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Review

A systematic review and recommendations on the use of plasma EBV DNA for nasopharyngeal carcinoma☆

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Clinical application;
Levels of evidence;
Recommendation;
Guideline

Abstract ***Introduction:*** Nasopharyngeal carcinoma (NPC) is an endemic malignancy in Southeast Asia, particularly Southern China. The classical non-keratinising cell type is almost unanimously associated with latent Epstein–Barr virus (EBV) infection. Circulating plasma EBV DNA can be a useful biomarker in various clinical aspects, but comprehensive recommendations and international guidelines are still lacking. We conducted a systematic review of all original articles on the clinical application of plasma EBV DNA for NPC; we further evaluated its strengths and limitations for consideration as standard recommendations.
Methods: The search terms 'nasopharyngeal OR nasopharynx', and 'plasma EBV DNA OR cell-free EBV OR cfEBV' were used to identify full-length articles published up to December 2020 in the English literature. Three authors independently reviewed the article titles, removed duplicates and reviewed the remaining articles for eligibility.
Results: A total of 81 articles met the eligibility criteria. Based on the levels of evidence and

☆ This article was written by members and invitees of the International Head and Neck Scientific Group (www.IHNSG.com).

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grades of recommendation assessed, it is worth considering the inclusion of plasma EBV DNA in screening, pre-treatment work-up for enhancing prognostication and tailoring of treatment strategy, monitoring during radical treatment, post-treatment surveillance for early detection of relapse, and monitoring during salvage treatment for recurrent or metastatic NPC. One major limitation is the methodology of measurement requiring harmonisation for consistent comparability.

Conclusions: The current comprehensive review supports the inclusion of plasma EBV DNA in international guidelines in the clinical aspects listed, but methodological issues must be resolved before global application.

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1. Introduction

Nasopharyngeal carcinoma (NPC) is an endemic malignancy in Southeast Asia, including Southern China [1]. The commonest histological cell type is non-keratinising carcinoma, which is almost unanimously associated with latent Epstein–Barr virus (EBV) infection [2]. Immunohistochemistry and in situ hybridisation studies have consistently demonstrated the incorporation of EBV genomes within NPC tumour cells [3–5]. In addition to the deep interest in studying the role of EBV in the oncogenesis of NPC and the feasibility of prevention [6], extensive research has been conducted to develop biomarkers based on this association.

The importance of developing a sensitive and accurate biomarker cannot be over-emphasised. Major issues in the current clinical management of NPC include (1) late presentation with more than 70% of patients diagnosed with advanced disease, (2) a one-size-fits-all treatment approach guided mainly by TNM staging and (3) difficulty in detecting early relapse for subsequent radical salvage treatment. Therefore, it is important to develop reliable and cost-effective biomarkers for screening/early detection, refinement of prognostication, tailoring of treatment stratification, as well as post-treatment disease surveillance.

Early studies were based on serology (IgA titre) to EBV, and focused primarily on early detection or diagnosis. However, this has limited prognostic value for treatment stratification or subsequent surveillance. The recognition that EBV-associated NPC releases secretory viral genomes into the host's circulation is a milestone discovery, leading to extensive research on the possible clinical applications of plasma EBV DNA [7–20]. Detectable cell-free EBV DNA in plasma/serum of NPC patients was first reported in the late 1990s by Mutirangura *et al.*, and Lo *et al.* [7,21]. The circulating EBV DNA detected is not the intact EBV DNA contained in the virions, but naked (cell-free) small viral DNA fragments of $\leq$82 base pairs (bp) [22]. Table 1 summarises the published studies on plasma/serum EBV DNA in NPC [7,8,21–38].

One of the key factors determining the sensitivity of the qPCR tests is the size of the EBV gene targets, and higher sensitivity can be attained by assays that target small amplicon sizes of either 76 bp or 59 bp when compared with those that target larger amplicon sizes. The method of plasma EBV DNA assay devised by Lo *et al.* [7,39], using RT-qPCR with either a commercial sequencing system or institution-derived system that amplifies a small DNA segment in the BamHI-W fragment region of the EBV genome, is now almost universally adopted; other assays which target EBNA1, EBNA2, BALF5, had been attempted, but did not show consistent superiority (Table 1).

A recent review by Trevisiol *et al.* showed that among the 16 guidance documents published between 2011 and 2017 [40], only one clinical practice guideline (CPG) had evaluated EBV DNA based on a systematic revision, and the recommendation on using plasma EBV DNA for different clinical aspects varies. Only 1 of 4 CPGs recommended that plasma EBV DNA may be considered for pre-treatment work-up and reassessment after initial curative treatment. None of the CPG recommended the test for diagnosis process (screening or differential diagnosis), early detection of relapse or monitoring of response to salvage therapy. Although international guidelines, including that for National Comprehensive Cancer Network (NCCN) and European Society for Medical Oncology, have included the recommendation of plasma EBV DNA in at least one clinical aspect [41,42], comprehensive evidence-based recommendations in other various clinical settings in NPC management are lacking. The present study will present the background information and a systematic review of published data on clinical applications and limitations of plasma EBV DNA for various clinical settings in the management of NPC. The goal is to provide comprehensive recommendations, based on the levels of evidence (LoEs) and grades of (GoRs), for consideration in future guidelines.

