Diagnostic and prognostic performance of circulating levels of the Epstein-Barr virus microRNAs BART 7-3p and BART 13-3p in nasopharyngeal carcinoma

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Abstract

Background Nasopharyngeal carcinoma (NPC) is closely associated with Epstein-Barr virus (EBV) infection. EBV BamHI A rightward transcripts (BART) encode microRNAs (EBV-miR-BARTs) abnormally highly expressed and play an essential role in NPC. Our previous study indicated that circulating EBV-miR-BARTs was potentially severed as a biomarker of NPC. This study aims to investigate the diagnostic and prognostic performance of miR-BART7-3p and miR-BART13-3p.

Methods Plasma levels of EBV DNA, miR-BART7-3p, and miR-BART13-3p were examined by quantitative PCR in 483 treatment-naïve NPC patients and 243 controls without NPC. The prognostic performance was examined by comparing plasma levels with rates of distant metastasis during follow-up.

Results Plasma EBV DNA was detected in 93.7% of NPC subjects vs. 8.6% of controls. The microRNAs BART7-3p and miR-BART13-3p were detected in 96.1% and 97.9% of NPC subjects vs. 3.39% and 3.3% of controls. The area under the receiver operating characteristic curve for diagnosing NPC was 0.926 for EBV DNA, 0.964 for miR-BART7-3p, 0.973 for miR-BART13-3p, and 0.997 for all three indices. Among 465 NPC patients without distant metastasis, the above-median miR-BART7-3p and EBV-DNA were independent risk for shorter distant metastasis-free survival (DMFS) (HR=2.94, 95%CI: 1.44-5.97, p=0.003; HR=2.27, 95%CI:1.26-4.10, p=0.006) in multivariate Cox regression. In the 245 patients who received radiotherapy, EBV DNA, miR-BART7-3p, and miR-BART13-3p were detectable immediately afterward in, respectively, 28.6%, 17.6%, and 54.7% of patients. Four-year DMFS rate was lower in patients with detectable miR-BART7-3p (73.0% vs. 89.7%, p<0.001), miR-BART13-3p (61.4% vs. 90.0%, p<0.001), and EBV-DNA (82.7% vs. 89.5%, p=0.035) after radiotherapy. In multivariate Cox regression, detectable miR-BART7-3p and EBV-DNA were independent risks for shorter DMFS (HR=4.13, 95%CI: 1.89-9.01, p<0.001; HR = 2.14, 95%CI: 1.04-4.42, p=0.039). Four-years DMFS rate was 92.0% in subjects (n=156) with neither detectable miR-BART7-3p nor EBV-DNA after radiotherapy, 80.0% in subjects (n=65) with either detectable miR-BART7-3p or EBV-DNA after radiotherapy, and 52.9% in subjects (n=24) with both detectable miR-BART7-3p and EBV-DNA after radiotherapy (p<0.001).

Conclusions Circulating levels of miR-BART7-3p and miR-BART13-3p show excellent diagnostic performance for NPC. The combination of plasma levels of miR-BART7-3p and EBV DNA at diagnosis and after radiotherapy may help stratify patients by risk of poor DMFS.

Introduction

Nasopharyngeal carcinoma (NPC) is common in Southeast Asia, especially in Southern China(1–3). Since early NPC is almost asymptomatic, 80% of patients present with locally advanced disease or distant metastasis at diagnosis(4). With the application of intensity-modulated radio- and chemotherapy, treatment outcomes have dramatically improved. The five-year overall survival of stage I NPC is as high as 95%, while the survival of stage IV cancer is over 60%(5–8). Nevertheless, 20–30% of patients suffer distant metastasis after radical chemo-radiotherapy(1, 4, 9). The survival rate and quality of life of these

patients are difficult to satisfy for patients with treatment failure, which is the bottleneck to improve the overall survival rate. Therefore, finding stable and reliable biomarkers for diagnosis, predicting prognosis, and monitoring treatment response were essential directions, which will help guide individualized treatment of NPC and further improve overall outcome.

The pathogenesis of NPC is closely associated with Epstein–Barr virus (EBV) infection; over 95% of tumors are EBV-positive in high-incidence areas(10). A current hypothesis proposes that EBV plays a crucial role in transforming nasopharyngeal epithelial cells into invasive cancer(11). Circulating cancer-derived EBV DNA has been established as a biomarker for NPC, with sensitivities ranging from 53–96% (12). One study suggested that plasma EBV DNA is useful for screening for early asymptomatic NPC, with 97.1% sensitivity and 98.6% specificity(13). EBV DNA level is also a strong predictor of NPC poor outcomes, especially high-risk of distant metastasis(14–18). However, current methods for assaying plasma levels of EBV DNA show high variability(19–23). The NRG-HN001 trial reported relatively poor inter-laboratory concordance for a PCR-based assay(19). At present, there is no standard assay for quantification of EBV DNA for clinical or analytical purposes. Establishing standard assay quantification of EBV DNA and searching other stable biomarkers for NPC are necessary.

EBV infection is typically latent in NPC. Only a few proteins are expressed in EBV-associated NPC, such as LMP1, LMP2, and EBNA, and they are expressed at low levels(13). microRNAs derived from the EBV gene BamHI A rightward transcripts (BARTs) are highly expressed in NPC tissues(24). These so-called miR-BARTs play essential roles in the development, invasion, metastasis, and immune escape of NPC, based on preclinical studies(19, 25–28). A previous study from our laboratory found high plasma levels of miR-BARTs in NPC patients(29). However, the diagnostic and prognostic performance of plasma miR-BART7-3p and miR-BART7-3p were not well confirmed.

In the current study, we examined the potential diagnostic performance of plasma levels of miR-BART7-3p and miR-BART13-3p in NPC by comparing levels in patients with levels in control individuals without NPC. We also examined prognostic performance by comparing plasma levels between patients with radiotherapy.

Materials And Methods

Study subjects and sample processing

This study was approved by the Hospital Review Board of Fujian Cancer Hospital, Fujian, China (2015-010-02). All participants provided written consent for their blood to be sampled and analyzed. The study was conducted according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines(30). From July 2012 to March 2015, 1,394 subjects at our hospital were diagnosed with NPC based on histology of biopsies, of whom 483 patients (18 with distant metastasis at diagnosis) were included in the study because plasma samples at diagnosis were available. Among the 465 NPC patients without distant metastasis at diagnosis, plasma samples within three days after completion of radiotherapy were available for 245. NPC was re-classified according to the 8th edition of the AJCC/UICC

staging system(8). From July 2012 to March 2015, 243 non-NPC controls were included in this study, included 207 healthy adults, 12 patients with chronic nasopharyngitis and 24 patients with histologically confirmed head and neck squamous cell carcinoma (HNSCC) excepting NPC.

Diagnostic and prognostic performance of biomarkers

The diagnostic performance of miR-BART7-3p, miR-BART13-3p and EBV DNA was examined by comparing plasma levels between NPC patients and non-NPC controls. The prognostic performance of miR-BART7-3p, miR-BART13-3p and EBV DNA was evaluated based on distant metastasis-free survival (DMFS) among the 465 NPC patients without distant metastasis at diagnosis, and among the 245 NPC patients for whom plasma samples were available immediately after radiotherapy. The workflow of data collection and analysis is shown in Fig. 1.

