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## Epstein Barr virus genomes reveal population structure and type 1 association with endemic Burkitt lymphoma [preprint]

Item Type	Preprint
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Citation	<p>bioRxiv 689216; doi: <a href="https://doi.org/10.1101/689216">https://doi.org/10.1101/689216</a> . <a href="https://doi.org/10.1101/689216" target="_blank"> Link to preprint on bioRxiv service.</a></p>
DOI	<a href="https://doi.org/10.1101/689216">10.1101/689216</a>
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Link to Item	<a href="https://hdl.handle.net/20.500.14038/29468">https://hdl.handle.net/20.500.14038/29468</a>

1 **Epstein Barr virus genomes reveal population structure and type 1 association with**  
2 **endemic Burkitt lymphoma**

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43 **Key words:** Epstein Barr virus, malaria, genome sequencing, genetic variation, endemic Burkitt  
44 Lymphoma, EBV type 1, EBV type 2.

45 Abstract: 237 words  
46 Text: 3,982 words  
47 Number of figures: 3  
48 Number of tables: 2  
49 Number of references: 50

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## 52 **Key Points**

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- 54 ● **EBV type 1 is more prevalent in eBL patients compared to the geographically**  
55 **matched healthy control group.**
- 56 ● **Genome-wide association analysis between cases and controls identifies 6 eBL-**  
57 **associated nonsynonymous variants in EBNA1, EBNA2, BcLF1, and BARF1**  
58 **genes.**
- 59 ● **Analysis of population structure reveals that EBV type 2 exists as two genomic**  
60 **sub groups.**

## 61            **Abstract**

62            Endemic Burkitt lymphoma (eBL), the most prevalent pediatric cancer in sub-Saharan  
63 Africa, is associated with malaria and Epstein Barr virus (EBV). In order to better understand the  
64 role of EBV in eBL, we improved viral DNA enrichment methods and generated a total of 98  
65 new EBV genomes from both eBL cases (N=58) and healthy controls (N=40) residing in the  
66 same geographic region in Kenya. Comparing cases and controls, we found that EBV type 1  
67 was significantly associated with eBL with 74.5% of patients (41/55) versus 47.5% of healthy  
68 children (19/40) carrying type 1 (OR=3.24, 95% CI=1.36 - 7.71,  $P=0.007$ ). Controlling for EBV  
69 type, we also performed a genome-wide association study identifying 6 nonsynonymous  
70 variants in the genes EBNA1, EBNA2, BcLF1, and BARF1 that were enriched in eBL patients.  
71 Additionally, we observed that viruses isolated from plasma of eBL patients were identical to  
72 their tumor counterpart consistent with circulating viral DNA originating from the tumor. We also  
73 detected three intertypic recombinants carrying type 1 EBNA2 and type 2 EBNA3 regions as  
74 well as one novel genome with a 20 kb deletion resulting in the loss of multiple lytic and virion  
75 genes. Comparing EBV types, genes show differential variation rates as type 1 appears to be  
76 more divergent. Besides, type 2 demonstrates novel substructures. Overall, our findings  
77 address the complexities of EBV population structure and provide new insight into viral  
78 variation, which has the potential to influence eBL oncogenesis.

## 79 Introduction

80 EBV infects more than 90% of the world's population and typically persists as a chronic  
81 asymptomatic infection.<sup>1</sup> While most individuals endure a lifelong infection with minimal effect,  
82 EBV is associated with ~1% of all human malignancies worldwide. EBV was first isolated from  
83 an endemic Burkitt lymphoma (eBL) tumor which is the most prevalent pediatric cancer in sub-  
84 Saharan Africa.<sup>2</sup> Repeated *Plasmodium falciparum* infections during childhood appear to drive  
85 this increased incidence.<sup>3</sup> Malaria causes polyclonal B-cell expansion and increased expression  
86 of activation-induced cytidine deaminase (AID) dependent DNA damage leading to the hallmark  
87 translocation of the *MYC* gene under control of the constitutively active immunoglobulin  
88 enhancer.<sup>4-6</sup> How EBV potentiates eBL is incompletely understood, however, the clonal  
89 presence of this virus in almost every eBL tumor suggests a necessary role.

90 EBV strains are categorized into two types based on the high degree of divergence in  
91 the *EBNA2* and *EBNA3* genes.<sup>7-9</sup> This long standing evolutionary division is also present in  
92 orthologous primate viruses,<sup>10</sup> yet remains unexplained. While EBV type 1 has been extensively  
93 studied,<sup>11,12</sup> because it causes acute infectious mononucleosis and other diseases in the  
94 developed world, type 2 virus studies have not kept pace since infected individuals are less  
95 frequent and found primarily in sub-Saharan Africa. While several recent studies have reported  
96 both types of EBV circulating in western countries,<sup>13,14</sup> the African context provides a better  
97 opportunity to examine viral variation because type 1 and type 2 are found in both eBL patients  
98 as well as healthy individuals.<sup>8,15,16</sup> Viral variation has been shown to impact differential  
99 transformation and growth, and capacity to block apoptosis or immune recognition.<sup>7,17,18</sup>  
100 However, studies focusing on only certain genomic regions/proteins potentially miss disease  
101 associations of other loci.<sup>19,20</sup> Although new studies have been conducted,<sup>21,22</sup> genome-wide  
102 examinations in case-control studies are few and often lack typing the virus.

