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Systematic comparison of plasma EBV DNA, anti-EBV antibodies and miRNA levels for early detection and prognosis of nasopharyngeal carcinoma

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Additional Supporting Information may be found in the online version of this article.

Key words: systematic comparison, biomarkers, early detection, prognosis, Epstein–Barr virus, nasopharyngeal carcinoma

Abbreviations: AJCC: American Joint Committee on Cancer; ASR: age standardized rate; AUC: area under curve; EA: early antigen; EBNA-1: EBV nuclear antigen 1; EBV: Epstein–Barr virus; ICC: intraclass correlation coefficient; LMICs: low- and middle-income countries; NPC: nasopharyngeal carcinoma; qPCR: quantitative polymerase chain reaction; ROC: receiver operating characteristic; RT: reverse transcription; VCA: viral capsid antigen

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Nasopharyngeal carcinoma (NPC) is originated from the epithelial cells of nasopharynx, Epstein–Barr virus (EBV)-associated and has the highest incidence and mortality rates in Southeast Asia. Late presentation is a common issue and early detection could be the key to reduce the disease burden. Sensitivity of plasma EBV DNA, an established NPC biomarker, for Stage I NPC is controversial. Most newly reported NPC biomarkers have neither been externally validated nor compared to the established ones. This causes difficulty in planning for cost-effective early detection strategies. Our study systematically evaluated six established and four new biomarkers in NPC cases, population controls and hospital controls. We showed that BamHI-W 76 bp remains the most sensitive plasma biomarker, with 96.7% (29/30), 96.7% (58/60) and 97.4% (226/232) sensitivity to detect Stage I, early stage and all NPC, respectively. Its specificity was 94.2% (113/120) against population controls and 90.4% (113/125) against hospital controls. Diagnostic accuracy of BamHI-W 121 bp and ebv-miR-BART7-3p were validated. Hsa-miR-29a-3p and hsa-miR-103a-3p were not, possibly due to lower number of advanced stage NPC cases included in this subset. Decision tree modeling suggested that combination of BamHI-W 76 bp and VCA IgA or EA IgG may increase the specificity or sensitivity to detect NPC. *EBNA1* 99 bp could identify NPC patients with poor prognosis in early and advanced stage NPC. Our findings provided evidence for improvement in NPC screening strategies, covering considerations of opportunistic screening, combining biomarkers to increase sensitivity or specificity and testing biomarkers from single sampled specimen to avoid logistic problems of resampling.

What's new?

Plasma Epstein–Barr virus (EBV) DNA is an established nasopharyngeal carcinoma (NPC) biomarker, but not all cases are associated with EBV and its sensitivity for stage I NPC remains controversial. Meanwhile, most newly-reported NPC biomarkers have neither been externally validated nor compared to established biomarkers. This study systematically evaluates six established and four new biomarkers in NPC cases, population controls, and hospital controls. The findings provide evidence to policymakers for improvement in NPC screening and monitoring strategies, covering considerations of opportunistic screening, combining biomarkers to increase sensitivity/specificity, and testing multiple biomarkers on single specimens to avoid the logistic problems of resampling.

Background

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy originating from the fossa of Rosenmüller of the nasopharynx. Its distribution is geographically distinct, with natives of Borneo Island, people in Southeast Asia and the Southern part of China having high age standardized rate (ASR) but is uncommon in most part of the world.[1,2] Among the top 20 countries with highest incidence and mortality rates of NPC,[3] 17 are low- and middle-income countries (LMICs), 10 of which are located in Southeast Asia. It is known that the family members of NPC patients have two to nine folds higher risk in developing NPC.[4–7] The lowest social class group had 4.1 odds ratio in developing NPC.[8] NPC is radiosensitive when treated early, with 5-year overall survival rate ranging from 78% to 100% (early stage) to as low as 26% (late stage and recurrent cases).[9–11] Recently, a study revealed that over 75% of cancer patients in Southeast Asia experienced death or financial catastrophe within 1 year of cancer diagnosis, mainly due to the lack of early detection and affordable cancer care.[12] As the majority of NPC patients present at late stage,[13] early detection could be the key to reduce the disease burden caused by NPC in LMICs.

Interaction among genes, environmental exposure and the Epstein–Barr virus (EBV) are the key events leading to NPC pathogenesis. Majority of NPC cases (>95%, except for the WHO keratinizing NPC subtype) are associated with EBV.[14] EBV is commonly detected in the tumor cells, blood and urine of NPC patients.[15] Over decades of research, EBV serology and plasma EBV DNA tests have become the established circulating biomarkers known to have high diagnostic performance in distinguishing NPC from controls.[15] Recent evidences showed that combination of serum viral capsid antigen (VCA) IgA and EBV nuclear antigen 1 (EBNA-1) IgA tests by ELISA could outperform single serology marker test in a case–control study[16] as well as in a cluster randomized screening trial[17] among the southern Chinese populations. The percentage of early stage NPC cases (Stages I and II) detected by the combination of these two serology markers during screening were higher (68.3%) as compared to unscreened populations in the screening towns (36.0%) and control towns (25.7%).[17] However, the seropositive rate of about 3% in a screening setting may still lead to a considerable burden on the resource low health care system in LMICs to conduct close follow-up for individuals with positive screening results. Meanwhile, plasma EBV DNA test is long known to have high sensitivity and specificity to distinguish NPC from controls when optimal experimental protocols were carried out, but there were concerns about its utility in detecting early stage NPC and recurrent NPC.[15,18] Of note, these EBV DNA case–control studies analyzed small sample size of Stage I NPC cases.[15,19] Recently, a large NPC screening study conducted in Hong Kong demonstrated that plasma EBV DNA test (BamHI-

W 76 bp) could identify a significantly higher proportion of participants with early stage NPC as compared to the unscreened historical cohort (70.6% *vs.* 19.2%).[20] The same study group subsequently reported that EBV DNA fragment size profiles of NPC patients are different from the small subset of general population who was transiently positive for plasma EBV DNA.[21]

According to the US National Cancer Institute's Early Detection Research Network, there are five phases for developing and validating biomarkers.[22] Despite the established EBV DNA tests and EBV serology tests which had already reach Phase 5 (Cancer Control), the pursuit of new NPC biomarkers continues for two main reasons: (*i*) keratinizing NPC subtype and recurrent NPC have reduced or absence of biomarkers originating from EBV,[15] and (*ii*) EBV is also associated with many other diseases[23] and biomarkers of non-EBV origin may help to reduce the false positive rate. Among the newly reported circulating biomarkers for NPC, serum ebv-BART2-5p, plasma ebv-miR-BART7-3p, ebv-miR-BART13-3p, hsa-miR-29a-3p, hsa-miR-103a-3p, hsa-miR-483-5p and hsa-let-7c had moderately good diagnostic accuracy (area under curve [AUC] > 0.7) in detecting NPC against controls.[24–26] Meanwhile, other newly reported circulating biomarkers had AUC < 0.7,[27–29] were identified from studies with normalization methods which are suboptimal for circulating biomarkers[30,31] and/or required additional processing or enrichment steps.[19,32] The reliability and diagnostic accuracy of these new biomarkers for early detection of NPC await validation by external independent studies (Phase 2, Clinical Assay and Validation) and should be evaluated together with the established EBV DNA and serology tests.

