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Regular Article

Evaluation of Malarial Var2CSA-Displaying Baculovirus Vector in Transduction Efficiency in Human Cancer Cells

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Baculovirus vectors (BVs) are able to use for gene transduction in mammalian cells and are recognized as growing viral vectors for cancer gene therapy applications. The transduction efficiency of BVs varies among cancer cell types. To improve the transduction efficiency of BVs in human cancer cells, BV displaying malarial variant surface antigen 2-chondroitin sulfate A (var2CSA) molecules was developed in this study. Var2CSA plays a critical role in the sequestration of *Plasmodium falciparum*-infected erythrocytes in the placenta. Moreover, var2CSA binds to cancer cells *via* placenta-like chondroitin sulfate A (CSA), but not to non-cancer cells. Var2CSA BV showed significantly higher gene transduction than control BV in HepG2 and Huh7 cells, human hepatic cancer cells as well as AsPC-1 cells, human pancreatic cancer cells. The transduction efficiency of var2CSA BV was significantly inhibited by the anti-gp64 antibody, free heparin, and CSA. The results of this study suggest that var2CSA BV would be an improved vector for cancer gene therapies, especially in the treatment of hepatic and pancreatic cancers.

Key words variant surface antigen 2-chondroitin sulfate A (var2CSA), baculovirus vector, transduction, cancer gene therapy

INTRODUCTION

Baculovirus vectors (BVs) are able to use for gene transduction in mammalian cells and possess many advantages over other viral vectors.¹⁾ For example, BVs are thought to be safer gene delivery vehicles than other viral vectors. *In vivo* administration of BVs induces innate immune responses in mice because BVs are DNA viruses that are recognized as exogenous pathogens *via* several immune receptors expressed in mammalian cells.^{2,3)} However, BVs cannot produce viral progeny in mammalian cells and do not induce severe pathological toxicities *in vivo*, in contrast to their toxicity to insects. Moreover, significant efforts have been made to overcome the vulnerability of BVs to human complement system of the innate immunity.⁴⁾ Given recent progress, BVs could be used for *in vivo* applications such as cancer gene therapies.^{5–8)}

The transduction efficiency of BVs varies among cancer cell types.⁹⁾ One of the most permissive cancer cell lines for BV-mediated gene transduction is hepatic cancer cell lines such as HepG2. Many cancer cells derived from various origins are able to exhibit significant BV-mediated gene transduction by increasing the amount of BVs available for transduction. BVs bind efficiently to the cell surface in many cancer cell types, but the efficacy of downstream steps necessary for gene transduction, including internalization of viral particles into cells, escape from endosomes, and transport of viral DNA into the nucleus, depends on the cancer cell species.¹⁰⁾ To improve transduction efficiency in cancer cells, modifications of the virion surface of BVs have been reported.¹¹⁾ The BV virions have envelopes derived from infected insect cells and can display desired proteins on the surface of virions using gene manipulation techniques. Further development of BVs to improve gene transduction efficiency is necessary to allow for the broad application of BVs within the cancer research field.

Sequestration of *Plasmodium falciparum* (Pf)-infected erythrocytes in the human placenta results in placental malaria, including placental dysfunction and low birth weight neonates.¹²⁾ The interaction between the placenta and Pf-infected erythrocytes is mediated by the malarial variant surface antigen 2-chondroitin sulfate A (var2CSA) protein expressed on the surfaces of Pf-infected erythrocytes. Var2CSA is a variant of the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). The var2CSA protein binds to chondroitin sulfate A (CSA), expressed in syncytiotrophoblasts in the placenta. The domains of var2CSA protein that are important for binding to CSA have been identified in several studies.^{13,14)} Recently, the three-dimensional structure of the var2CSA protein was revealed.^{15,16)}

While CSA is commonly expressed on the surfaces of mammalian cells, it is thought that placental CSA is different from CSA expressed in tissues other than the placenta. Recently, placental-like CSA (pl-CSA) was found to be expressed in many cancer cell types. In addition, var2CSA protein was shown to bind to cancer cells¹⁷⁾ including hepatic, pancreatic, lung, mammary, and prostate cancer cells, but not non-cancer cells. These findings suggest that var2CSA could be used for cancer-specific drug delivery and detection of circulating tumor cells. Var2CSA could also be useful for enhancing anticancer reagents *via* conjugation. However, var2CSA-displaying viral vectors have not yet been developed for gene transduction applications in cancer cells.

We previously developed a malarial circumsporozoite protein (CSP)-displaying BV, which enhanced transduction efficiency, especially in hepatic cells.¹⁸⁾ The main molecule binding to CSP is thought to be heparan sulfate on the surface of hepatocytes, which contributes to selective infection of sporozoite form of malaria parasites into hepatocytes.¹⁹⁾ These studies prompted us to adapt similar strategy to evaluate the

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capacity of var2CSA to enhance BV-mediated transduction efficiency in cancer cells. In this study, we constructed BVs displaying var2CSA to investigate gene transduction in cancer cells from several origins. The examination with a variety of cancer cells demonstrates that var2CSA type BV had the possibility of favored enhancement of gene transduction in hepatic and pancreatic cancer cells. These results show that var2CSA BV could join the ranks of available vectors for cancer research including gene therapy.

