

ORIGINAL ARTICLE

Targeted hepatocellular carcinoma proapoptotic BikDD gene therapy

L-Y Li^{1,2,3,8}, H-Y Dai^{1,8}, F-L Yeh¹, S-F Kan¹, J Lang⁴, JL Hsu⁴, L-B Jeng^{5,6}, Y-H Chen¹, Y-P Sher¹, W-C Lin⁷ and M-C Hung^{1,2,4}

¹Center for Molecular Medicine, China Medical University Hospital, Taichung, Taiwan; ²Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan; ³Department of Biotechnology, Asia University, Taichung, Taiwan; ⁴Department of Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; ⁵Department of Surgery, China Medical University Hospital, Taichung, Taiwan; ⁶Department of Medicine, China Medical University, Taichung, Taiwan and ⁷Department of Pharmacology, China Medical University, Taichung, Taiwan

Hepatocellular carcinoma (HCC), the third leading cause of cancer death in the world, is the most general type of primary liver cancer. Although current treatment modalities, such as liver transplantation, resection, percutaneous ablation, transarterial embolization, chemotherapy and radiotherapy are potentially curative, these methods are not universally applicable to all of HCC patients, especially for those with poor prognosis in which no effective remedy is available. Therefore, development of novel therapeutic approach for the treatment of HCC is urgently needed. In the current study, we developed a promising HCC-targeted gene therapy vector driven by liver cancer-specific α -fetoprotein promoter/enhancer coupled to an established platform technology. The activity of this expression vector is comparable with or even higher than that of strong cytomegalovirus (CMV) promoter and exhibits strong promoter activity in liver cancer cells/tumors, but has nearly no or very low activity in normal cells/organs *in vitro* and in orthotopic animal models *in vivo*. Its cancer specificity exceeds that of the CMV promoter, which expresses non-specifically in both normal and tumor cells. In addition, targeted expression of a therapeutic BikDD, a mutant of proapoptotic gene *Bik* effectively and preferentially killed liver cancer cells, but not normal cells and significantly repressed growth of HCC tumors, and prolonged survival in multiple xenograft and syngeneic orthotopic mouse models of HCC through intravenous systemic gene delivery. Importantly, systemic administration of BikDD by our expression vector exerted no systemically acute toxicity compared with CMV-BikDD in mice. Taken together, this study elucidates a relatively safe and highly effective and specific systemic gene therapy strategy for liver cancer, and is worthy of further development for future clinical trials.

Oncogene advance online publication, 13 December 2010; doi:10.1038/onc.2010.558

Keywords: liver cancer; α -fetoprotein; non-viral gene therapy; VISA

Introduction

Hepatocellular carcinoma (HCC) is the most general type of primary liver cancer and often diagnosed at late stage with the median survival rate of <3 months (Parkin *et al.*, 2005; El-Serag and Rudolph, 2007). Liver transplantation and surgical resection are considered as potential curative treatments for HCC, but not universally applicable to most HCC patients with concurrent liver cirrhosis, larger or metastatic liver tumor (Schafer and Sorrell, 1999; Hoofnagle, 2004). Most HCC patients experience liver cancer recurrence after resection or percutaneous ablation (Avila *et al.*, 2006). In addition, HCC has poor response to radiotherapy and is highly resistant to conventional chemotherapy (Llovet *et al.*, 2003; Avila *et al.*, 2006). Therefore, these therapeutic limitations make HCC a disease with no effective remedy and very poor prognosis.

Gene therapy is an attractive field that may have the potential to improve the therapeutic efficacy (Avila *et al.*, 2006). As HCC is a highly aggressive and metastatic disease, a systemic treatment is required for achieving an effective therapeutic outcome. Unfortunately, until recently, there has been no promising systemic therapy for HCC. Moreover, unlike other cancer types, such as head and neck or ovarian cancer in which regional treatment may be sufficient to improve therapeutic efficacy, liver cancer requires systemic gene delivery system to reach full effectiveness, which means that cancer-specificity will have a key role in limiting potential side effects in normal cells/tissues.