Patient treatment and follow-up

All patients received intensity-modulated radiotherapy according to our institutional protocols (5). In general, stage I disease was treated by radiotherapy alone, whereas stage II and IV tumors were treated by a combination of chemo- and radiotherapy. The main chemotherapy strategies are induction chemotherapy and concurrent chemotherapy. The most commonly used chemotherapy regimen for induction and adjuvant chemotherapy was platinum (cisplatin 80 mg/m^2, or nedaplatin 80 mg/m^2 intravenously in three daily doses), plus paclitaxel (135 mg/m2 intravenously on Day 1) or gemcitabine (1000 mg/m2 intravenously on Days 1 and 8). The concurrent chemotherapy regimen was cisplatin (80 mg/m^2 intravenously in three daily doses) or nedaplatin (80 mg/m^2 intravenously in three daily doses). After treatment completion, follow-up was typically conducted at three-month intervals for two years, and at six-month intervals thereafter.

RNA extraction

Venous blood samples were processed within six hours after collection in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Plasma was collected after centrifugation at 1,500 g for 10 min, then stored at -80 °C until use. RNAiso Plus (Takara, Japan) was used to purify total RNA from plasma samples. In brief, 480 μL of plasma was mixed thoroughly with 1000 μL of Trizol reagent (Takara, Japan) and 5 μL of Caenorhabditis elegans microRNA 39-3p (5 μmol/μL), then incubated for 10 min at room temperature, and finally mixed with 200 μL of chloroform. RNA was then purified according to the manufacturer's protocol(31), except that centrifugation was extended to 15 min following acid-phenol/chloroform extraction. RNA was eluted in 20.4 μL of RNAse-free water and stored at -80 °C until further processing.

Reverse transcription and quantification of miRNA

Reverse transcription of miRNA was performed using the TaqMan™ MicroRNA Reverse Transcription Kit (catalog no.4366597, Thermo Fisher Scientific, USA) and miRNA-specific stem-loop primers. The program

for reverse transcription involved 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, then a hold at 4 °C. Quantitative PCR was performed using TaqMan™ Universal Master Mix II, no UNG (catalog no. 4440048, Thermo Fisher Scientific, USA), and performed in duplicate on an the Applied Biosystems ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following conditions: 95 °C for 10 min, 45 cycles of 15 s at 95 °C and 1 min at 60 °C. To estimate the copy number of a particular miRNA in plasma samples, a standard curve was established by quantitative PCR using serially diluted synthetic miRNA mimics. Five μL of miRNA mimics (3×10^9 copies/μL) were added into the reaction system for reverse transcription, and then the cDNA of miRNA mimics was 10-fold diluted from 1×10^9 to 1×10^2 copies/μL. Data were collected and analyzed using the ViiA™ 7 DX Software (Applied Biosystems, Foster City, CA, USA). Serially diluted mimics were run along with the tested samples to generate the standard curve. Specific information on miRNA-specific stem-loop primers, Taqman Probes, PCR primers, and the reaction system of reverse transcription and PCR are described in Table S1 (Additional file 1). Multiple negative water blanks were included in every analysis.

Assay of plasma levels of EBV DNA

Plasma EBV DNA concentrations were measured by quantitative PCR as previously described (32). In brief, plasma samples (450 ∝L) were subjected to DNA extraction using a magnetic bead kit (catalog no. EA20160201, PerkinElmer, USA) using an automatic nucleic acid extraction workstation (Pre-NAT, PerkinElmer). DNA was eluted from the extraction column in 60 ∝L of nuclear-free water (catalog no 1902060, Invitrogen™, USA). Circulating EBV DNA concentrations were measured using a real-time qPCR system (catalog no. DA-D065, Daan gene, China) that amplified a DNA segment in the BamHI-W fragment region of the EBV genome. Data were collected using an ABI Prism 7500 Sequence Detector and analyzed using the 7500 Software (version 2.0.6, Applied Biosystems, Foster City, CA, USA). The sequences of the forward primers, reverse primers and probe were listed in Table S1. Results were expressed as copies of EBV genomes per mL of plasma. Multiple negative water blanks were included in every analysis.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla CA, USA), SPSS version 18.0 (SPSS, Chicago, USA) and R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Differences in miRNA and DNA levels between NPC and non-NPC controls were assessed for significance using the Mann-Whitney U-test. Differences in levels at diagnosis and after radiotherapy were assessed using the Wilcoxon test. DMFS, overall survival (OS), and locoregional recurrence-free survival (LRRFS) were analyzed using Kaplan-Meier survival analyses, and differences between groups were assessed using the log-rank test. Multivariate analyses using Cox proportional hazard modeling were performed to estimate the risk of distant metastasis, death, or locoregional recurrence. Potential confounders included sex, age, clinical stage, and number of chemotherapy cycles. $P < 0.05$ (two-sided) was considered statistically significant.

Results

Plasma EBV-miR BART7-3p and BART13-3p in NPC patients vs. non-NPC controls

The analysis included 483 NPC patients with a median age of 48 years (range, 18–83 years) and 243 control subjects (Table 1). EBV DNA was detected in 93.8% (453/483) of NPC patients, 7.2% (8/207) of healthy subjects, 16.7% (2/12) of subjects with chronic nasopharyngitis, and 12.5% (3/24) of HNSCC patients (Fig. 2A). MiR-BART7-3p and miR-BART13-3p were detected in 96.1% (464/483) and 97.9% (473/483) of NPC cases, but in only 3.9% (8/207) and 3.9% (8/207) of healthy controls (Figs. 2B and C). Neither miR-BART7-3p nor miR-BART13-3p was detected in patients with chronic nasopharyngitis or HNSCC.

Table 1
Demographic and clinical characteristics of nasopharyngeal carcinoma (NPC) patients and non-NPC controls.

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Demographic and clinical characteristics of nasopharyngeal carcinoma (NPC) patients and non-NPC controls.

Characteristic	Controls			NPC (n = 483)
	Healthy (n = 207)	Nasopharyngitis (n = 12)	HNSCC (n = 24)	
Age (years)				
Median	45	49	55	48
Range	18–75	19–69	26–72	18–89
Sex (n, %)				
Female	68 (32.9)	4 (33.3)	12 (50.0)	144 (29.8)
Male	139 (67.1)	8 (66.7)	12 (50.0)	339 (70.2)
Clinical stage (n, %)				
I				12 (2.50)
II				126 (26.1)
III				182 (37.7)
IVA				145 (30.0)
IVB				18 (3.70)
miR BART7-3p (copies/mL)				
Median (range)	0 (0-271)	0 (0–0)	0 (0–0)	3860 (0-635720)
miR BART13-3p (copies/mL)				
Median (range)	0 (0-1265)	0 (0–0)	0 (0–0)	10220 (0-1031760)
EBV DNA (copies/mL)				
Median (range)	0 (0-216)	0 (0–84)	0 (0-112)	5430 (0-6610000)

When compared with positive diagnosis by histology, miR-BART7-3p showed 96.1% sensitivity, 96.7% specificity and an area under the receiver operating characteristic curve (AUC) of 0.964 using the detection level as cut-off value; the corresponding values for miR-BART13-3p were 97.9%, 96.7%, and 0.973 (Fig. 1D and Table 2). Similarly, the sensitivity, specificity and AUC of EBV DNA were 93.7%, 91.4%, and 0.926, slightly inferior than those of miR-BART7-3p and miR-BART13-3p (Table 2). When the plasma levels of all three nucleic acid species were considered together, the sensitivity, specificity and AUC were 97.3%, 99.6%, and 0.997. In a subgroup analysis that included only the 138 patients with stage I-II NPC,

the AUC was 0.921 for EBV DNA, 0.965 for miR-BART7-3p, and 0.980 for miR-BART13-3p (Table 2). When plasma levels of the three nucleic acid species were considered together, the AUC was 0.994, higher than any single biomarker (Fig. S1 in Additional file 2).

