

# Establishment and characterization of gastric carcinoma cell clones expressing LMP2A of Epstein-Barr virus

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**Abstract.** Although Epstein-Barr virus (EBV) has been detected in 5-15% of gastric carcinoma (GC) cases, the mechanism by which EBV contributes to its tumorigenesis remains unclear. Only a subset of EBV latent proteins such as EBV nuclear antigen-1 and latent membrane protein 2A (LMP2A) are expressed in EBV-associated GC (EBVaGC) cases. In this study, to elucidate the role of LMP2A in the tumorigenesis of EBVaGC, we established permanent cell lines expressing LMP2A. The LMP2A gene was cloned from a naturally EBV-infected EBVaGC cell line, SNU-719 and transduced into an EBV-negative GC cell line, AGS, using a retroviral vector. The sequence of SNU-719 LMP2A showed several conserved variations compared to that of the prototype EBV strain B95-8 LMP2A. Four of seven established cell clones expressed LMP2A protein at detectable levels. These cell clones did not show enhanced cell growth compared to control cells in normal or low serum-containing medium. Furthermore, LMP2A expression had no effect on colony forming ability of the cell clones in soft agar. Our results suggest that LMP2A alone has little effect on tumorigenesis of EBVaGC.

## Introduction

Epstein-Barr virus (EBV) infection is associated with several human malignancies of lymphoid and epithelial origin, including Burkitt's lymphoma (BL), Hodgkin's lymphoma (HD), nasopharyngeal carcinomas (NPC) and gastric

carcinoma (GC) (1-3). Latent EBV infection can cause tumor initiation and propagation through the expression of viral genes that affect the cell cycle, apoptosis and immune response. EBV expresses several viral proteins with transforming ability including EBV nuclear antigen (EBNA)2, EBNA3A, EBNA3C and latent membrane protein 1 (LMP1).

EBV-positive gastric carcinoma (EBVaGC) is the most prevalent among EBV-associated malignancies and EBV association is found in 5-15% of GC worldwide (4-6). As EBV is found in almost all tumor cells of EBVaGC and the EBV genome is present as a monoclonal episomal form (5,7), EBV may act as an oncovirus to cause gastric carcinogenesis. However, it is still unclear whether the presence of EBV is the cause or the result of gastric carcinogenesis because none of the known viral oncogenes are expressed in EBVaGC (8,9).

EBVaGC exhibits a modulated latency I EBV infection, in that the expression of viral latent genes is restricted to EBNA-1, Epstein-Barr encoded RNAs (EBERs) and transcripts from the *Bam*HI-A region (BARTs). In addition, LMP2A is frequently expressed in EBVaGC but LMP1 and LMP2B are not (5,10). LMP2A is a transmembrane protein that inhibits normal B-cell signal transduction by mimicking an activated B-cell receptor (BCR) (11-13). LMP2A activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which normally provides a survival signal in response to BCR signaling (14,15). Previous studies reported that LMP2A contributes to maintenance of viral latency but it is not required for *in vitro* infection or growth transformation of primary B cells (16,17). However, it has been argued that LMP2A is essential for survival and continued proliferation of B cells, even though LMP2A is dispensable for B cell transformation if the cells express BCR (18). LMP2A may also be involved in EBV-positive gastric carcinogenesis; by enhancing transformation and epithelial cell motility as well as inhibiting epithelial cell differentiation (19-21). Conversely, LMP2A did not induce anchorage-independent cell growth in the human epithelial cell line (HaCaT) (22). LMP2A expression in the epidermis of transgenic mice had no effects on the growth and differentiation of epithelial cells (23). Therefore, there are inconsistent issues about the oncogenic role of LMP2A in EBV-associated epithelial tumors.

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Previously, we reported that SNU-719 is a gastric carcinoma cell line that is naturally infected with EBV and that this cell line expresses EBNA-1, LMP2A and EBERs but not LMP1 similar to most EBVaGC cases (24). To examine the oncogenic potential of LMP2A in gastric epithelial cells, the LMP2A gene from SNU-719 was cloned and transduced into an EBV-negative gastric cancer cell line, AGS, using a retroviral vector and the growth ability of cell clones expressing LMP2A was analyzed.

