CORRESPONDENCE

Re: Discrete Alterations in the BZLF1 Promoter in Tumor and Non-Tumor-Associated Epstein–Barr Virus

In their recent article, Gutiérrez et al. *(1)* identified three Epstein–Barr virus (EBV) variants in the BZLF1 promoter Zp (Zp-P [considered the prototypical sequence], Zp-V3, and Zp-V4) and postulated that these might result in different lytic potentials and be differentially expressed among individuals with nonmalignant and malignant diseases. Hong Kong and Southern China are considered to be endemic regions, with the EBV variants that are different from other localities.

We analyzed EBV type and sequence variations in EBV Zp domains by direct DNA sequencing in a total of 95 EBV-associated lesions (96 isolates) from individuals from Hong Kong and Southern China (Table 1). Eighty-seven of 95 (91.6%) lesions contained type A EBV. Eight (8.4%) lesions contained type B EBV, all of which carried Zp-V3. Of the 88 type A EBV isolates, 31 (35.2%) contained Zp-P and 53 (60.2%) contained Zp-V3. From 36 nonmalignant samples with type A EBV, 19 (52.8%) contained Zp-P, and 16 (44%) contained Zp-V3. From 51 malignant samples, we obtained 52 EBV isolates with type A EBV, of which 12 (23.1%) contained Zp-P and 37 (71.2%) contained Zp-V3. In addition, from one nonmalignant and three malignant samples with type A EBV, we identified four novel Zp-P variants. Three variants differed from Zp-P at one position only: Zp-V1_104 ($^{-104}$C-A), isolated from a healthy carrier; Zp-V1_105 ($^{-105}$C-T), isolated from a natural killer (NK)/T-cell lymphoma; and Zp-V1_119 ($^{-119}$G-A), isolated from a nasopharyngeal carcinoma (NPC). All these substitutions occurred within the ZIIIB domain of Zp, which can strongly bind ZEBRA (Z, EB replication activator) and contribute to the transactivation of Zp *(2).* A fourth variant (Zp-V3+118), isolated from an NPC, contained four nucleotide substitutions relative to Zp-P, three of which were identical to those found in Zp-V3 *(1),* and the fourth a C to T substitution at position –118. This position is also within the functional domain of ZIIIB. It is noteworthy that this variant with four nucleotide substitutions was different from the Zp-V4 variant described by Gutiérrez et al. *(1).* Taken together, these results suggest that the ZIIIB domain may be a mutational hotspot.

Table 1. Distribution of BZLF1 promoter variants in type A and type B Epstein–Barr virus (EBV) isolates from healthy carriers and various EBV-associated conditions*

	Total	Zp-P	Zp-V3	Zp-V3+118	Zp-V1
Type A EBV					
Non-malignant					
HC	21	11	9	0	1
IM	15	8	7	0	0
Total	36	19	16	0	1
Malignant					
NPC†	37	6	29	1	1
NK/T	12	6	5	0	1‡
PTLD	3	0	3	0	0
Total	52	12	37	1	2
Type B EBV					
Non-malignant					
HC	3		3		
IM	1		1		
Total	4		4		
Malignant					
NPC	4		4		
Total	4		4		

*A total of 96 EBV isolates from 40 non-malignant samples and 55 malignant samples were analyzed by direct sequencing. Briefly, amplification of position –221 to +12 (with reference to the transcription start site) of the BZLF1 promoter region was performed by using the polymerase chain reaction (PCR) as described by Gutiérrez et al. *(1).* Purified PCR products were sequenced using ABI PRISM Big Dye terminator cycle sequencing Kit (Applied Biosystems, Foster City, CA), and the sequencing products were run on an ABI 377 DNA auto-sequencer (Applied Biosystems). The non-malignant samples included 24 gargle specimens collected by rinsing the mouth with phosphate-buffered saline from healthy donors who were EBV carriers (HC) and 16 peripheral blood samples from subjects with infectious mononucleosis (IM). The malignant samples included biopsies of 39 nasopharyngeal carcinomas (NPC), 11 natural killer (NK)/T-cell lymphomas (NK/T), three EBV-positive post-transplant lymphoproliferative disease (PTLD) samples, and two NPC xenografts (Xeno-666 and Xeno-2117).