Table 1
Selected publications of plasma or serum EBV DNA quantitative polymerase chain reaction assays in nasopharyngeal carcinoma.

Year	Author	Reference	Number of patients	Sample	Assay	Target region	Amplicon size	DNA load (gene copies/ml) (median or mean)	Volume extracted	Sensitivity	Specificity
1999	Lo *et al.*	[7]	57	Plasma	TaqMan	BamH1-W	76	21,058	130–800	96	93
2000	Shotelersuk *et al.*	[22]	21	Plasma	Nested PCR	EBNA2	168	NA	200	71	87
2004	Shao *et al.*	[24]	120	Plasma	TaqMan	BamH1-W	76	6200	500–1000	96	92
2004	Lin *et al.*	[8]	99	Plasma	TaqMan	BamH1-W	76	1461	200–400	95	100
2004	Leung *et al.*	[25]	139	Plasma	TaqMan	BamH1-W	76	NA	400–800	95	98
2004	Shao *et al.*	[26]	150	Plasma	TaqMan	BamH1-W	76	82,500	500	92	88
2007	Twu *et al.*	[27]	114	Plasma	TaqMan	BamH1-W	76	4669	200–400	93	100
2007	O *et al.*	[28]	22	Plasma	TaqMan	BamH1-W	76	NA	NA	77	92
2008	Chan *et al.*	[23]	74	Plasma	TaqMan	BamH1-W	59	5560	800	99	96
2009	Luo *et al.*	[29]	160	Plasma	TaqMan	BamH1-W	NA	NA	NA	69	88
2012	Chai *et al.*	[30]	390	Plasma	TaqMan	BamH1-W	76	6582	400–800	90	88
1998	Mutirangura *et al.*	[21]	42	Serum	Conventional PCR	EBNA2	168 and 184	NA	2000–3000	31	100
2000	Shotelersuk *et al.*	[22]	146	Serum	Nested PCR	EBNA2	168	NA	200	57	87
2002	Hsiao *et al.*	[31]	36	Serum	Conventional PCR	EBNA1	241	NA	200	75	89
2003	Chan *et al.*	[32]	55	Serum	TaqMan	BamH1-W	192	NA	400	56	98
2004	Kondo *et al.*	[33]	64	Serum	TaqMan	BALF5	90	3625	100–200	86	89
2004	Fan *et al.*	[34]	93	Serum	Competitive PCR	EBER1	210	11,211	150–500	69	97
2004	Krishna *et al.*	[35]	29	Serum	Conventional PCR	EBNA1	262	NA	NA	58	83
2006	Yang *et al.*	[36]	19	Serum	TaqMan	BamH1-W	83	5138	200	74	95
2009	Mo *et al.*	[37]	62	Serum	TaqMan	BamH1-W	76	1062	200	87	100
2012	Baizig *et al.*	[38]	66	Serum	TaqMan	BXLF1	169	NA	200	53	100

2. Methods

2.1. Search strategy for systematic review and recommendation statement development

We performed a systemic literature search of all publications on plasma EBV DNA in NPC in accordance with PRIMSA guidelines (Fig. 1). PubMed-MEDLINE, Embase (Ovid), Scopus, Cochrane Library and CINAHL were searched using the search terms 'nasopharyngeal OR nasopharynx', and 'plasma EBV DNA OR cell-free EBV OR cfEBV'. Our search focused on full-length journal articles published in the English literature. Case reports or small series with less than 40 patients were excluded. The search date started from the inception of each database to December 31, 2020. Table 2 shows the articles that fulfilled the search criteria. These were categorised in accordance with the time point(s) when plasma EBV DNA was measured and whether the lowest detection limit of plasma EBV DNA was reported in the article. Overall, 81 articles were identified, including 4 meta-analysis, 36 prospective studies, 39 retrospective studies and 2 combined retrospective and prospective studies [7–17,19–21,24,25,30,39,43–105]. Two retrospective studies reported the use of plasma EBV DNA to monitor treatment outcomes in paediatric patients [58,100], while all others focused on adult patients. The LoEs and GoRs of each eligible article were gauged in accordance with the adapted Infectious Diseases Society of America-United States Public Health Service Grading System [106].

3. Results

3.1. Value of plasma EBV DNA in early detection of previously undiagnosed NPC

Eight articles reported on the role of plasma EBV DNA in NPC screening [7,21,24,25,49,52,68,87]. The pivotal prospective study by Lo *et al.* showed that 55 of 57 NPC patients (96%) had detectable plasma EBV DNA compared with 3 of 43 (7%) normal individuals [7]; the lowest detection limit was 60 copies/ml, the detection rate and false-positive rate were 96% and 7%, respectively. In addition, the study showed that plasma EBV DNA became undetectable in 7 of 15 patients (47%)

