

Original Article

Expression of miR-3182 and EBV-miR-BART8-3p in nasopharyngeal carcinoma is correlated with distant metastasis

Xianglan Mo¹, Wu Yin¹, Yongta Huang¹, Wenwen Guo¹, Minyan Zhou¹, Hongtao Ye^{1,2}

¹Department of Pathology, People's Hospital of Guangxi Province, Nanning, Guangxi, P. R. China; ²Department of Histopathology, Royal National Orthopaedic Hospital NHS Trust, Brockley Hill, Stanmore, Middlesex, HA7 4LP, United Kingdom

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Abstract: Nasopharyngeal carcinoma (NPC) is an EBV associated carcinoma showing prevalence in southeast China. Distant metastasis is the major cause of death. Herein, we investigated the expressions of microRNA-3182 (miR-3182) and EBV-miR-BART8-3p in 89 cases of NPC and evaluated their correlation with clinical outcomes. Fifty-one percent of NPC showed high level expression of miR-3182. Its expression was significantly correlated with distant metastasis ($P=0.005$). Fifty-two percent of NPC demonstrated high level expression of EBV-miR-BART8-3p and its expression was significantly correlated with distant metastasis ($P=0.006$). The overall survival was influenced by the expression of miR-3182 and EBV-miR-BART8-3p. The patients with a high-level expression of miR-3182 and EBV-miR-BART8-3p had worse overall survival ($P=0.005$ and $P=0.007$). Multivariable analysis demonstrated that EBV-miR-BART8-3p was an independent prognostic factor for overall survival ($P=0.018$). The expression of miR-3182 was significantly correlated with EBV-miR-BART8-3p ($P=0.045$). In conclusion, this is the first study examining the potential clinical utility of miR-3182 and EBV-miR-BART8-3p as prognostic biomarkers in NPC. EBV infection may promote NPC progression by disrupting the expression of miR-3182.

Keywords: Nasopharyngeal carcinoma, miR-3182, EBV-miR-BART8-3p, prognosis

Introduction

Undifferentiated nasopharyngeal carcinoma (NPC) is an Epstein-Barr virus (EBV) associated malignancy and prevalent in southeast China. About 80% of NPC patients are in Guangdong and Guangxi provinces which are located in the south of China. The incidence rate of NPC in Guangxi province is 30/100,000 to 50/100,000 [1]. The mortality rate of NPC in Guangxi province is about 5.98/100,000 in male and 2.72/100,000 in female patients [2]. However a proportion of patients with NPC are sensitive to radiotherapy and some of the patients who present with early stage disease can be cured. Despite limited treatment success, mortality is still frequent. Distant metastasis of NPC is the leading cause of death in these patients. The rate of distant metastasis is about 20%-30% with lung, liver and bone being the most common

sites of metastasis, which are not easily detected by routine examination. Therefore, there is an unmet need to identify robust molecular biomarkers for early prediction of distant metastasis and to understand the molecular mechanisms that underpin NPC.

It is well known that nearly all NPC samples are EBV positive, suggesting a strong etiological link between the malignant transformation and EBV infection. Latent membrane protein 1 (LMP1), which is encoded by EBV, is identified as the major oncoprotein. Meta-analysis has shown that LMP1 expression was positively associated with metastasis [3] and correlated with poorer overall survival (OS) in NPC [4]. LMP1 up-regulates epithelial-mesenchymal transition (EMT) and contributes to the highly metastatic features of NPC. Moreover, LMP1-associated EMT is accompanied by the expression of cancer stem cell (CSC)/cancer pro-

genitor cell (CPC) markers (CD44 high/CD24 low) and the acquisition of stem cell/progenitor cell-like properties [5]. Only 50% of NPC samples express LMP1, suggesting that there may be more important factors encoded by EBV that contribute to NPC progression and distant metastasis.

