Expression of Epstein-Barr virus in renal cell carcinoma

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Abstract. There have been few studies regarding the etiology of renal cell carcinoma. To examine the possible involvement of Epstein-Barr virus (EBV) in this disease, 9 renal cell carcinoma (RCC), 2 nephroblastoma (Wilms' tumor) and 2 RCC cell lines were subjected to mRNA *in situ* hybridization and indirect immunofluorescence staining. Messenger RNA *in situ* hybridization using *Bam*HIW, EBNA LP, EBNA 2 and EBER1 probes of EBV revealed signals in all the examined samples, although some samples showed weak signals using the EBNA LP probe. Indirect immunofluorescence staining using anti-EBNA LP, anti-EBNA2, anti-LMP1 and anti-BZLF1 antibodies showed definitive fluorescence. PCR also revealed EBV DNA in all 8 RCC specimens including 7 cases other than hybridization and fluorescence. EBV infected all the RCC and nephroblastoma irrespective of the histological or clinical stage. On the other hand, EBV expression was stronger in papillary and clear cell-type RCC than chromophobe cell-type, as well as being stronger in the higher grades of RCC. These results suggest that the expression of EBV may be involved in the pathogenesis of RCC and nephroblastoma.

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Abbreviations: EBV, Epstein-Barr virus; PCR, polymerase chain reaction; EBNA LP, Epstein-Barr virus nuclear antigen-leader protein; EBNA2, Epstein-Barr virus nuclear antigen-2; LMP1, latent membrane protein-1; EBER1, EBV encoded small non-polyadenylated RNAs; BZLF1, *Bam*HIZ coding leftward reading frame-1; RCC, renal cell carcinoma

Key words: human herpesvirus 4, *in situ* hybridization, RCC, Wilms' tumor

Introduction

Renal cell carcinoma (RCC) accounts for 3% of all malignancies and is the most lethal of the urologic cancers. RCCs are thought to arise primarily from the proximal convoluted tubules. Although a number of potential etiologic factors have been identified including viruses, lead compounds and chemicals, no specific agent has been established as causative in human RCC (1). In these etiologic agents, we studied Epstein-Barr virus (EBV) infection in RCC tissues, because the expression of EBV nuclear antigen 2 (EBNA2) in kidney tubule cells has been reported to induce renal tumors in transgenic mice (2).

EBV is a ubiquitous virus that infects both adults and adolescents throughout the world. It is a well-established causative agent of infectious mononucleosis, and is associated with endemic Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and diffuse large B-cell lymphoma in immunosuppressed hosts (3). We have shown that EBV is also related to many other human cancers (4-16). In thyroid carcinoma, EBV correlates with tumor progression of papillary to undifferentiated carcinoma (12). The transforming genes of EBV are suspected to be in the regions of EBV-determined nuclear antigen-2 (*EBNA2*) (17,18), and latent membrane protein-1 (LMP1) (19). EBNA-coding sequences commonly share the *Bam*HIW region which is repeated approximately 10 times in an EBV genome, and the *Bam*HIY1Y2 (EBNA LP) region. EBER1 is a non-polyadenylated RNA abundantly present in latently EBV-infected cells (20). BZLF1 protein initiates the switch from latent to lytic infection (21).

To examine the relationship between EBV and RCC, mRNA *in situ* hybridization, using 4 different EBV probes and indirect immunofluorescence staining using 4 different monoclonal antibodies against EBV, was performed. The *Bam*HIW and EBNA2 probes and anti-EBNA2 and anti-LMP1 antibodies were selected to detect transformation by EBV. Furthermore, the *Bam*HIY1Y2 (EBNA LP) probe and anti-EBNA LP antibody are also probably associated with EBV transformation (16). We examined 2 cases of nephroblastoma and 2 cell lines established from RCC. The EBV DNA was also confirmed by nested PCR.

EBV expression was detected in all RCC and nephroblastoma tissues. The results presented herein indicated that mRNAs and proteins of EBV were expressed in RCC and nephroblastoma, suggesting that EBV may be a causative agent of these disorders.

