Review Article

Prognostic value of Epstein-Barr virus biomarkers for nasopharyngeal carcinoma staging and post-treatment surveillance

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Abstract: The relationship between Epstein-Barr virus (EBV) infection and endemic nasopharyngeal carcinoma (NPC) has facilitated more than 40 years of biomarker-inspired translational research. Serologic and nucleic acid EBV biomarkers span the spectrum of this disease from population-level early detection, pre-treatment prognostication, response-adapted therapy, and long-term surveillance. Plasma EBV DNA remains the cornerstone of biomarker prognostication and surveillance for NPC, and there is increasing high-quality evidence that it merits inclusion in future staging systems. Recently-completed and ongoing biomarker-adapted clinical trials will determine whether biomarker-adapted management will become the standard of care. The HKNPCSG-0502 randomized trial demonstrated that post-treatment EBV DNA is prognostic but not predictive for response to adjuvant chemotherapy (AC), while the ongoing NRG-HN001 randomized trial may ultimately support the omission of AC in most patients. The next generation of biomarker-informed clinical trials may integrate early response to induction chemotherapy and/or immunotherapy. In this review, we discuss the clinical role and prognostic performance of EBV-based biomarkers for pre-treatment staging and post-treatment surveillance. In particular, we synthesize the available evidence which suggests that biomarker-informed staging systems might improve upon anatomic staging, but highlight the challenges in inter-laboratory reproducibility inherent to diagnostic assays without international standardization. Thereafter, we review a breadth of evidence which supports that undetectable post-treatment EBV biomarkers are highly specific for long-term cure. Finally, we contextualize emerging biomarkers that may further improve prognostication. Although these novel biomarkers have yet to supersede plasma EBV DNA in clinical performance, they may complement EBV DNA and identify the subset of patients at highest risk for clinical relapse.

Keywords: Nasopharyngeal carcinoma (NPC); Epstein-Barr virus (EBV); biomarkers; staging; surveillance

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Background

There is marked geographic variation in the incidence of nasopharyngeal carcinoma (NPC), with a lifetime risk as high as 1.8% in Guangdong, China (1). The Epstein-Barr virus (EBV)-associated variant of NPC comprises nearly all cases in endemic regions (2). This epidemiologic association between EBV and NPC was first observed decades ago, and since that time numerous EBV-based biomarkers have been developed

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to detect anti-EBV antibodies, circulating EBV nucleic acids, and circulating tumor cells (CTCs) (3-7). Although EBV is a nearly ubiquitous infection among adults worldwide, EBV-associated NPC has extreme regional and ethnic restriction that remains largely unexplained (8). Although human genome-wide association studies have identified human germline loci with modest attributable risk for NPC, select high-risk EBV variants appear to confer much more substantial risk in high-incidence populations (9,10).

Following primary infection, EBV undergoes lytic replication in the pharynx and establishes lifelong latent infection in B cells as an episome (11). EBV within latently-infected B cells can intermittently reactivate and replicate, shedding virions into the bloodstream that may reinfect epithelial cells. Importantly, EBV is not routinely detected in benign nasopharyngeal epithelium, suggesting that host or environmental factors must contribute to the persistent epithelial latent infection that precedes neoplasia. Clinically, the presence of EBV within NPCs can be demonstrated by fluorescence *in situ* hybridization of abundant non-coding RNAs (EBERs). It is this interaction between germline/environmental epithelial susceptibility and the oncogenic properties of EBV that is the current model of NPC pathogenesis (12).

Worldwide, NPC is the second leading cause of head and neck cancer mortality (13). Because NPC has a propensity for nodal metastasis, most unscreened patients present with locoregionally-advanced disease, for which the current standard of care is intensity-modulated concurrent chemoradiotherapy (CCRT) with or without induction (IC) or adjuvant chemotherapy (AC) (14-16). Although this approach achieves high rates of locoregional control, up to 30% of patients develop distant metastasis (14). Complex multidisciplinary care is required for treatment of these patients, and the aforementioned EBV-based biomarkers are increasingly recognized for their potential to guide risk-adapted treatment intensification or de-intensification. Similarly, many have proposed integrating these biomarkers into the traditional American Joint Committee on Cancer (AJCC) anatomic tumor, nodal, metastasis (TNM) staging system for improved prognostication, discrimination, and consistency (17,18).

In this review, we discuss the clinical role and prognostic performance of EBV-based biomarkers for pre-treatment staging and post-treatment surveillance. In particular, we synthesize the available evidence which suggests that biomarker-informed staging systems might improve upon anatomic staging, but highlight the challenges in inter-laboratory reproducibility inherent to diagnostic assays without international standardization. Thereafter, we review a breadth of evidence which supports that undetectable post-treatment EBV biomarkers are highly specific for long-term cure. Finally, we contextualize emerging biomarkers that may further improve prognostication.

