Multiplex Epstein-Barr virus BALF2 genotyping detects high-risk variants in plasma for population screening of nasopharyngeal carcinoma

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Abstract

Background: Epstein-Barr Virus (EBV)-associated nasopharyngeal carcinoma (NPC) exhibits unusual geographic restriction despite ubiquitous lifelong infection. Screening programs can detect most NPC cases at an early stage, but existing EBV diagnostics are limited by false positives and low positive predictive value (PPV), leading to excess screening endoscopies, MRIs, and repeated testing. Recent EBV genome-wide association studies (GWAS) suggest that EBV BALF2 variants account for more than 80% of attributable NPC risk. We therefore hypothesized that high-risk BALF2 variants could be readily detected in plasma for once-lifetime screening triage.

Methods: We designed and validated a multiplex genotyping assay to detect EBV BALF2 polymorphisms in human plasma. Targeted next-generation sequencing was used to validate this assay, conduct association studies with clinical phenotype, and longitudinally genotype plasma to assess within-host haplotype stability. We examined the association between NPC and BALF2 haplotypes in a large non-endemic population and three prior EBV GWAS. Finally, we estimated NPC mortality reduction, resource utilization, and cost-effectiveness of BALF2 variant-informed screening using a previously-validated cohort model.

Results: Following analytical validation, the BALF2 genotyping assay had 99.3% concordance with sequencing in a cohort of 24 NPC cases and 155 non-NPC controls. BALF2 haplotype was highly associated with NPC in this non-endemic population (I613V: odds ratio [OR] 7.9; V317M: OR 178.8). No other candidate BALF2 polymorphisms were significantly associated with NPC or hematologic disorders. Longitudinal genotyping revealed 97.8% within-host haplotype concordance, indicative of lifelong latent infection. In a meta-analysis of 755 NPC cases and 981 non-NPC controls, BALF2 I613V and V317M were significantly associated with NPC in both endemic and non-endemic populations. Modeled variant-informed screening strategies achieved a 46% relative increase in PPV with 7% decrease in effective screening sensitivity, thereby averting nearly half of screening endoscopies/MRIs among endemic populations in east/southeast Asia.
Conclusions: EBV BALF2 haplotypes are temporally stable within hosts and can be readily detected in plasma via an inexpensive multiplex genotyping assay that offers near-perfect sequencing concordance. In endemic and non-endemic populations, I613V and V317M were highly associated with NPC and could be leveraged to develop variant-informed screening programs that mitigate false positives with small reductions in screening sensitivity.

Keywords: Nasopharyngeal carcinoma, Epstein-Barr virus, Cancer screening, Plasma, BALF2, Cost-effectiveness

Background
Epstein-Barr Virus (EBV)-associated nasopharyngeal carcinoma (NPC) is unusually restricted to certain regions and populations despite nearly ubiquitous EBV infection early in life [1]. NPC is the second-leading cause of head/neck cancer mortality worldwide, and has no definite modifiable risk factors [2]. Without biomarker-based screening, most patients present with NPC at an advanced stage and have worse prognoses despite treatment intensification [3].

Screening high-risk populations can detect most NPC cases at an early stage, but existing serologic and molecular diagnostics are limited by low positive predictive value (PPV) secondary to benign EBV reactivation [4, 5]. These false positives result in excess screening imaging, endoscopies, biopsies, and/or repeated laboratory testing which increase screening costs and visits. Ancillary triage testing with nasopharyngeal EBV PCR and plasma EBV next-generation sequencing (NGS) can increase PPV [6, 7]. However, each screening algorithm has certain advantages and disadvantages with respect to performance, referral rates, cost, and complexity.

Human genome-wide association studies (GWAS) have previously identified susceptibility loci which are associated with NPC risk [8]. However, the effect sizes are modest relative to the marked variation in NPC incidence worldwide. In contrast, several recent EBV GWAS have identified viral polymorphisms with much greater attributable risk [9–11]. In particular, two non-synonymous polymorphisms within the EBV BALF2 gene (I613V, V317M) may contribute more than 80% of attributable risk in southern China. Because humans typically establish a single lifelong latent EBV infection, BALF2 genotyping could serve as an adjunctive tool for once-lifetime screening triage [9]. We therefore hypothesized that a noninvasive molecular diagnostic could detect high-risk EBV BALF2 variants in plasma and could serve to triage individuals for further screening work-up while remaining cost-effective in high-risk populations.

