p53, a key regulator in gemcitabine-induced Epstein-Barr virus lytic activation in EBV associated gastric carcinoma

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Directed by Professor Jae Myun Lee

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ABSTRACT

p53, a key regulator in gemcitabine-induced Epstein-Barr virus lytic activation in EBV associated gastric carcinoma

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Epstein-Barr virus (EBV), a dsDNA human gamma herpes virus, is associated with the development of malignancies including EBV-associated gastric carcinoma (EBVaGC) and constant presence of the viral genome in EBVaGC suggests the applicability of novel EBV-targeted therapies. An antiviral nucleoside drug, ganciclovir (GCV), is effective only in the context of the viral lytic cycle in the presence of EBV-encoded thymidine kinase (TK)/protein kinase (PK) expression.

For the treatment of EBVaGC, gemcitabine was identified as a candidate for combination with GCV by screening of the Johns Hopkins Drug Library. In this study, it was circumscribed that pharmacological induction of EBV-TK or -PK

by gemcitabine in EBVaGC-originated tumor cells might be useful for the combination treatment with GCV *in vitro* and *in vivo*. Gemcitabine induced EBV lytic activation via ataxia telangiectasia-mutated (ATM)/p53 genotoxic stress pathway, which was evaluated using an EBVaGC mouse model and a [^{125}I] fialuridine (FIAU)-based lytic activation imaging system. To determine the precise molecular mechanism of how p53 is involved in lytic activation in EBVaGC, I screened several EBVaGC cell lines, SNU-719, YCCEL1 and NCC-24, in which gemcitabine could induce lytic activation except in NCC24. NCC-24 has two-point mutations in the DNA binding sites of p53. Wild type TP53 transfection restored gemcitabine-induced lytic activation in NCC-24. Role of p53 status in lytic activation was also evaluated in *in vivo* gemcitabine-GCV combination therapy. It was found that gemcitabine-GCV combination treatment was effective in SNU-719. To determine how p53 is associated with gemcitabine-induced EBV lytic activation, interaction of p53 with EBV Z promoter (Zp) region and proteins capable of binding to it were investigated. p53 protein binding to ZID region of Zp is verified by ChIP and EBV Zp reporter assays in SNU-719, but not in NCC-24. ZID domain does not have p53 binding site but have Sp1 and MEF2D binding sites. Formation of p53 and Sp1 complex and p53 binding to EBV Zp via Sp1 in gemcitabine-induced lytic activation were shown by IP and ChIP only in SNU-719, but not in Sp1 knock-downed SNU-719 and NCC-24. Therefore, p53 binding ability is a key factor for Zp activation by gemcitabine.

Together, p53 is a key determining factor in gemcitabine-induced EBV lytic

activation and this viral enzyme-targeted anti-tumor strategy may provide a new therapeutic approach for EBVaGCs.

Key words: Epstein-Barr virus, EBV associated gastric carcinoma, gemcitabine, ganciclovir, EBV lytic activation, p53, ataxia telangiectasia-mutated (ATM)

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I. INTRODUCTION

Epstein-Barr virus (EBV) is a linear, double-stranded 172 kb DNA gamma herpes virus which encodes nearly 100 viral genes.[1] EBV is known as an ubiquitous virus because it has been estimated to be present in over 90% of the world's population.[2] Infection of naïve individuals occurs early in childhood and is usually asymptomatic. However, infection in developed countries often occurs following early adolescence and is associated with symptomatic disease presenting with clinical features of infectious mononucleosis (IM).[3]

EBV can establish two types of infection in cells, lytic and latent forms. In the latent infection, its genomic DNA that exists in the nucleus as an episome, is chromatinized with histones and expresses only a few latent genes.[4,5]

According to the gene expression, it was been identified to have at least four latency states.[6] Latency 0 is a state in which EBV-encoded ncRNAs, including EBERs and BARTs, are exclusively transcribed while viral proteins are not expressed.[7,8] This infection status was observed in resting memory B cells and have shown to aid there cells to evade T cell recognition due to the lack of EBV protein expression. However, it is still unknown how viral proteins are regulated in this pattern, as well as the kind of cellular transcription factors involved in the truly latent cells.[9] Latency III is a state of initial infection, in which the virus enters the lymphoid tissue within the pharyngeal lymphoid tissue, where it crosses the surface epithelium to infect naïve B cells, activating them into proliferating blasts.[10] In this stage, EBV expresses six nuclear antigen proteins (EBNA1, 2, 3A, 3B, 3C, and LP), latent infection integral membrane proteins (LMP1 and LMP2A, 2B) in addition to the BARTs, EBERs, and other miRNAs.[11-14] The expressed latent proteins play important roles in regulating cell cycle, cell proliferation and contribute to the oncogenic process.[15] EBV-associated malignancy is exemplified by EBV-infected B lymphocytes proliferating as long-term lymphoblastoid cell lines (LCLs) in cell culture and observed in post-transplant lymphoproliferative disease (PTLD).[9] Latency II is transit of these blasts into the germinal center, which allows B cells to undergo a germinal center reaction and the antigen-driven somatic hypermutation of their immunoglobulin genes.[16] In this latency, EBV expresses EBNA1, LMP1, and LMP2.[17] These two EBV oncogenes, LMP1 and LMP2, mimic key survival and proliferative signals in B cells. LMP1 mimics the constitutively active

CD40 receptor,[18] while LMP2A mimics the signaling through activated B cell Ig receptors according to tyrosine-based activation motif (ITAM).[19] Malignancies associated with this latency are Hodgkin's disease (HD), T and NK lymphoma, nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC).[20] Latency I is characterized by the expression of EBNA1, EBERs, BARTs, and BART microRNAs.[21] EBNA1, the only expressed viral protein in this latent infection, tags the EBV episome to the host chromosome, allowing it to segregate and be retained during cell division.[22] Furthermore, EBNA1 is essential for lymphoma survival by preventing cell death.[23] Burkitt's lymphoma (BL) is an associated disease in this latency. In brief, the EBV latency programs, related gene products and associated malignancies are shown in Table 1.[6,24]

Table 1. EBV latency gene expression pattern and associated malignancies

	EBERs	EBNA1	LMP1, 2	EBNA2,3,LP	Associated malignancies
Latency III	+	+	+	+	PTLD
Latency II	+	+	+		HD, T and NK lymphoma, NPC, GC
Latency I	+	+			BL
Latency 0	+				

PTLD; Post-transplant lymphoproliferative disease, HD; Hodgkin's disease, NPC; Nasopharyngeal carcinoma, GC; Gastric carcinoma, BL; Burkitt's lymphoma

GC is an epithelial malignancy that is frequently associated with EBV. The frequency of EBV infection in GC ranges from 2 to 20%, with a worldwide average of nearly 10%.[25] EBV associated gastric carcinoma (EBVaGC) is a

non-endemic disease that occurs throughout the world, however, similar to EBV positive NPC, EBVaGC shows substantial geographic variation which has been thought to be caused by ethnic and genetic differences.[26,27] The clinical features of EBVaGC include predominance among males and a predominant location in the proximal stomach and remnant stomach after partial gastrectomy for gastric ulcer or gastric carcinoma.[25] In gastric adenocarcinomas EBV may enter the gastric epithelium without the use of a receptor.[12] Direct cell-to-cell contact with B lymphocytes has been considered to be the main model of EBV infection in epithelial cells.[28] EBVaGC was originally proposed to display the same latency II as NPC; however, early studies were unable to demonstrate LMP1 expression, as such, viral gene expression was proposed to more closely represent a latency I, as observed in BL. Occasionally, there was additional expression of LMP2A at low levels.[29,30] In a recent study of EBV, gene expression in EBVaGC LMP1 was detected at a very low level, in addition to LMP2 and other EBV latent genes.[31] Cancer Genome Atlas Research Network characterized the genetics of EBV negative gastric carcinoma (EBVnGC) vs. EBVaGC, and interestingly found distinct mutations and epigenetic profiles in EBVaGC cases. It demonstrated that EBVaGCs have recurrent PIK3CA mutations, high DNA hypermethylation, and also amplification of JAK2, CD274, and PDCD1LG2.[9,32] EBV-associated epithelial malignancies are characterized by rare TP53 mutations[32,33]. Indeed, the TP53 pathway dysregulation is due to TP53 mutations in approximately 70% of all cases of GCs,[34,35] however, TP53 mutations are infrequent in EBVaGC infrequent.[32,35]

Replication of EBV requires lytic induction with the expression of over 80 viral gene products that leads to the production and release of new, infectious viral particles.[36] Lytic replication starts by first transcribing viral immediate-early (IE) lytic genes, BamHI Z fragment leftward open reading frame 1 (BZLF1) and BamHI R fragment leftward open reading frame 1 (BRLF1). They encode the transcription factors, Zta (Z, ZEBRA) and Rta (R), respectively and these two transactivators are both required for lytic DNA replication.[37,38] Lytic induction triggers the temporal and ordered cascade of viral gene expression; early (E) genes, are required for viral DNA replication and nucleotide metabolism, and subsequent expression of late (L) viral genes, which produce the structural components, are required for virion packaging.[36,39] BZLF1 is regulated by Z promoter that contains at least three kinds of important cis-acting motifs: ZI, ZII and ZIII. The ZI motifs are bound by SP1 and MEF2 family transcription factors. The ZII motifs are targeted by various cellular b-Zip type transcription factors such as CREB, ATF, AP-1, and CCATT-enhancer binding protein (C/EBP). The ZIII motif is targeted by BZLF1 itself.[40] Typically, ZI retains an A/T-rich motif and either a GC-rich motif or a CACC motif, which are bound by MEF2[41] and SP1 transcription factor,[42] respectively. ZII is the second cis-acting element essential for activation of Zp in response to inducers; it is located near the TATA box of the Zp region.[43] The ZII motif shares similarity with the AP-1 binding consensus and actually binds c-JUN and c-FOS. In addition to AP-1, other cellular b-Zip type transcription factors, such as CREB, ATF, C/EBP and a spliced form of X-box-binding protein 1,[44-46]

reportedly associate with the ZII motif and enhance BZLF1 transcription.[40] Finally, BZLF1 protein itself can target its own promoter at ZIII motifs. There are two ZIII boxes located adjacent to one another in Zp (ZIIIA and B); ZIIIB reportedly has higher affinity with BZLF1 protein.[47] This positive feedback loop appears to be required for full promoter activation of BZLF1 **(Fig 1)**.[48]

Figure 1. Regulatory elements within the Z promoter (Zp).[49] The diagram shows Z promoter and its interacting transactivators which start lytic replication of EBV.