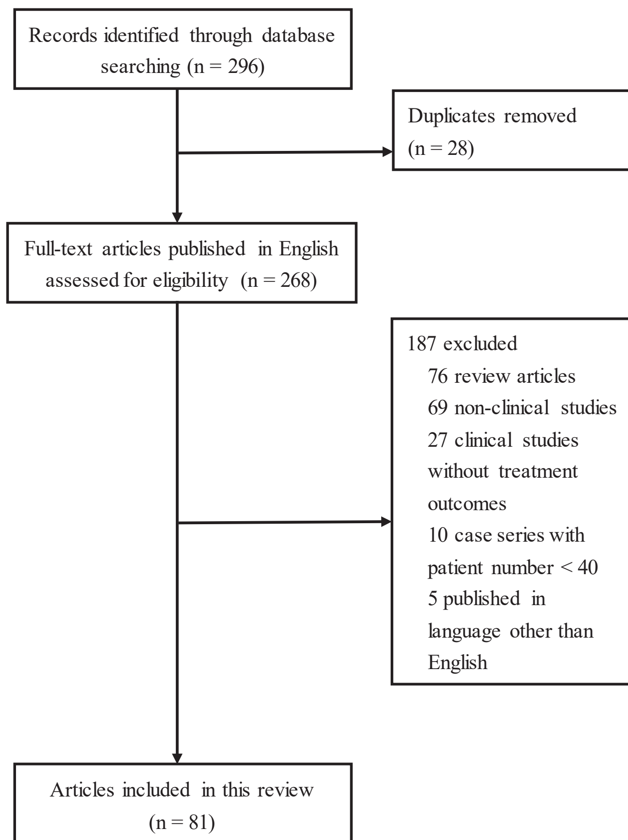

Fig. 1. PRISMA flow chart showing the identification and selection of full-length journal articles published in English.

after radical radiation therapy for non-metastatic NPC, and they all showed complete tumour regression.

Studies have been conducted to compare the value of plasma EBV DNA versus EBV antibodies for NPC screening and diagnosis. Shao *et al.,* in 2004 reported that IgA anti-viral capsid antigen (VCA) was superior for detecting stage I disease, as plasma EBV DNA was not detected in more than 50% of these early cases [24]. However, plasma EBV DNA level correlated with T-category, suggesting that this biomarker reflects tumour burden. Leung *et al.* also investigated the accuracy of plasma EBV DNA and IgA anti-VCA in detecting NPC [25]. With a detection limit of 60 copies/ml, EBV DNA was detected in 132 of 139 patients (95%). Compared with IgA anti-VCA, plasma EBV DNA improved the sensitivity from 72% to 90% for stage I/II disease and from 85% to 98% for stage III/IV disease. The diagnostic sensitivity can further be improved to 99% when both plasma EBV DNA and IgA anti-VCA are combined as a marker panel.

There are at least two large-scale NPC screening programmes using plasma EBV DNA as the screening tool. The first study by Ji *et al.* of 825 participants in two cities (Zhongshan and Sihui) of Guangdong province of China showed that, with the cutoff value set at zero copies/ml, plasma EBV DNA had a sensitivity of 86.8% (33/38 patients) for NPC detected within the first year of follow-up, giving a positive predictive value of 30% (33/110 participants) and a negative predictive value of 99.3% (696/701 participants) [52]. The sensitivity of plasma EBV DNA in detecting NPC was lower in early-stage patients (22/27, 81.5%) than those with advanced NPC (11/11, 100%). For the 14 patients who had NPC detected after 1 year of follow-up, 50% (7 of 14 patients) had their plasma EBV DNA elevated at the baseline. The authors concluded that EBV DNA load may improve the accuracy of diagnosing NPC in high-risk individuals, but its lower sensitivity in early-stage tumours may lead to a false-negative result.

A territory-wide screening study on 20,349 male participants in Hong Kong showed that, with the cutoff value set at 20 copies/ml, the sensitivity and specificity were 97.1% and 98.6%, respectively [68]. In this study with a median follow-up duration of 22 months, 308 participants had elevated plasma EBV DNA on 2 consecutive tests were further investigated, and 34 patients were confirmed to have NPC within 1 year of their follow-up. Among the 19,865 participants without elevated plasma EBV DNA, 1 patient was diagnosed with NPC within 1 year. Although the negative predictive value almost approached 100%, the positive predictive value was only 11.0%. This screening programme did achieve a stage shift towards more stage I–II disease (70% versus 20%; $P < 0.001$), leading to a better 3-year progression-free survival [PFS] (97% versus 70%; $P < 0.001$) [68].

A recently published decision-analytic model study [107], including a hypothetical cohort in China, showed that screening by the combination of plasma EBV DNA and EBV serology was cost-effective in endemic regions: screening could improve 10-year survival from 71.0% to 86.3%, corresponding to a median 10-year reduction of NPC mortality of 52.9% [107].

3.2. Value of pre-treatment plasma EBV DNA for prognostication and staging

More than 30 studies on prognostic outcomes based on plasma EBV DNA at various pre-treatment time points have been reported [8–10,12,15,20,30,42–45,47,48,50,53–55,57,58,60,62,63,67,72–74,76,78–81,88,92,95–97,99,102]. Different titres were suggested as the optimal cutoff values for risk segregation. The study by Lin *et al.* identified 1500 copies/ml to be the best cutoff to segregate long-term survivors from poor survivors [8], whereas Chan *et al.* and Leung *et al.* from the same institution, showed that 4000 copies/ml was a significant prognostic factor for overall survival (OS) [10,14].