MATERIALS AND METHODS

Cell Culture Sf9, an insect cell line derived from *Spodoptera frugiperda* as a host for baculovirus expression systems, was maintained in Sf-900II SFM (Invitrogen, Carlsbad, CA, U.S.A.) in the incubator in which the temperature was set to 27°C. HepG2 and Huh7 human hepatocellular carcinoma cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), non-essential amino acids, and antibiotics at 37°C. AsPC-1 (pancreatic cancer cell line), A549 (lung cancer cell line), and MCF7 (breast cancer cell line) cells were maintained in RPMI1640 supplemented with 10% heat-inactivated FBS and antibiotics at 37°C.

Baculovirus Production The construction of BVs was based on the Bac-to-bac system (Invitrogen). Control type BV and full-length CSP type BV were constructed as previously described.¹⁸⁾ The var2CSA BV was constructed using the same method as the CSP type BV.¹⁸⁾ A DNA fragment of DBL1X-DBL3X (a.a.59–1576), derived from *Plasmodium falciparum* 3D7 strain,¹³⁾ was obtained by PCR using Phusion high fidelity DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.) and subcloned into pENTR/D-TOPO vector (Invitrogen). The primers used for PCR were synthesized (Fasmac Co., Ltd., Kanazawa, Japan). The primer sequences were 5'-CACC GAATTC GATTC CGGCACAAATGATCC-3' (*EcoRI* restriction enzyme site was underlined) and 5'-CCC GGG CAAGCTT GTTATTTTCTTTTCGGC-3' (*XmaI* restriction enzyme site was underlined). The DNA sequence was confirmed by standard DNA sequencing method. Next, DBL1X-3X sequence replaced the full length CSP sequence of the previously-constructed pFast-CMV-EGFP-GL3-p10-DsRed-full length CSP¹⁸⁾ using the *EcoRI* and *XmaI* restriction enzyme sites, resulting in pFast-CMV-EGFP-GL3-p10-DsRed-DBL1X-3X vector (CMV: cytomegalovirus, EGFP: enhanced green fluorescent protein, DsRed: Discosoma sp. red fluorescent protein). The constructed pFast vector was then transformed into DH10Bac to obtain the corresponding bacmid, after which the bacmid was transfected into Sf9 cells. Culture medium containing var2CSA BV was collected. The BVs were purified by ultracentrifugation (Beckman Coulter, Brea, CA, U.S.A.) for experiments in this study, and virus titers were calculated based on the DsRed positive wells of Sf9 cells seeded 96 well plates with maximum dilution as previously described.¹⁸⁾ In details, Sf9 cells (7×10^4 cells/well) were seeded into 96-well plates. After one hour, culture medium was removed, and medium containing serially diluted BVs was added. After incubation of plates for six days at 27°C, DsRed expression was examined using a BZ-X710 microscope (Keyence, Osaka, Japan). Virus titers were expressed as TCID₅₀/mL determined using Reed-Muench method to detect

DsRed expression. The titers of BVs used in this study were approximately 10^8 – 10^9 TCID₅₀/mL.

Western Blot Analysis BV samples were separated on an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel, transferred to an Immobilon FL membrane (Millipore, Darmstadt, Germany), and probed with 1:5000 diluted mouse anti-flag antibody (M2; Sigma, St. Louis, MO, U.S.A.) or 1:500 diluted mouse anti-var2CSA monoclonal antibody (reacts with DBL1X)²⁰⁾ with 1:1000 diluted rabbit anti-VP39 antibody, followed by 1:10000 diluted secondary antibodies (IRDye 800CW goat anti-mouse and 680LT goat anti-rabbit; LI-COR Biotechnology, Lincoln, NE, U.S.A.) as previously described.¹⁸⁾ The membrane was analyzed using an Odyssey IR imaging system (LI-COR Biotechnology).

Transduction Cells (1×10^4 cells/well) were seeded into 96-well plates. The next day, the virus, as indicated in each figure legend, was added to the cells. After a further 24h of culture, EGFP expression was examined using a BZ-X710 microscope. Luciferase assays were performed as previously described,¹⁸⁾ according to the experimental protocol of Luciferase Assay System (Promega, Madison, WI, U.S.A.). Briefly, the culture medium was removed, and cell culture lysis buffer (50 μ L/well) was added. The extracts collected from the cells (20 μ L) were mixed with luciferase assay reagent (100 μ L) in wells of luciferase assay plate, and were immediately subjected to luciferase activity measurements using a Glomax Plate Reader (Promega). The value of measured luciferase activity of each sample was represented as relative light unit (RLU). To investigate transduction mechanisms, BVs were pre-incubated with the indicated dose of anti-gp64 antibody (AcV1, BioLegend, San Diego, CA, U.S.A.), heparin (Mochida Pharmaceutical, Tokyo, Japan), or CSA (from bovine trachea; Sigma) for 15 min at room temperature, then added to cells for gene transduction.