Table 2
Diagnostic performance of plasma biomarkers for detection of nasopharyngeal carcinoma (NPC) (cut-off = 0 copies/mL).

Table 2

Diagnostic performance of plasma biomarkers for detection of nasopharyngeal carcinoma (NPC) (cut-off = 0 copies/mL).

Comparison	TP (n)	FN (n)	TN (n)	FP (n)	SE (%)	SP (%)	PPV (%)	NPV (%)	AUC
EBV DNA									
Early stage vs. control	128	10	222	21	92.8	91.4	85.9	95.7	0.921
NPC vs. control	453	30	222	21	93.7	91.4	95.6	88.1	0.926
miR-BART7-3p									
Early stage vs. control	133	5	235	8	96.4	96.7	94.3	97.9	0.965
All NPC vs. control	464	19	235	8	96.1	96.7	98.3	92.5	0.964
miR-BART13-3p									
Early stage vs. control	137	1	235	8	99.3	96.7	94.5	99.6	0.980
All NPC vs. control	473	10	235	8	97.9	96.7	98.3	95.9	0.974
All three nucleic acid species combined									
Early stage vs. control	137	1	242	1	99.3	99.6	99.3	99.6	0.994
All NPC vs. control	471	13	242	1	97.5	99.6	99.7	94.9	0.997

Abbreviations: TP, true positive; FN, false negative; TN, true positive; FP, false positive; SE, sensitivity; SP, specificity; PPV, positive predictive values; NPV, negative predictive values; AUC, area under the receiver operating characteristic curve.

EBV DNA levels correlated moderately with levels of both miR-BART7-3p ($r = 0.453$, $p < 0.001$) and miR-BART13-3p ($r = 0.420$, $p < 0.001$) (Figs. 2E and F). Levels of miR-BART7-3p correlated positively with those of miR-BART13-3p ($r = 0.748$, $p < 0.001$) (Fig. 2G).

Association between plasma miR-BARTs and NPC burden

Patients with advanced NPC stage had higher plasma levels of miR-BART7-3p ($r = 0.354$, $p < 0.001$), miR-BART13-3p ($r = 0.329$, $p < 0.001$) and EBV DNA ($r = 0.316$, $p < 0.001$) (Figs. 2H-J). There was a trend towards higher plasma levels of miR-BART7-3p, miR-BART13-3p and EBV DNA with advanced N- and T-classification (Fig. S2 in Additional file 2). In a majority of the 245 NPC study subjects with plasma samples after radiotherapy, miR-BART7-3p and miR-BART13-3p levels were undetectable or significantly

reduced in comparison with pre-treatment levels (Figs. 2L and M). Similar results were observed for EBV DNA (Fig. 2K).

Prognostic performance of plasma levels of miR-BARTs

The prognostic performance of circulating miR-BARTs was first examined in the 465 NPC patients without distant metastasis at initial diagnosis. Clinicopathological features of these subjects are listed in Table 3. The median follow-up time was 55 (range 2–83) months. Median circulating levels at diagnosis were 4770 (range: 0-6610000) for EBV DNA, 3669 (0-635730) for miR-BART7-3p, and 9441 (0-1031760) copies/ml for miR-BART13-3p. The median expression value is used as the cut-off value for high and low expression of miR-BARTs and EBV DNA. The four-year DMFS rate was 81.3% in patients with high EBV DNA expression vs. 92.6% in those with low expression ($p < 0.001$; Fig. 3A), 80.9% in subjects with high miR-BART7-3p expression vs. 93.2% with low expression ($p < 0.001$; Fig. 3B), and 83.1% in subjects with high miR-BART13-3p expression vs. 90.8% with low expression ($p = 0.005$; Fig. 3C). In the multivariate analysis, short DMFS was independently associated with high levels of miR-BART7-3p (hazard ratio [HR] 2.94, 95% confidence interval [CI] 1.44–5.98, $p = 0.003$) and EBV DNA (HR 2.27, 95%CI 1.26–4.10, $p = 0.006$), but not high levels of miR-BART13-3p (HR 0.67, 95%CI 0.35–1.28, $p = 0.227$) (Table 4).

Table 3
Demographic and clinical characteristics of nasopharyngeal carcinoma patients before and after treatment.

Table 3
Demographic and clinical characteristics of nasopharyngeal carcinoma patients before and after treatment.

Covariate	Pre-treatment (n = 465) (n, %)	Post-treatment (n = 245) (n, %)
Age (year)		
≤ 45	189 (40.6)	109 (44.5)
> 45	276 (59.4)	136 (55.5)
Sex		
Male	324 (69.7)	172 (70.2)
Female	141 (30.3)	73 (29.8)
Pathology		
Keratinizing squamous cell	2 (0.40)	2 (0.80)
Non-keratinizing, differentiated	45 (9.70)	22 (9.00)
Non-keratinizing, undifferentiated	418 (89.9)	221 (90.2)
T-category		
T1	120 (25.8)	70 (28.6%)
T2	86 (18.5)	46 (18.8%)
T3	158 (40.0)	83 (33.9%)
T4	91 (19.7)	46 (18.8%)
N-category		
N0	37 (8.00)	22 (9.00)
N1	226 (48.6)	117 (47.8)
N2	138 (29.7)	73 (29.8)
N3	64 (13.7)	33 (13.5)
Clinical stage		
I	12 (2.60)	7 (2.90)
II	126 (27.1)	68 (27.8)
III	182 (39.1)	95 (38.8)
IVa	145 (31.2)	75 (30.6)

Covariate	Pre-treatment (n = 465) (n, %)	Post-treatment (n = 245) (n, %)
Chemotherapy (cycles)		
≤ 3	155 (33.3)	70 (28.6)
> 3	310 (66.7)	175 (71.4)
Follow-up time (months)		
Median (range)	55 (2–83)	55 (2–82)

Table 4 Univariate and multivariate analysis of distant metastasis-free survival (DMFS) according to pre-treatment levels of miR-BART7-3p, miR-BART13-3p and EBV DNA.

Table 4

Univariate and multivariate analysis of distant metastasis-free survival (DMFS) according to pre-treatment levels of miR-BART7-3p, miR-BART13-3p and EBV DNA.

Covariate		Univariate analysis		Multivariate analysis	
		5-year DMFS (%)	p	HR (95% CI)	p
miR-BART7-3p	Low	93.2	< 0.001	1.0	0.003
	High	80.8		2.94 (1.44–5.97)	
miR-BART13-3p	Low	90.8	0.005	1.0	0.227
	High	83.1		0.67 (0.35–1.28)	
EBV DNA	Low	92.6	< 0.001	1.0	0.006
	High	81.3		2.27 (1.26–4.10)	
Age (years)	≤ 45	87.7	0.151	1.0	0.204
	> 45	82.1		1.41 (0.83–2.40)	
Sex	Male	81.9	0.004	1.0	0.009
	Female	92.1		0.40 (0.20–0.80)	
Chemotherapy cycles	≤ 3	87.1	0.302	1.0	0.620
	> 3	83.1		1.16 (0.65–2.05)	
Clinical stage	I-II	95.1	< 0.001	1.0	0.018
	III-IV	81.0		2.83 (1.19–6.72)	

Combining levels of miR-BART7-3p with those of EBV DNA levels improves prognostic performance

The four-year DMFS rate of subjects with both high levels of EBV DNA and miR-BART7-3p, subjects with high EBV DNA expression but low miR-BART7-3p expression, and subjects with high miR-BART7-3p expression but low EBV DNA expression were similar (78.7/ 83.3%/ 84.8%, all $p > 0.05$ for comparison between any two groups) (Fig. 3D). While the four-year DMFS of subjects with both low EBV-DNA and miR-BART7-3p expression (n = 150) was significantly better than other 3 groups ($p < 0.001$) (Fig. 3D). Based on these findings, we classified as “high-risk” those subjects with high levels of EBV DNA and/or miR-BART7-3p. This high-risk group showed a four-year DMFS rate of 82.3% (Fig. 3E) and four-year OS rate of 85.8% (Fig. 3F). The four-year LRRFS did not differ between the high- and low-risk groups (92.1% vs. 91.6%, p = 0.329, Fig. S3 in Additional file 2). In multivariate analysis, the abovementioned “high-risk” status was independently associated with short DMFS (HR 8.74; 95%CI 2.73–27.98; $p < 0.001$) and short OS (HR 2.45, 95%CI 1.16–5.19; p = 0.019), but not RFS (HR 1.24, 95%CI 0.61–2.50, p = 0.550) (Table 5).

