Preclinical Evaluation of Transcriptional Targeting Strategy for Human Hepatocellular Carcinoma in an Orthotopic Xenograft Mouse Model

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Abstract

Gene regulation of many key cell-cycle players in S-, G2 phase, and mitosis results from transcriptional repression in their respective promoter regions during the G0 and G1 phases of cell cycle. Within these promoter regions are phylogenetically conserved sequences known as the cell-cycle-dependent element (CDE) and cell-cycle genes homology regions (CHR) sites. Thus, we hypothesize that transcriptional regulation of cell-cycle regulation via the CDE/CHR region together with liver-specific apolipoprotein E (apoE)-hAAT promoter could bring about a selective transgene expression in proliferating human hepatocellular carcinoma. We show that the newly generated vector AH-6CC-L2C could mediate hepatocyte-targeted luciferase gene expression in tumor cells and freshly isolated short-term hepatocellular carcinoma cultures from patient biopsy. In contrast, normal murine and human hepatocytes infected with AH-6CC-L2C expressed minimal or low luciferase activities. In the presence of prodrug 5-fluorocytosine (5-FC), AH-6CC-L2C effectively suppressed the growth of orthotopic hepatocellular carcinoma patient-derived xenograft mouse model via the expression of yeast cytosine deaminase (yCD) that converts 5-FC to anticancer metabolite 5-fluorouracil. More importantly, we show that combination treatment of AH-6CC-L2C with an EZH2 inhibitor, DZNep, that targets EpCAM-positive hepatocellular carcinoma, can bring about a greater therapeutic efficacy compared with a single treatment of virus or inhibitor. Our study showed that targeting proliferating human hepatocellular carcinoma cells through the transcriptional control of therapeutic gene could represent a feasible approach against hepatocellular carcinoma. Mol Cancer Ther; 12(8); 1–14. ©2013 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer in the world (1). Although curative treatments are available for patients with hepatocellular carcinoma such as liver transplantation, surgical resection, and radiofrequency ablation (2), these treatment regimens are not always possible due to the size and location of the tumors. Even after liver resection, tumor recurrence remains high due to intrahepatic metastases via the portal veins or metachronous multicentric hepatocellular carcinogenesis (3). Currently, the most effective medical treatment for advanced hepatocellular carcinoma is with the antiangiogenic drug sorafenib. Despite the success of sorafenib to serve as the only approved medical drug for treating advanced hepatocellular carcinoma, only 2.3 months improvement in median overall survival was observed in Asian hepatocellular carcinoma patients (4). Therefore, treatment options for hepatocellular carcinoma remained universally dismal and require a concerted effort from research and innovative medical interventions.

Viral gene therapy has emerged as a potential alternative treatment modality for cancer treatment as evidenced by the significant number of reported clinical trials in recent years (5). Diverse strategies and a plethora of cell-type-specific promoters have been used to achieve targeted cancer gene therapy. Recent advances in molecular biologic techniques such as cDNA microarray have unraveled differentially expressed genes in human hepatocellular carcinoma versus normal controls (6, 7). Among the differentially expressed gene is a prominent overlap of cell-cycle genes including the Aurora kinase family members A and B (AURKA and AURKB, respectively), cyclin A2 (CCNA2), cyclin B1 (CCNB1), proliferating cell nuclear antigen, and polo-like kinase 1. Interestingly, all of these genes are regulated by phylogenetically conserved...
sequences known as the cell-cycle–dependent element (CDE) and cell cycle genes homology regions (CHR) sites in their promoters (8). Thus, cell-cycle-dependent transgene regulation is achieved through the binding of transcriptional repressors, such as cell-cycle–dependent factor 1 (CDF-1) and cyclin A CHR-binding factor (CHF), to the CDE and/or CHR elements within the cyclin A2 promoter (9, 10). Depending on the phase of the cell cycle, the MMB and DREAM complexes could regulate cell-cycle–dependent gene transcription through the CHR element of mouse and human cyclin B2 promoter (11). Furthermore, the transitional switch from MMB to DREAM bound to CHR sites is dependent on the activation status of p53, which subsequently regulates downstream transcriptional targets (12). Mutation of either CDE or CHR or both elements leads to a substantial deregulation of cell-cycle control (8, 13). Multimerization of CDE/CHR elements within the cyclin A2 promoter could also improve the kinetics of gene regulation (13). On the basis of this concept, we have previously generated a herpes simplex virus type 1 (HSV-1) ampiclon viral vector whereby the chimeric gene consisting of nuclear transcription factor Y subunit A (NF-YA) fuses with the DNA-binding domain of Gal4 and is expressed under the liver-specific hybrid 4×ApoE/hAAT promoter (14). This strategy is independent of exogenous stimulation for gene expression regulation; activation of the transgene expression is triggered by the proliferation status of the host cells. Thus, in proliferating liver cells, the binding of Gal4/NF-YA fusion proteins to Gal4-binding sites cloned upstream of the minimal cyclin A2 promoter will transactivate the promoter, as measured by the luciferase reporter gene activity. In nondividing or quiescent cells, the binding of the Gal4/NF-YA proteins will be prevented by repressor proteins occupying the CDE/CHR site, thus suppressing transcriptional activation of the reporter gene.