## Materials and methods

**Cell culture.** SNU-719 is a naturally derived EBV-infected GC cell line (24,25). AGS is an EBV-negative GC cell line (26) that was used to transduce the LMP2A gene from SNU-719 using a retroviral vector. SNU-719 and AGS cell lines were obtained from Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (100 units/ml (Invitrogen, Carlsbad, CA, USA), and fungizone (250  $\mu$ g/ml, Gibco BRL). The AGS-EBV cell line, which is AGS infected with a recombinant Akata virus (27), was maintained in RPMI-1640 medium supplemented with 10% FBS and 400  $\mu$ g/ml G418 (Gibco BRL).

**Cloning of LMP2A from SNU-719 in a retroviral vector.** Coding sequence of SNU-719 LMP2A was amplified by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, RNA was extracted from the SNU-719 cells using RNeasy B (Tel-Test, Friendswood, TX, USA) and RT was conducted in 20  $\mu$ l reaction volumes at 37°C for 50 min. For PCR, 5  $\mu$ l of RT product was added to 50  $\mu$ l of PCR mixture containing 10 mM Tris-HCl (pH 9.0), 10 mM KCl, 15 mM MgCl<sub>2</sub>, 8 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1% NP-40, 1 mM dNTPs, 1 unit of pfu DNA polymerase (Invitrogen) and 25 pmol of PCR primers specific for LMP2A (5'-AGAATTCATGGGGTCCCTAGA AATG-3' and 5'-ATACTCGAGCTTTATACAGTGTT GCG-3'). The PCR mixtures were subjected to 30 cycles of PCR in a thermocycler (Perkin Elmer, Foster City, CA, USA). Each cycle consisted of a denaturing step at 94°C for 30 sec, an annealing step at 55°C for 60 sec and an extension step at 72°C for 120 sec. The amplified DNA products were separated by electrophoresis in a 1% agarose gel with 0.5X Tris-acetate-EDTA (TAE) buffer and stained with 0.5  $\mu$ g/ml ethidium bromide. Bands of interest were excised from the gel, purified using a gel extraction kit (Qiagen, Valencia, CA, USA) and ligated into pGEM easy T-vector (Promega, Madison, WI, USA). The LMP2A gene was then subcloned into a retroviral vector, pMIG, which contains enhanced green fluorescence protein (EGFP) as a reporter (28). The constructed plasmids were sequenced by the dideoxynucleotide chain termination method using an ABI prism™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 3770 Genetic analyzer (Perkin Elmer).

**Virus infection.** To produce retrovirus encoding LMP2A, 293T cells (1x10<sup>6</sup>/ml) were seeded in a 60-mm-diameter dish and cultured for 24 h prior to transfection. A LMP2A gene expression construct or the empty pMIG vector was then transfected into the cells by a calcium phosphate method (29).

After 24 h, the medium was removed and fresh medium added. The conditioned medium was harvested following an additional 72 h of incubation and filtered through a 0.45- $\mu$ m-pore size membrane to collect virus solution. AGS cells (0.5x10<sup>5</sup>/well) were seeded in a 6-well plate 24 h before infection. The cells were then incubated with 4 mg/ml of polybrene and 1 ml of virus solution for 24 h. The medium was then changed and GFP expression was evaluated.

**Isolation of EBV-infected cell clones.** AGS cells expressing the LMP2A gene were sorted using fluorescence-activated cell sorting (FACS) based on the expression of the GFP marker. GFP-expressing cells were then serially diluted and seeded in 96-well plates. A microscope was used to determine the wells containing only a single cell. These wells were then marked and maintained at 37°C until the cells grew to ~70% confluence, at which time they were transferred to 12-well plates and expanded for further experiments.

