Methylation Status of the Epstein-Barr Virus Major Latent Promoter C in Iatrogenic B Cell Lymphoproliferative Disease

Application of PCR-Based Analysis

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The Epstein-Barr virus (EBV) major latent promoter C drives the expression of viral nuclear proteins important in lymphocyte immortalization and as targets for immune surveillance by cytotoxic T cells. Hypermethylation of the C promoter silences its transcription. This promoter is methylated and silent in Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and nasal lymphoma. However, it is never methylated in the EBV-immortalized lymphoblastoid cell lines that serve as a model for EBV-associated lymphoproliferative disease. We have analyzed C promoter methylation in iatrogenic EBV-associated B-cell lymphoproliferative disease, mainly posttransplant lymphoma, using a sensitive polymerase chain reaction-based C promoter methylation assay. Our results showed heterogeneity in lymphoproliferative disease with methylation of viral DNA in specimens from 3 of 13 patients. In specimens from two of these patients, only methylated viral DNA was detected and viral nuclear antigen expression was correspondingly restricted. Heterogeneity in C promoter methylation and expression of associated transcripts may be an important determinant of the growth properties of lymphoproliferative lesions and may provide an explanation for the failure of some tumors to respond to withdrawal or reduction of immunosuppressive therapy. (*Am J Pathol* 1999, 155:619–625)

The Epstein-Barr virus (EBV) latent C promoter drives expression of six EBV nuclear antigens (EBNAs): 1, 2, 3A, 3B, 3C, and LP.^{1,2} The ability of the virus to immortalize B cells is one of the properties that suggest a role for the

virus in the pathogenesis of various neoplasia. Among the viral proteins required for lymphocyte immortalization and transformation, four are expressed from the C promoter. All of the immunodominant viral antigens expressed during latency (EBNA3A, 3B, and 3C) are expressed from the C promoter (Figure 1).^{3,4} Thus regulation of the C promoter is likely to be a critical determinant of the growth characteristics of infected cells and of their susceptibility to lysis by cytotoxic T cells. In Burkitt's lymphoma (BL), the C promoter is silent, the growth characteristics of the tumor appear to largely reflect the *c-myc* translocation rather than viral gene expression, and there is no evidence that this tumor is sensitive to control by virus-specific cytotoxic T cells.^{5–7} In contrast, in posttransplant lymphoproliferative disease (PTLD), the C promoter in many cases is active,^{8,9} *c-myc* translocations are the exception rather than the rule,¹⁰ and viral genes are believed to play a central role in driving cellular proliferation. Similarly, expression of the immunodominant EBNA3s from the C promoter in PTLD is presumably responsible for PTLD's exquisite sensitivity to immune manipulations. These include withdrawal of immunosuppressive medicines and adoptive transfer of EBV-specific cytotoxic lymphocytes.^{11–14} However, the patterns of viral antigen expression in PTLD and thus of C promoter usage are not uniform. Several groups of investigators, including our own, have documented heterogeneity of antigen expression in these tumors.^{9,15–19}

One of the mechanisms important in the regulation of the C promoter is CpG methylation. *In vitro* studies have identified two promoter regions that are especially sensitive to the transcriptional repression of CpG methylation.^{20,21} Studies of lymphocytes from healthy EBV seropositive blood donors and in BL, Hodgkin's disease

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Figure 1. Schematic drawing of the structure of EBV C promoter and the location of methylation-specific PCR analysis region. IR1, internal repeat 1; TR, terminal repeat; EBNA2 RE, EBNA2 response element; OriP, origin of the replication of EBV plasmid DNA. The positions of methylation-specific PCR primers (arrows) and the two CAAT boxes are shown. The immunodominant viral antigens, EBNA3A, 3B, and 3C, are underlined.

