Yonago Acta medica 1996;39:171–176

Improvements in the Detection of Epstein-Barr Virus DNA on Paraffin-Embedded Gastric Carcinoma Tissues: Treatment of Extracted Cellular DNA with a Restriction Enzyme Prior to Polymerase Chain Reaction

Noriko Takasaka, Yukio Satoh, Yoshiko Hoshikawa, Mitsuhiko Osaki*, Hisao Ito*, Jen-Yang Chen† and Takeshi Sairenji

*Department of Biosignaling and *First Department of Pathology, Faculty of Medicine, Tottori University, Yonago 683 and †Department of Bacteriology and Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan*

An association between Epstein-Barr virus (EBV) and a certain gastric carcinoma has been suggested by the presence of EBV-DNA in the carcinoma cells. We tried to detect EBV-DNA in paraffin-embedded tissues of gastric carcinoma by polymerase chain reaction (PCR) followed by Southern analysis. The EBV-DNA sequence was not amplified sufficiently with a *Bam*H I-W primer pair to the extracted DNA itself from the tissues which were detected for the expression of EBV-encoded small RNA (EBER), while the sequence was amplified to the DNA from Raji cells as a positive control. However, when the extracted DNA was digested with a restriction enzyme, *Bam*H I or *Eco*R I and then subjected to PCR, the *Bam*H I-W region was amplified in the DNA from EBER-positive but not -negative tissues. In the extracted DNA from an EBV genome-carrying cell line derived from a gastric carcinoma, the amplification with PCR was increased slightly by the digestion with *Bam*H I. Thus, the pretreatment for template DNA increased the sensitivity of PCR for the detection of EBV-DNA from paraffin-embedded tissues.

Key words: Epstein-Barr virus; gastric carcinoma; PCR; restriction enzyme

In recent studies, the Epstein-Barr virus (EBV) genome was detected in about 7% of gastric carcinomas in Japan and was detected in carcinoma cells of most cases of gastric carcinoma with lymphoid stroma (Shibata et al., 1991; Min et al., 1991; Tokunaga et al., 1993; Fukayama et al., 1994; Imai et al., 1994). These results suggest that EBV might be associated with the pathogenesis of some gastric carcinomas. It is a significant issue to detect the EBV genome in carcinoma tissues with high sensitivity and specificity for the diagnosis of EBV-related gastric carcinoma. The use of polymerase chain reaction (PCR) for rapid diagnosis of infectious agents has been reported in several studies (Shibata et al., 1988; Saito et al., 1989; Sallie et al., 1992). It has been reported that the EBV genome has been detected in DNA extracted from paraffin-embedded tissues by PCR (Shibata et al., 1991; Min et al., 1991; Fukayama et al., 1994; Imai et al., 1994). There are reports which describe the improvement of PCR detection from paraffin-embedded tissues (Kiene et al., 1992; Sepp et al., 1994). We tried to detect the EBV genome more effectively in DNA extracted from paraffin-embedded gastric carcinoma tissues by PCR-Southern analysis and found that the detection of EBV-DNA was improved by the pretreatment of the restriction enzyme for extracted DNA.

Abbreviations: bp, base pair; EBER, EBV-encoded small RNA; EBV, Epstein-Barr virus; PCR, polymerase chain reaction

Materials and Methods

Specimens

The tissues were from surgical specimens of gastrectomy. Formalin-fixed paraffin-embedded tissues from gastric carcinoma with lymphoid stroma were tested for the expression of EBV-encoded small RNA (EBER; Glickman et al., 1988) as previously described (Tokunaga et al., 1993), and used for the detection of EBV-DNA by PCR.

Cells

Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37˚C in a CO_2 incubator. Epithelial cells were passaged with 0.25% trypsin and 0.02% EDTA. EBV genome-carrying Raji cells (Pulvertaft, 1965) and the genome-free Ramos cells (Klein et al., 1975) were used as positive and negative controls respectively for PCR analysis.

DNA extraction

Twenty pieces of a 10 μm section were cut from the paraffin blocks and transferred into sterile 15 mL plastic tubes. Paraffin was removed by treatment with xylene. The sections were hydrated with a graded alcohol series of 100%, 70% and 50% ethanol and deproteinated with 200 μg/mL proteinase K at 37˚C overnight. The DNA was purified by phenol-chloroform extraction and ethanol precipitation and dissolved in 1 mL TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

For DNA extraction from cultured cells, 10^6 cells were washed with 10 mM phosphate buffered saline, pelletted, resuspended in distilled water and lysed by 2 cycles of freezing-thawing. The DNA was extracted by 50 mM NaOH at 95˚C for 10 min, neutralized with 1 M Tris-HCl (pH 7.5) and purified by phenol-chloroform extraction and ethanol precipitation. It was then dissolved in 50 μL TE.