Table 5
Multivariate analysis based on the pre-treatment levels of both miR BART7-3p and EBV DNA in nasopharyngeal carcinoma.

Table 5
Multivariate analysis based on the pre-treatment levels of both miR BART7-3p and EBV DNA in nasopharyngeal carcinoma patients.

Covariate		DMFS		OS		LRRFS	
		HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
Combination	Low-risk	1	< 0.001	1.0	0.019	1	0.550
	High-risk	8.74 (2.73–27.98)		2.45 (1.16–5.19)		1.24 (0.92–4.51)	
Age (years)	≤ 45	1	0.239	1.0	0.447	1	0.512
	> 45	1.38 (0.81–2.35)		1.24 (0.71–2.15)		1.25 (0.64–2.42)	
Sex	Male	1	0.014	1.0	0.135	1	0.572
	Female	0.43 (0.22–0.85)		0.62 (0.33–1.16)		1.21 (0.63–2.31)	
Chemotherapy cycles	≤ 3	1	0.337	1.0	0.493	1	0.058
	> 3	1.32 (0.75–2.33)		0.82 (0.47–1.43)		0.54 (0.28–1.02)	
Clinical stage	I-II	1	0.010	1.0	0.001	1	0.078
	III-IV	3.06 (1.31–7.16)		7.05 (2.19–22.71)		2.04 (0.92–4.51)	

Abbreviations: DMFS, distant metastasis-free survival; OS, overall survival, LRRFS, locoregional recurrence-free survival; HR, hazard Ratio; CI, confidence interval.

Plasma miR-BART7-3p levels at the end of radiotherapy can predict outcomes

This analysis included 245 NPC patients (Table 3) followed up for a median of 55 months. At the end of radiotherapy, EBV DNA was detectable in 28.6% (70/245) patients; miR-BART7-3p, in 17.6% (43/245) patients; and miR-BART13-3p, in 54.7% (134/245) patients. The four-year DMFS rate was 73.0% in subjects with detectable miR-BART7-3p vs. 89.7% in those without ($p < 0.001$), 61.4% in subjects with detectable miR-BART13-3p vs. 90.0% ($p < 0.001$), and 82.7% in subjects with detectable EBV DNA vs. 89.5% ($p = 0.035$) (Figs. 4A-C). In multivariate analysis, independent risk factors for short DMFS included detectable levels of miR-BART7-3p (HR 4.13, 95%CI 1.89–9.01, $p < 0.001$) and EBV DNA (HR 2.14, 95%CI

1.04–4.42, p = 0.039) at the end of radiotherapy, but not of BART13-3p (HR 1.19, 95% CI 0.52–2.72, p = 0.672) (Table 6).

Table 6
Multivariate analysis of distant metastasis-free survival (DMFS) according to post-treatment levels of miR-BART7-3p, miR-BART13-3p and EBV DNA.

Table 6
Multivariate analysis of distant metastasis-free survival (DMFS) according to post-treatment levels of miR-BART7-3p, miR-BART13-3p and EBV DNA.

Covariate		DMFS	
		HR (95%CI)	p
EBV DNA	Undetectable	1.0	0.039
	Detectable	2.14 (1.04–4.42)	
miR-BART7-3p	Undetectable	1.0	< 0.001
	Detectable	4.13 (1.89–9.01)	
miR-BART13-3p	Undetectable	1.0	0.672
	Detectable	1.19 (0.52–2.72)	
Chemotherapy cycles	≤ 3	1.0	0.762
	> 3	0.89 (0.42–1.90)	
Age (years)	≤ 45	1.0	0.059
	> 45	2.10 (0.97–4.54)	
Gender	Male	1.0	0.011
	Female	0.25 (0.09–0.73)	
Clinical stage	I-II	1.0	0.060
	III-IV	2.78 (0.96–8.09)	
Abbreviations: miR, microRNA ; EBV, Epstein–Barr virus ; HR, hazard Ratio; CI, confidence interval.			

The four-year DMFS rate were similar between those with only EBV DNA detectable (83.6%) and those with only miR-BART7-3p detectable (71.8%, p = 0.293) (Fig. 4D). Based on these findings, we divided the patients into a low-risk group (neither EBV DNA nor miR-BART7-3p detectable), an intermediate-risk group (either detectable) and a high-risk group (both detectable). The groups showed respective four-year DMFS rates of 92.0%, 80.0% and 52.9% (p < 0.001) (Fig. 4E), and respective four-year OS rates of 94.6%, 79.8%, and 60.4% (p < 0.001) (Fig. 4F).

Discussion

This retrospective study found that plasma levels of miR-BART7-3p and miR-BART13-3p may be useful as diagnostic and prognostic biomarkers for NPC, and their biomarker performance appears to be at least as good as that of the well-established EBV DNA biomarker. Furthermore, the combination of miR-BART7-3p, miR-BART13-3p and EBV DNA levels at diagnosis may show better diagnostic performance than any

of the biomarkers on their own. The combination of miR-BART7-3p and EBV DNA at diagnosis and at the end of radiotherapy may perform better than any of the biomarkers on their own for predicting risk of distant metastasis in NPC.

Our previous study found that EBV-positive NPC cells release miR-BARTs into culture supernatants, and high expression of miR-BARTs can be detected in the plasma of NPC patients, especially miR-BART7-3p and miR-BART13-3p(29). Our present study validates these findings in an expanded patient cohort, confirming that miR-BART7-3p and miR-BART13-3p can be used as diagnostic biomarkers for NPC. The AUC of miR-BART7-3p and miR-BART13-3p for the diagnosis of early NPC was as high as 0.90. Consistent with this, another study(33) identified levels of plasma miR-BART7-3p and miR-BART13-3p as potential biomarkers. The detection of miR-BARTs is both a qualitative (detectable or undetectable) and quantitative determination, which is significantly different from human miRNAs that show relatively high or low expression.

In addition, the AUC of miR-BART7-3p (0.964) and miR-BART13-3p (0.973) in our study were higher than that of EBV DNA (0.926) and they also showed higher positive and negative predictive values. These results indicate that miR-BART7-3p and miR-BART13-3p are at least as effective as EBV DNA for the diagnosis of NPC. A study from Hong Kong demonstrated the value of plasma EBV DNA screening for NPC with sensitivity and specificity of 97.1% and 98.6%(13). A meta-analysis of 15 studies found pooled sensitivity of 89.1% (95%CI 87.0%-90.9%) and specificity of 85.0% (95%CI 83.0%-86.9%)(20). The sensitivity and specificity of EBV DNA in our study were 93.8% and 91.4%, slightly lower than in reference(13), but higher than in reference(20), suggesting that the detection level of EBV DNA in our study was comparable to that in other studies. Although sequencing analysis of the EBV DNA may lead to even more accurate diagnosis (34), this may still be too expensive for routine screening in many medical centers. We were able to inexpensively improve performance by combining plasma levels of miR-BART7-3p, miR-BART13-3p, and EBV DNA, yielding diagnostic sensitivity of 97.5% and specificity of 99.6%. The combination of DNA as a genomic biomarker and miRNAs as transcribed biomarkers may better capture EBV presence and activity in NPC. This possibility should be explored in larger population cohorts.

