of 22 NPC patients and 7 controls. Blood samples were collected and processed as described in Materials and Methods. RNA was extracted and reverse transcribed into the first strand of cDNA. Then, RT-qPCR was performed to measure the expression levels of these miRNAs in patient's plasma. Expression levels were compared in relative to internal controls as described in Materials and Methods, and analyzed by the two-independent parametric Student *t* test. As shown in **Table 2**, 7 miRNAs had increased plasma expression levels in NPC patients compared with control. Among them, miR-16, miR-21, miR-24, and miR-155 were changed by 6- to 28-fold. The other 14 miRNAs had lower plasma expression levels in NPC patients compared with control. Most of the changes were subtle except miR-378, which was less than 10% of the control ( $P < 0.001$ ). Therefore, miR-16, 21, 24, 155, and 378 were subjected to further analysis.

To monitor PCR conditions, graphical analysis on amplification curves and melting peaks of miR-16, 21, 24, 155, and 378 and miRNA controls are shown in **Figure 2**. All PCR amplification of miRNAs including internal control U6 and positive control Cel-miR-39, but not the negative control (water only), had a single melting peak, indicating that the amplification was specific. Moreover, amplification curves of miRNA covered initial phase, amplification phase, and stationary phase, which was essential for quantification analysis.

### Expression of miR-16, 21, 24, 155, and 378 in large scale analysis

Given the PCR condition in pilot experiment, we examined the expression of miR-16, 21, 24, 155, and 378 on the plasma specimen of 217 NPC patients and 73 NPI controls followed by covariance analysis. As shown in **Table 3**, expression of miR-16, 21, 24, and 155 in NPC patients had remarkable increase compared with control, which was ranging from about 5- to 10-fold. The expression of miR-378 in NPC patients remarkably decreased at similar magnitude, which was less than 25% of the control. In all cases, the difference between NPC and control groups was statistically significant ( $P < 0.01$ ). To analyze the expression of miRNAs in relation to age, we used the Pearson test and found that miR-378 but not 16, 21, 24, and 155, had



**Figure 1.** Distribution of NPC patients and control. (A) Among age. (B) Between sexes. NPC, nasopharyngeal carcinoma; NPI, nasopharyngitis.

strong association with age ( $P = 0.007$ ). In both groups, miR-378 expression decreased in elderly people. Meanwhile, we performed a two-independent parametric Student  $t$  test for sex association and found that none of these 5 miRNAs had any significant difference between males and females (data not shown).

#### Association of plasma miRNA expression with cancer progression

To examine the expression of plasma miRNAs in relation to cancer progression, we used Spearman test for non-parametric correlation analysis between each miRNA expression and cancer progression stages. As shown in Table 4, we found that the expression of all 5 miRNAs was somewhat negatively correlated with the progression of NPC disease in T stages, where in the case of miR-21 the correlation was statistically significant ( $P < 0.05$ ). We observed a similar situation in N staging categories, where miR-16, 21, and 24 gave strong but negative correlation to the progression of NPC. No significant correlation was found in M stages. Based on the Chinese NPC 2008 staging system, it also appears that the expression of all these 5 miRNAs was negatively correlated with cancer progression. Among them,

miR-21 had the highest level of correlation ( $P = 0.056$ ), which indicated that it might be associated with the early stage of NPC.

#### Receiver operating characteristics (ROC)

To examine the diagnostic value of these 5 miRNAs, we performed statistical analysis on their receiver operating characteristics (ROC).<sup>32</sup> The ROC curves were calculated and shown in Figure 3, while area under ROC curve (AUR) was given in Table 5. As what we can see, miR-16, 21, 24, 155, and 378 demonstrated fairly good (>60%) specificities and sensitivities for NPC diagnosis. In particular, miR-21 and 155 yielded 79.2% and 76.3% of AUR, respectively (Fig. 3A). A combination of 5 miRNAs yielded 90.8% of AUR, in which it provided 87.7% of sensitivity and 82.0% of specificity for NPC diagnosis. In the absence of miR-16, combination of the rest 4 miRNAs had no change in diagnostic sensitivity, while the diagnostic specificity only dropped slightly from 82.0% to 80.2% (Fig. 3B).