Malaysia is a country inhabited by multiethnic groups with different ASRs of NPC. Highest ASR of NPC (30 per 100,000) was observed in Bidayuh males, followed by Bidayuh females, Chinese males, Iban males and Kadazan males (10–20 per 100,000). Malay males, Chinese females, Iban females and Kadazan females have intermediate ASR of NPC (3.3–5.9 per 100,000), while lowest ASR of NPC (0.6–1.3 per 100,000) was observed in Malay females, Indian males and females.[33–35] According to the Malaysian National Cancer Registry Report 2007–2011, NPC was the cancer with the highest ASR among Malaysian men between 26 and 45 years old.[35] Despite the progress of NPC screening studies in southern China, NPC screening is yet to be adopted in Malaysia, due to less characterized population baseline values and uncertainty in the application of single or combination of biomarkers for screening. In Malaysia, histological examination of nasoendoscopic biopsy samples remains the gold standard to diagnose NPC. Computerized tomography is limited to major centers while magnetic resonance imaging and positron emission tomography are not routinely available to most NPC patients. Due to the confusing and nonspecific nature of early stage NPC symptoms,[13] as well as the invasive and difficult accessibility of nasoendoscopic biopsy tests mandatory to confirm the presence of tumor (nasoendoscopy is only performed by trained otorhinolaryngologists in major centers), late presentation is a common issue.[13]

Our study aimed to evaluate the diagnostic performance of six established NPC biomarkers, consisting of two EBV DNA (BamHI-W 76 bp and *EBNA1* 99 bp) and four anti-EBV antibodies (early antigen [EA] IgA, EA IgG, EBNA-1 IgA and VCA IgA), in local NPC cases, population controls and hospital controls. In addition, the performance of four newly reported NPC biomarkers, including one EBV DNA (BamHI-W 121 bp) and three miRNAs (ebv-miR-BART7-3p, hsa-miR-29a-3p and hsa-miR-103a-3p) were evaluated in a subset of our study. It is hoped that single or combination of tests optimal for early detection and prognosis of NPC can be identified to improve strategies for NPC screening and monitoring.

Materials and Methods

Participants and blood samples collection

Participants were recruited from hospitals and National Blood Bank from year 2008 to 2017. Ethics approval was obtained from the Medical Research and Ethics Committee, Ministry of Health Malaysia. Signed informed consent was obtained from histologically confirmed NPC patients, population controls (apparently healthy asymptomatic individuals) and hospital controls (patients without any cancer, EBV related diseases or ear-nose-throat diseases). Blood samples were collected in EDTA tubes and processed within 4 hr. Blood tubes were centrifuged at room temperature for 10 min at 2,500 RPM, and plasma aliquoted into separate cryogenic tubes and stored at −80°C. The numbers of samples analyzed for each test are stated in Table 1. Staging for NPC was based on the American Joint Committee on Cancer (AJCC) 7th edition and completion of radical treatment was defined as receiving a minimum of 66 Gy of radiotherapy. Survival information was retrieved from National Registration Department, Ministry of Home Affairs.

Measurement of plasma anti-EBV antibodies using ELISA

Plasma VCA IgA, EBNA-1 IgA, EA IgA and EA IgG were measured according to manufacturer's instructions (IBL International, Hamburg, Germany). The microtiter strips of VCA IgA (RE57341), EBNA-1 IgA (RE57321), EA IgA (RE56211) and EA IgG (RE57311) ELISA kits were precoated with VCA gp 125 affinity purified from P3HR1 cells, recombinant EBNA-1 p72 antigen expressed in Sf9-cells, an immunodominant region of EA-D which was affinity purified from RAJI cells, and recombinant EA p54 expressed in *Escherichia coli*, respectively. First, plasma samples were diluted in diluent buffer (1:401). Standard, control or diluted samples were aliquoted (100 µl each) into duplicate wells of microtiter plates, followed by 60 min incubation at 25°C or 37°C and three times washing (each time with 350 µl wash buffer per well). Then, 30 min or 1 hr incubation with 100 µl of enzyme conjugate was carried out at 25°C or 37°C, followed by another washing step as described previously. Twenty or 30 min incubation with 100 µl of 3,3′,5,5′-tetramethylbenzidine substrate solution was subsequently carried out in the dark and the reaction was stopped by addition of 100 µl stop solution. Optical density was measured at 450 nm and average results from duplicate wells were calculated. Levels of anti-EBV antibodies in Unit/ml were interpolated from standard curve.