PCR-Southern analysis

The DNA (1 μg) in 5 μL TE was diluted into 50 μL of a solution containing 200 μM each of deoxyribonucleoside triphosphates (dNTPs), 2 mM $MgCl_2$, 500 nM each primer, 2.5 U Taq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan). The primers were designed to amplify a 129-base pair (bp) segment in the *Bam*H I-W region (5'-CCAGACAGCAGCC AATTGTC-3' and 5'-GGTAGAAGACCC CCTCTTAC-3') of the EBV genome as previously described (Tomita et al., 1995). PCR was performed in one cycle for 3 min at 94˚C, 1 min at 58˚C and 1 min at 72˚C, and in 34 cycles for 1 min at 94˚C, 58˚C and 72˚C, respectively. The amplified products were electrophoresed in 2% agarose gel and transferred to a Hybond N^+ membrane (Amersham Japan Co., Ltd., Tokyo, Japan). A 40-base oligonucleotide probe (5'-CCCTGGTATAAAGTGGTCCTGCAGCTA TTTCTGGTCGCATC-3') (Uhara et al., 1990), which hybridized to intervening sequence between 2 EBV primers was 5'end-labeled with a [γ-^{32}P]ATP. The membrane was hybridized with the labeled probe at 42˚C overnight and

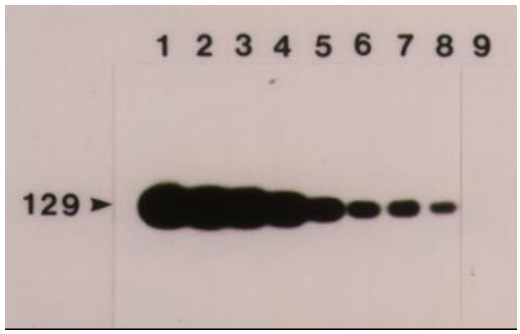

Fig. 1. Detection of *Bam*H I-W region in the EBV genome on Raji cells by PCR-Southern analysis. The serial 10-fold diluted cellular DNA of Raji (10^5 cells or 5×10^6 copies of viral genomes) were subjected to PCR (lane 1, 5×10^6 copies of EBV genome; lane 2, 5×10^5 copies; lane 3, 5×10^4 copies; lane 4, 5×10^3 copies; lane 5, 5×10^2 copies; lane 6, 5×10^1 copies; lane 7, 5×10^0 copies; lane 8, 5×10^{-1} copies). Ramos cells were used as the negative control (lane 9). The same amount (5 μL) of PCR reaction mixture was electrophoresed.

washed in washing buffer (2 × SSC, 0.1% SDS) at 50˚C. Autoradiography was carried out at –80˚C with X-ray film.

Bam*H I or* Eco*R I digestion*

The DNA (1.8 μg) extracted from the paraffin blocks were dissolved in 10 μL of buffer K (20 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl) or buffer H (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1mM DTT, 100 mM NaCl) containing 10 U *Bam*H I or *Eco*R I (Takara Shuzo Co.), and incubated at 30˚C or 37˚C, respectively for 1 h. The reaction mixtures were incubated at 60˚C for 15 min to inactivate the enzyme, and the DNA was ethanol-precipitated and dissolved in 5 μL TE.

Shearing or alkali treatment of DNA

The DNA in TE was sheared mechanically by passing through a 26 gauge needle 10 times. Part of the DNA sample (1.8 μg) in 5 μL TE was then mixed with 50 μL 50 mM NaOH, layered by 50 μL mineral oil, heated at 95˚C for 10 min and neutralized by the addition of 8 μL 1 M Tris-HCl, pH 7.5. After removal of the mineral oil by treatment with chloroform, the DNA was precipitated with ethanol and dissolved in 5 μL TE.

Results

Sensitivity of PCR

The sensitivity of PCR-Southern analysis to detect EBV-DNA was tested in serially 10-fold diluted template DNA obtained from Raji cells that have 50 copies of EBV-DNA per cell (zur Hausen et al., 1972) (Fig. 1). A band at 129 bp was detected in all the PCR of the diluted template DNA (lanes 1–8), with *Bam*H I-W probe, but not in the PCR of DNA from EBV genome-free Ramos cells. The PCR amplification could detect 5×10^{-1} copies of EBV genome (lane 8) which was estimated from the copies of viral genome in diluted Raji DNA. This result showed that the PCR had high sensitivity enough to detect even one copy in the template DNA.

