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Epstein–Barr virus is integrated between *REL* and *BCL-11A* in American Burkitt lymphoma cell line (NAB-2)

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Epstein–Barr virus (EBV) initially isolated from the cultured Burkitt lymphoma (BL) cells, is one of the well-known oncogenic virus. The NAB-2 line, which was established from a North American Burkitt's tumor, was indicated to contain one copy of EBV DNA as the integrated form into chromosome 2p13 of the host genome. To demonstrate the integration site of EBV directly, and to clarify the relation between the integration sites and the oncogenes, fragments containing the nucleotide sequence of NAB-2 integration sites were cloned. EBV was integrated via the terminal repeats (TR), and integration sites located in the clone RP11-440P5 on chromosome 2, between two oncogenes, *REL* and *BCL11A*, which is apart from approximately 350 kbp from each other. Expression level of *REL* in NAB-2 was increased. The flanking region of chromosome 2 at the bilateral junction sites showed no homology to the junction sites of EBV. The integration site 2p13 overlaps with common fragile site, FRA2E. NAB-2 cells expressed almost all latent genes but LMP-2A that flanks the TR, indicating the type III of latent infection of EBV. Integration event in NAB-2 might alter the regulation of the oncogenes and provide advantage for continuous cell proliferation.
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Epstein–Barr virus (EBV) is a human herpesvirus with a double-stranded DNA genome, and is reported to be associated with development of malignancies including Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC).[1–3] EBV DNA is detected as a linear form in cells at the lytic phase of EBV infection. During the latent phase of infection, viral genome is maintained as an episomal form through the fusion of genomic termini.[4–6] Most BL and NPC tumors harbor episomal EBV genomes representing the persistently latent infection. Chromosomal integration of EBV DNA provides an alternative way of persistent infection, which may represent another state of virus–cell interaction.[7–10] Integration of EBV into the host genome might be a common event in lymphoma cells, but analysis of integrated EBV DNA is complicated because of the presence of an episomal form of EBV in the nucleoplasm.

A North American BL (NAB) line was established from a Burkitt's tumor derived from a 14-year-old female Caucasian.[11] The NAB-2 line, a clonal derivative of the original culture, exhibited t(8;22)(q22;q11–12) involving the c-*myc* gene and lambda light chain of immunoglobulin genes.[12] NAB-2 was reported to contain one copy of EBV DNA, which is integrated into the host genome.[10,12] The latency pattern of the EBV in such a condition is so far not clarified. The previous study with *in situ* hybridization method revealed the presence of EBV genome in chromosome 2p13.[10] It is known that at least three oncogenes, *REL*, transforming growth factor-α and *BCL-11A* gene are located around the region. To demonstrate the integration site of EBV in the chromosome 2p13 directly, and to clarify the relation between the integration sites and the oncogenes, fragments containing the nucleotide sequence of NAB-2 integration sites were cloned. Furthermore, the EBV latent gene expressions were analyzed.

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Materials and methods

Cell Lines

A Burkitt cell line NAB-2 was a kind gift from Dr PW Tucker (University of Texas at Austin). Other Burkitt

cell lines, BJAB, Namalwa, Raji, and Ramos, were purchased from Japanese Collection of Research Bioresources (JCRB). A lymphoblastoid cell line IB4 was a kind gift from Dr E Kieff (Brigham and Women's Hospital). Another lymphoblastoid cell line (LCL) was established in our laboratory by infection of B-lymphocytes from healthy volunteer with EBV. They were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS at 37°C in 5% CO_2 in air.

Southern Blot Analysis and DNA Probes

A measure of 10 μg of DNA from the cell lines were digested with *Bam*HI or *Bam*HI + *Bgl*II, electrophoresed on 0.7% agarose gels, and transferred to a Hybond N^+(Amersham) nylon membrane. The filters were hybridized with two probes that contain the unique DNA sequences at either end of the EBV genome. The EBV *Bam*HI–*Nhe*t fragments in the vector pUC119 to make the probes-L and -R were kind gifts from Dr Kenzo Takada (Hokkaido University). Probes-L and -R are a portion of the *Nhe*t-containing nucleotides 4–3957 and 166615–169424, respectively (GenBank ACC M80517). Each probe was radiolabeled by the random prime method according to the indications of the manufacturer (Amersham).

