Polymorphisms of Epstein-Barr virus BHRF1 gene, a homologue of bcl-2

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Abstract

Background and Objective: EBV BamHI fragment H rightward open reading frame 1 (BHRF1), the Epstein-Barr virus (EBV) early gene, is structurally and functionally homologous to the oncogene bcl-2 and may play an important role in the development of EBV-associated tumors. To characterize the polymorphisms of BHRF1 in EBV-associated tumors, we analyzed the sequences of BHRF1 in isolates from nasopharyngeal carcinoma (NPC) and EBV-associated gastric carcinoma (EBVaGC) biopsies as well as throat washing (TW) samples from healthy donors. Methods: BHRF1 DNA sequences were analyzed by polymerase chain reaction (PCR) and sequencing for 39 NPC samples, 40 EBVaGC samples, and 53 EBV-positive TW samples from healthy donors. The variants of BHRF1 gene were classified according to the signature changes. The EBV types 1 and 2 at nuclear antigen (EBNA) 3C locus were determined by PCR. Results: Compared with EBV standard cell line B95-8, all isolates carried a silent mutation at amino acid (AA) 80 (nucleotide 54616 T→C), the AA88 L→V mutation was found in most isolates, and the AA79 V→L mutation in a few isolates. Other mutations were sporadically distributed. Based on the mutations at AA88 and AA79, 3 distinct variants of BHRF1 genes, designated as 79V88V, 79L88L, and 79V88L, were identified. The 79V88V was the most common variant. The distribution of the BHRF1 variants among the NPC, EBVaGC, and TW samples was not significant. The corresponding regions of bcl-2 homologues were conserved in all isolates except for 3 samples. The distribution of BHRF1 variants in type 1 and type 2 strains was significant different (P<0.001, contingency coefficient was 0.554). Conclusions: The 79V88V is the dominant variant in NPC, EBVaGC, and TW samples from healthy donors and preferential linkages between BHRF1 and EBNA3C variants exist. Conserved BHRF1 in Bcl-2 homologous domains is helpful to remain the important role of BHRF1.

Keywords: Epstein-Barr virus, nasopharyngeal neoplasm, gastric neoplasm, BHRF1, polymorphism

Epstein-Barr virus (EBV), a well-known tumor virus, is closely related with several human lymphoid and epithelial tumors, such as Burkitt’s lymphoma (BL), Hodgkin’s disease (HD), AIDS-associated lymphoma, nasopharyngeal carcinoma (NPC) and gastric cancer 1. Because the incidences of NPC, BL, and other EBV-associated tumors are obviously regional and the EBV strains from different origins have different genetic variations and biological functions, the relationship between EBV gene variation and tumorigenesis has always been the hot issue in the study of EBV carcinogenic mechanism. EBV frequently exists in the form of latent infection in EBV-associated tumor tissues. Studies on EBV gene polymorphisms mainly focused on the latent genes, such as the well recognized oncogene of latent membrane protein 1 (LMP1), EBV nuclear antigen 2 (EBNA2), EBNA3, EBNA1, and other necessary genes for transformation. According to the linked polymorphisms of EBNA2 and EBNA3 genes, EBV is divided into type 1 and type 2, also known as types A and B 2,3. Although the B-cell transformation capability of type 1 strain is more powerful than that of type 2 strain 4, it is generally believed that types
1 and 2 are regional variants and their distributions have regional differences. Type 1 EBV is the dominantly epidemic strain in all regions, whereas type 2 EBV only has a relatively high infection rate in Central Africa and New Guinea[8].

In recent years, the role of EBV BamHI fragment H rightward open reading frame 1 (BHRF1), an EBV early gene, in the EBV-associated tumors has received much attention. BHRF1 is the homologous gene of human proto-oncogene bcl-2, sharing 25% amino acids (AA) sequence identity and 42% amino acid sequence similarity with bcl-2[9,10]. BHRF1 is functionally similar to bcl-2 and can inhibit the apoptosis of B lymphocytes and epithelial cells[11]. Because BHRF1 mRNA contains the leading sequence of EBNA family, BHRF1 can also be expressed in virus latency phase by using the C or W promoter and in lytic phase by using Hp promoter[12]. BHRF1 mRNA can be detected in B-cell lymphoma, NPC, HD, T-cell lymphoma, NK/T-cell lymphoma, and gastric cancer tissues[13-16] and BL cell lines[17]. Although the detection rate in different studies varied, most of the results suggested that BHRF1 may play an important role in EBV-associated tumors. In the present study, we analyzed the BHRF1 gene polymorphism in EBV-positive NPC and EBV-associated gastric cancer (EBVaGC) tissues and the throat washing (TW) samples from healthy donors in Shandong Province, compared with the standard EBV strain and among different populations to clarify BHRF1 gene variation patterns in NPC, EBVaGC, and healthy donors and its significance.