103           To address this shortfall, whole genome sequencing of EBV is now attainable from  
104 tumor, blood, or saliva using targeted viral DNA capture methods.<sup>23–28</sup> However, studying EBV  
105 from the blood of healthy individuals remains challenging due to low viral abundance relative to  
106 human DNA (1-10 EBV copy/ng blood DNA). In addition, EBV's GC-rich genome is inefficiently  
107 amplified using conventional library preparation methods. Here, we present improved methods  
108 for EBV genome enrichment that allow us to sequence virus directly from eBL patients and  
109 healthy children. Leveraging these samples, we sought to define the viral population structure  
110 and characterize viral subtypes collected from children hailing from the same region of western  
111 Kenya. Additionally, we performed the first genome wide association study to identify viral  
112 variants that correlate with eBL pathogenesis.

## 113 **Materials and Methods**

### 114 **Ethical approval and sample collection**

115 For this study, we recruited children between 2009 and 2012 with suspected eBL,  
116 between 2-14 years of age, undergoing initial diagnosis at Jaramogi Oginga Odinga Teaching  
117 and Referral Hospital (JOOTRH; Kisumu), which is a regional referral hospital for pediatric  
118 cancer in western Kenya.<sup>29</sup> We obtained written informed consent from children's parents or  
119 legal guardians to enroll them in this study. Ethical approval was obtained from the Institutional  
120 Review Board at the University of Massachusetts Medical School and the Scientific and Ethical  
121 Review Unit at the Kenya Medical Research Institute. For this study, primary tumor biopsies  
122 were collected using fine needle aspirates (FNA) and transferred into RNAlater at the bedside,  
123 prior to induction of chemotherapy. In addition, peripheral blood samples were collected and  
124 fractionated by centrifugation prior to freezing into plasma and cell pellets. All samples were  
125 stored at -80°C prior to nucleic acid extraction.

### 126 **Improved enrichment of GC-rich EBV in low abundance samples**

127 We used Allprep DNA/RNA/Protein mini kit (Qiagen) for DNA isolations from FNAs and  
128 QIAamp DNA Kit for blood and plasma. We developed an improved multi-step amplification and  
129 enrichment process for the GC-rich EBV genome, particularly in samples with low viral copies.  
130 We used EBV-specific whole genome amplification (sWGA) to provide sufficient material and  
131 targeted enrichment with hybridization probes after the library preparation. For this, we designed  
132 3'-protected oligos following the instructions from Leichty et al.<sup>30</sup> (detailed in Supplemental  
133 Methods). For low viral load samples, we added a multiplex long-range PCR amplification  
134 (mlrPCR) step, comprising two sets of non-overlapping EBV-specific primers<sup>31</sup> tiling across the  
135 genome. We improved the amplification yield for low copy EBV input (**Supplemental Table 1**)  
136 by optimizing buffers and reaction conditions (**Supplemental Figure 1A and 1B**).

137           **Sequencing library preparation and hybrid capture enrichment**

138           Illumina sequencing library preparation steps consisted of DNA shearing, blunt-end  
139 repair (Quick Blunting kit, NEB), 3'-adenylation (Klenow Fragment 3' to 5' exo-, NEB), and  
140 ligation of indexed sequencing adaptors (Quick Ligation kit, NEB). We PCR amplified libraries to  
141 a final concentration with 10 cycles using KAPA HiFi HotStart ReadyMix and quantified them  
142 using bioanalyzer. We then pooled sample libraries balancing them according to their EBV  
143 content and proceeded to target enrichment hybridization using custom EBV-specific  
144 biotinylated RNA probes (MyBaits, Arbor Biosciences). We sequenced the libraries using  
145 Illumina sequencing instruments with various read lengths ranging from 75bp to 150bp.

146           **Sequence preprocessing and de novo genome assembly**

147           We checked the sequence quality using FastQC (v0.10.1) after trimming residual adapter and  
148 low quality bases (<20) using cutadapt (v1.7.1)<sup>32</sup> and prinseq (v0.20.4),<sup>33</sup> respectively. After  
149 removing reads that mapped to the human genome (hg38), we de novo assembled the  
150 remaining reads into contigs with VelvetOptimiser (v2.2.5)<sup>34</sup> using a kmer search ranging from  
151 21 to 149 to maximize N50. We then ordered and oriented the contigs guided by the reference  
152 using ABACAS, extended with read support using IMAGE,<sup>35</sup> and merged the overlapping  
153 contigs to form larger scaffolds (using in-house scripts). By aligning reads back to scaffolds, we  
154 assessed contig quality requiring support from  $\geq 5$  unique reads. We created a final genome by  
155 demarcating repetitive and missing regions due to low coverage with sequential ambiguous "N"  
156 nucleotides. We excluded minor variants (<5% of reads) in final assemblies. Deposited  
157 genomes can be accessed from GenBank ([accession #](#)) and raw reads can be downloaded  
158 from SRA ([SRA accession #](#)).