As a proof-of-concept that these vectors hold promise as novel viral vectors against hepatocellular carcinoma, the reporter gene is substituted with a bicistronic cassette encoding the yeast cytosine deaminase gene (yCD) fused in-frame with the luciferase reporter gene. The CDE/CHR regulatory motifs within the minimal cyclin A2 promoter are also multimerized to improve the kinetics of cell-cycle regulation. The yCD is a well-characterized suicide gene that converts nontoxic antifungal agent 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU; ref. 15). The toxic metabolites could freely diffuse to surrounding uninfected cells to exert additional bystander killing effect (16). In view of future clinical application, the vectors were studied in hepatocellular carcinoma patient-derived xenograft mouse models and freshly isolated human hepatocellular carcinoma samples with matched controls. Recently, Chiba and colleagues have also shown that 5-FU–resistant hepatocellular carcinoma cells as a result of continuous 5-FU treatment can be efficiently abolished by an EZH2 inhibitor called 3-Deazaneplanocin A (DZNep; ref. 17). DZNep is a pharmacologic inhibitor of the enhancer of zeste homolog 2 (EZH2; ref. 18). EZH2 is a histone methyltransferase that plays an important regulatory role in the self-renewal and differentiation of murine hepatic stem cells (19) and cancer stem cells (20). In a recent study, DZNep was shown to diminish the self-renewal capability of hepatocellular carcinoma, as indicated by the reduced EpCAM-positive hepatocellular carcinoma cells (17). Interestingly, the biologic effects of DZNep on hepatocellular carcinoma are distinct from those of 5-FU and thus we postulate that the combination treatment of our vectors with DZNep would bring about a greater therapeutic efficacy than a single treatment.

Materials and Methods

Cell lines and tissue specimens

A human hepatocellular carcinoma cell line (PLC/PRF/5) and a human lung adenocarcinoma cell line (A549) were obtained from the American Type Culture Collection (ATCC). A human hepatocellular carcinoma cell line (HuH-7) was obtained from the Japanese Collection of Research Bioresources (JCRB cell bank). Human ΔGli36 glioma cells (also known as Gli36EGFR) were kindly provided by M. Sena-Esteves (University of Massachusetts Medical School, Worcester, MA). African green monkey kidney (2–2) cells, used for vector packaging, were derived from Vero cells that constitutively expressed the HSV-1 ICP27 proteins (kindly provided by R.M. Sandri-Goldin, University of California, Irvine, CA). All cells were maintained at 37°C in a 5% CO2–95% air atmosphere and cultured in Dulbecco’s modified Eagle medium supplemented with 10% FBS (Hyclone Laboratories), penicillin (100 U/mL; Life Technologies), streptomycin (100 μg/mL; Life Technologies), and 2 mmol/L l-glutamine (Life Technologies). The ΔGli36 cells were further supplemented with 1 μg/mL puromycin (Invivogen), whereas the 2–2 and PLC/PRF/5-DsRed2 (PLC/PRF/5 cell stably expressed DsRed2 protein) cells were cultured in the presence of 500 μg/mL of Geneticin (Life Technologies). The immortalized untransformed human neonatal liver cell line (NeHepLxHT) was kindly provided by Lee CG (National Cancer Centre, Singapore). Human primary hepatocellular carcinoma and its adjacent matched normal liver tissues were obtained from the SingHealth Tissue Repository following approval from the SingHealth Centralized Institutional Review Board, and with patient informed consent. Preparation of short-term culture of hepatocellular carcinoma tumor or matched normal liver specimens can be found in Supplementary Materials and Methods. All cell lines except 2–2 used in this study were verified by using short tandem repeat analysis by Genetica DNA laboratories, Inc. NeHepLxHT cell line authentication was conducted by ATCC.

HSV-1 amplicon plasmid DNA constructs

The generation of pAH-6CC-L2C plasmid DNA construct was constructed from pApoE/hAAT-cc-Luc (14), pC8-6CC-Luc (13), and pHGCX-L2C (21) that were previously constructed in our laboratory. 6CC promoter was first PCR from pC8-6CC-Luc using the forward primer...
5'-CTGTCCCTCGTCTAGATAGTCGGGATAAC and reverse primer 5'-GATATGAAATTCCAGACCACGGCAGGCGCGGAG with the introduction of XbaI and EcoRI sites. The PCR product of 6CC was then used to replace the minimal cyclin A2 promoter of pApoE/hAAT-cc-Luc using the same restriction sites to generate pApoE/hAAT-6CC-Luc. Finally, luciferase foot and mouth disease virus 2A-yeast cytosine deaminase (Luc2ACD) cassette was released from the pHGCX-L2C construct and substituted with luc gene in pApoE/hAAT-6CC-Luc using the NotI and PacI restriction site to generate pApoE/hAAT-6CC-L2C. This construct is later renamed as pAH-6CC-L2C. Subsequent helper virus-free packaging methods are described in Supplementary Materials and Methods.

Luciferase reporter gene assay

Hepatocellular carcinoma cells (HuH-7 and PLC/PRF/5) or non–hepatocellular carcinoma cells (Gli36 and A549) were seeded at 3 x 10^5 cells in 6-well plate before being transfected with 3 μg of pAH-6CC-L2C plasmid using 8 μL of Lipofectamine (Life Technologies) and 5 μL of Plus reagent (Life Technologies) according to manufacturer’s instructions. Completed culture medium with 10% FBS was added to cells after transfection to maintain its proliferation status, whereas fresh culture medium supplemented with 0.2% FBS and 70 μmol/L of lovastatin (Merck) was added to cells to induce G1 arrest for 48 hours before subjecting to luciferase assay. For single-cell suspensions isolated from tumors or matched normal liver, the cells were seeded at 1 x 10^5 cells in 24-well plate and infected with AH-6CC-L2C at the appropriate multiplicity of infection (MOI) for 24 hours before subjecting to luciferase assays. All cells were harvested and lysed in 120 μL of lysis buffer (50 mmol/L Tris-Cl, 150 mmol/L NaCl, 1% Triton X-100). Luciferase activity and protein concentration were measured as described previously (14).