EBV is one of the viruses that can encode microRNAs (EBV-miRs) which can regulate the expression of EBV proteins and the gene expression of EBV infected cells. MicroRNA (miR) is a short, non-coding single-stranded RNA molecule that post-transcriptionally regulates gene expression by recruiting the RNA-induced silencing complex (RISC) to target mRNAs. MiRs dysregulation is associated with the development of many human diseases including cancer as well as NPC [6-8]. Abnormal expression of several miRs contribute to the distant metastasis of NPC by modifying the important steps of cancer invasion and metastasis, such as EMT, cell movement, and angiogenesis [8, 9]. There are 25 EBV miRs precursors and 44 mature EBV miRs coded by BHR-F1 and BART clusters, and they regulate the expression of EBV-encoded oncogenes and cellular miRs as well as host genes [10]. EBV-miR promotes NPC cell metastasis by modifying the expression of genes which regulate the EMT and cell proliferation as well as invasion. Du et al. showed that EBV-miR promotes NPC cell metastasis by up-regulating the expression of miR-155, which has been identified as an oncogene, and miR-155 induces the EMT by up-regulating the expression of TGF- β [11]. The expression of EBV-MiR-BART9 is up-regulated in NPC cells. It can promote NPC cell migration by suppressing E-cadherin expression [12]. NDRG1 (N-myc downstream-regulated gene 1) which has been identified as an important metastasis suppressor and is the target gene of EBV BART22 and BART miR cluster 2. Its expression in NPC cells is low and associated with metastasis [13]. However, it is still unknown whether the mechanism of EBV-miRs plays an important role in promoting the distant metastasis of NPC in Guangxi Province.

In order to clarify the underlying mechanisms of distant metastasis in NPC, our group previously used miRs microarray hybridization to identify NPC metastasis-associated biomarkers. Our study in a small number of samples showed that the expressions of miR-3182

and EBV-miR-BART8-3p were significant higher in NPC tissue than those in normal nasopharyngeal mucosa tissue. The expression levels of miR-3182 and EBV-miR-BART8-3p in NPC tissue were correlated with distant metastasis. To further investigate their roles to predict NPC patients' clinical outcome, we used RT-PCR to detect the expression of miR-3182 and EBV-miR-BART8-3p in NPC samples and analyzed the correlations between their expression levels, clinicopathologic features, and overall survival.

Patients and methods

Patients

Eighty-nine NPC patients from Guangxi Province were recruited between 2008 and 2012 in People's hospitals of Guangxi Province. All of them were initially diagnosed with NPC but without any other diseases. All pathologic specimens were reviewed and re-classified by three experienced pathologists using the WHO criteria for pathological diagnosis. The carcinoma cells were EBER infection positive as detected by in situ hybridization. This study was approved by the institutional review board of People's Hospital of Guangxi Province and all patients provided informed consent in compliance with institutional guidelines.

Seventy-nine patients were treated with combination therapy using a radiotherapy (RT) followed by a chemotherapeutic regimen. 10 patients had RT alone. The follow-up period of 83 patients varied from 4 to 82 months with a median of 32 months. Twenty-two patients (27%) died during their follow-up, and 19 (23%) had distant metastasis. Overall survival (OS) was measured from the date of diagnosis to the date of death or the last follow-up visit.

Quantitative real-time PCR

Paraffin embedded NPC tissue biopsies were cut and total RNA was isolated using the Hi-Pure FFPE MIRNA KIT (Qiagen, R4313-02) according to the manufacturer's instructions. First-strand cDNA was synthesized from polyadenylated RNA using a REVERTRA ACE QPCR RT KIT (FSQ-101, TOYOBO) in a 20- μ L reverse transcription reaction mixture containing 2 μ g of total. U6 was used for an endogenous control. The primers of miR-3182, EBV-miR-BART8-3p and U6 were designed using Primer ex-

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Table 1. Correlation among clinical features and miR-3182 as well as EBV-miR-BART8-3p expressions

Clinical features	n	miR-3182 expression			EBV-miR-BART8-3p expression		
		High	Low	<i>p</i> -value	High	Low	<i>p</i> -value
Sex							
Male	64	31	33	0.638	34	30	0.814
Female	25	14	11		12	13	
Age							
≥50	34	15	19	0.387	18	16	1.000
<50	55	30	25		28	27	
Stage							
I/II	15	5	10	0.167	6	9	0.400
III/IV	74	40	34		40	34	
Distant metastasis							
Yes	27	20	7	0.005*	20	7	0.006*
No	62	25	37		26	36	

**P*<0.05.

press 2.0 software. The Primers were miR-3182 (Sense: 5'-CAC TCA GCT GGC TTC TGT AGT G-3'); EBV-miR-BART8-3p (Sense: 5'-CAC TCA GCT GGT CAC AAT CTA TGG-3'); U6 (Sense: 5'-CAC TCA GCT CAC GCA AAT TCG TG-3'). Universal antisense primers were 5'-CTG GT-G TCG TGG AGT CG-3'. Universal probes were FAM-CCT GTC ACG ACA CGA CGT CAG TTG AG-BHQ1. qRT-PCR was performed using the KAPA PROBE FAST qPCR according to the manufacturer's instructions, in a 20 µL reaction mixture containing 2 µL of cDNA. PCR runs and melting temperature analysis were carried out in an Agilent MX3005P Real Time PCR System (Agilent, Strata Gene, US). The reaction condition followed was: 95°C 3 min, 95°C 3 s, 55°C 30 s, 40 cycles, 30°C, 30 s. Each reaction was performed in duplicate to evaluate the reproducibility of data. Calculations were made using the comparative 2^{-ΔΔCt} method.