(HD), nasopharyngeal carcinoma (NPC), and nasal lymphoma tumor tissues have shown hypermethylation and transcriptional silence in this promoter.^{22–29} In Burkitt's cell lines treated with 5-azacytidine, an inhibitor of DNA methyltransferase, the C promoter is activated.^{20,30} In the investigations reported here, we sought to determine whether methylation of the C promoter occurred in post-transplant and similar iatrogenic EBV-associated lymphoproliferative disease and whether its occurrence correlated with patterns of viral antigen expression. In the past, analysis of C promoter methylation has been performed by Southern blot hybridization with methylation-sensitive and -insensitive restriction enzyme isoschizomers or with bisulfite genomic sequencing. The former approach requires substantial quantities of high quality genomic DNA (10–20 μ g) and provides information only about potential methylation sites that happen to coincide with restriction enzyme sites.^{24,25} The latter approach is so laborious as to prohibit its application to large numbers of clinical specimens.^{20,22} Therefore, we have developed an alternative rapid polymerase chain reaction (PCR)-based assay for C promoter methylation analysis that requires only small amounts of DNA and specifically targets a region of the C promoter shown to be hypersensitive to the transcriptional inhibitory effects of CpG methylation *in vitro*.

Materials and Methods

Cell Lines

B95-8 is an EBV-immortalized lymphoblastoid cell line. Rael, Akata, Wanyonyi (Wan), Raji, and AG876 are EBV-positive BL cell lines. BJAB is an EBV-negative B-lymphoma cell line. All cell lines were cultivated at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L glutamine, 100 U/ml penicillin and streptomycin. 5-azacytidine was used to treat the Rael cell line (Rael-AzaC).²⁰ The EBV latency types and C promoter methylation status of these cell lines are listed in Table 1.

Table 1. Latency Types, Methylation Status, and C Promoter Activities in EBV-Positive Cell Lines

Cell line	Latency type	C promoter methylation status	C promoter activity
B95-8	III	u	on
Rael	I	m	off
Rael-AzaC*	I + III	m + u	off + on
Akata	I	m	off
Wan†	II/III	m + u	off + on
Raji‡	III	m	off

*Depending on the dosage and the treatment length of 5-azacytidine to Rael cell line, C promoter can be partially to completely demethylated and active in Rael-AzaC.^{20,30}

†Wan was originally classified as a type I/II cell line.⁵ However, the Wan cell line we obtained showed a drift to type III in some cells.²⁷

‡In the Raji cell line, C promoter is methylated and silent and EBNA3A, 3B, and 3C are driven by an unknown promoter.

u, unmethylated; m, methylated.

Tumor Specimens

The sources of endemic BL, NPC, HD, and nasal lymphoma specimens have been described previously,^{22,27–29} as have 14 lymphoproliferative disease specimens from 12 organ transplant recipients and one lymphoproliferative disease specimen from a patient with aplastic anemia arising after treatment with immunosuppressive therapy (B-LPD).^{15,22} Genomic DNA was extracted from frozen tumor blocks by conventional methods.

Bisulfite Treatment of Genomic DNA

DNA (2–5 μ g) was denatured in 33 μ l of 0.3 mol/L NaOH at 37°C for 15 minutes, without using restriction endonuclease. Denatured DNA was mixed directly with 333 μ l of bisulfite solution and treated in darkness. The bisulfite solution was prepared as either 2.4 mol/L sodium metabisulfite (pH 5.0–5.2) (Sigma S-1516, St. Louis, MO)/0.5 mmol/L hydroquinone (Sigma H-7148) for a 4-hour treatment³¹ or 3.1 mol/L sodium bisulfite (pH 5.0–5.2) (Sigma S-8890)/0.5 mmol/L hydroquinone for a 16-hour treatment.³² DNA was desalted and purified using the Wizard DNA Clean-Up system (Promega, Madison, WI). DNA was then treated with 0.3 mol/L NaOH at 37°C for 15 minutes and precipitated with 3 mol/L ammonium acetate and 3 volumes of ethanol. Recovered DNA was dissolved in 20–50 μ l of TE buffer (pH 8.0) and stored at –20°C.