Methods
Multiplex BALF2 genotyping assay design
We designed a multiplex allele-specific real-time polymerase chain reaction (qPCR) genotyping assay to detect three non-synonymous polymorphisms in the EBV BALF2 gene (NCBI RefSeq NC_007605.1 Aug 2018: V700L [162215C > A], I613V [162476 T > C], V317M [163364C > T]). To permit single reaction multiplexing, we designed three conserved primer sets flanking the single nucleotide variants (SNVs), with one allele-specific propynyl-modified dual-labeled hydrolysis probe for each SNV (Biosearch Technologies, Petaluma, USA). A fourth allele-specific probe detecting the wild-type V700 allele (162215C) served as an additional internal control for samples lacking these polymorphisms (Fig. S1, Table S1).

Recognizing the potential for off-target polymorphisms in primer/probe regions, on November 23, 2021 we identified 1050 EBV GenBank sequences aligning to the EBV BALF2 region of interest (NC_007605.1:162115–163,464) with ≥98% coverage. Each primer was conserved in ≥98.7% of sequences. The 162215C, 162215C > A, 162476T > C, and 163364C > T alleles were present in 78.3, 20.9, 37.2, and 29.2% of sequences, respectively.

Two synthetic dsDNA gene fragments (gBlocks, Integrated DNA Technologies, Coralville, USA) served as either the NPC risk-associated (V700, I613V, V317M) or non-risk-associated (V700L, I613, V317) controls (Table S2). Supernatant from the EBV-infected B95–8 cell line served as an additional wild-type whole-virus control (ATCC, Catalog #VR-1492). Further methodological details are available in the Supplementary Methods and Tables S1–3. Assay interpretation and example amplification curves are presented in Table S4 and Fig. S1.

BALF2 genotyping qPCR analytical validation
The 95% lower limit of detection (LLOD) was assessed in replicates of 20 from 0.1–5.0 copies/μL template (1.0–50.0 copies/reaction) using the risk and non-risk dsDNA controls. Any amplification crossing the fluorescence threshold was regarded as detection. Linearity was assessed from 0.0 to 6.0 log10 copies/μL template in replicates of three. Because a minority of individuals may be latently infected with multiple distinct EBV variants, we evaluated the assay’s performance with mixed risk and non-risk dsDNA controls ranging from 0 to 100% allele frequency at a fixed total template concentration of 100 copies/μL in replicates of three.
Clinical specimens
This study included human plasma specimens collected between July 1, 2019 and November 1, 2020 as part of routine clinical care for detection of EBV EBNA-1 by qPCR. Clinical EBV DNA qPCR was conducted as previously described [12, 13]. Approximately 3mL whole blood was collected in EDTA tubes, centrifuged, and at least 1.25mL plasma aliquoted into separate tubes within six hours of collection. Total nucleic acids were extracted from 1000μL plasma using the QIAsymphony DSP Virus/Pathogen Midi kit and eluted into 60μL buffer AVE. After development and analytical validation of our genotyping qPCR, we retrospectively genotyped specimens meeting the following criteria: 1) EBV positive by EBNA-1 qPCR (Ct ≤ 45), 2) ≥20μL residual extracted nucleic acid, and 3) highest viral load for a given patient within the study period. No diagnoses or indications for testing were excluded. Specimens were collected from patients with a range of benign and neoplasic EBV-associated disorders (Table S5).

NGS validation of BALF2 genotyping qPCR
We validated the genotyping qPCR assay with targeted NGS using a subset of specimens from NPC cases and controls. We sequenced a region of the BALF2 gene (NC_007605.1.162126–163,483) spanning the three non-synonymous polymorphisms of interest (Supplementary Methods). Sequences with a depth of at least 10 reads at the three SNV positions of interest were accepted for interpretation. We filtered out variants with the parameter 'QUAL<30 | MQ<40 | DP<10 | MQ0F<4 | DV<3'. Specimens selected for sequencing were either the highest viral load specimen for a given patient or were specimens with residual extracted nucleic acid included in the longitudinal sequencing subset described below.

Within-host longitudinal genotyping
To assess whether EBV BALF2 haplotypes persisted over time, we longitudinally genotyped plasma specimens collected over the study period from a subset of individuals with multiple EBV-positive specimens.