Table 1. Diagnostic performance of 10 plasma biomarkers for detection of NPC

	BamHI-W 76 bp								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>0 copy/ml	29	1	113	7	94.7%	96.7%	94.2%	0.9726
Stages I and II NPC *vs*. PC	>0 copy/ml	58	2	113	7	95.0%	96.7%	94.2%	0.9756
All NPC *vs*. PC	>0 copy/ml	226	6	113	7	96.3%	97.4%	94.2%	0.9832
Stage I NPC *vs*. HC	>0 copy/ml	29	1	113	12	91.6%	96.7%	90.4%	0.9615
Stages I and II NPC *vs*. HC	>0 copy/ml	58	2	113	12	92.4%	96.7%	90.4%	0.9679
All NPC *vs*. HC	>0 copy/ml	226	6	113	12	95.0%	97.4%	90.4%	0.9796
	EBNA1 99 bp								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>0 copy/ml	22	8	119	1	94.0%	73.3%	99.2%	0.8650
Stages I and II NPC *vs*. PC	>0 copy/ml	48	12	119	1	92.8%	80.0%	99.2%	0.8988
All NPC *vs*. PC	>0 copy/ml	200	32	119	1	90.6%	86.2%	99.2%	0.9303
Stage I NPC *vs*. HC	>0 copy/ml	22	8	124	1	94.2%	73.3%	99.2%	0.8608
Stages I and II NPC *vs*. HC	>0 copy/ml	48	12	124	1	93.0%	80.0%	99.2%	0.8941
All NPC *vs*. HC	>0 copy/ml	200	32	124	1	90.8%	86.2%	99.2%	0.9281
	EA IgA								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	<1,006 U/ml[1]	4	4	83	29	72.5%	50.0%	74.1%	0.5368
Stages I and II NPC *vs*. PC	>1,852 U/ml	17	13	81	31	69.0%	56.7%	72.3%	0.6226
All NPC *vs*. PC	>1,510 U/ml	136	53	71	41	68.8%	72.0%	63.4%	0.6835
Stage I NPC *vs*. HC	>823.0 U/ml	7	1	44	9	83.6%	87.5%	83.0%	0.8514
Stages I and II NPC *vs*. HC	> 815.0 U/ml	27	3	44	9	85.5%	90.0%	83.0%	0.9094
All NPC *vs*. HC	>1,002 U/ml	172	17	48	5	90.9%	91.0%	90.6%	0.9567
	EBNA-1 IgA								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>6,147 U/ml	4	4	107	6	91.7%	50.0%	94.7%	0.6565
Stages I and II NPC *vs*. PC	>4,409 U/ml	10	19	100	13	77.5%	34.5%	88.5%	0.6196
All NPC *vs*. PC	>5,217 U/ml	58	130	104	9	53.8%	30.9%	92.0%	0.6476
Stage I NPC *vs*. HC	>5,080 U/ml	4	4	50	3	88.5%	50.0%	94.3%	0.7476
Stages I and II NPC *vs*. HC	>2,988 U/ml	15	14	48	5	76.8%	51.7%	90.6%	0.7586
All NPC *vs*. HC	>1,791 U/ml	117	71	43	10	66.4%	62.2%	81.1%	0.7886
	EA IgG								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>1,605 U/ml	8	0	60	52	56.7%	100.0%	53.6%	0.7031
Stages I and II NPC *vs*. PC	>1,575 U/ml	28	2	60	52	62.0%	93.3%	53.6%	0.7991
All NPC *vs*. PC	>5,322 U/ml	156	34	84	28	79.5%	82.1%	75.0%	0.8612
Stage I NPC *vs*. HC	>1,642 U/ml	8	0	50	3	95.1%	100.0%	94.3%	0.9670
Stages I and II NPC *vs*. HC	>1,481 U/ml	28	2	49	4	92.8%	93.3%	92.5%	0.9415
All NPC *vs*. HC	>1,642 U/ml	185	5	50	3	96.7%	97.4%	94.3%	0.9765
	VCA IgA								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>731.5 U/ml	8	0	71	42	65.3%	100.0%	62.8%	0.7378
Staged I and II NPC *vs*. PC	>731.5 U/ml	27	2	71	42	69.0%	93.1%	62.8%	0.7667
All NPC *vs*. PC	>731.5 U/ml	180	8	71	42	83.4%	95.7%	62.8%	0.7979
Stage I NPC *vs*. HC	>1,055 U/ml	6	2	51	2	93.4%	75.0%	96.2%	0.9033
Stages I and II NPC *vs*. HC	>964.5 U/ml	25	4	50	3	91.5%	86.2%	94.3%	0.9115
All NPC *vs*. HC	>1,022 U/ml	170	18	51	2	91.7%	90.4%	96.2%	0.9498

(Continues)

Table 1. Diagnostic performance of 10 plasma biomarkers for detection of NPC (Continued)

	BamHI-W 121 bp								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>0 copy/ml	14	6	48	1	89.9%	70.0%	98.0%	0.8459
Stages I and II NPC *vs*. PC	>0 copy/ml	28	8	48	1	89.4%	77.8%	98.0%	0.8861
All NPC *vs*. PC	>0 copy/ml	48	14	48	1	86.5%	77.4%	98.0%	0.8845
Stage I NPC *vs*. HC	>0 copy/ml	14	6	8	4	68.8%	70.0%	66.7%	0.6333
Stages I and II NPC *vs*. HC	>0 copy/ml	28	8	8	4	75.0%	77.8%	66.7%	0.6736
All NPC *vs*. HC	>0 copy/ml	48	14	8	4	75.7%	77.4%	66.7%	0.7218
	ebv-miR-BART7-3p								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>5.565 FCOD	15	4	38	5	85.5%	78.9%	88.4%	0.8550
Stages I and II NPC *vs*. PC	>4.145 FCOD	30	5	31	12	78.2%	85.7%	72.1%	0.8399
All NPC *vs*. PC	>4.085 FCOD	52	19	31	12	72.8%	73.2%	72.1%	0.7737
Stage I NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
Stages I and II NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
All NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
	hsa-miR-29a-3p								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>9.760 FCOD[1]	6	12	23	0	70.7%	33.3%	100.0%	0.6763
Stages I and II NPC *vs*. PC	>9.760 FCOD[1]	9	23	23	0	58.2%	28.1%	100.0%	0.5639
All NPC *vs*. PC	<8.200 FCOD	25	21	15	8	58.0%	54.3%	65.2%	0.5071
Stage I NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
Stages I and II NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
All NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
Stages II to IVC NPC *vs*. PC	<8.300 FCOD	22	6	14	9	70.6%	78.6%	60.9%	0.6250
	hsa-miR-103a-3p								
Comparison	**Cutoff**	**TP**	**FN**	**TN**	**FP**	**Accuracy**	**Sensitivity**	**Specificity**	**AUC**
Stage I NPC *vs*. PC	>10.89 FCOD[1]	5	13	20	3	61.0%	27.8%	87.0%	0.5060
Stages I and II NPC *vs*. PC	<9.270 FCOD	19	13	15	8	61.8%	59.4%	65.2%	0.5618
All NPC *vs*. PC	<9.390 FCOD	31	15	15	8	66.7%	67.4%	65.2%	0.6144
Stage I NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
Stages I and II NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
All NPC *vs*. HC	ND	ND	ND	ND	ND	ND	ND	ND	ND
Stages II to IVC NPC *vs*. PC	<9.390 FCOD	22	6	15	8	72.5%	78.6%	65.2%	0.6918

All cutoff values were calculated based on Youden index from ROC analysis except BamHI-W 76 bp, EBNA-1 99 bp and BamHI-W 121 bp which had cutoff set as >0 copy/ml.
[1]Cutoff is not practical due to biomarker not suitable for detection of early stage NPC.
Abbreviations: FCOD, fold change over detection limit; HC, hospital controls; PC, population controls; ND, not determined.

Plasma DNA and RNA extractions

Frozen plasma samples were thawed and centrifuged at room temperature for 10 min at 3,000 RPM to remove any cell debris prior to DNA or RNA extractions. DNA extraction from 200 to 400 µl plasma per sample was performed using QIAamp DNA Mini kit, while automated extraction of RNA from 400 µl plasma per sample was carried out using miRNeasy Micro Kit with QIAcube according to manufacturer's protocols (Qiagen, Hilden, Germany). In order to account for possible plasma RNA extraction bias, 500 attomole of synthetic miRNA cel-miR-39 (Integrated DNA Technologies, Coralville, IA) was spiked into all plasma samples after mixing with QIAzol from the miRNeasy Micro Kit (Qiagen). All DNA and RNA samples were eluted in 50 and 25 µl of nuclease free water (Qiagen), respectively.