Cloning of the EBV Junction Sites

Two genomic DNA libraries were prepared from NAB-2 DNA; one was digested with *Bam*HI for cloning the junction site adjacent to the right end of EBV and another was digested with *Bam*HI + *Bgl*II for cloning the junction site adjacent to the left end of EBV. DNA around 10 kbp, obtained through separation of the DNA on 0.7% agarose gel electrophoresis, was ligated to DASH II vector, packaged with the Gigapack III Gold packaging system (Stratagene, La Jolla, CA, USA). The libraries were plated on *Escherichia coli* XL1-blue MRA (P2) strain. Plaques (1×10^5) were screened with either Probe-L or Probe-R. Positive plaques were purified using the Qiagen Lambda Mini Kit, digested with either *Bam*HI or *Xba*I, and cloned into pCR 2.1 (Invitrogen, Carlsbad, CA, USA). Sequencing was performed by the dideoxy chain termination method using the DNA sequencing kit (Applied Biosystems). The samples were analyzed using the Genetic Analyzer (ABI PRISM 310′, Applied Biosystems).

RT-PCR for Detection of Expression

RNA from 10^5 to 2×10^7 cells was isolated using TRIzol reagent (Invitrogen), and reverse transcribed as described previously.[13] Expression levels of *REL* and *BCL-11A* gene in the Burkitt lymphoma cell lines, NAB-2, BJAB, Namalwa Raji, and Ramos, were analyzed using Assays-on-Demands™ according to the protocol of the manufacturer. Standard curves for the quantitation of the molecules were constructed from the results of simultaneous amplification of serial dilutions of cDNA from lymphoblastoid cell line, IB4. Real-time PCR and subsequent calculation were performed with an ABI Prism 7700 Sequence Detector System (Applied Biosystems). To normalize the differences in RNA degradation and RNA loading for RT-PCR in individual samples, the expression level of each molecule was divided by that of β-actin in the same samples. All experiments were performed in triplicate.

EBV latent genes, LMP1, LMP2A, EBNA1, EBNA2, BARF0, and ZEBRA, were amplified with primer pairs shown in Table 1. All primer pairs were designed across the splice sites; thus, could distinguish complementary DNA (cDNA) from genomic DNA by their sizes. Thermocycling conditions were 30 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 15 s, and extension at 72°C for 45 s, followed by a final 7-min extension at 72°C.

Table 1 Oligonucleotide primers used for PCR reactions

Primer		*Nucleotide sequence (5′–3′)*	*Size (bp)*
Primers for amplification of the EBV genes			
BARF0	A3	5′-AGAGACCAGGCTGCTAAACA-3′	240
	A4	5′-AACCAGCTTTCCTTTCCGAG-3′	
BZLF1	Z1	5′-CGCACACGGAAACCACAACAGC-3′	227
	Z2	5′-CGGCGGATAATGGAGTCAACATCC-3′	
LMP2A	L2F	5′-ATGACTCATCTCAACACATA-3′	280
	L2R	5′-CATGTTAGGCAAATTGCAAA-3′	
LMP1	F	5′-TGTACATCGTTATGAGTGAC-3′	240
	R	5′-ATACCTAAGACAAGTAAGCA-3′	
EBNA1	Y3F	5′-TGGCGTGTGACGTGGTGTAA-3′	Y3*U*K*spliced 265
	QF	5′-GTGCGCTACCGGATGGCG-3′	Q*U*K*spliced 236
	KR	5′-CATTTCCAGGTCCTGTACCT-3′	

In Situ Hybridization

RNA *in situ* hybridization using EBER1 probe was performed as previously described with some modification.[14] Briefly, 30-base oligonucleotide probes (5′-AGACACCGTCCTCACCACCCGG GACTTGTA-3′),[15] which were sense and antisense for a portion of the EBER1 gene, a region of the EBV genome that is actively transcribed in latently infected cells, were synthesized using a DNA synthesizer. As positive control, the Raji cell line was used. As negative controls, the hybridizing mixture containing sense probe or antisense probe after RNase treatment was used.