159           **Diversity and variant association analysis**

160           We used Mafft (v7.215)<sup>36</sup> for multiple sequence alignment (msa) of genomes, and  
161           constructed phylogenetic neighbor-joining trees with Jukes-Cantor substitution model using  
162           MEGA (v6.0).<sup>37</sup> We determined variant sites relative to consensus using snp-sites (v2.3.2)<sup>38</sup>  
163           then projected variant loci on EBV type 1 reference. For principal coordinate analysis (PCoA),  
164           we used dartR (v1.0.5).<sup>39</sup> We calculated dN/dS rates per gene using SNAP (v2.1.1) after  
165           excluding frameshift insertions and ambiguous bases.<sup>40</sup> For variant association analysis, we  
166           used 'v-assoc' function from PSEQ/PLINK. To control for multiple testing, we calculated  
167           empirical p-values with one million permutations (pseq proj v-assoc --phenotype eBL --fix-null --  
168           perm 1000000) with EBV type stratification which permutes within types (--strata EBVtype).

## 169           **Results**

### 170           **Study participant characteristics**

171           The objective of this study was to examine EBV genetic variation in a region of western  
172 Kenya with a high incidence of eBL<sup>29</sup> and determine if any variants are associated with eBL  
173 pathogenesis. We leveraged specimens from eBL patients and healthy children residing in the  
174 same geographic area (**Figure 1A**).<sup>29</sup> We sequenced the virus isolated from 58 eBL cases and  
175 40 healthy Kenyan children, as controls. Patients aging between 1 and 13 years were  
176 predominantly male (74%), consistent with the sex ratio of eBL (**Table 1**).<sup>29</sup> Healthy controls had  
177 similar levels of malaria exposure based on previous epidemiologic studies.<sup>41</sup> Control samples  
178 ranged in age from 1 to 6 years. This difference in age was necessary due to the finding that  
179 younger, healthy yet malaria-exposed children have higher average viral loads compared to  
180 older children who have developed immune control over this chronic viral infection.<sup>42</sup>

### 181           **Sequencing and assembly quality**

182           EBV is a large GC-rich double stranded DNA virus with 172 kb genome of which ~20%  
183 is repetitive sequence. For the majority of eBL patients, we prepared sequencing libraries  
184 directly from tumor DNA followed by hybrid capture enrichment. For low copy viral samples,  
185 such as eBL plasma and healthy control blood, we designed and implemented additional viral  
186 whole genome amplification and enrichment prior to library preparation and sequencing (**Figure**  
187 **1A; Supplementary Figure 1**). We generated a study set of 114 genomes including replicates  
188 from cell lines and primary clinical samples, representing 98 cases and controls. In addition, we  
189 sequenced 20 technical replicates for quality control purposes such as estimation of re-  
190 sequencing error or sWGA bias, and sensitivity of detection of mixed infections. The baseline  
191 re-sequencing error rate was limited to  $\sim 1.1 \times 10^{-5}$  bases when our assemblies are compared  
192 with high-quality known strain genomes<sup>43</sup> (**Supplemental Table 2**). The mean error rate was

193 ~2.1x10<sup>-5</sup> bases for sWGA with GenomiPhi, while it is ~1.1x10<sup>-4</sup> bases when we used more  
194 sensitive mlrPCR-sWGA (Methods). We obtained an average of ~5 million reads, resulting in an  
195 average 9,688 depth of coverage across assemblies (**Supplemental Table 3**). De novo  
196 sequence assembly created large scaffolds covering non-repetitive regions, except three  
197 isolates with low coverage, yielded a median of 137,887bp genomes (ranging 47,534bp -  
198 146,920bp). We determined the types of each isolate by calculating the nucleotide distance to  
199 both reference types in addition to read mapping rates against type-specific regions. Despite our  
200 ability to experimentally detect mixed types at levels as low as 10% (**Supplemental Figure 2A**),  
201 we found no evidence of mixed infections in our cases and controls. Also, to ensure that our  
202 sample inclusion was unbiased when selecting healthy individuals with high enough viremias to  
203 sequence, we compared the viral loads and found no significant difference between type 1 and  
204 2 ( $P=0.126$ , **Supplemental Figure 2B**).

#### 205 **Equivalence of tumor and plasma viral DNA in eBL cases**

206 The viral genomes from eBL cases included virus reconstructed from plasma and tumor  
207 samples. We confirmed that viral DNA in the plasma was representative of the virus in the tumor  
208 cells by sequencing plasma-tumor pairs from 6 eBL patients (**Figure 1B**). Accounting for the  
209 sequencing errors, the pairs appeared to be identical. Besides these plasma-tumor pairs, we  
210 further confirmed identical EBV types with additional pairs from 8 separate patients using type-  
211 specific PCRs. Overall, these findings demonstrate that viral DNA isolated from plasma  
212 represents the tumor virus.

#### 213 **Structural variation and intertypic recombinants**

214 First, we looked for large deletions within our viral genomes, but did not detect any of the  
215 previously described deletions in EBNA3C deletion in Raji and the EBNA2 deletion in Daudi cell lines. However, in one  
216 sample we did detect a novel 20kb deletion, spanning from 100 kb to 120 kb in the genome  
217

218 **(Figure 1C)**, which contains lytic phase genes *BBRF1/2*, *BBLF1/3*, *BGLF1/2/3/4/5*, and  
219 *BDLF2/3/4*. Interestingly, none of the latent genes were affected by this deletion.

