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Original Article

Up-regulation of serum miR-744 predicts poor prognosis in patients with nasopharyngeal carcinoma

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Abstract: Background: MiRNAs has been shown to be implicated in the pathogenesis of many human diseases including cancer. Dysregulation of miR-744 is common in a number of cancers, indicating miR-774 might be closely correlated with the tumorigenesis process. However, the role and clinical significance of miR-774 in nasopharyngeal carcinoma (NPC) is poorly known. Thus the aim of this study is to investigate whether there was any clinical value of serum miR-744 in detecting and predicting the prognosis of NPC. Materials and methods: Real-time PCR was used to examine the expression level of serum miR-744 in patients with NPC and the healthy volunteers. The changes in serum miR-744 expression level of NPC patients after receiving chemo-radiotherapy were also evaluated. The association between pre-treatment serum miR-744 expression level and NPC clinicopathological parameters was investigated. Finally we employed Kaplan-Meier method and Cox proportional hazards model to evaluate the clinical value of serum miR-744 in predicting the prognosis of NPC. Results: Our study showed the expression level of serum miR-744 was significant higher in patients with NPC in comparison with healthy controls ($P<0.01$). The serum miR-744 expression level was down-regulated significantly in NPC patients after receiving chemo-radiotherapy ($P<0.01$). The Pre-treatment Serum miR-744 expression level was correlated with various important NPC clinicopathological parameters including N stage, clinical stage and grade. In addition, NPC patients with higher serum miR-744 expression had poorer 5 year overall survival rate and relapse-free survival rate. What was more, serum miR-744 was showed to be an independent factor for predicting the prognosis of NPC. Conclusion: Serum miR-744 was up-regulated in NPC patients. Higher expression level of serum miR-744 was closely correlated with was associated with poor prognosis in NPC and it might be employed as a potential biomarker for predicting the clinical outcome of NPC patients.

Keywords: Nasopharyngeal carcinoma, serum miR-744, prognosis

Introduction

Although nasopharyngeal carcinoma (NPC) is a relatively rare malignance in most parts of the world, it is a leading cause of death in Southern Asia especially in Southern China [1]. Currently chemo-radiotherapy currently represents the standard approach for treating NPC and the clinical outcome is mostly depended on the tumor stages. Tissue biopsy and cancer imaging are the common methods for detecting NPC. However, it is difficult to diagnosis NPC at an early stage due to its anatomical location [2]. Thus, exploring sensitive molecular biomarkers for early detecting and predicting the prognosis of NPC is extremely important.

MicroRNAs are small, highly conserved non-coding RNA molecules that negatively regulate gene expression at the post-transcriptional level. MicroRNAs play important roles in regulation of various biological processes such as proliferation, differentiation, survival and development [3]. They have also been shown to be involved in a variety of pathological processes including cancer pathogenesis [4]. MiRNAs might function as oncogenes or tumor suppressors in the process of tumorigenesis depending on the tumor microenvironment and the targets that they regulated. In addition, dysregulation of miRNAs has been found to be closely correlated with the initiation and progression of various types of cancers including NPC. Peng et al

Table 1. The association between serum miR-744 level and NPC clinicopathological parameters

Variable	No. of patients (n)	miR-774 expression (n)		P
		Low	High	
Gender				
Male	46	26	20	0.887
Female	40	22	18	
Age				
<60	51	29	22	0.813
≥60	35	19	16	
T stage				
T1-T2	45	29	16	0.091
T3-T4	41	19	22	
N stage				
N0-N1	50	35	15	0.002
N2-N3	36	13	23	
M stage				
M0	47	30	17	0.100
M1	39	18	21	
Clinical stage				
I-II	44	33	11	0.000
III-IV	42	15	27	
Grade				
G1-G2	47	32	15	0.012
G3	39	16	23	
EBV infection				
Y	79	43	36	0.385
N	7	5	2	