Plasma EBV DNA PCR testing and harmonization

Although serologic EBV assays have been widely studied for early detection of preclinical NPC, these assays have had limited utility in pre-treatment prognostication and surveillance, in contrast to nucleic acid amplification tests (19). A quantitative real-time polymerase chain reaction (qPCR) to noninvasively detect cell-free EBV DNA in plasma was first described more than 20 years ago (4). This specific method amplifying the *BamHI-W* tandem-repeated fragment has been the cornerstone of biomarker-informed pre-treatment prognostication, post-treatment surveillance, and ongoing risk-adapted clinical trials. In contrast to commercial assays which amplify single-copy regions of the EBV genome encoding viral proteins (*EBNA-1*, *LMP-1*), the tandem-repeated nature of the *BamHI-W* sequence facilitates increased analytical and clinical sensitivity, although the number of copies per EBV genome may vary (20,21). After development of the *BamHI-W* qPCR assay by Lo and colleagues (4), Chan and colleagues further characterized the nature of EBV DNA in plasma and demonstrated that it remains in the supernatant and not the pellet after ultracentrifugation (22). With additional experiments that revealed the majority of DNA fragments in plasma were shorter than 181 nucleotides, it was deduced that EBV DNA in plasma is naked and not contained within intact virions. As such, EBV DNA in the plasma of patients with NPC does not suggest the presence of circulating virions but rather reflects cell-free DNA shed from infected neoplastic epithelium. Although whole blood may have certain advantages over plasma for detection of EBV DNA in the post-transplantation setting, the presence of latently-infected lymphocytes in whole blood would result in false positives in the NPC patient population, as cell-free EBV DNA is derived from epithelium (23-25).

While plasma and serum have been the primary matrices for population-level screening, nasopharyngeal PCR has also been investigated to triage screen-detected patients or detect local recurrences after radiotherapy (26,27). However, nasopharyngeal or salivary swabs alone are inadequate for surveillance or pre-treatment prognostication due to the

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propensity for NPC to metastasize to lymph nodes and distant organs. Accordingly, the presence of cell-free DNA in plasma facilitates monitoring of nearly all tissues in totality, which is in contrast to pharyngeal specimens. Many investigators have leveraged this to explore plasma EBV DNA as not only a qualitative biomarker for presence of malignancy but also a quantitative biomarker as a surrogate for disease burden before, during, and after radiotherapy (28). For example, Lv and colleagues described four prognostic NPC phenotypes based on plasma EBV DNA response during induction chemotherapy, and propose that these phenotypes be leveraged in treatment intensification or de-intensification protocols (29). As the burden of neoplastic cells decreases or increases in a given patient, there is a concomitant proportional change in cell-free EBV DNA that is detected in the quantitative PCR reaction.

Notably, EBV PCR testing has yet to be standardized across institutions and myriad approaches to prognostication before, during, and after radiotherapy have been assessed. The critical importance of assay harmonization for biomarker-informed management has been highlighted in multi-institutional studies that demonstrate significant inter-laboratory quantitative variability (6). Typically, a log-transformed coefficient of variation below 20% within and between laboratories is desirable for quantitative PCR assays. Reproducibility can be improved with harmonization of external calibrators and reagents used in qPCR, and formal recommendations from a National Cancer Institute-convened workshop have been made to promote assay standardization (30). While post-transplantation EBV monitoring is routinely reported in WHO international units traceable to the NIBSC EBV international standard, this practice has not been adopted for NPC despite variability introduced by external calibrators (21,31). Although digital PCR may permit absolute quantitation without these external calibrators, the inter-laboratory performance of digital *vs.* real-time PCR remains unknown (*Figure 1*). With increasing multi-institutional evidence that a biomarker-informed staging system offers superior prognostication, assay standardization is of paramount importance.

Pre-treatment prognostication and biomarker-informed staging

The earliest studies quantifying EBV DNA in plasma noted differences in pre-treatment copy number among patients with early *vs.* advanced anatomic stage and presence or absence of post-treatment relapse (4,32). These investigators recognized that EBV DNA copy number in plasma correlated with tumor burden and was also prognostic for long-term outcome. Since that time, numerous studies have assessed the prognostic value of plasma EBV DNA copy number adjusted for anatomic stage, and several large studies have proposed biomarker-informed staging systems based on partitioning analyses.

Before assessing the prognostic value of pre-treatment EBV DNA, it is important to consider differences among studies in inclusion criteria, staging systems, imaging modalities, treatment, and plasma EBV DNA quantitation (*Figure 2*) (3,18,32-37). While all studies amplify the *BamHI-W* fragment, variable volume of extracted plasma (200–800 µL), elution volumes (50–100 µL), eluate volume in the qPCR reaction (2–10 µL), and reagents/calibrators could contribute to imprecision or systematic differences in quantitative accuracy that yield differing prognostic cutoffs (500–40,000 copies/mL). With the exception of the earliest series, most studies propose that 1,500–4,000 cp/mL is associated with an increased risk of relapse, with generally similar hazard ratios. Importantly, the optimal cutoff may vary among these studies due to inherent differences in stage distributions and treatment paradigms across institutions and time periods. The pooled results of these select studies across institutions, staging systems, and treatment paradigms indicate a relatively consistent progression-free survival (PFS) hazard ratio (*Figure 2*). These findings are similar to a systematic review and meta-analysis including studies published from 2001–2014 (37).