Materials and methods

Patients. Formalin-fixed paraffin-embedded tissue samples from 9 patients with RCC and 2 patients for control with renal diseases other than RCC and 1 with a bladder tumor were selected from the 1994 to 2004 files at Osaka National Hospital; 2 patients with nephroblastoma were selected from the 1994 to 2004 files at Wakayama Medical College. As a control, 2 samples of normal kidney were selected from the 2003 to 2005 autopsy files at Osaka Prefectural Medical Center for Respiratory and Allergic Diseases. The pathological classification was in accordance with the general rule indicated in previously reported literature (22). All were Japanese and none had features indicative of an immunocompromised state. Informed consent was obtained from each patient.

Probes. *Bam*HIW probes were transcribed from 2.27 kb EBV *Bam*HIW fragments from which the '*Alu*-family'-like sequence had been deleted. The *Bam*HIW fragment of EBV is a highly repetitive sequence that contains the mRNA leader sequence for EBNAs (23). The fragment was cloned into the pBluescript II SK$^+$ vector. cDNA of the *Bam*HIY1Y2 (EBNA LP) region was also cloned into pBluescript II SK$^+$, and the size of this cDNA was 153 bp. The antisense and sense probes were labelled with digoxigenin-11-UTP by *in vitro* transcription with T3 and T7 polymerases, respectively, using a commercial kit (Boehringer Mannheim, Mannheim, Germany). EBNA2 cDNA (14802-48583 including a spliced sequence) and EBER1 cDNA (6629-6795) were synthesized with RT-PCR according to the method reported by Tierney *et al* (24), and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). The sizes of these cDNAs were 386 and 167 bp, respectively. The antisense and sense RNA probes were labelled with digoxigenin-11-UTP by *in vitro* transcription with SP6 and T7 polymerases using a commercial kit (Boehringer Mannheim). The labelled *Bam*HIW and EBNA2 riboprobes were then fragmented to about 100 bases in length with alkaline hydrolysis. The sense probe served as a negative control.

Messenger RNA in situ hybridization. Paraffin sections were prepared from formalin-fixed tissues from biopsied or surgically resected materials. Serial sections were cut to 3-5 μm. After dewaxing and dehydration with graded ethanol, slides were treated with 0.2 N HCl for 15 min at room temperature (RT) and rinsed with phosphate-buffered saline (PBS) for 5 min at RT, then treated with 50 μg/ml proteinase K in PBS for 15 min at 37˚C, and immersed in 2 mg/ml glycine in PBS for 10 min at RT. They were then refixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed twice with PBS for 3 min at RT, then treated with 0.1 M triethanolamine (pH 8.0) for 10 min at RT. After washing with PBS, the sections were dehydrated with ethanol and hybridized for 40 h at 37˚C for EBNA2 and EBER1, 39˚C for *Bam*HIY1Y2 (EBNA LP) and at 45˚C for *Bam*HIW in 4X SSC, 50% formamide, 1X Denhardt's solution, 5% dextran sulfate, 0.5 mg/ml salmon sperm DNA, 0.5 mg/ml yeast tRNA and 10 mM dithiothreitol. After hybridization, the sections were washed twice with 2X SSC for 30 min and then twice with 0.5X SSC for 20 min with gentle shaking at RT. They were then blocked with 1% skim-milk (Difco) in 100 mM Tris, 0.15 M NaCl (pH 7.5) for 30 min at RT. Next, they were reacted with 1:100 diluted (for EBNA2) and 1:200 diluted (for *Bam*HIW, *Bam*HIY1Y2 and EBER1) alkaline phosphatase-labelled anti-DIG antibody (Boehringer Mannheim) in blocking buffer for 2 h at RT. After washing, the sections were incubated with Nitroblue tetrazolium and X-phosphate (Boehringer Mannheim) in buffer containing 0.1 M Tris, 0.1 M NaC1, 0.005 M $MgCl_2$ and 1 mM levamisole (pH 9.6) for 16 h at RT. Visualization was stopped with EDTA, and then the slides were dehydrated with graded ethanol and xylene, and sealed with malinol. These methods are a modified version of those previously reported by us (4-16).