Flow Cytometry Analysis Transduced HepG2 cells were detached from culture plates by treatment with TrypLE Select enzyme (Thermo Fisher Scientific, Waltham, MA, U.S.A.), then collected. Cells were analyzed using a BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA, U.S.A.). EGFP expression in transduced cells was analyzed using BD FACSsuite software (BD Biosciences).¹⁸⁾ In some experiments, detached cells were resuspended in DMEM supplemented with 10% FBS on ice to avoid cell death during further experimental procedure. 7-Amino-actinomycin D (7-ADD) solution (BioLegend; final concentration was 0.5 μ g/mL) was added to stain dead cells before analysis by flow cytometer.

Statistical Analysis Results are expressed as mean \pm standard error (S.E.). Statistical analysis was performed using the Mann-Whitney test for comparisons between two experimental groups, and calculated using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, U.S.A.). Statistical significance was set at $p < 0.05$.

RESULTS

Construction of BV Displaying var2CSA Var2CSA is a 350kDa transmembrane protein, and is composed of an extracellular domain containing six Duffy-binding-like (DBL) domains with several interdomains, transmembrane, and cytoplasmic domains.¹³⁾ Attempts to construct BV expressing the

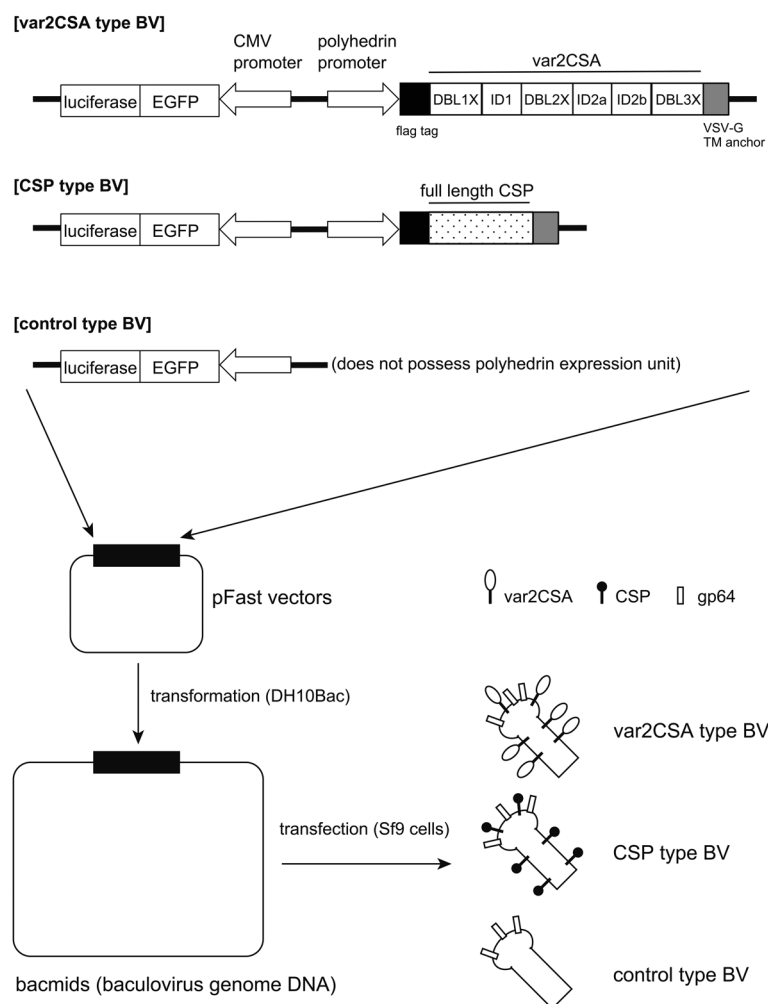


Fig. 1. Construction of BV Displaying var2CSA

Schematic representation of the recombinant BVs constructs used in this study. EGFP-luciferase fusion protein is expressed under the control of the CMV promoter, which is an indicator of transduction efficacy in mammalian cells. The proteins expressed under the polyhedrin promoter are fused to the flag tag and VSV-G TM domain and anchored to BV virions.

full-length extracellular portion of var2CSA were unsuccessful, perhaps because the length of the DNA sequences (over 9 kb) was too long to be inserted into the pFast plasmid vector. Therefore, we trimmed the var2CSA sequence to be expressed in the BV by approximately half. The truncated fragment used in this study contained the DBLX1–DBLX3 (a.a.59–1576) domains (Fig. 1). This portion of the var2CSA protein includes a minimal CSA-binding domain.^{14,21)} This fragment was successfully inserted into the pFast vector to obtain the bacmid derived from it. The bacmid was then transfected into Sf9 cells to generate BV displaying var2CSA. As shown in Fig. 1, the var2CSA protein was fused to the flag tag and vesicular stomatitis virus (VSV)-G membrane anchor (total 1645a.a., 189kDa). Because CSP BV increased the transduction efficiency in hepatic cancer cells in previous study,¹⁸⁾ CSP BV was used in parallel with var2CSA BV to justify research design in this study (Fig. 1). The expression of var2CSA was confirmed by Western blot analysis using anti-flag and anti-var2CSA antibodies, as well as the expression of VP39, the major capsid protein of BVs as loading control (Figs. 2A left, B). CSP expression in CSP BV was also confirmed (Fig. 2A right).