Since 2015, there have been several large studies conducted with the specific objective of improving upon the AJCC anatomic staging system via integration of pre-treatment plasma EBV DNA. In 2015, Tang *et al.* published a study including more than 6,300 patients treated at Sun Yat-sen University Cancer Center (SYSUCC) spanning a training cohort (n=3,113), internal validation cohort (n=1,556), and prospective validation cohort (n=1,668) (36). Patients were treated according to the existing evidence at that time, with either radiotherapy alone (AJCC 7 stage I, select stage II), concurrent chemoradiotherapy alone (select stage II), or chemoradiotherapy with induction or AC (stage III-IV). Based on prior evidence, a cutoff of 4,000 cp/mL was assessed, which was independently prognostic for disease-free survival, distant-metastasis-free survival, and overall survival (OS) after adjusting for other prognosticators in both the training and validation datasets. In addition, high-sensitivity CRP (hs-CRP) was

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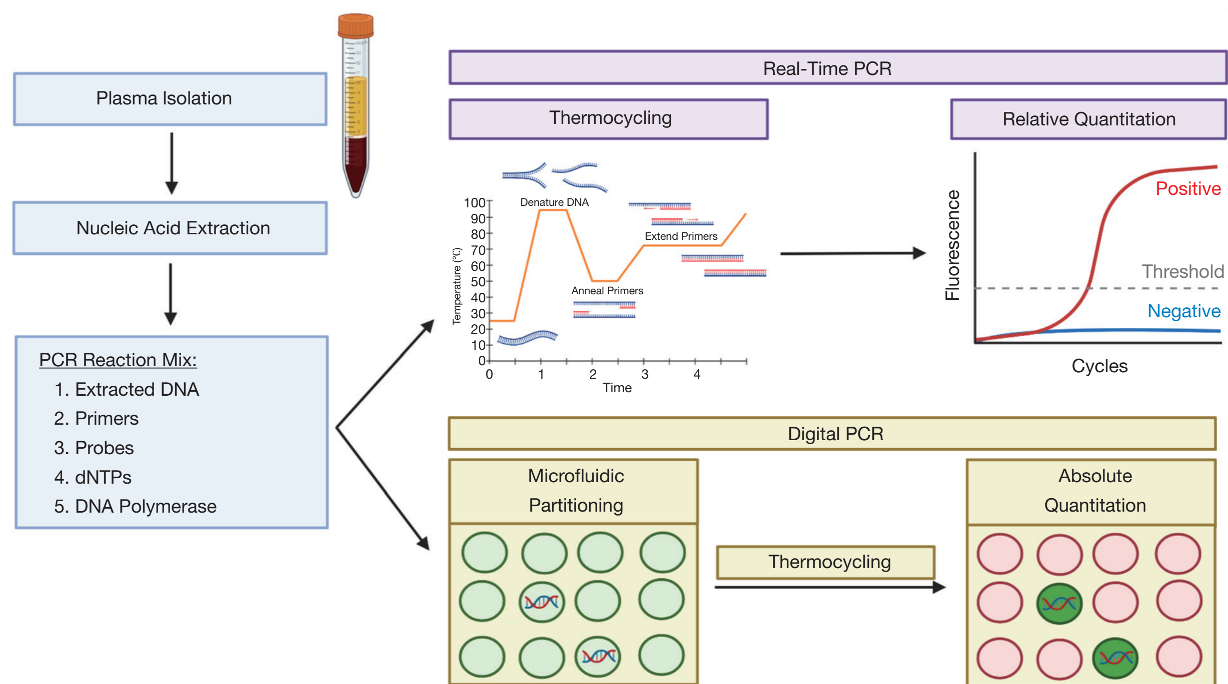

Figure 1 Similarities and differences between real-time and digital polymerase chain reaction in the detection of plasma EBV DNA. Real-time and digital PCR require centrifugation of whole blood for isolation of plasma, followed by extraction of nucleic acids that include cell-free EBV DNA. A buffered PCR reaction mix of extracted nucleic acid, primers, probes, dNTPs, and thermostable DNA polymerase is prepared for both assays. In conventional real-time PCR, simultaneous polymerase chain reaction thermocycling and fluorescence detection facilitates relative quantitation of the amplification target using external calibrators. In digital PCR, thermocycling is preceded by partitioning of the reaction mix into thousands of similarly-sized droplets or plate-based microwells. Thermocycling is then performed, and after thermocycling each partition is assessed for presence or absence of cleaved probe fluorescence. The original concentration of EBV DNA can be calculated from the number of positive and negative partitions using Poisson statistics, thereby permitting absolute quantitation without external calibrators. PCR, polymerase chain reaction; dNTP, deoxynucleoside triphosphate; EBV, Epstein-Barr virus.

also independently prognostic in this study. Based on these findings, the authors proposed and validated a new risk stratification wherein patients with early (stage I-II) or advanced (stage III-IV) disease were categorized by low *vs.* high hs-CRP and/or EBV DNA. An open question is whether hs-CRP (HR 1.82) has sufficient additive prognostic value to EBV DNA (HR 2.99) for widespread adoption, as it is not routinely collected during workup.