Indirect immunofluorescence staining. Formalin-fixed paraffin-embedded tissue samples and control specimens were cut in serial sections of 3-5 μm. After dewaxing and dehydration, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) for 10 min at 120˚C to detect EBNA2, EBNA LP and BZLF1. Alternatively, the samples were digested for 15 min with proteinase K at a concentration of 1 mg/ml in 50 mM Tris-HCl (pH 7.6) at RT to detect LMP1. The sections were then blocked with 10-times-diluted EBV-negative human serum and 20% normal goat serum for 16 h at 4˚C. Monoclonal anti-EBNA2 antibody PE2, anti-LMP1 antibody CS1-4, anti-BZLF1 antibody (Dako, Glostrup, Denmark) and anti-EBNA LP antibody JF186 (25) were diluted 10 times with PBS and reacted for 90 min at 37˚C. After washing with PBS, biotinylated goat anti-mouse IgG (Vector, Burlingame, CA, USA) was diluted 75 times with PBS and reacted for 60 min at 37˚C. After washing, streptavidin-fluorescein isothiocyanate conjugate (Bethesda Research Laboratory, Gaithersburg, MD, USA) diluted 200 times was reacted for 45 min at RT. After washing, the slides were sealed with Perma Fluor (Japan Tanner, Kobe). Negative controls with tissue samples were reacted with 10-times-diluted normal mouse IgG. These methods are identical to those we have used to study other human cancers (4-16).

Nested PCR. DNA was extracted from frozen tissues. The primers used for the 1st round of PCR were: 5'CCAGACAG CAGCCAATTCTC3' (nucleotide positions of *Bam*HIW 1087-1106), and 5'CCTAAGAAGGCACCGGTC3' (1503-1520). The primers used for the 2nd round of PCR were: 5'GTAAGAGGGGGTCTTCTAC3' (1196-1214), and 5'CCA GAGGTAAGTGGACTT3' (1399-1416). The resulting 2nd round product was 220 bp. The PCRs were carried out in a thermal cycler GeneAmp 9600-R (Perkin-Elmer). The reaction mixture contained 100 ng to 1 μg of template DNA or 10 ng of positive control DNA (Namalwa and Raji), 0.1 nmol of each primer, and 200 μM each of dATP, dGTP, dCTP and dTTP in 1X amplification buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% W/V gelatin) and 2.5 U of EX Taq polymerase (Perkin-Elmer/Takara) in a total volume of 100 μl. The samples were treated for 5 min at 94˚C

Table I. Summary of results.

Diseases	Case	Histology	Grade	Age	Sex	ISH				IF			
						*Bam*W[d]	LP[e]	E2[f]	E1[g]	LP	E2	LMP1	BZ1[h]
RCC	1	Clear cell	G1	41	M	+	w+	+	+	-	+	+-	-
	2	Clear cell	G1	39	F	+	w+	w+	+	+-	+-	+-	-
	3	Clear cell	G2	59	M	+	w+	-	+	+	+-	w+	w+
	4	Clear cell	G3	59	F	++	+	+	+	++	+	w+	++
	5	Clear cell	G3	73	M	++	+	w+	w+	+	w+	++	+-
	6	CC with sarc[a]	G3	48	M	++	+	w+	w+	++	+	+	+-
	7	Chromophobe	G2	42	F	w+	-	w+	w+	-	w+	+	+-
	8	Chromophobe	G3	48	M	+	-	-	-	-	+-	+	-
	9	Papillary	G3	53	M	++	w+	+	+	w+	+	+	-
Wilms	1	Nephroblastoma		2	F	+-	+	+	-	+	+	++	w+
	2	Nephroblastoma		10M	F	+	++	+	w+	w+	w+	+	w+
RCC lines		NC65				+	++	+	++	+	+	w+	+-
		ACHN				+	++	+	+	w+	w+	w+	+-
Controls	1	Glomerulosc[b]		43	M	+-	-	-	+-	-	+-	-	-
	2	Ischemic[c]		59	M	+-	-	-	+-	+-	-	-	-
	3	Bladder tumor		68	M	-	-	NT	NT	NT	NT	NT	NT
	4	Normal kidney				-	-	-	-				
	5	Normal kidney				-	-	-	-				

[a]CC with sarc, clear cell RCC with sarcomatoid change; [b]Glomerulosc, Glomerulosclerosis; [c]Ischemic, ischemic renal disease; [d]*Bam*W, *Bam*HIW; [e]LP, EBNA LP; [f]E2, EBNA2; [g]E1, EBER1; [h]BZ1, BZLF1; w+, weakly positive; +-, equivocal.

and amplified by 40 cycles of PCR, each consisting of 1 min of denaturing at 94˚C, 30 sec of annealing at 55˚C, and 1 min of extension at 72˚C. After treating for 10 min at 72˚C, the DNA was concentrated by ethanol precipitation, electrophoresed on a 1.8% agarose gel in TAE buffer (40 mM Tris-HCl, pH 8.0, 5 mM sodium acetate, 1 mM EDTA) and stained with 0.5 μg/ml ethidium bromide. This method has already been described (4,5,12).