220 Next, we interrogated our isolates by comparing the pairwise similarities of each genome  
221 against EBV type 1 and type 2 references. By traversing through the genome with a window, we  
222 were able to delineate regions that were more similar to one type over the other **(Figure 1D)**. As  
223 expected, Jijoye, a type 2 strain, displayed less similarity against type 1 reference around its  
224 *EBNA2* and *EBNA3* genes, the most divergent region between types, while Namalwa as a type  
225 1 strain shows the same pattern of dissimilarity against type 2 reference around the same  
226 regions. Interestingly, we found three patient-derived genomes, eBL-Tumor-0012, eBL-Tumor-  
227 0033, and eBL-Plasma-0049, with mixed similarity trends. Similar to a previously detected  
228 recombinant strain (LN827563.2\_sLCL-1.18),<sup>43</sup> all of the intertypic isolates carried type 1  
229 *EBNA2* and type 2 *EBNA3* genes. Although not significant ( $P=0.268$ ), these new intertypic  
230 hybrids were all isolated from eBL patients while we did not detect any in healthy controls.

### 231 **Genomic population structure is driven by type differences with distinct** 232 **substructure in type 2 viruses.**

233 Our samples present a unique opportunity to study population structure of EBV types  
234 and their co-evolution within a geographically defined region. As expected, the major bifurcation  
235 within the phylogenetic tree based on the entire genome occurs between type 1 and type 2  
236 viruses **(Figure 2A)**. Viruses from eBL patients as well as healthy controls appeared to be  
237 intermixed almost randomly within the type 1 branch. Interestingly, within type 2 genomes 8  
238 eBL-associated isolates formed a sub-cluster. The hybrid genomes clustered with type 2s,  
239 which is consistent with type 2 *EBNA3s* representing a greater amount of sequence than type 1  
240 *EBNA2* region.

241 We further explored viral population structure with principal coordinate analysis (PCoA)  
242 of variation across the genome. While the first three components cumulatively explain 57.2% of

243 the total variance, the first component, which solely accounted for 43.9% of the variance,  
244 separates genomes based on type 1 and type 2 (**Figure 2B, upper plot**). Similar to the  
245 phylogenetic tree, intertypic genomes positioned more closely to type 2s. Interestingly, the  
246 second and predominantly third components separate type 2 viruses into two distinct clusters,  
247 group A and B (**Figure 2B, lower plot**). These clusters were reflected, although not as  
248 distinctly, in the structure of the tree as well. The PCoA loading values, which accounts for  
249 37.1% of the variance between the type 2 groups, are predominantly driven by correlated  
250 variation spanning 70kb upstream of EBNA3C (**Supplemental Figure 3A and B**). Together  
251 these findings suggest that there are two EBV type 2 strains circulating within this population.  
252 We also examined viral variation from the perspective of LMP1. Interestingly, the vast majority  
253 of viruses were grouped into Alaskan and Mediterranean strains (**Supplemental Figure 4**). For  
254 all available LMP1 type 2 sequences, group A and group B correlated with Mediterranean and  
255 Alaskan, respectively.

### 256 **EBV type 2 has less diversity compared with type 1**

257 We further explored the pattern and nature of genomic variation across the genome  
258 comparing and contrasting EBV type 1 and type 2. Examining the pairwise divergence of coding  
259 genes for all viral genomes, we found that the divergence was the highest in the type-specific  
260 *EBNA* genes (*EBNA2* and *EBNA3s*), in particular, with *EBNA2* showing the greatest divergence  
261 ( $d=0.1313 \pm 2.3 \times 10^{-3}$ ) (**Figure 2C, upper panel**). Investigating each type separately, the  
262 diversity within types was low for *EBNA2* and *EBNA3Cs*, consistent with type 1 and 2 being  
263 separated by many fixed differences (**Figure 2C, middle panel**). In both types, intra-type  
264 divergence was greatest for *EBNA1* and *LMP1*. Most remarkable was the fact that type 2  
265 generally showed lower levels of divergence across the genome ( $0.0047 \pm 3.7 \times 10^{-3}$  and  $0.0025$   
266  $\pm 2.7 \times 10^{-3}$  for type 1 and type 2, respectively). Overall, these measures suggest that EBV gene  
267 evolutionary rates differ by types.

268 To explore signatures of evolutionary selection, we examined the dN/dS ratios within  
269 coding sequences (**Figure 2C, lower panel**). Overall most genes showed signals of purifying  
270 selection, as indicated by  $\omega < 1.0$ , except *LMP1*, *BARF0*, and *BKRF2* (only type 2).  
271 Interestingly, with dN/dS measures, *EBNA2*, *BSLF1*, *BSLF2*, and *BLLF2* genes had relatively  
272 higher rates in type 2 compared to type 1 suggestive of differential evolutionary pressure.  
273 Overall, the magnitude of average nonsynonymous and synonymous changes per gene,  
274 normalized by gene length, reflect the high-level diversity accumulated in certain genes  
275 (**Supplemental Figure 5**). Latency-associated genes generally have the highest non-  
276 synonymous variant rates, but they also have the highest synonymous rates consistent with  
277 longstanding divergence (**Figure 2D**). Other functional categories, including lytic genes, have  
278 relatively low levels of nonsynonymous mutations suggesting stronger purifying selection.