Determination of transfection and infection efficiency

*In vitro* transfection efficiency of pAH-6CC-L2C and *in vivo* tumor infectivity of AH-6CC-L2C were determined on the basis of the quantification of the percentage of enhanced green fluorescence proteins (eGFP)-positive cells determined using FACS Calibur (Becton Dickinson). All generated data were analyzed using FlowJo software (version 7.6.4; TreeStar Inc.). *In vitro* AH-6CC-L2C infectivity in 24-well plate was determined by counting the total eGFP-positive cells per well using Eclipse TE300 inverted fluorescence microscope (Nikon).

Cell viability assay

Cell viability was assayed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories). A total of 5 x 10^5 cells were seeded in 96-well plate with 100 μL complete medium per well. After incubation for 24 hours at 37°C, 5% CO2, these cells were infected with AH-6CC-L2C at MOI of 1 for 6 hours and replenished with fresh complete culture medium or fresh complete culture medium containing 2 mmol/L 5-FC (Sigma-Aldrich) and/or 10 μmol/L DZNep (Sigma-Aldrich). These cells were then incubated at 37°C, 5% CO2 for another 72 hours before cell viability assays were carried out. To conduct an assay for cell viability, 10 μL of CCK-8 solution was added to each well and the plate was incubated for another 1 to 2 hours at 37°C, 5% CO2 depending on the cells. The absorbance was finally read at 450 nm with a reference at 650 nm using a Victor 3V plate reader (PerkinElmer Life Sciences).

Establishment of PLC/PRF/5-DsRed2 tumor xenografts and primary patient-derived tumor model in SCID mice

PLC/PRF/5-DsRed2 tumor xenografts were established in the left lateral liver lobe of severe combined immunodeficient (SCID) mice as previously described (21). HCC 26-1004 tumors were derived, with informed consent, from patients undergoing liver resection and histology analysis with confirmed diagnosis of hepatocellular carcinoma (22). Samples for xenografting were taken from morphologically viable and non-necrotic tumor regions. hepatocellular carcinoma diagnoses were subsequently verified in all cases by our pathologists. Detailed procedure for the establishment of subcutaneous HCC 26-1004 tumor xenograft in SCID mice was described previously (21). Cell-cycle analysis is described in Supplementary Materials and Methods. Orthotopic HCC 26-1004 tumor was established using the subcutaneous HCC 26-1004 tumor. When a subcutaneous HCC 26-1004 tumor size reached about 1,000 mm^3, the tumor was harvested and cut into 1 to 2 mm^3 tumor cubes. Using 8-0 absorbable suture (Johnson & Johnson), the tumor cubes were orthotopically implanted into the left lateral liver lobe of 6-week-old female immunodeficient SCID mice (Animal Resource Centre).

Animal works

All animal experiments were carried out according to the guidelines and protocols approved by the SingHealth Institutional Animal Care and Use Committee (Singapore). After 4 to 6 days of HCC 26-1004 or RCC 09-1210C tumor implantation, 3-4 x 10^6 transduction units (TU) of AH-6CC-L2C in 100 μl Hank’s balanced salt solution (HBSS) were intratumorally injected. This was followed by intraperitoneal injection of 5-FC at 500 to 1,000 mg/kg doses, twice a day at 6-hour interval. At various designated time points, tumor growth was monitored using calipers, and the volumes were calculated using the formula V (volume) = π/6 x [L x (W)^2], where L is the length and W is the width. After acquiring the measurements, mice were subjected to noninvasive bioluminescence imaging using the Xenogen IVIS Lumina (Caliper Life Sciences) system as previously described (21). All image analyses to quantitate the bioluminescence signals were conducted using Living Image software version 3.0 (Caliper Life Sciences) where regions of interest (ROI) were drawn at sites of vector injection. Background signal corrections were conducted with an ROI drawn over the regions on mice where no vector had been administered.
Statistical analysis

Data are presented throughout this study as means ± SEM. Student unpaired t test was used for comparing the statistical significance for study involving two groups. One-way ANOVA followed by the Bonferroni multiple comparisons test were used for comparing the statistical significance for more than two groups. Two-way ANOVA followed by the Bonferroni multiple comparisons test were used for comparing the statistical significance for study involving two groups. P < 0.05 was considered statistically significant.

Results

AH-6CC-L2C mediates preferential transgene expression in proliferating orthotopic hepatocellular carcinoma tumors compared with normal livers

Previous studies from our laboratory have shown that a basic HSV-1 amplicon vector engineered with cell-cycle–regulatory elements could confer luciferase activities in a proliferation-dependent manner (14). In this study, additional modifications were made to this vector which included the use of (i) multimerized CDE/CHR regulatory motifs within the minimal cyclin A2 promoter to improve the kinetics of cell-cycle regulation and (ii) a bicistronic 2A-mediated therapeutic and reporter gene expression cassette for assessing the preclinical therapeutic efficacy while providing real-time monitoring of the vector activities. The newly generated vector is denoted as pAH-6CC-L2C in its amplicon plasmid form (Fig. 1A) and AH-6CC-L2C when it has been packaged into amplicon viral vectors.