In 2019, Lee and colleagues published results from an institutional prospective cohort study with the intention of improving upon the AJCC 8 staging system (18,38). In this cohort of 518 patients with nonmetastatic NPC treated at The University of Hong Kong with definitive intensity-modulated radiotherapy (IMRT), recursive partitioning analysis (RPA) identified 536 cp/mL as the optimal cutoff. Out of simplicity, the authors reasonably selected 500 cp/mL for further evaluation and internal validated this cutoff in bootstrap analysis. As shown in *Table 1*, the final RPA created five stage groups [AJCC 8 T1-4N0-2 with EBV <500 cp/mL (I), T1-4N0-2 with EBV ≥500 cp/mL (II), T1-2N3 (III), T3-4N3 (IVA), and T1-4N0-3M1 (IVB)].

In a rigorous comparison of the biomarker-informed RPA against the AJCC 8 system, the RPA offered superior hazard consistency, hazard discrimination, explanation of variance, and overall performance for PFS, OS, and cancer-specific survival (CSS). Performance of the RPA was also similar or superior to a biomarker-informed multivariable Cox model, which would be one alternative to partitioning analysis. A clinically-relevant consequence of this RPA was the observation that RPA stage I patients using their proposed staging system (AJCC 8 T1-4N0-2 with EBV <500 cp/mL) appeared to derive marginal benefit from the addition of chemotherapy to radiotherapy, highlighting opportunities for risk-adapted clinical trials.

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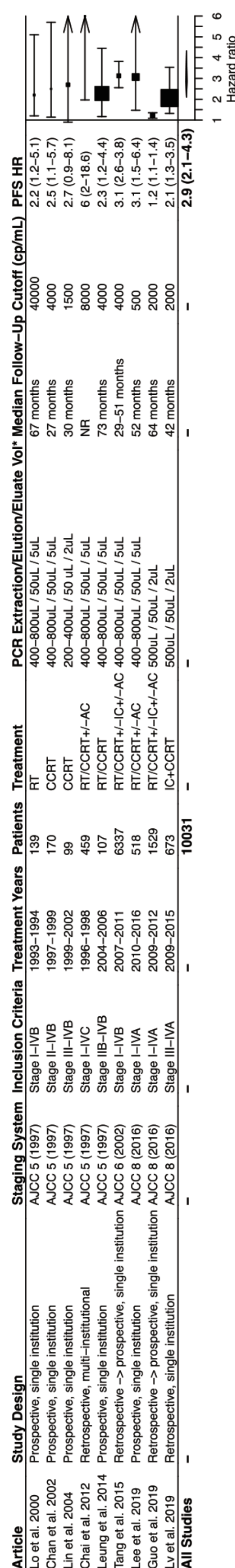

Figure 2 Select publications assessing prognostic value of high *vs.* low pre-treatment EBV DNA. Hazard ratio point estimate and 95% confidence interval are plotted for each study and for weighted average of all studies. Study designs with "retrospective → prospective" notation indicate retrospective training cohorts and prospective validation cohorts. Cutoff denotes the EBV DNA copy number per mL plasma threshold above which is considered to be high *vs.* low. All PCR assays amplified the EBV *BamHI-W* fragment and the human Beta-globin gene as per Lo *et al.* (4). *, select details from these PCR assays include the volume of plasma extracted, elution volume, and volume of eluate used for real-time PCR. RT, radiotherapy; CCRT, concurrent chemoradiotherapy; AC, adjuvant chemotherapy; IC, induction chemotherapy; PCR, polymerase chain reaction; Vol, volume; NR, not reported; PFS, progression-free survival; HR, hazard ratio; EBV, Epstein-Barr virus.

Finally, Guo and colleagues reported results from a similarly-designed study which included 979 patients in a retrospective training cohort and 550 patients in a prospective validation cohort treated at SYSUCC (*Table 1*) (17). An EBV DNA cutoff of 2,000 cp/mL was selected based on prior institutional experience (39). Following RPA, five revised biomarker-informed stages were proposed: T1N0M0 (I), T1-3N0-1M0 and EBV DNA ≤2,000 (IIA), T1-3N0-1M0 and EBV DNA >2,000 or T1-3N2M0 and EBV DNA ≤2,000 (IIB), T1-3N2M0 and EBV DNA >2,000 or T4N0-2M0 (III), T1-4N3M0 (IVA), and T1-4N0-3M1 (IVB). As similarly reported by Lee *et al.*, the biomarker-informed system offered improved hazard consistency, hazard discrimination, explanation of variance, and overall performance. The authors propose several specific recommendations for a future biomarker-informed system. With the routine use of IMRT, the authors observed minimal independent discrimination between T2 and T3 tumors, both of which had high rates of locoregional control. Furthermore, patients with bilateral (N2) nodal disease and low EBV DNA (<2,000 cp/mL) did not exhibit higher rates of distant metastasis relative to patients with N0-1 disease. These findings suggest that the T4 and N3 categories are the most pertinent anatomic staging factors followed by EBV DNA, whereas the prognostic value of the remaining anatomic factors (T1-3N0-2) are relatively minor and conditional on EBV DNA.