EBV reactivation can be triggered in human cells by either chemical agents or biological stimuli, including TPA, sodium butyrate, HDAC inhibitors, phorbol esters, calcium ionophores, chemotherapeutic agents, BCR engagement, TGF-β, and hypoxia.[37] EBV encodes a thymidine kinase (TK) and a protein kinase (PK), which was encoded by an early lytic BXLF1 gene and BGLF4 gene, respectively. These kinases can convert the nucleoside analogs 2'-fluoro-2'-deoxy-1-β-D-arabinofuransyl-5-iodo-uracil (FIAU) and ganciclovir (GCV) into cytotoxic drugs that both kill the EBV-positive tumor cells and prevent release of infectious virus from them.[50,51] Since the

phosphorylated form of GCV can also be transferred to nearby tumor cells through gap junctions, activation of GCV phosphorylation in even a small percentage of tumor cells results in “bystander killing” of a much greater percentage of them[37]. The use of kinases from the EBV lytic activation stage in EBV-associated malignancies is called "lytic-induction therapy".[2,52,53] This therapy requires identification of drugs and other treatment modalities that induce viral reactivation in EBV-positive tumor cells without causing unacceptable toxicity to normal cells.[37] Several pharmacological agents are known to induce EBV lytic activation via the endoplasmic reticulum (ER) or genotoxic stress response in EBV-infected cells.[54,55] Genotoxic stress activates ataxia telangiectasia-mutated (ATM) pathway and its downstream target, p53, playing key roles in promoting EBV lytic activation.[56]

p53, a tumor suppressor, is known for its critical role in coordinating a wide-range of cellular stress responses, such as apoptosis, DNA repair, cell-cycle arrest, senescence, and autophagy when cells sense particular stress signals. The critical mechanistic contribution of p53 to this process is that it acts as a signal transducer or a potent transcription regulator, controlling the transactivation of its downstream genes, which encode indispensable factors for each stress response.[57] For instance, p53 transactivates p21 and Bax, which control the initiation of cell-cycle arrest and apoptosis, respectively, leading to the corresponding biological consequences.[58-60] TP53 is mutated or lost in over 50% of human cancers,[61] representing the most commonly mutated gene in human tumors. In unstressed cells, p53 is kept at low levels by its negative regulator

MDM2 (HDM2) through the ubiquitin-dependent proteasome pathway.[62] Upon DNA damage, p53 is phosphorylated to escape from proteasomal degradation,[63] and then is stabilized and activated to function primarily as a transcription factor, consequently leading to cell cycle arrest or apoptosis through the p53-mediated gene expression cascades.[64,65] These cellular outcomes after stresses, including DNA damage, oncogene activation, hypoxia, nucleotide imbalance, and oxidative damage, are tightly linked to p53 dynamics mediated by both the p53 levels and post-translational modifications of p53.[66,67] In viral replication p53 acts as a double-edged sword. For example, in DNA viruses such as polyomaviruses and papillomaviruses, activation of p53 would lead to cell cycle arrest and thus impede viral replication.[68,69] On the other hand, p53 may participate positively in the replication of other viruses by promoting adenoviral replication and production of virions.[70] For herpesviruses, several studies have indicated that p53 is recruited to viral replication compartments, suggesting its possible involvement in viral replication.[71,72] Recently, p53 was also found to be important in permissive human cytomegalovirus (HCMV) replication, probably by affecting the formation of replication foci and hence promoting viral replication.[73]

Recently a clinically applicable new drug, gemcitabine, was identified by a drug repositioning approach.[56] Gemcitabine (2, 2-difluorodeoxycytidine, dFdC; Gemzar®) has been used in various cancer therapeutic regimens[74-76] and also shown to be a lytic inducer with therapeutic potential in EBV-positive B cell lymphoma cell lines and NPC cell lines.[77,78] However, this drug had not been

examined with respect to the precise mechanism of lytic activation in the context of EBVaGC. In this study, I investigated the role of p53 in the gemcitabine-induced EBV lytic activation in EBVaGC and evaluate the feasibility of p53 status as a biomarker for ‘gemcitabine-GCV combination therapy’.

II. MATERIALS AND METHODS

1. Cell lines

EBV-positive gastric carcinoma cell lines, SNU-719 and NCC-24, were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (complete RPMI-1640; HyClone, South Logan, UT, USA). Another EBV-positive gastric carcinoma, YCCEL1, was obtained from the Yonsei Cancer Center at Yonsei University College of Medicine (Seoul, Korea). YCCEL1 cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids (Gibco, Grand Island, NY, USA) at 37ºC in a humidified CO_2 incubator.

2. Chemical reagents and siRNAs

Gemcitabine (Eli Lilly, Indianapolis, IN, USA), ganciclovir (Sigma-Aldrich, St Louis, MO, USA), were used. The siRNAs targeting *TP53* (NM_000546) and *SP1* (NM_138473) were designed and generated (Integrated DNA Technologies; IDT, Coralville, IA, USA). The target sequences of the selected siRNAs are shown in Table 2. The siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Table 2. siRNA sequences used to target

Target	siRNA sequence
TP53	CCA CCA UCC ACU ACA ACU ACA UGT G
SP1	GUU CUG CCA GCU UGG UAU CAU CAC A

3. Induction of EBV lytic activation in EBVaGCs

Cells were treated with the indicated doses of gemcitabine (0 – 200 ng/ml). Briefly, Cells were incubated with gemcitabine for 24 hr, then washed with PBS, followed by additional culture for 48 hr. Expression of EBV lytic genes or proteins were checked in gemcitabine treated cells.

4. Immunofluorescence assay

Cells were fixed in cold 4% paraformaldehyde for 30 min and blocked with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hr at room temperature. Cells were stained with anti-Zta or anti-gp350 Ab (gift from Professor Song, Y. J., Gachon University, Gyeonggi-do, Korea) and Rhodamine Red-X-AffiniPure donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch), and then visualized by LSM 700 confocal microscopy (Cal Zeiss, Oberkochen, Germany). In addition, 4, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) staining was also performed to visualize cell nuclei.

5. Western blot analysis

Cells were lysed with RIPA buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Samples were separated on a 10% SDS-PAGE gel and transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked in TBS containing 5% non-fat milk and 0.05% Tween 20 solution and incubated with primary antibody. The following antibodies were used for western blot: anti-Zta (Santa Cruz Biotechnology, Dallas, TX, USA), anti-p53 (Do-1; Santa Cruz Biotechnology), anti-p53 pSer15 (Cell Signaling Technology, Beverly, MA, USA), anti-ATM (Cell Signaling Technology), anti-ATM pSer1981 (Cell Signaling Technology), anti-GRP78 (Santa Cruz Biotechnology), anti-CHOP (Santa Cruz Biotechnology), anti-Flag (Sigma-Aldrich), anti-Sp1 (EMD Millipore, Temecula, CA, USA) and anti-β-actin (Sigma-Aldrich).

6. Reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen) and RNase-free DNase (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. The cDNA was synthesized using 1 μg total RNA, SuperScript III reverse transcriptase (Invitrogen) and oligo dT primers (Invitrogen). Quantitative RT-PCR was performed on a StepOne-plus™ Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). The specific primer pairs are shown in Table 3. GAPDH expression was used as an internal control. Without reverse

transcriptase was performed to rule out contamination by EBV genomic DNA.

Table 3. The primer sequences for real time PCR

Gene names		Sequences
BZLF1	Forward	ACC AAG CCG GGG GAG AAG CA
	Reverse	CCA GGC TTG GGC ACA TCT GC
BGLF4	Forward	CGC TCG GCT ACT CGC TGC TC
	Reverse	CGG AGG AAG CGG GCA AAC GT
BXLF1	Forward	TTA CCC TGC CCA GGG GAG CC
	Reverse	GTC ATC GAG CCC AAG GCC GG
GAPDH	Forward	GAT GGC ATG GAC TGT GGT CA
	Reverse	GCA ATG CCT CCT GCA CCA CC
Zp -221-80 (for ChIP)	Forward	CCA TGC ATA TTT CAA CTG GGC
	Reverse	TGA TGT CAT GGT TTG GGA CGT G

7. [^{125}I] FIAU cellular uptake assay

Cells, which were induced into lytic activation as described, were incubated with 1 μCi/2 ml [^{125}I] FIAU at 37ºC for 4 hr. Then cells were washed and harvested with 200 μl trypsin-EDTA (0.25%, Thermo Fisher Scientific Inc., Rockford, IL, USA), followed by addition of 400 μl PBS. The radioactivity was measured using a γ-counter (Perkin Elmer, Waltham, MA, USA). The accumulation of [^{125}I] FIAU was calculated as the percentage of the input dose added to the medium (%AD).

8. Cell viability assay

Cell viability was analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo Lab, Kumamoto, Japan). SNU-719 (3×10^3 cells/100 μl) and MKN-74 (2×10^3 cells/100 μl) cells were plated in 96-well plates and incubated at 37ºC in 5% CO_2 overnight. Then, the cells were treated as described in Figure 4D. Then, 10 μl CCK-8 solution (2-[2-methoxy- 4-nitrophenyl]-3-[4-nitrophenyl]-5-[2, 4-disulfophenyl]-2H-tetrazolium) was added to each well. The plates were incubated at 37ºC for 3 hr, and the absorbance at 450 nm was measured using a microplate reader (Perkin Elmer).

9. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed essentially as described. [79] Cells were fixed with 1% formaldehyde (Sigma-Aldrich) for 20 min at room temperature. The cells were then washed with cold PBS 3 times. Cells were double cross-linked with EGS (ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester), Thermo Fisher Science)[80] for 1 hr at room temperature and washed with cold PBS 3 times. Cells were pelleted and lysed in lysis buffer (5 mM PIPES [pH 8.0], 85 mM KCl, 0.5% NP40, and 1× protease inhibitor cocktail (Gene Depot, Barker, TX, USA)) for 10 min on ice. Nuclei were pelleted and lysed in nuclei lysis buffer (10 mM Tris-HCl [pH 8.0], 2 mM EDTA, 0.2% SDS, and 1× protease inhibitor cocktail). Chromatin was sheared using Covaris M220 (Covaris, Woburn, MA, USA) until the average DNA fragment was ~400 bp and centrifuged at 14000 rpm for 15 min at 4°C.

Supernatant was collected and resuspended in sonication buffer to a final volume of 700 μl; samples were precleared by incubating with 25 μg/ml salmon sperm DNA (Invitrogen), 100 μg/ml BSA, and 40 μl of protein A/G magnetic beads (Dynal, Lake Success, NY, USA) for 1 hr at 4 °C with rotation. Immunoprecipitations were performed with anti-p53, anti-Sp1 and control IgG (sc-2027; Santa Cruz Biotechnology) at 4°C overnight with rotation. Immune complexes were harvested with magnetic beads, and washed twice for 5 min in each of the following buffers: 1× RIPA-140 buffer (0.1 % SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0], and 140 mM NaCl), 1× RIPA-300 buffer (0.1% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0], 300 mM NaCl, and 1% deoxycholic acid), and LiCl buffer (0.25 M LiCl, 0.5 % NP40, 0.5% deoxycholic acid, 10 mM Tris-HCl [pH 8.0], and 1 mM EDTA), followed by a final wash with TE (10 mM Tris-HCl [pH 7.4] and 0.5 mM EDTA). Immune complexes were disrupted with elution buffer (100 mM $NaHCO_3$ and 1% SDS), and covalent links were reversed by adding 300 mM of NaCl and 400 μg of RNase A (QIAGEN) at 37°C for 30 min. Then, 100 μg of proteinase K (New England Biolabs, Ipswich, MA, USA) were added and incubated at 65°C for overnight. DNA was eluted by MinElute PCR purification kit (QIAGEN). DNA was dissolved in 40 μl water and used as template for PCR. The specific primer pairs are shown in Table 3.

10. Plasmid construction and transient transfections

Flag tagged WT-TP53 was constructed by several subcloning steps. Wild-type TP53 was amplified by PCR using pcDNA3-p53 plasmid (gift from professor Kim, J. S., Korea Institute of Radiological and Medical Sciences, Seoul, Korea) as a template. The PCR primer sequences were as follows: 5'-CCG GAA TTC TAT GGA GGA GCC GCA GTC A-3', 5'- CGC GGA TCC TCA GTC TGA GTC AGG CCC TTC-3'. The amplified fragments were gel purified, digested (*EcoR*I and *Bam*HI) cloned into Flag tagged Myc-CMV-26 (Sigma-Aldrich).