Previously, our laboratory developed a gene therapy approach that successfully addressed issues relating to cancer-specificity, expression efficiency and systemic toxicity, which are some of the major factors in determining therapeutic benefit under a gene therapy setting. The system consists of a cancer-specific promoter and transcriptional amplification module called VISA

Correspondence: Assistant Professor L-Y Li, Center for Molecular Medicine and Graduate Institute of Cancer Biology, China Medical University & Hospital, Taichung 40447, Taiwan.

E-mail: lyl@mail.cmu.edu.tw or Professor M-C Hung, Department of Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Unit 079, Houston, TX 77030, USA.

E-mail: mhung@mdanderson.org

⁸These authors contributed equally to this work.

Received 31 July 2010; revised 20 October 2010; accepted 4 November 2010

(VP16-GAL4-WPRE4 Integrated Systemic Amplifier), which allows expression of target gene at a level comparable with or even higher than the cytomegalovirus (CMV) promoter (Xie *et al.*, 2007). When coupled with a potent proapoptotic therapeutic gene, BikDD, under a cancer-specific expression vector and encapsulated in DOTAP:cholesterol (Templeton *et al.*, 1997), a liposome formulation that has been tested in a phase I clinical trial (NCT00059605), the VISA system demonstrated significant reduction of tumor without toxicity in pancreatic cancer model (Xie *et al.*, 2007). On the basis of the encouraging results, relevant toxicity studies to fulfill requirement for approval by the Food and Drug Administration to initiate a Phase I human clinical trial is completed and an investigator-initiated Investigational New Drug is currently under preparation. At the time of initial design, the versatility of the expression system was also taken into consideration so that it can be used in various cancer types by replacing promoter and therapeutic gene accordingly. Thus far, in addition to the pancreatic model mentioned above (Xie *et al.*, 2007), we have also demonstrated success in tumor reduction in both lung (Sher *et al.*, 2009) and ovarian (Xie *et al.*, 2009) cancer preclinical models.

In an attempt to develop a system that can also be applied to liver cancer, we searched for a liver cancer-specific promoter and identified several potential liver cancer-specific promoter candidates, including α -fetoprotein (AFP) basic promoter and AFP basic promoter combined with AFP enhancers (eAFP), both of which have already been known for being highly liver cancer specific (Kim and Wang, 2003; Lu *et al.*, 2003; Shi *et al.*, 2004; Suriawinata and Xu, 2004; Lemken *et al.*, 2005; Guan *et al.*, 2006). AFP, produced by the yolk sac and liver, is expressed at high levels during early development in fetus and decreases to a low or undetected level in adults (Abelev and Erasler, 1999). However, AFP expression is reactivated in liver neoplastic transformation. In about 70% of HCC, AFP is over-expressed and has been used as a biomarker for diagnosis of HCC (Stillwagon *et al.*, 1991; Izumi *et al.*, 1992). We integrated the AFP promoter with and without its enhancer elements into the VISA vector to express BikDD, a mutant that mimics constitutively active proapoptotic Bik protein from the Bcl-2 family that was shown to possess potent antitumor activity, and tested the newly constructed vector for expression efficiency and cell-killing activity as well as performed a safety study on its toxicity in DOTAP:cholesterol liposome nanoparticles. Here, we report an effective HCC gene therapy and show that this approach significantly induced apoptosis, which reduced tumor volume without systemic toxicity and prolonged survival in animal models, further validating the success of the VISA-based gene therapy approach as an alternative option for liver cancer treatment.

Results

AFP promoter is selectively activated in liver cancer cells

To test the promoter activity of AFP, we generated two expression systems, AFP-VISA-Luc and eAFP-VISA-