Statistical analysis

The median of miR-3182 and EBV-miR-BART8-3p expressions in nasopharyngeal carcinoma tissue was calculated, and those above the median were defined as high expression, while those equal or below the median were defined as low expression. The Chi-square and the Fisher exact test were used to compare differences among clinical features and the expressions of miR-3182 and EBV-miR-BART8-3p. The Spearman method was used to analyze the correlations between the expressions of

miR-3182 and EBV-miR-BART8-3p. The Kaplan-Meier method was used to estimate OS and down survival curves. The prognostic parameters were identified by a forward stepwise Cox regression analysis. A *p*-value less than 0.05 was considered statistically significant.

Results

Patients' characteristics

Sixty-four patients with NPC were male, 25 were female. Their ages ranged from 25 to 67 years old, and the median age was 46. Thirty-four of them were over 50, and 55 were under 50. According to the AJCC classification system, 15 (17%) patients with NPC were stage II, 21 (23%) were stage III, 21 (23%) were stage IV A, 6 (7%) were stage IV B, and 26 (30%) with stage IV C. 27 (30%) of them had distant metastasis, and 62 (69%) did not have distant metastasis. 83 patients had follow-up from 4 months to 82 months; the medium follow-up was 32 months. The end point was calculated for the initial diagnosis to when the patient died or had recurrent cancer or metastasis. 22 of them died of the disease. 19 had recurrent cancer or metastasis. 42 survived free of disease. The patients' clinical and biological characteristics are summarized in **Table 1**.

Correlation of clinical features with miR-3182 and EBV-miR-BART8-3p expression

High expression of miR-3182 and EBV-miR-BART8-3p was observed in 45 cases (51%) and 46 cases (52%), respectively and their expressions were correlated with distant metastasis (*P*=0.005 and *P*=0.006, respectively) (**Table 1**). A Spearman correlation analysis showed that there was a significant correlation with EBV-miR-BART8-3p expression in NPC (*R*=0.213, *P*=0.045).

Patient survival rate based on miR-3182 and EBV-miR-BART8-3p expression

The 3-year overall survival rate in these 89 patients with NPC was 68%. Clinical factors predicting poor survival by the univariate analysis were advanced clinical stage and distant

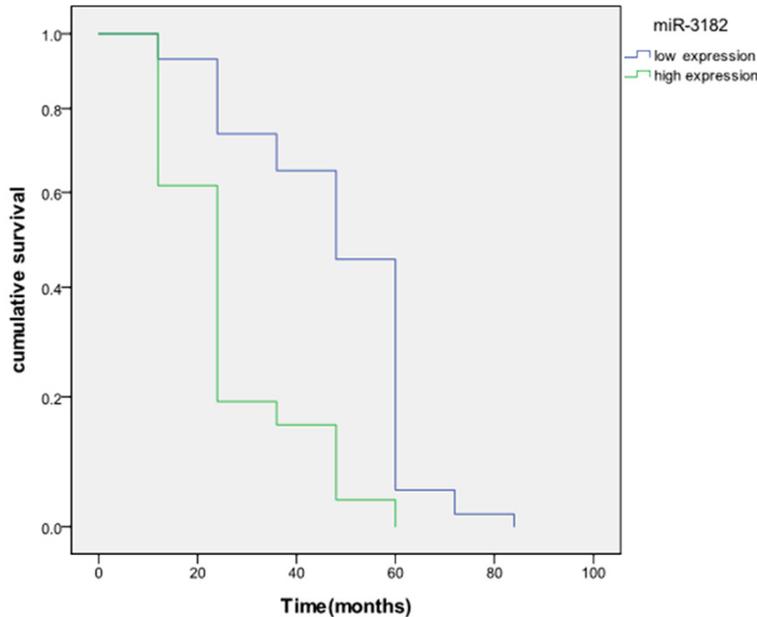


Figure 1. Cumulative survival based on the expression of miR-3182 for the 89 patients with nasopharyngeal carcinoma.

metastasis. High expressions of miR-3182 and EBV-miR-BART8-3p were inverse features for survival. NPC patients with high miR-3182 and EBV-miR-BART8-3p expressions had a worse survival rate than those with low miR-3182 and EBV-miR-BART8-3p expressions ($P=0.005$ and $P=0.007$) (Figures 1, 2).