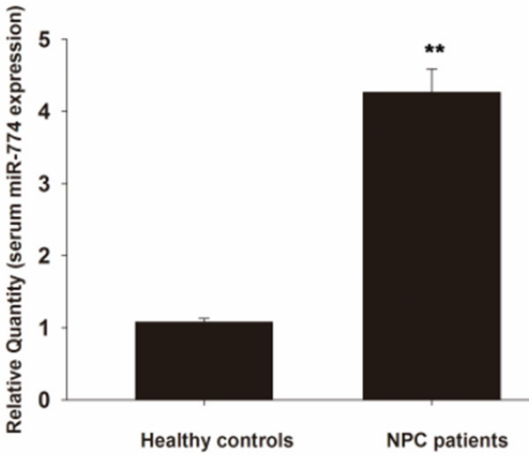

Figure 1. The expression level of serum miR-744 in patients with NPC and healthy controls.

showed that miR-124 expression was down-regulated NPC tissues and cell lines. Over-

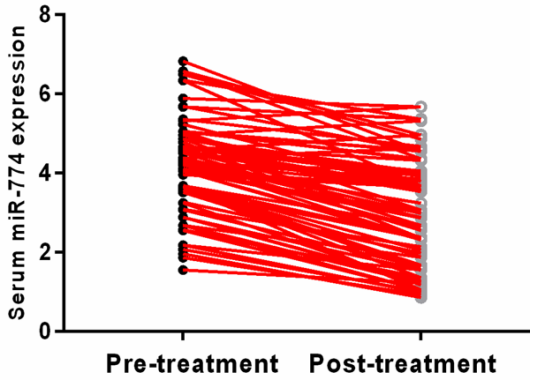

Figure 2. The association between serum mir-744 expression level and treatment response.

expression of miR-124 could suppress NPC growth and metastasis by inhibiting Foxq1 expression both in vitro and in vivo, indicating miR-124 played a tumor suppressive role in NPC [5]. Lu et al revealed that there were 33 differently expressed plasma miRNAs between NPC patients and healthy controls. The significant down-regulation of plasma miR-9 in NPC patients was closely correlated with higher chance of tumor metastasis [6]. Wang et al used deep sequence method to screen the differentially expressed serum microRNAs between patients with NPC and healthy controls; they explored a set of miRNAs that might be employed to predict survival in NPC patients [7].

Previous studies have showed miR-744 dysregulation was a common feature in cancer. The expression level of miR-744 was significantly down-regulated HCC [8]. However, serum miR-744 expression was up-regulated in patients with gastric cancer [9]. The deregulation of miR-744 in cancer indicated that it may play an important role in tumorigenesis. Currently, little information about the role of miR-744 in NPC is available. In the present study, we first performed real-time PCR to evaluate the expression level of miR-744 in NPC patients and healthy controls. Then we investigate whether there was any clinical value of miR-744 in detecting and predicting the prognosis of NPC.

Materials and methods

Study population

The study was approved by the Research Ethics Committee of Union Hospital, Tongji Medical