Quantification of plasma EBV DNA level

Three EBV DNA tests with different primers and hydrolysis probes were conducted in our study (Supporting Information Table S1). Quantitative polymerase chain reaction (qPCR) was carried out using TaqMan Fast Advanced Master Mix in the ABI7500 Fast Real-Time PCR system (Applied Biosystems, Foster

Table 2. Comparison of decision tree models and single tests for the detection and prognosis of NPC

Biomarker test												Diagnostic performance								Cutoff			
	Decision tree algorithm	Growing method	BamHI-W as first testing criteria	Validation	BamHI-W 76 bp	EBNA1 99 bp	EA IgG	EA IgA	VCA IgA	EBNA-1 IgA	Name	True negative	False negative	False positive	True positive	Accuracy	Sensitivity	Specificity	AUC	BamHI-W 76 bp (copy/ml)	EBNA1 99 bp (copy/ml)	EA IgG (U/ml)	VCA IgA (U/ml)
Detection of NPC[1]	Classification and Regression Trees with Gini impurity measure	PN ≥ 50, CN ≥ 2, TD ≤ 5	No	20-fold CV	+	–	–	–	+	–	Model 1	106	7	0	180	97.6%	96.3%	100.0%	ND	›0.2459	NA	NA	›512.6
			Yes	50% train	+	–	–	–	–	–	Model 2	45	2	0	102	98.7%	98.1%	100.0%	ND	›0.1810	NA	NA	NA
				50% test								58	2	3	81	96.5%	97.6%	95.1%			NA	NA	NA
	Classification and Regression Trees with Twoing impurity measure		No	20-fold CV	+	–	–	–	+	–	Model 3	106	7	0	180	97.6%	96.3%	100.0%	ND	›0.2459	NA	NA	›512.6
			Yes	50% train	+	–	–	–	+	–	Model 4	60	0	0	88	100.0%	100.0%	100.0%	ND	›0.6786	NA	NA	›541.8
				50% test								46	10	0	89	93.1%	89.9%	100.0%					
	CHAID with likelihood ratio Chi-square statistics	PN ≥ 50, CN ≥ 2, TD ≤ 3	No	20-fold CV	+	–	–	–	–	–	Model 5	102	4	4	183	97.3%	97.9%	96.2%	ND	›0	NA	NA	NA
					+	–	+	–	–	–	Model 6	94	1	12	186	95.6%	99.5%	88.7%	ND	›0	NA	›97,619	NA
			Yes	50% train	+	–	–	–	–	–	Model 7	44	1	1	91	98.5%	98.9%	97.8%	ND	›0	NA	NA	NA
				50% test								58	3	3	92	96.2%	96.8%	95.1%					
	BamHI-W 76 bp											102	4	4	183	97.3%	97.9%	96.2%	0.987	›0	NA	NA	NA
Prognosis of NPC[2]	Classification and Regression Trees with Gini impurity measure	PN ≥ 50, CN ≥ 2, TD ≤ 5	No	20-fold CV	–	+	–	–	–	–	Model 8	31	8	16	25	70.0%	75.8%	66.0%	ND	NA	›14.0598	NA	NA
			Yes	50% train	–	+	–	–	–	–	Model 9	15	5	10	16	67.4%	76.2%	60.0%	ND	NA	›14.0451	NA	NA
				50% test								16	3	6	9	73.5%	75.0%	72.7%					
	Classification and Regression Trees with Twoing impurity measure		No	20-fold CV	–	+	–	–	–	–	Model 10	31	8	16	25	70.0%	75.8%	66.0%	ND	NA	›14.0598	NA	NA
			Yes	50% train	–	+	–	–	–	–	Model 11	19	3	7	14	76.7%	82.4%	73.1%	ND	NA	›14.0598	NA	NA
				50% test								12	5	9	11	62.2%	68.8%	57.1%					
	CHAID with likelihood ratio Chi-square statistics	PN ≥ 50, CN ≥ 2, TD ≤ 3	No	20-fold CV	–	+	–	–	–	–	Model 12	31	9	16	24	68.8%	72.7%	66.0%	ND	NA	›14.4270	NA	NA
			Yes	50% train	–	+	–	–	–	–	Model 13	24	13	3	6	65.2%	31.6%	88.9%	ND	NA	›137.998	NA	NA
				50% test								16	7	4	7	67.6%	50.0%	80.0%					
	EBNA1 99 bp											31	8	16	25	70.0%	75.8%	66.0%	0.709	NA	›14.0600	NA	NA
	BamHI-W 76 bp											ND	ND	ND	ND	ND	ND	ND	0.680	ND	NA	NA	NA
	EA IgA											ND	ND	ND	ND	ND	ND	ND	0.518	ND	NA	NA	NA
	EA IgG											ND	ND	ND	ND	ND	ND	ND	0.545	ND	NA	NA	NA
	EBNA-1 IgA											ND	ND	ND	ND	ND	ND	ND	0.525	ND	NA	NA	NA
	VCA IgA											ND	ND	ND	ND	ND	ND	ND	0.516	ND	NA	NA	NA

[1]Data set included 187 NPC patients and 106 population controls who had test results of six established biomarkers. Positive = NPC; Negative = Population control.
[2]Data set included 80 NPC patients who completed radical treatment, had overall survival information and test results of six established biomarkers. Positive = Dead; Negative = Alive.
Abbreviations: +, included in decision tree; –, not included in decision tree; AUC, area under curve; CHAID, Chi-square Automatic Interaction Detector; CN, child node; CV, cross validation; NA, not applicable; ND, not determined; PN, parental node; TD, tree depth.

City, CA) according to manufacturer's instructions. A total of 5 µl eluted DNA was used in 20 µl total reaction volume in each qPCR well, and each sample was analyzed in triplicate wells. Each qPCR plate contained no-template-control and serially diluted Namalwa cell DNA samples as standard points for the construction of EBV DNA copy number standard curve. Namalwa cells are known to have two integrated EBV genomes per cell.[36] Accurate dilution of Namalwa cell DNA standard points and quantification of EBV copy numbers by *EBNA1* 99 bp test were validated by calibrating these Namalwa cell DNA standard points to the 1st WHO International Standard for EBV for Nucleic Acid Amplification Techniques[37] (NIBSC code: 09/260, Supporting Information Table S2). Thermal cycling conditions include 50°C for 2 min, 95°C for 20 sec, and 40 cycles of 95°C for 3 sec and 56°C for 30 sec. EBV DNA copy number was interpolated from the Namalwa cell DNA standard curve and plasma EBV DNA level was calculated using the following formula:

$$\text{Plasma EBV DNA level, copy/ml} = \left[\left(\text{average } C_q - c\right)/m\right] \times \left(V_e/V_f\right) \times 1/a$$

where c = intercept, m = slope of the standard curve, V_e = DNA elution volume, V_f = final DNA volume used per qPCR well, a = ml of plasma used for DNA extraction.