**Detection of LMP2A expression by RT-PCR.** The expression of LMP2A gene in the established cell clones was examined by RT-PCR using specific primers for LMP2A (5'-TACTGA GTAGAGTTGTGTAT-3' and 5'-CATGTTAGGCAAATTG CAAA-3', 280 bp). The RT-PCR product was separated by electrophoresis in a 2% agarose gel. The integrity of the RNA and RT-PCR reaction was evaluated by a parallel amplification of glyceraldehydes 3-phosphatedehydrogenase (GAPDH).

**Detection of LMP2A protein by Western blot analysis.** To detect LMP2A protein, cell lysate in RIPA buffer (50-100  $\mu$ g) was mixed with 2X sodium dodecyl sulfate (SDS) sample buffer and heated at 70°C for 5 min. Next, the samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and the separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Rat polyclonal antibody against LMP2A (15F9, GSF-Forschungszentrum fur Umwelt und Gesundheit, Munchen, Germany) was used as a primary antibody. After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated anti-rat (Amersham Biosciences, Piscataway, NJ, USA) secondary antibody for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection system (Amersham Bioscience). A 1:1000 dilution of anti- $\alpha$ -tubulin antibody (Calbiochem, San Diego, CA, USA) was used to confirm comparable loading.

**Colony formation assay on soft agar.** A single cell suspension in 3 ml of 0.3% SeaKem LE agarose (Cambrex Bio Science Rockland, Inc., ME, USA) was over-laid onto 3 ml of 0.6% base agarose in 6-well plates (1.5x10<sup>4</sup>/well). To prevent drying out and to supply nutrients, 0.5 ml of complete medium was added to the top of the agarose layers once a week. After 3 weeks of incubation at 37°C, the resulting colonies were stained with 0.005% crystal violet for 30 min at room temperature and then washed with de-ionized water. The colonies were observed under an inverted microscope and counted using the UTHSCSA Image Tool 3.0 program (<http://ddsdx.uthscsa.edu>). The colony-forming efficiency was calculated by dividing the number of colonies with the number of plated cells.

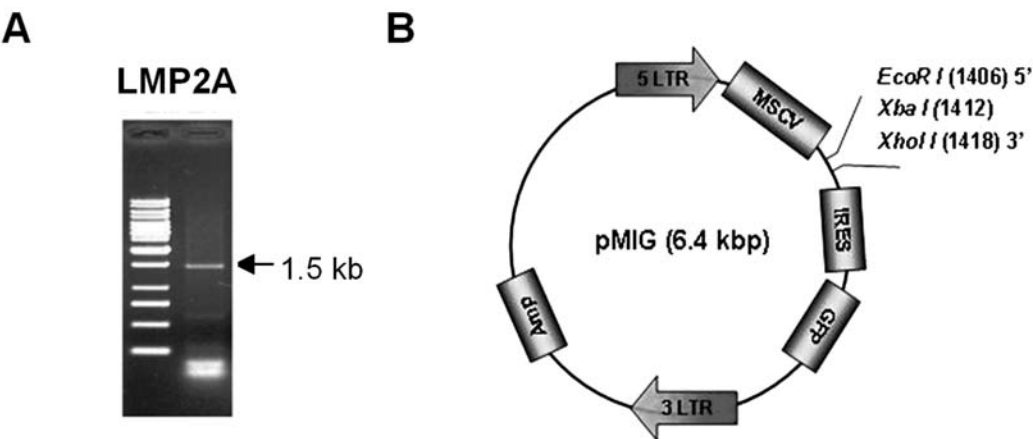


Figure 1. Cloning of LMP2A gene from SNU-719 in a retroviral vector. Total RNA was extracted from SNU-719 cells. The cDNA of LMP2A was amplified by RT-PCR. The expected size of the PCR product is marked on the right (A). Schematic diagram of the retroviral vector, pMIG. The pMIG has an internal ribosomal entry site (IRES) cassette and downstream enhanced GFP cDNA which can be used to select transduced cells by FACS (B).