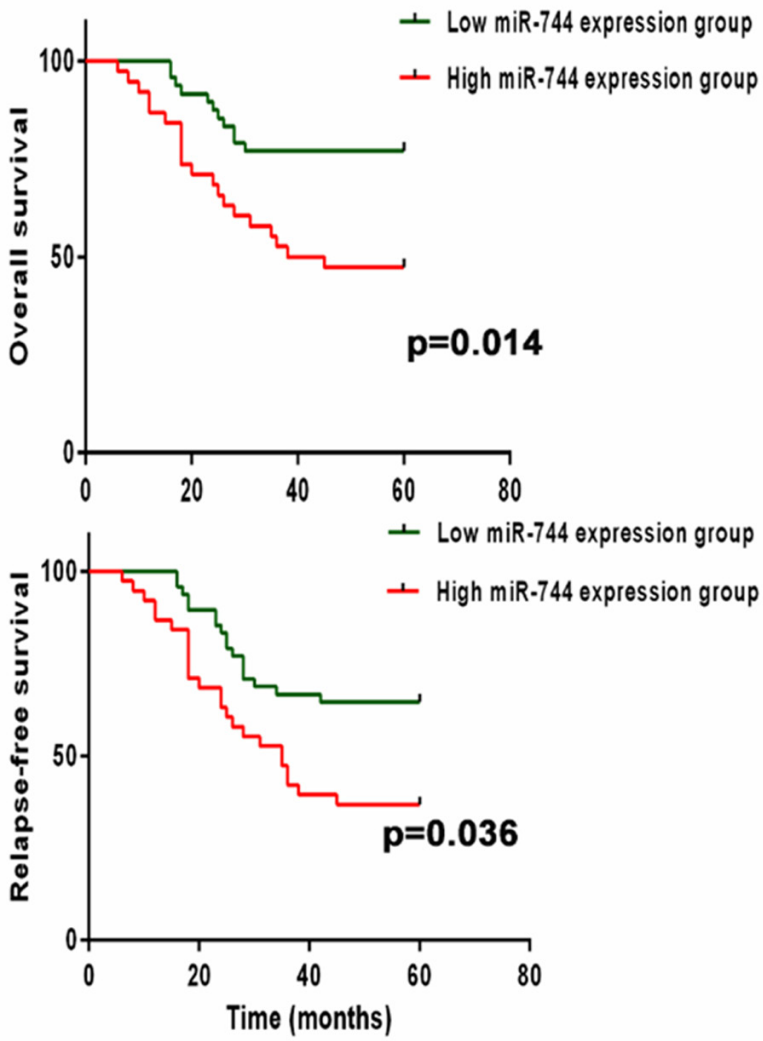

Figure 3. The relationship between serum miR-744 expression level and 5-year survival rates.

College, Huazhong University of Science and Technology. 86 patients with NPC and 40 healthy volunteers were recruited from the Department of Otolaryngology, Union Hospital. All the participants in this study gave their written consent. The NPC patients did not receive any kinds of therapy before sample collection. All the NPC patients were diagnosed and confirmed by biopsy. Up to 6 ml whole blood was collected from each participant, and the serum was isolated from the blood by centrifuging at 3,000 rpm for 5 min at room temperature; then centrifuged at 12,000 g at 4°C for 5 min. The serum samples were stored at -80°C and were not thawed until use. The age of the 86 patients were ranged from 22y to 75y and there were 46 male and 40 female respectively. The clinical feature of NPC patients was summarized in **Table 1**.

Real-time PCR

Real-time PCR was performed to detect the expression level of serum miR-744 in patients with NPC and healthy volunteers. Briefly, QIAamp RNA Blood kit (Qiagen, Hilden, Germany) was employed to extract the total RNAs from cells according to the instructions. cDNA was then synthesized from 2 ug of RNA and it served as the template for amplification of PCR with sequence-specific primers (Sangon Biotech, Shanghai, China) using SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co. Ltd, Dalian, China) on the 7500 Real-Time PCR systems (Applied Biosystems, Carlsbad, CA, USA). The PCR conditions were 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 54°C for 30 s and 72°C for 34 s. Each sample was examined in triplicate and RNU6B was used as internal control for normalization. The cycle threshold (C_T) value was calculated. The $2^{-\Delta CT}$ (ΔC_T = $C_{TmiR744}$-$C_{TU6\ RNA}$) method was used to quantify relative amount of miR-744. Real-timePCRprimers:miR-744:F5'-AATGCGGGGCTAGGGCTA-3'; R: 5'- GTGCAGGGTCCGAGGT-3'; U6: F: 5'-GCGCGTCGTGAAGCGTTC-3'; R: 5'-GTGCAGGGTCCGAGGT-3'

Statistical analyses

The differential expression of serum miR-744 between NPC patients and healthy controls was evaluated by independent t test. Paired sample t test was employed to compare the difference between pre-treatment and post-treatment serum miR-744 expression level. The association between serum miR-744 expression and NPC clinicopathological parameters was revealed using chi-square tests. Kaplan-