Transduction in Hepatic Cancer Cells Using var2CSA-Displaying BV BV displaying var2CSA was evaluated for

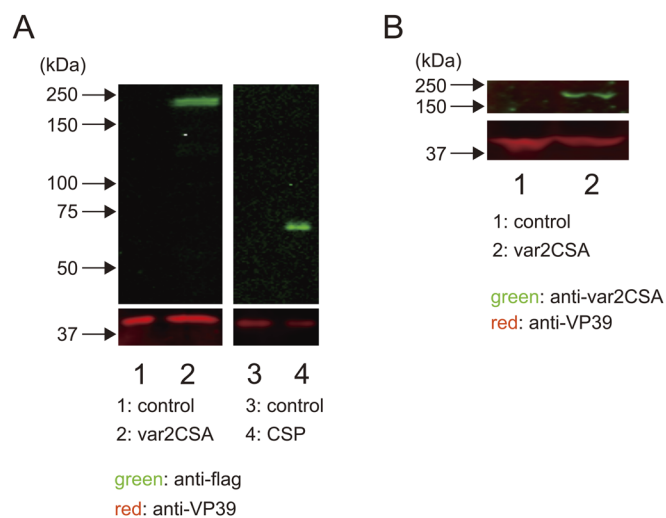


Fig. 2. Expression of the var2CSA Protein in the Constructed BV

(A) var2CSA protein incorporation in purified virions was detected using Western blot analysis. The samples were run on the gels, transferred to membranes, and probed with an anti-flag antibody to detect the var2CSA protein as well as anti-VP39 antibody as loading control. (left) lane1: control BV, lane2: var2CSA BV. (right) lane3: control BV, lane4: CSP BV (flag tag was added to the var2CSA and CSP protein). (B) The cell lysates from control or var2CSA BV infected Sf9 cells were subjected to Western blot analysis. The transferred membrane was probed with anti-var2CSA monoclonal antibody and anti-VP39 antibody.