RT-qPCR validation of differential miRNA expression

Pooled reverse transcription (RT) of cel-miR-39, hsa-miR-29a-3p and hsa-miR-103a-3p was carried out using commercially available assays (Applied Biosystems) according to optimized protocol which showed high reliability and consistency.[38,39] RT protocol, primers and probe sequences of ebv-miR-BART7-3p were according to Zhang *et al.*[24] RT products of each sample, negative and positive controls were analyzed in duplicate wells using TaqMan 2X Universal PCR Master Mix, No AmpErase UNG in ABI7500 Fast Real-Time

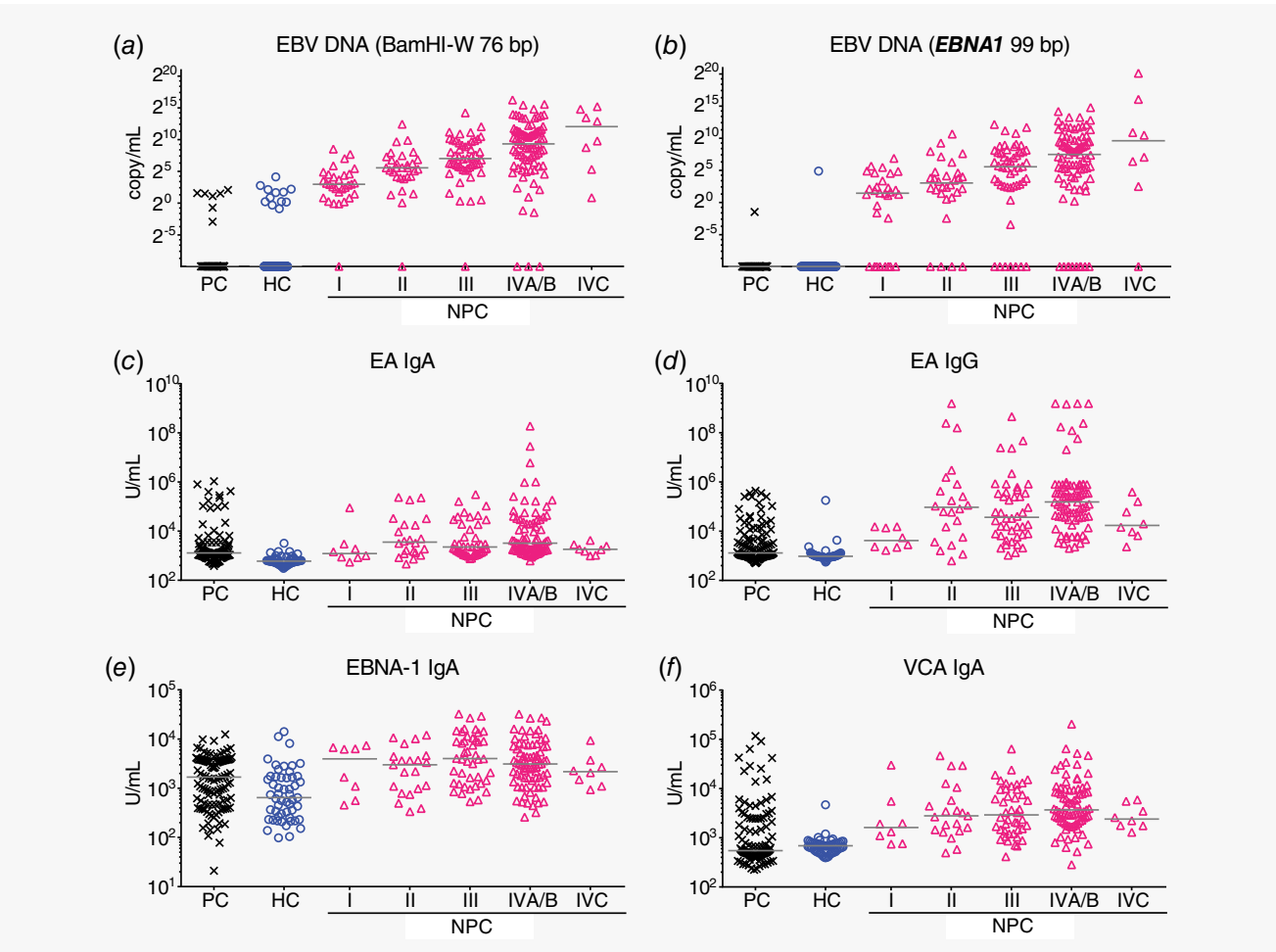

Figure 1. Evaluation of established plasma biomarkers to detect NPC against controls in our study. (*a*, *b*) Only low levels of plasma EBV DNA was observed in small subset of population controls and hospital controls. The levels of plasma EBV DNA increased with the stages of NPC. (*c*–*f*) NPC patients generally had higher plasma levels of anti-EBV antibodies as compared to controls but no obvious trend within NPC subgroups was observed. Samples with undetectable plasma BamHI-W 76 bp and plasma EBNA1 99 bp were arbitrarily set as 0.001 copy/ml. Abbreviations: HC, hospital control; PC, population control. [Color figure can be viewed at wileyonlinelibrary.com]

PCR system (Applied Biosystems) according to manufacturer's instructions. Data were normalized to cel-miR-39 (spike-in control) and fold change over detection limit was calculated.[38,39]

Statistical analysis

In GraphPad Prism software, Mann–Whitney test was used to compare the mean rank differences between NPC and controls. AUC values were generated from receiver operating characteristic (ROC) curve analysis. In SPSS software, intraclass correlation coefficient (ICC) was obtained from average-measurement, absolute-agreement, two-way mixed-effects model. Decision tree models for NPC detection and prediction of overall survival were built with sample size, decision tree growing methods, criteria and validation parameters stated in Table 2.

Data availability

The data that support the findings of our study are available from the corresponding author upon reasonable request.

Results

Plasma EBV DNA

Demographic and clinicopathological characteristics of NPC patients and controls are shown in Supporting Information Table S3. All 10 plasma biomarkers analyzed in our study did not correlate with age and were not significantly different between different sex and ethnic groups (Supporting Information Table S4). In our hands, results of EBV DNA test from DNA extraction replicates had excellent test–retest reliability (ICC > 0.95, Supporting Information Fig. S1*a*). We also found that prior to plasma processing, plasma EBV DNA load was fairly stable up to 6 hr in EDTA blood tube kept on bench at room temperature (Supporting Information Fig. S1*b*).