To evaluate the cell-cycle–dependent gene expression property of the newly generated vector, PLC/PRF/5 hepatocellular carcinoma cells were either transected with pAH-6CC-L2C plasmids or infected with AH-6CC-L2C amplicon viral vectors at a MOI equivalent to 1. In both cases, luciferase activities were significantly enhanced in proliferating cells versus G1-arrested cell population (5.9-fold under transfection and 4.2-fold under infection conditions; Supplementary Fig. S1A and S1B, respectively). Flow cytometric analysis confirmed the proliferation and G1 arrest status of the cells (Supplementary Fig. S1C).

We next generated an orthotopic PLC/PRF/5-DsRed2 hepatocellular carcinoma tumor model to study the functionality of viruses in malignant hepatocellular carcinoma tumors versus normal liver. Before viral vector injection, the proliferating status of the implanted hepatocellular carcinoma tumors was confirmed by analyzing the cell-cycle profiles of cells prepared from the tumor and normal liver in representative animal. The results showed that cells originated from an orthotopic tumor region consisted of 9.7-fold higher fractions of S-phase compared with normal liver tissues that consisted of about 95% of quiescent cells in the G0 state (Supplementary Fig. S2A).

Equal viral doses were subsequently intrahepatically injected into normal mice or mice bearing orthotopic hepatocellular carcinoma tumors. Luciferase activities were measured at 24 hours after viral injection. As shown in Supplementary Fig. S2B, luciferase activities were significantly enhanced in liver regions that corresponded to the site of implanted tumors, as indicated by the red colored fluorescent proteins from tumors (Supplementary Fig. S2C; left). This was further confirmed using the luciferase assays on lysates prepared from the corresponding PLC/PRF/5-DsRed2 tumors and normal livers (Supplementary Fig. S2C; right).

The activities of AH-6CC-L2C in proliferating versus postmitotic hepatocytes were further confirmed in primary patient-derived HCC 26-1004 xenograft mouse models. The tumor was derived from a patient diagnosed with hepatocellular carcinoma and should be a better representation of the human hepatocellular carcinoma architecture because the tumors, stromal fibroblast cells, and inflammatory cells are retained (22). Rapid proliferation of the tumor cells in these xenografts was further confirmed by the enhanced endogenous cyclin A expression (Fig. 1B). It is therefore not surprising that high levels of luciferase activities were detected in the tumors compared with normal liver tissues when both regions were injected with the same amount of AH-6CC-L2C (Fig. 1C and D). To exclude tissue difference, AH-6CC-L2C-transduced primary cells derived from HCC 26-1004 tumors were compared with those in NeHepLxHT, a human telomerase reverse transcriptase immortalized human liver cell line. At 24 hours after infection, the number of eGFP-positive cells was similar in HCC 26-1004 versus NeHepLxHT (Fig. 1E). Luciferase activities mediated by AH-6CC-L2C were significantly higher in HCC 26-1004 compared with NeHepLxHT (Fig. 1F) as well as after normalizing against the number of infected eGFP-positive cells (Fig. 1G). Taken together, our results showed that AH-6CC-L2C could mediate elevated transgene expression in proliferating hepatocellular carcinoma in preference to normal mouse liver and immortalized normal human hepatocytes.

AH-6CC-L2C confers hepatocellular carcinoma specificity using hepatocellular carcinoma patient-derived xenograft mouse models