At least three meta-analyses confirmed that pre-treatment plasma EBV DNA was prognostic of OS [54,60,72]. Other studies attempted to use plasma EBV DNA in conjunction with other clinicopathological parameters (such as tumour volume, nodal size, and cervical node necrosis, metabolic parameters of

Table 2
List of full publications on plasma EBV DNA for nasopharyngeal carcinoma categorised by the timing of measurement.

Year	Author	Reference	Setting	Category[a]									
				1	2	3	4	5	6	7	8	9	Lowest detected limit reported
1998	Mutirangura *et al.*	[21]	P	√									No
1999	Lo *et al.*	[7]	P	√									Yes
1999	Lo *et al.*	[39]	P			√			√		√	√	Yes
2000	Lo *et al.*	[43]	P			√							Yes
2000	Lo *et al.*	[13]	P					√					Yes
2002	Chan *et al.*	[14]	P							√			Yes
2003	Leung *et al.*	[44]	P			√							Yes
2004	Shao *et al.*	[24]	P	√									No
2004	Leung *et al.*	[25]	P	√	√								Yes
2004	Lin *et al.*	[8]	P			√				√			No
2004	Wei *et al.*	[19]	P								√		No
2006	Leung *et al.*	[10]	P		√	√							Yes
2007	Lin *et al.*	[45]	R			√			√				No
2010	Wang *et al.*	[16]	P								√	√	No
2011	Hou *et al.*	[9]	R			√			√				No
2011	An *et al.*	[17]	R								√	√	No
2011	Wang *et al.*	[46]	P							√			Yes
2012	Ferrari *et al.*	[12]	P			√				√	√	√	Yes
2012	Hsu *et al.*	[15]	P			√		√					No
2012	Chai *et al.*	[30]	R			√							No
2012	Chang *et al.*	[47]	P			√							No
2012	Jin *et al.*	[48]	R			√							No
2013	Chan *et al.*	[49]	P	√									Yes
2013	Wang *et al.*	[50]	R + P			√			√				No
2014	Leung *et al.*	[11]	P					√					Yes
2014	Twu *et al.*	[51]	P						√				No
2014	Ji *et al.*	[52]	R	√									No
2014	Wei *et al.*	[53]	R			√							No
2015	Zhang *et al.*	[54]	M			√		√	√				No
2015	Liu *et al.*	[55]	P			√	√						No
2015	Shen *et al.*	[56]	R								√	√	No
2015	Chen *et al.*	[57]	R			√							Yes
2015	Shen *et al.*	[58]	R			√							No
2016	Lee *et al.*	[59]	P						√				Yes
2016	Peng *et al.*	[20]	R			√			√				No
2016	Zhang *et al.*	[60]	M			√			√				No
2016	Tang *et al.*	[61]	R		√								No
2016	Chen *et al.*	[62]	P			√							No
2016	Du *et al.*	[63]	R			√							No
2016	Wang *et al.*	[64]	R						√				No
2016	Zhang *et al.*	[65]	R		√								No
2016	Zhang *et al.*	[66]	R							√			No
2016	Zhao *et al.*	[67]	R			√			√				No
2017	Chan *et al.*	[68]	P	√									Yes
2017	Lee *et al.*	[69]	P							√			Yes
2017	Xu *et al.*	[70]	R		√								No
2017	Li *et al.*	[71]	R							√			No
2017	Liu *et al.*	[72]	M			√							No
2017	Chen *et al.*	[73]	P			√							No
2017	Prayongrat *et al.*	[74]	R			√		√	√	√			No
2018	Chan *et al.*	[75]	P						√				Yes
2018	Lertbutsayanukul *et al.*	[76]	P					√	√				Yes
2018	Zhou	[77]	R			√							No
2018	Li	[78]	R		√								No
2018	Chen	[79]	P			√							No
2018	He	[80]	R			√		√	√	√			Yes
2018	Liang	[81]	R			√							No
2019	Xie	[82]	M			√		√		√			No
2019	Lee	[83]	P		√								Yes
2019	Guo	[84]	R + P		√								No
2019	Lv	[85]	R				√	√					No
2019	Liang	[86]	R						√				No

(*continued on next page*)

Table 2 (*continued*)

Year	Author	Reference	Setting	Category[a]									
				1	2	3	4	5	6	7	8	9	Lowest detected limit reported
2019	Nicholls	[87]	P	√									Yes
2019	Sun	[88]	R						√				No
2019	Du	[89]	R			√							No
2019	Kitpanit	[90]	P		√								No
2019	Liu	[91]	R								√		No
2019	Huang	[92]	R					√					No
2019	Chen	[93]	P			√		√					No
2019	You	[94]	P									√	No
2020	Chan	[95]	P				√	√					Yes
2020	Liu	[96]	R			√							No
2020	Li	[97]	R			√							No
2020	Zhang	[98]	R			√							No
2020	Shen	[99]	R		√								No
2020	Qiu	[100]	R			√							No
2020	Hui	[101]	P						√				Yes
2020	Zheng	[102]	R		√								No
2020	Chen	[103]	R							√			No
2020	Lin	[104]	R									√	No
2020	Zhou	[105]	R									√	No
Total			M = 4 P = 36 R = 39 R + P = 2	9	11	37	2	12	18	11	7	8	Yes = 23 No = 58

M, meta-analysis; P, prospective; R, retrospective.