Comparison of plasma EBV DNA load as measured by two established EBV DNA tests (BamHI-W 76 bp and *EBNA1* 99 bp, Figs. 1*a* and 1*b*) were carried out between NPC patients and controls. In general, plasma EBV DNA loads were significantly higher in NPC patients compared to controls, and only low levels of plasma EBV DNA load was observed in a small subset of controls (Figs. 1*a* and 1*b*). The level of plasma EBV DNA increases with more advanced stages (Figs. 1*a* and 1*b*). Similar to the large cohort NPC screening study in Hong Kong,[20] plasma EBV DNA load of >0 copy/ml was set as positive for both plasma EBV DNA tests (Table 1). This resulted in 94.2% and 99.2% specificity, respectively for BamHI-W 76 bp and *EBNA1* 99 bp to identify NPC against population controls. Specificity for BamHI-W 76 bp and *EBNA1* 99 bp to identify

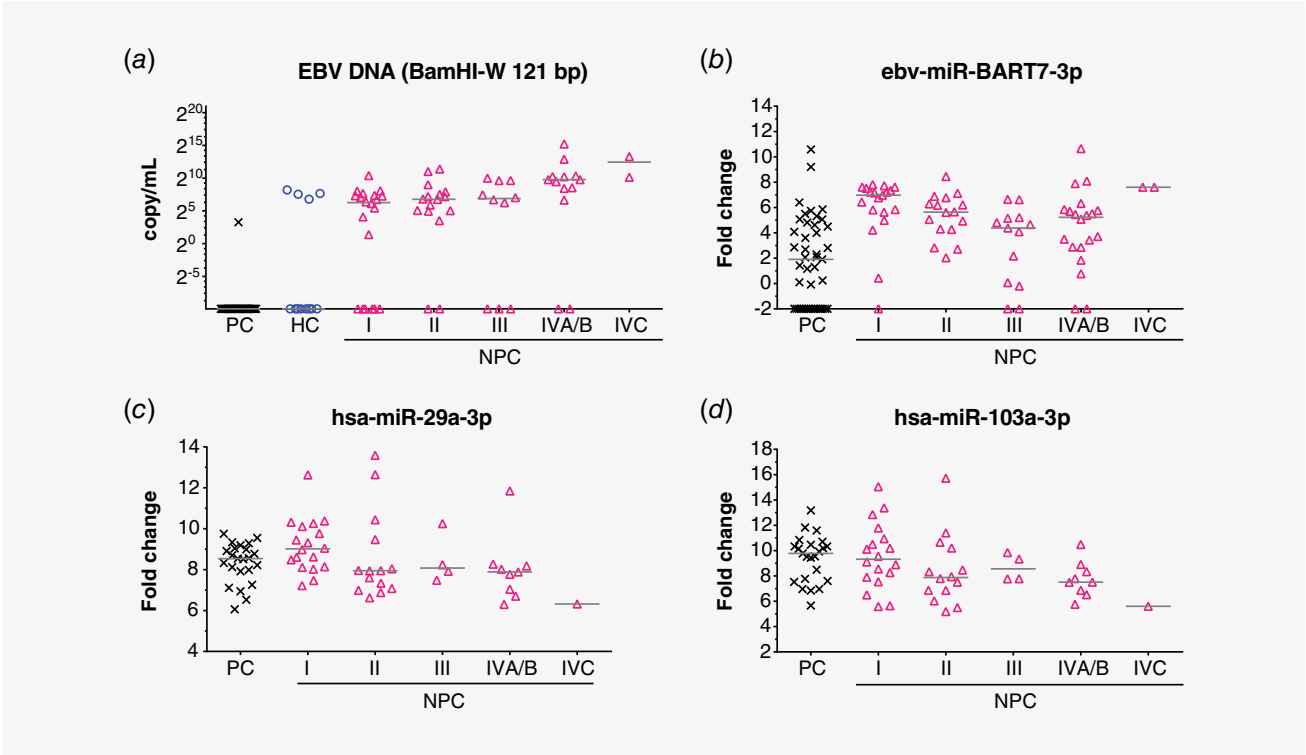

Figure 2. Evaluation of newly reported plasma biomarkers to detect NPC against controls in our study subset. (*a*) Plasma EBV DNA trend as measured by BamHI-W 121 bp is similar to the other two EBV DNA tests in Figure 1. (*b*) NPC patients generally had higher plasma levels of ebv-miR-BART7-3p. A portion of healthy donors also had detectable level of plasma ebv-miR-BART7-3p. (*c*, *d*) Decreasing plasma levels of hsa-miR-29a-3p and hsa-miR-103a-3p were observed from early stage NPC to advanced stage NPC. Plasma levels of these two human miRNAs were not significantly different between population controls and Stage I NPC ($p > 0.05$). Samples with undetectable plasma BamHI-W 121 bp were arbitrarily set as 0.001 copy/ml and samples with undetectable plasma ebv-miR-BART7-3p were arbitrarily set as −2 fold change over detection limit. Abbreviations: HC, hospital control; PC, population control. [Color figure can be viewed at wileyonlinelibrary.com]

NPC against hospital controls were 90.4% and 99.2%, respectively (Table 1). BamHI-W 76 bp being the EBV DNA test with highest sensitivity to detect NPC had 96.7% (29/30) sensitivity to detect Stage I NPC, 96.7% (58/60) sensitivity to detect early stage (Stages I and II) NPC and 97.4% (226/232) sensitivity to detect all NPC (Table 1). Based on recent findings that NPC patients had significantly longer fragment lengths of plasma EBV DNA compared to non-NPCs,[21] the new BamHI-W 121 bp test was evaluated in a subset of our study samples with more early stage NPC cases as well as cases with false positive results as determined by the two common EBV DNA tests (Fig. 2*a*). When testing NPC against controls, improved specificity but decreased sensitivity was found with BamHI-W 121 bp as compared to BamHI-W 76 bp (Supporting Information Fig. S2).

Plasma anti-EBV antibodies

Moderately good to excellent test–retest reliability (ICC of 0.837–0.998) was achieved by commercially available ELISA tests measuring plasma VCA IgA, EBNA-1 IgA, EA IgA and EA IgG (Supporting Information Fig. S1*c*).

Comparison of ELISA results between NPC patients and controls showed that plasma level of anti-EBV antibodies was generally higher in NPC patients as compared to controls. No obvious trend was observed across different NPC stages and high levels of plasma anti-EBV antibodies were observed in some controls (Figs. 1*c*–1*f*). Among these four anti-EBV antibody tests evaluated in our study, VCA IgA and EA IgG consistently had higher AUC values to detect early stage NPC against all controls, while EBNA-1 IgA consistently showed the lowest AUC values among the established biomarkers (Fig. 3 and Table 1).

Plasma miRNAs

Plasma ebv-miR-BART7-3p, hsa-miR-29a-3p and hsa-miR-103a-3p were shortlisted for validation in a subset of our study samples enriched with more early stage NPC cases (Table 1). In general, plasma ebv-miR-BART7-3p levels were higher in NPC compared to population controls and a portion of population controls also had detectable plasma ebv-miR-BART7-3p (Fig. 2*b* and Table 1). Similar median levels of plasma hsa-miR-29a-3p and hsa-miR-103a-3p were observed between population controls and Stage I NPC (Figs. 2*c* and 2*d*). It appeared that there was a decreasing trend in plasma hsa-miR-29a-3p and hsa-miR-103a-3p with the advancement of NPC stage (Figs. 2*c* and 2*d*).