Methylation-Specific PCR

C promoter methylation status was determined by using methylation-specific PCR³³ (Figure 1). In this method, bisulfite treatment converts unmethylated cytosine to uracil but does not affect the methylated cytosine. Thus, PCR primers can be designed that anneal selectively to methylated or unmethylated DNA after bisulfite conversion.^{32,33} The sequences of the unmethylated DNA and methylated DNA-specific primers are listed in Table 2. The primer pair ub3/ub4 was designed specifically for amplification of the bisulfite-converted unmethylated C promoter, while the mb3/mb4 primer pair was designed

Table 2. Primers for Methylation-Specific PCR Analysis of EBV C Promoter

Primers	DNA sequence (5' → 3')	PCR annealing temperature	Coordinate in B95-8 genome
ub3	CATCCAAAAACCAACAACCTCA	55°C	10,702-10,723
ub4	AGTAAGGTGTAATTAATTTGTTT		11,217-11,194
mb3	GTCC*GAAAACC*GAAC*GACTC*G	58°C	10,703-10,723
mb4	GTAAGGC*GTAATTAATTC*GTTC*		11,216-11,194

The cytosine nucleotides in each CpG site that are susceptible to methylation are marked by asterisks.

specifically for the amplification of the bisulfite-converted methylated C promoter.

One microliter of bisulfite-treated DNA (around 40 ng) was PCR-amplified by using 1.875 U of *Taq* Gold polymerase (Perkin Elmer-Cetus, Norwalk, CT) with 1.5 mmol/L MgCl₂ and 0.2 mmol/L dNTP in a 25-μl reaction volume. Primers were used at a final concentration of 0.4 μmol/L each. The PCR involved an initial denaturation at 94°C for 10 minutes, followed by 40 cycles consisting of 94°C for 30 seconds, predetermined optimal annealing temperature for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 3 minutes. Twenty microliters of PCR product were analyzed on a 1.8% agarose gel. Methylation-specific PCR yields a 516-bp product for the unmethylated and a 514-bp product for the methylated C promoter.

Results

Development of the Assay

Primers were designed to amplify a region immediately adjacent to the EBNA2 response element of the C promoter, with the reverse primers (ub4 and mb4) located just within the previously identified methylation hypersensitive region II, which is flanked by two CAAT boxes (Figure 1).²¹ *In vitro* studies had previously shown that C promoter transcription was hypersensitive to CpG methylation at this locus.²⁰⁻²² Differential amplification of methylated and unmethylated sequences by methylation-specific PCR relies on cytosine to uracil conversion after bisulfite treatment but no conversion of cytosine methylated at the 5-position (^{met}C) to uracil after the same treatment.^{32,33} This amplification is strand-specific because bisulfite conversion renders DNA strands non-complementary. Primer pairs were designed to amplify only bisulfite-reacted DNA sequences of the bottom strand (Table 2).

Application of the technique to EBV-positive cell lines whose C promoter methylation status had been previously characterized by bisulfite genomic sequencing verified that the amplification was selective (Tables 1 and 3). There was no amplification of untreated DNAs from B95-8, Akata, Raji, or AG876 cell lines (Figure 2). When these primers were applied to bisulfite-treated DNAs from these cell lines, there was amplification of DNA (Figure 2), indicating that the primer pairs ub3/ub4 and mb3/mb4 were specific for bisulfite-treated DNA. Rael DNA (methylated C promoter)²⁰ yielded a band with primers (mb3/mb4) specific for bisulfite-treated methylated DNA but