Given that most proliferating tumor cells would display elevated Ki-67 and cell-cycle proteins, we next determine whether AH-6CC-L2C could function preferentially in patient-derived hepatocellular carcinoma versus non-hepatocellular carcinoma xenograft mouse models. This was first conducted in vitro with cell line–derived cells by comparing the pAH-6CC-L2C–transfected hepatocellular carcinoma cells to non–hepatocellular carcinoma cells where all of these cells consisted of high percentage of proliferating cells in S-phase (Supplementary Fig. S3). Using A549 as a more stringent representative of non-hepatocellular carcinoma lines, HuH-7 and PLC/PRF/5 cells exhibited a corresponding 6.8- to 12.3-fold increase in transgene expression (Fig. 2A). One-way ANOVA analysis indicated that the differences between all cell lines were statistically significant (P < 0.0001) where the Bonferroni multiple comparisons test also confirmed that
Figure 1. Transcriptional targeting of AH-6CC-L2C in orthotopic patient-derived HCC 26-1004 tumor compared with adjacent matched normal liver and immortalized untransformed human neonatal liver cell line NeHepLxHT. A, schematic representation of pAH-6CC-L2C plasmid construct containing (i) human α-1-antitrypsin promoter (hAATp) with 4 copies of apolipoprotein E enhancer (4 × ApoE enh) driving a fusion protein of Gal4-DNA binding domain (Gal4 bd) and nuclear transcription factor Y, alpha (NF-YA); (ii) 8 copies of Gal4-DNA binding sites (8 × Gal4 bs) upstream of 6CC promoter (6CCp); and (iii) 6CCp consisted of minimal cyclin A2 promoter with 6 tandem repeat of cell-cycle–dependent element (CDE)/cell-cycle homology element (CHR) region that drive the expression of luciferase-FMDV 2A- yeast cytosine deaminase (Luc2ACD) gene cassette. Other common elements in the construct included ampR, ampicillin-resistant gene; bGHp(A), bovine growth hormone polyadenylation signal; OriS, HSV origin of replication; SV40 p(A), SV40 polyadenylation signal; pac, HSV-1 packaging signal; and IE4/5p, HSV-1 immediate early gene 4/5 promoter. B, immunofluorescent staining of cyclin A expression in tissue sections obtained from normal liver of SCID mouse and HCC 26-1004 tumor. Similar concentrations of rabbit IgG antibodies were used as negative control. Scale bar, 50 μm. C, bright field image of orthotopic HCC 26-1004 tumors (day 7 after tumor implantation) and right medial normal liver lobes 24 hours post-intratumoral or -intrahepatic injection with 3 × 10^6 TU of AH-6CC-L2C each site, respectively (left). Corresponding noninvasive imaging of luciferase gene expression from the injection sites (right). Red circles indicate the ROI where the AH-6CC-L2C was injected. The total flux measurements of photon emitted per second are stated above the ROI circles. D, corresponding quantifications of the luciferase signals emitted from livers and tumors in C. E, in vitro infectivity (indicated by eGFP-positive cells) of HCC 26-1004 short-term culture and NeHepLxHT 24 hours after infection with AH-6CC-L2C at MOI of 1. F, luciferase activities conferred by AH-6CC-L2C in HCC 26-1004 and NeHepLxHT 24 hours after infection at MOI of 1. G, specific luciferase activities conferred by AH-6CC-L2C in F after normalizing with infectivity of cells. All data are presented as mean ± SEM; n = 3.
Figure 2. Hepatocellular carcinoma specificity of AH-6CC-L2C in cell line-derived or patient-derived human hepatocellular carcinoma and non–hepatocellular carcinoma cells. A, luciferase activities conferred by pAH-6CC-L2C in cell line–derived hepatocellular carcinoma (HuH-7 and PLC/PRF/5) and non–hepatocellular carcinoma (AGL36 and A549) cells at 48 hours after transfection. Total relative luciferase units (RLU) were normalized with total protein contents and transfection efficiency (indicated by eGFP-positive cells). B, luciferase gene expression in in vitro cell cultures of patient-derived HCC 26-1004 and RCC 09-1210C (non–hepatocellular carcinoma) 24 hours after infection with AH-6CC-L2C at MOI of 0.5, 1.0, 1.5, and 2.0. Total RLU was normalized with total protein contents. C, comparison of in vitro transduction efficiency of HCC 26-1004 (infected with AH-6CC-L2C at MOI of 0.5) with RCC 09-1210C (infected with AH-6CC-L2C at MOI of 0.5, 1.0, and 1.5) as indicated by eGFP-positive cells. D, specific luciferase activities conferred by AH-6CC-L2C in HCC 26-1004 and RCC 09-1210C after normalizing with total protein content and transduction efficiency. All in vitro data are presented as mean ± SEM; n = 3. E, noninvasive imaging of luciferase gene expression conferred by AH-6CC-L2C in HCC 26-1004 and RCC 09-1210C subcutaneous primary tumor xenograft 24 hours after injection with 3 \times 10^6 TU and 6 \times 10^6 TU of AH-6CC-L2C, respectively. Red circles indicate the ROI where the AH-6CC-L2C was injected intratumorally. The total flux measurements of photon emitted per second are stated above the ROI circles. F, corresponding quantifications of the luciferase signal emitted from tumors in E. All in vivo data are presented as mean ± SEM; n = 5. G, immunofluorescent staining for hAAT and cyclin A expression in HCC 26-1004 and RCC 09-1210C tumor sections. Similar concentrations of rabbit IgG antibodies were used as negative control. Scale bar, 50 μm.
Luciferase expression in non–hepatocellular carcinoma cells is significantly different than in hepatocellular carcinoma cells.

The cell-type specificity of AH-6CC-L2C was further confirmed in primary patient-derived HCC 26-1004 and RCC 09-1210C mice xenografts. Single-cell suspensions of HCC 26-1004 and RCC 09-1210C tumors were prepared. Given that these two tumor types may express different receptors that resulted in differential HSV-1 infectivity, we tested a range of MOI of vectors on primary cells derived from RCC 09-1210C against HCC 26-1004 cells infected at a constant MOI of 0.5 (Fig. 2B). The results indicated that twice as much of the vector was required to infect RCC 09-1210C cells to achieve similar infectivity with HCC 26-1004 (Fig. 2C). However, after normalizing the luciferase activities against infected cells, the functional expression of luciferase reporter activities remained constant regardless of MOIs, and AH-6CC-L2C still mediate significantly increased transgene expression for about 4.7-fold higher in hepatocellular carcinoma 26-1004 compared with RCC 09-1210C (one-way ANOVA: \( P < 0.0001 \); Fig. 2D).

On the basis of the in vitro virus optimization study, twice the amount of AH-6CC-L2C viral vectors was subsequently injected subcutaneously into RCC 09-1210C tumor-bearing mice. The human origin and proliferation status of both HCC 26-1004 (Supplementary Fig. 5A) and RCC 09-1210C (Supplementary Fig. 5B) primary patient-derived xenografts were validated through positive staining against human nuclei and a corresponding high Ki-67–labeling index. As represented in Fig. 2E, the luciferase activities were 4.6-fold higher in HCC 26-1004 than in RCC 09-1210C (Fig. 2F) despite the fact that mice bearing renal cell carcinoma tumors were injected with twice the amount of viruses. Primary cell suspensions were prepared from tumors in representative animal and the similar infectivity of AH-6CC-L2C in the two tumor types was further confirmed by flow cytometric analysis (data not shown). In an attempt to address the differential luciferase expression, the tumor sections were stained against human \( \alpha-1 \)-antitrypsin (hAAT) and cyclin A (see Supplementary Materials and Methods for details). Both tumor types expressed high levels of endogenous cyclin A protein but only hepatocellular carcinoma tumors expressed hAAT proteins (Fig. 2G). Taken together, these results consistently showed that AH-6CC-L2C mediate enhanced transgene expression in proliferating tumors expressing elevated levels of hAAT proteins.