A multivariate analysis was performed to analyze the independent prognostic factors among the included variables. It showed that the clinical stage and metastasis as well as EBV-miR-BART8-3p were independent prognostic factors for NPC patients ($P=0.033$, 0.004 and 0.018 , respectively).

Discussion

MiRs dysregulation contributes to the progression of NPC. Our present study showed that miR-3182 had high expression in NPC and its expression level was correlated with distant metastasis and poor survival. MiR-3182 may promote distant metastasis of NPC by down-regulating its target gene expression, such as TP53INP1, ITGA2 and CADM2, which inhibit cell proliferation, invasion and metastasis.

Studies have identified that several miRs may promote NPC development and progression by disrupting the functions of tumor associat-

ed genes or signal transduction pathways. MiR-144 can down regulate the expression of the tumor suppressor PTEN, which can increase the expression of cyclin D1 and pAkt, promote cell cycle G1 progression, down regulate E-cadherin expression, and promote NPC cell metastasis and invasion [14]. miR-93 directly regulates transforming growth factor- β receptor II and promotes the proliferation, invasion, and metastasis of NPC cells [15]. miR-744 expression was upregulated in NPC specimens and associated with TNM stage, tumorigenesis and metastasis [16]. miR-23b is key in promoting NPC cell proliferation, migration, and invasion by targeting E-cadherin [17]. MiR-205 over-

expression downregulates E-cadherin and up-regulates Snail expression via the down regulation of PTEN and the upregulation of AKT in the human NPC cell line CNE2 *in vitro* [18]. The expression of microRNA-92a (miR-92a) was significantly upregulated in NPC and associated with adverse clinicopathological features, including the advanced tumor-node-metastasis stage and distant metastasis, and conferred poor prognosis of patients. *In vitro* assays showed miR-92a exerted its promoting effects on the metastatic behaviors of NPC cells by suppressing the PTEN/AKT pathway [19]. Our previous study showed that miR-451a demonstrates high expression in NPC and is correlated with distant metastasis, overall survival and disease-free survival [20], which is inconsistent with the findings of other studies [21], suggesting that the mechanism of NPC distant metastasis in Guangxi are different from those in other districts.

In contrast, some miRs can act as tumor suppressors which can inhibit metastasis. MiR-9 is downregulated in nasopharyngeal carcinoma tissue, which can inhibit the proliferation, metastasis and invasion of NPC cells [22]. A decrease in serum miR-9 levels was significantly associated with lymph node metastasis and cancer progression [23]. The miR-223 expres-

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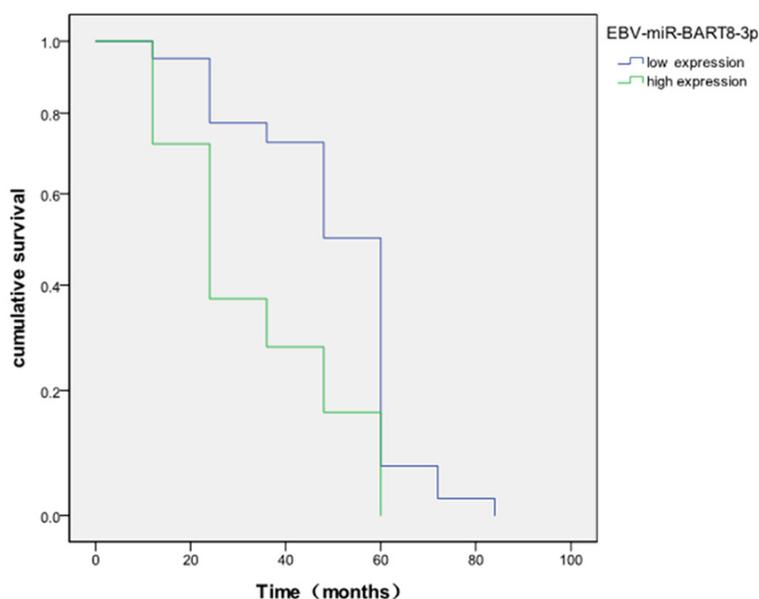


Figure 2. Cumulative survival based on the expression of EBV-miR-BART8-3p for the 89 patients with nasopharyngeal carcinoma.