not with primers (ub3/ub4) specific for bisulfite-treated unmethylated DNA, whereas B95-8 DNA (unmethylated C promoter) yielded a band with ub3/ub4 primers but not mb3/mb4 primers (Table 3). Other cell lines yielded bands with both sets of primers, reflecting the presence of a mixture of methylated and unmethylated DNA. The C promoter is active in the Wan cell line, which explains the bands obtained with both methylated and unmethylated primers.²⁸ The Raji cell line is a latency III cell line that expresses the immunodominant EBNA_s.²⁷ It might have been anticipated that the C promoter would be unmethylated. However, bisulfite genomic sequencing studies carried out previously by our group have shown that the C promoter is methylated in Raji, and the immunodominant EBNA_s expressed in this cell line derive not from the C promoter but from an alternate uncharacterized promoter (KD Robertson, unpublished observations). The very weak unmethylated PCR band in Raji probably resulted from the presence of unmethylated C promoter or spontaneous lytic activation in a very small proportion of cells. Although Akata is a latency I cell line,^{5,27} the spontaneous lytic activation of EBV in a fraction of cells produces a substantial amount of unmethylated EBV virion DNA,^{27,28,34} which explains the bright PCR band obtained with ub3/ub4 primers.

Bright PCR bands with both primer pairs were obtained using as little as 2 ng of bisulfite-treated Rael cell line DNA in a 25-μl PCR volume. For most tumor samples, 1 μl (around 40 ng) of treated DNA was enough to produce a visible band although up to 4 μl were needed in some cases, indicating a sensitivity appropriate to the analysis of clinical specimens available in limited quantity.

Validation of the Assay in Characterized Tumors

We studied the methylation status of the C promoter in a variety of EBV-positive tumor specimens that had previously been characterized either by bisulfite genomic sequencing of the C promoter or by reverse transcription PCR.^{22,27,29} The tumors studied include endemic BL, NPC, HD, and nasal natural killer and T cell lymphoma (NTL). Methylation-specific PCR analysis showed the presence of methylated C promoter sequences in all of these tumors (Figure 3a and Table 3). A weak to very faint PCR band indicating the presence of unmethylated viral DNA was also detected in some cases (BL1, HD1, 4, 5 and 6, NPC-C15), probably reflecting either the presence of unmethylated C promoter in a very small fraction of tumor cells or a very low level of lytic viral infection, as

Table 3. Correlation of Methylation-Specific PCR, Bisulfite Sequencing Results, and Activities of the C Promoter

Samples		Methylation-specific PCR*	Bisulfite genomic sequencing or promoter activity as determined by RT-PCR ^{20,22,27,29}
EBV(+) cell lines	B95-8	u	u
	Rael	m	m
	Rael-AzaC	u + m	u + m
	Akata	u + m	u + m
	Wan	u + m	u + m
	Raji	((u)) + m	m
Endemic BL	BL1	(u) + m	m
	BL2	m	m
	BL3	m	(u) + m
	BL5	m	(u) + m
	BL6	m	C promoter off
	BL7	m	C promoter off
	BL8	m	C promoter off
	BL9	m	C promoter off
	BL10	m	C promoter weakly positive
	NPC1	m	C promoter off
NPC	NPC2	m	C promoter off
	C15	(u) + m	(u) + m
Nasal lymphoma	NK1	m	C promoter off
	NTL	m	C promoter off
HD	HD1	((u)) + m	m
	HD2	m	(u) + m
	HD4	(u) + m	m
	HD5	((u)) + m	m
	HD6	((u)) + m	m
PTLD	#19	m	m
B cell lymphoma	B-LPD	u	u

*For methylation-specific PCR: u, PCR band obtained only by primers for unmethylated C promoter; m, PCR band obtained only by primers for methylated C promoter; u + m, PCR bands obtained by primers for both unmethylated and methylated C promoter; ((u)) + m, weak unmethylated band together with methylated band; u + (m), unmethylated band together with weak methylated band, ((u)), very faint unmethylated band.