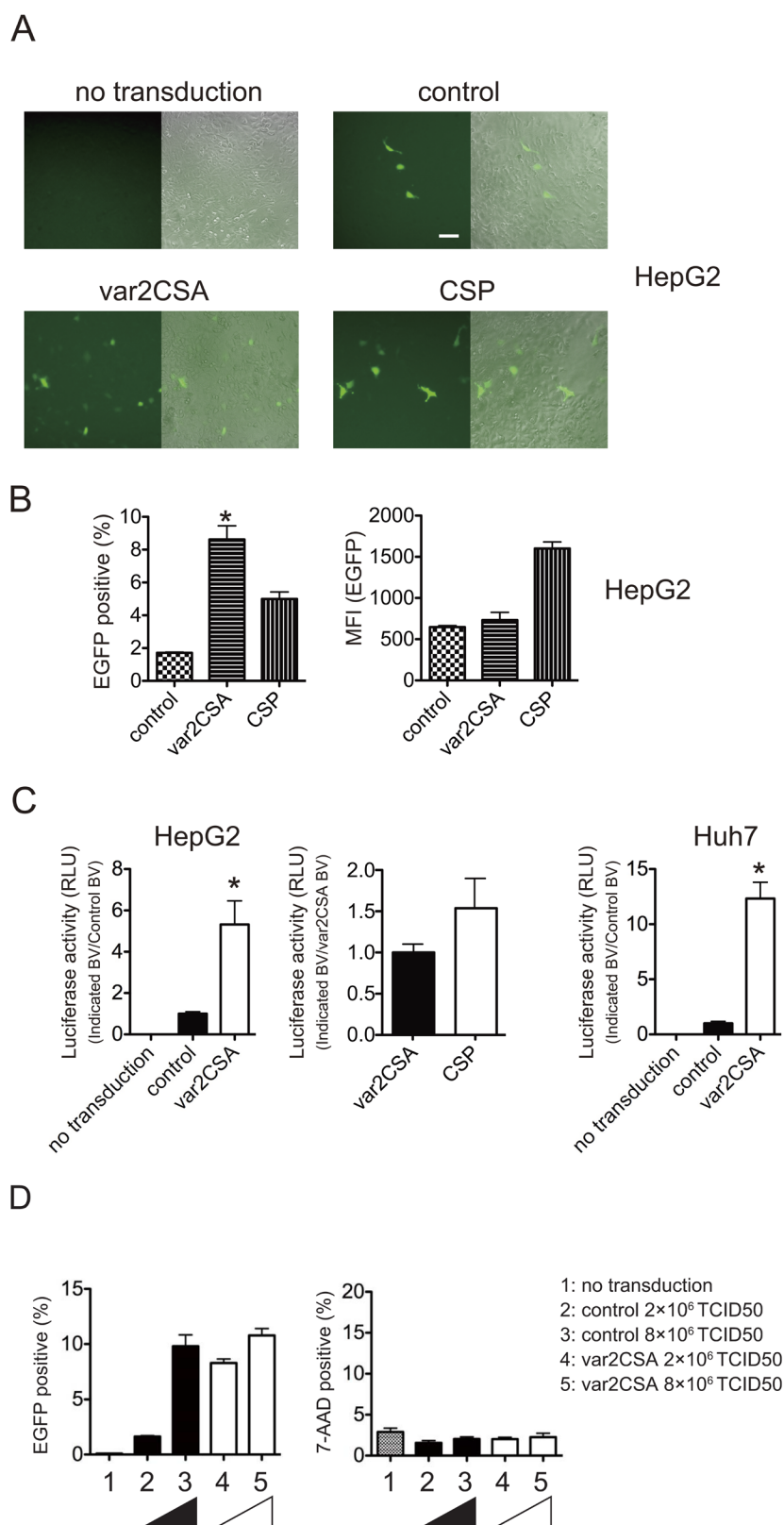


Fig. 3. Transduction of Hepatic Cancer Cells Using var2CSA-Displaying BV

(A) The constructed BVs (1×10^6 TCID₅₀) were transduced to HepG2 cells. After 24h, microscopic images of EGFP expression in transduced cells were taken and shown here. Scale bar: $100 \mu\text{m}$. (B) Transduced HepG2 cells were analyzed by flow cytometry analysis. The left panel shows the percentage of EGFP expressing cells within the total cell population ($n=3$ for each group). Asterisks indicate a significant difference compared with control or CSP type virus ($p<0.05$). The right panel shows MFI of EGFP expressing cells. (C) The left panel shows fold activation of luciferase activity of indicated BVs compared with control BV ($n=3$ for each group) in HepG2 cells. BVs (1×10^6 TCID₅₀) were added to hepatic cancer cells. After 24h, luciferase activity of the cell extracts was measured. Bars (the RLU of indicated BV transduced cells/the RLU of control BV transduced cells) represent the mean \pm S.E. The middle panel shows the luciferase activity of CSP BV compared with var2CSA BV. The right panel shows fold activation of luciferase activity of indicated BVs compared with control BV ($n=3$ for each group) in Huh7 cells as same as the left panel. Asterisks indicate a significant difference between control virus and the indicated virus ($p<0.05$). The experiments were performed twice and representative data are shown. (D) Transduced HepG2 cells with higher dose of BVs were stained with 7-AAD, followed by flow cytometry analysis. The left panel shows the percentage of EGFP expressing cells within the total cell population ($n=3$ for each group). The right panel shows the percentage of 7-AAD stained cells within the total cell population ($n=3$ for each group).

their efficient transduction into HepG2 human hepatocellular carcinoma cells. In this study, the expression of EGFP-luciferase fusion as reporter gene is driven under CMV promoter (Fig. 1) as previously study.¹⁸⁾ Therefore, the activity of EGFP and luciferase in transduced cells could be measured at same time, which enables to analyze the features of var2CSA BV-mediated transduction. As previously reported, var2CSA protein can bind to the surface of HepG2 cells.²²⁾ As shown in Fig. 3A, var2CSA BV showed enhanced EGFP expression compared to that of control BV. Flow cytometric analysis revealed that var2CSA BV induced transgene expression in larger percentages within cells than control BV (Fig. 3B). The mean fluorescence intensity (MFI) of the EGFP-expressing cell population was calculated. The MFI in var2CSA BV-transduced cells was not significantly higher than that in control BV, which is in contrast to CSP BV. Luciferase activity in cells was also measured, which is an indicator of total transgene expression within the transduced cells. The var2CSA BV significantly increased transduction efficiency compared to that of control BV (Fig. 3C left). As previously published, CSP BV also significantly increased transgene expression in HepG2 cells, and slightly more than var2CSA BV (Fig. 3C middle). We also examined another hepatic cancer cell line, Huh7. In addition to HepG2 cells, var2CSA BV induced transgene expression in Huh7 cells more than control BV (Fig. 3C right). To examine the cell viability after transduction, dose-escalated BVs were added to HepG2 cells. 7-AAD staining of transduced cells were analyzed as well as EGFP expression. As shown in Fig. 3D, EGFP expression was increased according to the amounts of BVs added to cells, although 7-AAD stained cells remained low percentage even in the case of higher dose. These results demonstrate that var2CSA BV significantly increased transduction efficacy in hepatic cancer cells.