#### 279 **Global context of Kenyan viruses**

280 To more broadly contextualize our viral population from western Kenya, we examined  
281 the phylogeny of the Kenyan viruses along with other publicly available genomes from across  
282 the world (**Supplemental Table 4**). Among all isolates, the most polymorphic genomic regions  
283 appeared to be around *EBNA2* and *EBNA3* genes (**Supplemental Figure 6A**). Phylogenetic  
284 tree shows that the major types, type 1 and type 2, are the main demarcation point regardless of  
285 the source or geographic location. The three intertypic genomes from our sample set neatly  
286 cluster with the previously isolated intertypic hybrid, sLCL-1.18 (**Supplemental Figure 6B**).  
287 Type 1 genomes from our study were split into two groups, with one forming a sub-branch only  
288 with Kenyan type 1, including Mutu, Daudi, and several Kenyan LCLs. The second group  
289 interspersed with other African (Ghana, Nigeria, North Africa) and non-African isolates. In  
290 addition, a few of our genomes from healthy carriers clustered with a group of mainly Australian  
291 isolates, however; none of them clustered with South Asian group. Our Kenyan EBV type 2s  
292 generally intermixed with other type 2 genomes.

## 293 **Viral Genomic Variants and Associations with eBL**

294 After excluding the intertypic hybrids, we compared type frequencies of EBV genomes  
295 isolated from eBL patients and healthy controls. We observed a significant difference in  
296 frequencies with 74.5% of eBLs carrying type 1 while only 25.5% carried type 2 infections. In  
297 contrast, 47.5% vs. 52.5% of type 1 and type 2, respectively were found in healthy controls.  
298 EBV type 1 was associated with eBL (OR=3.24, 95% CI=1.36 - 7.71,  $P = 0.007$ , Fisher's exact)  
299 (**Figure 3A**), independent of age and gender (all  $P > 0.05$ , **Supplemental Figure 7**). We then  
300 expanded the association analysis to all 2198 non-synonymous single nucleotide variations  
301 across the entire genome (**Figure 3B**). We did an initial association test for each  
302 nonsynonymous variant and detected 133 significant associations (**Supplemental Table 5 &**  
303 **Methods**). The vast majority of these variants were located within the type1-type2 region given  
304 the highly correlated nature of this region. We then stratified by type to detect variation  
305 independent of viral type. This yielded 6 variants solely associated with eBL (**Table 2,**  
306 **Supplemental Table 5**). Variant 37668T>C represents a serine residue change to a proline at  
307 the C-terminus of *EBNA2* (S485P) which is carried by 24/54 eBL cases; while this variant was  
308 present in only 2/36 healthy controls. Two variants in *EBNA1* at 95773A>T and 95778T>G  
309 (N38Y and H39Q, respectively) were both observed in 3/57 eBL isolates while their  
310 corresponding frequencies were 11/36 and 12/37 among healthy controls.

311 Nucleotide variants in non-coding and promoter regions can affect regulation of viral  
312 gene expression and activity within host cell. *BZLF1* is a regulator gene of lytic reactivation and  
313 classified based on its promoter as prototype Zp-P (B95-8) and Zp-V3 (M81 strain).<sup>44</sup> We  
314 determined variants at seven positions in the upstream promoter region of *BZLF1*  
315 (**Supplemental Table 6**). Interestingly, all of the Kenyan viruses carried C at positions both -525  
316 and -274 (as in Zp-P) regardless of promoter type. We also found that -532 and -524 are  
317 variable in our isolates while these two are not variant in both promoter types. Our results show  
318 that only 12.5% (5/40) type 1 promoter sequences fully resembled Zp-V3 in eBL group as

319 opposed to 22% (2/9) healthy genomes, while all of the type 2 genomes, without exception,  
320 carried Zp-V3 type promoter regardless of disease status.

## 321 Discussion

322 In this study, we investigated genomic diversity of EBV by sampling virus from children  
323 in western Kenya where eBL incidence is high.<sup>41</sup> Our improved methods allowed us to  
324 sequence asymptotically infected healthy controls with relatively low peripheral blood viral  
325 loads, and thereby examine the virus in the population at large.<sup>42</sup> We performed the first  
326 association study comparing viral genomes from eBL patients and geographically matched  
327 controls, without the need for viral propagation in LCLs; thus showing that type 1 EBV, as well  
328 as potentially several non-type specific variants, are associated with eBL. Furthermore, as the  
329 first study that characterized significant numbers of EBV type 2, we were able to compare and  
330 contrast both types and explore the viral population, thus discovering novel differences including  
331 population substructure in EBV type 2.

332 Our sequencing data demonstrated that EBV from plasma is representative of the tumor  
333 virus in eBL patients. This is consistent with the premise that peripheral EBV DNA originates  
334 from apoptotic tumor cells given that cell-free EBV DNA in eBL patients are mostly unprotected  
335 against DNase<sup>45</sup>, as opposed to being encapsidated during lytic reactivation, and that plasma  
336 EBV levels are associated with tumor burden and stage.<sup>46</sup> These findings support the use of  
337 plasma viremia as a surrogate biomarker and the development of plasma-based prognostic  
338 tests with predictive models that could be used during clinical trials.<sup>46</sup> The lack of mixed  
339 infections observed in our healthy controls could be due to the limit of detection in blood  
340 compared to virus isolated from saliva.<sup>14</sup> Further studies are needed to understand the  
341 coevolution and dynamics of both EBV types.