sion was decreased in CNE-1, CNE-2 cells, and its level was also reduced in NPC patients' plasma. The exogenous expression of miR-223 in CNE-2 cells could inhibit cell proliferation both *in vitro* and *in vivo*. Exogenous miR-223 in CNE-2 cells would decrease the ability of colony formation and migration. MAFB, a transcription factor of Maf family members, was identified as a target gene of miR-223. The migration and invasion abilities were inhibited by MAFB silencing [24]. MiR-24 was obviously downregulated in NPC cell lines and tissue samples. Ectopic expression of miR-24 inhibited the cell viability, proliferation, migration, and invasion *in vitro*, and suppressed the xenograft tumor growth and lung metastasis formation *in vivo*. Fascin homologue 1 (FSCN1) was verified as a direct target of miR-24, and silencing FSCN1 expression with small interfering RNA inhibited NPC cell proliferation and invasion [25]. MiR-491-5p was downregulated in NPC tissues and cell lines compared with the corresponding normal counterparts. Overexpression of miR-491-5p significantly inhibited cell proliferation, migration and invasion *in vitro* and suppressed tumor growth *in vivo*. MiR-491-5p suppressed Notch3 expression both at the mRNA and protein level through directly targeting the 3' untranslated region (3'-UTR) of Notch3 mRNA. Over expression of Notch3 significantly reversed the tumor-suppressive effects of miR-491-5p [26]. MiR-1275 was mark-

edly down regulated in NPC tissues and cell lines. MiR-1275 markedly repressed cell growth as confirmed by CCK8 and the colony formation assay via the inhibition of HOXB5 in NPC cell lines. Moreover, miR-1275 suppressed G1/S transition via the inhibition of HOXB5. Further, oncogene HOXB5 was a potential target of miR-1275, and its expression was conversely correlated with miR-1275 expression in NPC [27].

Studies indicated that EBV-miR-BART had high expression in EBV associated malignancies, including Burkitt's lymphoma [28], nature killer/T cell lymphoma [29], gastric carcinoma [30] and naso-

pharyngeal carcinoma [31]. Some EBV-miR-BARTs expression in NPC tissues were correlated with cancer metastasis and poor prognosis [11-13]. The plasma ebv-miR-BART7 level was significantly higher in patients with NPC in comparison with that from healthy individuals. The ebv-miR-BART7 was detectable in all the patient plasma samples and was independent of the EBV DNA level. *In vitro*, expression of ebv-miR-BART7 enhanced proliferation, migration, and invasion of NPC cells [32]. EBV-miR-BART1 was highly expressed in NPC and closely associated with pathological and advanced clinical stages of NPC. Alteration of EBV-miR-BART1 expression results in an increase in migration and invasion of NPC cells *in vitro* and causes tumor metastasis *in vivo*. Mechanistically, EBV-miR-BART1 directly targets the cellular tumor suppressor PTEN. Reduction of the PTEN dosage by EBV-miR-BART1 activates PTEN-dependent pathways including PI3K-Akt, FAK-p130 (Cas) and Shc-MAPK/ERK1/2 signaling, drives EMT, and consequently increases migration, invasion and metastasis of NPC cells. Reconstitution of PTEN rescues all phenotypes generated by EBV-miR-BART1, highlighting the role of PTEN in EBV-miR-BART-driven metastasis in NPC [33].

Our present study shows that EBV-miR-BART8-3p is highly expressed in NPC tissue and cor-

related with distant metastasis and poor prognosis. EBV-miR-BART8-3p is an independent prognostic factor of NPC. The expression of EBV-miR-BART8-3p is significantly correlated with miR-3182 expression. Our present study indicates that neither the miR-3182 nor the EBV-miR-BART8-3p expression level in NPC is correlated with clinical stage which is associated with OS. This may partially be due to the small proportion of patients in the early clinical stage in our cohort.

In conclusion, miR-3182 and EBV-miR-BART8-3p are highly expressed in nasopharyngeal carcinoma and correlated with distant metastasis and poor prognosis. EBV-miR-BART8-3p is a useful biomarker predicting distant metastasis and poor survival of NPC. EBV infection may promote NPC progression by disrupting the expression of miR-3182.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xianglan Mo, Department of Pathology, People's Hospital of Guangxi Province, 6 Taoyuan Road, Qing Xiu District, Nanning 530021, Guangxi, P. R. China. Tel: 0086-771-2186131; Fax: 0086-771-2802018; E-mail: moxianglan@21cn.com

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