Transduction in Additional Cancer Cell Lines Using var2CSA-Displaying BV Var2CSA recognizes pl-CSA, which is expressed in many cancer cell types.¹⁷⁾ Therefore, we examined cancer cells other than hepatic cancer cells for var2CSA BV-mediated gene transduction. Pancreatic ductal adenocarcinoma (PDAC) is one of var2CSA-binding cancer cells.²³⁾ In this study, AsPC-1, the human PDAC cell line, was used to investigate gene transduction by var2CSA BV. In the case of control BV, the transduction level (measured luciferase activity) in AsPC-1 cells was lower than that in HepG2 cells, which is consistent with previous reports.⁹⁾ In contrast, as shown in Fig. 4A, var2CSA BV significantly enhanced gene transduction in AsPC-1 cells, in which the transduction level was comparable to that of HepG2 cells transduced with control BV. Interestingly, the effect of var2CSA BV was significantly higher than that of CSP BV. This may be consistent with the fact that CSP BV has a preference for hepatic cells.¹⁸⁾

Previous studies have demonstrated that A549 human lung carcinoma cells and MCF7 human breast cancer cells also express pl-CSA and have an affinity for the var2CSA protein.¹⁷⁾ Therefore, the transduction efficiency in these cell lines was also examined. As shown in Figs. 4B and C, the increase in gene transduction in A549 and MCF7 cells by var2CSA BV was much less than that in AsPC-1 cells. These results suggest that the effect of var2CSA BV on gene transduction may depend on the type of cancer cell and may be more prominent in hepatic and pancreatic cancer cells.

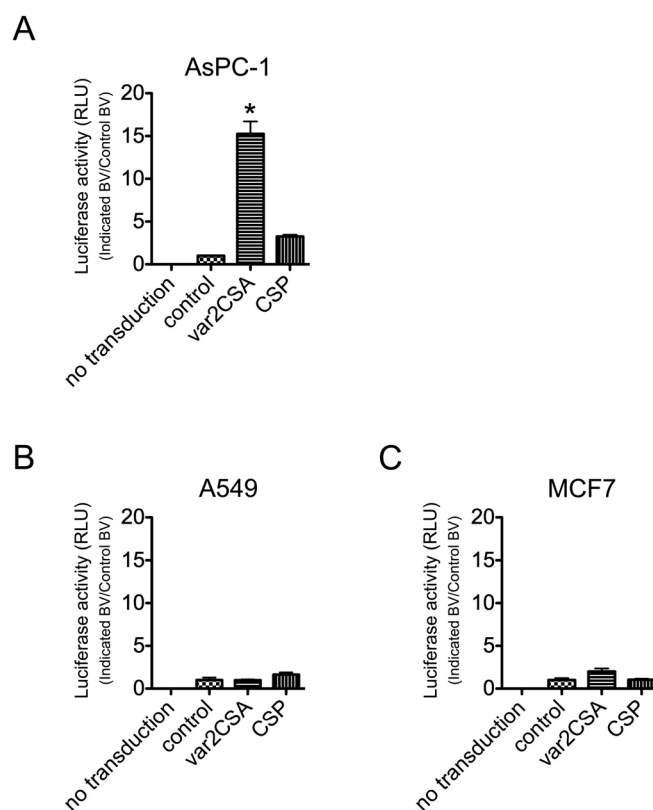


Fig. 4. Transduction of Additional Cancer Cell Lines Using var2CSA-Displaying BV

(A) AsPC-1, pancreatic cancer cell line, (B) A549, lung cancer cell line, and (C) MCF7, breast cancer cell line. BVs (1×10^6 TCID₅₀) were added to the cells. After 24h, the luciferase activity of the cell extracts was measured. Fold activation of luciferase activity of indicated BVs compared with control BV ($n = 3$ for each group) was calculated as the RLU of indicated BV transduced cells/the RLU of control BV transduced cells. Bars, indicating fold activation, represent the mean \pm S.E. Asterisks indicate a significant difference compared to the control or CSP type virus ($p < 0.05$). The experiments were performed three times, and representative data are shown.

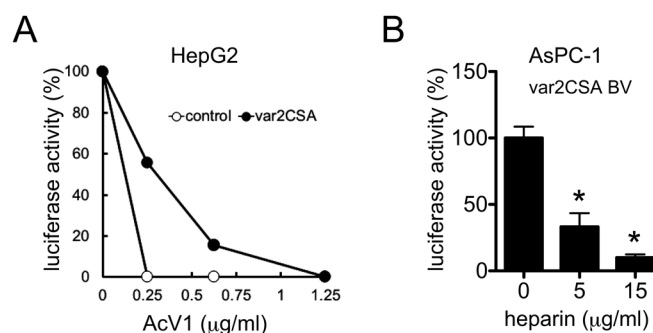


Fig. 5. Transduction Using var2CSA-Displaying BV Is Dependent on Viral gp64 Activity

(A) BVs (3.3×10^5 TCID₅₀) pre-incubated with dose-escalated AcV1 antibody were added to HepG2 cells ($n = 1$ for each group). After 24h, luciferase activity of cell extracts was measured. The value of luciferase activity in the graph was calculated as the RLU of samples with AcV1/the RLU of that without AcV1 in each BV. (B) var2CSA type BV (2×10^6 TCID₅₀) pre-incubated with heparin (final concentration: 5, or 15 μ g/mL) were added to AsPC-1 cells ($n = 3$ for each group). After 24h, luciferase activity of cell extracts was measured. The value of luciferase activity in the graph was calculated as the RLU of samples with heparin/the RLU of that without heparin. Bars represent the mean \pm S.E. Asterisks indicate a significant difference compared to the group without heparin ($p < 0.05$). The experiments were performed twice, and representative data are shown.