Modeled NPC mortality and resource utilization with variant-informed screening strategies
We estimated population-level NPC mortality reduction, resource utilization, and cost-effectiveness of BALF2 variant-informed screening strategies using a previously-validated time-inhomogeneous decision-analytic cohort model (Table S6) [14]. This analysis was limited to high-risk populations with endemic NPC in southern China and southeast Asia. First, we conducted a meta-analysis of three prior EBV GWAS to model BALF2 haplotype prevalence among NPC cases and non-NPC controls [9–11]. Thereafter, we compared variant-agnostic screening strategies from prospective studies to variant-informed screening strategies which triage positive plasma/nasopharyngeal EBV DNA with the BALF2 genotyping qPCR. Full details regarding the model framework, population selection, screening strategies, and sensitivity analyses are detailed in the Supplementary Methods and Tables S6–11.

Statistical analysis
Positive percent agreement (PPA) and negative percent agreement (NPA) were reported with Clopper-Pearson score 95% binomial confidence intervals using NGS as the reference method. The 95% LLOD was calculated using probit regression for each target. Linear regression was used to fit Ct values against nominal concentrations. Odds ratios for high-risk haplotypes (C-C-T and/or C-C-C at positions 162,215–162,476-163,364) were calculated using the common low-risk haplotypes as reference (sum of A-T-C and C-T-C). For EBV-positive NPC cases, the reference group includes all non-NPC controls for each individual study (present cohort, Xu et al., Hui et al., Lam et al.) [9–11]. Fisher exact tests were used to calculate p-values for SNV and haplotype associations with NPC. For targeted NGS, the p-value threshold for statistical significance was adjusted for the number of evaluated positions using the Bonferroni correction (α = 3.68 × 10^-5). Analyses were conducted using the R statistical software package.

Results
High-risk EBV variants are readily detected in plasma via a single-reaction genotyping assay
We designed and validated a multiplex allele-specific real-time polymerase chain reaction (qPCR) genotyping assay to detect three EBV BALF2 variants (V700L, I613V, V317M; Supplementary Methods, Fig. S1, Tables S1–4). The wild-type V700 allele was selected as an internal control for samples lacking these polymorphisms. The assay’s 95% lower limit of detection was 2.0 copies/reaction (95% CI 1.4–2.6) with < 20% coefficient of variation across six orders of magnitude (R^2 ≥ 0.992, Fig. 1A-B, Tables S12–13). Non-specific amplification was not observed for off-target alleles, and replicates of the B95–8 wild-type whole-virus control also confirmed specificity. In mixing experiments ranging from 0 to 100% allele frequency, the assay detected allele frequencies as low as 10% for each of the four targets, below the host heterozygosity threshold (Fig. 1C, Table S14) [9].
Multiplex BALF2 genotyping qPCR has near-perfect concordance with next-generation sequencing
We sequenced the BALF2 region in 258 clinical plasma specimens genotyped by qPCR, and 152 had adequate sequencing depth and coverage (Supplementary Methods). Samples with adequate sequencing depth and coverage had higher viral load (median 1600 vs. 201 IU/mL, p < 0.01). There was a single discordant genotyping call between qPCR and NGS. In a 43-year-old immunosuppressed woman with heart/lung transplantation, the sixth of six plasma specimens collected over 4.9 months showed qPCR loss of I613V which was detected on all five prior specimens. The specimen was sequenced and revealed the I613V mutation in 35/36 (97.2%) reads, reflecting false negative qPCR, possibly due to low viral load (EBNA-1 < 100 IU/mL). Positive and negative percent agreements for V700L, I613V, and V317M were otherwise 100%, and overall haplotype concordance between qPCR and NGS was 99.3% (151/152, Table S15).

BALF2 haplotypes are associated with NPC in a non-endemic cohort
We genotyped plasma specimens from 179 unique patients in a non-endemic population, including 155 non-NPC controls and 24 EBV-positive NPC cases (Table S5, Fig. 1G). Among controls, the most common indication for plasma EBV PCR was monitoring after solid organ transplant (44%) or bone marrow transplant (33%). Seventy-six control patients (49%) had hematologic neoplasms with (66%) or without (33%) prior bone marrow transplant, including EBV-positive lymphomas/leukemias. Nineteen patients (12%) had no history of transplant or neoplasm, including ten patients with primary EBV infection. There was no significant association between plasma EBV EBNA-1 viral load and disease phenotype (Fig. 1H).

High-risk BALF2 haplotypes, defined by the presence of I613V with or without V317M, were rare among non-NPC controls (Fig. 1D-E, Table S16). The C-C-C and C-C-T high-risk haplotypes were present in 5.8 and 1.3% of controls, compared with 12.5 and 62.5% of NPC cases. Using the low-risk A-T-C and C-T-C haplotypes as reference, both the C-C-C (odds ratio [OR] 7.9 95% confidence interval [CI] 1.7–37.1) and C-C-T (OR 178.8, 95% CI 33.1–965.3) haplotypes were highly associated with NPC in this non-endemic population (Fig. 1F, Table S16). We observed no association between these haplotypes and other diseases, including hematologic neoplasms.