342 In addition, we detected three intertypic recombinant EBV genomes solely found within  
343 our eBL patients; similar to those previously described in other cancers.<sup>47</sup> It is unclear whether  
344 the intertypic genomes represent a common event with subsequent mutation and recombination  
345 or multiple independent events. If the latter is true, it supports more frequent mixed-type

346 infections given that both parents have to be present in the same cell.<sup>48–50</sup> It is interesting that all  
347 four intertypics observed to date carry the same type *EBNA2/EBNA3* combinations with the type  
348 2 genes being so closely related (**Supplemental Figure 8**). Thus, if multiple events have  
349 generated these viruses, it suggests that certain strains may have a greater proclivity to  
350 recombine. Further studies will be needed to better define the intertypic population, their origins  
351 and their association with disease.

352       Importantly, we were able to explore EBV population genetics and compare and contrast  
353 type 1 and type 2 because of their co-prevalence in Africa. As well described, the major  
354 differentiation in terms of genetic variability was the variation correlated with type 1 and type 2  
355 viruses. These viral types showed distinct population characteristics with type 1 harboring  
356 greater diversity especially in functionally important latent genes. Combined with the observed  
357 nucleotide diversity, latency genes appear to have long standing divergence that has  
358 accumulated significant synonymous changes (as opposed to recent sweeps on  
359 nonsynonymous changes that would erase synonymous variants). Global phylogenetic analysis  
360 emphasizes this diversity by providing two main subgroups for type 1 genomes in our  
361 sequencing set. One group represents core local Kenyan viruses while the second group is a  
362 mixture of viruses from across the globe, with the exception of South Asian viruses that group  
363 apart. While previously sequenced type 2 viruses intermingle with western Kenya isolates, the  
364 majority of these originated from East Africa with only a few from West Africa. Interestingly,  
365 intermingling is also true for type 2 as we observed two distinct groups. This is more apparent in  
366 PCA where type 2 virus forms 2 clusters. Examination via PCA, the loading values are  
367 determined by a broad stretch of the genome from the end of *EBNA3C* to *LMP1*, where  
368 Mediterranean and Alaskan designations correlate. It remains to be determined whether this  
369 substructure might be due to the introduction of previously geographically isolated viruses or  
370 distinct evolutionary trajectories within the population. Further study is needed with broader

371 samplings to understand its significance but our findings suggest that there may be significant  
372 epistasis potentially including *LMP1*.

373         By sequencing virus directly from healthy controls, we were able to address the question  
374 of relative tumorigenicity between type 1 and 2. We tested the long-standing hypothesis that  
375 type 1 virus is more strongly associated with eBL, in contrast to type 2. Our work was able to  
376 more definitely answer this question as we were not reliant on LCLs from healthy controls where  
377 type 1 bias in transformation might explain the lack of previous associations. We earlier  
378 demonstrated, by mutational profiling of EBV positive and negative eBL tumors, that the virus,  
379 especially type 1, might mitigate the necessity of certain driver mutations in the host genome.<sup>16</sup> In  
380 addition, our genome-wide results controlling for viral type substantiates investigations of non-  
381 type associated variation that could also impart oncogenic risk, as we found suggestive trends  
382 for several nonsynonymous variants as well. Only a small subset of type 1 viruses from eBL  
383 patients carried *BZLF1* promoter variant, which leads to a gain of function,<sup>44</sup> while all type 2  
384 viruses carried this variant suggesting this promoter might be beneficial for type 2 but makes it  
385 unlikely to be a driver of oncogenesis.

386         Overall, this population-based study provides the groundwork to unravel the complexities  
387 of EBV genome structure and insight into viral variation that influences oncogenesis. Genomic  
388 and mutational analysis of BL tumors identified key differences based on viral content  
389 suggesting new avenues for the development of prognostic molecular biomarkers and the  
390 potential for antiviral therapeutic interventions.

## 391 **Acknowledgements**

392 This work was supported by the US National Institutes of Health, National Cancer  
393 Institute R01 CA134051, R01 CA189806 (A.M.M., J.A.B, C.I.O, Y.K.) and The Thrasher  
394 Research Fund 02833-7 (A.M.M.), UMCCTS Pilot Project Program U1 LTR000161-04 (Y.K.,  
395 J.A.B., and A.M.M.), Turkish Ministry of National Education Graduate Study Abroad Program  
396 (Y.K.). We would like to thank the Kenyan children and their families who participated in this  
397 study. Patrick Marsh for helping with EBV genotyping assays, Mercedeh Movassagh for sharing  
398 genotyping primers. This publication was approved by the Director of KEMRI.

## 399 **Authorship Contributions**

400 Contribution: Y.K., C.I.O., and O.A. designed and performed experiments; Y.K. and  
401 C.I.O analyzed and interpreted results; Y.K. made the figures; Y.K., J.A.B. and A.M.M. designed  
402 the research and wrote the paper, C.I.O, J.A.O., J.M.O., and A.M.M. organized clinical sample  
403 acquisition.