#### Treatment and follow-ups

All patients were given treatment based on the most recent NPC treatment guidelines. A total of 40 NPC patients had complete records of follow-up check, where 31 patients were males

**Table 1.** Primer design (<http://www.mirbase.org>)

Name	RNA sequence (5'–3')	Primer (5'–3')
Cel-miR-39	UCACCGGGUG UAAAUACAGCU UG	TCACCGGGTG TAAATCAGCT TG
Let-7a	UGAGGUAGUA GGUUGUAUAG UU	TGAGGTAGTA GGTGTATAG TT
miR-009	UCUUUGGUUA UCUAGCUGUA UGA	TCTTTGGTTA TCTAGCTGTA TGA
miR-010b	UACCCUGUAG AACCGAAUUU GUG	TACCTGTAG AACCGAATTT GTG
miR-015a	UAGCAGCACA UAAUGGUUUG UG	TAGCAGCACA TAATGGTTTG TG
miR-016	UAGCAGCACG UAAUAUUGG CG	TAGCAGCACG TAAATATTGG CG
miR-021	UAGCUUAUCA GACUGAUGUU GA	TAGCTTATCA GACTGATGTT GA
miR-024	UGGCUCAGUU CAGCAGGAAC AG	TGGCTCAGTT CAGCAGGAAC AG
miR-026a	UUCAAGUAAU CCAGGAUAGG CU	TTCAAGTAAT CCAGGATAGG CT
miR-029c	UAGCACCAUU UGAAUCGGU UA	TAGCACCATT TGAATCGGT TA
miR-031	AGGCAAGAUG CUGGCAUAGC U	AGGCAAGATG CTGGCATAGC T
miR-034a	UGGCAGUGUC UUAGCUGGUU GU	TGGCAGTGTC TTAGCTGGTT GT
miR-092a	UAUUGCACUU GUCCCGGCCU GU	TATTGCACTT GTCCCGCCT GT
miR-130a	CAGUGCAAUG UAAAAAGGGC AU	CAGTGCAATG TAAAAGGGC AT
miR-141	UAACACUGUC UGGUAAAAGAU GG	TAACACTGTC TGGTAAAGAT GG
miR-143	UGAGAUGAAG CACUGUAGCU C	TGAGATGAAG CACTGTAGCT C
miR-145	GUCCAGUUUU CCCAGGAAUC CCU	GTCCAGTTTT CCCAGGAATC CCT
miR-155	UUAAUGCUAA UCGUGAUAGG GGU	TTAATGCTAA TCGTGATAGG GGT
miR-184	UGGACGGAGA ACUGAUAGG GU	TGGACGGAGA ACTGATAAGG GT
miR-211	UUCCCUUUGU CAUCCUUCGC CU	TTCCTTTGT CATCCTTCGC CT
miR-221	AGCUACAUUG UCUGCUGGGU UUC	AGCTACATTG TCTGTGGGT TTC
miR-378	ACUGGACUUG GAGUCAGAAG G	ACTGGACTTG GAGTCAGAAG G
U6	CGCAAGGAUG ACACGCAAU UCGU	CGCAAGGATG ACACGCAAAT TCCT

and 10 were females. Among them, 12 patients were followed up for more than 24 mo, 9 were between 12 to 24 mo, 7 were between 3 to 12 mo, and another 7 patients were less than 3 mo. Five patients had cancer relapse and displayed metastasis. To predict the patient's survival rate based on miRNA information, we performed regression analysis using Cox model. As a result, none of these 5 miRNAs had any association with any side effects that had been tested in the present study, and all of them failed to predict the outcome of treatment, which was given by the patient's survival rate (data not shown). Therefore, the prognostic values of these plasma miRNAs alone or in combination are, if any, very limited. However, all 5 miRNA expressions were altered after therapeutic treatment (Table 6). The Student *t* test for paired samples indicated that expression levels of miR-16, 21, 24, and 155 in plasma were significantly reduced after treatment in cancer survivors without cancer relapse ( $P < 0.05$ ), but they were increased in patients with cancer relapse and metastasis. On the contrary, miR-378 level in plasma increased to a certain extent in survivors without relapse but further decreased in patients with cancer relapse and metastasis (data not shown). Therefore, the changing pattern of plasma miRNA somewhat reflected the disease development tendency. For all miRNAs in test, their expression levels seemed to go back to normal after treatment in patients without cancer relapse, but deregulate when the disease became

worse. Analysis by the two-independent parametric Student *t* test indicated that in those 5 patients with cancer relapse, expression levels of miR-16, 21, 24, and 155 were somewhat correlated with cancer clinical manifestations but not statistically significant probably due to the small size of samples (data not shown).