Combination of plasma biomarkers for the detection of NPC

In order to evaluate if combination of plasma biomarkers may improve NPC detection, decision tree modeling was carried out on our data set comprising of 187 NPC cases and 106 population controls with available results of six plasma biomarkers (Table 2 and Supporting Information Table S3). BamHI-W 76 bp test alone appeared to be sufficient for the detection of NPC, and appeared to be essential in all seven decision tree models (Table 2). Models 2, 5

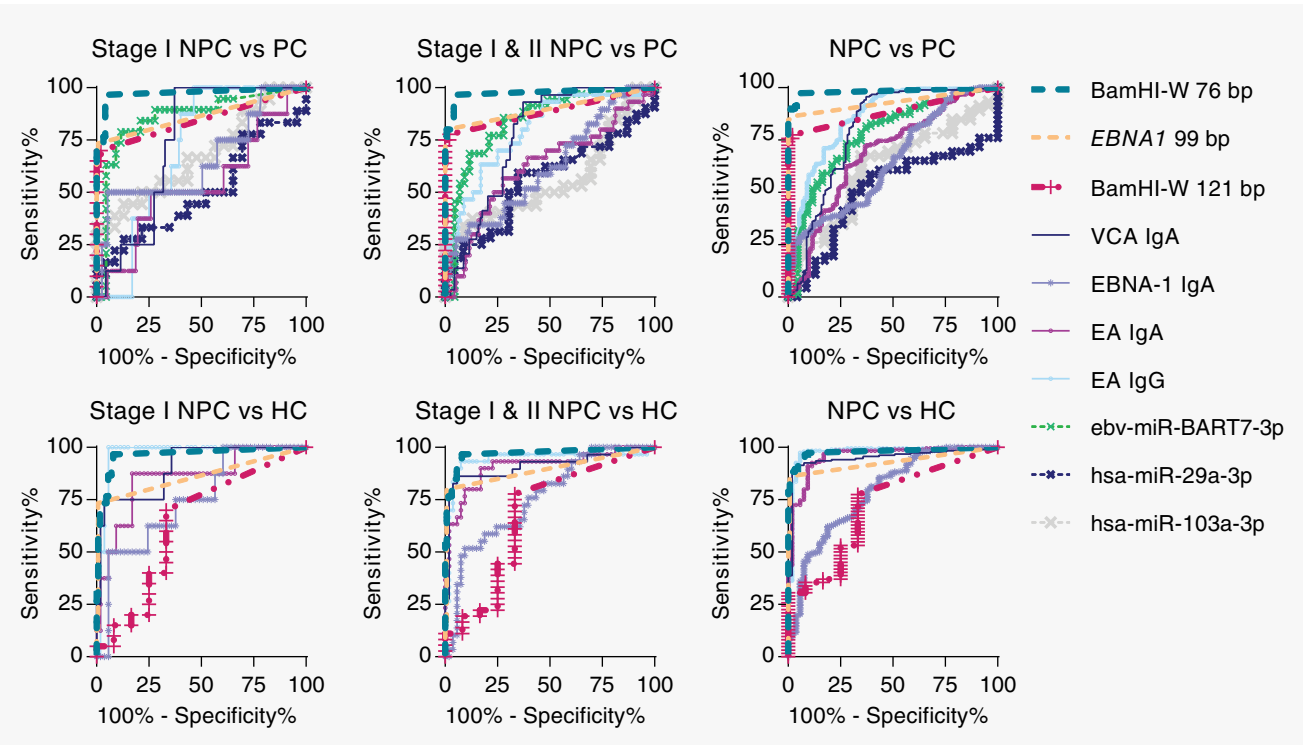

Figure 3. ROC analysis of 10 plasma biomarkers. BamHI-W 76 bp test (dark green dash line) consistently appeared to be the test with highest AUC values while EBNA-1 IgA (purple line) consistently appeared to be the test with lowest AUC values among the six established biomarkers. AUC values and numbers of test subjects can be viewed in Table 1. [Color figure can be viewed at wileyonlinelibrary.com]

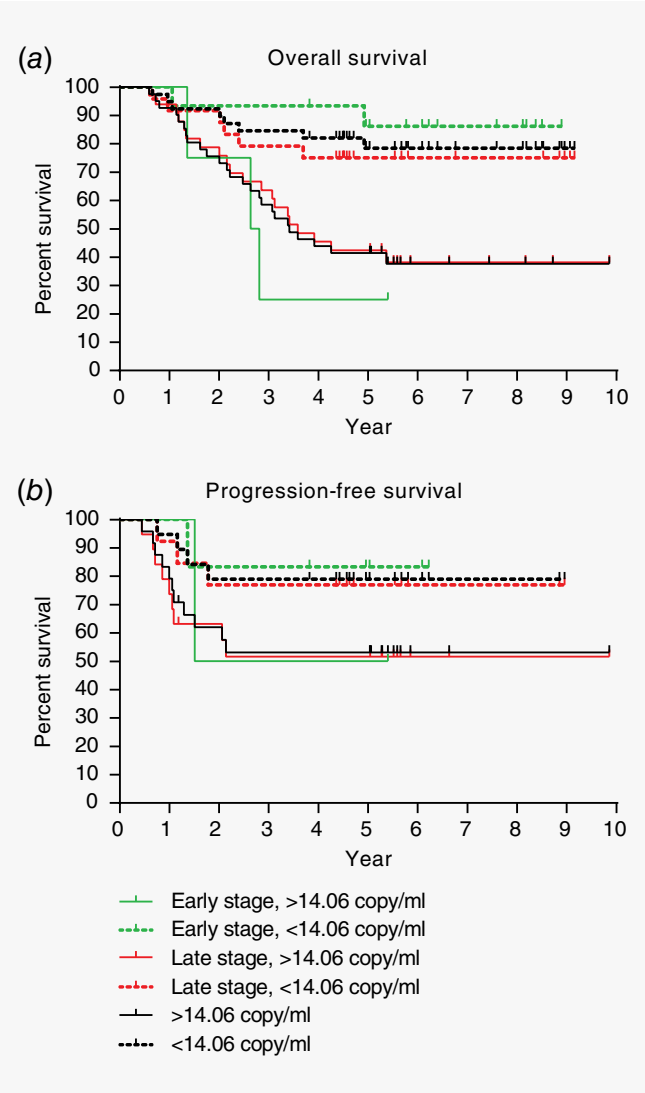

Figure 4. Prognostic value of EBNA1 99 bp test. NPC patients with plasma EBNA1 99 bp >14.06 copy/ml had poorer (*a*) overall survival and (*b*) progression-free survival as compared to those with less plasma EBNA1 99 bp level. EBNA1 99 bp is a good prognostic marker regardless of early or late stages. [Color figure can be viewed at wileyonlinelibrary.com]

and 7 suggested that BamHI-W 76 bp test alone is sufficient (Table 2). Models 1, 3, and 4 suggested that combining VCA IgA with BamHI-W 76 bp test can improve specificity at the expense of reduced sensitivity while Model 6 suggested that combining EA IgG with BamHI-W 76 bp test can further increase sensitivity at the expense of decreased specificity (Table 2 and Supporting Information Fig. S3).