A previous study showed that miR-BART7-3p can promote tumor cell metastasis in vitro and in vivo(20). In the present work, pre-treatment plasma levels of miR-BART7-3p were a prognostic marker for NPC, with high expression indicating higher risk of distant metastasis. In contrast, detectable post-treatment miR-BART7-3p levels were a poor predictor of DMFS and OS, as confirmed in multivariate analysis(14–18). 28.6% (70/245) of patients was still detectable EBV DNA at post-treatment, which were with higher risk of metastasis. Several studies have established the prognostic value of EBV DNA levels pre- and post-treatment in NPC. A multi-center trial found that, in 27.4% of patients, EBV DNA was detectable at 6–8 weeks after radiotherapy, and detectable levels were associated with 3.14-fold greater risk of distant metastasis risk(16). Another study(18) detected EBV DNA in 13.4% of NPC patients at one week after completion of radiotherapy, and detectable levels were associated with higher risk of distant metastasis.

These two studies(16, 18), like ours, suggest that post-treatment EBV DNA may be a useful prognostic factor for NPC.

We found that the combination of pre-treatment plasma levels of miR-BART7-3p and EBV DNA may reliably classify patients as being at low or high risk of distant metastasis. This may help identify individuals in whom more aggressive treatment and close follow-up may inhibit distant metastasis and improve outcomes, which may also help reduce over-treatment of those at low risk of distant metastasis. Similarly, the combination of circulating levels of miR-BART7-3p and EBV DNA at the end of radiotherapy may improve patient stratification by risk of distant metastasis. It will be necessary to perform clinical trials to explore the treatment value of adjuvant chemotherapy, maintenance chemotherapy, and immunotherapy for patients at intermediate or high risk of distant metastasis after treatment.

Our study presents several limitations. First, this is a retrospective, single-center analysis and therefore we could not avoid potential selection biases. Multi-center and prospective studies should be performed to validate the diagnostic and prognostic performance of miR-BARTs in NPC. Second, the detection method of miR-BARTs was quantitative PCR, which is susceptible to some variability in factors such as plasma volume, extraction method, and PCR reagents. Even if the diagnostic and prognostic value of EBV DNA has been confirmed in several studies, the detection level is still inconsistent in different centers(23). Therefore, in order to obtain stable and consistent results, a standard detection system should be established and confirmed in multi-center studies.

Conclusions

This study confirmed that the diagnostic performance of circulating miR-BART7-3p and miR-BART13-3p was at least as good as that of circulating EBV DNA, and the combination of the three nucleic acid markers may further improve performance. We also found that circulating miR-BART7-3p levels before and after treatment can be used as prognostic markers for NPC, and the combination of EBV DNA and miR-BART7-3p may further improve performance.

Abbreviations

NPC
nasopharyngeal carcinoma;EBV:Epstein-Barr virus; BART:BamHI A rightward transcripts; miR:microRNA; PCR:polymerase chain reaction, EDTA:ethylenediaminetetraacetic acid; HNSCC:head and neck squamous cell carcinoma; AUC:area under the receiver operating characteristic curve; DMFS:distant metastasis-free survival; OS:overall survival; LRRFS:locoregional recurrence-free survival; HR:hazard Ratio; CI:confidence interval.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Review Committee of Fujian Cancer Hospital (approval no. FJZLYY2015-010-02). Written informed consent was obtained from all participants following a detailed description of the purpose of the study. All experiments described in this study were conducted in accordance with international and national laws, regulations and guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

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References

1. Lee AW, Ma BB, Ng WT, Chan AT. Management of Nasopharyngeal Carcinoma: Current Practice and Future Perspective. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33(29):3356-64.

2. Chen YP, Chan ATC, Le QT, Blanchard P, Sun Y, Ma J. Nasopharyngeal carcinoma. Lancet (London, England). 2019;394(10192):64-80.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018;68(6):394-424.
4. Agulnik M, Epstein JB. Nasopharyngeal carcinoma: current management, future directions and dental implications. Oral oncology. 2008;44(7):617-27.
5. Lin S, Pan J, Han L, Guo Q, Hu C, Zong J, et al. Update report of nasopharyngeal carcinoma treated with reduced-volume intensity-modulated radiation therapy and hypothesis of the optimal margin. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology. 2014;110(3):385-9.
6. Liu YP, Lv X, Zou X, Hua YJ, You R, Yang Q, et al. Minimally invasive surgery alone compared with intensity-modulated radiotherapy for primary stage I nasopharyngeal carcinoma. Cancer communications (London, England). 2019;39(1):75.
7. Tang LL, Chen YP, Mao YP, Wang ZX, Guo R, Chen L, et al. Validation of the 8th Edition of the UICC/AJCC Staging System for Nasopharyngeal Carcinoma From Endemic Areas in the Intensity-Modulated Radiotherapy Era. Journal of the National Comprehensive Cancer Network : JNCCN. 2017;15(7):913-9.
8. Pan JJ, Ng WT, Zong JF, Chan LL, O'Sullivan B, Lin SJ, et al. Proposal for the 8th edition of the AJCC/UICC staging system for nasopharyngeal cancer in the era of intensity-modulated radiotherapy. Cancer. 2016;122(4):546-58.
9. Guo Q, Lu T, Hui Huang S, O'Sullivan B, Zong J, Xiao Y, et al. Depicting distant metastatic risk by refined subgroups derived from the 8th edition nasopharyngeal carcinoma TNM. Oral oncology. 2019;91:113-20.
10. Pathmanathan R, Prasad U, Chandrika G, Sadler R, Flynn K, Raab-Traub N. Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein-Barr virus-infected neoplasia. The American journal of pathology. 1995;146(6):1355-67.
11. Young LS, Yap LF, Murray PG. Epstein-Barr virus: more than 50 years old and still providing surprises. Nature reviews Cancer. 2016;16(12):789-802.
12. Fung SY, Lam JW, Chan KC. Clinical utility of circulating Epstein-Barr virus DNA analysis for the management of nasopharyngeal carcinoma. Chinese clinical oncology. 2016;5(2):18.
13. Chan KCA, Woo JKS, King A, Zee BCY, Lam WKJ, Chan SL, et al. Analysis of Plasma Epstein-Barr Virus DNA to Screen for Nasopharyngeal Cancer. The New England journal of medicine. 2017;377(6):513-22.
14. Lv J, Chen Y, Zhou G, Qi Z, Tan KRL, Wang H, et al. Liquid biopsy tracking during sequential chemo-radiotherapy identifies distinct prognostic phenotypes in nasopharyngeal carcinoma. Nature communications. 2019;10(1):3941.