A luciferase reporter plasmid driven by Zp (-221 to +12), which was generated by gene synthesis service (IDT), was constructed in the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA).

The inserted DNA sequence of each vector was confirmed by direct DNA sequencing.

Plasmid DNA transfection performed with Lipofectamine™ 2000 or 3000 (Invitrogen) according to manufacturer's instructions.

11. Promoter luciferase assay

Cells were transfected with EBV Zp luciferase reporter constructs including pRL-SV40 (Promega) as a transfection control. Twenty-four hours after transfection, cells were lysed with 1x passive lysis buffer (Promega). The luciferase activities were measured using the Dual-Luciferase™ Reporter Assay System (Promega) and a VICTOR™ X4 luminometer

(Perkin Elmer), and were analyzed based on the ratio of Firefly (luciferase constructs): Renilla (pRL-SV40 vector) and normalized to the cell number and transfection efficiency.

12. Co-immunoprecipitation (Co-IP) assay

Nuclear extracts from SNU-719 and NCC-24 cells cultured in the presence or absence of gemcitabine for 24 hr were isolated using a Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) following the manufacturer's instructions. Nuclear extracts were precleared by incubating with 30 μl of protein G magnetic beads (Dynal) for 1 hr at 4°C with rotation. Protein G magnetic beads were pre-coupled with anti-p53, anti-Sp1, mouse IgG or rabbit IgG (EDM Millipore) for 1 hr at room temperature with rotation. The beads were washed three times with washing buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, and 10 % NP-40), added to nuclear extracts and incubated overnight at 4°C with rotation. The beads were washed three times with washing buffer and were resuspended in 2X sodium dodecyl sulfate (SDS)-polyacrylamide gel loading buffer, incubated at 100°C for 10 min. Lysed beads were placed on magnet and transferred supernatant to a clean tube and analyzed by Western blotting.

13. Generating EBV-positive or -negative GC cell line-implanted mice and gemcitabine-GCV combination treatment

All mice were maintained under specific pathogen-free conditions, and the experiments involving animals were approved by the Institutional Animal Care and Use Committees (IACUC) at Yonsei University College of Medicine (2015-0131). Six to seven-weeks-old female NOD-SCID mice (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) were used for all experiments. SNU-719, MKN-74 or NCC-24 cells were suspended in a 200 μl solution that containing 100 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and 100 μl PBS and then injected subcutaneously into the right or left flank of mice. Approximately 3 wks after implantation of SNU-719, MKN-74 or NCC-24 cells, mice carrying tumors that reached a volume of approximately 1,000 mm^3, were used for *in vivo* experiments. The mice were treated with the indicated dose of gemcitabine or GCV as schedule described in Figure 6A. Tumor size was measured approximately every third day with calipers, and tumor volume was calculated as $l \times w^2$ (l: long axis, w: width).

14. Molecular imaging of *in vivo* lytic activation

SNU-719 and MKN-74 implantation mice were treated with gemcitabine once, and 200 μCi [^{125}I] FIAU was administered to each tumor-carrying mouse through the tail vein. The mice were imaged by [^{125}I] FIAU-based SPECT planar imaging as previously described.[81] Briefly, the mice were placed in a posterior position on a warm-bed and anesthetized with 2% isoflurane (Choongwae, Seoul, Korea) before injection of [^{125}I] FIAU and 1.5% isoflurane during imaging. For thyroid-blocked images, 1 ㎎ sodium perchlorate (Sigma- Aldrich) was injected intraperitoneally into the mice before injection of [^{125}I] FIAU.

15. Statistical analysis

Statistical analysis was performed with unpaired Student's *t*-tests using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). All *in vitro* experiments were performed for at least three times. Values represent means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

III. RESULTS

1. The expression of EBV-TK during gemcitabine-induced lytic activation in SNU-719 cells

To identify new chemical reagents that could induce lytic activation in EBVaGCs, high-throughput screening of the Johns Hopkins Drug Library was performed using EBV BZLF1 promoter-transfected human gastric carcinoma (AGS) cells[82]. Among 2,687 drugs, 188 candidates showing significant increased luciferase activity when compared with the control were discovered, then validation experiments were performed with the top 15% candidates. Gemcitabine was finally identified as an ideal candidate for further evaluation. Treatment of an EBVaGC cell line, SNU-719, and an EBV-negative gastric cancer (EBVnGC) cell line, MKN-74, with gemcitabine as scheduled in **Figure 2A** revealed that the EBV IE lytic protein Zta was induced in SNU-719 cells even at a low dose (5 ng/ml; **Fig. 2B**), which was confirmed by immunofluorescence assay (IFA) (**Fig. 2C**). This effect was observed 48 hr after gemcitabine treatment (**Fig. 2D and 2E**). To determine whether the low dose of gemcitabine may efficiently induce other lytic genes, *BGLF4* (EBV-PK) and *BXLF1* (EBV-TK), RT-PCR was performed. These genes exhibited a similar expression pattern to that of *BZLF1,* which encodes the Zta (**Fig. 2F**). Additionally, a component of capsid gp350 was detected only in lytic activation-induced SNU-719 cells (**Fig. 2G**).

Recently, it was reported that the ER or genotoxic stress response is associated with EBV lytic activation.[55,83] Moreover, the ATM kinase/p53

pathway is activated during genotoxic stress-induced EBV lytic activation.[55] Therefore I first screened for the involvement of the ER stress response during gemcitabine-induced lytic activation, but C/EBP-homologous protein (CHOP) and glucose-regulated protein-78 (GRP78), which are known ER stress markers, exhibited no differences between SNU-719 and MKN-74 cells following gemcitabine treatment (**Fig. 3A and 3B**). Next, I evaluated lytic activation in the context of ATM/p53 activation. SNU-719 cells have wild-type *TP53,*[84] yielding an intact ATM/p53 pathway. Serine 1981 of ATM was phosphorylated 3 hr after gemcitabine treatment, and serine 15 of p53 was phosphorylated subsequently (**Fig. 3C**). Phosphorylated p53 was decreased following treatment with the ATM inhibitor KU55933 (**Fig. 3D**), which may have suppressed Zta expression as previously reported.[55] To further evaluate the involvement of the ATM/p53 pathway in lytic activation, I performed siRNA-based knock-down experiments. Phosphorylation of p53 was decreased by si-*ATM*, resulting in a decrease of Zta protein expression (**Fig. 3E**). Moreover, this finding was confirmed by si-*TP53* (**Fig. 3F**). Collectively, these results suggest that gemcitabine induces lytic activation via the ATM/p53-mediated genotoxic stress pathway in SNU-719 cells.

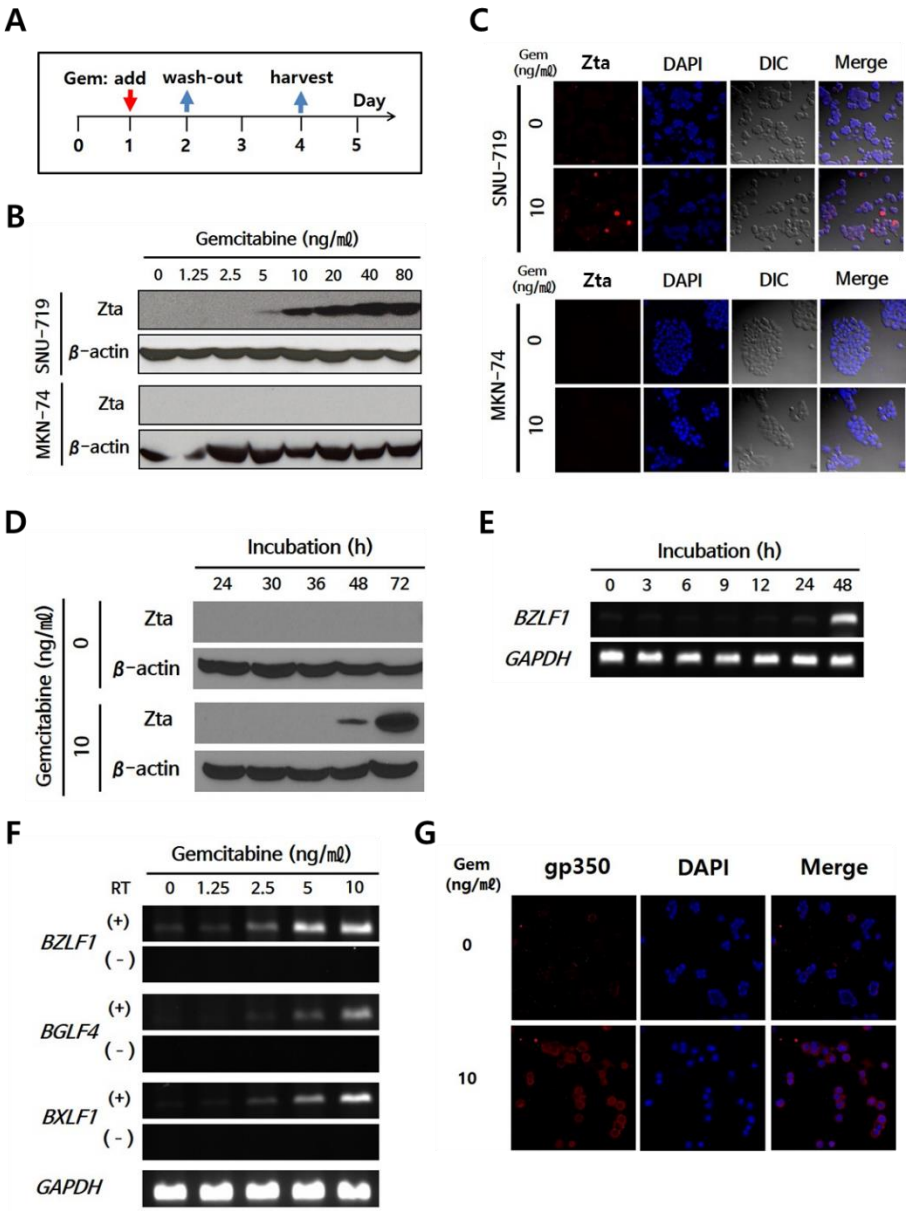


Figure 2. Expression of EBV-TK/PK during gemcitabine-induced lytic activation in EBVaGC cells. (A) EBVaGC cells (SNU-719) or EBVnGC cells (MKN-74) were treated with gemcitabine (0 - 80 ng/ml) for 24 hr and washed, then were cultured for another 2 days. (B) Zta expression was evaluated by western blot and (C) IFA. Blue, DAPI; Red, Zta. SNU-719 cells were treated with 10 ng/ml gemcitabine and then harvested after the indicated incubation times. (D) Western blot or (E) RT-PCR was performed to visualize Zta expression. (F) RT-PCR for BZLF1, BGLF4, and BXLF1 was performed on gemcitabine (0 - 10 ng/ml)-treated SNU-719 cells. RT (-) lane is a negative control to determine contamination by EBV genomic DNA. (G) gp350 was visualized by IFA in gemcitabine (10 ng/ml)-treated SNU-719 cells. Blue, DAPI; Red, gp350. β-actin and GAPDH was used as a loading control for western blot and RT-PCR, respectively.