**Intratumoral administration of AH-6CC-L2C inhibits growth of patient-derived human hepatocellular carcinoma xenograft mouse models**

Next, we evaluate the therapeutic efficacy of AH-6CC-L2C viral vectors in patient-derived subcutaneous and orthotopic hepatocellular carcinoma tumor xenograft models. The functionality of the \( \gamma \text{CD} \) suicide gene in the presence of its prodrug (5-FC) was confirmed in vitro in PLC/PRF/5 cells (Supplementary Fig. 5S). Because AH-6CC-L2C vector comprised of \( \gamma \text{CD} \) gene fused in-frame with the luciferase reporter, we were able to monitor the estimated levels of therapeutic proteins produced in vivo, and the mice were injected with a booster shot of vectors when minimal luciferase activities were detected (Supplementary Fig. S6A; red arrows). Similar volume of HBSS to those of infected vectors was used as control. As shown in Fig. 3A, significant tumor growth inhibition was observed in AH-6CC-L2C/5-FC–treated hepatocellular carcinoma patient-derived subcutaneous xenograft compared with HBSS/5-FC control group (3.1-fold). Measurement of tumor volumes from each group of animals corresponded to the size of the tumors in the representative animal sacrificed at the endpoint of study (Fig. 3B). Furthermore, animals treated with AH-6CC-L2C/5-FC exhibited marked reduction in the levels of Ki-67, which was accompanied by elevated caspase-3 expression (Fig. 3C and D). The Ki-67 index of AH-6CC-L2C/5-FC–treated tumors were about 10-fold lower when compared with all other control groups (Fig. 3D).

To confirm the functionality of these viruses in the lysosome-enriched liver microenvironment, similar experiment was carried out in orthotopically implanted HCC 26-1004 animals injected with AH-6CC-L2C viral vectors as outlined in Fig. 4A. The results showed that there was a significant decrease in tumor volume and weight in animals treated with AH-6CC-L2C/5-FC when compared with HBSS/5-FC group (Fig. 4B) at the endpoint of the study. Expression of \( \gamma \text{CD} \) was monitored indirectly through the reporter activities with booster shots of vectors administered at the indicated times (Supplementary Fig. S6B). Corresponding luciferase activities and tumors in the representative animals were shown in Fig. 4C. Similar to previous findings, high levels of Ki-67 were observed in control animals but not in AH-6CC-L2C/5-FC group (Fig. 4D and E). In contrast, active caspase-3 was detected only in animals treated with AH-6CC-L2C/5-FC (Fig. 4D and E). Taken together, we have clearly shown that AH-6CC-L2C could effectively suppress the growth of patient-derived human hepatocellular carcinoma in subcutaneous and orthotopic xenograft mouse models.

**Combination treatment of AH-6CC-L2C and DZNep exerts synergistic cytotoxic effects against hepatocellular carcinoma**

For clinical relevance, we asked whether AH-6CC-L2C would enhance transgene expression in short-term cultures consisting of patient-derived hepatocellular carcinoma compared with the corresponding matched normal adjacent liver cells. Representative liver and tumor sections showed increased proliferation as characterized by the Ki-67 labeling index (Fig. 5A), which may be used as prognostic marker for hepatocellular carcinoma associated with poor survival (23). In two representative short-term cultures, AH-6CC-L2C infected hepatocellular carcinoma slightly better than matched normal control (Fig. 5B and E). However, after normalizing to the number of
of eGFP-positive cells, the luciferase activities were still significantly higher in hepatocellular carcinoma tumors compared with matched normal controls, ranging from 5.1-fold in Fig. 5D to 3.3-fold in Fig. 5G. The overall trend is similar when luciferase activities in the samples were normalized to total proteins (Fig. 5C and F). Thus, AH-6CC-L2C could mediate preferential transgene expression in primary tumor cells derived from patients with hepatocellular carcinoma.

In view of tumor cells that may be resistant to 5-FU, we determine the effects of DZNep (Supplementary Fig. S7) in combination with AH-6CC-L2C/5-FC treatment. Control groups were either injected intratumorally with HBSS or injected intraperitoneally with PBS. Red arrows indicate the time points when $3 \times 10^6$ TU of AH-6CC-L2C were intratumorally injected. Blue double-head arrow indicates the period during which 5-FC (1000 mg/kg) or PBS was administered via intraperitoneal injection twice a day at 6-hour interval. Data are presented as mean ± SEM; n = 6. B, indirect noninvasive imaging of eGFP suicide gene expression (via luciferase imaging) and corresponding bright field images of subcutaneous tumors harvested at endpoint from representative mice of each experiment group. C, immunohistochemical analysis of AH-6CC-L2C and 5-FC-treated tumors harvested at endpoint with cell proliferation marker (Ki-67) and apoptosis marker (active caspase-3) compared with control tumors treated with HBSS and 5-FC. Scale bar, 50 μm. D, quantification of Ki-67 and active caspase-3 index of tumors harvested at endpoint from each experiment group. Data are presented as mean ± SEM; n = 3. *, P < 0.05; ***, P < 0.01.