15. Lin JC, Wang WY, Chen KY, Wei YH, Liang WM, Jan JS, et al. Quantification of plasma Epstein-Barr virus DNA in patients with advanced nasopharyngeal carcinoma. The New England journal of medicine. 2004;350(24):2461-70.
16. Chan ATC, Hui EP, Ngan RKC, Tung SY, Cheng ACK, Ng WT, et al. Analysis of Plasma Epstein-Barr Virus DNA in Nasopharyngeal Cancer After Chemoradiation to Identify High-Risk Patients for Adjuvant Chemotherapy: A Randomized Controlled Trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2018:Jco2018777847.
17. Leung SF, Zee B, Ma BB, Hui EP, Mo F, Lai M, et al. Plasma Epstein-Barr viral deoxyribonucleic acid quantitation complements tumor-node-metastasis staging prognostication in nasopharyngeal carcinoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006;24(34):5414-8.
18. Wang WY, Lin TY, Twu CW, Tsou HH, Lin PJ, Liu YC, et al. Long-term clinical outcome in nasopharyngeal carcinoma patients with post-radiation persistently detectable plasma EBV DNA. Oncotarget. 2016;7(27):42608-16.
19. Le QT, Zhang Q, Cao H, Cheng AJ, Pinsky BA, Hong RL, et al. An international collaboration to harmonize the quantitative plasma Epstein-Barr virus DNA assay for future biomarker-guided trials in nasopharyngeal carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2013;19(8):2208-15.
20. Liu Y, Fang Z, Liu L, Yang S, Zhang L. Detection of Epstein-Barr virus DNA in serum or plasma for nasopharyngeal cancer: a meta-analysis. Genetic testing and molecular biomarkers. 2011;15(7-8):495-502.
21. Tan LP, Tan GW, Sivanesan VM, Goh SL, Ng XJ, Lim CS, et al. Systematic comparison of plasma EBV DNA, anti-EBV antibodies and miRNA levels for early detection and prognosis of nasopharyngeal carcinoma. International journal of cancer. 2019.
22. Sanosyan A, Fayd'herbe de Maudave A, Bollore K, Zimmermann V, Foulongne V, Van de Perre P, et al. The impact of targeting repetitive BamHI-W sequences on the sensitivity and precision of EBV DNA quantification. PloS one. 2017;12(8):e0183856.
23. Kim KY, Le QT, Yom SS, Pinsky BA, Bratman SV, Ng RH, et al. Current State of PCR-Based Epstein-Barr Virus DNA Testing for Nasopharyngeal Cancer. Journal of the National Cancer Institute. 2017;109(4).
24. Raab-Traub N. Nasopharyngeal Carcinoma: An Evolving Role for the Epstein-Barr Virus. Current topics in microbiology and immunology. 2015;390(Pt 1):339-63.
25. Cai LM, Lyu XM, Luo WR, Cui XF, Ye YF, Yuan CC, et al. EBV-miR-BART7-3p promotes the EMT and metastasis of nasopharyngeal carcinoma cells by suppressing the tumor suppressor PTEN. Oncogene. 2015;34(17):2156-66.
26. Xu YJ, Zhou R, Zong JF, Lin WS, Tong S, Guo QJ, et al. Epstein-Barr virus-coded miR-BART13 promotes nasopharyngeal carcinoma cell growth and metastasis via targeting of the NKIRAS2/NF-kappaB pathway. Cancer letters. 2019;447:33-40.

27. Lin C, Zong J, Lin W, Wang M, Xu Y, Zhou R, et al. EBV-miR-BART8-3p induces epithelial-mesenchymal transition and promotes metastasis of nasopharyngeal carcinoma cells through activating NF-kappaB and Erk1/2 pathways. Journal of experimental & clinical cancer research : CR. 2018;37(1):283.
28. Zheng X, Wang J, Wei L, Peng Q, Gao Y, Fu Y, et al. Epstein-Barr Virus MicroRNA miR-BART5-3p Inhibits p53 Expression. Journal of virology. 2018;92(23).
29. Zhang G, Zong J, Lin S, Verhoeven RJ, Tong S, Chen Y, et al. Circulating Epstein-Barr virus microRNAs miR-BART7 and miR-BART13 as biomarkers for nasopharyngeal carcinoma diagnosis and treatment. International journal of cancer. 2015;136(5):E301-12.
30. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumour MARKer prognostic studies (REMARK). British journal of cancer. 2005;93(4):387-91.
31. Chomczynski P, Mackey K. Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. BioTechniques. 1995;19(6):942-5.
32. Lo YM, Chan LY, Lo KW, Leung SF, Zhang J, Chan AT, et al. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer research. 1999;59(6):1188-91.
33. Ramayanti O, Verkuijlen S, Novianti P, Scheepbouwer C, Misovic B, Koppers-Lalic D, et al. Vesicle-bound EBV-BART13-3p miRNA in circulation distinguishes nasopharyngeal from other head and neck cancer and asymptomatic EBV-infections. International journal of cancer. 2019;144(10):2555-66.
34. Lam WKJ, Jiang P, Chan KCA, Cheng SH, Zhang H, Peng W, et al. Sequencing-based counting and size profiling of plasma Epstein-Barr virus DNA enhance population screening of nasopharyngeal carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 2018;115(22):E5115-e24.

Figures

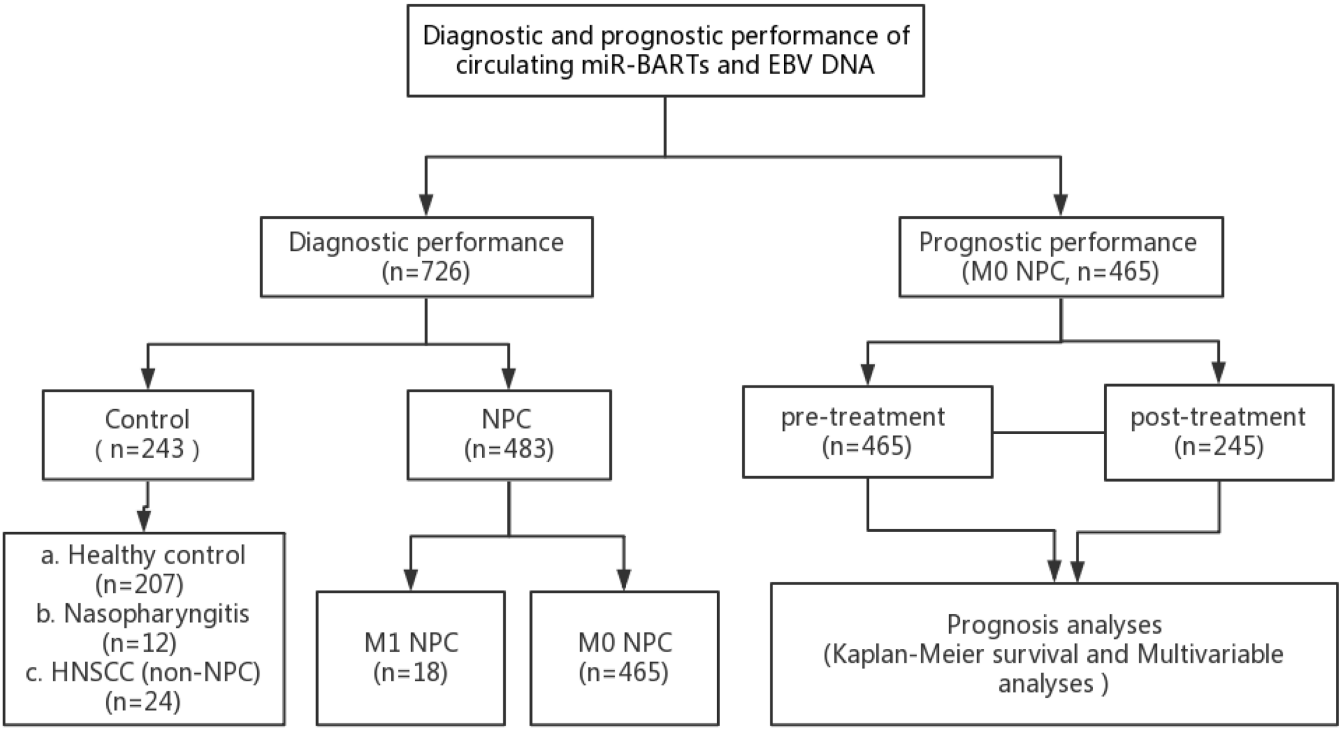

Figure 2

The workflow of data generation and analysis. EBV DNA, Epstein-Barr virus DNA; HSNCC, head and neck squamous cell carcinoma; M0, without distant metastasis; M1, with distant metastasis; miR-BARTs, microRNA BARTs; NPC, nasopharyngeal carcinoma.