Materials and Methods

Specimens and DNA extraction

This study was approved by the Ethics Committee of Affiliated Hospital of Medical College, Qingdao University. We collected 175 samples of paraffin-embedded NPC tissue, 1656 samples of paraffin-embedded and freshly resected gastric cancer tissue from 10 hospitals in Shandong Province. The diagnoses of all samples were confirmed by pathologic examination. The EBV-encoded small RNA1 (EBER1) in NPC and gastric cancer tissues was detected by in situ hybridization[17]. TW samples were collected from 268 healthy adults. EBV BamHI-W fragment in these samples was detected by polymerase chain reaction (PCR) and southern hybridization to select EBV-positive samples[18]. All samples were from natives of Shandong Province with at least three generations living at there. The positive rate of EBV was 80.6% in NPC tissues, 6.1% in gastric cancer tissues, and 40.3% in TW samples. BHRF1 gene polymorphism and EBV1/2 type in 39 NPC samples, 40 EBVaGC samples, and 53 TW samples from healthy donors were detected.

DNA from fresh tumor tissues and TW samples was extracted using the standard method with proteinase K digestion and phenol-chloroform purification. DNA of paraffin-embedded tissues was extracted using DNA extraction kit of QIAamp formalin-fixation and paraffin-embedding (FFPE, QIAGEN GmbH, Hilden, Germany).

BHRF1 gene sequencing

BHRF1 sequences were determined by nested PCR and direct sequencing. The primers of BHRF1 gene were designed according to the sequence of EBV standard strain B95-8 using the Primer premier5 software. Primer sequences and the position in the genome (B95-8, GenBank accession No. V01555.1) were 5'-TGTAGGTCCGGCTTGGTTTGGTTCG-3' (54 274–54 297) for BHRF1-1, 5'-ACCCCGGCTAATCTGCTGCTG-3' (54 321–54 344) for BHRF1-2, 5'-TCTTTGGTTTGGAGCTAGTCT-3' (55 035–55 052) for BHRF1-3, and 5'-GGCCCAATGACGCTGATCC-3' (54 997–54 981) for BHRF1-4. BHRF1-1 and BHRF1-2 were the first round primers, and the amplified fragment was 762 bp. BHRF1-3 and BHRF1-4 were the second round primers, and the amplified fragment was 677 bp. The first round PCR reaction system was 25 μL, including 2.5 μL 10× Buffer, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs, 0.4 μmol/L of each primer, 1.0 U Taq DNA polymerase (Takara Biotechnology Co., Company), and 100 ng DNA template. Cycle conditions were pre-denaturation at 94°C for 5 min, then 35 cycles of 94°C 30 s, 55°C 30 s, and 72°C 1 min, and final elongation at 72°C for 10 min. The first round PCR product was diluted by 1 : 20 to 1 : 100, then 2 μL of the diluted product was amplified in the second round PCR as described above with 50 μL reaction volume. After the second round amplification, 3 μL product was identified by 2% agarose gel electrophoresis. Water, EBV-positive cell line B95-8, and EBV-negative cell line Ramos were used as the blank control, positive control, and negative control, respectively, in each PCR reaction.

The product of the second round PCR was sequenced by Beijing Genomics Institute Co., Ltd. with the second round primers. After sequencing, the peak files were opened by the Chromas software, and the sequences were analyzed and spliced using the DNAStar software (Lasergene, version7.0), then, compared with the sequences of B95-8 and other related sequences in the GenBank.

EBV 1/2 type detection

Based on the type-specific regions of EBNA3C gene, EBV 1/2 type was detected by PCR in the EBV-positive samples with primers EBNA3C-1 (5'-AGAAGGGGAGCGT-
GTGTTGT-3') and EBNA3C-2 (5'-GGCTCGTTTTTGCA-CGTCGGC-3'), as previously reported [9]. EBV-positive cell lines B95-8 and P3HR1 were used as the controls of type 1 and type 2 strains, respectively. The size of products was 153 bp for type 1 strain, 246 bp for type 2 strain, and a mixture of 153 bp and 246 bp for co-infection of type 1 and type 2 strains.