Results

Southern Blot Analysis

Existence pattern of EBV in cells, circular, linear, or integrated, could be distinguished through analysis of the viral genome structure with probes to the unique DNA sequences at either end of the EBV genome. Enzymatic digestion of episomal DNA with an enzyme such as *Bam*HI that spares the terminal repeats (TR) sequences produces a single fused terminal fragments in monoclonal cell lines and human tumor specimens. If the virus is integrated into chromosome through the TR, distinct restriction fragments containing viral/cellular junction sites must be detected. Using this method, genomic Southern blotting analysis was performed with probes at either end of the EBV genome; that is, Probe-L and -R. The Probe-L and -R were hybridized with 23.5 kbp and 10 kbp fragments, respectively, that were consistent with the report by Popescu *et al*[10] (Figure 1). Any additional bands were not detected. These findings indicate that EBV genome does not present as circular form, but are integrated into host genome through TR.

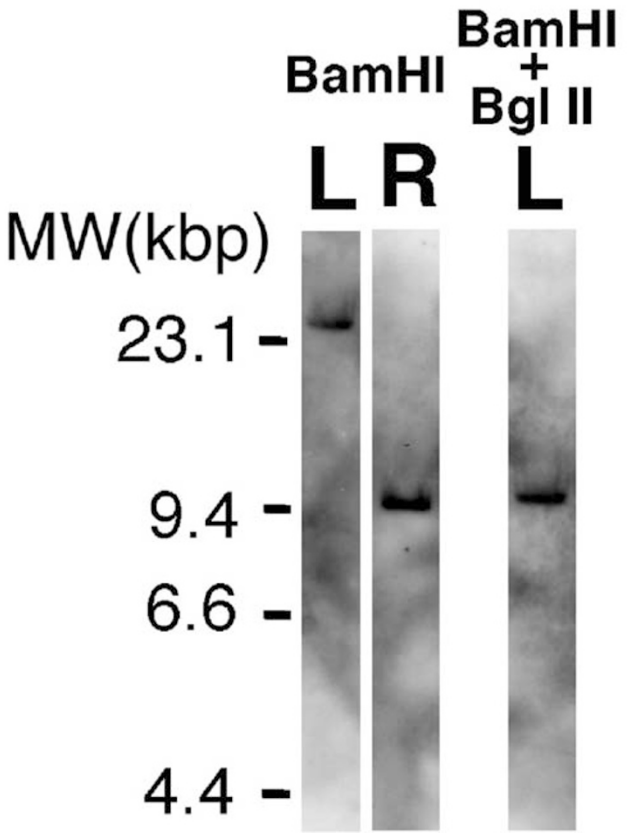

Figure 1 A measure of 10 μg of DNA from the NAB-2 cell line were digested with *Bam*HI or *Bam*HI + *Bgl*II, electrophoresed on 0.7% agarose gels, transferred to a Hybond N^+ (Amersham) nylon membrane. The filters were hybridized with either Probe-R or Probe-L.

Cloning the Junction Sites Adjacent to Terminal End of EBV

Two genomic DNA libraries were prepared from NAB-2 DNA. One was digested with *Bam*HI for cloning the junction site adjacent to the right end of EBV. Another was digested with *Bam*HI + *Bgl*II for cloning the junction site adjacent to left end of EBV, because Probe-L was hybridized with 10 kbp of *Bam*HI + *Bgl*II digested fragments, which are of suitable size for the library (Figure 1).

Plaques (1×10^5) were screened with either Probe-L or Probe-R, and four and three positive phage plaques were obtained, respectively. The phage DNA was purified from these plaques and the inserts were recloned to plasmid vector, and named $pNBL_{1–4}$ and $pNBR_{1–3}$. Sequencing analysis revealed that each plasmid had identical sequences, thus derived from the same library. The pNBR contained approximately 4.5 kbp of unknown sequence adjacent to the EBV right end (Figure 2). BLAST analysis revealed that the unknown sequences corresponded to nucleotides 113504–117973 of clone RP11-440P5 on chromosome 2 (ACC AC009970), indicating that nucleotide 113504 of the clone RP11-440P5 being one of the junctions. The pNBL contained approximately 4.9 kbp of unknown sequence adjacent to the EBV right end (Figure 2). BLAST analysis revealed that the unknown sequences corresponded to nucleotides 100248–101796 of clone RP11-440P5 on chromosome 2 (ACC AC009970), indicating that nucleotide 101796 of the clone RP11-440P5 being another junction.