Detection of EBV-DNA in paraffin-embedded tissues of gastric carcinoma by PCR

The PCR-Southern analysis was carried out to detect the EBV genome in the DNA from paraffin-embedded tissues of gastric carcinoma (Fig. 2A). However the *Bam*H I-W region was not amplified to a detectable level to the extracted DNA by PCR in EBER-positive (lanes 1–3) or -negative tissues (lanes 4–7), while the region was amplified in DNA from the EBV genome-positive Raji cells (lane 9). In order to find the problem for no amplification, an extracted DNA from an EBER-positive tissue was pretreated by several methods prior to PCR (Fig. 2B). The treatments were as follows: i) the shearing of DNA through a needle, ii) digestion with *Bam*H I, iii) boiling with an alkali solution, and iv) the addition of Raji DNA to test the presence of any inhibitor(s) to PCR. The amplified EBV-DNA band was detected in *Bam*H I digestion (lane 3), alkali treatment (lane 4) or the DNA mixture with Raji DNA (lane 5) but not in the shearing of DNA (lane 2). The amplification of DNA in the mixture with Raji DNA indicated that there was no inhibitor for PCR in the extracted DNA.

DNA samples which were not detected for EBV-DNA by PCR in Fig. 2A were digested with *Bam*H I and then subjected to PCR (Fig. 2C). The band to *Bam*H I-W was detected in all DNAs from EBER-positive (lanes 1–3) but not in -negative tissues (lanes 4–7).

The effect of another restriction enzyme, *Eco*R I was examined for DNA extracted from an EBER-positive tissue (Fig. 2D). The *Bam*H I-W region was also amplified similarly.

Effect of Bam*H I digestion of DNA extracted from a cultured EBV positive cell line derived from a gastric carcinoma*

DNA was extracted from an EBV-positive cell line (Yanoma et al., unpublished data) derived