Broadly, these select studies in combination with other institutional experiences suggest that a pre-treatment plasma EBV DNA cutoff ranging from 500–4,000 cp/mL is an independent prognosticator for PFS despite variable inclusion criteria, staging systems/modalities, treatment paradigms, and qPCR assays. The aforementioned biomarker-informed staging systems developed via partitioning analysis have the potential to identify groups of patients with similar prognosis despite differing anatomic stage. For example, a recently-presented phase II trial randomized patients with AJCC 7 stage III-IVB NPC and EBV DNA <4,000 cp/mL to either two or three cycles of 100 mg/m^2 cisplatin with concurrent radiotherapy (40). With a median follow-up of nearly three years, two cycles of cisplatin was non-inferior to the standard three cycles and had reduced toxicity. Additional clinical trials are awaited to validate high- and low-risk biomarker groups through selective treatment intensification and de-intensification.

Post-treatment prognostication and surveillance

Circulating cancer antigen biomarkers have long been used

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Table 1 AJCC 8th edition prognostic group stage and select validation studies proposing biomarker-informed staging systems

AJCC 8 T stage	AJCC 8 N stage	AJCC 8 M stage	Plasma EBV DNA (copies/mL)	AJCC 8 TNM	Lee *et al.* 2019	Guo *et al.* 2019
T1	N0	M0	Any	I	–	–
T1	N1	M0	Any	II	–	–
T2	N0–1	M0	Any	II	–	–
T1-2	N2	M0	Any	III	–	–
T3	N0–2	M0	Any	III	–	–
T4	Any N	M0	Any	IVA	–	–
Any T	N3	M0	Any	IVA	–	–
Any T	Any N	M1	Any	IVB	–	–
Any T	N0–2	M0	<500	–	I	–
Any T	N0–2	M0	≥500	–	II	–
T1-2	N3	M0	Any	–	III	–
T3-4	N3	M0	Any	–	IVA	–
Any T	Any N	M1	Any	–	IVB	–
T1	N0	M0	Any	–	–	I
T2-3	N0	M0	≤2,000	–	–	IIA
T1-3	N1	M0	≤2,000	–	–	IIA
T2-3	N0	M0	>2,000	–	–	IIB
T1-3	N1	M0	>2,000	–	–	IIB
T1-3	N2	M0	≤2,000	–	–	IIB
T1-3	N2	M0	>2,000			III
T4	N0–2	M0	Any	–	–	III
Any T	N3	M0	Any	–	–	IVA
Any T	Any N	M1	Any	–	–	IVB

TNM, tumor, node, metastasis; AJCC, American Joint Committee on Cancer; EBV, Epstein-Barr virus.

for pre-treatment prognostication, response assessment, and post-treatment surveillance in prostate specific antigen (PSA), ovarian (CA-125), and gastrointestinal [carcinoembryonic antigen (CEA), CA 19-9] cancers. Similarly, the phenomenon of plasma EBV DNA clearance after radiotherapy has long been recognized as a favorable prognosticator (32). Although most patients do achieve EBV DNA clearance, a subset experience biomarker persistence/recurrence which generally precedes clinical relapse by weeks to months. The sensitivity and specificity of post-treatment EBV DNA for relapse has therefore motivated cooperative-group clinical trials and staging systems for post-treatment prognostication.

Although many institutions practice routine EBV DNA surveillance, it is important to contextualize this biomarker alongside standard imaging-based response assessment. In conjunction with physical examination and nasoendoscopy, NCCN guidelines recommend post-treatment FDG-PET/CT for confirmation of clinical complete response, with EBV DNA surveillance as a category 2B recommendation (41). In a systematic review and meta-analysis of studies assessing the diagnostic accuracy of FDG-PET, CT, and MRI for diagnosis of residual/recurrent NPC, the sensitivity/specificity of PET (95%/90%) exceeded both CT (76%/59%) and MRI (78%/76%) (42). While EBV DNA is a relatively sensitive and specific biomarker for recurrence,

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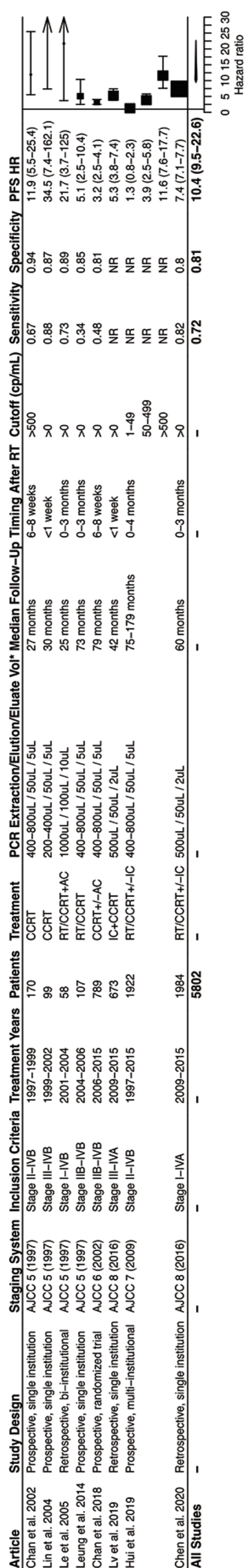