According to the information of GenBank of National Center for Biotechnology Information (NCBI), integration sites locate between two oncogenic genes, *REL* and *BCL-11A*, which is apart from approximately 350 kbp from each other. *PAPOLG* gene is located 200 kbp apart from the junction. *PAPOLG* gene is recently reported to play an essential role in polyadenylation of mRNA precursors.[16] The genome of the cell at the junction continued repetitive sequences such as Alu, L2 sequences, for over hundred kilobasepairs.

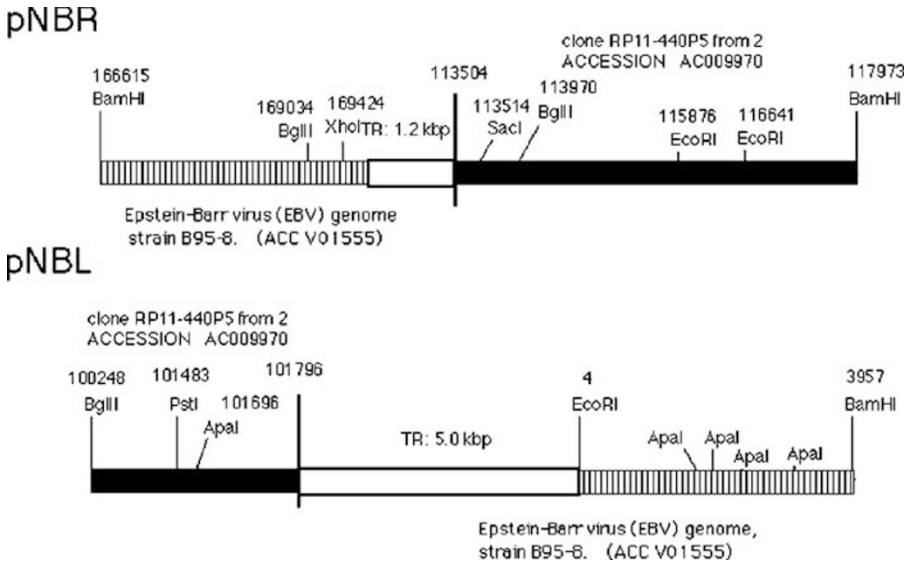

Figure 2 Schematic representation of pNBR and pNBL. Plaques (1×10^5) were screened with either Probe-L or Probe-R, and four and three positive phage plaques were obtained, respectively. They were recloned to plasmid vector. Both pNBR and pNBL contained EBV and host genome sequence on clone RP11-440P5 from chromosome 2.