Transduction Using var2CSA-Displaying BV Depends on gp64 Activity Previous studies have demonstrated that the viral gp64 protein on BV virions plays a critical role in gene

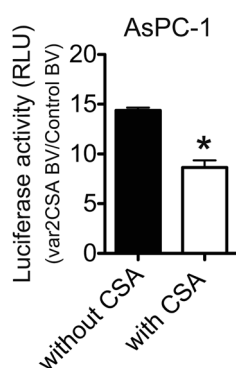


Fig. 6. Transduction Activity of var2CSA-Displaying BV Is Reduced by the Addition of Free CSA

BVs (2×10^6 TCID₅₀) pre-incubated with CSA (final concentration: $1 \mu\text{g/mL}$) were added to AsPC-1 cells. After 24 h, the luciferase activity of the cell extracts was measured. Bars represent the ratio of var2CSA BV to control BV with or without CSA ($n = 3$ for each group). Asterisks indicate a significant difference compared to those without CSA ($p < 0.05$). The experiments were performed twice, and representative data are shown.

transduction in mammalian cells.^{18,24}) After gp64 interacts with heparan sulfate, one of the extracellular glycosaminoglycans expressed on the cell surface,²⁵) virion particles enter cells *via* endocytosis, and viral DNA molecules are imported into the nucleus. To investigate the role of gp64 in the transduction of var2CSA-displaying BV, the activity of gp64 on BV virions was inhibited using an AcV1 monoclonal antibody. AcV1 binds to gp64 and blocks the transduction ability of BVs.^{18,24}) As shown in Fig. 5A, transduction activity of var2CSA BV as well as control BV was abolished in HepG2 cells following the addition of AcV1, demonstrating that gene transduction by var2CSA BV is gp64-dependent. Similar results showing gp64-dependent gene transduction by var2CSA BV were also obtained in AsPC-1 cells (data not shown). The addition of free heparin molecules competes with heparan sulfate on the cells for BVs interactions, resulting in a reduction in transduction efficiency.^{18,26}) As shown in Fig. 5B, the addition of free heparin significantly inhibited the transduction activity of var2CSA-displaying BV in AsPC-1 cells. These results demonstrate that var2CSA-displaying BV may use the transduction pathway of gp64-heparan sulfate in cancer cells.

Transduction Activity of var2CSA-Displaying BV Was Reduced by the Addition of Free CSA Previous studies have demonstrated that there is a structural difference between pl-CSA and typical CSA molecules,²⁷) but the typical CSA molecule (for example, those which are commercially available) in soluble form could compete with pl-CSA for binding of var2CSA protein to cancer cells.¹⁷) To investigate the effect of CSA on transduction by var2CSA type BV, commercially available CSA molecules were used. If var2CSA BV interacts with pl-CSA expressed by cancer cells, the addition of free CSA molecules should reduce the transduction efficacy of var2CSA BV. In the transduction experiments, free CSA molecules with BVs were added to the AsPC-1 cells. As shown in Fig. 6, under conditions without CSA addition, var2CSA BV significantly increased transduction activity in AsPC-1 cells. In contrast, with the addition of free CSA molecules, while the transduction efficiency of control BV did not decrease (data not shown), the transduction efficiency of var2CSA BV was significantly decreased. These results suggest that the interaction between var2CSA BV and pl-CSA molecules in can-

cer cells is involved in the augment of transduction efficiency by var2CSA BV.

DISCUSSION

In this study, we developed var2CSA-displaying BV to improve gene transduction in cancer cells. This var2CSA BV exhibited several features that differentiated it from the previously developed control BV or CSP BV.¹⁸) Application of var2CSA BV resulted in a larger percentage of cells that were successfully transduced, even when compared with CSP BV. This increase in transduction could confer significant advantages in the case of delivering suicide genes or apoptosis-inducing genes into target of cancer cells, as it could enhance cytotoxic efficacy of the cancer gene therapy. These studies could provide further valuable information about usefulness of var2CSA type BV. Moreover, var2CSA BV has the possibility of significant enhancement of gene transduction in hepatic and pancreatic cancer cells compared with that in control BV. These features of var2CSA BV may provide advantages in the development of new cancer gene therapies, especially in hepatic and pancreatic cancers. Previous studies have suggested the use of the var2CSA protein to detect pancreatic cancer cells in patient samples.²³) We previously demonstrated that the conferment of complement regulatory proteins to CSP BV induced resistance against inactivation by the human complement system.^{28,29}) A similar strategy could be applied to var2CSA BV, which would increase gene transduction as well as efficacy of therapeutics *in vivo*.