Results

The results are summarized in Table I. In *Bam*HIW mRNA *in situ* hybridization, signals were detected in the cytoplasm in all the sections hybridized with the antisense probe (Fig. 1B), but not with the sense probe or in the normal part (Fig. 1C). This result indicates that EBV infected all RCC and nephroblastoma, irrespective of histological or clinical diagnosis. Normal kidney tissues and cancer cells of bladder tumor for the negative control showed few signals with the antisense probe. In *Bam*HIY1Y2 (EBNA LP) mRNA *in situ* hybridization, signals were detected in the cytoplasm in the sections hybridized with the antisense probe (Fig. 1D), but not with the sense probe (Fig. 1E). Signals were weak in low-grade RCC, and were not observed in chromophobe cell-type RCC. In EBNA2 mRNA *in situ* hybridization, signals were also detected in the cytoplasm in many cases hybridized with the antisense probe (Fig. 1G), but not with the sense probe, and the signals were weak in 4 cases. In EBER1 RNA *in situ* hybridization, signals were detected in the nuclei in many cases that were hybridized with the antisense probe (Fig. 1H), but not with the sense probe, and the signals were weak in 4 cases. Two cell lines established from RCC also showed frequently strong hybridization signals with all these antisense probes, but not with sense probes. An example is shown in Fig. 1F.

We further examined the EBV protein expression by indirect immunofluorescence staining. Immunofluorescence staining using anti-EBNA2 antibody was prominent in the nuclei of RCC cells (Fig. 2A and B), but not in the normal region (Fig. 2C). Immunofluorescence staining using anti-LMP1 antibody revealed fluorescence in the membranes of RCC cells (Fig. 2D). Immunofluorescence staining using anti-EBNA LP antibody was prominent in the nuclei of RCC cells (Fig. 2E). Immunofluorescence staining using anti-BZLF1 antibody revealed fluorescence in the nuclei of RCC cells (Fig. 2F). Immunofluorescence was also observed in cases of nephroblastoma.

Hybridization signals and fluorescence were observed in all these experiments of renal cancer specimens, while normal kidney and renal diseases other than neoplasm showed few signals or fluorescence (Table I). This showed that EBV was