LMP2A

LMP2A_B95-8	MGSLEMVPMGAGPPSPGGDDPGYDGGNNSQYPSASGSSGNTPTPPNDEERESNEEPPPPY
LMP2A_SNU-719	MGSLEMVPMGAGPPSPGGDDPGYDGGNNSQYPSASGSSGNTPTPPNDEERESNEEPPPPY
	*****
LMP2A_B95-8	EDPYWGNGDRHSDYQPLQTDQSLYLGLQHDGNDGLPPPPYSPRDDSSQHIYEEAGRGS
LMP2A_SNU-719	EDLDWGNGDRHSDYQPLQNDPQLYLGLQHDGNDGLPPPPYSPRDDSSQHIYEEAGRGS
	** *****
LMP2A_B95-8	NPVCLPVIVAPYLFWLAIAASCFTASVSTVVTATGLALSLLLLAAVASSYAAAQRKLLT
LMP2A_SNU-719	NPVCLPVIVAPYLFWLAIAASCFTASVSTVVTATGLALSLLLLAAVASSYAAAQRKLLT
	*****
LMP2A_B95-8	PVTVLTAVVTFFAICLTWRIEDPPFNLSLLFALLAAAGGLQGIYVLVMLVLLILAYRRWR
LMP2A_SNU-719	PVTVLTAVVTFFAICLTWRIEDPPFNLSLLFALLAAAGGLQGIYVLVMLVLLILAYRRWR
	*****
LMP2A_B95-8	RLTVCGGIMFLACVLVLIIDDAVLQLSPLLGAFTVVSMTLLLLAFVLWSSPGGLGTLGAA
LMP2A_SNU-719	RLTVCGGIMFLACVLVLIIDDAVLQLSPLLGAFTVVSMTLLLLAFVLWSSPGGLGTLGAA
	*****
LMP2A_B95-8	LLTLAAALALLASLILGTLNLTTFLLMLLWTLVLLICSSCSCPLSKILLARFLYAL
LMP2A_SNU-719	LLTLAAALALLASLILGTLNLTTFLLMLLWTLVLLICSSCSCPLTKILLARFLYAL
	*****
LMP2A_B95-8	ALLLASALIAGGSILQTNFKSLSTEFIPNLFMCLLLIVAGILFILAILTEWGSNRTY
LMP2A_SNU-719	ALLLASALIAGGSILQTNFKSLSTEFIPNLFMCLLLIVAGILFILAILTEWGSNRTY
	*****
LMP2A_B95-8	GPVFMCLGGLLTMVAGAVLTVMTNTLLSAWILTAGFLIFLIGFALFGVIRCCRYCCYYC
LMP2A_SNU-719	GPVFMCLGGLLTMVAGAVLTVMTNTLLSAWILTAGFLIFLIGFALFGVIRCCRYCCYYC
	*****
LMP2A_B95-8	LTLESEERPPTPYRNTV
LMP2A_SNU-719	LTLESEERPPTPYRNTV
	*****

Figure 2. Sequence alignment of SNU-719 LMP2A with B95-8 LMP2A. Open boxes indicate changed amino acids of SNU-719 LMP2A compared to the B95-8 LMP2A sequences. There are eight amino acid differences. The asterisks (\*) indicate tyrosine residues. Grey boxes indicate 12 transmembrane domains.

*Cell growth in reduced serum medium.* Cells (7x10<sup>3</sup>/well) in a medium containing 10% FBS were seeded in a 12-well plate. After one day, the cells were washed once with a serum-free medium and supplied with a fresh medium containing 1% FBS. The cells were then counted once every other day for 5 days.