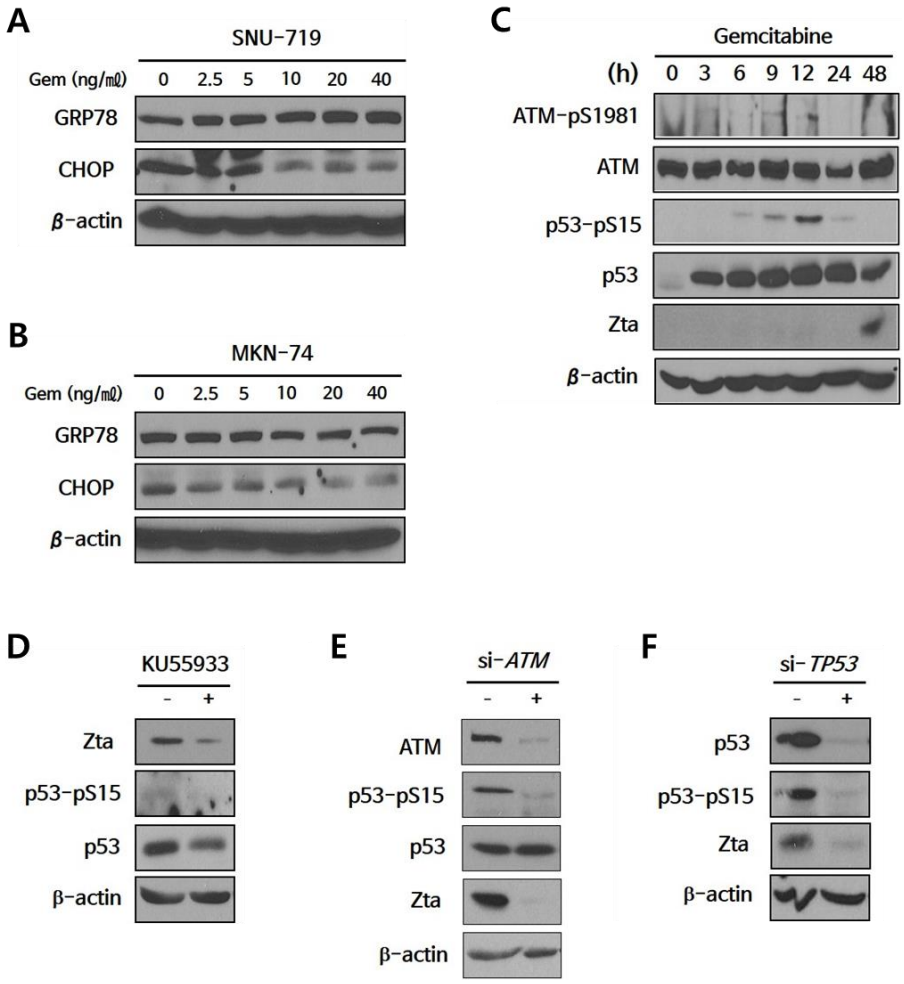


Figure 3. Gemcitabine-induced EBV lytic activation via ATM/p53 genotoxic stress pathway in EBVaGC cells. (A) SNU-719 and (B) MKN-74 cells were treated with gemcitabine (0 - 40 ng/ml). Western blot was performed to visualize GRP78 and CHOP expression. (C) Changes in phosphorylated ATM (pSer 1981) and p53 (pSer 15) during gemcitabine treatment were evaluated by western blot. (D - F) An ATM inhibitor (KU55933) was treated twenty-four hours after gemcitabine treatment, while si-*ATM* and si-*TP53* were transfected twenty-four hours before gemcitabine treatment. Inhibition of lytic activation by KU55933, si-*ATM*, or si-*TP53* was evaluated by changes in Zta, ATM, phosphorylated ATM, p53, and phosphorylated p53 using western blot. β-actin was used as a loading control.

2. Gemcitabine confers GCV susceptibility on EBVaGC cells

To confirm the induction of EBV-TK/PK by gemcitabine, enzymatic activity was measured using the radio-isotope labeled-nucleoside analogue, [^{125}I] FIAU.[85] Cellular accumulation of [^{125}I] FIAU showed a positive correlation with the dose of gemcitabine in SNU-719 cells but not in MKN-74 cells (**Fig. 4A**).

I determined the concentration of gemcitabine that induced lytic activation while minimizing cell death, as gemcitabine is currently used as a chemotherapeutic drug in various kinds of cancers.[74-76,86] The inhibition of cell proliferation by 50% (IC_{50}) for MKN-74 cells was 2.2 - 3.8 ng/ml, which is similar to the concentration previously reported,[75] while that of SNU-719 cells (8.4 - 16.8 ng/ml) was slightly higher (**Fig. 4B**). Thus, the induction of lytic activation in SNU-719 cells by 5 ng/ml gemcitabine occurred at a level below the IC_{50}. To establish a combination treatment protocol with gemcitabine and GCV, I first treated cells with GCV alone. There was little difference in the response of SNU-719 and MKN-74 cells to GCV treatment, and GCV had little influence on both cell types even at a relatively high concentration (100 μg/ml; **Fig. 4C**).

The cytotoxicity of the combination treatment was evaluated under an optimized schedule as described in **Figure 4D**. This schedule was based on the toxicity and short half-life (8 - 17 min) of gemcitabine.[87] Gemcitabine conferred cytotoxicity on GCV in SNU-719 cells (**Fig. 4E)** but not in MKN-74 cells (**Fig. 4F**).

GCV worked more efficiently in concert with the low concentration of gemcitabine. That is, the decrease in cell survival was more profound at 0.1 - 10 ng/ml than at 10 - 100 ng/ml. GCV did not exert an additional effect at gemcitabine concentrations of 1 μg/ml. GCV induced maximum efficacy when 100 μg/㎖ GCV was combined with lytic activation-inducible concentrations (1 - 100 ng/㎖) of gemcitabine *in vitro*. Taken together, a low dose of gemcitabine combined with GCV which is below the IC_{50} is an efficacious combination treatment option in SNU-719 cells.

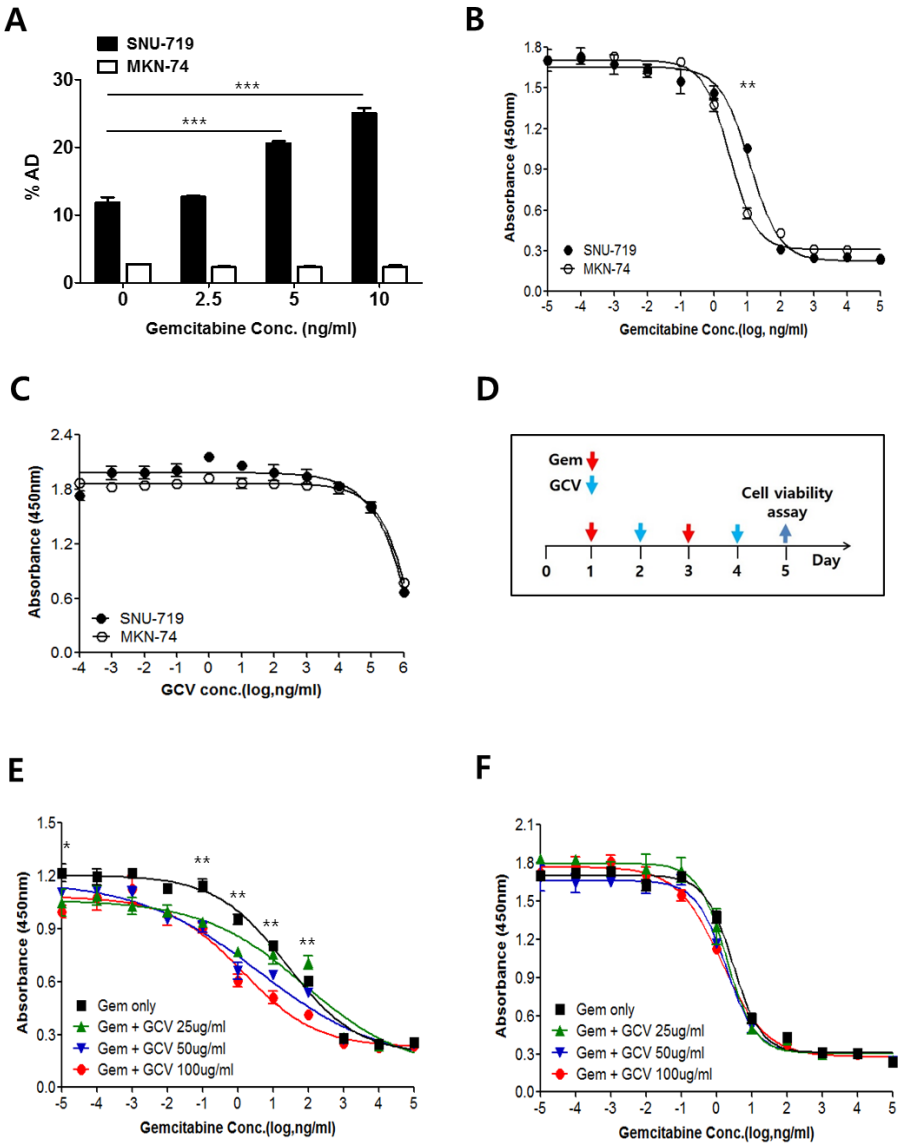
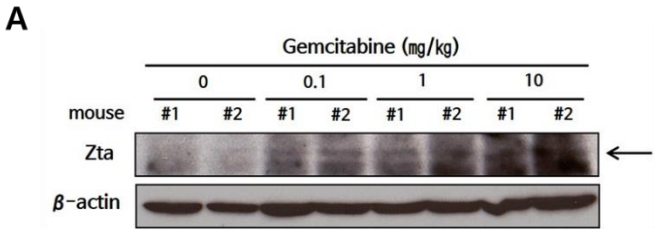
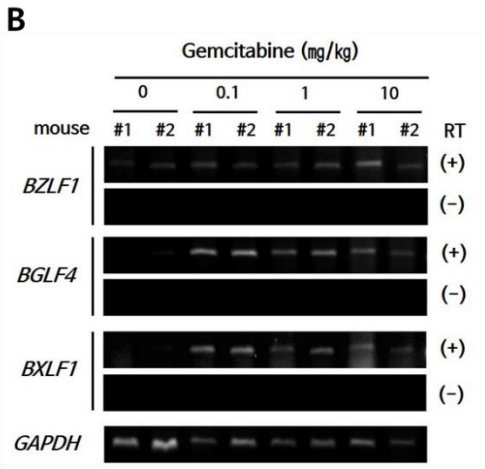


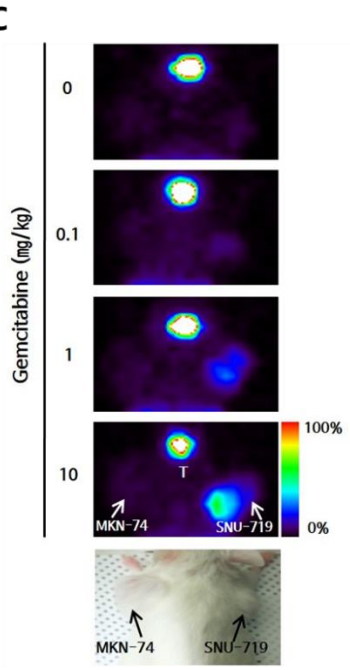
Figure 4. Gemcitabine confers GCV susceptibility on EBVaGC cells. (A) Gemcitabine-treated SNU-719 or MKN-74 cells were incubated with 1 μCi/2 ml of [^{125}I] FIAU for 4 hr. The radioactivity of harvested cells was determined by a γ-counter. SNU-719 and MKN-74 cells were treated with either (B) gemcitabine or (C) GCV for 4 days. Then the viable cells were determined by the CCK-8 assay. (D) Experiment schedule of gemcitabine and GCV treatment and cell viability assay. Dose response of (E) SNU-719 cells and (F) MKN-74 cells to gemcitabine. Serially diluted gemcitabine and indicated doses of GCV were added as administration schedule. The 95% confidence intervals of the slopes, which were determined using best-fit four-parameter regression, are shown. The statistical analysis was performed between Gem only and Gem + 100 μg/ml GCV. Values represent means ± SEM. *P<0.05, **P<0.01, ***P<0.001.