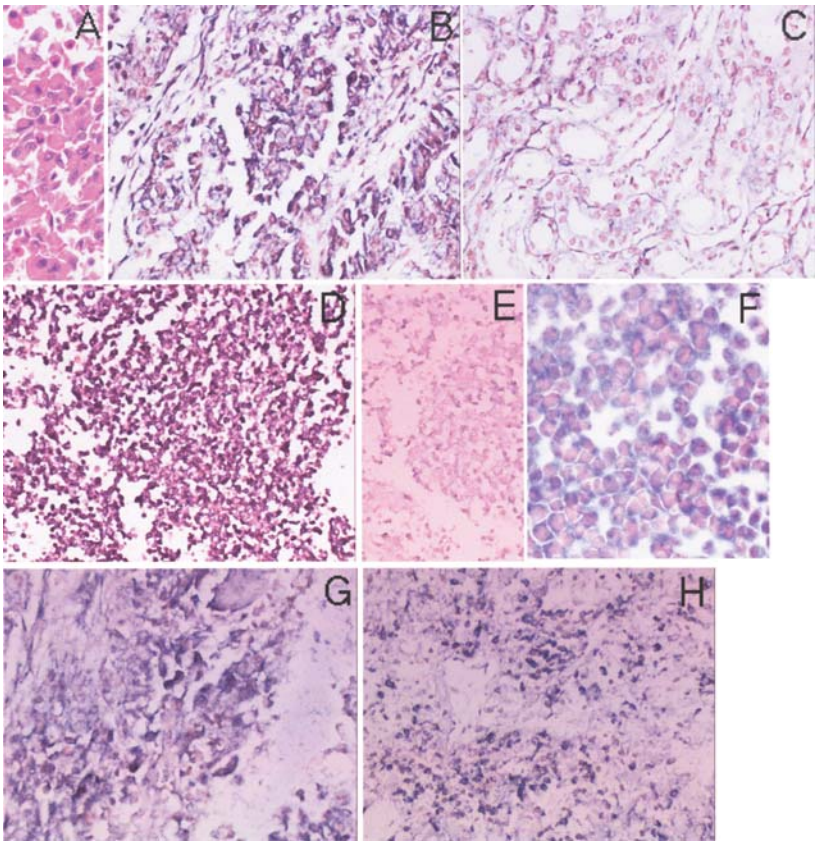

Figure 1. Results of mRNA *in situ* hybridization. (A) Hematoxylin-eosin staining of RCC case 9, papillary cancer area, x40; (B) mRNA *in situ* hybridization of RCC case 9 with *Bam*HIW antisense probe, papillary cancer area, x40; (C) mRNA *in situ* hybridization of RCC case 9 with *Bam*HIW antisense probe, normal area, x40. Hybridization signal was far clearer in papillary cancer area than normal area. (D) mRNA *in situ* hybridization of Wilms case 1 with EBNA LP antisense probe, x40; (E) mRNA *in situ* hybridization of Wilms case 1 with EBNA LP sense probe, x40. Hybridization signal was very weak with the sense probe. (F) mRNA *in situ* hybridization of RCC cell line NC65 with *Bam*HIW antisense probe, x40; (G) mRNA *in situ* hybridization of RCC case 6 with EBNA 2 antisense probe, x40; (H) mRNA *in situ* hybridization of RCC case 4 with EBER 1 antisense probe, x40.

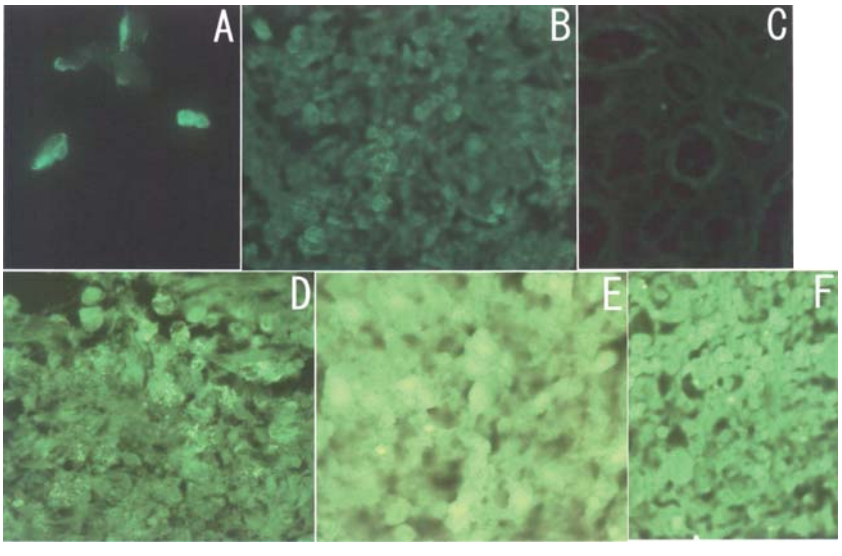

Figure 2. Results of indirect immunofluorescence. (A) Immunofluorescence with monoclonal anti-EBNA2 antibody PE2 of RCC cell line NC65, x40; (B) Immunofluorescence with monoclonal anti-EBNA2 antibody PE2 of RCC case 9, papillary carcinoma area, x40; (C) Little fluorescence was observed with the same antibody of RCC case 9, normal area, x40; (D) Immunofluorescence with monoclonal anti-LMP1 antibody CS1-4 of RCC case 5, x40; (E) Immunofluorescence with monoclonal anti-LP antibody JF186 of RCC case 4, x40; (F) Immunofluorescence with monoclonal anti-BZLF1 antibody BZ1 of RCC case 4, x40.

expressed in cells with renal cancer, but that EBV expression seldom occurred in the normal kidney or other renal diseases than RCC.