## 404 **Disclosure of Conflicts of Interest**

405 The authors declare no competing financial interests. The current affiliation for Yasin  
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528 sequences in the region of BamHI fragments Y and H. *J. Virol.* 1985;55(2):286–297.

529 **Tables:**

530 **Table 1. Characteristics of children included in EBV sequencing analysis.**

		eBL Patients (N=58)	Healthy Controls (N=40)
<b>Age at collection, N (%)</b>	<6 (yrs)	16 (27.6)	39 (97.5)
	7 - 13 (yrs)	42 (72.4)	1 (2.5)
<b>Sex, N (%)</b>	Female/Male	15/43 (25.9/74.1)	20/20 (50.0/50.0)
<b>Obtained Specimen, N (%)</b>	Tumor biopsy	41 (41.8)	-
	Blood	-	40 (100.0)
	Plasma	14 (14.2)	-
	New cultured eBL	3 (3.0)	-

531

532

533 **Table 2. Single nucleotide variants associated with eBL.**

Gene	Position	Ref	Alt	AA Change	eBLs		Healthy Controls		P	OR
					Genotypes*	Alt Count	Genotypes*	Alt Count		
EBNA2	37668	T	C	S485P	54	24	36	2	0.000328	0.1
EBNA1	95773	A	T	N38Y	57	3	36	11	0.001322	6.67213
EBNA1	95778	T	G	H39Q	57	3	37	12	0.000538	7.16129
BcLF1	124703	T	G	K159T	56	1	34	7	0.003178	12.7377
BcLF1	124709	G	A	A157V	56	1	34	7	0.003092	12.7377
BARF1	165131	T	C	V29A	57	36	36	10	0.004082	0.349462

534 Single nucleotide variant association test results with  $P < 0.01$  after type stratification. Table

535 summarizes the statistically significant single nucleotide variant associations and their effects in the

536 coding regions. Reference is the genotype based on the consensus of all genomes in the sequencing

537 set and variant position denotes the projection to type 1 reference genome (NC\_007605). The  
538 association test has been performed for every variant position comparing the frequency of reference  
539 and alternative (minor allele) bases among eBL patient and healthy control children (Fisher's exact  
540 test). Empirical p values were based on one million permutations. \*Genomes with missing data (Ns,  
541 lack of coverage) were excluded. Ref: reference allele, Alt: alternative/variant allele, AA: amino acid, P:  
542 p-value, OR: odds ratio.

## 543 **Figure Legends**

### 544 **Figure 1. EBV genome sequencing from tumors and primary clinical samples.**

545 **A)** Overview of sample collection and methods for sequencing virus from Kenyan children  
546 diagnosed with eBL and healthy children as controls. Hybrid capture was universally performed  
547 along with additional amplification and enrichment steps to overcome low amounts of virus and  
548 input DNA. mlrPCR-sWGA; multiplexed long range PCR - specific whole genome amplification.  
549 **B)** Comparison of virus from paired tumor (brown circles) and plasma samples (pink circles) at  
550 diagnosis shows viral DNA circulating in the peripheral blood represents the virus in the tumor.  
551 The neighbor-joining tree is scaled (0.001 substitutions per site) and includes standard  
552 reference genomes for type 1 (NC007605, blue diamond) and type 2 (NC009334, red diamond).  
553 **C)** The depth of coverage showing an absence of reads from approximately 100 kb to 120 kb is  
554 indicative of a large deletion in the virus from an eBL tumor (top panel). In the middle and lower  
555 panels, although we did not detect any in our tumor or control viruses, we had the power to  
556 detect deletions previously described in tumor lines including EBNA3C deletion in Raji and  
557 ENBA2 deletion in Daudi strains. **D)** Three intertypic viruses were detected by scanning across  
558 the genomes for percent identity in 1kb windows to both type 1 and type 2 references  
559 (NC\_007605, NC\_009334, respectively). Top two graphs (grey) represent controls, Jijoye and  
560 Namalwa, followed by 3 intertypic viruses from this study and one publicly available intertypic  
561 virus (LN827563.2\_sLCL-1.18 in grey).

### 562 **Figure 2. Diversity analysis of EBV genomes and coding genes in Kenyan population.**

563 **A)** Phylogenetic tree of the Western Kenya EBV genomes demonstrating the major type 1 and  
564 type 2 demarcation (blue and red branches, respectively). Pairwise distance calculations were  
565 based on Jukes-Cantor nucleotide substitution model, and the tree was constructed with the  
566 simple Neighbor-Joining method. Genomes are colored based on sample type: healthy children

567 blood (green squares), eBL tumors (brown circles), plasma of eBL children (pink circles), and  
568 new and previous cell lines (brown and yellow triangles, respectively). Low coverage genomes  
569 are excluded. **B)** Principal coordinates analysis plots of nucleotide variations among whole  
570 genome sequences with first and second axes (upper plot, colored by sample type), and second  
571 and third axes (lower plot, colored by EBV subtype and shapes represent case and control). **C)**  
572 Genetic distance metrics of each EBV gene calculated based on Kimura-2-parameter method  
573 averaged across all genomes (upper panel) or type 1 / type 2 separately (middle panel). Lower  
574 panel shows nonsynonymous to synonymous change (dN/dS) ratios of viral protein coding  
575 genes averaged across all pairwise comparisons with in each group separately. Error bars  
576 represent standard error of mean. (Three intertypic genomes are excluded). **D)** Average  
577 synonymous and non-synonymous variants in genes are summarized as functional categories  
578 of genes. Variant level represents the number of variants per gene normalized by gene length in  
579 kb.