Statistical analysis

The distributions of BHRF1 variants and 1/2 types among NPC tissues, EBVaGC tissues, TW samples from healthy donors and the relationship between BHRF1 variants and 1/2 types were analyzed by Fisher's exact test using the SAS 6.12 software. Differences were considered significant when \( P < 0.05 \).

Results

BHRF1 sequence variations

We detected the polymorphisms of BHRF1 gene full-length sequence (54 376–54 951) in 132 EBV-positive samples: 39 NPC tissues, 40 EBVaGC tissues, and 53 TW samples from healthy donors. DNA direct sequencing showed the presence of a single BHRF1 sequence variant in each sample. All isolates exhibited single nucleotide mutations compared with standard strain B95-8 (Figure 1). Totally, 22 amino acid mutations and 4 silent mutations were identified in all samples: 4 amino acid mutations (AA 24, 75, 79, and 88) and 3 silent mutations (AA 41, 80, and 112) were detected in at least 2 samples; others were sporadically distributed in only one sample. Silent mutation 54616nt T→C (AA80) was found in all isolates. Amino acid mutation AA88 L→V (54637nt T→G) was found in 26 (66.7%) of 39 NPC tissues, 34 (85.0%) of 40 EBVaGC tissues, and 41 (77.4%) of 53 TW samples from healthy donors. Amino acid mutation 79 V→L (54610nt G→T) was found in 2 EBVaGC and 4 NPC samples.

AA88 L→V and AA79 V→L mutations were not detected simultaneously in any isolates. According to the presence and/or absence of these two mutations, three BHRF1 variants were classified. The first variant was 79V88V, with AA88 L→V mutation but no AA79 mutation, and both sites were valine (V). Most of this variant exhibited AA88 L→V mutation and 54616nt T→C silent mutation, and the sequences were consistent with Guangzhou NPC strain GD1 [10]. The second variation was 79L88L, with AA79 V→L mutation but no AA88 L→V mutation, and both sites were leucine (L). Amino acid mutation and silent mutation were found at one or several sites in addition to the AA79 V→L mutation and 54616nt T→C mutation in this variant.

Silent mutation 54711nt C→T (AA112) was found in 4 samples, 54498nt G→A (AA41) in 2 samples, and amino acid mutations AA24 T→S or T→A in 3 NPC samples. The third variant was 79V88L, without AA79 and AA88 mutations. Most isolates only had 54616nt T→C silent mutation, and the sequences were consistent with the African BL strain AG876 [9].

BHRF1 gene contains 5 bcl-2 homologous domains NH1 (AA60-67), NH2 (AA13-22), BH1 (AA92-103), BH2 (AA142-150), and BH3 (AA50-59), in which AA99 glycine and AA143 tryptophan were equivalent to AA145 glycine and AA188 tryptophan of Bcl-2 protein, respectively. Except that 2 EBVaGC samples (G77 and G78) had an AA mutation in the NH2 domain and 1 NPC sample (N34) had an AA mutation in the BH1 domain. All these 5 bcl-2 homologous domains in the remaining strains were conserved. Both AA99 and AA143 were not changed in all isolated strains (Figure 1).

The distribution of BHRF1 variants in three groups

The frequency of 79V88V, 79L88L, and 79V88L was 76.5%, 4.6%, and 18.9%, respectively, in the 132 samples. 79V88V was the most common variant and no significant difference was observed in the distribution of these 3 variants among 3 groups (\( P = 0.052 \)) (Table 1).

Types of EBV strains present in samples

EBV 1/2 type was determined in 39 NPC, 40 EBVaGC, and 44 TW samples. The detection rates of type 1, type 2, and type 1/2 were 83.7%, 14.7%, and 1.6%, respectively. The distribution of type 1/2 in NPC, EBVaGC and healthy populations was not significantly different (\( P = 0.192 \)) (Table 1).

The relationship between BHRF1 variations and EBV type 1/2

The distribution of BHRF1 variations in 103 type 1 strains and 18 type 2 strains was significantly different (direct probability \( P < 0.001, \) contingency coefficient \( = 0.554 \)) (Table 2). Type 2 EBNA3C mainly was 79V88L BHRF1, accounting for 83.3% (15/18), whereas type 1 EBNA3C mainly exhibited 79V88V BHRF1, accounting for 85.4% (88/103), and 79L88L variant was only found in 6 type 1 strains.