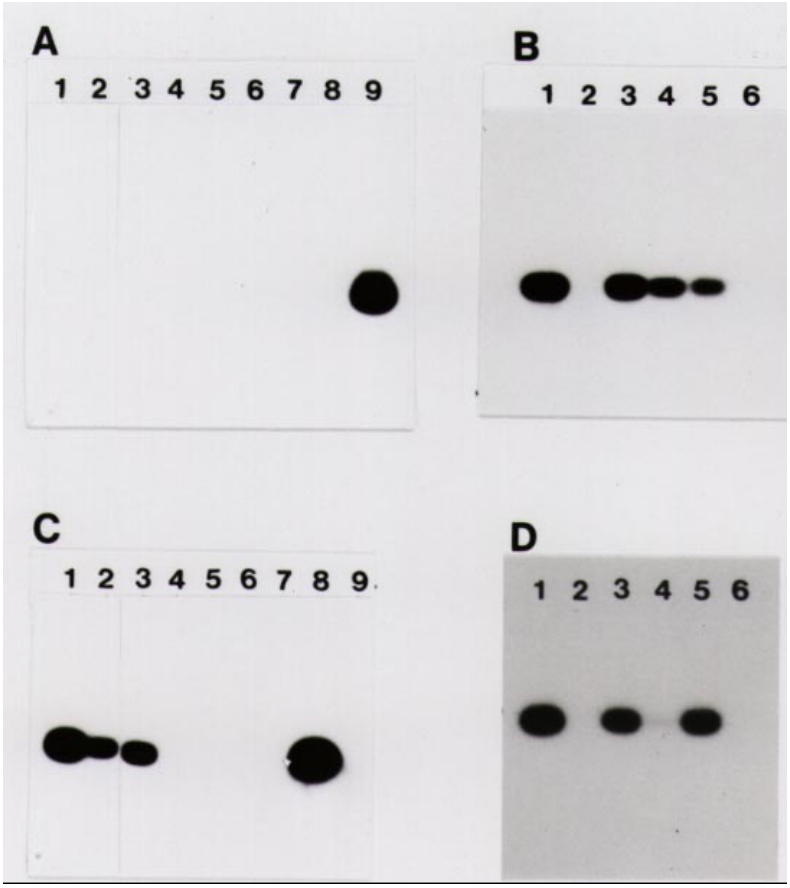

Fig. 2. PCR-Southern blot analysis of amplified DNA with *Bam*H I-W primer. (**A**) Detection of EBV genomes in gastric carcinoma with lymphoid stroma. The DNA extracted from paraffin-emdedded tissues was amplified by PCR and detected with Southern blot. (lanes 1–3, EBER positive tissues; lanes 4–7, EBER negative tissues; lane 8, Ramos; lane 9, Raji). (**B**) PCR amplification of DNA pretreated by several procedures as described in Materials and Methods. (lane 1, Raji DNA; lane 2, shearing; lane 3, *Bam*H I digestion; lane 4, treated with alkali solution; lane 5, Raji DNA (the same as lane 1) plus DNA extracted from a paraffin block (the same as lane 2); lane 6, Ramos DNA). (**C**) Effect of *Bam*H I digestion on PCR. The extracted DNA was digested with *Bam*H I and analyzed by PCR-Southern blot. (lanes 1–3, EBER positive; lanes 4–7, EBER negative; lane 8, Raji; lane 9, Ramos). (**D**) Effect of *Eco*R I digestion for detection of EBV-DNA in PCR. The extracted DNA from an EBER-positive tissue was digested with *Eco*R I and amplified with *Bam*H I-W primer by PCR. (lane 1, Raji; lane 2, no digestion; lane 3, *Bam*H I digestion; lane 4, no digestion; lane 5, *Eco*R I digestion; lane 6, Ramos)

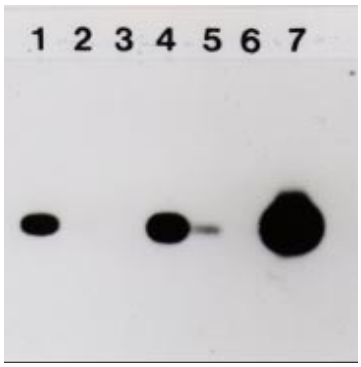

Fig. 3. Effect of *Bam*H I digestion on DNA from cultured EBV-genome-positive cells. DNA was extracted from an EBV-positive cell line derived from a gastric carcinoma and digested with *Bam*H I and analyzed by PCR-Southern analysis. (lane 1, no digestion; lanes 2–3, 10- and 100-fold dilutions of PCR product of lane 1; lane 4, *Bam*H I digestion; lanes 5–6, 10- and 100-fold dilution of PCR products of lane 4; lane 7 Raji).

from a gastric carcinoma and digested with *Bam*H I and analyzed with PCR. The amplification for *Bam*H I-W was increased slightly in digested DNA than non-digested DNA (Fig. 3).

Discussion

In the present study, we demonstrated that digestion of extracted DNA with restriction enzymes, *Bam*H I or *Eco*R I was a useful procedure to detect the EBV genome for DNA extracted from paraffin-embedded tissues by PCR. Restriction enzymes, *Bst*X I, *Cla* I, *Kpn* I or *Sal* I, which have no cleavage site(s) in the *Bam*H I-W fragment, were similarly effective (data not shown). We do not know the reason why the amplification of PCR is increased by the digestion with a restriction enzyme; however, these results may indicate that PCR reactivity could be increased by the exposure of the *Bam*H I-W region to the fragmentation of cellular DNA. Taq polymerase containing the associated 5' to 3' exonuclease activity facilitates a nick translational DNA synthesis (Longley et al., 1990). If the DNA from paraffin-embedded tissues has many nicks, then the nicks could serve as primers for the DNA synthesis. Shortening of DNA by digestion will result in quicker release of the polymerase from DNA and a more frequent reassociation to the next primer for DNA amplification.

We observed no amplification of EBV-DNA in DNA from paraffin-embedded gastric carcinoma tissues without the pretreatment of restriction enzyme, although the sensitivity of PCR was very high to detect EBV-DNA for EBV-positive Raji cells. A possibility may depend on the configuration of EBV-DNA in gastric carcinoma cells. The configuration of EBV-DNA has not been known in cells of gastric carcinoma. EBV-DNA may exist in a different mode, such as the integration in cellular DNA which differs from the episomal DNA as seen in most EBV-positive B cell lines. PCR amplification may be increased by the digestion of cellular DNA with a restriction enzyme which exposes the integrated EBV-DNA.

Detection of the EBV genome by PCR has been used more frequently for diagnosis of EBV infection for paraffin-embedded tissues from several malignant diseases (Tomita et al., 1995; Van Rensburg et al., 1995; Vasef et al., 1995). The treatment of a restriction enzyme will be useful in improving the detection of EBV-DNA for such materials.