†Including two NPC xenografts, both carried type A EBV and contained Zp-V3.

‡This sample was co-infected with a variant containing Zp-P.

It was suggested that only Zp-P and Zp-V4, but not Zp-V3, were found in the nonmalignant samples *(1).* However, in the 40 nonmalignant samples from our locality, Zp-P and Zp-V3 occurred with similar frequency (19 of 40 and 20 of 40, respectively). None of our 95 samples contained Zp-V4, suggesting that the Zp-V4 identified by Gutiérrez et al. *(1)* may be restricted to people in a particular geographic region. In our malignant NPC samples, 80.5% (33/41) contained Zp-V3 and 14.6% (6/41) contained Zp-P, whereas in the nonmalignant samples, 50% (20/40) contained Zp-V3 and 47.5% (19/40) contained Zp-P. Thus, Zp-V3 was more prevalent in NPC samples than in nonmalignant samples ($P = .001$, chi-square test). If we considered only type A EBV isolates, then the association between NPC and Zp-V3 was still statistically significant ($P = .001$).

In our locality, the distribution of EBV variants with respect to Zp in various EBV-associated conditions is distinct from other geographic regions. Although Zp-V3 is detectable in nonmalignant samples, it is statistically significantly associated with NPC. Whether these variants have a functional consequence remains to be determined. However, the possible link(s) between different variants and lytic potential or latency-to-lytic cycle switch requires further investigation.

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RESPONSE

Tong et al. have extended the study related to BZLF1 polymorphic variants of Epstein–Barr virus (EBV) to samples from Hong Kong and Southern China. This study is of special interest because it incorporates malignant and nonmalignant samples from regions that are endemic for nasopharyngeal carcinoma (NPC). Their observations both confirm and contradict several conclusions from our study *(1)*. First, they provide independent confirmation of the existence of the Zp-P and Zp-V3 variants in another geographic region. Our earlier observations suggested that Zp variants segregated strongly by EBV type, with type B virus exclusively associating with Zp-V3 and type A virus often associating with Zp-P. Exceptions were noted in samples from Far East Asia, which contained type A virus with a Zp-V3 sequence, suggesting that type A Zp-V3 may be prevalent in this geographic region. Tong et al. confirm these findings and validate the existence and the prevalence of the type A Zp-V3 variant in the Southeastern Asian population.

Type B virus is generally considered to be more readily inducible than the type A virus *(2)*. We have speculated that the association of a virus with malignant and nonmalignant lesions is influenced by the lytic potential of the viral types, which in turn may be dictated by the variation in Zp. Tong et al. found a statistically significant association between type A Zp-V3 and NPC. One possible interpretation of these observations would be that a type A Zp-P variant is more responsive to lytic induction than a type A Zp-V3 variant. However, additional regulatory elements beyond Zp also modulate BZLF1, and the possibility that variations in these regions occur between type A Zp-P and type A Zp-V3 needs to be considered.

Several important differences also arise between our observations *(1)* and those of Tong et al. For example, there is the complete absence of Zp-V4 in their samples. We identified 53 Zp-V4 variants among our nonmalignant samples. It is highly unlikely that these differences are merely technical. An important element in these two studies pertains to the origin of the samples. Our samples from South and North America, Saudi Arabia, and Japan—all regions non-endemic for NPC *(3)*—contained EBV with a Zp-V4 variant. If indeed the prevalence of Zp-V4 is geographically restricted, it raises important epidemiologic considerations that may affect the pathogenesis of EBV-associated malignancies. If the absence of Zp-V4 in tumors reflects lower oncogenic potential, the diminished prevalence of this strain in a population endemic for EBV-associated malignancies is of notable interest. Additional studies from other endemic and non-endemic areas are required to establish these associations.

The apparent mutual exclusivity of Zp-V4 and Zp-V3 EBV in non-endemic and endemic populations, respectively, is intriguing. Given the low levels of EBV DNA in nonmalignant samples, the possibility that Zp-V3 and Zp-V4 variants are present at undetectable levels cannot be ruled out. Furthermore, the identification of other variants—albeit at low frequency—raises the possibility that ongoing mutational events may indeed play a role in the genesis of such variations *(4)*.

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