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 BamHIW, EBNA LP, EBNA2 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-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.

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 and 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 BamHIW region which is repeated approximately 10 times in an EBV genome, and the BamHIY1Y2 (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 BamHIW and EBNA2 probes and anti-EBNA2 and anti-LMP1 antibodies were selected to detect transformation by EBV. Furthermore, the BamHIY1Y2 (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.** BamHIW probes were transcribed from 2.27 kb EBV BamHIW fragments from which the ‘Alu-family’-like sequence had been deleted. The BamHIW 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 BamHIY1Y2 (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 BamHIW 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 and xylene, the sections 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).

**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 mCi 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 37°C. 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 Tannner, 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 CAGCCAAATTCTC3’ (nucleotide positions of BamHIW 1087-1106), and 5’CTTAAGAAAGCCACCGGTC3’ (1503-1520). The primers used for the 2nd round of PCR were: 5’TGAAGAGGGGTTCTCTACTC3’ (1196-1214), and 5’CCA GAGTGAAGTGACTT3’ (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 (Namdaw 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 MgCl2; 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
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 BamHIW mRNA in situ hybridization, signals were 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 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

Table I. Summary of results.

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<td>48</td>
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aCC with sarc, clear cell RCC with sarcomatoid change; bGlomerulosc, Glomerulosclerosis; cIschemic, ischemic renal disease; dBamW, BamHIW; eLP, EBNA LP; fE2, EBNA2; gE1, EBER1; hBZ1, BZLF1; w+, weakly positive; +-, equivocal.
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 immunofluorescence.
EBV DNA was amplified in the region of BamHIW 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 BamHIW 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 BamHIW 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 BamHIW fragment of EBV contains a 10-times redundant sequence (23), this probe is very sensitive for the detection of EBV. Furthermore, the BamHIW fragment is the leader sequence of mRNAs of EBNA3s, which are suspected to be oncogenes of EBV; therefore, the BamHIW 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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