Acknowledgments: This work was supported by a Grant-in-Aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan. We thank Dr. Shunsuke Yanoma for providing an EBV-positive gastric carcinoma-derived cell line.

References

1 Fukayama M, Hayashi Y, Iwasaki Y, Chong J, Ooba T, Takizawa T, et al. Epstein-Barr virus-associated gastric carcinoma and Epstein-Barr virus infection of the stomach. Lab Invest 1994; 71:73–81.

2 Glickman JN, Howe JG, Steitz JA. Structural analysis of EBER 1 and EBER 2 ribonucleoprotein particles present in Epstein-Barr virus-infected cells. J Virol 1988;62:902–911.

3 Imai S, Koizumi S, Sugiura M, Tokunaga M, Uemura Y, Yamamoto N, et al. Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein. Proc Natl Acad Sci USA 1994;91:9131–9135.

4 Kiene P, Milde-Langosch K, Runkel M, Schulz K, Loning T. A simple and rapid technique to process formalin-fixed, paraffin-embedded tissues for the detection of viruses by the polymerase chain reaction. Virchows Archiv A Pathol Anat 1992;420:269-273.

5 Klein G, Giovanella B, Westman A, Stehlin JS, Mumford D. An EBV-genome-negative cell line established from an American Burkitt lymphoma: Receptor characteristics. EBV infectibility and permanent conversion into EBV-positive sublines by in vitro infection. Intervirology 1975; 5:319–334.

6 Longley MJ, Bennett SE, Mosbaugh DW. Characterization of the 5' to 3' exonuclease associated with Thermus aquaticus DNA polymerase. Nucleic Acids Res 1990;18:7317–7322.

7 Min K-W, Holmquist S, Peiper SC, O'Leary TJ. Poorly differentiated adenocarcinoma with lymphoid stroma (lymphoepithelioma-like carcinomas) of the stomach. Am J Clin Pathol 1991;96:219–227.

8 Pulvertaft RJV. A study of malignant tumors in Nigeria by short-term tissue culture. J Clin Pathol 1965;18:261–273.
9 Saito I, Servenius B, Compton T, Fox RI. Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome. J Exp Med 1989;169:2191–2198.
10 Sallie R, Rayner A, Portmann B, Eddleston ALWF, Williams R. Detection of hepatitis "C" virus in formalin-fixed liver tissue by nested polymerase chain reaction. J Med Virol 1992;37: 310–314.
11 Sepp R, Szabo I, Uda H, Sakamoto H. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. J Clin Pathol 1994;47:318–323.
12 Shibata DK, Arnhem N, Martin WJ. Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. J Exp Med 1988;167:225–230.
13 Shibata D, Tokunaga M, Uemura Y, Sato E, Tanaka S, Weiss LM. Association of Epstein-Barr Virus with undifferentiated gastric carcinomas with intense lymphoid infiltration: lymphoepithelioma-like carcinoma. Am J Pathol 1991; 139:469–474.
14 Tokunaga M, Land CE, Uemura Y, Tokudome T, Tanaka S, Sato E. Epstein-Barr virus in gastric carcinoma. Am J Pathol 1993;143:1250–1254.
15 Tomita Y, Ohsawa M, Mishiro Y, Kubo T, Maeshiro N, Kojya S, et al. The presence and subtype of Epstein-Barr virus in B and T cell lymphomas of the sino-nasal region from the Osaka and Okinawa districts of Japan. Lab Invest 1995;73:190–196.
16 Uhara H, Sato Y, Mukai K, Akao I, Matsuno Y, Furuya S, et al. Detection of Epstein-Barr virus DNA in Reed-Sternberg cells of Hodgkin's disease using the polymerase chain reaction and in situ hybridization. Jpn J Cancer Res 1990;81: 272–278.
17 Van Rensburg EJ, Engelbrecht S, Van Heerden W, Raubenheimer E, Schoub BD. Detection of EBV DNA in oral squamous cell carcinomas in a black African population sample. In Vivo 1995; 9:199–202.
18 Vasef MA, Kamel OW, Chen YY, Medeiros LJ, Weiss LM. Detection of Epstein-Barr virus in multiple sites involved by Hodgkin's disease. Am J Pathol 1995;147:1408–1415.
19 zur Hausen H, Diehl V, Wolf H, Schulte-Holthausen H, Schneider U. Occurrence of Epstein-Barr virus genomes in human lymphoblastoid cell lines. Nature New Biol 1972;237: 189–190.

(Received September 2, Accepted September 30, 1996)