Table 2. Univariate analysis of prognostic factors in NPC

Parameter	Hazard ratio	*P* value
Gender (male/female)	1.06	0.931
Age (>60/≤60)	1.11	0.876
T stage (T3-T4/T1-T2)	1.56	0.127
N stage (N2-N3/N0-N1)	3.28	0.012
M stage (M1/M0)	1.51	0.185
Clinical stage (III-IV/I-II)	5.87	0.001
Grade (G2/G0-G1)	2.68	0.024
EBV infection (Yes/No)	1.42	0.364
miR-744 expression (High/Low)	3.54	0.007

Table 3. The independent prognostic factors of NPC in multivariate analysis model

Parameter	Hazard ratio	*P* value
N stage	2.39	0.038
Clinical stage	4.12	0.009
Grade	1.56	0.082
MiR-744 expression	2.87	0.016

Meier method was then employed to investigate whether serum miR-744 expression level was correlated with 5-year overall survival and relapse-free survival. Univariate and multivariate analysis was used to find out the independent factors for predicting the prognosis of NPC. The software of SPSS version 21.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. Data were expressed as means ± standard deviation (SD). Differences were considered statistically significant when p was less than 0.05.

Results

The expression level of serum miR-744 in patients with NPC and healthy controls

The serum miR-744 expression level was evaluated using real-time PCR. The results showed that the patients with NPC had a significantly higher serum miR-774 expression level compared with healthy volunteers (P<0.01) (**Figure 1**).

The association between serum miR-744 expression level and treatment response

We compared the pre-treatment and posttreatment serum miR-774 expression level in patients with NPC. The results revealed that the serum miR-774 expression level was down-regulated significantly after the patients had received the standard chemo-radiotherapy (P<0.01) (**Figure 2**), suggesting serum miR-744 might be employed as a biomarker to monitor treatment response.

The correlation between serum miR-744 expression level and clinicopathological parameters of NPC

The mean expression level of serum miR-774 in patients with NPC was used as a cut-off point to group all the NPC patients into two groups (High serum miR-744 expression group/Low serum miR-744 expression group). Our results showed that serum miR-744 expression was associated with N stage (P=0.002), clinical stage (P=0.000) and grade (0.012); and it was not correlated with gender (P=0.887), age (P=0.813), T stage (P=0.091), M stage (P= 0.100) and EBV infection (P=0.385) (**Table 1**).

The relationship between serum miR-744 expression level and 5-year survival rates

We used Kaplan-Meier method to evaluate whether there was any association between serum miR-744 expression and survival rates. Our results showed that the NPC patients who had a higher expression level of serum miR-744 suffered both poorer 5 year overall survival rates (P=0.014) and 5 year relapse-free survival rates (P=0.036) (**Figure 3**).

Serum miR-744 was an independent factor for predicting the prognosis of NPC

The univariate and multivariate analyses were conducted to identify factors related to patient prognosis. The univariate analysis showed that N stage (P=0.012), clinical stage (P=0.001), grade (P=0.024) and serum miR-744 expression level (P=0.007) were significantly correlated with post-treatment survival (**Table 2**). What was more, the multivariate regression analysis indicated that N stage (P=0.038), clinical stage (P=0.009) and serum miR-744 expression level (P=0.016) were independent prognostic factors for patients with NPC (**Table 3**).

Discussion

NPC is a malignant tumor in head and neck region are derived from nasopharyngeal epithe-

lium and it is more sensitive to radiation therapy than other types of cancers. The tumor stage is closely correlated with prognosis. The 5-year survival rates of stages III and IV NPC patients are significantly lower than that of stages I and II NPC patients [10]. The etiology of NPC is associated with complex risk factors including genetic factors, environmental factors and virus infections, which are responsible for lacking of sensitive and robust biomarkers for the early detection of NPC [11]. Thus screening new biomarkers for NPC is extremely urgent and important.