[a] Category is based on the time points of plasma EBV DNA measurement as follows: (1) for screening and diagnosis; (2) for staging; (3) at the baseline before treatment; (4) before and during induction chemotherapy; (5) during concurrent chemoradiation (e.g. mid-course); (6) after radical RT and or chemotherapy; (7) surveillance after completion of RT and/or chemotherapy; (8) at relapse; and (9) at metastasis.

positron-emission tomography with integrated computed tomography (PET-CT), cumulative chemotherapy dose intensity) to improve prognostication [47,53,57,63,89,93].

Eleven articles have been published on the use of pre-treatment plasma EBV DNA for refinement of staging for NPC [10,25,61,65,70,78,83,84,90,99,102]. Leung *et al.* showed that plasma EBV DNA had a better correlation with clinical stage than IgA anti-VCA [25]; furthermore, pre-treatment plasma EBV DNA was an independent prognostic factor for OS. Stage I and II patients with a high EBV DNA load of ≥4000 copies/ml had an OS similar to stage III patients [14]. This was the first evidence showing that we can use pre-treatment plasma EBV DNA to segregate poor-risk subgroups among patients with early stages.

Xu *et al.* developed a nomogram based on the incorporation of pre-treatment plasma EBV DNA with the current UICC/AJCC staging system (TNM-8) and showed that this could identify NPC patients who would benefit from induction plus concurrent chemoradiotherapy [70]. Two other studies showed that using recursive partitioning analysis (RPA) to incorporate pre-treatment plasma EBV DNA with TNM-8 could lead to more refined risk groups for prognostication. Lee *et al.* proposed that EBV DNA with a cutoff value of 500 copies/ml could segregate low-risk from intermediate-risk groups [83], whereas Guo *et al.* used a higher cutoff value of 2000 copies/ml [84]. Interestingly, both studies concurred that N3 disease was a stand-alone poor prognostic factor with a 5-year PFS of 63%, regardless of the plasma EBV DNA titres.

Although there is little doubt that pre-treatment plasma EBV DNA has significant prognostic value, a universally accepted prognostic cutoff has yet to be determined. As discussed below, international harmonisation to standardise EBV DNA assays is crucial to ensure reproducibility and clinical applicability worldwide.

3.3. Value of plasma EBV DNA for monitoring response, predicting outcome and stratifying treatment

3.3.1. Response at mid-course of radiotherapy

Plasma EBV DNA is a valuable tool for revealing treatment response both in primary and recurrent settings [11,13,18,19]. Lo *et al.* showed that the median half-life clearance was 3.8 days for patients between the third and seventh week of radiation therapy after an initial rise of plasma EBV DNA due to therapy-induced tumour cell death in the initial radiotherapy period [13]. Patients with more rapid clearance of plasma EBV DNA reflected a better tumour response and subsequently showed better survival outcomes. A recent prospective study by Chan *et al.* on patients treated with radical intensity-modulated radiation therapy revealed that half-time clearance of plasma EBV DNA >15 days was associated with lower distant metastasis-free survival, PFS and OS [95].

Leung *et al.* showed that detectable plasma EBV DNA at midpoint during radiation therapy carried a worse outcome on distant failure, PFS and OS [11]. In this study, 74% of all failures were associated with detectable plasma EBV DNA at midpoint during radiation therapy. Furthermore, plasma EBV DNA concentration positively correlated with urine EBV DNA concentrations, suggesting that urine EBV DNA analysis can be adopted as an ultra–non-invasive test for treatment response monitoring and prognostication [108].

3.3.2. Response to induction chemotherapy for a risk-adapted strategy to determine adjuvant therapy

The retrospective study of 673 patients by Lv *et al.* showed that early responders with a more rapid drop of EBV DNA during induction chemotherapy had a better prognosis than late responders and non-responders [85]. In this study, four distinct groups of patients were identified based on the kinetic of EBV DNA clearance: early responders had the best DFS, followed by intermediate responders, late responders, and the least for those who were treatment-resistant. Following this classification, a biomarker-guided risk adopted treatment strategy was proposed (NCT04072107, currently recruiting patients): the intermediate responders (detectable EBV DNA after one cycle of induction chemotherapy but undetectable EBV DNA after subsequent induction chemotherapy) would receive adjuvant metronomic capecitabine for six months, while high-risk patients (detectable EBV DNA after three cycles of induction chemotherapy or early bounce of EBV DNA during induction phase) would receive concurrent anti-PD-1 therapy (sintilimab) with cisplatin and IMRT followed by adjuvant sintilimab for six months.

3.3.3. Post-treatment response for a risk-adapted strategy to determine adjuvant therapy

Studies have shown that detectable plasma EBV DNA after radical treatment was associated with a very poor prognosis [51,69,101]. Two studies revealed that detectable plasma EBV DNA immediately after radical treatment was prognostic of survival [45,50]. However, the optimal post-treatment time point(s) of EBV DNA measurement remains to be defined. A prospective study by Lee *et al.* on 260 patients treated by radical IMRT with or without concurrent chemoradiation showed that 30 patients (11.5%) still had persistently elevated plasma EBV DNA at eight weeks after IMRT [59], but 20 of them had subsequent complete clearance at later time points without evidence of persistent disease. However, an update of this study revealed that detectable plasma EBV DNA at eight weeks and six months after completion of IMRT were prognostic of PFS, cancer-specific survival and OS [69].