Article	Study Design	Staging System	Inclusion Criteria	Treatment Years	Patients	Treatment	PCR Extraction/Elution/Eluate Vol*	Median Follow-Up	Timing After RT	Cutoff (cp/mL)	Sensitivity	Specificity	PFS HR
Chan et al. 2002	Prospective, single institution	AJCC 5 (1997)	Stage II–IVB	1997–1999	170	CCRT	400–800uL / 50uL / 5uL	27 months	6–8 weeks	>500	0.67	0.94	11.9 (5.5–25.4)
Lin et al. 2004	Prospective, single institution	AJCC 5 (1997)	Stage III–IVB	1999–2002	99	CCRT	200–400uL / 50uL / 5uL	30 months	<1 week	>0	0.88	0.87	34.5 (7.4–162.1)
Le et al. 2005	Retrospective, bi–institutional	AJCC 5 (1997)	Stage I–IVB	2001–2004	58	RT/CCRT+AC	1000uL / 100uL / 10uL	25 months	0–3 months	>0	0.73	0.89	21.7 (3.7–125)
Leung et al. 2014	Prospective, single institution	AJCC 5 (1997)	Stage IIB–IVB	2004–2006	107	RT/CCRT	400–800uL / 50uL / 5uL	73 months	0–3 months	>0	0.34	0.85	5.1 (2.5–10.4)
Chan et al. 2018	Prospective, randomized trial	AJCC 6 (2002)	Stage IIB–IVB	2006–2015	789	CCRT+/–AC	400–800uL / 50uL / 5uL	79 months	6–8 weeks	>0	0.48	0.81	3.2 (2.5–4.1)
Lv et al. 2019	Retrospective, single institution	AJCC 8 (2016)	Stage III–IVA	2009–2015	673	IC+CCRT	500uL / 50uL / 2uL	42 months	<1 week	>0	NR	NR	5.3 (3.8–7.4)
Hui et al. 2019	Prospective, multi–institutional	AJCC 7 (2009)	Stage II–IVB	1997–2015	1922	RT/CCRT+/–IC	400–800uL / 50uL / 5uL	75–179 months	0–4 months	1–49	NR	NR	1.3 (0.8–2.3)
										50–499	NR	NR	3.9 (2.5–5.8)
										>500	NR	NR	11.6 (7.6–17.7)
Chen et al. 2020	Retrospective, single institution	AJCC 8 (2016)	Stage I–IVA	2009–2015	1984	RT/CCRT+/–IC	500uL / 50uL / 2uL	60 months	0–3 months	>0	0.82	0.8	7.4 (7.1–7.7)
All Studies	–	–	–	–	**5802**	–	–	–	–	–	**0.72**	**0.81**	**10.4 (9.5–22.6)**

Figure 3 Select publications assessing prognostic value of high *vs.* low/undetectable post-treatment EBV DNA. Hazard ratio point estimate and 95% confidence interval are plotted for each study and for weighted average of all studies. Cutoff denotes the EBV DNA copy number per mL plasma threshold above which is considered to be high *vs.* low/undetectable. All PCR assays amplified the EBV *BamHI-W* fragment and the human Beta-globin gene as per Lo *et al.* (4). *, select details from these PCR assays include the volume of plasma extracted, elution volume, and volume of eluate used for real-time PCR. RT, radiotherapy; CCRT, concurrent chemoradiotherapy; AC, adjuvant chemotherapy; IC, induction chemotherapy; PCR, polymerase chain reaction; Vol, volume; NR, not reported; PFS, progression-free survival; HR, hazard ratio; EBV, Epstein-Barr virus.

select series have suggested low sensitivity (52%) for local recurrence, highlighting the importance of confirming clinical complete response and continuing clinic-based surveillance (43).

Multiple studies have identified post-treatment EBV DNA clearance (0 cp/mL) as most prognostic for PFS (*Figure 3*). These publications also differed in study design, inclusion criteria, staging systems/modalities, treatment paradigms, timing of plasma collection, and EBV PCR assays. EBV DNA clearance generally has higher specificity for relapse than pre-treatment EBV DNA, and therefore is more prognostic but perhaps less actionable than pre-treatment EBV DNA. In a meta-analysis of six studies published between 2002 and 2014, the pooled hazard ratios for OS and PFS were 4.26 and 5.21 among patients with detectable post-treatment EBV DNA, relative to 2.81 and 2.74 for high *vs.* low pre-treatment EBV DNA. Ongoing efforts seek to integrate longitudinal biomarker response before, during, and after chemoradiotherapy for adaptive treatment (29).