BALF2 haplotypes are associated with NPC in a meta-analysis of endemic and non-endemic cohorts
In a meta-analysis of 755 NPC cases and 981 non-NPC controls from this study and three previously-published EBV GWAS, the NPC odds ratios for the C-C-C and C-C-T haplotypes were 4.0 (95% CI 2.6–6.0) and 15.4 (95% CI 11.2–21.0), respectively (Fig. 1D-F, Table S16). While I613V and V317M were common (> 75%) in NPC cases across cohorts, they were uncommon in non-endemic controls (7.1%) relative to endemic controls (60.5%), suggesting that variable NPC incidence could be explained by underlying BALF2 haplotype prevalence.
Fig. 1 (See legend on previous page.)
We also evaluated the association between clinical phenotypes and other \textit{BALF2} SNVs. For example, the previously-described 162507C > T and 162852G > T synonymous polymorphisms have been rarely observed (3%) in NPC cases but are common in endemic controls (41–43%). Among 108 unique patients with sequenced specimens, neither mutation was significantly associated with NPC. Beyond I613V and V317M, only the synonymous 163287G > A SNV reached statistical significance (Fig. 1I–J, Table S17). We observed no \textit{BALF2} SNVs which were significantly associated with EBV-positive leukemias/lymphomas or post-transplant lymphoproliferative disorders. We also assessed whether other SNVs were associated with high-risk \textit{BALF2} haplotypes, and identified multiple variants which were significantly correlated with I613V and V317M. For example, seven BALF2 SNVs occur with 100% frequency in the I613V/V317M haplotype and with 0–2% frequency in low-risk haplotypes ($p \leq 1.31 \times 10^{-7}$). This supports the hypothesis that high-risk EBV variants are transmitted locally rather than developing de novo after primary infection (Table S18).

\textbf{Longitudinal genotyping within hosts confirms temporal stability of BALF2 haplotypes}

Because EBV establishes lifelong latent infection, \textit{BALF2} genotyping could facilitate once-lifetime screening triage. To assess whether EBV \textit{BALF2} haplotypes persisted over time, we genotyped 90 EBV-positive plasma specimens collected from a subset of 16 patients. These patients had a median of 5 (range, 2–7) specimens genotyped over a median period of 8.6 months (range, 2.8–13.9). Among the 90 genotyped specimens, 88 (97.8%) haplotype calls were concordant within a given individual over time (Fig. 2A). The two discordant specimens both occurred in individuals with solid organ transplantation (Fig. 2A, Patients #2 and #10) and may represent mutagenesis under immunosuppression or reactivation of distinct latent infections from the host and donor tissue.

\textbf{Variant-informed NPC screening strategies reduce false positives and unnecessary procedures}

We estimated population-level NPC mortality reduction, resource utilization, and cost-effectiveness of \textit{BALF2} variant-informed screening strategies using a previously-validated time-inhomogeneous decision-analytic cohort model (Fig. S2) [14]. Full details regarding the model framework, population selection, screening strategies, and sensitivity analyses are provided in the Supplementary Methods and Tables S6–9.

First, we conducted a meta-analysis of three prior EBV GWAS to model endemic \textit{BALF2} haplotype prevalence among NPC cases and non-NPC controls [9–11]. Thereafter, we compared seven variant-agnostic screening strategies from prospective studies to seven variant-informed strategies wherein positive plasma/nasopharyngeal EBV PCR are triaged using the \textit{BALF2} genotyping qPCR (Table S10). Twelve high-risk populations in southern China, Hong Kong SAR, Macao SAR, Republic of China, and Singapore met inclusion criteria (Fig. 2B, Table S11).

Variant-informed screening increased PPV by a median of 46% (range, 26–51%) with an absolute decrease in screening sensitivity of 7%. Variant-informed screening reduced referrals for endoscopy and/or MRI by approximately 40% relative to the corresponding variant-agnostic strategy (Table S10). This reduction in referrals for further screening steps averted a median of 2969 screening visits per 100,000 subjects (Table S19).