A recently published RPA which incorporated post-treatment plasma EBV DNA of patients recruited into Hong Kong NPC-0502 study as the training set and the retrospective cohort of Sun Yat-Sen University Cancer Center as the validation set revealed that low-risk groups (either stage II/III patients with post-treatment EBV DNA 0 copies/ml, or stage II patients with post-treatment EBV DNA 1–49 copies/ml) had an OS similar to that for stage II disease [101]. This would help to identify low-risk groups who could potentially be spared from adjuvant chemotherapy.

Several randomised-controlled trials (RCT) aim to investigate if patients with detectable EBV DNA post-radical chemoradiation would benefit from additional adjuvant chemotherapy. A retrospective study by Twu *et al.* on patients with persistently detectable plasma EBV DNA one week after completion of radiation therapy demonstrated that the addition of adjuvant oral tegafur-uracil for one year reduced distant relapse and improved OS [51]. However, The Hong Kong NPC-0502 RCT on patients with persistent detectable plasma EBV DNA at 8 weeks following radical chemoradiation showed that the addition of 6 cycles of gemcitabine and cisplatin failed to achieve OS benefit [74]. The NRG-HN001 combined phase 2 and 3 multicentre RCT (NCT02135042) is still ongoing to investigate whether adjuvant chemotherapy can be safely omitted in those who had undetectable EBV DNA after radical chemoradiation and whether adjuvant chemotherapy by gemcitabine and paclitaxel can achieve better survival compared with cisplatin and 5-fluorouracil [109]. Several other phase II and III trials are also ongoing based on a similar strategy: including NCT03544099 (adjuvant pembrolizumab for 2 years), NCT02874651 (adjuvant apatinib for 2 years), NCT02958111 (adjuvant metronomic capecitabine for 1 year) and NCT02363400 (immediate versus delayed chemotherapy with mitomycin, epirubicin and cisplatin followed by tegafur-uracil).

3.4. Value of plasma EBV DNA for post-treatment surveillance

Sixteen articles showed that plasma EBV DNA is valuable for disease surveillance after completion of radical treatment for early detection of relapse [9,20,39,45,50,51,54,59,60,64,67,74–76,80,86,88,101]. At least four studies reported that 51%–67% of patients with local/locoregional recurrence had elevated plasma EBV DNA, and between 86% and 96% of those with distant metastases had detectable plasma EBV DNA [30,110–112]. Therefore, it is logical to postulate that plasma EBV DNA elevation after prior normalisation following radical treatment may herald the onset of recurrence.

A recent retrospective study of 1984 non-disseminated NPC patients after prior radical treatment showed that the recurrence rate was 63.8% in patients with elevated EBV DNA during surveillance compared with 8.6% among those without detectable EBV DNA after a median follow-up of 5 years [103]. It

also showed that EBV DNA elevation preceded radiological and/or clinical manifestation of NPC recurrence by a median of 2.3 months. However, the frequency of EBV DNA monitoring is yet to be determined because it is subject to the sensitivity and lowest detection limits of the assay, which allow physicians to detect early subclinical relapse.

3.5. Value of plasma EBV DNA in recurrent or metastatic NPC

Several articles described the role of kinetic changes of plasma EBV DNA in recurrent or metastatic NPC, demonstrating that faster clearance of EBV DNA during and after chemotherapy predicted better response and survival [15–17]. Besides, one trial including synchronous (n = 190) and metachronous (n = 817) metastatic NPC patients showed that using RPA, combined EBV DNA titres (with 33,000 copies/ml as a cutoff) with the number of metastatic lesions could segregate M1 disease into subcategories with different prognosis [102]. Another study showed that EBV DNA and some PET parameters were unfavourable prognostic factors [104]. However, one study showed that post-treatment EBV DNA titres after 1st-line platinum-based chemotherapy was not prognostic of OS [105].

3.6. Limitations of plasma EBV DNA

Plasma EBV DNA assay by RT-qPCR is not yet a routine investigation in many centres, especially in low-income countries. As the assay was previously a laboratory-derived test (DLT) devised by individual institutions using different master mixes and calibrators, the measurement method must be standardised to ensure that the results are comparable. With enormous efforts on international harmonisation of plasma EBV DNA assays [113,114], interclass correlation coefficients could improve from 0.72 to 0.96 [113]. However, most studies are still based on DLT of the individual institute: the lowest detection limit varies widely among different institutions, resulting in variation in false-negative rates and recommended cutoff values [113].

