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Basic Investigations

THE INTRACELLULAR FORM OF EPSTEIN-BARR VIRUS GENOME IN NASOPHARYNGEAL CARCINOMA*

Wang Huimin[1] 汪慧民 Chen Jun[1] 陈军 Zeng Musheng[1] 曾木圣
Li Manzhi[1] 李满枝 Jian Shaowen[1] 简少文 Pan Wentong[2] 潘文彤
Zhang Ling[1] 张玲 Wu Yintang[1] 吴荫棠

[1]*Cancer Institute and* [2]*Department of Microbiology, Sun Yat-sen University of Medical Sciences, Guangzhou 510060*

Object: To study the existent form of EBV genome in nasopharyngeal carcinoma (NPC) biopsies, in a transplanted NPC tumor SUNT-1 and its corresponding epithelial cell line SUNE-1. Methods: By using polymerase chain reaction (PCR) amplification of Epstein-Barr virus (EBV) *BamHI* W fragment, EBV DNA was detected in 20/20 biopsy specimens of poorly-differentiated, as well as in a nude mouse xenografted NPC tumor (SUNT-1, from passage 1 to 34) and in the corresponding epithelial cell line (SUNE-1, from passage 1 to 62). The intracellular form of EBV genome was studied by analyzing the terminal structure using a LMP2A probe and an "*in situ* lysing gel" technique. Results: A single EBV fused terminal DNA fragment was detected in 19 biopsy specimens, two hybridized bands were seen in one specimen. These results indicate that an episomal form of EBV genome is predominantly present in most NPC biopsy specimens, but insertion of the genome into the host chromosome could not be excluded. Conclusion: The finding suggests that EBV infection precedes clonal amplification of transformed cells, or in a rare case, that a single EBV infected clone is predominant in the development of NPC. Linear form of EBV DNA was detected in the 20th passage of SUNE-1; this may imply the *in vitro* activation of the productive cycle of EBV.

Key words: Nasopharyngeal neoplasm, Epstein-Barr virus gene.

The linkage of Epstein-Barr virus (EBV) with nasopharyngeal carcinoma (NPC) has been well documented by a large number of studies. The EBV genome can be detected in 100% poorly-differentiated or undifferentiated cancer. However, only a few of the EBV genes are known to be expressed in NPC. They are EBV nuclear antigen-1 (EBNA-1), latent membrane protein-1 (LMP-1), LMP2A and LMP2B, as well as two small early RNA (EBERs) which do not code for protein.[1]

Two types of EBV infection, productive infection and latent infection, were identified.[2] The productive infection represent an entire process of DNA replication, transcription, translation and virus assembly, leading to virus releasing and target cells lysis. On the other hand, latent infection does not result in virus release, while viral DNA consistently maintains in the host cells either as a circular episomal form or as linear form integrated into the host chromosomal DNA.[3] In this study, we examine the existent form of EBV genome in NPC biopsies, in a transplanted NPC tumor SUNT-1 and its corresponding epithelial cell line SUNE-1, which was established in our laboratory.

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MATERIALS AND METHODS

Target Tissues and Cells

Biopsies from 20 cases of poorly-differential NPC were provided by the Department of NPC. Cancer Hospital of Sun Yat-sen University of Medical Sciences. SUNT-1 is a nude mice transplanted tumor, established from a poorly-differentiated NPC in our laboratory. SUNE-1 is an established epithelial cell line from the same patient.[4] The following cell lines were used as EBV production or latent infection target cell control: P3HR-1, B95-8 and Raji.

Primers and Probes

The oligonucleotides of 5'CCAGACAGCAG-CCAATTGTC-3' and 5'GGTAGAAGACCCCCTCT-TAC-3', which flank the *Bam*HI W fragment in the internal repeat sequence (IR1) of EBV genome, were used as primers for PCR amplification of a 129 bp fragment.[5] To detect different EBV fragments, the following plasmids were used to prepare probes: 1. pLMP2A e1–e5, an EBV coded LMP2A exon 1–5 cDNA cloned in pBR322 *Eco*RI site; 2. pNhet, an EBV DNA *Eco*RI fragment inserted in an ampicillin resistant plasmid (a gift from Dr. M. H. Ng, Hong Kong University, Hong Kong); 3. pLMP2Ae1, a pMa1C2 plasmid carrying EBV coded LMP2A exon1 between the *Eco*RI and *Hin*dIII restriction sites; 4. pEBW, a plasmid containing the EBV DNA *Bam*HI W fragment (a gift from Dr. Dolly Wong, Hong Kong Chinese University, H. K.); 5. pLMP1, an EBV LMP1 coded cDNA cloned into the *Eco*RI site of pUC vector (a gift from Dr. Cesar Nadana, IMCB, National University of Singapore, Singapore.