For a hypothetical cohort of 50-year-old men and women who develop NPC in southern China, 10-year survival improved from 70.4% (95% CI 68.1–72.5%)
Fig. 2 (See legend on previous page.)
in an unscreened cohort to a median of 85.7% (range, 85.4–87.0%) with variant-agnostic screening and 85.2% (range, 84.3–85.9%) with variant-informed screening (Fig. 2C, Table S19). In the highest incidence region, the small reduction in screening sensitivity after BALF2 triage resulted in approximately 3.4 excess NPC deaths and 600 fewer false-positives requiring endoscopy/MRI per 100,000 subjects screened.

**Variant-informed NPC screening is cost-effective and facilitates once-lifetime testing**

The base case screened adult men and women once at age 50 years (Fig. 2D, Table S19–20). Variant-informed screening was cost-effective in all populations except Hengdong, China (due to lower NPC incidence). Across the 12 populations and 14 screening strategies, an initial screening age of 40–45 tended to be most cost-effective irrespective of screening interval (Table S21). Screening intervals as short as every two years could be cost-effective. Variant-informed screening became more cost-effective as the number of lifetime screens increased due to the increasing proportion of subjects known to have low-risk BALF2 haplotypes that were never subsequently screened (Fig. S3). Sensitivity analysis identified parameters that most impacted cost-effectiveness (Fig. S3, Tables S22–23).

**Discussion**

Existing NPC screening strategies typically utilize EBV serology or plasma PCR as the initial screening assay [4–7, 10]. These programs achieve PPV ranging from 2 to 16% and do not currently leverage EBV genotyping to mitigate false positives. In light of existing laboratory screening infrastructure, we developed and validated our assay using specimens from a non-endemic population that had few healthy controls. Although we observed no association between BALF2 haplotypes and other non-NPC diseases, it is possible that non-NPC controls had different haplotype prevalence relative to the healthy population. Second, the ability to triage individuals once in their lifetime with BALF2 genotyping is predicated on haplotype stability over time and absence of multiple EBV co-infections. Because longitudinal specimens were not available over a years- or decades-long period, it is uncertain whether haplotypes may change over longer time scales. Third, BALF2 haplotype
distributions for NPC cases and controls were derived from a meta-analysis of three studies which predominantly included subjects in southern China. The degree to which these distributions vary within southeast Asia is unknown, and would impact effective screening sensitivity.

Conclusions
Approximately 93% of endemic nasopharyngeal carcinoma harbors high-risk EBV BALF2 haplotypes. These haplotypes are stable over time within hosts and readily detectable in plasma using an inexpensive single-reaction multiplex genotyping assay. The BALF2 1613V and V317M polymorphisms are rare in non-endemic controls, supporting the hypothesis that regional EBV genomic diversity contributes to differential NPC risk worldwide. Triaging subjects who test positive for plasma/nasopharyngeal EBV DNA using BALF2 genotyping could substantially reduce referrals for more complex and expensive endoscopy/MRI. Across seven prospectively-evaluated screening strategies in 12 high-risk endemic populations, these variant-informed strategies maintain high screening sensitivity while averting 40% of referrals for endoscopy/MRI. In suitable populations, this may be a low-cost and readily accessible alternative to higher-complexity triage algorithms, and could identify low-risk individuals who require no further lifetime screening.

Abbreviations
AML: acute myeloid leukemia; C<sub>t</sub>: cycle threshold; dsDNA: double-stranded DNA; EBV: Epstein-Barr Virus; ELISA: enzyme-linked immunosorbent assay; GDP<sub>ppp</sub>: purchasing power parity-adjusted per-capita gross domestic product; GWAS: genome-wide association study; ICER: incremental cost-effectiveness ratio; IMRT: intensity-modulated radiotherapy; IU: international units; LLOD: lower limit of detection; MPN/MDS: myeloproliferative neoplasm/myelodysplastic syndrome; MRI: magnetic resonance imaging; NED: no evidence of disease; NGS: next-generation sequencing; NPC: nasopharyngeal carcinoma; OS: overall survival; PCR: polymerase chain reaction; PPA: positive percent agreement; PPV: positive predictive value; qPCR: real-time polymerase chain reaction; SNV: single nucleotide variant; VAF: variant allele fraction; WHO: World Health Organization; WTP: willingness-to-pay.

Supplementary Information
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Authors’ contributions
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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files. Sequence data that support the findings of this study have been deposited in the NCBI Sequence Read Archive with the primary accession code PRJNA848410.

Declarations
Ethics approval and consent to participate
This study was conducted with Stanford University institutional review board approval. Individual consent was waived.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests related to this work.

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