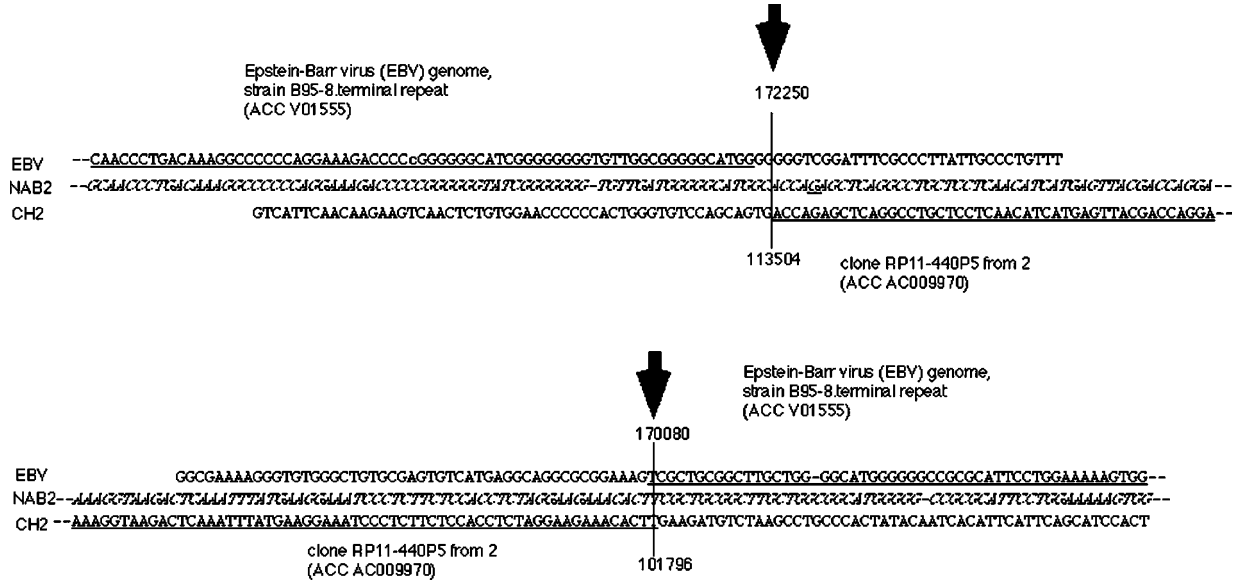

Figure 3 Sequences of the NAB-2-EBV junction sites. Sequence from the NAB-2 cell line is shown in italic letters. The unrecombined sites obtained from clone RP11-440P5 are shown in the lower line and terminal repeats fragments of EBV genome from strain B95-8 in the lower line. The arrows indicate the junction between chromosome 2 and EBV.

Sequence Analysis of the NAB-2-EBV Junction Sites

Sequences of the NAB-2-EBV junction sites were compared to those of chromosome 2 (Figure 3). The flanking region of chromosome 2 at the bilateral junction sites showed no homology to the junction sites of EBV. G/C content in the TRs were about 70%, whereas, that in each junction site of chromosome 2 was 68 and 46%, respectively.

Expression of the Host Genes around the Junction Sites

Expression level of *REL* in NAB-2 was significantly higher than that in other cell lines examined (Table 2). Expression level of *BCL-11A* was more than hundred times higher in Burkitt lymphoma cell lines than in IB4. Among the Burkitt cell lines, expression level of *BCL-11A* was slightly higher in

Table 2 Expression of *Bcl-11* and *REL* gene in cell lines

	Bcl11A	REL	
IB4	1	1	
NAB2	475.5±211.4	4.67±0.46	*
BJAB	326.7±104.4	0.60±0.27	
Namalwa	419.0±280.7	0.37±0.26	
Raji	317.5±203.3	0.43±0.32	
Ramos	144.1±64.1	0.57±0.31	

*$P<0.05$ by Student's *t*-test.

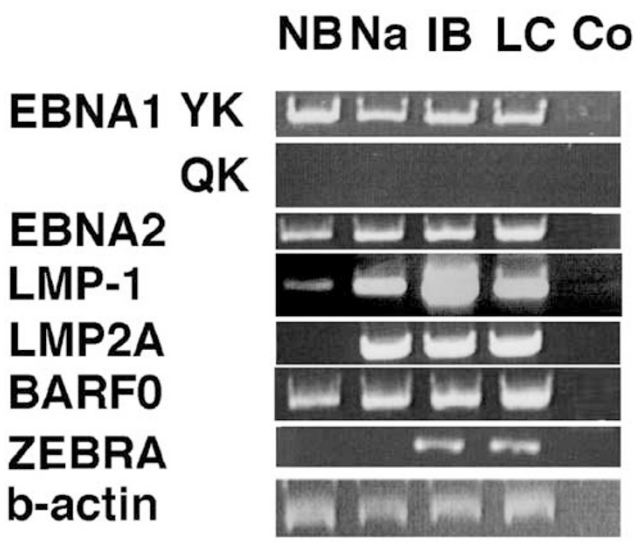

Figure 4 Expression of EBV genes in NAB2 and other cell lines determined by RT-PCR. β-actin was used as a control. Expression of the EBNA1 was detected using the primer pairs of Y3F and KR, indicating that Cp/Wp promoter was used for the latent gene expression. LMP1 was detectable but LMP2A was not at all in NAB-2. These results indicated that NAB-2 cells had a type III latent infection of EBV. NB, NAB2; Na, Namalwa; IB, IB4; LCL, EBV-transformed lymphoblastoid cell line, Co; without template DNA.

NAB-2 than in other cell lines, although the difference was not significant.