We examined the effects of var2CSA BV in A549 cells (lung cancer cell line) and MCF7 cells (breast cancer cell line), both of which the var2CSA protein has been shown to bind to. Depending on the amount of virus used, control BV increased gene transduction in A549 and MCF7 cells (data not shown). Therefore, these cancer cells are unlikely to be resistant to BV-mediated gene transduction, even though A549 and MCF7 cells are considered to be less permissive than HepG2 cells. The reason why var2CSA BV did not show a significant effect on A549 and MCF7 cells is unknown. Previous studies have demonstrated that the var2CSA protein binds to many different cancer cell types.¹⁷) However, a direct comparison of var2CSA binding affinity between cancer cells has not yet been reported. The varying experimental conditions and the expression level of pl-CSA in each cancer cell line may explain the differences between binding and transduction efficiency in cancer cells. Therefore, the enhanced transduction efficacy of var2CSA BV seen in this study may not be observed in all cancer cell lines in which var2CSA binding has been demonstrated in previous studies. In many of these studies, a truncated-type var2CSA protein containing DBL1X-ID2a (called rVAR2) was used,¹⁷) which is different from the var2CSA construct used in this study. We are now investigating DBL1X-ID2a type (rVAR2 type) BV as well as ID1-DBL2Xb type BV. ID1-DBL2Xb region is the minimum region for CSA binding.²¹) Further analysis using these new BVs together with this study could provide additional information about the possibility of using var2CSA BV to transduce cancer cells, and in potential gene therapies. The previous study¹¹) demonstrates that BVs displaying tumor-homing peptides enhanced transduction efficiency in MDA-MB-435 cells (breast cancer cell line, now recognized as melanoma cell line) and

HepG2 cells, although the data of other cancer cells have not been shown there. The tumor-homing peptides by themselves are capable of being internalized by cancer cells, suggesting it would be a different mechanism from that of transduction by var2CSA type BV in this study. The recombinant BVs which have the combination of var2CSA with other cancer cell-tropic molecules could enhance transduction efficiency in wider types of cancer cells.

This study demonstrates that the mechanisms of transduction by var2CSA BV are dependent on the gp64-heparan sulfate pathway and CSA-involved pathway, although these have not been fully elucidated. The activity of gp64 is diverse and essential for BV-mediated gene transduction in mammalian cells,^{18,24)} including binding to heparan sulfate on the cell surface, and fusion of viral and endosomal membranes. Previous studies have demonstrated that the var2CSA protein itself has internalization activity in cancer cells, whereas internalized var2CSA protein is released outside of cells.³⁰⁾ We investigated the dependency of gene transduction by var2CSA BV on gp64 function. The results in Fig. 5A show the indispensable role of gp64 in var2CSA BV transduction and that it is unlikely for var2CSA type BV to utilize the gp64-independent pathway as well as control type BV. In contrast, transduction efficiency by var2CSA BV was partly inhibited by the addition of free CSA molecules (Fig. 6). This result suggests that the interaction between the var2CSA protein on the BV virions and CSA (pl-CSA) expressed on cancer cells may increase successful entry of BV particles into the gp64-dependent transduction pathway, which may lead to a larger percentage of successfully transduced cells. Partial inhibition by CSA could be due to the difference between the typical CSA and pl-CSA molecules, which are derived from cancer cells or the placenta. There are many species of heparinase and chondroitinase, varying the substrate specificity. The analysis using these enzymes would provide more information about the requirement of glycosaminoglycan structure for the transduction pathway by var2CSA type BV.

The reasons why var2CSA type BV increased EGFP positive percentage within cells but not MFI of EGFP-expressing cells (Fig. 3B) have not been fully elucidated yet. In our previous study, CSP type BV increased transport of BV genome DNA into nucleus.¹⁸⁾ This could contribute to increase transgene expression in cells with BV entry. It is known that CSP interacts with heparan sulfate on the surface of cells as same as viral gp64.^{19,25)} The facilitation of gp64-dependent transduction pathway by CSP type BV would result in MFI increase within EGFP positive cells as well as percentage of EGFP positive cells compared with control BV as shown in Fig. 3B.

In contrast, var2CSA type BV may use the transduction pathway, which is different from control or CSP type BV as discussed above. In general, the phenotypes of cancer cells are not necessarily homogeneous in each cell level. Some cell population may be easy to accept BVs, but some population may not be so easy. The susceptibility of BVs mediated-gene transduction may be varied within total cell population. Var2CSA would interact with CSA on the surface of cells and this event may increase the chance of passing of BVs particles into gp64-dependent transduction pathway. As result of cooperation of var2CSA and gp64, var2CSA type BV could enter into cells in which BVs (e.g. control or CSP type BV) are usu-

ally not efficiently acceptable.

However, expression level of transgene in transduced cells may be low in those cases, because gp64-dependent transduction pathway originally may be not efficiently worked in transduction-refractory cells. As other possibility, the addition of var2CSA may exert negative effect on transgene expression beside positive effect discussed above. As example, the interaction between var2CSA and CSA on cells may be too rigid to enter gp64-dependent transduction pathway. Collectively, the percentage of transgene (EGFP) transduced cells by var2CSA BV was increased compared with control BV, although MFI in transduced cells was not so increased as shown in Fig. 3B. This is consistent with the increase of measured luciferase activity, which means total gene transduction in Fig. 3D.

In sum, in this study, BV displaying var2CSA was developed and examined for gene transduction ability in several human cancer cell lines. The expression of var2CSA increased BV-mediated gene transduction, a finding which has implications for the application of var2CSA molecules in cancer research, therapeutics, and diagnostics.

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Conflict of Interest The authors declare no conflict of interest.

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