## Discussion

To date, close to 2000 miRNAs have been identified that affect cell growth at certain levels in human.<sup>7</sup> It is difficult to examine every miRNA for cancer association at one time. First, clinical specimens are limited so that we do not have enough RNA sample for high-throughput screening. Second, making microchips for a whole set of miRNAs for real-time PCR analysis is costly. In this regard, we used candidate approach to select miRNAs for detail analysis. These miRNA candidates were chosen for several reasons: First, they have been previously shown differential expression in nasopharyngeal carcinoma by others. We want to validate these results in the present study. Second, we selected genes that have been well studied in our lab. Third, these miRNAs are expressed in plasma, and some are known to associate with angiogenesis for cancer growth. In the present study, we found that miR-16, 21, 24, and 155 had elevated levels in the plasma specimen of NPC patients, whereas the level of miR-378

**Table 2.** Pilot study on plasma miRNA expression

Name	$\Delta Ct$		$2^{-\Delta\Delta Ct}$	t	P
	NPC (n = 22)	NPI (n = 7)			
miR-16	-11.102 ± 3.828	-6.251 ± 1.489	28.860	4.893	<0.001**
miR-155	-4.061 ± 5.777	-0.036 ± 1.986	16.287	2.791	0.010*
miR-24	-5.807 ± 3.416	-2.589 ± 1.808	9.305	2.369	0.025*
miR-21	-8.120 ± 3.555	-5.387 ± 3.803	6.648	1.744	0.093
miR-143	6.810 ± 6.789	8.484 ± 2.613	3.191	0.631	0.533
miR-141	-4.425 ± 2.489	-4.236 ± 1.291	1.140	0.191	0.850
miR-10b	-4.474 ± 2.463	-4.386 ± 1.838	1.063	0.087	0.931
miR-15a	0.207 ± 3.700	0.060 ± 2.162	0.903	-0.099	0.922
miR-26a	0.616 ± 3.802	0.380 ± 2.903	0.849	-0.150	0.882
miR-130a	-4.345 ± 3.195	-4.609 ± 2.001	0.833	-0.204	0.840
let-7a	4.708 ± 3.132	4.350 ± 3.162	0.780	-0.263	0.795
miR-145	-4.561 ± 2.522	-4.940 ± 1.765	0.769	-0.364	0.718
miR-221	-2.914 ± 2.294	-3.333 ± 2.653	0.748	-0.406	0.688
miR-92a	9.005 ± 3.637	8.297 ± 1.834	0.612	-0.491	0.627
miR-211	-2.734 ± 2.426	-3.733 ± 3.215	0.500	-0.878	0.388
miR-34a	-1.731 ± 2.686	-2.790 ± 3.042	0.480	-0.881	0.386
miR-31	-4.014 ± 3.060	-5.137 ± 1.841	0.459	-0.913	0.369
miR-9	-4.143 ± 3.234	-5.316 ± 2.331	0.443	-0.884	0.385
miR-184	-1.216 ± 2.845	-2.939 ± 2.780	0.303	-1.402	0.172
miR-29c	-4.024 ± 3.750	-5.967 ± 2.360	0.260	-1.622	0.124
miR-378	-1.922 ± 3.173	-5.970 ± 3.656	0.091	-2.839	0.009**

\* $P < 0.05$ ; \*\* $P < 0.01$ .

was significantly reduced (Tables 2 and 3). Surprisingly, the expression of these miRNAs in plasma did not persistent when cancer progressed into late stages and instead it was negatively correlated with most of the cancer staging categories (Table 4). This finding suggested that these 5 miRNAs in test may play a role in the early stage of cancer development prior to metastasis. Accordingly, it would be also interesting to examine the protein levels of some of these miRNAs' downstream targets in the future for their associations with cancer development.