Plasma EBV DNA load as a prognosis marker for NPC overall survival

Survival information and test results of six plasma biomarkers were available for a subset of our NPC cases who had completed radical treatment (n = 80, Supporting Information Table S3). ROC analysis and decision tree modeling were carried out to evaluate if any of these six plasma biomarkers had prognostic value for the survival of these NPC patients (Table 2). According to ROC analysis, *EBNA1* 99 bp was the only biomarker with AUC > 0.7 (Table 2). With a cutoff at 14.06 copy/ml, *EBNA1* 99 bp could identify NPC patients with poor overall survival (Fig. 4*a*) as well as poor progression-free survival (Fig. 4*b*) in both early stage and late stage NPC (Fig. 4). Decision tree modeling supported findings from this ROC analysis, revealing that *EBNA1* 99 bp with cutoff at about 14 copy/ml (Models 8–12) is sufficient for prognosis of survival while increasing *EBNA1* 99 bp cutoff to 138 copy/ml (Model 13) led to higher specificity but lower sensitivity in prognosis of survival (Table 2). Notably, *EBNA1* 99 bp with cutoff at about 14 copy/ml was still the only biomarker chosen by decision tree modeling even though additional information including age, sex, ethnicity, WHO type and AJCC staging were added into the analysis (data not shown).

Discussion

In our study, 10 plasma biomarkers (BamHI-W 76 bp, BamHI-W 121 bp, *EBNA1* 99 bp, EA IgA, EA IgG, EBNA-1 IgA, VCA IgA, ebv-miR-BART7-3p, hsa-miR-29a-3p and hsa-miR-103a-3p) were systematically analyzed for early detection and prognosis of NPC. These included established and newly reported NPC biomarkers of EBV and human origin.

To our knowledge, published case–control studies which reported 50–86% sensitivity of plasma EBV DNA test for Stage I NPC had only analyzed two to 22 cases.[15,19] Our study which include larger sample size of Stage I NPC (n = 30) for plasma EBV DNA test revealed 96.7% (29/30) sensitivity to detect Stage I NPC. Besides larger sample size, our improved sensitivity findings may be due to lower qPCR platform detection limit (25 copy/ml) achieved with usage of more advanced qPCR master mix in our study as compared to other studies.[15,19] Our findings from comparison of BamHI-W 121 bp test and BamHI-W 76 bp test (Supporting Information Fig. S2) support the notion that the larger the qPCR amplicon size, the more specific but less sensitive is the EBV DNA qPCR test. This is consistent with the findings reported earlier which compared the performance of *EBNA1* 213 bp test and *EBNA1* 99 bp test.[40] It is estimated that increase in input volume by eight times may compensate the sensitivity issue of BamHI-W 121 bp test as compared to BamHI-W 76 bp test, hypothetically from qPCR C_q 40 (undetected) to C_q 37, but will incur higher cost and larger effort in sample processing and DNA extraction. Interestingly, four hospital controls were positive in both plasma BamHI-W 76 bp and BamHI-W 121 bp tests (Supporting Information Fig. S2). It is possible that these tests were sensitive enough to detect NPC in cases which were too early to be detected clinically. A follow-up on these individuals to check on event of NPC will be interesting.

In the EBV genome, there is only one copy of *EBNA1* gene while BamHI-W region may be reiterated by 7 to 11 repeats.[41] Prevalent EBV in different populations may differ in the numbers of BamHI-W region repeats, making prognostic cutoff value of

pretreatment plasma BamHI-W 76 bp level deduced from one cohort not optimal for another cohort.[42–44] If plasma BamHI-W 76 bp test results are intended to be used for prognosis, EBV DNA clearance rate calculated from pretreatment and posttreatment plasma EBV DNA load may be analyzed to rule out interindividual variability. Indeed, in a systematic review and meta-analysis on the prognosis of NPC by plasma BamHI-W 76 bp test, Zhang *et al.* showed that cutoff for EBV DNA clearance rate was comparable among studies cohort.[42] In our study, pretreatment plasma *EBNA1* 99 bp and BamHI-W 76 bp tests had similar prognostic values (AUC 0.709 and 0.680, respectively). Unlike BamHI-W 76 bp test, pretreatment plasma *EBNA1* 99 bp test is not affected by interindividual variability and do not require multiple sampling to calculate EBV DNA clearance rate. It would be interesting to investigate if the cutoff value of pretreatment plasma *EBNA1* 99 bp level deduced in our study is applicable to future follow-up studies.

Our study had served as an external independent study and validated the diagnostic performance of two newly reported biomarkers (BamHI-W 121 bp and ebv-miR-BART7-3p). Specificity of ebv-miR-BART7-3p appeared to be less optimal as it was detected in about 28% (12/43) of population controls (Table 1), which is in line with recent findings from Ramayanti *et al.*[32] but not Gao *et al.*[29] The discrepancy may be due to the differences in PCR primers. Meanwhile the diagnostic performance of hsa-miR-29a-3p and hsa-miR103a-3p reported elsewhere[25] could not be reproduced in our study, possibly due to inclusion of more early stage NPC and less advanced stage NPC in our analysis. Consistent with findings from a previous report,[25] differences in plasma hsa-miR-29a-3p levels seemed to be more apparent only when comparing controls to advanced stage NPC (Fig. 2*c*). It is possible that four other miRNAs (ebv-BART2-5p, ebv-miR-BART13-3p, hsa-miR-483-5p and hsa-let-7c) that are not included for validation in our study may perform well as early diagnosis markers for NPC. EBV DNA markers are already well established for NPC screening. From a clinical utility viewpoint, the additional value of including non-EBV markers may be higher than the additional value of including another EBV marker in the NPC detection panel. Our study indicates that much effort is still needed to identify a combination panel of EBV markers and non-EBV markers that will benefit the detection of not only the majority of NPC cases which are EBV positive but also the small subset of NPC cases which are EBV negative.

Conclusions

Our study provides important information to policy makers in LMICs who have limited health care resources to plan a more cost-effective NPC screening and monitoring strategy for the apparently healthy asymptomatic controls. We showed that the diagnostic performance of established biomarkers to detect NPC in local general population were comparable to findings of studies from another NPC endemic area[15] and plasma BamHI-W 76 bp test is superior for early detection of NPC. Comparison of plasma biomarkers in NPC patients and local hospital controls suggests that plasma EBV DNA test could identify NPC cases among individuals who visit the hospital for other conditions in local setting, thus allowing for opportunistic screening. Combined biomarker tests from single sampled specimens can improve NPC detection specificity (with slight decrease in sensitivity) and avoid logistic problems of resampling. Plasma *EBNA1* 99 bp test may have important prognostic value and could be used to stratify NPC patients for different clinical management.

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