In Situ Hybridization

Digoxingenin-labeled DNA probes were prepared using a PCR extension procedure. 20 μg/ml of template DNA, 25 pmol of each primer, 200 μmol of each dATP, dCTP, dGTP, 140 μmol of dTTP and 60 μmol of dig-dUTP (Boehringer Mannheim) were mixed, incubate at 94℃ for 5 min; 1.5 μl of Taq DNA polymerase was then added. The final volume of reactions were adjusted to 50 μl with water. The conditions for reactions were 1 min at 94℃, 1 min at 55℃, 1.5 min at 72℃ for 35 cycles, with the last extension went for 5 min at 72℃. DNA probes were purified using low melting temperature agarose gel electrophoresis and quantitated with spot hybridization. Hybridization was carried out as follows: 1. Cell smears were fixed with 4% paraformaldehyde diluted in 0.1 M PBS for 10 min. After 2×10 min rinse in PBS, the smears were digest with 40 μg/ml protein K at room temperature for 10 min, rinsed with PBS for 10 min, and then subject to the post-fixing for 3 min with 4% paraformaldehyde following by two 5 min PBS rinses. The slides were dehydrated by incubation in gradually increasing concentrations of ethanol and dried at room temperature; 2. Prehybridization was performed at 37℃ for 1 h with 50 μl of hybridization solution (4×SSC, 50% formamide, 1×Denhardts solution, 150 μg/ml of denatured salmon sperm DNA, 5% dextran sulfate); 3. For hybridization, hybridization solution containing 0.125–0.25 μg/ml denatured probe was used. Slides were incubated at 40℃for 16 h in a humidified box; 4. After hybridization, slides were rinsed to remove the probe using the following steps: 4×SSC, 2×10 min at RT; 2×SSC 20 min, at 55℃; 0.1×SSC 20 min, at 55℃; 0.05×SSC 20 min, at 55℃. Hybridization signal was detected following the instruction of manufacturer.

Southern Blotting and Hybridization

Total DNA extracted from NPC tissues and cultured cells were digested with *Bam*HI to completion, then electrophoresed on an agarose gel, following by Southern transfer to a nitrocellulose membrane (Amersham). The membrane was baked for 2 h at 80 ℃. LMP2A e1–e5 DNA fragments were prepared for producing radioactive DNA probe using a labeling kit (Pharmacia). The specific activity was around 1×10^8 cpm/μg DNA. Membrane was prehybridized in prehybridization solution (5×SSPE, 5×Denhardts solution, 0.5% SDS, 20 mg/ml salmon sperm DNA) for 1 h at 65℃. Labeled probe was then added, hybridize 12 h at 65℃. After hybridization, membrane was washed twice with 2×SSPE, 0.1% SDS at room temperature for 10 min, followed by 1×SSPE, 0.1% SDS for 15 min at 65℃ and 0.1×SSPE, 0.1% SDS for 10 min at 65℃. The membrane was then air dried and exposed to X-ray film for 36–48 h at -20℃.

In Situ Lysing Gel Electrophoresis

To determine the intracellular forms, linear or circular, of EBV DNA in different host cells, a simple

gel technique was adopted with minor modification.[6] Briefly, 5×10^5 cell pellets were resuspended in 75 μl of sample buffer (containing 15% Ficoll, 2 Kunitz units of *RNase* type 1A, and 0.01% bromophenol blue in buffer TBE), and were pipetted into the well of a vertical 0.8% agarose gel prepared in buffer TBE (120 ×120×1.5 mm). Lysis buffer (100 μl, containing 5% Ficoll, 1% SDS, 1 mg/ml of proteinase K) was then carefully layered over the cell sample layers. Electrophoresis was performed at 7.5 V/cm for 8 h. DNA was then Southern transferred to a nitrocellulose membrane for hybridization with *Bam*HI fragment of EBV DNA as probe.

RESULTS

Detection of EBV Genome in NPC Tissues, SUNT-1 and SUNT-1 Cells

PCR amplification is a sensitive technique to detect low copy number of EBV genome in infected cells. Using PCR technique, an amplified 129 bp *Bam*HI fragment was detected in all biopsies of the 20 cases of poorly-differentiated NPC, in the 32nd passage of SUNT-1 nude mouse transplanted tumor, and in the SUNE-1 epithelial cell line until the 62nd passage. However, EBV DNA was no longer detected after the 64th passage of SUNE-1 by this method (Figure 1). Failure to detect EBV DNA in the extracted total cellular DNA after long term culture may be attributed to the decrease of EBV DNA copy number. To further trace the EBV DNA in individual cells of the epithelial cell line, *in situ* hybridization was used to detect the LMP1 mRNA in the cells. We have been able to confirm the persistence of EBV genome in cells until p33rd of SUNT-1 and until p133rd of SUNE-1 (Figure 2).