Results

*Cloning and sequence analysis of LMP2A gene from SNU-719.* A 1,511 bp long LMP2A transcript was RT-PCR amplified

from the SNU-719 cell line (Fig. 1A). The RT-PCR product was cloned into the retroviral vector pMIG (Fig. 1B). *Escherichia coli* transformed with the LMP2A construct, was used to purify DNA for the sequencing of the construct. The resulting LMP2A sequence comparison between SNU-719 and B95-8 prototype strain is shown in Fig. 2. The LMP2A of SNU-719 showed six changes at amino acids 23, 38, 63, 64, 79 and 82 in the N-terminus and two changes (S to T) at amino acids 348 and 444 in the inter-membrane domains compared to the LMP2A of B95-8.



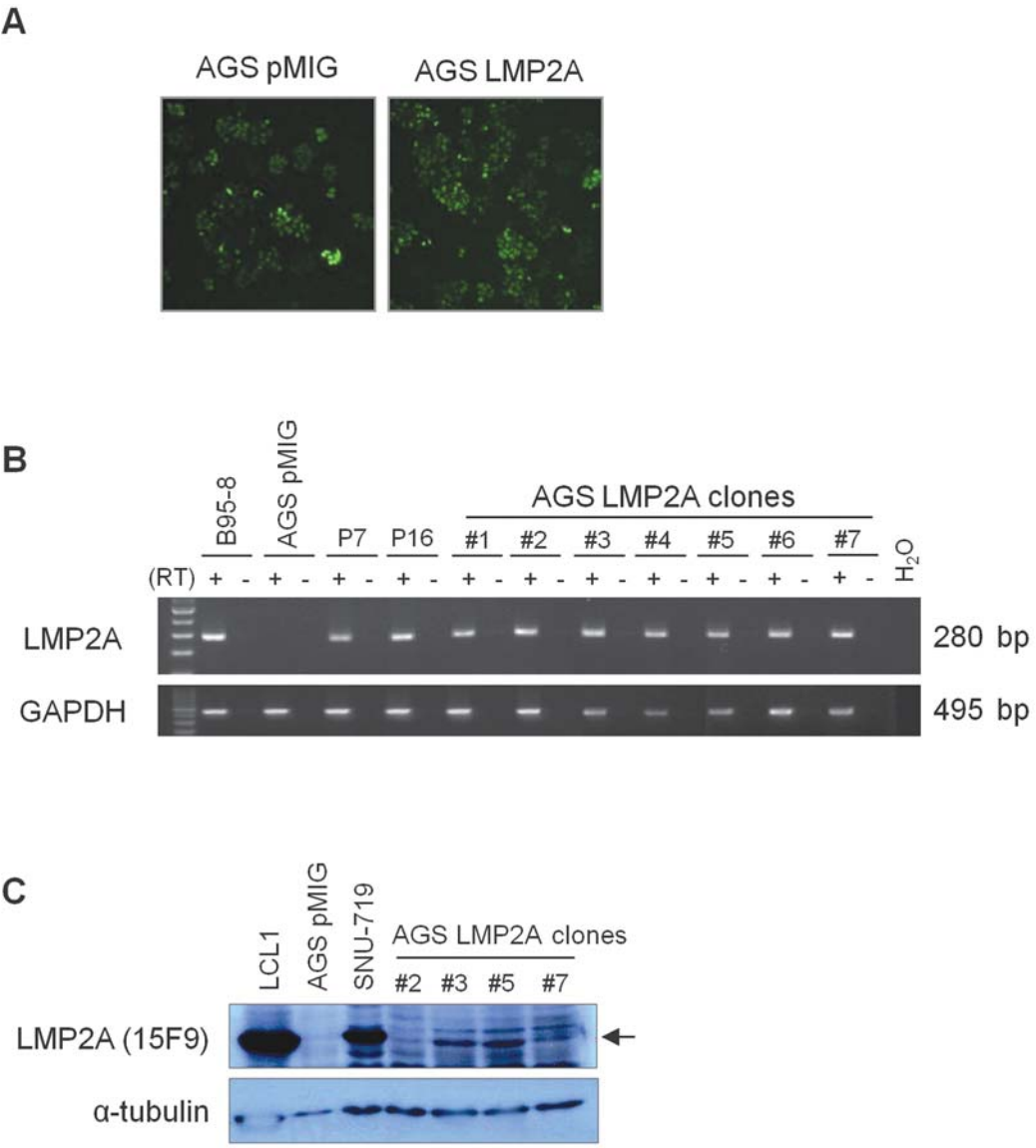


Figure 3. Detection of LMP2A gene expression in the AGS cell clones transduced with the LMP2A construct. Permanent cell clones were obtained by serial dilution. Detection of GFP expression in the AGS cell clones transduced with LMP2A gene after FACS sorting (A). To analyze the expression of LMP2A in each cell clone, total RNA was extracted and then RT-PCR was performed. For comparison, RT-PCR was also performed for GAPDH. P7 and P16 indicate passages 7 and 16 of subculture (B). LMP2A protein was detected by Western blot assay with anti-15F9 antibody. The arrow indicates the LMP2A band (C).

*LMP2A gene transduction into AGS cells and isolation of single cell clones.* The LMP2A gene cloned in the pMIG retroviral vector was transduced into the EBV-negative gastric cancer cell line, AGS. After GFP expression was confirmed (Fig. 3A), the transduced cells were maintained for one week and the GFP expressing cells were collected by FACS. The mRNA expression of LMP2A gene in the sorted cells was verified by RT-PCR after four passages (data not shown). Cell clones expressing LMP2A were then isolated by serial dilution. Seven