EDITORIAL

The evolution of Epstein-Barr virus detection in nasopharyngeal carcinoma

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The detection of Epstein-Barr virus (EBV) in nasopharyngeal carcinoma (NPC) has evolved over the last 40 years, transitioning from simple serological tests of latent viral infection to extremely sensitive measurements of the circulating tumor virome. Compared to the former, cell-free (cf) EBV DNA quantification is considered superior for population-based screening, and possesses additional advantages of clinical prognostication and surveillance for subclinical recurrences. However, despite its broad utility, the clinical value of cf EBV DNA for prediction of treatment response remains uncertain, and is currently being investigated in prospective clinical trials. These lessons from EBV and NPC have since been tested in another emerging viral-associated head and neck cancer that is linked to the human papillomavirus (HPV).

EBV and nasopharyngeal carcinoma

The first association of EBV with tumorigenesis was reported by Woodliff through his observation in African endemic Burkitt's lymphoma[1]. Since this seminal finding, associations of EBV with several other lymphoid and epithelial cancers have been reported, including NPC, as reported by Old and Clifford *et al.*[2]. Owing to this invariable etiological linkage (especially for NPC from endemic regions), serological markers of EBV infection, such as anti-IgA antibodies for early antigen (EA) and viral capsid antigen (VCA), have been investigated for screening of NPC. Following acute infection, EBV enters a phase of latency within B-lymphocytes of the host, and antibodies such as EBV-EA, -VCA, and -NA (nuclear antigen) persist at detectable levels throughout this phase. It is thus plausible that these serological markers potentially represent precursor signals of the eventual onset of NPC[3]; however, arguments against this notion relate to the ambiguous involvement of EBV during the process of NPC tumorigenesis. First, it is contentious if early or delayed exposure to the virus after birth determines the individual risk of developing NPC later in adult life[4]. Second, this conundrum is further compounded by the observation that EBV is not detected in pre-malignant lesions in high-risk individuals, thus suggesting that either 1) the downstream effects of EBV infection may be inconsequential in the irreversible malignant transformation of the epithelium or 2) EBV resides in other tissue types apart from the epithelium[5]. Of note, other molecular pathways such as p16 dysregulation have been implicated in the maintenance of the virome within a cell[3]. Third, EBV is especially resistant to transfecting epithelial cells, as opposed to lymphoid cells. For these reasons, the optimal screening strategy for NPC using serological markers of latent EBV infection remains elusive until today.

Historical studies using EBV serology

The focus of cancer screening has always been on early detection, coupled with the simplistic logic that inducing stage migration from advanced to early-stage disease will eventually improve population survival rates in the long term. This may hold true in NPC, since the prognosis of this disease is highly correlated with disease stage[6]. In this background, early screening studies in high-risk populations were first designed using EBV serological assays (**Table 1**). In these studies, high titers of IgA-VCA, IgA-EA, and EBV DNAse at baseline, with subsequent consecutive rises during follow-up, were predictive of an NPC diagnosis; however, false positive rates of 2%–18% were also reported using serological tests alone[7-10]. Expectedly, accuracy was enhanced

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Table 1 Summary of NPC screening studies.

Study	Sample size	Screening marker	Results
Zeng et al.[7]	12,932	VCA/IgA and EA/IgA	39 NPC detected; PPV for VCA/IgA & EA/IgA 1.0%; PPV for VCA/IgA 1.9%
Zong et al.[8]	52,450	VCA/IgA	136 NPC detected; Sensitivity 97.84%; Specificity 93.59%; PPV 4.82%; NPV 99.99%
Chien et al.[9]	9,699	VCA/IgA and EBV DNase neutralising	22 NPC detected; Sensitivity of VCA/IgA 18%; Specificity of VCA/IgA 98%; PPV of VCA/IgA 3%; NPV of VCA/IgA 99% Sensitivity of Anti-EBV DNAse 31%; Specificity of Anti-EBV DNAse 88%; PPV of Anti-EBV DNAse 0.6%; NPV of Anti-EBV DNAse 99%
Cao et al.[10]	18,986	VCA/IgA & EA/IgA	125 NPC detected; Sensitivity 33.6%; Specificity 93.0%; PPV 3.0%; NPV 99.5%
Ji et al.[17]	862	EBNA1/IgA & VCA/IgA; EBV DNA	33 NPC detected; Sensitivity of EBV DNA 86.8%; Specificity of EBV DNA 90%; PPV of EBV DNA 30%; NPV of EBV DNA 99.3%
Chan et al.[16]	1,318	VCA/IgA & EBV DNA	3 NPC detected; Sensitivity of EBV DNA 100%; Specificity of EBV DNA 98.7%; PPV of EBV DNA 15%; NPV of EBV DNA 100%
Chan et al.[18]	20,174	EBV DNA	34 NPC detected; Sensitivity 97.1%; Specificity 98.6%; PPV 11.0%; NPV 99.9%

PPV: positive predictive value; NPV: negative predictive value.

when combinatorial markers were tested, compared to that observed using a single marker; the use of dual IgA-VCA and DNAse markers resulted in a higher rate of detection in a population of 9,699 Taiwan individuals (371 *vs*. 45 cases per 100,000 person-years)[9]. Similarly, Liu et al.[11] also showed an improved area under the curve (AUC) for NPC detection using dual marker selection as opposed to that of using a single marker in an independent Chinese cohort of 5,481 cases (AUC range of 0.87–0.97 *vs*. 0.77–0.95). While this may be true and heralds promise, a crucial issue that remains unresolved pertains to the uncertainty regarding the optimal combination of serological markers that would yield the highest accuracy for NPC screening. Owing to these

limitations, coupled with the advent of circulating tumor EBV DNA testing, enthusiasm to implement EBV serological screening gradually waned over time.