For bisulfite genomic sequencing:^{20,22,28} u, all colonies with unmethylated C promoter; m, all colonies with methylated C promoter; u + m, half unmethylated and half methylated colonies; (u) + m, at least one but <50% unmethylated colonies together with other methylated ones; u + (m), at least one but <50% methylated colonies together with other unmethylated ones.

reported previously in these tumors.^{27,35-37} Previously reported RT-PCR results for EBNA2 and EBNA3C were negative in BL1; therefore, the C promoter was most probably fully methylated in this case and the weak unmethylated band derived from a very low level of lytic infection in this tumor.²⁷

Analysis of C Promoter Methylation in Tumors Arising in Iatrogenically Immunosuppressed Patients

B-cell lymphoproliferative lesions from 13 patients were studied, including 12 PTLD patients. In contrast to results of EBV-positive tumors from immunocompetent patients, methylation-specific PCR analysis showed that 11/13 cases carried viral genomes with unmethylated C promoter, whereas three cases showed methylation in the C promoter with a very faint methylated band in one case (PTLD #12) (Figure 3b). In two of these cases (PTLD #19 and PTLD #20), only methylated DNA was detected and EBNA2 was not expressed (Table 4). One of the C promoter unmethylated lesions (PTLD #16) also lacked EBNA2 expression, whereas EBNA2 was expressed in all of the other C promoter unmethylated lesions in at least a fraction of cells.¹⁵

The clinical course of patients with C promoter methylation and absence of EBNA2 expression was reviewed to determine whether patients with restricted patterns of viral gene expression regressed after withdrawal or reduction of immunosuppression. One of these patients with C promoter methylation (PTLD #19) died during the biopsy procedure that established the diagnosis of PTLD. The other patient with C promoter methylation (PTLD #20)

Figure 2. Validation of the methylation-specific PCR assay for C promoter in EBV-positive cell lines. Methylated or unmethylated PCR products were obtained as expected in bisulfite-treated DNA samples, but not in any of the untreated DNA samples.

Figure 3. Methylation-specific PCR analysis of the C promoter in EBV-positive tumors from patients with Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD), and nasal lymphoma (NL) showing that methylation of the C promoter predominates (A), and from iatrogenically immunosuppressed, mainly PTLT, patients showing absence of methylation of the C promoter to predominate (B). 4a and 4b or 7a and 7c are PTLT specimens from the same patients at different times.

and the patient with unmethylated C promoter but no expression of EBNA2 protein (PTLT #16) failed to respond to withdrawal of immunosuppression and required cytotoxic chemotherapy.

Table 4. Methylation Status and Activity of the C Promoter in Lymphoproliferative Lesions from Iatrogenically Immunosuppressed Patients

PTLT cases studied in this report		Corresponding cases in ref. 15		Corresponding cases in ref. 27
Case	C promoter methylation status*	Case no.	EBNA2 staining†	Case
#2	u	20	+	
#4	u	11	+	
#6	u	10	+	
#7	u	6	+	
#8	u	18	+	
#10	u	7	+	
#12	u + ((m))	1	+	
#15	u	9	+	
#16	u	19	—	PTLT2‡
#17	u	12	+	
#19	m	5	—	
#20	m		—	PTLT1‡
B-LPD	u		+	

*CpG methylation determined by methylation-specific PCR; u, unmethylated C promoter; m, methylated C promoter; u + ((m)), unmethylated together with very faint methylated band.

†Determined by immunostaining on paraffin sections by using monoclonal antibody PE2 against EBNA2.¹⁵