cell clones were obtained and the expression of LMP2A mRNA was detected by RT-PCR (Fig. 3B). To confirm the expression of LMP2A protein in the cell clones, Western blot assay was carried out. As shown in Fig. 3C, four (#2, #3, #5, and #7 clones) out of seven LMP2A-transduced cell clones expressed LMP2A protein at a detectable level. However, the expression level of LMP2A in the cell clones was lower than the levels observed in SNU-719 and LCL1 used as positive controls.

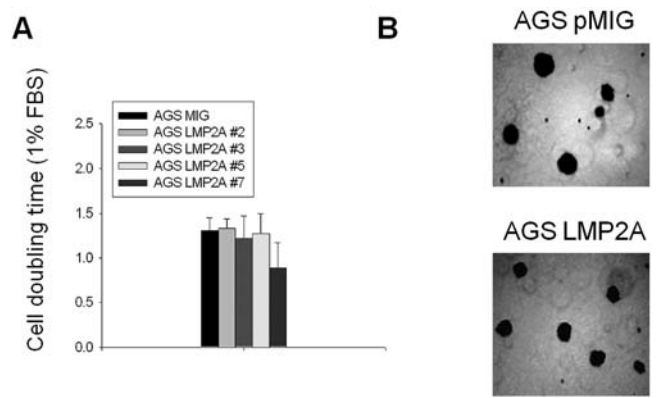


Figure 4. Effect of LMP2A gene expression on cell growth. LMP2A clones were cultured in medium containing 1% FBS and counted after 5 days. The data represent mean  $\pm$  SEM of three independent experiments (n=3) (A). To investigate an anchorage-independent growth ability, cells were seeded in soft agar and then cultured for 3 weeks. Colonies were stained with 0.005% crystal violet for visualization and then washed with de-ionized water (B).



**LMP2A on the cell growth.** The effect of LMP2A on the cell growth was analyzed by culturing the cells in medium containing low serum (1% FBS). LMP2A expressing cell clones had little or no effect on cell growth when compared with the AGS pMIG (Fig. 4A). The growth rate of these cell clones was not different from that of AGS pMIG cells in 10% FBS-containing medium, either (data not shown). The colony formation efficiency (CFE) of the cell clones expressing LMP2A gene was evaluated in soft agar in duplicate experiments (Fig. 4B). EBV-negative AGS cells that were transduced with pMIG (AGS pMIG) formed  $201 \pm 4$  colonies (mean  $\pm$  standard deviation, CFE=0.13%). EBV-positive AGS cells (AGS-EBV) formed  $193 \pm 2$  colonies (CFE=0.13%). The LMP2A expressing cell clone formed  $218 \pm 2$  colonies (CFE=0.15%), which was comparable with AGS-EBV and AGS pMIG cells.