The meteoric rise of circulating EBV DNA in NPC

Cell-free EBV DNA (cf EBV DNA) in the plasma was first reported as a biomarker for NPC by Lo and colleagues in 1999[12]; briefly, these are short fragments of the EBV virome (<181 bp) that are supposedly released by cancer cells during apoptosis. Using real-time quantitative PCR targeted to the *Bam*HI-W and *EBNA-1* regions, Lo et al.[12] were able to

demonstrate the presence of a circulating virome in a majority of NPC cases (55 of 57), but only in a few healthy controls (3 of 43). Importantly, this biomarker has clinical relevance; EBV DNA copy number was correlated to clinical disease stage, and in the post-treatment surveillance setting, persistent or detectable EBV DNA was predictive of an eventual tumor recurrence[13]. These findings were corroborated by Lin et al.[14] in a subsequent study. An interesting observation from these studies is the temporal sequence of cf EBV DNA detection relative to the onset of clinical disease (50–150 days in 6 cases), which suggests that cf EBV DNA is likely a surrogate for occult NPC tumor clones. This intuitively broadens the potential utility of EBV DNA in population-based screening, where the ultimate clinical goal is to detect early-stage disease.

cf EBV DNA was first combined with serum IgA-VCA antibody for screening, and the former method identified 75% of the false-positive cases detected by serology[15]. A subsequent moderate-sized population-based study of 1,318 volunteers in Hong Kong, SAR, China evaluated cf EBV DNA as a screening modality for NPC. Of the 69 individuals (5.2%) with a baseline positive test, only 3 early-stage NPC cases were identified by nasal endoscopy and MRI[16]. In a replicate study of 862 individuals from Southern China, the investigators reported a sensitivity of 86.8% (33 of 38 NPC cases) for NPC diagnosis using EBV DNA, but the sensitivity for early-stage disease was only 81% compared to 100% for cases with advanced disease[17].

Against this background, the current study by Chan et al.[18] is seminal, since it represents the largest sample size considered until today, where 20,174 individuals were prospectively screened for NPC using cf EBV DNA from 2013 to 2016. Of the 309 cases with persistently elevated EBV DNA, 34 were eventually diagnosed with NPC (11.2% positive predictive value). The reported sensitivity and specificity of the assay were 97.1% and 98.6%, respectively. Crucially, 24 of the 34 (71%) cases diagnosed by screening presented with stage I and II disease, which compares favorably to an unscreened cohort in the same period [only 149 of 773 (19%) presented with stage I/II disease]; although not without potential selection bias, the early detection also corresponded to a superior progression-free survival [HR 0.10 (95% CI = 0.05–0.18)]. Overall, this study provides strong level IIA evidence to suggest implementing the cf EBV DNA assay as a method of NPC screening in high-risk individuals from endemic regions.

Nonetheless, to advocate caution, the short follow-up duration of the present study precludes an assessment of the long-term clinical impact, particularly for long-term survivorship. It is inconclusive if stage migration through screening will improve long-term overall survival, since survival rates of even advanced stages of NPC exceed 75%–80% with contemporary treatment of combination chemotherapy and intensity-modulated radiotherapy[6]. Next, the trajectory of tumorigenesis of NPC is unknown; it is possible that a subset of NPC cases directly progress to malignancy through a punctuated evolutionary process, and therefore screening will not alter the natural history of the disease in such cases. Third, the positive predictive value of the assay based on the current study is low at 11.0%, which is not unexpected given the gradual decline in NPC incidence even in the endemic parts of the world[19]. Should this declining trend continue, there would be a lesser need for NPC screening. Finally, the cost effectiveness of cf EBV DNA requires further investigation, especially when 593 individuals have to be screened to detect 1 NPC case; of note, the estimated cost burden has to include not only the cost of the assay but also that of subsequent investigations such as MRI and endoscopic examination, all of which are against life-years saved by screening.

Future of EBV DNA and cf tumor DNA technologies in head and neck cancers

Despite its multipurpose utility, cf EBV DNA remains, at best, a prognostic biomarker. Its role is limited for predicting therapeutic efficacy and influencing treatment recommendation, since it does not inform on the molecular vulnerabilities of the circulating occult tumor clones. The concept of using cf EBV DNA as a predictive biomarker is presently being tested in a prospective clinical trial (NRG-HN001; ClinicalTrials.gov, NCT02135042); in this study, patients are stratified based on the presence or absence of this biomarker at the end of chemoradiotherapy, and patients harboring persistent cf EBV DNA copies will be referred to a randomized phase 2 study of adjuvant gemcitabine-paclitaxel compared to conventional cisplatin-5-fluorouracil. While the results of this clinical trial are awaited, Chan and colleagues[20] reported their findings of NPC-0502, which unexpectedly revealed no benefit of adjuvant gemcitabine-cisplatin over observation in patients with persistent cf EBV DNA after-chemoradiotherapy. Hence, a novel approach might be required. In this context, we now possess a catalog of mutational events occurring in NPC[21-23]. This opens the possibility of designing novel assays that can capture mutations of circulating tumor clones, which could then be exploited for designing novel paired drug-mutational targets

studies in NPC.

Finally, it would be intuitive to transpose the findings observed in EBV-associated NPC onto another emerging virus-associated head and neck cancer - HPV oropharynx squamous cell carcinoma (HPV-OPSCC). However, several practical issues, including harmonization of the assay to measure cf HPV DNA, still need to be addressed prior to its clinical implementation. Nonetheless, few groups have reported an association between such a biomarker and advanced nodal status and overall TNM stage, with potential utility in clinical prognostication and monitoring of treatment response[24,25].

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Conflict of interest statement

No potential conflicts of interest are disclosed.

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