Expression of EBV Genes

In situ hybridization showed that all NAB-2 cells were positive for EBERs (data not shown). Expression of EBNA1, EBNA2, BARF0, LMP1, and LMP2A gene transcripts was examined using RT-PCR (Figure 4). EBNA2 and BARF0 transcripts were detected at the compatible level with control lymphoblastoid cell lines. Expression of the EBNA-1 was detected using the primer pairs of Y3F and KR, indicating that promoter Cp/Wp was used for the latent gene expression.[17] Expression of LMP1 was detected but that of LMP2A not at all. These results indicated that NAB-2 cells showed the type III of latent gene expression of EBV according to Rowe *et al*.[18]

The activation of EBV from latency is known to be initiated by expression of the BZLF1 immediate-early gene product ZEBRA (also referred to as Zta or EB1).[19] Then transcription of the BZLF1 gene was examined in the cell lines by RT-PCR (Figure 4). IB4 and another LCL constantly expressed the BZLF1 mRNA, but NAB-2 and Namalwa cells did not. The lack of ZEBRA production indicated that EBV did not replicate in NAB-2 cells.

Discussion

Previous studies demonstrated the integration of EBV in the chromosomes of tumor cells in biopsy specimens or cell lines of BL,[7,10,11] other kinds of B-cell lymphomas,[8,20] and NPC.[9,21] In these studies, integration sites of EBV were indirectly identified by Gardella gels[22] and/or by Southern blot analysis. Localization of EBV on the chromosomes was also shown by *in situ* hybridization method. Although it is proposed that integration is an important mechanism for interaction of EBV with cellular genes, particularly those involved in cell-growth regulation and tumorigenesis, neither direct evidence of EBV integration nor inactivation of suppressor genes through integration were demonstrated until recently. This might be due to the presence of highly methylated DNA, which hinders mapping of EBV genomes and multiple copies of the viral episomes which give interfering noise at detection of EBV integration sites.[9,23] Large genome size of EBV compared to other viruses such as papilloma virus, hepatitis B virus, polyoma virus, and retrovirus also make identification of the integration site and analysis of its influence difficult.

Integration sites of two Burkitt cell lines, Namalwa and Raji, were cloned and analyzed previously. The Namalwa line, which was originally thought to contain only one copy of EBV, proved to actually harbor two closely neighboring copies integrated at chromosome 1p35.[24,25] According to nucleotide sequence of the junction sites and the information of GenBank of NCBI, EBV was integrated into the *MACF1* gene in the Namalwa line. *MACF-1 (ACF7)* is a member of the spectraplakin family of cytoskeletal crosslinking proteins possessing actin and microtubule binding domains.[26] *MACF-1* is important for controlling microtubule dynamics and reinforcing links between microtubule and polarized F-actin, so that cellular polarization and coordinated cell movements can be sustained. Recently, integration of EBV into chromosome 6 of the Raji cell line and the resultant loss of *BACH2* gene was reported.[13]

In the present study, junction sites of EBV in NAB-2 cells were cloned: the results showed that two oncogenes, *REL* and *BCL-11A*, were located near the integration sites. *REL* encodes a nuclear factor (NF)-kappaB transcription factor and is frequently amplified in various neoplasias of B-cell lineage,[27–30]

including extranodal diffuse large cell lymphoma. *BCL-11A* was identified in B-cell lymphoma with t(2;14)(p13;q32) and encodes a zinc-finger transcription factor. *BCL-11A* is overexpressed in B-cell lymphomas with t(2;14) and Hodgkin's lymphoma (HL) cell lines.[31] Gain and amplification of the region covering chromosome 2p13 have been reported in extranodal non-Hodgkin's lymphoma (NHL) of B-cell type, follicular NHL, mediastinal B-NHL, and HL.[27–31] *BCL-11A* is coamplified with *REL* in B-NHL cases and HL cell lines with gains and amplifications of 2p13, suggesting that *REL* and *BCL-11A* might be involved in lymphomagenesis through either chromosomal translocation or amplification.[31] In NAB-2 line, expression levels of *Rel* oncogenes were increased. Further study is necessary to determine whether regulation of *REL* was altered by the integration event.

Fragile sites (FSs) are loci that are prone to breakage, recombination as well as becoming preferential targets for mutagens, carcinogens and integration of oncogenic viruses.[32,33] Circumstantial evidences linking FSs and cancer development have been accumulated. Consistent with the pattern of integration of other DNA viruses, the EBV integration site at 2p13 in NAB-2 overlaps with the one of common fragile sites, FRA2E.