A critical consideration when evaluating these studies is the timing of post-treatment plasma collection for EBV DNA PCR. Similar to the timing of post-treatment imaging for response assessment in head and neck carcinomas, the timing of response assessment could impact sensitivity and specificity, with a greater proportion of false positives if early response assessment is conducted (44). To date, existing studies have collected plasma for EBV DNA PCR as early as seven days and as late as four months after radiotherapy (29,33,45). Furthermore, many studies report collection of plasma "within three months" after radiotherapy, which may introduce heterogeneity in biomarker performance (35,46,47). The prospective randomized HKNPCSG-0502 trial and an early prospective observational study mandated plasma collection 6–8 weeks after radiotherapy, whereas the ongoing NRG-HN001 trial mandates collection within one week after radiotherapy (3,48,49).

Among the earliest publications identifying post-treatment EBV DNA clearance as prognostic was a prospective observational series reported by Chan *et al.* (3) The authors recruited 170 patients with AJCC 5th edition stage II-IVB NPC treated with concurrent chemoradiotherapy. With a median follow-up of 27 months at the time of publication, post-treatment EBV DNA >500 cp/mL was highly prognostic for PFS (HR 11.9). The sensitivity and specificity for relapse at this cutoff were 67% and 94%, respectively. This study, among others, hypothesized that patients with a favorable biomarker response might be spared AC given low rates of relapse in

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this group.

The HKNPCSG-0502 randomized trial is the highest level of evidence thus far for the prognostic and predictive role of post-treatment EBV DNA (49). This trial enrolled 789 patients with AJCC 6th edition stage IIB-IVB NPC across all six oncology centers in Hong Kong. The primary objective of the study was to determine if adjuvant gemcitabine/cisplatin, which has efficacy in the metastatic setting, would improve relapse-free survival among patients without EBV DNA clearance (16). The study was designed with separate prospective observational and randomized arms, wherein patients with undetectable EBV DNA 6–8 weeks after radiotherapy were observed and patients with persistently-detectable EBV DNA were randomly assigned to observation or six cycles of adjuvant gemcitabine/cisplatin. Patients were permitted to receive radiotherapy alone (19%), concurrent chemoradiotherapy (81%), and/or neoadjuvant chemotherapy (25%).

After radiotherapy, 573 (73%) patients had no detectable EBV DNA and were observed. This cohort had excellent 5-year OS (87%) which was not significantly different than patients with 1–49 cp/mL (83%). In contrast, patients with 50–499 cp/mL or ≥500 cp/mL had significant worse 5-year survival (51% and 27%, respectively).

Among the remaining 216 patients (27%) with persistently-detectable EBV DNA, 112 were excluded for randomization (patient refusal, residual disease, distant metastasis, or renal/hematologic function) and 104 (13%) were randomized. In the 52 patients randomized to adjuvant gemcitabine/cisplatin, 50% completed all six cycles and 65% completed at least four cycles. Tolerance to AC was lower than in the metastatic setting (83% four cycles, 58% six cycles), likely due to radiotherapy-associated acute toxicities. With a median follow-up of 6.6 years, there was no improvement in relapse-free survival between the randomized arms. The authors postulated that several factors might have contributed to the trial results, including AC compliance, duration between completing radiotherapy and initiation of chemotherapy (median 91 days), exclusion of patients at higher risk for relapse in the prerandomization evaluation, and inability to eradicate platinum-resident clones with gemcitabine/platinum.

The ongoing NRG-HN001 trial randomizes a similar population of patients with persistently-detectable EBV DNA to non-cross-resident paclitaxel/gemcitabine at an earlier time point (28 days after radiotherapy), which will add further clarity to the predictive *vs.* prognostic significance of post-treatment EBV DNA. Similar to HKNPCSG-0502, The National Health Research Institutes of Taiwan are also enrolling patients with detectable post-treatment EBV DNA on a randomized phase III trial of observation *vs.* adjuvant MEP chemotherapy followed by oral Tegafur-uracil (50). Finally, SYSUCC is enrolling a similar population of patients on a II randomized trial of observation *vs.* oral apatinib (51).

Across the selected studies in *Figure 3*, the pooled sensitivity and specificity for relapse of post-treatment EBV DNA is 72% and 81%, highlighting opportunities for further improvements to biomarker-informed surveillance programs. Chen *et al.* reported results from a large series of 1,984 patients treated at SYSUCC, among which 767 (39%) had detectable EBV DNA after radiotherapy (47). Importantly, the sensitivity for detection of local recurrence (69%) was lower than for regional (80%) or distant (91%) recurrence, concordant with Leung *et al.* (43). Biomarker relapse preceded clinical relapse by a median of 2.3 months, and 82% of patients who had detectable EBV DNA but did not develop relapse cleared EBV DNA during long-term monitoring. These findings have implications for surveillance programs, as a significant proportion of patients with local recurrence can be successfully salvaged, highlighting the importance of early endoscopic and/or imaging-based detection (52).