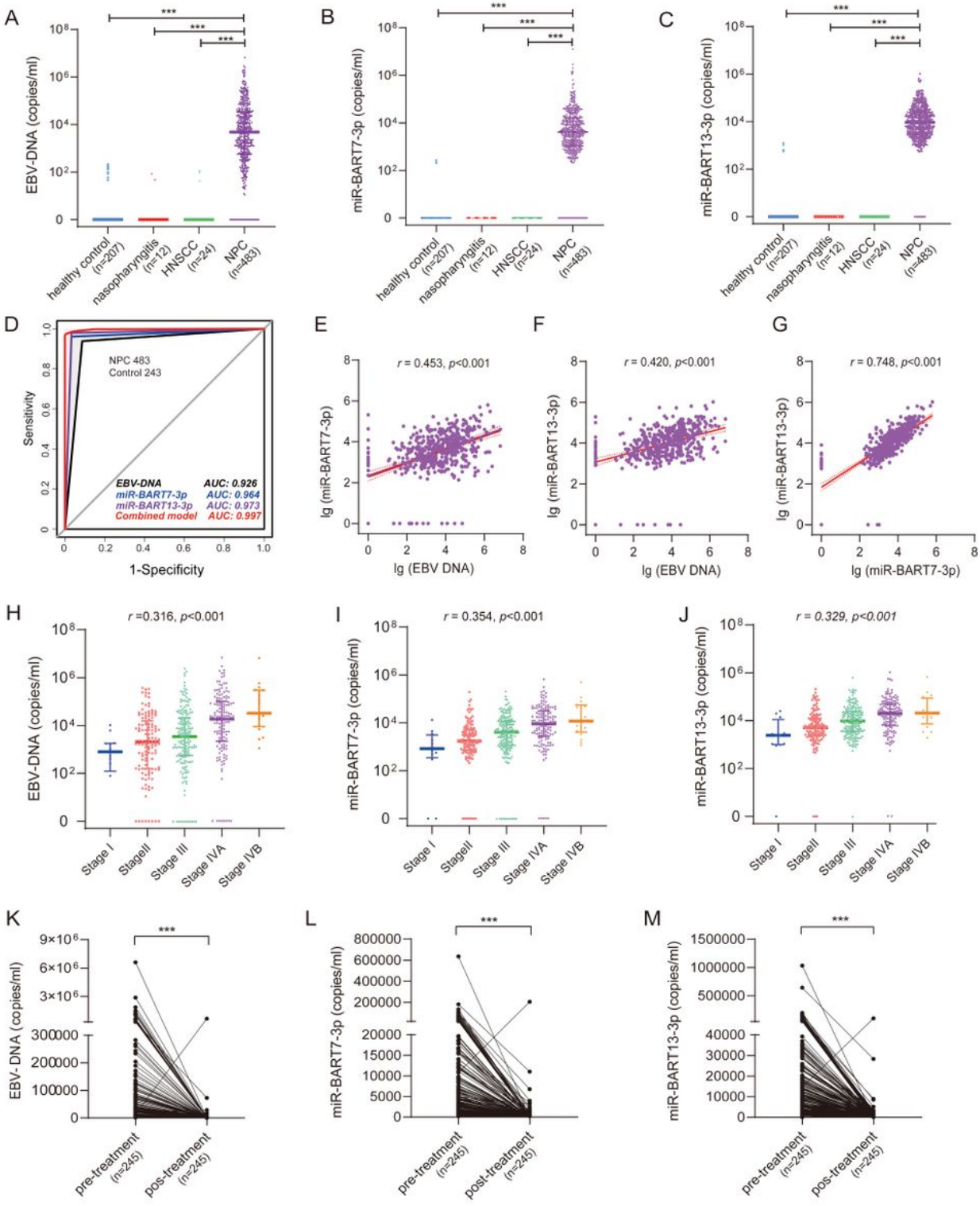

Figure 4

Diagnostic performance of plasma levels of EBV DNA, miR-BART7-3p, and miR-BART13-3p. (A-C) Plasma EBV DNA, miR-BART7-3p and miR-BART13-3p levels in healthy control (n=24), subjects with nasopharyngitis (n=12), head and neck squamous cell carcinoma (HNSCC), and nasopharyngeal carcinoma (NPC); (D) The receiver operating characteristic curve of EBV DNA, miR-BART7-3p, miR-BART13-3p and combination of the three nucleic acid species. All the three nucleic acid molecules can be

detected as 1 point, and undetectable as 0 points. The total score = 3.188×EBV DNA + 4.165×miR-BART7-3p + 5.445×miR-BART13-3p□5.531. A score greater than 0 is positive, and a score less than 0 is negative; (E-G) the correlation analysis of the expression of EBV DNA, miR-BART7-3p and miR-BART13-3p. The expression of the three nucleic acid species was transformed by log10; (H-J) the correlation of levels of EBV DNA, miR-BART7-3p, and miR-BART7-3p with clinical stage of NPC patients. A correlation coefficient (r) and the corresponding p value for this correlation were estimated by Spearman's correlation; (K-M) Difference in plasma EBV DNA, miR-BART7-3p, and miR-BART7-3p between pre-treatment and post-treatment samples.