Figure 3. Effectiveness of AH-6CC-L2C in delivering and expressing eCD suicide gene in vivo for treatment of subcutaneous HCC 26-1004 tumor xenografts in SCID mice. A, in vivo killing efficacy of the AH-6CC-L2C on subcutaneous HCC 26-1004 tumor xenografts after 3 optimum doses of intratumoral injection of AH-6CC-L2C and 5-FC treatment. Control groups were either injected intratumorally with HBSS or injected intraperitoneally with PBS. Red arrows indicate the time points when $3 \times 10^6$ TU of AH-6CC-L2C were intratumorally injected. Blue double-head arrow indicates the period during which 5-FC (1000 mg/kg) or PBS was administered via intraperitoneal injection twice a day at 6-hour interval. Data are presented as mean ± SEM; n = 6. B, indirect noninvasive imaging of eGFP suicide gene expression (via luciferase imaging) and corresponding bright field images of subcutaneous tumors harvested at endpoint from representative mouse of each experiment group. C, immunohistochemical analysis of AH-6CC-L2C and 5-FC-treated tumors harvested at endpoint with cell proliferation marker (Ki-67) and apoptosis marker (active caspase-3) compared with control tumors treated with HBSS and 5-FC. Scale bar, 50 μm. D, quantification of Ki-67 and active caspase-3 index of tumors harvested at endpoint from each experiment group. Data are presented as mean ± SEM; n = 3. *, P < 0.05; ***, P < 0.01.
Discussion

Over the last decade, advanced technologies such as DNA microarrays have enabled us to characterize the aberrant expression profiles with respect to tumor types and/or stages in comparison with the adjacent normal tissues. For example, comparison of differentially regulated genes in hepatocellular carcinoma compared with normal liver using microarray analysis has enabled the identification of several hepatocellular carcinoma-specific promoters which may be a valuable tool for the generation of hepatocellular carcinoma-specific viral vectors (24). Herein, we have incorporated multimerized CDE/CHR modules within the minimal cyclin A2 promoter of a HSV amplicon vector as a mean of regulating transgene expression in response to the proliferation status of the host cells. When coupled with 4/C2 ApoE/hAAT liver-specific promoter, the results obtained are encouraging in that these AH-6CC-L2C viral vectors could mediate specific transgene expression in hepatocellular carcinoma but not renal cell carcinoma (RCC) patient-derived xenografts despite both tumor types originated from patients with advanced grade 3 tumor and expressed high Ki-67 proliferating index. Taken together, these data suggest that AH-6CC-L2C vectors could represent a novel therapeutic vector platform for hepatocellular carcinoma.

In the clinical setting, overexpression of cyclin A and cyclin E proteins have been associated with tumor relapse of human hepatocellular carcinoma and are independent predictive markers for their recurrence and prognosis (25, 26). Silencing of β-catenin gene in hepatocellular carcinoma induces changes in the expression of cyclin A and cyclin E, suggesting that the Wnt/β-catenin signaling pathway may be involved in the hepatocellular carcinoma pathogenesis through cell-cycle proteins (27).
B virus (HBV)-induced hepatocarcinogenesis, HBV pre-S2 mutant has been found to induce chromosome instability through the induction of aberrant cyclin A expression (28). A recent study has indicated that a functional single-nucleotide polymorphism at cyclin A2 promoter region associates with increased risk of hepatocellular carcinoma which further indicates that cyclin A2 activation may directly involve in hepatocellular carcinoma hepatocarcinogenesis (29). While the mechanism responsible for its complex hepatocarcinogenesis is still under investigation, rapid hepatocellular carcinoma tumor proliferation due to dysregulation of the cell-cycle proteins is clear. Herein, we have taken advantage of the rapid proliferating nature of tumor cells in general and the high levels of hAAT expression found in hepatocellular carcinoma (30–32) and generated an effective vector system whereby transgene expression is dependent on the activation of two endogenously regulated promoters, i.e., 4/ε/C2 ApoE/hAAT liver-specific promoter and recombinant human cyclin A2. In the context of oncolytic HSV-1, the 4/ε/C2 ApoE/hAAT liver-specific promoter has also been shown to drive the expression of complementary sequences of selected miRNAs exclusively in hepatocellular carcinoma (33).

Enzyme/prodrug strategy with yCD/5-FC has been extensively used as a therapeutic gene for cancer gene therapy in viral vectors (34–36) and cellular vehicles such as mesenchymal/neural stem cells (37, 38) to treat various...
mouse cancer models including colon carcinoma, hepatocellular carcinoma, ovarian cancer, breast cancer cells, lung cancer, and glioma. In our study, the expression of yCD gene is fused with a bioluminescence reporter gene so as to provide real-time information on viral kinetics in vivo, which is critical information, but usually not included in clinical trials with viral vectors. With the optimum intratumoral doses of AH-6CC-L2C as guided by indirect monitoring of yCD expression, significant suppressive effect on the growth of both subcutaneous and orthotopic hepatocellular carcinoma tumor growth in patient-derived hepatocellular carcinoma tumor models was successfully observed (Fig. 3A and Fig. 4B, respectively). Interestingly, despite the drastic reduction in Ki-67 proliferation index in these animal studies (ranging from 90.5% to 96.2% after treating with AH-6CC-L2C/5-FC), the corresponding reduction in tumor volumes approximate to 65%; the difference may be attributed by nonproliferating cells or cells resistant to 5-FU. Given that human Ki-67 proteins are often associated with biologically aggressive HCC phenotypes and poor overall survival rates (23), the observed reduction in cell proliferation index in AH-6CC-L2C–treated animals is nevertheless encouraging. Another observation worth noting is that the Ki-67 proliferating index in short-term cultures of fresh hepatocellular carcinoma samples (24.9% ± 1.6%) is lower when compared with untreated patient-derived hepatocellular carcinoma tumors implanted in control mice (74.8% ± 4.3%). This corresponded to the overall lower fold change in cell-cycle–dependent gene expression in short-term culture (3–5-fold; Fig. 5D and G) compared with orthotopic patient-derived hepatocellular carcinoma xenograft in SCID mice (25-fold; Fig. 1D). The difference in Ki-67 proliferation index could have arisen from the procedure of serial transplantation of patient-derived biopsy tissues in immunodeficient mice that have given rise to more aggressive tumors. This has also been reported by other group (39). In a recent study with oncolytic HSV-1 expressing yCD, the levels of in vivo 5-FU generated from 5-FC within tumor has been shown to be insignificant in plasma using liquid chromatography/tandem mass spectrometry measurement when compared with the systemic delivery level of 5-FU (34). These findings suggest that the anticipated intestinal toxicity derived from the yCD/5-FC system should be much reduced in a clinical application. In addition, the
use of yCD/5-FC system can be further improved in the presence of radiation as 5-FU is a potent radiosensitizer (40, 41). Alternatively, a humanized yCD has shown 3-fold increase in specific activities compared with the original prototype due to an improvement in thermostability (42).