MiRNAs have been found to be involved in most, if not all, signaling pathways related to cancer development and progression [12]. It plays important roles in the initiation and progression process of various cancers. In addition to the deregulation of miRNAs expression in NPC, various studies have demonstrated that miRNAs might be indispensable for the nasopharyngeal tumorigenesis process both in vitro and in vivo [2]. In the past few years, screening new biomarkers for early detection of many diseases in the body fluids (serum, plasma, saliva and urine etc) has become a hot research area. As miRNAs is implicated in a number of human diseases including cancer, thus miRNAs are promising biomarkers without doubt.

Currently miR-744 was generally regarded as an oncosuppressor miRNA. Several molecular targets of miR-744 have been identified (TGF-beta and cyclin B1). Martin et al showed that miR-744 can negatively regulate TGF-beta activity [13]. Huang et al revealed that overexpression of miR-744 increased cyclin B1 activity, while miR-744 down-regulation decreased the expression level of cyclin B1; indicating miR-774 might promote tumor cell proliferation through in regulation of cyclin B1 [14]. Vislovukh reported that overexpression of miR-744 could inhibit proliferation of breast cancer cells by down-regulating eukaryotic translation elongation factor 1A2 (eEF1A2), suggesting the tumor suppressive role of miR-744 [15].

In the present study, our study showed the expression level of serum miR-744 was significant higher in patients with NPC in comparison with healthy controls. The serum miR-744 expression level was down-regulated significantly in NPC patients after receiving chemo-radiotherapy. Serum miR-744 expression was correlated with various important NPC clinicopathological parameters including N stage, clinical stage and grade. In addition, NPC patients with higher serum miR-744 expression had poorer 5 year overall survival rate and relapse-free survival rate. What was more, serum miR-744 was showed to be an independent factor for predicting the prognosis of NPC. Consistent with our study, miR-744 was overexpressed in NPC tissues compared to nasopharyngeal normal tissue. Moreover, tissue miR-744 overexpression was significantly correlated with TNM stage, tumorigenesis and metastasis. They also reported that miR-744 might promote progression and metastasis of NPC through in regulation of Rho GTPase activating protein 5, suggesting that miR-744 might act as an oncogene in NPC [16]. Similarly, Nurul et al screened 10 differentially expressed tissue miRNAs between patients with head and neck cancer and normal controls and tissue miR-744 was found to be overexpressed in cancer patients, indicating that miR-744 might be involved in the pathogenesis of head and neck cancer [17]. As regards to other types of cancers; Song et al identified the differentially expressed serum miRNA between patients with gastric cancer and healthy controls using TaqMan low density array. A panel of three miRNAs (miR-221, miR-744, and miR-376c) which were significantly up-regulated in the serum of gastric cancer patients was showed to be as potential biomarkers for early detection of gastric cancer [9].

However, the expression level of miR-744 has also been reported to be down-regulated in a number of cancers. Tan et al showed that lower expression of miR-744 was detected in hepatocellular carcinoma (HCC) tissue and miR-744 underexpression could be employed as an independent factor for predicting poorer prognosis for the patients with HCC [8]. Recently Lin et al reported that miR-744 exerted its tumor suppressive function by targeting c-Myc directly in HCC cell lines [18]. Similarly, the expression level of miR-774 was found to be significantly lower in HER2-positive breast tumors compared with HER2-negative tumors [19]. Kubiczkova et al revealed that lower expression lev-

els of serum miR-744 indicated shorter overall survival and remission of myeloma patients, suggesting that miR-744 might as tumor suppressor in multiple myeloma [20]. There might be two reasons accounting for the contradictory role of miR-744 in different cancers. First, it is possible that the function of miR-744 in cell type dependent. MiR-744 might work as oncogene in a certain cell type, but might also act as tumor suppressor in another cell type. Secondly, the concrete function of miR-744 in cancer might be closely correlated with the tumor microenvironment. In that case, it is reasonable to observe the phenomenon that miR-744 plays a diverse role in different cancers.

Conclusion

Up-regulation of serum miR-744 was detected in patients with NPC and it was associated with important clinicopathological parameters and survival rates. What was more, the serum miR-744 was proven to be an independent factor for predicting the prognosis of NPC, suggesting it might have potential clinical value.

Disclosure of conflict of interest

None.

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