580 **Figure 3. Significant associations of EBV type 1 genomes and single nucleotide variants**  
581 **with eBL.**

582 **A)** The frequency of type 1 and type 2 genomes identified from eBL patients and healthy control  
583 children (excluding the three intertypic hybrid genomes is significantly different ( $P=0.007$ ,  
584 Fisher's exact). **B)** Manhattan plot for genome-wide associations of non-synonymous single  
585 nucleotide variants tested for frequency differences between cases and controls controlling for  
586 type specific variants. The significance of each locus association is represented with an  
587 empirical p-value (negative log<sub>10</sub> scale) that was calculated by 1 million permutations with  
588 random label swapping. Permutations were stratified for EBV genome type and adjusted for the  
589 missing genotypes due to lack of coverage. All significant variants associated with eBL cases  
590 are shown in red ( $P < 0.01$ ). Nucleotide positions are according to type 1 reference genome.

## Figure 1

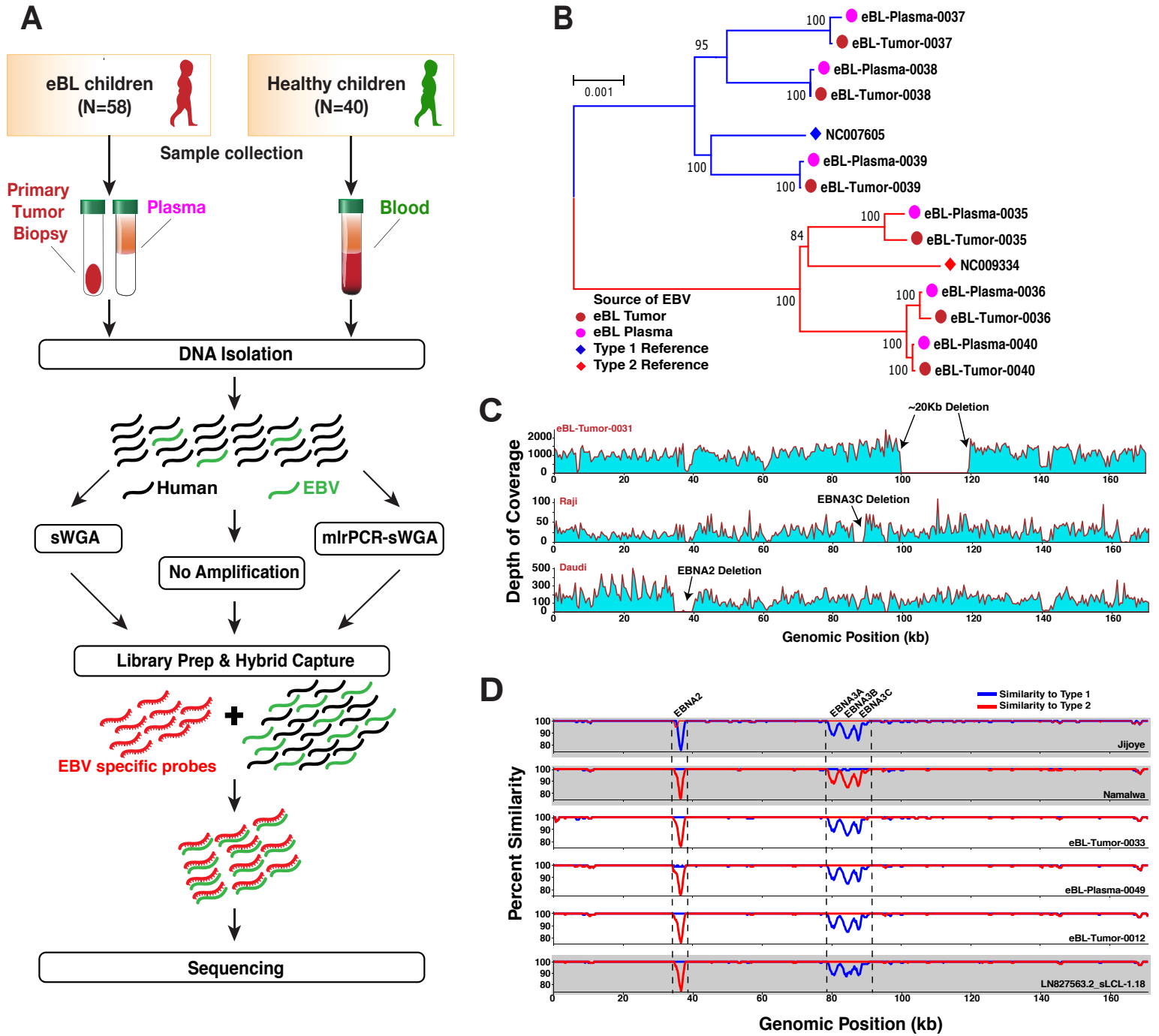




Figure 3