‡Cases with silent C promoter and EBNA2 negative by RT-PCR.²⁷

Discussion

The investigations reported show that the EBV C promoter is methylated in a minority of iatrogenic lymphoproliferative lesions. In these lesions EBNA2, one of the most readily assayed C promoter-driven antigens, is not expressed. These findings are consistent with reports from many groups that PTLT lesions demonstrate much greater heterogeneity with regard to patterns of viral antigen expression than do BL, HD, NPC, and nasal lymphoma, although some heterogeneity has been recognized in each of these tumors.^{8,9,15–19} PTLT #16 was associated with unmethylated C promoter as determined by methylation-specific PCR but failed to express any EBNA2 protein.¹⁵ In this case, nine CpG sites around the EBNA2-response element in the C promoter have been assessed by bisulfite genomic sequencing and show great heterogeneity in methylation status with no methylation from CpG sites 1 to 6 (including the EBNA2 response element) but complete methylation at CpG sites 8 and 9, which are proximal to the first CAAT box and the methylation hypersensitive region II (KD Robertson, unpublished observations)^{21,22} The lack of correlation between methylation-specific PCR results and EBNA2 expression in this case can be explained by either a high heterogeneity in methylation at some critical CpG sites (maybe the methylation-hypersensitive region II) missed by methylation-specific PCR assay, or other levels of C promoter transcriptional control besides methylation.^{38–40}

The method used to characterize the methylation status of the C promoter here was adapted from a technique first described to assess the methylation status of CpG islands of tumor suppressor genes.³³ This PCR-based assay detects the CpG methylation status of the PCR primer annealing regions and only represents an average of the methylation status of the studied promoter. It does not provide information about CpG sites located between the PCR primers. Heterogeneity in patterns of methylation have been recognized. The CpG methylation status of particular regions of some promoters, even adjacent to the transcription start site, does not always correlate well with the promoter activity. We have previously investigated methylation and transcription of the EBV F promoter in detail.²⁸ Heterogeneity in the F promoter might frustrate a similar PCR-based approach to analysis of methylation in the F promoter. However, as demonstrated in our previous work, there is much less heterogeneity of methylation within the C promoter.²² The presence of unmethylated virion DNA might also interfere with the interpretation of the methylation status of the C promoter, as seen in the Akata cell line. However, many studies have shown that latent viral infection predominates in EBV-associated tumors.² To validate the methylation-specific PCR assay of the C promoter, we compared the results of this technique with those of bisulfite genomic sequencing and RT-PCR previously carried out on a variety of EBV-positive cell lines and tumor tissues. Results of the different assays were consistent (Tables 3 and 4), indicating that it is appropriate to draw inferences from

the methylation-specific PCR assay about the C promoter.

In comparison with other assays of C promoter methylation (Southern hybridization and bisulfite sequencing methods), the present assay requires less material and is more appropriate for the investigation of collections of clinical specimens. Methylation-specific PCR appeared to be slightly more sensitive than bisulfite genomic sequencing, which can be seen from the data in Table 3. Because this assay amplifies only a small region of the EBV genome, DNA extracted from paraffin-embedded tissues, which is normally not of high-molecular weight, can also be used for the analysis. In an ongoing clinical study, we have successfully used this assay to monitor the EBV C promoter methylation status of paraffin-embedded tumor specimens from patients treated with 5-azacytidine (RF Ambinder, manuscript in preparation).

Determination of the methylation status of the C promoter in PTLD lesions may facilitate identification of a population of patients with tumors with a distinctive biology insofar as they lack C-promoter-driven immunodominant viral proteins. Cellular proliferation of tumors in these patients, may, as in BL, predominantly reflect dysregulated expression of cellular genes. Regression following immune reconstitution may be less likely. Although our series is too small to confirm these expectations, we note that three patients with PTLD (PTLD #16, #19, #20) showed no EBNA2 expression. Immunosuppression was withdrawn in two, but lesions failed to regress. The third (#19) could not be assessed due to perioperative death at the time of diagnostic biopsy. It seems likely that lesions with methylated C promoter and no EBNA2 expression are relatively resistant to immune manipulations. Recent investigations have suggested that mutations in the cellular *bcl-6* gene correlate with clinical outcome after changes in immunosuppressive therapy.⁴¹ Future studies are needed to elucidate the relationship between cellular genes and viral genes in these lesions.

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