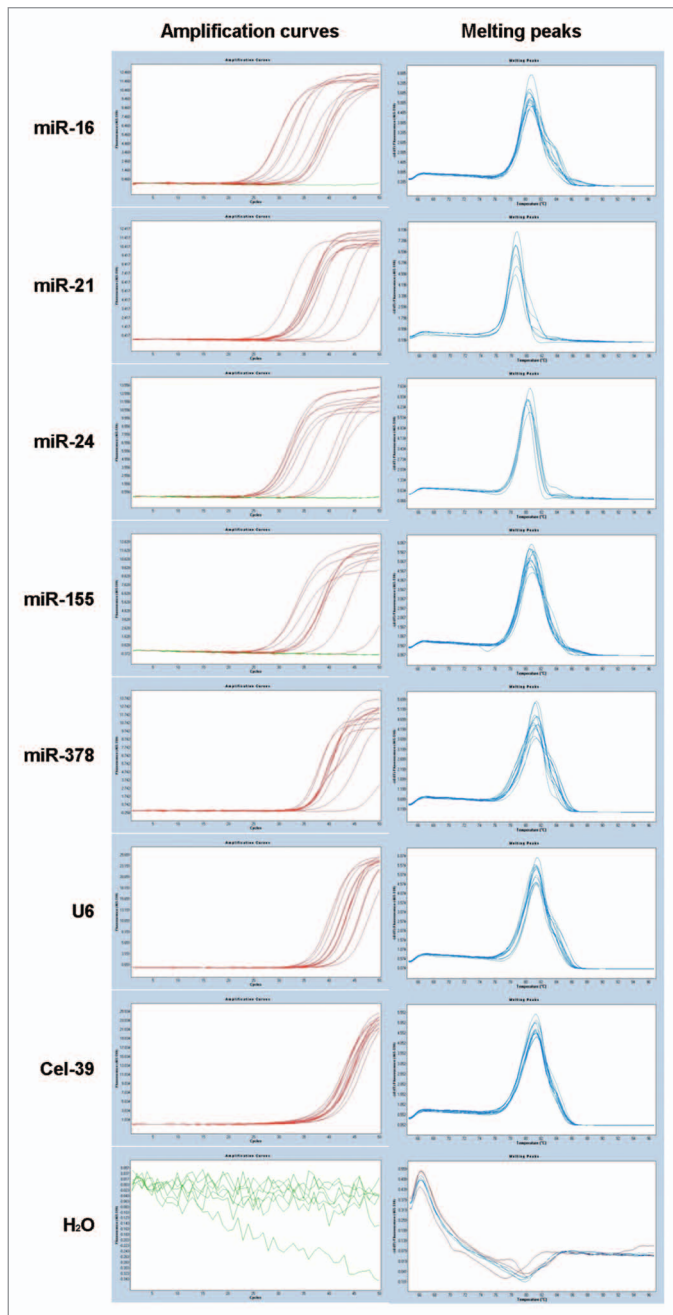
Since its discovery in 2001, miR-21 has been found associated with cancer development. In fact, it is one of the most studied miRNAs in human cancers. MiR-21 was highly expressed in solid tumors and leukemia cells,<sup>33</sup> and targets PDCD4, PTEN, SPRY, ERCK, and Bcl-2 family proteins. It was regulated at transcription by AP-1, STAT3, and TGF- $\beta$ , but the mechanism of dysregulation in cancer cells is not clear.<sup>34</sup> Biopsy analysis indicated that miR-21 expression was sensitive and specific for diagnosis of cholangiocarcinoma, glioblastoma, and colorectal cancer. Meanwhile, miR-21 expression in serum had proved to be a useful diagnostic marker in breast, lung, gastric, and ovarian cancers, and correlated with the survival rate of B lymphoma patients. In the present study, miR-21 was highly expressed the plasma of NPC patients, but reduced in parallel to cancer progression based on traditional TNM cancer staging, as well as the

Chinese 2008 NPC staging system (Table 4). The molecular mechanism is not clear, which deserves further studies.

The function of miR-24 in cells is far more obscure. In some studies, it inhibited cancer growth, while in others it had the opposite effect. Such discrepancy may be explained, in part, by the variable targets of miR-24 in cell signaling. These downstream effectors include but not limited to FAF1, p16, Bcl-2, H2AX, Net1A, and Notch1. The latter two are involved in regulation of EMT in cells, which depends on the TGF- $\beta$ /Smad/Net1A signaling. In patients with oral carcinoma, miR-24 expression was increased both inside the cancer cells and in the plasma of peripheral blood.<sup>35</sup> In consistence with these findings, we found that the expression of miR-24 was higher in the plasma of NPC patients. Statistical analysis indicated that the measurement of plasma miR-24 resulted in a diagnostic value, which was slightly lower than that of miR-21 (Table 4). Meanwhile, somewhat similar to miR-21 the expression of miR-24 was reduced when cancer progressed into higher clinical stages.