3. Establishment of a lytic activation-inducible SNU-719 cell-implanted NOD-SCID mouse model

An EBVaGC animal model system is indispensable for *in vivo* evaluation of the gemcitabine-GCV combination treatment. Therefore, I developed a mouse model for this purpose using NOD-SCID. SNU-719 cell-implanted mice developed measurable tumors in 40-45 days after implantation. Then, I tested whether gemcitabine induced functional EBV lytic proteins in this mouse model. The gemcitabine concentration commonly used in cancer therapy for a human is 20-60 μM (*i.e.,* 5.2 - 15.6 μg/ml) in plasma, and such levels are achieved by infusion of a drug at a dose of 1,000-1,200 mg/m^2.[87] In the mouse model, gemcitabine was used with a much lower dose than used in cancer therapy, and induced Zta in SNU-719 cell-implanted tumors (referred to as SNU-719 tumors) in a dose-dependent manner (**Fig. 5A**). *BGLF4* and *BXLF1* were also induced in SNU-719 tumors (**Fig. 5B**) as observed in the *in vitro* system. I used [^{125}I] FIAU-based single-photon emission computed tomography (SPECT) planar imaging to confirm the induction of functional EBV-TK/PK in SNU-719 tumors. The intensity of the [^{125}I] FIAU signal showed a positive correlation with the dose of gemcitabine, whereas this signal was not detected in MKN-74 cell-implanted tumors (referred to as MKN-74 tumors) (**Fig. 5C**). These were also observed by imaging of isolated tumors. As a result, gemcitabine induced EBV lytic activation was observed in SNU-719 tumor mouse model but not in MKN-74 tumor (**Fig. 5D**).







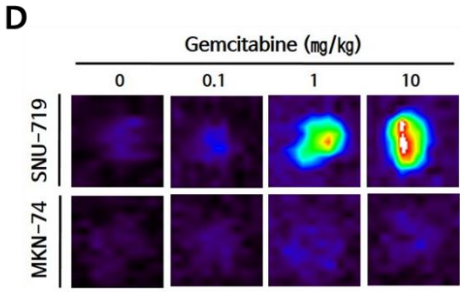


Figure 5. Establishment of a lytic activation-inducible EBVaGC mouse model. (A) Western blot and (B) RT-PCR of EBV lytic genes in isolated tumors from gemcitabine-treated mice. β-actin or *GAPDH* was used as a loading control. (C-D) Tumor cell-engrafted mice were injected with gemcitabine (0.1, 1, or 10 mg/kg) and administrated 200 μCi [^{125}I] FIAU 24 hr after drug injection. The mice (C) or isolated tumors (D) were imaged using SPECT. Color bar indicates the range of [^{125}I] FIAU uptake as a percentage. T, Thyroid.

4. Gemcitabine-GCV combination treatment is efficient in the EBVaGC mouse model

The ability of gemcitabine to induce EBV-TK/PK in the mouse model system led to test for the combination treatment of gemcitabine with GCV. Approximately $1x10^7$ SNU-719 cells or $2x10^6$ MKN-74 cells were engrafted subcutaneously in the right flank of NOD-SCID mice. When the tumors reached approximately 1,000 mm^3, the mice were treated with the indicated dose of gemcitabine or GCV as scheduled (**Fig. 6A**). A dose of 10 mg/kg gemcitabine and 25 mg/kg GCV had little influence on tumor growth in MKN-74 cell-engrafted mice; however, the same dose of gemcitabine, alone or in combination with GCV, resulted in gradual regression of tumors until the tumors were no longer palpable in SNU-719 cell-engrafted mice (**Fig. 6B and 6C**). Mice treated with gemcitabine alone and the gemcitabine-GCV combination lost body weight severely, which led to the discontinuation of the experiment after 3 cycles in accordance with IACUC guidelines (**Fig. 6D and 6E**). Therefore, I sought to identify a gemcitabine concentration that produced synergism with GCV without inducing adverse effects. The combination of 0.5 mg/kg gemcitabine and 25 mg/kg GCV with the same schedule suppressed the growth of tumors significantly (**Fig. 7A**), accompanied by a significant but tolerable body weight loss (**Fig. 7B**).

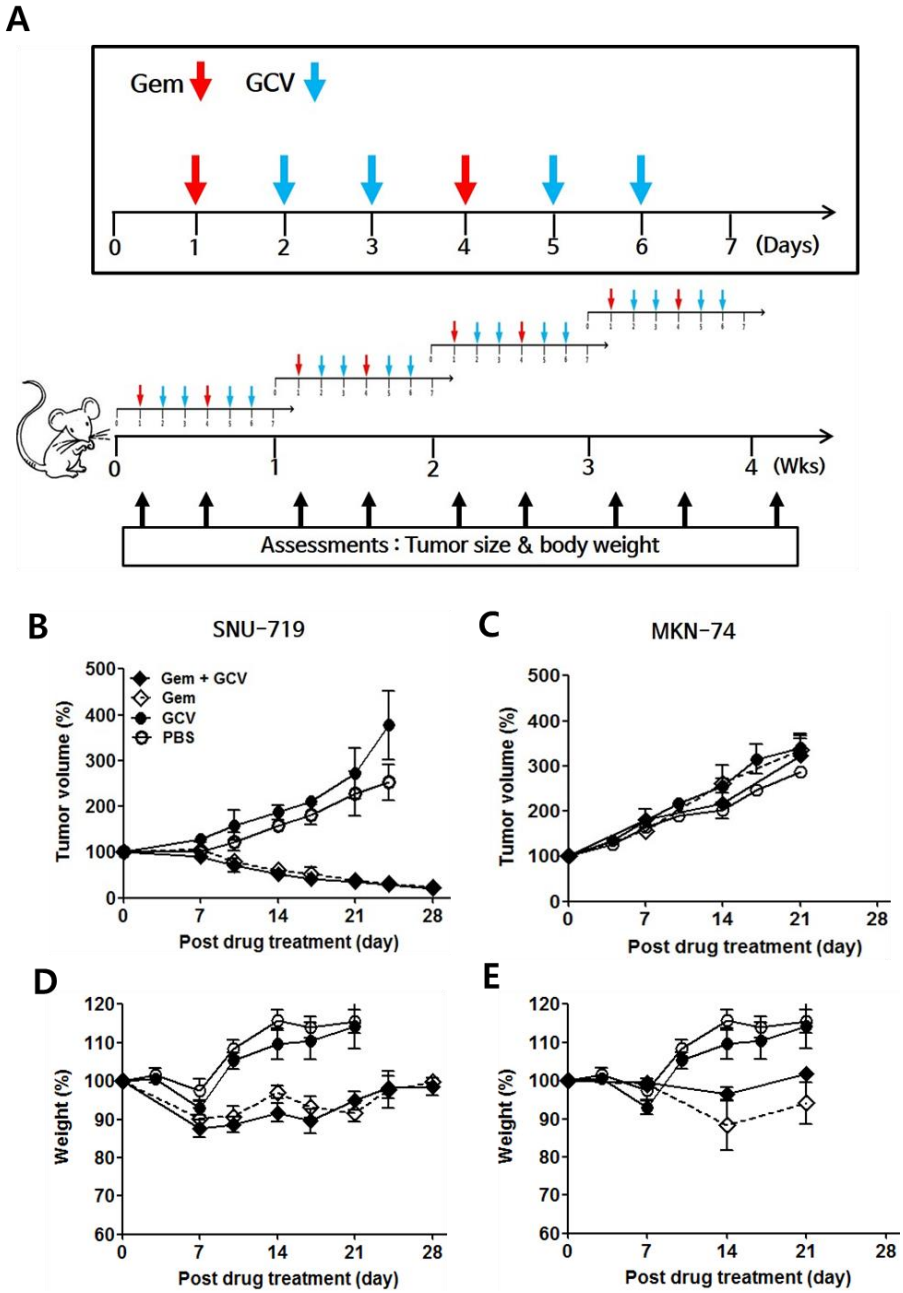


Figure 6. Gemcitabine-GCV combination treatment in EBVaGC cell-implanted mice. (A) Gemcitabine/GCV administration and experiment schedule. (B) SNU-719 or (C) MKN-74 cells implanted mice were injected intraperitoneally with 10 mg/kg gemcitabine, 25 mg/kg GCV, or both according to the schedule shown in the above. Body weight in (D) SNU-719- or (E) MKN-74-implanted mice injected intraperitoneally with 10 mg/kg gemcitabine, 25 mg/kg GCV, or both according to the schedule shown as above. Tumor mass and body weight were measured before every gemcitabine injection. Each data point reflects observations from five mice. Both values were calculated as the percentage of the initial value for each mouse. Values represent means ± SEM.

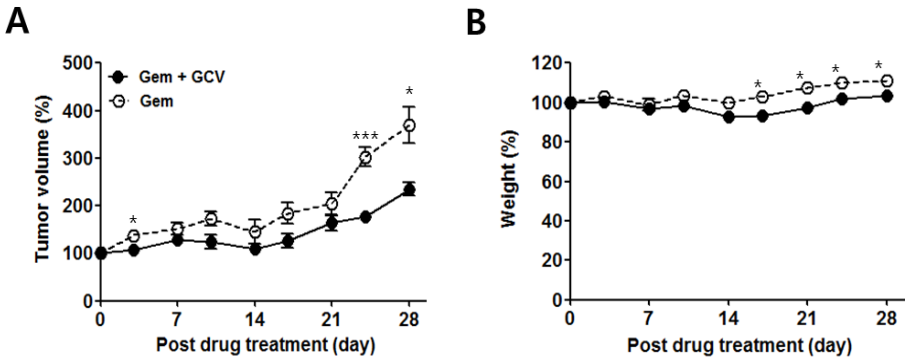


Figure 7. Optimization of gemcitabine-GCV combination treatment in EBVaGC mice. Mice were injected with 0.5 mg/kg gemcitabine alone or 25 mg/kg GCV in combination with 0.5 mg/kg gemcitabine as scheduled in Figure 6A. (A) Tumor mass and (B) body weight were measured before every gemcitabine injection. Each data point reflects observations obtained from five mice. Both values were calculated as the percentage of the initial value for each mouse. Values represent means ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

5. p53 is a determinant of gemcitabine-induced EBV lytic activation in EBVaGCs

To date, three naturally EBV infected gastric carcinoma cell lines have been reported, such as SNU-719, YCCEL1 and NCC-24. I examined the p53 status of SNU-719, YCCEL1 and NCC-24 cell lines and found that SNU-719 and YCCEL1 have wild-type p53. But, NCC-24 have two missense mutations from glycine (GGA) to arginine (AGA) at codon 266 and arginine (CGT) to histidine (CAT) at codon 273 (**Table 4**).[88]

Table 4. p53 status in EBVaGC cell lines

Cell line	EBV	P53	Amino-acid change
SNU-719	Positive	Wild type	-
YCCEL1	Positive	Wild type	-
NCC-24	Positive	Mutant	Gly266Arg/Arg273His

To determine whether gemcitabine-induced EBV lytic activation occurred in EBVaGC cell lines other than SNU-719, various concentrations of gemcitabine were treated with YCCEL1 and NCC-24 (**Fig. 8A**). As a result, Zta expression was increased by activation of p53-pS15 according to gemcitabine concentration, and EBV lytic gene expression was decreased when transfected with p53 siRNA in YCCEL1 cell line (**Fig. 8B and 8C**). On the other hand, Zta expression was not observed in NCC-24 even under treatment with a higher concentration of gemcitabine (**Fig. 8A**). To determine whether wild type *TP53* affect EBV lytic activation in NCC-24, I tested the

expression of Zta and other EBV lytic genes after transfecting with 3xFlag-WT-*TP53* or its empty vectors as a control. NCC-24 cells transfected with wild type *TP53* resulted in the induction of EBV lytic activation by gemcitabine (**Fig. 8D and 8E**). Thus, gemcitabine-induced EBV lytic activation occurs not only in SNU-719 but also in YCCEL1, yet, not in p53 mutated NCC-24, confirming the importance of p53 status in lytic activation.