Expression patterns of latent genes was compared among three cell lines; NAB-2, Namalwa and IB4, all contain EBV as the integrated form.[10,24,34] NAB-2 integrates one EBV copy through TR, Namalwa two EBV tandem copies through TR,[25] and IB4 four to five copies through *Eco*RI-I and *Bam*HI C fragments of EBV.[34,24] All three lines use Cp/Wp promoter and express almost all latent genes examined, showing the Latency III type of latent gene expression.[17] Integration event through TR is expected to result in the disruption of the LMP2A gene in NAB-2. Indeed, LMP2A was not expressed at al in NAB-2 line, with no episomal EBV and tandem integration. Whereas in Namalwa line with tandem integration of EBV through TR, LMP2A was expressed. Whereas EBV is integrated through a part of EBV other than TR in IB4, thus the structure of LMP2A gene is not disrupted. ZEBRA production was not observed in NAB-2 and Namalwa cells, indicating that EBV did not replicate in those cells.[19,35]

Previous studies emphasized the oncogenic capacities of EBV through expression of its latent genes, such as EBNAs and LMPs. Integration of EBV could be another mode of oncogenic pathway because integration sites occasionally overlap genes encoding proteins necessary for maintenance of normal characters of cells such as *MACF1* for cell mobility in the Namalwa line, *BACH2* for cell differentiation thus function as cancer suppressor gene in Raji, and *REL* and *BCL-11A* in NAB-2 as shown in this study. Integration sites in NAB-2 overlap with common, fragile sites, which might give advantages for sequential genetic alterations.

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References

1 Kieff E. Epstein–Barr virus, its replication. In: Fields BN, Knipe DM, Howley PM (eds). Virology Aspects. Lippincott-Raven: Philadelphia, PA, 1996, pp 2343–2396.
2 Rickinson AB, Kieff E. Epstein–Barr virus. In: Fields BN, Knipe DM, Howley PM (eds). Virology Aspects. Lippincott-Raven: Philadelphia, PA, 1996, pp 2397–2446.
3 zur Hausen H. Viruses in human cancers. Science 1991;254:1167–1173.
4 Hurley EA, Thorley LD. B cell activation and the establishment of Epstein-Barr virus latency. J Exp Med 1988;168:2059–2075.
5 Kaschka DC, Adams A, Lindahl T, *et al.* Intracellular forms of Epstein–Barr virus DNA in human tumour cells *in vivo*. Nature 1976;260:302–306.
6 Raab-Traub N, Flynn K. The structure of the termini of the Epstein–Barr virus as a marker of clonal cellular proliferation. Cell 1986;47:883–889.
7 Delecluse HJ, Bartnizke S, Hammerschmidt W, *et al.* Episomal and integrated copies of Epstein–Barr virus coexist in Burkitt lymphoma cell lines. J Virol 1993; 67:1292–1299.
8 Gulley ML, Raphael M, Lutz CT, *et al.* Epstein–Barr virus integration in human lymphomas and lymphoid cell lines. Cancer 1992;70:185–191.
9 Kripalani JS, Law HY. Identification of integrated Epstein–Barr virus in nasopharyngeal carcinoma using pulse field gel electrophoresis. Int J Cancer 1994;56: 187–192.
10 Popescu NC, Chen MC, Simpson S, *et al.* A Burkitt lymphoma cell line with integrated Epstein–Barr virus at a stable chromosome modification site. Virology 1993;195:248–251.
11 Gravell M, Levine PH, McIntyre RF, *et al.* Epstein–Barr virus in an American patient with Burkitt's lymphoma: detection of viral genome in tumor tissue and establishment of a tumor-derived cell line (NAB). J Natl Cancer Inst 1976;56:701–704.
12 Popescu NC, Dahlberg JE, Ablashi DV, *et al.* Oncogene expression and immunoglobulin synthesis in a North American Burkitt (NAB-2) lymphoma cell line with a 8;22 chromosome translocation. Oncogene Res 1990;5:295–303.
13 Takakuwa T, Luo WJ, Ham MF, *et al.* Integration of Epstein–Barr virus into chromosome 6q15 of Burkitt lymphoma cell line (Raji) induce loss of *BACH2* expression. Am J Pathol 2004;164:967–974.
14 Weiss LM, Jaffe ES, Liu XF, *et al.* Detection and localization of Epstein–Barr viral genomes in angioimmunoblastic lymphadenopathy and angioimmunoblastic lymphadenopathy-like lymphoma. Blood 1992;79:1789–1795.