Our results have shown that a single miRNA could be useful for diagnosis purpose in human cancer patients, but a combination of several miRNA would significantly increase the sensitivity and specificity of their diagnosis value (Fig. 3; Table 5). This is also true in some studies by others. For example, a combination of miR-122, 192, 21, 223, 26a, 27a, and 801





**Figure 2.** Illustration of amplification plots and melting curves from RT-qPCR. Representative results are shown for amplification of plasma miR-16, 21, 24, 155, and 378, while the snRNA U6 serves as internal control. A set of PCR includes synthetic miRNA Cel-miR-39 and distilled water as positive and negative controls, respectively.

were very useful for diagnosis of liver cancer,<sup>36</sup> whereas combination of miR-21, 486, 375, and 200b gave 80.6% of sensitivity, and 91.7% of specificity in the diagnosis of human lung cancer.<sup>37</sup> In the present study, miR-21 was the best among all miRNAs in test, which gave 76% of sensitivity and 70% of specificity for diagnosis of NPC. However, in combination with miR-16, 24, 155, and 378 these values can be increased to 87.7% and 82%, respectively. Without miR-16, the sensitivity

remains unchanged, and the specificity only slightly decreased to 80.2%.<sup>38</sup> Therefore, we concluded that grouping of miRNA markers is a promising strategy to yield clinically relevant information in the future study.

Early detection and new treatments have largely improved survival rates in human cancer patients. This is especially true in the case of NPC. The presence of certain cancer markers can help to predict cancer diagnosis, prognosis and therapeutic efficacy, and even help to determine whether adjuvant drug treatment should be given. In the present study, we found that miR-16, 21, 24, 155, and 378 exhibited deregulation of expression in the plasma of NPC cancer patients, and 4 or 5 of them demonstrated strong association with cancer staging. After treatment, all of them were somewhat back to normal levels, except those in patients that cancer recurrence took place (data not shown). It appears that using those 4 or 5 biomarkers that have been identified in this study, alone or in combination, should increase the chance of success in early detection, which will help in the battle against this type of cancer in human.

## Materials and Methods

### Patients

Between December 2007 and June 2011 a total of 217 previously untreated patients, who were initially diagnosed with nasopharyngeal carcinoma in Nanfang Hospital, were included in this study. All patients were given questionnaires about their symptoms and medical history before taking a physical exam. When tumor was found by electric nasopharyngoscope, the patient was hospitalized and tissue biopsy samples were collected and sent for pathological examination. All patients gave blood, urine, and stool samples, which were tested for infectious diseases including HIV, hepatitis B and C viruses, and syphilis as routine. Electrocardiogram examination was also performed. Diagnoses were confirmed by CT, magnetic resonance imaging (MRI), chest X-ray, emission CT (ECT), and whole abdomen ultrasound, urinary tract ultrasound, and/or full body bone scan. In some cases, patients were given whole body PET-CT exam for a complete TNM tumor staging. In addition, all patients received oral and dental care prior to radiation therapy. In the control group, there are 73 patients who suffered chronic suppurative otitis media and chronic sinusitis, which were collectively named nasopharyngitis (NPI).

All patients have been informed and signed a letter of consent for this study. The protocol was approved by Ethical Committee of Nanfang Hospital, and registered at NIH Clinical Trials (NCT01171235) in the United States.

### Blood samples

Approximately 6–8 ml whole blood sample was drawn from each patient into EDTA-containing Vacutainer® tube, mixed well and kept at room temperature. Then, blood samples were centrifuged at  $1000 \times g$  for 5 min to separate cellular fractions and plasma. The plasma-containing supernatant was transferred into micro-centrifuge tubes, followed by centrifuge again at  $13000 \times g$  for 10 min. to completely remove cellular components. Finally, plasma was aliquoted and stored at  $-80^\circ\text{C}$  until use. All

**Table 3.** Expression of 5 miRNAs in the plasma of NPC patients and control

Name	$\Delta Ct$		$2^{-\Delta\Delta Ct}$	F	P
	NPC (n = 217)	NPI (n = 73)			
miR-16	-6.295 ± 3.721	-3.840 ± 3.140	5.483	25.369	<0.001**
miR-21	-6.687 ± 2.915	-3.364 ± 3.252	10.001	72.535	<0.001**
miR-24	-3.851 ± 2.881	-1.586 ± 2.594	4.808	34.710	<0.001**
miR-155	-2.001 ± 3.137	0.475 ± 2.257	5.561	40.680	<0.001**
miR-378	-1.286 ± 1.842	-3.302 ± 3.173	0.247	37.960	<0.001**

\*\*P &lt; 0.01.