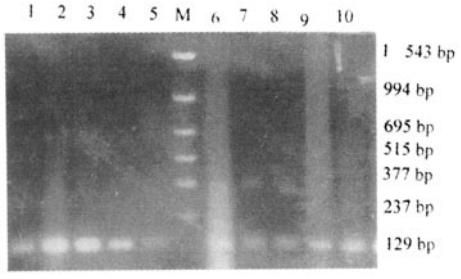

Fig.1. PCR amplification of EBV *Bam*HI W fragment from DNA of 1–5: NPC tissues; 6 and 7: SUNE-1 8th and 60th passage; 8–10: SUNT-1 1st, 15th and 32nd passage. M: molecular weight markers.

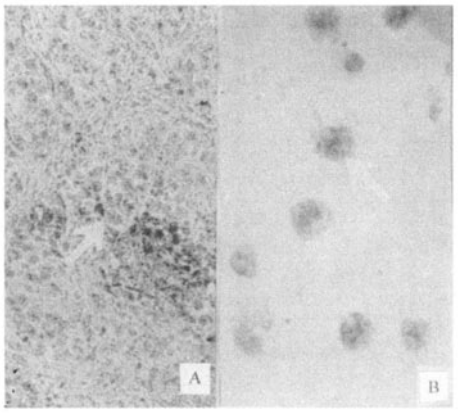

Fig. 2. Detection of EBV LMP1-el gene fragment by *in situ* hybridization. A: SUNT-1 32nd passage. B: SUNE-1 133th passage.

Intracellular Form of EBV Genome in NPC Biopsy

The linear viral genome form was seldom reported in NPC specimens, implying that the latent infection state is predominant. For latent infections, EBV genomes may either exist in the cells as episomes or integrate into the host chromosomes. We used ^{32}P labeled LMP2A exon 1–5 DNA fragments as probes to explore the form of EBV in NPC. Figure 3 and 4 outline the rational of experimental design. The LMP2A gene, flanked by two *Bam*HI sites, is located at both ends of a linear EBV DNA; Exon 1 is

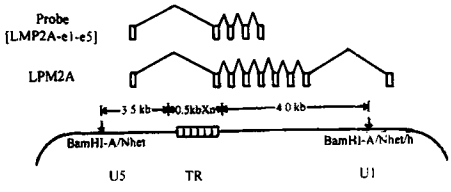

Fig. 3. Terminal structure of EBV DNA circular form.

at the U5 area, while Exon 2–8 are located at the opposite end of U1 area. Digestion of the episomal EBV DNA with *Bam*HI will produce joined terminal fragments. A single fragment will be resulted from tissue of single cell origin. In the case of hetero-

geneous origin, there will be a heterogeneous array of joined terminal fragments. They can be identified by the labeled probe. Two bands will be detected with tissues carrying integrated EBV genomes. To analyze the existing form of EBV genome in NPC, the following cells and DNA were used as controls: B95-8 cells as a productive infection and linear DNA control, Raji cells as a latent infection and episomal form control, *Bam*HI Nhet and pMa1-LMP2A el DNA as the U5 terminal control (Figure 5, 6).

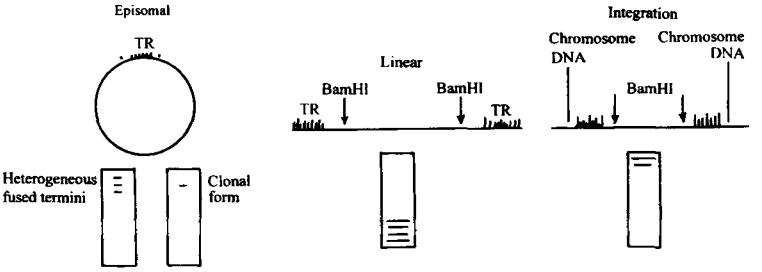

Fig. 4. Scheme for detection of linear or circular form of EBV genome by hybridization with LMP2A-e1–e5 probe (see Reference 8).

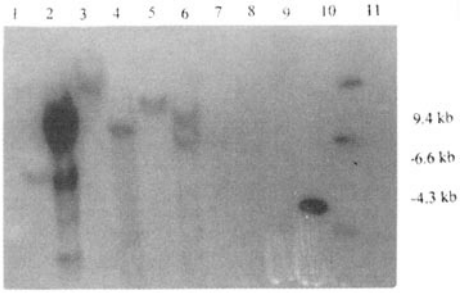

Fig. 5. Analysis of EBV DNA terminus. 1, λ DNA/*Hin*dIII; 2. B95-8; 3. Raji; 4–8, NPC biopsy tissues; 9. SUNE-1 passage 67; 10. EBV *Bam*HI Nhet fragment; 11. pMal-LMP2A-exon 1.