15 Chang KL, Chen YY, Shibata D, *et al.* Description of an *in situ* hybridization methodology for detection of Epstein–Barr virus RNA in paraffin-embedded tissues, with a survey of normal and neoplastic tissues. Diagn Mol Pathol 1992;1:246–255.
16 Topalian SL, Kaneko S, Gonzales MI, *et al.* Identification and functional characterization of neo-poly(A) polymerase, an RNA processing enzyme overexpressed in human tumors. Mol Cell Biol 2001;21:5614–5623.
17 Trivedi P, Spinsanti P, Cuomo L, *et al.* Differential regulation of Epstein–Barr virus (EBV) latent gene expression in Burkitt lymphoma cells infected with a recombinant EBV strain. J Virol 2001;75:4929–4935.
18 Rowe M, Lear AL, Croom CD, *et al.* Three pathways of Epstein–Barr virus gene activation from EBNA1-positive latency in B lymphocytes. J Virol 1992;66: 122–123.
19 Countryman J, Miller G. Activation of expression of latent Epstein–Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc Natl Acad Sci USA 1985;82: 4085–4089.
20 Daibata M, Taguchi T, Nemoto Y, *et al.* Epstein–Barr virus (EBV)-positive pyothorax-associated lymphoma (PAL): chromosomal integration of EBV in a novel CD2-positive PAL B-cell line. Br J Haematol 2002;117: 546–557.
21 Chang Y, Cheng SD, Tsai CH. Chromosomal integration of Epstein–Barr virus genomes in nasopharyngeal carcinoma cells. Head Neck 2002;24:143–150.
22 Gardella T, Medveczky P, Sairenji T, *et al.* Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J Virol 1984; 50:248–254.
23 Anvret M, Karlsson A, Bjursell G. Evidence for integrated EBV genomes in Raji cellular DNA. Nucleic Acids Res 1984;12:1149–1161.
24 Matsuo T, Heller M, Petti L, *et al.* Persistence of the entire Epstein–Barr virus genome integrated into human lymphocyte DNA. Science 1984;226:1322–1325.
25 Lawrence JB, Villnave CA, Singer RH. Sensitive, high-resolution chromatin and chromosome mapping *in situ*: presence and orientation of two closely integrated copies of EBV in a lymphoma line. Cell 1988;52:51–61.
26 Kodama A, Karakesisoglou I, Wong E, *et al.* ACF7. An essential integrator of microtubule dynamics. Cell 2003;115:343–354.
27 Houldsworth J, Mathew S, Rao PH, *et al.* REL proto-oncogene is frequently amplified in extranodal diffuse large cell lymphoma. Blood 1996;87:25–29.
28 Joos S, Otano-Joos MI, Ziegler S, *et al.* Primary mediastinal (thymic) B-cell lymphoma is characterized by gains of chromosomal material including 9p and amplification of the REL gene. Blood 1996;87: 1571–1578.
29 Goff LK, Neat MJ, Crawley CR, *et al.* The use of real-time quantitative polymerase chain reaction and comparative genomic hybridization to identify amplification of the REL gene in follicular lymphoma. Br J Haematol 2000;111:618–625.
30 Rao PH, Houldsworth J, Dyomina K, *et al.* Chromosomal and gene amplification in diffuse large B-cell lymphoma. Blood 1998;92:234–240.
31 Satterwhite E, Sonoki T, Willis TG, *et al.* The BCL11 gene family: involvement of BCL11A in lymphoid malignancies. Blood 2001;98:3413–3420.
32 Sutherland GR, Baker E, Richards RI. Fragile sites still breaking. Trends Genet 1998;14:501–506.
33 Popescu NC. Genetic alterations in cancer as a result of breakage at fragile sites. Cancer Lett 2003;192:1–17.
34 Hurley EA, Klaman LD, Agger S, *et al.* The prototypical Epstein–Barr virus-transformed lymphoblastoid cell line IB4 is an unusual variant containing integrated but no episomal viral DNA. J Virol 1991;65:3958–3963.
35 Chevallier GA, Manet E, Chavrier P, *et al.* Both Epstein–Barr virus (EBV)-encoded *trans*-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J 1986;5: 3243–3249.