The challenge of using plasma EBV DNA as a screening tool for NPC is the trade-off between sensitivity and specificity. It must be cautioned that both false-negative and false-positive cases are not infrequent. Factors such as the histology types, small-volume disease in early-stage, recurrent/metastatic NPC with a defective secretion of viral genomes, and environmental factors may all affect the sensitivity and specificity. Chan *et al.* further showed that increasing age, decreasing ambient temperature and smoking status also correlate with the presence of detectable plasma EBV DNA: every 5 °C decrease in ambient temperature and 5-year increase in age would lead to a rise in the positive rate of plasma EBV DNA by 0.85% and 0.6%, respectively [115], while smokers were 1.59 times more likely to have detectable EBV DNA when compared with non-smokers. These three factors were thought to play a role in promoting viral replication, leading to transient elevation.

One way to reduce false-positive rates is to repeat the plasma EBV DNA test a few weeks later to rule out transient EBV infection as 5% of the general population can harbour EBV DNA in their plasma during their recent EBV infection [7,116]. Those with a mild and brief elevation of plasma EBV DNA may not need further investigation, but those with persistently elevated plasma EBV DNA should be meticulously investigated to exclude NPC [49]. The screening study by Chan *et al.* showed that among those who had an initial detectable EBV DNA, 70% would test negative four weeks later, and none of them was diagnosed with NPC within 1 year. By repeating the EBV DNA test 4 weeks later and proceeding with investigations only among with persistent elevation could reduce the false-positive rates from 5.4% to 1.4% [68]. Furthermore, sequencing-based analyses revealed that NPC patients tended to have a higher proportion of EBV DNA reads and longer plasma EBV DNA fragments than non-NPC individuals [117]. By devising an algorithm that considered EBV DNA reads and size profiles, the specificity and positive predictive value improved to 99.3% and 19.5%, respectively. The obvious drawback of this sequencing-based analysis is the expensive cost (US 3000 dollars per test) compared with the PCR-based assay (US 50 dollars). Hopefully these sequencing-based assays will be more easily accessible when they become less expensive.

A recent review on the usefulness of liquid biopsy for tumour detection showed that the weight or volume of the tumour was a significant factor affecting the effectiveness of a screening test [118]. In patients with a tumour weighing 10 g or more, and sampling 10 ml of blood, the cancer screening test appears effective, but in patients with a smaller tumour, the effectiveness becomes questionable. It is not surprising that patients with small-volume NPC, both for primary and recurrent tumours, do not have detectable plasma EBV DNA levels [118].

Another reason for lower sensitivity is the lowest detection limit of the assay. From our systematic review, only 23 (28.4%) journal articles clearly stated the lower detection limit of their EBV DNA assays, ranging from 0 to 1000 copies/ml (Table 2). Genome copy number below the detection threshold in some assays was reported as 0 copy/ml or undetectable. The lowest detection limit of the assay devised by Lo *et al.* has improved from 60 copies/ml to 20 copies/ml for the past 20 years [7,68]. As demonstrated in the screening programme by Chan *et al.*, the negative predictive value was 99.995% since only 1 of 19,865 individuals without elevated plasma EBV DNA was diagnosed with NPC within a year [68].

However, it must be cautioned that not all NPC patients had detectable plasma EBV DNA. In a study of 518 histologically confirmed NPC patients recruited into the prospective observational study on serial plasma EBV DNA monitoring in Hong Kong, plasma EBV DNA titres ranged from 0 to 20 copies in 78 patients (15.1%) [87], with 62 patients (12.0%) tested 0 copies/ml. Furthermore, 55.1% of EBV DNA-negative patients actually had locoregionally advanced stage III to IVA disease. Similarly, other studies also showed that 17.2%–29.3% of NPC patients in endemic areas had undetectable plasma EBV DNA at initial diagnosis [114,119]. Therefore, although plasma EBV DNA is thus far the most valuable biomarker for NPC, this is not a reliable tool for the ideal goal of detecting small tumour within the nasopharynx. Using plasma EBV DNA as the sole screening tool may miss 15% of NPC. With the current problem of unavailability in many countries (especially endemic regions), lack of consensus on optimal cutoff value, and the fact that more than 15% of patients with confirmed NPC do not have detectable levels, it is difficult to incorporate pre-treatment plasma EBV DNA into the TNM staging system for global application.

4. Discussion

NPC in endemic regions is highly associated with EBV infection. There is increasing evidence that plasma EBV DNA is a highly sensitive and specific biomarker for various clinical aspects in the management of EBV-associated NPC. However, few CPGs have included this biomarker in their recommendations, and the selected clinical indications vary [40]. In the latest NCCN Guideline (Version 1, 2021), the only recommendation on plasma EBV DNA is stated under work-up ‘consider EBV DNA testing’ with the footnote ‘EBV DNA load may reflect prognosis and change in response to therapy’.