Discussion

In this study, we analyzed BHRF1 gene polymorphism...
Figure 1  BHRF1 polymorphisms in Epstein-Barr virus (EBV)-positive nasopharyngeal carcinoma and gastric carcinoma biopsies as well as throat washing samples from healthy donors

The listed BHRF1 amino acid (AA) sequence corresponds to the B95.8 sequence under which the AA changes and the corresponding nucleotide changes in wild isolates are shown. An asterisk indicates a silent mutation in the corresponding AA residue. “N” means nasopharyngeal carcinoma (NPC) isolates, “G” means EBV-associated gastric carcinoma (EBVaGC) isolates, and “T” is throat washing samples from healthy donors. BHRF1 were classified into 3 variants, 79V88V, 79L88L, and 79V88L, based on the mutations at amino acids 79 and 88 (which are shaded). The positions of all known bcl-2 homology domains are shown in boxes. Gly-99 in BH1 corresponds to the functionally important Gly-145 in bcl-2, and Trp-143 corresponds to the functionally important Trp-188 in bcl-2. The corresponding regions of bcl-2 homologues were conserved in all isolates except for 2 EBVaGC isolates and 1 NPC isolate, which showed a mutation in NH2 and BH1 domain, respectively. Neither Gly-99 in BH1 nor Trp-143 in BH2 was altered in all the sequences.

Table 1  Distribution of BHRF1 variants and Epstein-Barr virus (EBV) types in EBV-positive nasopharyngeal carcinoma (NPC) and EBV-associated gastric carcinoma (EBVaGC) biopsies as well as throat washing (TW) samples from healthy donors

<table>
<thead>
<tr>
<th>Gene</th>
<th>NPC (n=39) [cases (%)]</th>
<th>EBVaGC (n=40) [cases (%)]</th>
<th>TW (n=53) [cases (%)]</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHRF1</td>
<td></td>
<td></td>
<td></td>
<td>0.052</td>
</tr>
<tr>
<td>79V88V</td>
<td>26 (66.7)</td>
<td>34 (85.0)</td>
<td>41 (77.4)</td>
<td></td>
</tr>
<tr>
<td>79L88L</td>
<td>4 (10.2)</td>
<td>2 (5.0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>79V88L</td>
<td>9 (23.1)</td>
<td>4 (10.0)</td>
<td>12 (22.6)</td>
<td></td>
</tr>
<tr>
<td>EBNA3C*</td>
<td></td>
<td></td>
<td></td>
<td>0.192</td>
</tr>
<tr>
<td>Type 1</td>
<td>32 (82.1)</td>
<td>37 (92.5)</td>
<td>34 (77.3)</td>
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<tr>
<td>Type 2</td>
<td>7 (17.9)</td>
<td>3 (5.0)</td>
<td>8 (18.2)</td>
<td></td>
</tr>
<tr>
<td>Type 1/2</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (4.5)</td>
<td></td>
</tr>
</tbody>
</table>