## Discussion

EBV infection is associated with ~10% of the GC worldwide. However, the causal relationship between EBV and EBVaGC remains unclear. EBVaGC exerts a modified latency I and expresses only a limited set of latent EBV proteins such as EBNA-1 and LMP2A. LMP2A is routinely detected in HD, BL, NPC and GC, suggesting that it may contribute to EBV-associated tumor formation and/or maintenance. However, the exact role(s) of LMP2A in EBV-associated tumors is contentious. To better understand the role of LMP2A in EBVaGC, we constructed LMP2A-expressing cell clones by transducing LMP2A gene into an EBV-negative gastric carcinoma cell line AGS.

SNU-719 LMP2A showed six sequence variations at the LMP2A N-terminal domain. Among eight tyrosine residues within the N-terminus of LMP2A, Y23 and Y64 were changed (Y to D) while Y31, Y60, Y74, Y85, Y101, and Y112 were not altered. Y74, Y85 and Y112 have been demonstrated to be essential for LMP2A-mediated blockade of protein phosphorylation, calcium mobilization, and induction of BZLF1 expression after BCR activation, while the other tyrosine residues did not affect LMP2A function *in vitro* (30-32). Thus, Y to D changes at Y23 and Y64 are expected to have little effect on the function of LMP2A in SNU-719. SNU-719 LMP2A showed additional amino acid substitutions at amino acids 348 and 444 of the inter-membrane domains. The S→T substitution at the amino acid 348 of LMP2A which is located in the CTL-epitope region, is a frequent event in EBVaGC cases (33). Such alterations in CTL-epitopes of LMP2A may have helped to avoid immune attack of the EBV-associated gastric carcinoma from which SNU-719 was derived.

After transduction with the retrovirus co-expressing EGFP and LMP2A, cell clones continued to express EGFP for at least 20 passages (120-140 days after gene transduction). This suggests the integration of the LMP2A gene as well as the EGFP gene into the cellular chromosome. However, the intensity of GFP expression was variable in the established cell clones, implying different integration sites or extents of integration.

In our experiments, LMP2A expression did not contribute a growth advantage to AGS cells in low serum medium and LMP2A did not enhance anchorage-independent growth of

AGS cells. There are several plausible reasons for this. First, LMP2A does not have any effect on cell growth, transformation, and tumorigenesis of gastric epithelial cells. Second, the oncogenic effect of the LMP2A gene may have been masked by the basal cell activity because AGS itself is a GC cell line. Indeed, AGS cells showed comparable growth ability in soft agar not only with AGS LMP2A expressing cell clones but also with EBV-positive AGS cells. Third, there might not be enough LMP2A expressed to function properly. We observed that LMP2A was expressed only at low level in all the AGS LMP2A clones compared to the EBV infected cell lines. Compared to the episomal EBV genomes present at up to 50 copies within a cell (34,35), the retroviral vector we used is expected to be integrated to the chromosomal DNA at one or a few different sites. Gene silencing may also have contributed to low LMP2A expression in AGS cell clones, as retroviral vectors often suffer from gene silencing after they are integrated to the chromosomal DNA (36).

In addition, expression of LMP2A gene by itself may not sufficient to affect cell growth leading to oncogenesis, rather they might co-operate to cause tumors with other EBV genes. Related to this point, EBV has been recently shown to express over 20 viral microRNAs (miRNA) (37,38). miRNAs have variable effects and they may cooperate with the latent EBV genes to drive tumorigenesis. In order to identify possible contribution of the latent EBV genes to the development of epithelial malignancies, further analysis of cell lines co-expressing two or more EBV genes would be required.

In conclusion, we established LMP2A gene-expressing cell clones and found that LMP2A by itself does not enhance cell growth any further in an EBV-negative GC cell line.

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