A single joined terminal fragment in different sizes was detected in 19 cases of NPC biopsies, suggesting a clonal origin of the tumors but infected with different viruses containing variable terminal repeats. In one case, two hybridizing bands were detected. This result suggests an integrated form of the EBV DNA in this biopsy.

Intracellular Form of EBV Genome in SUNE-1 Cells

A simple technique, *in situ* lysing gel electrophoresis, was reported to be sensitive in examining the intracellular state of herpesvirus genome in immortalized cell lines — as few as 0.25 EBV circular genome per cell could be detected. Linear DNA can also be detected and be discriminated form the circular form. We adopted this technique in SUNE-1 cells. Immortalized cell lines P3HR-1 and B95-8 cells were used as the EBV productive infection controls, while Raji cells were used as the latent infection control. Figure 7 shows the linear state of EBV DNA in SUNE-1 cells.

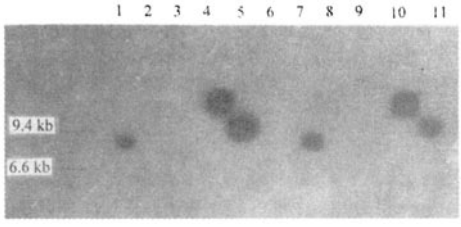

Fig. 6. Analysis of EBV DNA terminus. 1, 4, 5, 7, 10 and 11, NPC biopsy tissues; 6. normal nasopharyngeal tissue; 2 and 3, SUNE-1 pasage 70 and 80; 8 and 9, NIH 3T3 cells.

DISCUSSION

The association of EBV and poorly-differentiated NPC has been previously well-documented. It is generally accepted that EBV is in a latent infection form in the cancer cells,[7] since the expression of latent infection-related proteins, EBNA-1 and LMP, as well as the abundant expression of EBER-1 small RNA, were found in NPC biopsy specimens. However, it is still unclear whether EBV in cancer cells is in a linear

or an episomal form, and whether the cancer is polyclonal or monoclonal in origin. The detection of single fused terminal fragments, which are heterogeneous in size, in 19 out of 20 NPC biopsy specimens strongly suggests that EBV genome is most likely episomal in the cancer cells. The number of terminal repeats (approximately 500 bp each) in different virus strain is variable, so *Bam*HI-digested fragments are heterogeneous in size.[8] In addition, because NPC biopsy includes multiple cancer nets with lymphocyte infiltration, the single band with different sizes in different specimens strongly implies that NPC is monoclonal in origin. If all tumor cells carried a clonal viral genome, it means that the infection occurred at the very beginning of carcinogenesis, and the virus may play an important role in the early state of this event. On the other hand, if the infection occurred after the cancer has developed, polyclonal genomes will be identified.

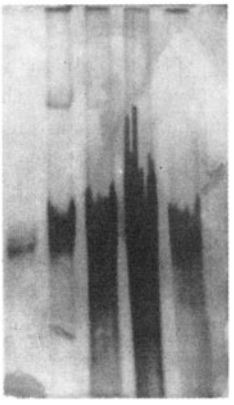

Fig. 7. Detection of EBV DNA in 1: P3HR-1 cells, 2: Raji cells, 3 B95-8 cells (cultured in 37℃), 4: B95-8 cells (cultured in 33℃) and 5:

One of the NPC specimens showed two hybridized bands that are larger than 9.4 kb, suggesting a possible integration of the EBV genome into host chromosome.

Epithelial cell lines established from NPC, such as CNE2 and HK1, were reportedly harboring EBV DNA in their early passages, but lose them after a period of continuing passage. The events leading to deletion and their influences on the host cells are unknown. Nevertheless, this deletion did not occur in long term cultured SUNE-1 cells from passages 1 to 133. Even though PCR amplification could not detect EBV DNA W fragment in SUNE-1 cells after 64 passages, *in situ* hybridization could identify the existence of LMP1 gene transcription in p133 of SUNE-1, as well as in p32 of SUNT-1. This result suggests that both transplanted tumor and the corresponding cell line are consistent EBV carriers, but the copy number of EBV genome is gradually decreased by *in vitro* passage. The finding of linear virus form in the 20th passage of SUNE-1, together with the observation of viral particles in SUNT-1 and SUNE-1 cells by electromicroscopy,[4] suggests that an *in vitro* virus productive activation and the subsequent death of the cultured cells may be the reason for gradual viral genomic deletion. Since LMP may be associated with cellular transformation, the continuing expression of EBV genome is critical to maintaining the characteristics of cancer cells.

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