* Nine TW samples have not been detected for EBNA3C.
of EBV strains isolated from 39 NPC samples, 40 EBVaGC samples, and 53 TW samples from healthy donors. The results showed that sequences of BHRF1 in all isolated strains were highly conserved in the functional homologous domain of bcl-2. BHRF1 is structurally and functionally similar to bcl-2, a human anti-apoptosis gene. A variety of Bcl-2 family members have been found, including many cellular proteins (Bcl-x, Bax, Bak, Bad, and so on) and some viral proteins such as the adenovirus E1B 19K protein and the Kaposi sarcoma herpes virus ORF16-encoded protein.[1,2] The key motifs of the gene family are bcl-2 homology domains 1 and 2 (BH1, BH2), in which the Gly-145 at BH1 and Trp-188 at BH2 are critical to anti-apoptotic activity and forming dimmers with Bax.[3] In addition, conserved homologous domains NH1, NH2, and BH3 have also been found by comparing the sequences of adenovirus E1B 19K protein and cellular protein Bik with the sequences of Bcl-2.[4,5] These homologous domains can mediate the interaction among Bcl-2 family members and then affect cell apoptosis. In the present study, except the EBV strains isolated from 2 EBVaGC and 1 NPC samples had BHRF1 gene mutations in the bcl-2 homologous domains, all isolates were conserved in the 5 bcl-2 homologous domains. Furthermore, no mutation was found at 2 key sites of the bcl-2 homologous domains: AA99 glycine, which correspond to Gly-145; AA143 tryptophan, which correspond to Trp-188. All the consensus mutations and sporadic mutations were located out of 5 bcl-2 homologous domains. All isolates showed a silent mutation (AA80 TGC→TGT), most isolates also had AA88L→V mutation, and a few had AA79V→L mutation. According to the presence or absence of AA88 L→V and AA79 V→L mutations, BHRF1 gene variations were divided into 3 types: 79V88V, 79L88L, and 79V88L. This result was consistent with the findings of Khamin et al.[6] and Liu et al.[7]. Khamin et al.[8] detected BHRF1 sequences in 15 EBV strains isolated from different sources and different regions: 5 Chinese samples [2 normal human lymphoblastoid cell lines (LCL), 2 NPC LCL, and 1 NPC], 6 African samples (4 BL cell lines, 1 normal LCL, and 2 SCID mouse passed NPC), and 4 European HD samples. Liu et al.[9] detected the BHRF1 sequences in EBV strains isolated from 6 Taiwanese NPC samples, the soft tissues or lymph nodes from 5 patients with NPC and metastasis, 2 lymph node hyperplasia samples, and 1 adenoid hyperplasia sample. They all found that 5 bcl-2 homologous domains of BHRF1 in all isolated strains were conserved, and that most of the detected mutations were AA79 and AA88 mutation. Among the 14 samples detected by Liu et al.[10], 8 showed AA79 V→L mutation and 6 showed AA88 L→V mutation. Similar to the 79V88V and 79L88L variants in our study, AA79 and AA88 exhibited the same AA residues in the same isolated strain. The symmetry of these two AA residues may be necessary to maintain the protein structure.

BHRF1 is also functionally similar to bcl-2 which can inhibit the apoptosis of B lymphocytes and many epithelial cells. In vitro experiments demonstrated that the BHRF1 protein could enhance cell resistance to apoptosis induced by various external factors, such as removal of growth factors, serum-free culture, heterologous virus infection, chemotherapeutic drugs and radiation.[11] Theodorakis et al.[12] confirmed the importance of structural integrity of bcl-2 homologous domain to BHRF1 function. They found that any mutation in bcl-2 homologous domain of BHRF1 can lead to loss of the ability that encoded proteins protect rat kidney cell line BRK against the apoptosis induced by p53. BHRF1 is not persistently expressed in all EBV-associated tumors. However, early expression of the protein after virus infection may contribute to tumorigenesis and development. Dawson et al.[13] found that BHRF1 could delay the polarization differentiation of epithelial cells by inhibiting the apoptosis. This function may be related to the formation of epithelial tumor. BHRF1 gene plays a role in virus replication and the release of mature virus particles. It is highly expressed in lytic cycle and contributes to generate a large number of viruses by inhibiting cell apoptosis, and lowly expressed in virus latency to maintain the persistent infection of virus.[14]. Thus, the conservation of BHRF1 sequences is significant to maintain its functional stability.

Most of our samples had BHRF1 79V88V and 79L88L mutations, in which the AA residues were the same. Compared with the results of Liu et al.[15], the detection rate AA88 L→V mutation was higher in our samples than in Taiwanese samples [76.5% (101/132) vs. 42.9% (6/14)], whereas the detection rate 79L88L was lower in our samples than in Taiwanese samples [4.6% (6/132) vs. 57.1% (8/14)]. The difference, on the one hand, may be due to a relative small sample size studied by Liu et al.[16], on the other hand, may reflect the regional distribution. The distribution of BHRF1 subtypes had no significant difference among NPC samples, EBVaGC samples, and TW samples from healthy donors, suggesting no disease-associated variation. Our results also showed that 1/2 types was related to BHRF1 variation types. Type 2 EBNA3C mainly corresponded to 79V88L BHRF1, whereas type 1 EBNA3C
mainly corresponded to 79V88V BHRF1, indicating that 79V88L variation occurs mainly in type 2 strain, whereas 79V88V variation occurs mainly in type 1 strain. The correlation between 1/2 types and BHRF1 variations was consistent with that in EBNA1 gene in the same samples, which showed that type 2 isolate was mainly correlated with the P-thrV subtype, and type 1 isolate was mainly linked to the V-val subtype in Shandong Province\(^{39}\).

In summary, we found that EBV bcl-2 homologue BHRF1 is highly conserved in the functional domain, which is very important to maintain its role in the biological activity of virus.

References