**Table 4.** Statistical analysis of miRNA expression in plasma among clinical stages

Clinical stage	N	miR-16	miR-21	miR-24	miR-155	miR-378
T1	41	107.48	128.84	115.60	111.88	112.68
T2	55	128.55	117.69	125.88	124.28	120.09
T3	66	96.48	92.91	87.88	91.72	99.63
T4	55	105.60	104.83	112.55	112.31	106.41
Spearman	r	-0.085	-0.159	-0.090	-0.064	-0.075
	P	0.214	0.019*	0.188	0.348	0.269
N0	34	127.50	133.88	134.57	121.76	105.37
N1	100	115.61	108.54	107.60	112.02	117.02
N2	50	88.51	98.61	97.53	94.21	101.16
N3	33	100.95	100.50	104.27	109.11	100.32
Spearman	r	-0.187	-0.160	-0.149	-0.105	-0.068
	P	0.006**	0.018*	0.028*	0.122	0.318
M0	200	107.26	110.36	108.57	109.83	110.80
M1	17	129.53	93.06	114.12	99.24	87.88
Spearman	r	0.096	-0.074	0.024	-0.045	-0.098
	P	0.161	0.277	0.727	0.505	0.149
I	10	115.30	126.35	135.60	105.20	94.30
II	52	131.70	122.88	125.90	123.77	123.90
III	69	92.55	104.41	90.90	99.59	105.08
IV	86	107.74	102.27	110.21	108.06	104.84
Spearman	r	-0.106	-0.130	-0.086	-0.061	-0.076
	P	0.118	0.056	0.205	0.369	0.264

\*P &lt; 0.05; \*\*P &lt; 0.01.

blood samples were processed and completed within 4 h after collection.

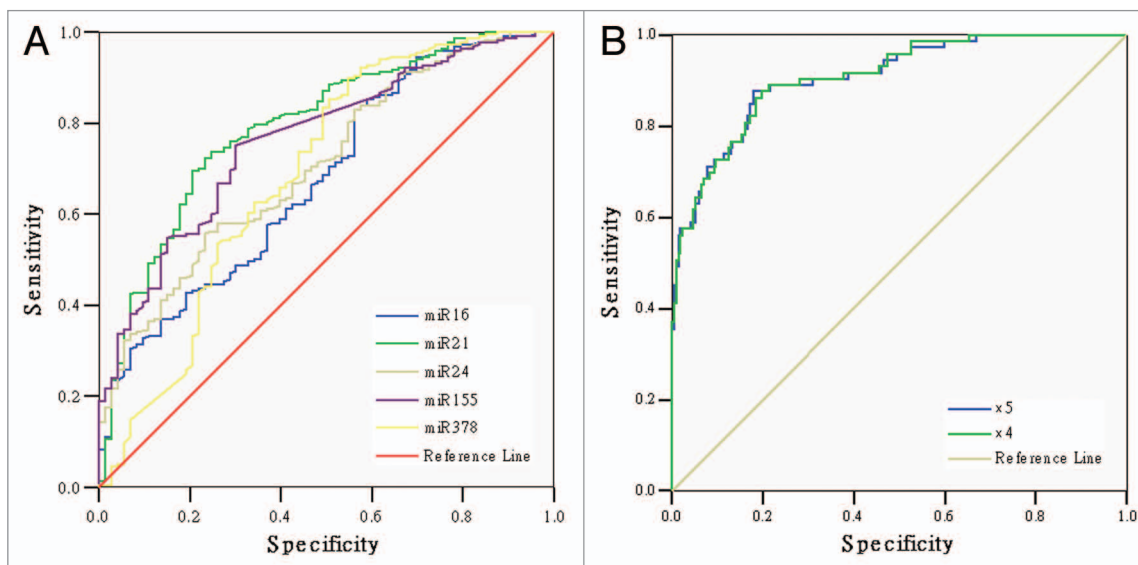
#### RT-qPCR

Total RNA was extracted from plasma specimen with Trizol® LS RNA Extraction Kit (Invitrogen) and reverse transcribed using All-in-One™ miRNA First-strand cDNA Synthesis Kit (Gene Copoeia). Quantitative real-time PCR (RT-qPCR) was performed in duplicate using LightCycler® 480 SYBR Green II real-time PCR system (Roche Applied Science) equipped with LightCycler® 480 software (Release 1.5.0 SP3) according to manufacturer's instruction for a total of 45 cycles at an annealing temperature of 60 °C. When the reactions were finished,