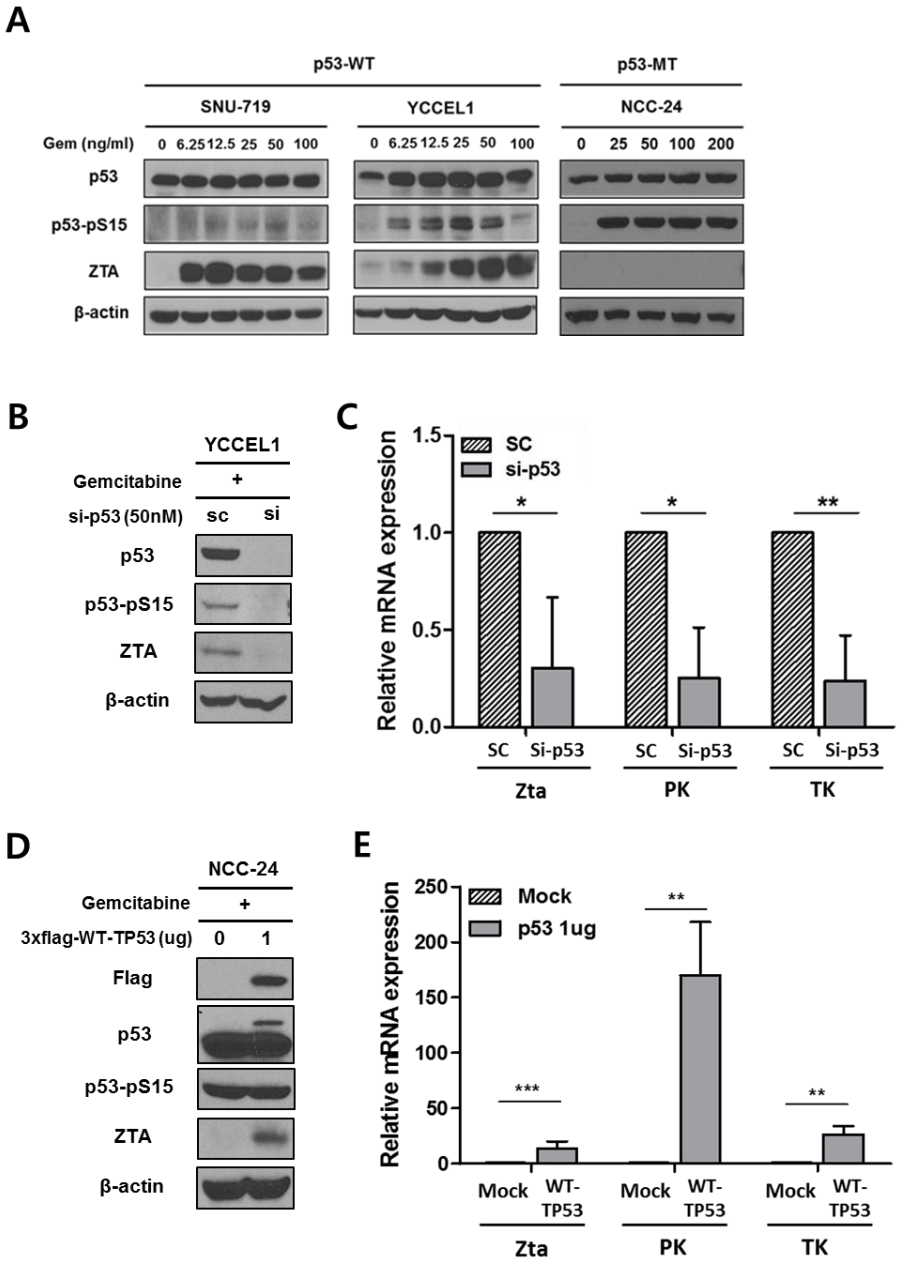


Figure 8. p53 is a determinant of gemcitabine-induced EBV lytic activation in EBVaGCs. (A) EBVaGC cells (SNU-719, YCCEL1, and NCC-24) were treated with gemcitabine (0 - 200 ng/ml for 24 hr and were cultured for another 2 days. Zta expression was evaluated by western blot. (B, C) YCCEL1 cells were transfected with si-*TP53* before gemcitabine treatment. Inhibition of lytic activation by si-*TP53* was evaluated by changes in Zta, p53, and phosphorylated p53 using western blot and expression of lytic genes including *BZLF1*, *BGLF4*, and *BXLF1* using quantitative RT-PCR. (D, E) NCC-24 cells were transfected with 3xFlag-WT-TP53 construct before gemcitabine treatment. Induction of lytic activation by wild-type *TP53* was evaluated by changes in Flag, Zta, p53, and phosphorylated p53 using western blot and expression of lytic genes including *BZLF1*, *BGLF4*, and *BXLF1* using quantitative RT-PCR. β-actin was used as a loading control. Values represent means ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

6. p53 is important to *in vivo* EBV lytic activation

To confirm that the importance of p53 in *in vivo* mouse model, I engrafted SNU-719 and NCC-24 cells subcutaneously in the right and left flank of NOD-SCID mice. When the tumors reached approximately 1,000 mm^3, the mice were treated with the indicated dose of gemcitabine or GCV as scheduled (**Fig. 9A**). In SNU-719 tumors, the GCV combination group showed a gradual decrease in tumor size when compared with gemcitabine alone and gemcitabine combination with GCV (**Fig. 9B**). However, in the case of NCC-24 tumors, the regression effect on tumor was not observed either in gemcitabine alone or GCV combination group (**Fig. 9C**). This confirmed the importance of p53 status in gemcitabine-GCV combination therapy.

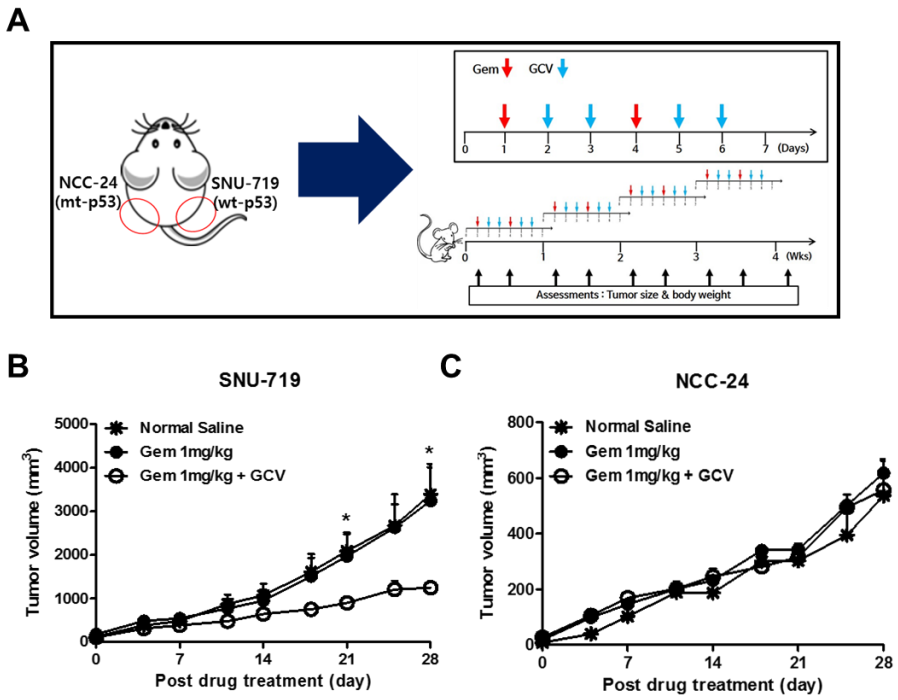


Figure 9. Importance of p53 *in vivo* EBV lytic reactivation. (A) SNU-719 and NCC-24 cells were engrafted subcutaneously in the right and left flank of NOD-SCID mice. Gemcitabine/GCV was administrated as shown above. (B) SNU-719 or (C) NCC-24 tumor bearing mice were injected intraperitoneally with 1 mg/kg gemcitabine, 25 mg/kg GCV, or both according to the schedule shown in the above. A tumor mass was measured before every gemcitabine injection. Each data point reflects observations from five mice. Values represent means ± SEM. $^{*}P<0.05$.

7. p53 binds to the EBV Z promoter

I confirmed that the p53 pathway is important in gemcitabine-induced EBV lytic activation. Next, I determined how p53 regulates EBV lytic activation in EBVaGC. I hypothesized that the p53 will play a key role in the regulation of the Z promoter by gemcitabine. Therefore, to determine the potential p53 binding to Z promoter (Zp) after EBV lytic activation in SNU-719 and NCC-24, ChIP was performed. In SNU-719, the binding of p53 to Zp increased after gemcitabine treatment. (**Fig. 10A**). However, NCC-24, which has mutations in the DNA-binding domain region of p53, did not show the binding of p53 to Zp after gemcitabine treatment. (**Fig. 10B**). These observations indicate that p53 protein interacts with Zp via its DNA-binding ability during EBV reactivation.

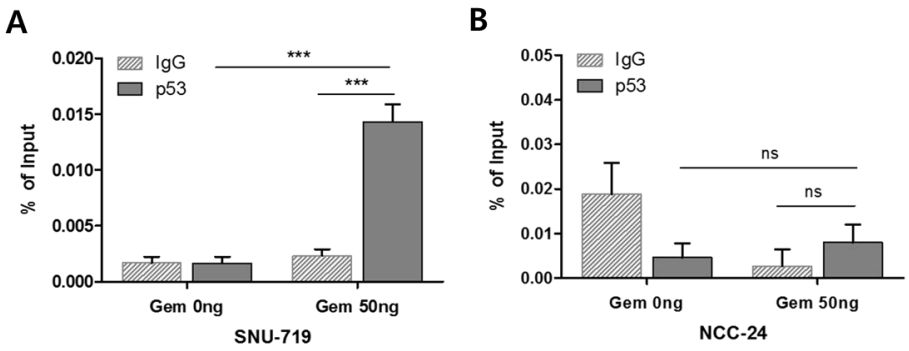


Figure 10. p53 binds to the EBV Z promoter. (A) SNU-719 and (B) NCC-24 cells were incubated for 24 hr with or without gemcitabine 50 ng/ml prior to processing for ChIP analysis. Quantitative-PCR analyses of the chromatin obtained from the cells following precipitation with anti-p53 or anti-IgG antibodies were done. The results are expressed as % of input ± SEM. $^{***}P<0.001$.