EBV DNA was detected by nested PCR. DNA was extracted from resected RCC tissues of other RCC samples than those used for *in situ* hybridization and immunofluore-

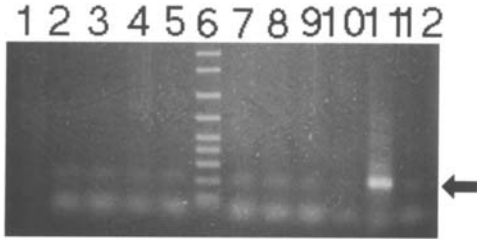

Figure 3. Results of nested PCR. Lane 1, distilled water for negative control; lane 2, clear cell RCC, G2, 52-year-old male; lane 3, chromophobe cell RCC, G2, 42-year-old female; lane 4, clear cell RCC, G2, 86-year-old female; lane 5, clear cell RCC, G2, 70-year-old male; lane 6, molecular weight marker; lane 7, clear cell RCC, G2, 48-year-old male; lane 8, clear cell RCC, G2, 57-year-old male; lane 9, clear cell RCC, G2>G3, 71-year-old male; lane 10, same as RCC case 5, clear cell RCC, G3, 73-year-old male; lane 11, EBV-carrying lymphocyte cell line Namalwa; lane 12, EBV-negative infantile tonsilla for negative control.

scence except one case. EBV DNA was amplified in the region of *Bam*HIW in all cases examined (Fig. 3).

Discussion

In this study, we showed that EBV infected all samples of RCC, nephroblastoma and RCC cell lines. This would be the first study to demonstrate a direct association of EBV with RCC. In the literature, EBV infection of renal proximal tubule cells has been described in chronic interstitial nephritis (26). The report mentioned that EDV DNA was detected in renal proximal cells by *in situ* hybridization using a DNA probe of *Bam*HIW and by PCR, and further, CD21, which is a cellular receptor of EBV, was also detected to be co-localized (26). Although RCC has not been described to occur after the course of interstitial nephritis, there may be a link between chronic interstitial nephritis and RCC, because RCC has been described to be derived from the region of proximal tubule cells (1). Inflammation and cancer have been discussed for many years (27,28). We have demonstrated EBV expression in lymphoproliferative disorders in the lung, which contained long-term interstitial pneumonia, and lymphomas thought to originate from cells generated in response to various types of chronic inflammation (29). Therefore, it may be easily inferred that chronic interstitial nephritis infected with EBV causes RCC. A report on induced renal tumors in transgenic mice expressing EBNA2 in kidney tubule cells (2) also supported our supposition.

EBV expression was observed stronger in papillary and clear cell RCC than chromophobe cell RCC (Table I). Chromophobe cell RCC is described to be less malignant than other RCC (1). Further, EBV was expressed more strongly in high-grade RCC than low-grade RCC (Table I). This suggested that EBV expression correlated with RCC malignancy.

Our *Bam*HIW probes did not contain an '*Alu*-family'-like sequence, and so the reaction was considered to be specific to EBV. The antisense probe did not react with EBV-negative cells. As the *Bam*HIW fragment of EBV contains a 10-times redundant sequence (23), this probe is very sensitive for the detection of EBV. Furthermore, the *Bam*HIW fragment is the leader sequence of mRNAs of EBNAs, which are suspected to be oncogenes of EBV; therefore, the *Bam*HIW probe is important for the detection of EBV transformation. Moreover, we confirmed the expression of oncogenic proteins EBNA2 and LMP1 in this study. Our results with *in situ* hybridization and immunofluorescence suggest an oncogenic and tumor progressive role of EBV in RCC and nephroblastoma.

Nephroblastoma occurs mostly in children, and there seems no correlation with chronic EBV infection. In this experiment, we showed EBV proliferation in RCC, nephroblastoma and RCC cell lines through the examination of immunofluorescence staining with BZLF1. Although the fluorescence was weak, both cases of nephroblastoma showed BZLF1 fluorescence. Accordingly, nephroblastoma may be induced by primary infection with EBV in the childhood. Tornell *et al* described that Wilms tumor is associated with the overexpression of insulin-like growth factor II (IGF-II) and that EBNA2 can induce transcription of the IGF-II gene (2); therefore, the mechanism of tumorigenesis with EBV between RCC and nephroblastoma might be different. Further study is necessary to solve this problem.

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