expression levels of miRNA were automatically calculated using the number of cycle threshold (Ct) and normalized to internal control U6 snRNA ( $\Delta Ct$ ). The Ct value in each reaction was negatively correlated with the log value of initial copy number of the template. Fold change in miRNA expression after normalization was calculated using equation  $2^{-\Delta\Delta Ct}$ .<sup>38</sup> In each experiment we included synthetic Cel-miR-39 as positive control and H<sub>2</sub>O as negative control. Forward primers are listed in Table 1.

#### Treatment

All patients received standard radiation therapy according to the Practice Guidelines in Oncology for Head and



**Figure 3.** Diagnostic value of plasma miRNAs. (A) ROC curve regression analysis was performed for expression of miR-16, 21, 24, 155, and 378 in the plasma of NPC patients. (B) ROC curve Binary Logistic regression analysis for combination of 4 or 5 miRNAs. ROC, receiver operative characteristic.

**Table 5.** Diagnostic value of 5 plasma miRNAs

Name	AUR	SD	95% confidence		Sensitivity	Specificity	P
			Lower	Upper			
miR-16	0.677	0.036	0.606	0.747	0.687	0.507	<0.001**
miR-21	0.792	0.030	0.733	0.852	0.760	0.699	<0.001**
miR-24	0.707	0.034	0.641	0.773	0.668	0.575	<0.001**
miR-155	0.763	0.031	0.703	0.823	0.751	0.699	<0.001**
miR-378	0.688	0.040	0.609	0.766	0.671	0.604	<0.001**
× 5	0.908	0.020	0.869	0.948	0.877	0.820	<0.001**
× 4	0.909	0.020	0.871	0.948	0.877	0.802	<0.001**

× 5, Combination of 5 miRNAs 16, 21, 24, 155, and 378; × 4, Combination of 4 miRNAs 21, 24, 155, and 378; AUR, area under ROC curve; SD, standard deviation. \*\* $P < 0.01$ .

Neck Cancers, v.2.2008, which was written by the National Comprehensive Cancer Network (NCCN). Radiation consisted of 70 Gy in total with a once daily fractionation schedule of 2 Gy per fraction, administered five days per week over 35 treatment days. Sometimes intensity-modulated radiation therapy (IMRT) was performed, which consisted of 66–70 Gy in total with 2.2 Gy per fraction over 30 treatment days. In the case that cancer had spread to neck lymph nodes, 66 Gy in total was used with 2 Gy per fraction over 30 treatment days. Otherwise, 50–56 Gy in total was used with 2 Gy per fraction over 25–30 treatment days.

Patients in Stage I according to Chinese NPC 2008 staging system (see description in Results) received radiation therapy only, whereas those in Stages II–IV received induction chemotherapy prior to radiation or adjuvant chemotherapy after radiation.

#### Follow-up

All patients provided contact information including telephone and cell phone numbers. Follow-up check was scheduled and delivered to patient by direct phone call. The first return visit usually took place 3 mo after treatment, and then once every half year. The efficacy of treatment was evaluated by video laryngoscope and diagnostic imaging. About 5–6 ml blood samples were collected each time for molecular biology analysis.

#### Statistical analysis

Statistical analyses were performed by using SPSS 13.0 software (IBM, USA). A  $P$  value lower than 0.05 was considered statistically significant.

#### Disclosure of Potential Conflict of Interests

There were no conflict of interests in the present study.



**Table 6.** Expression of 5 miRNAs in the plasma of NPC patients before and after treatment

Name	ΔCt			t	P
	Before	After	Change		
miR-16	-6.746 ± 4.460	-3.032 ± 3.182	-3.715 ± 5.088	-4.380	<0.001**
miR-21	-6.664 ± 3.228	-2.404 ± 2.912	-4.259 ± 3.993	-6.400	<0.001**
miR-24	-4.077 ± 3.328	-1.404 ± 2.718	-2.674 ± 4.474	-3.586	0.001**
miR-155	-2.482 ± 3.209	0.315 ± 2.455	-2.796 ± 3.823	-4.389	<0.001**
miR-378	-1.508 ± 1.980	-2.150 ± 3.173	0.643 ± 3.476	1.109	0.275

\*\*P &lt; 0.01.

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