8. ZID domain of Z promoter is important to p53 binding

To understand how p53 activation induces transcriptional activation of Zp, I asked what regions of Zp are needed for it to occur. To identify the possible p53 binding region on Zp, a series of Z promoter deletion constructs were expressed in an EBVnGC cells, AGS cells. The Zp activity was observed and found to have significantly decreased in the -80 Zp construct in which the ZIA to ZID domain was deleted when compared with the -221 Zp construct (**Fig. 11A**). To confirm the importance of p53 binding, luciferase activity was confirmed by expressing Z promoter luciferase deletion constructs in AGS cells with wild-type p53 and NCC-24 cells with mutant p53. Z promoter activity of p53 mutated NCC-24 cells were significantly lower than wild-type p53 AGS cells (**Fig. 11B and 11C**). This suggests that ZID region is important for Z promoter activation and p53 binding ability affects Zp activation.

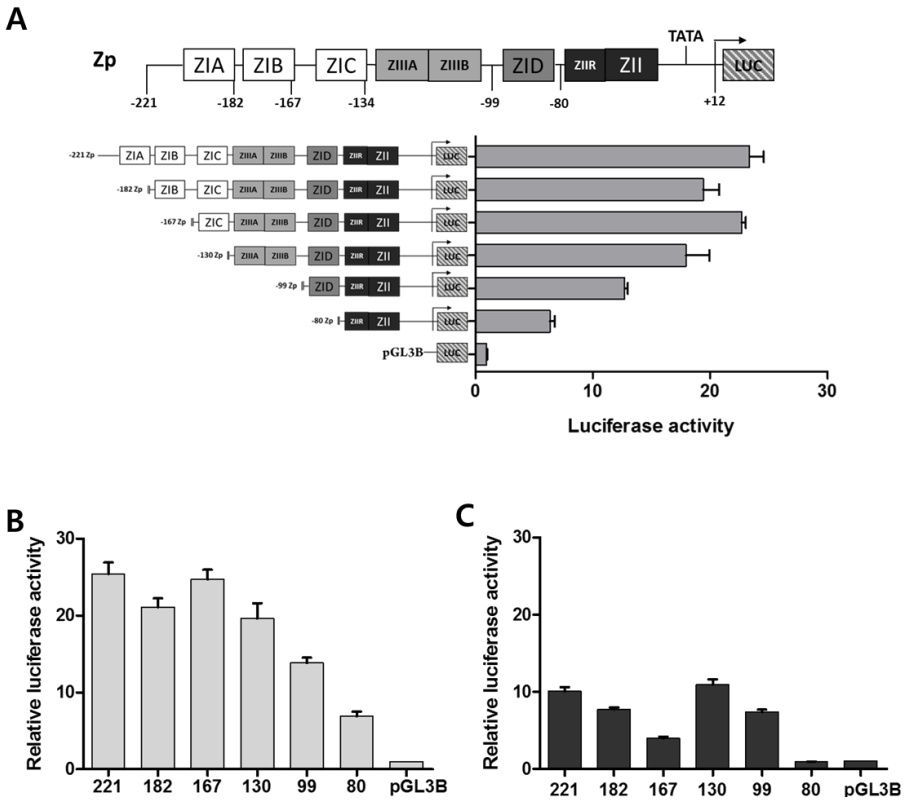


Figure 11. ZID domain of Z promoter is important to p53 binding. (A) Diagram representation of the EBV Zp reporter constructs and their luciferase activity are depicted. (B) AGS and (C) NCC-24 cells were co-transfected with Zp or its deletion constructs and pRL-SV40 as an internal control. The luciferase activities were measured 24 hr after transfection. Relative luciferase activity normalized to *Renilla* is shown.

9. p53 bind to Zp through Sp1 in gemcitabine-induced EBV lytic activation

Z promoter does not have p53 binding consensus sequence and the ZID domain of the Z promoter has binding sites for the transcription factors, Sp1 and MEF2D.[42,45] It has also been reported that Z promoter activity is conferred by binding of Sp1 to the ZID domain.[89] I examined the Sp1 knockdown experiment to determine the importance of Sp1 in gemcitabine-induced EBV lytic activation. Zta expression levels were decreased in SNU-719 cells after si-Sp1 transfection (**Fig. 12A**). As shown above, p53 binds to Zp (**Fig. 10A**) and Sp1 is related to Zp (**Fig. 12A**). Thus, co-IP was performed to investigate the possibility that Sp1 and p53 form complexes in gemcitabine-induced EBV lytic activation. As a result, it was found that Sp1 was co-precipitated with anti-p53 antibody and p53 was co-precipitated with anti-Sp1 antibody (**Fig. 12B and 12C**). As shown above, NCC-24 cell line mutated at the p53 DNA binding domain did not show Zp binding (**Fig. 10B**), and that the ZID domain binding ability was poor (**Fig. 11C**). This suggests that the DNA-binding ability of p53 may be a significant factor in the binding of Sp1 and p53. To confirm this, co-IP was performed on NCC-24 cells, and it was not observed that p53 or Sp1 co-precipitates in any of the antibodies (**Fig. 12D and 12E**). These data suggest that, Sp1 and p53 form a complex in gemcitabine-induced EBV lytic activation, and the DNA-binding ability of p53 may be a key factor in the interaction between Sp1 and p53.

Finally, to confirm whether p53 binds to EBV Zp through Sp1, ChIP was

performed after EBV lytic activation by gemcitabine in si-*Sp1* transfected SNU-719 cells. The result revealed that p53 bound to Zp with gemcitabine treatment in scrambled RNA transfected SNU-719 cells but, binding of p53 to Zp was decreased when Sp1 was knock-downed (**Fig. 12F**). Taken together, Sp1 and p53 form a complex in EBV lytic activation by gemcitabine, and p53 binds to Zp via Sp1.

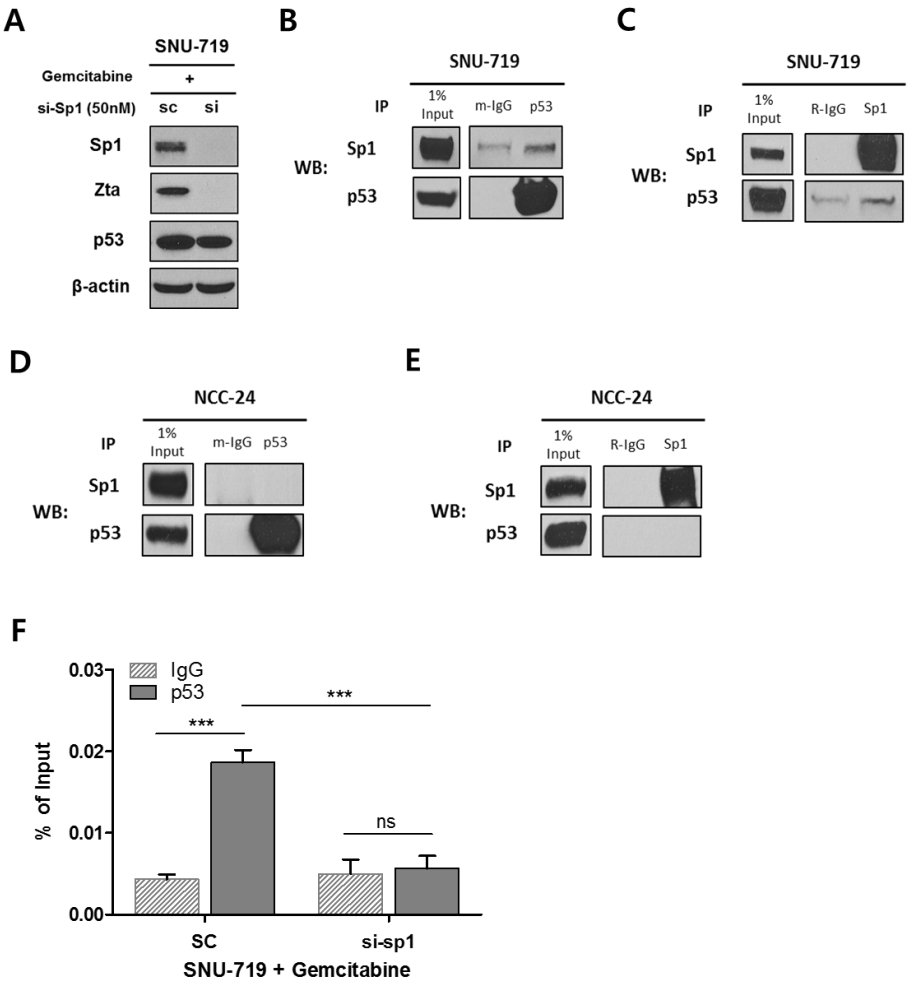


Figure 12. p53 bind to Zp through Sp1 in gemcitabine-induced EBV lytic activation. (A) SNU-719 cells were transfected with si-*Sp1* before gemcitabine treatment. Inhibition of lytic activation by si-*Sp1* was evaluated by changes in Zta, Sp1, and p53 using western blot. SNU-719 (B, C) and NCC-24 (D, E) cells were incubated with gemcitabine 50 ng/ml for 24 hr prior to processing for co-IP analysis. Co-IP was performed using nuclear extract and immunoprecipitated with anti-p53 and anti-Sp1 antibody. The Immunocomplexes were then revealed by Western blotting. (F) Scrambled RNA or si-*Sp1* transfected SNU-719 cells were treated with gemcitabine 50 ng/ml for 24 hr prior to processing for ChIP analysis. Quantitative-PCR analyses of the chromatin obtained from the cells following precipitation with anti-p53 or anti-IgG antibodies. The results are expressed as % of input ± SEM. $^{***}P<0.001$.

IV. DISCUSSION

The recent remarkable progress in cancer research has produced new target-oriented drugs and treatment strategies.[90] Three decades ago, virus-targeted therapies were tested for the treatment of virus-associated cancers.[91] An anti-viral prodrug, GCV, selectively phosphorylated by HSV-TK, was suggested for the treatment of virus-associated cancers.[52] EBV also encodes EBV-TK and EBV-PK that have similar function and are only expressed during lytic activation. Therefore, chemicals that act as lytic inducers have been sought to facilitate treatment with GCV in EBV-associated cancers.

Epigenetic modifying agents (5-azacytidine, trichostatin A, sodium butyrate, and valproic acid) and some anti-cancer drugs (5-fluorouracil [5-FU], cis-platinum, and taxol) induce lytic activation in EBV-positive cell lines that originate from various tumors.[54,77,92] The effectiveness of these reagents differs among different cell types. For instance, 5-FU and cis-platinum efficiently induce lytic activation in EBV-positive epithelial cell tumors but not in lymphoblastoid cell lines (LCLs) or B cell tumors. These differences make it difficult to generalize the outcome of EBV-lytic induction treatments, and therefore only a few clinical trials of these agents have been performed.[93,94]

Recently, EBV lytic activation was reportedly induced by the ER or genotoxic stress response. Moreover, the ATM/p53 pathway that is activated during the genotoxic stress response directly influences Zta induction.[55,83] The different sensitivity on lytic inducers among cell types may be due to the difference of underlying molecular mechanisms during lytic activation.

In this study, I attempted to find new drugs that induce lytic activation more efficiently in EBVaGCs and do not affect healthy cells and tissues by screening JHDL with BZLF1 promoter-transfected AGS cells. This library consists of drugs that are already used in patients and have been approved for safety and toxicity, allowing a bypass of phase I/II clinical trials.[95] Gemcitabine was selected and was confirmed as a lytic inducer by induction of lytic gene expression and EBV-TK/PK activity in the EBVaGC SNU-719 cells. Moreover, I observed that an extremely low dose (5 ng/ml) of gemcitabine induced Zta in SNU-719 cells compared to the dose required in LCLs or B cell lines (1 μg/ml),[77] which was checked by an optimized schedule. The dose discrepancy between EBVaGC-derived cells and B cell lymphoma-derived cells requires further evaluation with respect to molecular mechanisms.