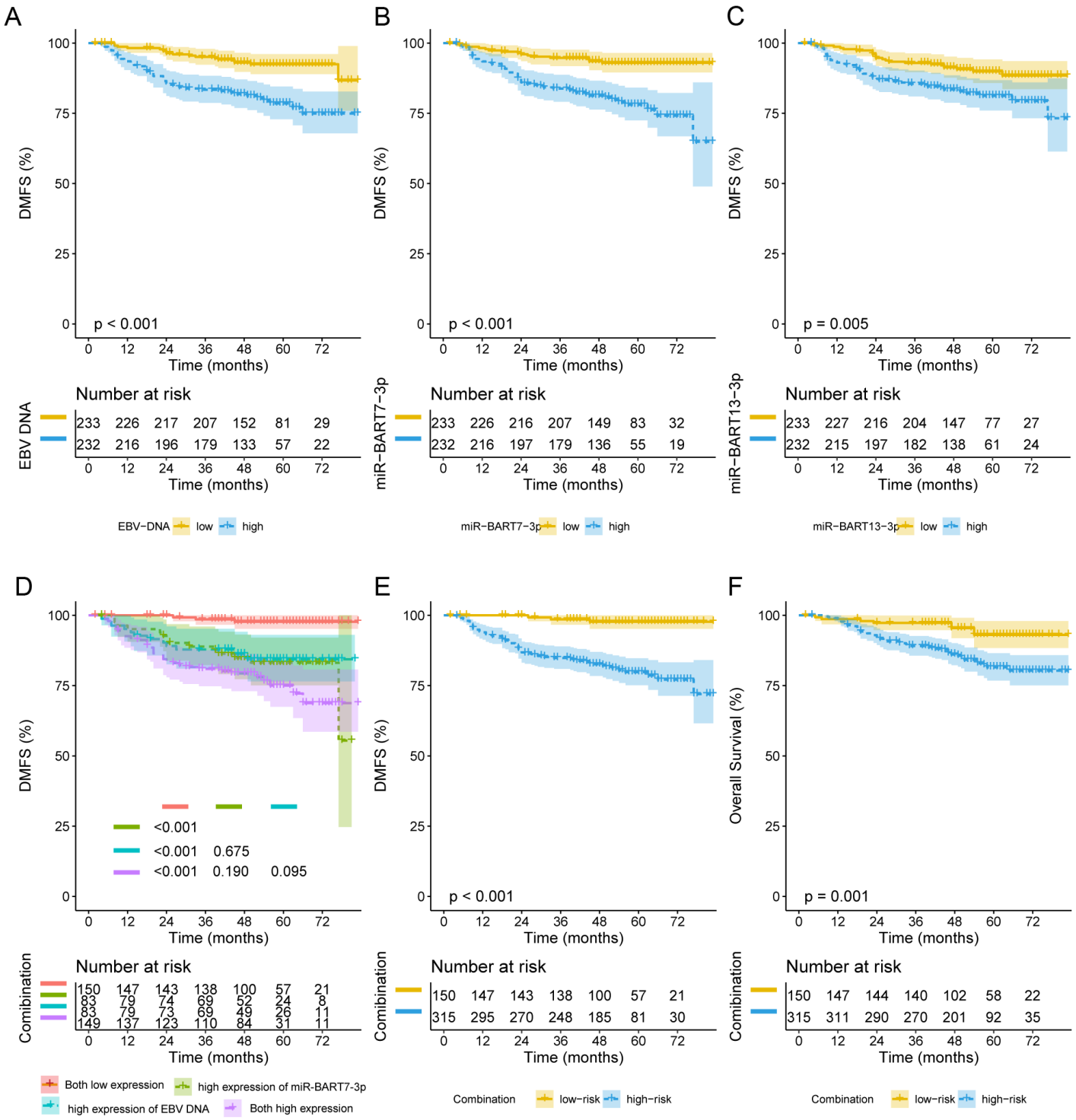

Figure 6

. Kaplan-Meier curves for distant metastasis-free survival (DMFS) and overall survival (OS) in nasopharyngeal carcinoma patients. (A) DMFS of patients with detectable EBV DNA pre-treatment. (B) DMFS of patients with detectable miR-BART7-3p pre-treatment. (C) DMFS of patients with detectable miR-BART13-3p pre-treatment. (D) DMFS of four patient subgroups based on the combination of EBV DNA and miR-BART7-3p. The color coding is explained at the bottom of the panel. (E) DMFS of patient

groups at low or high risk of poor DMFS based on the combination of EBV DNA and miR-BART7-3p. (F) OS of patient groups at low or high risk of poor OS based on the combination of EBV DNA and miR-BART7-3p.

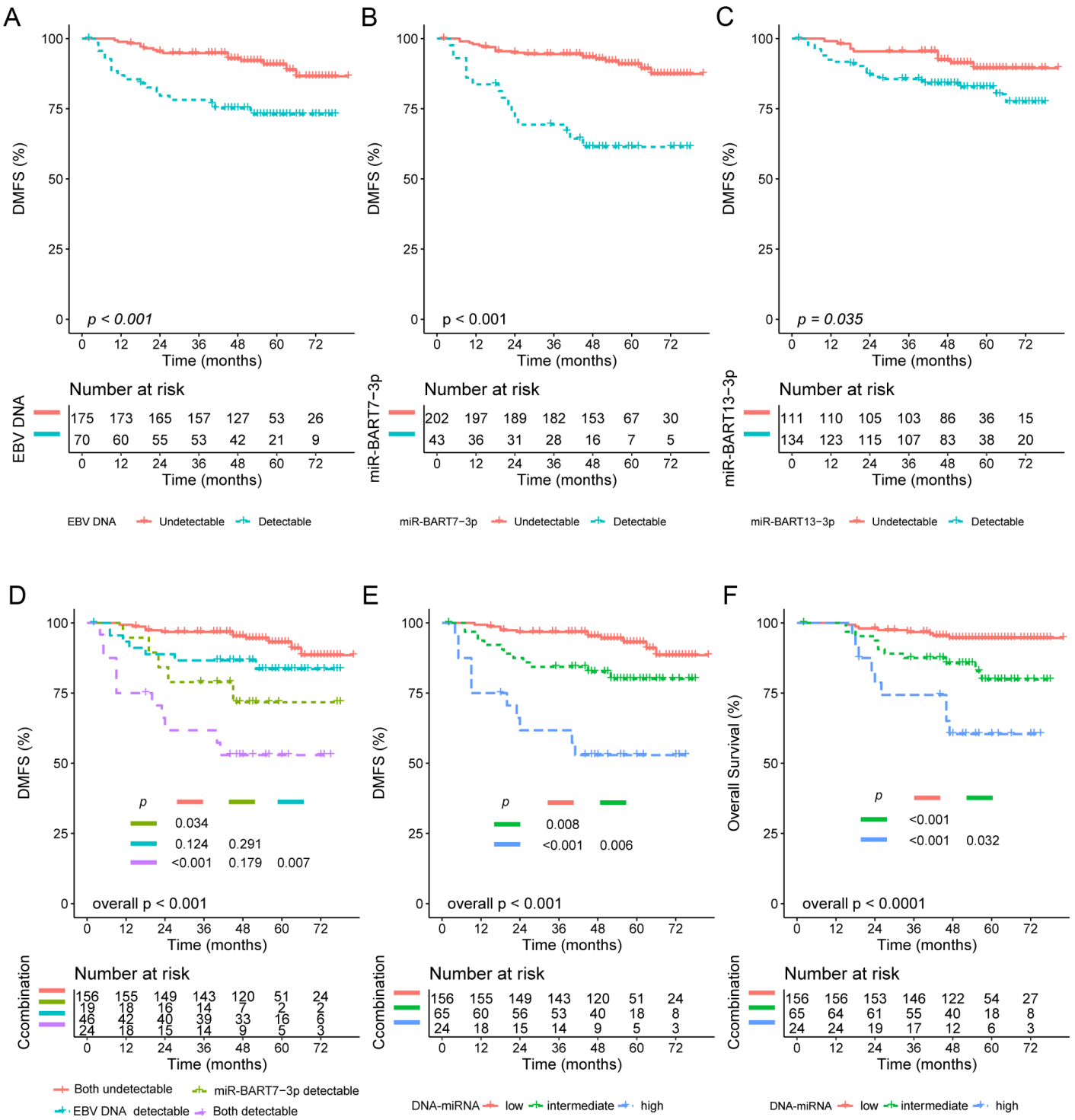

Figure 8

Kaplan-Meier curves of the three biomarkers for distant metastasis-free survival (DMFS) and overall survival (OS) in nasopharyngeal carcinoma patients. (A) DMFS of patients with detectable EBV DNA post-

treatment. (B) DMFS of patients with detectable miR-BART7-3p post-treatment. (C) DMFS of patients with detectable miR BART13-3p post-treatment. (D) DMFS of patients at four subgroups based on the combination of EBV DNA and miR-BART7-3p. The color coding is explained at the bottom of the panel. (E) DMFS of patients at different risk of poor DMFS based on the combination of EBV DNA and miR-BART7-3p. Low, intermediate, and high-risk was defined as undetectable EBV DNA and miR-BART7-3p, detectable EBV DNA or miR-BART7-3p, and detectable EBV DNA and miR-BART7-3p, respectively. (F) OS of patients at different risk of poor OS based on the combination of EBV DNA and miR-BART7-3p.

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