Novel biomarkers for improved prognostication

Plasma EBV *BamHI-W* DNA remains the most widely utilized biomarker for EBV-associated NPC before, during, and after definitive therapy. However, given the opportunity to improve upon its sensitivity and specificity for relapse, many groups have explored novel or complementary biomarkers that include microRNAs (miRNA), CTCs, and serologic assays.

The EBV genome encodes dozens of BART and BHRF1 miRNAs related to viral gene expression and post-transcriptional modification. In 2012, Liu and colleagues published findings from a miRNA expression analysis of 312 paraffin-embedded NPC tissue specimens collected at SYSUCC, which were compared against 18 specimens of benign nasopharyngitis (53). Forty-one miRNAs were differentially expressed in NPC, and a risk score comprising five miRNAs (miR-93, miR-142-3p, miR-29c, miR-26a, miR-30e) was found to be independently prognostic for disease-free, distant metastasis-free, and OS after adjusting for anatomic stage and other clinical prognosticators. It remains to be determined whether this miRNA signature

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has prognostic value in addition to plasma EBV DNA, and whether this panel of miRNAs can be detected in plasma or standardized across laboratories.

Several groups have also studied circulating EBV miRNAs and their diagnostic and prognostic performance against *BamHI-W* EBV DNA. Zhang *et al.* profiled EBV miRNA expression in EBV latently-infected cell lines, an NPC-derived cell line, and an artificially-infected NP epithelium cell line (54). After identifying differentially-expressed miRNAs, a case-control series demonstrated that a combination of miR-BART7-3p and miR-BART13-3p had 90% accuracy for distinguishing 89 NPC patients from 28 healthy controls. The author subsequently validated these findings in a larger study of 465 NPC nonmetastatic patients and 243 healthy controls (55). In this study, the sensitivities and specificities of miR-BART7-3p and miR-BART13-3p for NPC were each 96–98%, which was marginally improved over EBV DNA (94% sensitive, 91% specific). The combination of these three nucleic acids yielded better diagnostic accuracy than any single biomarker, with an AUC of 0.997 for NPC. Similar to EBV DNA, most NPC patients achieved miR-BART7-3p clearance (82%) and miR-BART13-3p clearance (45%) after radiotherapy. Persistently-detectable EBV DNA and miR-BART7-3p, but not miR-BART13-3p, were independently prognostic for poorer distant metastasis-free survival relative to patients achieving biomarker clearance. Moreover, the combination of EBV DNA and miR-BART7-3p had greater prognostic value than either nucleic acid alone, highlighting opportunities for further risk stratification in clinical trials.

In contrast to nucleic acid amplification techniques, CTCs have potential utility as both prognostic and functional biomarkers. Few studies have characterized CTCs in NPC, but preliminary evidence suggests they may be detectable in most patients. Zhang and colleagues enumerated NPC-associated CTCs using subtraction-enrichment fluorescence *in situ* hybridization, and observed that 92% of patients had identifiable CTCs (56). Importantly, CTCs were defined only by the presence of nucleated cells positive for EpCAM and/or chromosome 8 aneuploidy without CD45, which may not be specific to NPC and might be observed in non-NPC controls or other epithelial malignancies. The investigators observed differential CTC dynamics among patients with or without response to induction chemotherapy, and also reported that aneuploidy appeared to functionally correlate with response to chemotherapy. Because the equipment and technical expertise for CTC detection are not widely available, further studies will be required to determine the role of this biomarker in pre-treatment prognostication and post-treatment surveillance.

Due to heterogeneity in study design, laboratory methodology, inclusion criteria, and statistical analysis, it remains difficult to discern the relative performance of these novel biomarkers in the context of longstanding biomarkers such as *BamHI-W* DNA, *EBNA-1* DNA, and anti-VCA/EA IgA. For this reason, Tan and colleagues conducted a systematic comparison of ten distinct EBV DNA, miRNA, and serologic biomarkers characterized from the same specimens (57). In a large cohort of 251 healthy controls and 232 patients with NPC, the 76-nucleotide *BamHI-W* amplicon had the highest accuracy (96%), which was greater than the 121-nucleotide *BamHI-W* amplicon (90%), *EBNA*-1 (94%), miRNA-BART7-3p (86%), and anti-VCA/EA IgA (57–65%), among others. Because *BamHI-W* may be detected in healthy controls, partitioning analysis suggested that a combination of *BamHI-W* and either anti-VCA IgA or anti-EA IgG may slightly improve specificity. This approach has previously been studied in the context of early NPC detection during population-level screening (58).

Conclusions and future directions

The relationship between EBV infection and endemic NPC has facilitated more than 40 years of biomarker-inspired translational research. Serologic and nucleic acid EBV biomarkers span the spectrum of this disease from population-level early detection, pre-treatment prognostication, response-adapted therapy, and long-term surveillance. Plasma EBV DNA remains the cornerstone of biomarker prognostication and surveillance, and there is increasing high-quality evidence that it merits inclusion in future staging systems. However, inter-laboratory reproducibility remains largely unaddressed, while imperfect sensitivity and specificity highlight opportunities for novel complementary biomarkers to further risk stratify patients. Ongoing and future clinical trials will determine whether biomarker-adapted management will be the standard of care.

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