Furthermore, gemcitabine-induced lytic activation was evaluated to determine whether the ER or genotoxic stress pathway was involved. The ATM inhibitor KU55933, si-*ATM*, or si-*TP53* treatment induced a decrease in Zta protein expression. Since the duration of ATM inhibitor activity is very short, suppression by the ATM inhibitor was relatively weaker than that of si-*ATM*. Most Zta expression was diminished by si-*ATM* and si-*TP53*. Therefore, the ATM/p53 pathway may be a key regulator involved in lytic activation by gemcitabine. In this reason, p53 may be applicable as a biomarker to determine whether EBVaGC patient is a candidate for gemcitabine-GCV combination treatment. TP53 mutation is frequently observed in cancers of various types, TP53 mutation is frequently observed in various types of cancers, however, is

rarely observed in EBVaGCs.[32,96] Moreover, the stability of p53 is also regulated by the interaction with EBNA1.[97] Thus, the status of p53 may determine the responsiveness of gemcitabine-induced lytic activation, although this notion requires further evaluation.

The final goal of this study was to apply GCV to the EBV-TK/PK-induced EBVaGC cells. I observed that functional EBV-TK/PK was efficiently induced by gemcitabine. The enzymatic activity of EBV-TK/PK was demonstrated via a positive correlation between gemcitabine concentration and the accumulation of [^{125}I] FIAU. The accumulation of [^{125}I] FIAU in the gemcitabine-untreated SNU-719 cells may be due to abortive lytic activation as discussed previously.[77] There are some data supporting abortive lytic activation in this study. BZLF1 was observed by RT-PCR in SNU-719 cells and in mouse-implanted SNU-719 cells. Even with an extremely low dose of gemcitabine, the survival of SNU719 cells was decreased by GCV treatment (Fig. 4E), and this is consistent with a previous report.[77] Despite these observations, it remains unclear whether these observations are a direct read-out of abortive lytic activation.

I also evaluated the toxicity of gemcitabine and GCV, and then examined the effects of an *in vitro* combination treatment. Gemcitabine exhibited a narrow safety window in SNU-719 and MKN-74 cells, but GCV was safe at a relatively high concentration (100 μg/ml). Synergistic effects of combination treatment were observed with a range of 0.1 - 100 ng/ml gemcitabine, as reported previously.[77,92] The concentration of gemcitabine was the most important factor for efficacy of the combination treatment. In a previous report, the efficiency

of GCV-induced cytotoxicity may have been underestimated due to the high dose of gemcitabine (1 μg/ml),[77] as at this dose, I observed no beneficial outcomes in combination with GCV. To overcome this problem, I utilized multiple treatments of low-dose gemcitabine. Gemcitabine-GCV combination treatment has previously been reported as a treatment for cancers, albeit via a completely different mechanism.[98] Gemcitabine was utilized as a ribonucleotide reductase inhibitor, which reduces endogenous dGTP to increase the incorporation of phosphorylated GCV into DNA. Moreover, gemcitabine is also known to increase the sensitivity of bystander cytotoxicity, although the concentration of gemcitabine needed for such effects (10 μM or 2.6 μg/ml) is 100 times higher than the concentration used in this study.

To date, only a few reports have described mouse models bearing EBVaGC-originated cell lines due to the rareness of EBV-naturally infected GC cell lines and the limited establishment of tumors in immune competent animals [92,99] or nude mice.[100] In this study, I established a lytic activation-inducible EBVaGC mouse model using NOD-SCID mouse, and furthermore, [^{125}I] FIAU-based EBV lytic activation monitoring system. Then, I evaluated a gemcitabine-GCV combination treatment using this EBVaGC mouse model. As Ghosh et al. performed with butyrate,[101] I modified the dosing schedule with the consideration of gemcitabine toxicity and rapid turnover rate and observed the efficient induction of lytic activation with multiple low dose schedule. As a result, a combination treatment of 0.5 mg/kg gemcitabine and 25 mg/kg GCV suppressed tumor growth more effectively than gemcitabine alone without

adverse effects. Furthermore, recently, it was reported that the combination treatment with gemcitabine, valproic acid, and GCV was efficient in EBV-positive refractory nasopharyngeal carcinoma patients.[78] Thus, additional combination with valproic acid could be considerable.

The importance of p53 in gemcitabine-induced EBV lytic activation was confirmed *in vitro* and *in vivo* experiments. And I wondered how p53 involves in Zta expression of EBV. Until now, three naturally EBV infected gastric carcinoma cell lines have been reported, such as SNU-719, YCCEL1 and NCC-24. They have different p53 status that SNU-719 and YCCEL1 have wild-type p53 but, NCC-24 have two missense mutations from glycine (GGA) to arginine (AGA) at codon 266 and arginine (CGT) to histidine (CAT) at codon 273 (**Table 4**). Gemcitabine also activates the EBV lytic cycle in YCCEL1 by activation of p53-pS15; however, this was not observed in NCC-24 even with a higher concentration of gemcitabine. On the other hand, EBV lytic activation was induced in wild type *TP53* transfected NCC-24 cells by gemcitabine. When gemcitabine-GCV combination therapy was tested in SNU-719 and NCC-24 transplant mouse models, SNU-719 tumors were effective for gemcitabine-GCV treatment but not for NCC-24 tumors. Kenney et.al, reported that ATM is required for efficient viral reactivation even in cells with mutant p53,[55] but I have conflicting results with previous results. That is, gemcitabine-induced EBV lytic activation occurs not only in SNU-719 but also in YCCEL1, but not in p53 mutated NCC-24. As a result, the importance of p53 status in lytic activation was confirmed *in vitro* and *in vivo*.

After confirming the importance of p53 in gemcitabine induced EBV lytic activation in EBVaGCs, next I wondered how p53 regulates EBV lytic activation in EBVaGCs. EBV reactivation from latency is determined by the balance between positive and negative transcription factors.[24] Various transcription factors contribute to EBV lytic activation. I account that Z promoter could regulate Zta expression by numerous transcription factors. The Z promotor has a short sequence of about 240 bp and there are various binding regions for various transcriptional factors within the cell. When p53 was knock-downed, a decrease in the expression level of Zta was shown; therefore, I expected that p53 will play a key role in the regulation of the Z promoter by gemcitabine. p53 binding to Z promoter and especially ZID domain of Zp was verified by ChIP and EBV Zp reporter assay in EBVaGC or EBVnGC, AGS cells. However, NCC-24, which has mutations in the DNA-binding domain (DBD) regions of p53, did not show the binding of p53 to Zp and ZID domain of Zp. The promoter activity of NCC-24 was significantly lower than that of AGS cells with wild-type p53. These observations indicate that p53 protein interacts with Zp via its DNA-binding ability during reactivation, shows the importance of ZID region for Z promoter activation, and that p53 binding ability affects Zp activation.

The ZID domain of the Z promoter has binding sites for the transcription factors Sp1 and MEF2D.[42,45] It has also been reported that Z promoter activity is conferred by binding of Sp1 to the ZID domain.[89] A region of p53 defined by amino acids 145 - 292 is capable of interacting physically with Sp1.[102] My result

showed that Sp1 forms a complex with p53 in gemcitabine-induced EBV lytic activation in SNU-719 cells, but not in NCC-24 cells due to its mutation in DBD regions of p53. Finally, binding of p53 to Zp via Sp1 was confirmed by ChIP in the Sp1 knock-downed SNU-719 cell line.

V. CONCLUSION

Gemcitabine was selected from a screen of JHDL for its ability to induce EBV lytic activation *in vitro* and *in vivo* in an EBVaGC cell line, SNU-719. ATM/p53 genotoxic stress pathway is a key regulator of gemcitabine-induced lytic activation. I also developed a lytic activation-inducible EBVaGC mouse model to evaluate the efficacy of gemcitabine-GCV combination treatment *in vivo* in concert with an imaging system for evaluating lytic activation. EBV lytic activation-based GCV combination therapy showed promising results in EBVaGC mouse model.

Furthermore, I examined EBVaGC cell lines that are reported to date and the p53 status was identified. Gemcitabine-induced EBV lytic activation occurs in SNU-719 and YCCEL1 but not in NCC-24, explaining the importance of p53 status in lytic activation *in vitro* and *in vivo* gemcitabine-GCV combination therapy. In gemcitabine-induced EBV lytic activation, p53 binds to Zp and ZID region of Zp, and p53s' DNA-binding ability is important for Zp activation. Also, Sp1 and p53 form a complex and p53 binds to EBV Zp via sp1 in gemcitabine-induced EBV lytic activation.

Consequently, p53 status is a key factor of ATM/p53 pathway and p53 uses Sp1 to regulate EBV Z promoter in gemcitabine-induced EBV lytic activation. Also, p53 might be available as a biomarker for 'gemcitabine-GCV combination therapy'.

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ABSTRACT (IN KOREAN)

p53, EBV 양성 위암에서 젬시타빈에 의해 유도 되는 엡스타인-바 바이러스의 용해 감염 활성의 주요 조절 인자

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엡스타인-바 바이러스 (EBV)는 이중 나선 DNA를 갖는 인간 감마 헤르페스 바이러스로 엡스타인-바 바이러스 연관 위암 (EBVaGC)을 포함한 악성 종양의 발병과 관련 되어있다. EBVaGC에서 지속적으로 존재하는 바이러스 게놈을 이용하여 EBV 표적 치료가 가능하고, EBV의 용해감염 주기에서 발현하는 thymidine kinase (TK)/protein kinase (PK)의 존재 시, 항바이러스성 약물인 ganciclovir (GCV)를 치료에 이용 할 수 있다. 이 논문에서는 EBVaGC의 치료를 위해 Johns Hopkins Drug Library를 스크리닝하여 GCV와의 병용 후보 물질로 젬시타빈을 선정하였다. 젬시타빈에 의한 EBV의 용해감염

유도는 ataxia telangiectasia-mutated (ATM)/p53 기전을 통해 일어나고, EBVaGC 세포주와 마우스 모델에서 젬시타빈-GCV 병용 요법이 효과적임을 확인하였다. 현재 알려진 EBVaGC, SNU-719, YCCEL1 그리고 NCC-24, 세포주를 확보하여 젬시타빈으로 용해감염을 유도한 결과, p53 유전자 변이형을 가진 NCC-24 세포주만 용해감염이 유도되지 않음을 시험관내에서 확인하였다. 야생형 p53을 NCC-24 세포주에 도입하였을 때, 젬시타빈에 의한 용해감염이 회복되었고, SNU-719와 NCC-24 종양 마우스 모델에서 젬시타빈-GCV 병용 처리시, SNU-719 종양에서만 치료 효과가 있었다. 젬시타빈에 의한 EBV 용해감염 유도에서 p53의 역할을 확인하기 위해 ChIP과 EBV Z 프로모터 reporter assay를 수행한 결과, p53이 EBV의 Zta 발현을 조절하는 Z 프로모터에 결합하고, Z 프로모터 중 특히 ZID 도메인에 결합하여 Zta의 발현을 조절하였다. 그리고 용해감염 활성화 시, Sp1과 p53이 복합체를 형성하고 p53이 Sp1을 통해 EBV Z 프로모터에 결합한다. 따라서, 젬시타빈에 의한 EBV 용해감염 활성화에서 p53이 중요한 결정 요소이고, EBV 양성 위암의 치료법으로 젬시타빈-GCV 병용 항암 요법을 제안할 수 있다.

핵심되는 말: Epstein-Barr virus, EBV 양성 위암, 젬시타빈, ganciclovir, EBV 용해감염 활성화, p53, ataxia telangiectasia-mutated

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