The following summarises our recommendations on the use of plasma EBV DNA in different clinical aspects based on the current review and assessment on LoE:

1. Plasma EBV DNA can be considered for NPC screening in endemic regions—this is a useful biomarker with overall high sensitivity and specificity for the early detection of NPC. The diagnostic sensitivity and cost-effectiveness can be further improved by combination with IgA anti-VCA (LoE and GoR: II, B).
2. Plasma EBV DNA should be included in the pre-treatment work-up for newly diagnosed NPC—this is useful for enhancing prognostication, identifying poor-risk cohorts among patients with apparently stage I-II disease, considering thorough metastatic work-up with PET-CT and addition of concurrent chemotherapy, and providing baseline for monitoring of response (LoE and GoR: III, B).
3. Plasma EBV DNA monitoring could be considered during radical treatment as its clearance can provide additional information on prognosis and a risk-adapted strategy to determine adjuvant therapy (LoE and GoR: III, B).
4. Plasma EBV DNA should be tested after completion of radical treatment for better prognostication, thorough investigation to exclude residual disease, and consideration of adding adjuvant therapy (LoE and GoR: II, B)
5. Plasma EBV DNA could be used for disease surveillance during subsequent follow-up for early detection of relapse (LoE and GoR: III, B).
6. Plasma EBV DNA could be considered as a biomarker to monitor response to salvage treatment for recurrent or metastatic NPC and predict prognosis (LoE and GoR: IV, B).

However, it must be cautioned that the current application of plasma EBV DNA still has some limitations:

Screening—False positive is not uncommon as 5% of the general population can harbour EBV DNA in their plasma during their recent EBV infection [7,116]. One possible solution to avoid unnecessary investigations due to false-positive results as adopted in the study from Hong Kong [68], is to repeat EBV DNA measurement 4 weeks after the initial positive result. Only those with persistently elevated EBV DNA need to be further investigated. On the other hand, the sensitivity of plasma EBV DNA in detecting NPC was lower in early-stage patients (81.5%) than those with advanced NPC (100%) [52]. Using plasma EBV DNA as the sole screening tool may miss 15% of NPC [87]. One possible solution, besides serial regular EBV DNA measurement, is the addition parallel screening with IgA anti-VCA which can also enhance diagnostic sensitivity [25]. Sequencing-based assays, when they are more financially affordable and widely available, can also improve positive predictive values.

Prognostication—Not all confirmed NPC cases had detectable plasma EBV DNA; the proportion of NPC patients in endemic areas with undetectable plasma EBV DNA at initial diagnosis ranged from 12% to 29% [87,114,119]. Furthermore, the recommended cutoff value for segregating patients into different risk cohorts vary widely from 500 copies/ml [83], to 2000 copies/ml [84] and 4000 copies/ml [14]. Hence, it is difficult to incorporate pre-treatment plasma EBV DNA into the TNM staging system. International consortium and task groups should be established to advocate and promote international harmonisation and improve the lowest level of detection.

Disease surveillance—the sensitivity is low for small-volume local recurrence. Elevation of plasma EBV DNA was detected in 86%–96% of patients with distant metastases, but only 51%–67% of those with local/locoregional recurrence [30,110–112]. A multidisciplinary surveillance programme with frequent follow-up by surgeons, oncologists, radiologists and pathologists is crucial to earlier detection of relapse leading to more effective and efficacious early salvage treatment.

All these limitations are related mainly to the methodological problems requiring international harmonisation and the need for further enhancement of sensitivity. There have been researches to improve the accuracy, sensitivity and specificity of plasma EBV DNA, including droplet and digital PCR such as Beads Emulsion Amplification and Magnetics (BEAMing), sequencing-based assays or exploring other potential biomarkers. BART microRNAs encoded by BamHI region of EBV have recently emerged as new biomarkers for NPC. A recent study showed that BART 2-5p demonstrated a sensitivity and specificity of 93.9%–94.2% and 83.5%–89.8%, respectively, in the Hong Kong and Guangzhou cohort [120]. Circulating tumour cells (CTCs) have also been extensively investigated in NPC with initially promising results [121–123]. A small prospective study on using CTCs and PET-CT scan to monitor treatment response in metastatic NPC patients showed that CTCs were detectable in patients with a complete metabolic response by PET-CT [123]. The implication was that CTCs could be employed to monitor the minimal residual disease, which is not readily shown by imaging. The drawback of the clinical applications of CTCs is the very low number of CTCs (<5) identified in early stage I/II disease [124]. Further analyses on the cost-effectiveness and accessibility of these biomarkers are warranted.

5. Conclusions

Plasma EBV DNA has established itself as an accurate and reliable biomarker for NPC. It is now routinely used in major centres, providing valuable information for screening, prognostication/staging, tailoring treatment stratification, gauging treatment response and post-treatment surveillance. The recent advances in technology have made this assay more sensitive and specific, but international harmonisation of assay methodology and consensus on optimal cutoff value is crucial. It should be noted that there are limitations, particularly for the detection of early primary or recurrent tumours. Concerted efforts to further devise complementary diagnostics for improving accuracy is needed. Despite these limitations, the current evidence support consideration of plasma EBV DNA in different clinical aspects; inclusion into international CPGs to provide useful references to physicians is warranted.

Author contributions

Anne W. M. Lee, Victor H. F. Lee and Wai Tong Ng were responsible for study concepts, study design and data acquisition. Anne W. M. Lee, Victor H. F. Lee, Wai Tong Ng and Alfio Ferlito were responsible for quality control of data and algorithms. All authors were involved in formal analysis and interpretation of data. Anne W. M. Lee, Victor H. F. Lee, Wai Tong Ng and Alfio Ferlito were involved in statistical analysis. Alfio Ferlito was responsible for project administration, resources, software, supervision and validation. All authors wrote the first draft, reviewed, edited and approved the final manuscript before submission.

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