For clinical relevance, we have also tested the functionality of AH-6CC-L2C in short-term culture of fresh hepatocellular carcinoma samples versus its matched normal total liver cell population. Primary hepatocellular carcinoma and normal cells were isolated immediately after surgery resection. These short-term cultures did not stain against human fibroblast markers but in hepatocellular carcinoma cultures were immunopositive against cytokeratin-18, cyclin A, and hAAT (data not shown). It may be worth noting that hepatocellular carcinoma tumors are 1.3-fold more susceptible to AH-6CC-L2C infection when compared with its matched normal liver cells (Fig. 5B and E). This differential in infectivity may be attributed to the overexpression of glypican-3 (GPC3) which is a cell surface heparin sulfate proteoglycan required by the HSV-1 viruses for adsorption (43). Increasing studies have reported on the use of replication-competent HSV-1 against hepatocellular carcinoma (44, 45) and hepatic metastases (46). We and others (47, 48) have shown that replication-defective HSV-1 ampiclon viral vectors are equally efficient in delivering genes to the cells of hepatic origin. For clinical application of AH-6CC-L2C, it may be worthwhile to study whether these vectors could preferentially infect GPC3-positive hepatocellular carcinoma tumors when delivered intratumorally with the aid of a catheter and ultrasound imaging. Unfortunately, the clinical applications of these amplicon vectors are restricted by the current technology platform for clinical scale-up manufacturing and production.

Although our results show that AH-6CC-L2C could inhibit hepatocellular carcinoma tumor growth efficiently, most treatments would be faced with problems of chemotherapeutic drug resistance including those of 5-FU (49). Prolonged 5-FU treatment has been shown to increase in EpCAMhigh and CD133high 5-FU–resistant hepatocellular carcinoma cells (HuH-7; ref 17). Hepatocellular carcinoma cells expressing EpCAM and CD133 proteins have been implicated to possess features characteristic of tumor-initiating cells (50). Interestingly, EpCAMhigh and CD133high 5-FU–resistant hepatocellular carcinoma cells could be selectively killed by DZNep (17). Our results supported the finding in that combined treatment of viral vectors and DZNep significantly enhances cell kill in Hep3B (Fig. 6A) and PLC/PRF/5 (Fig. 6B) when compared with a single treatment. We anticipated that after treatment with AH-6CC-L2C/5-FU, the residual 5-FU–resistant hepatocellular carcinoma cells could increase the fractions of EpCAM expression in hepatocellular carcinoma cells. Instead, our results showed that EpCAM level was slightly reduced in hepatocellular carcinoma treated with AH-6CC-L2C in 5-FU (Supplementary Fig. S8) which could imply that the time point chosen for FACS analysis was suboptimal, thus 5-FU–resistant cells had not been enriched. DZNep has been reported to selectively inhibit the trimethylation of lysine 27 on histone H3 (H3K27me3) and lysine 20 on histone H4 (H4K20me3) and could induce demethylation of CpG sites on genes such as FBXO32, which subsequently lead to efficient apoptosis (51). In acute myeloid leukemia, the decrease in H3K27me3 after DZNep treatment resulted in an upregulation of thioredoxin-binding protein 2 (TXNIP) which resulted in an overexpression of reaction oxygen species and led to apoptosis (52). A recent study showed that combination treatment of DZNep and gemcitabine in pancreatic cancer cells significantly reduce EZH2 and H3K27me3 expression as well as depleted the intracellular adenosine content (53). This synergist interaction resulted in the reduction of another cancer stem cell (CSC) marker–expressing cells, CD133, suggesting that dual targeting of CSC and non-CSC could represent an effective treatment modality in human tumors. Having said this, the specificity and possible toxicity of DZNep or similar compound in vivo require further investigation.

In conclusion, we have shown that AH-6CC-L2C vectors could mediate elevated transgene expression in proliferating hepatocellular carcinoma tumors in preference to normal mouse or human hepatocytes. Selective transgene expression in hepatocellular carcinoma may be attributed to the combined transcriptional regulation of cell-cycle regulation and liver-specific promoters. In the presence of produg 5-FC, AH-6CC-L2C effectively suppressed the growth of orthotopic hepatocellular carcinoma patient-derived xenograft mouse model. More importantly, we show that combination treatment of AH-6CC-L2C with an EZH2 inhibitor, DZNep, can bring about a greater therapeutic efficacy compared with a single treatment. Taken together, we showed that targeting proliferating human hepatocellular carcinoma cells through transcriptional control of therapeutic gene could represent a feasible approach against hepatocellular carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Sia et al.

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