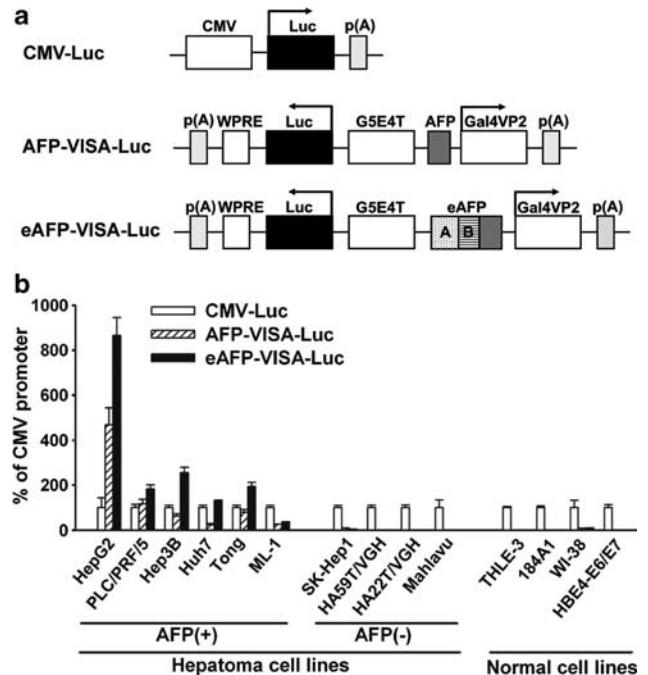


Figure 1 Molecularly engineered eAFP-VP16-Gal4-WPRE4 integrated systemic amplifier (VISA) is highly and specifically active in AFP-producing liver cancer cells. (a) Schematic sketch of engineered α -fetoprotein (AFP) promoter-based VISA constructs. CMV, cytomegalovirus promoter; eAFP, human AFP promoter combined with domain A and B of the AFP enhancer; Luc, luciferase gene; Gal4VP2, Gal4 DNA-binding domain fused with two copies of transcriptional activation domain of HSV-1 VP16; G5E4T, five copies of Gal4 responsive element in a minimal promoter; WPRE, the posttranscriptional regulatory element of the woodchuck hepatitis virus. (b) The activities and specificities of AFP-VISA and eAFP-VISA promoters. The CMV-Luc, AFP-VISA-Luc and eAFP-VISA-Luc were separately cotransfected with pRL-TK (an internal control) into the cells as indicated. After 48 h, the dual luciferase ratios were measured. The data represent the percentage of the dual luciferase ratio relative to the dual luciferase ratio for CMV-Luc, which was set as 100%. AFP (+), AFP-producing; AFP (-), non-AFP-producing. Each value represents the mean \pm s.d. of the three independent experiments performed in triplicate.

Luc that includes domain A and B of the eAFP (Figure 1a). Using luciferase (Luc) reporter assay in multiple liver cancer cell lines, we found that the human AFP promoter combined with enhancers (eAFP-VISA) was the most active in the majority of AFP-producing (AFP⁺) HCC cell lines (Figure 1b). To ensure that the constructed eAFP-VISA-Luc is still liver cancer specific and has an enhanced basal activity in liver cancer cells, but low or no activity in normal cells, we compared luciferase expression of AFP-VISA-Luc, eAFP-VISA-Luc and CMV-Luc in a panel of liver cancer cell lines, such as Huh7, HepG2, Hep3B, PLC/PRF/5, HA22T/VGH, HA59T/VGH, Tong, Mahlavu, SK-Hep-1 (from human), ML-1 (from mouse) and cell lines derived from normal tissues, for example, THLE-3 (hepatocyte), WI-38 (lung fibroblasts), 184A1 (breast epithelial) and HBE4-E6/E7 (tracheal epithelia). Indeed, the activity of eAFP is consistently higher than that of AFP and CMV promoters in AFP⁺ liver cancer cell lines, with the exception of the mouse ML-1 cancer cells (Figure 1b).

Remarkably, we observed signals of eAFP-VISA in AFP⁺, but virtually no activity in non-AFP-producing (AFP⁻) liver cancer cells and normal cells, indicating that this expression system is specific in AFP⁺ liver cancer cells. Contrary to AFP, the CMV promoter exhibited high activity not only in cancer, but also in normal cells. On the basis of these results, eAFP-VISA

displayed desirable features of both liver cancer-specificity and high basal activity features *in vitro*.

To determine whether eAFP-VISA targets transgene expression in liver cancer in mouse models *in vivo*, we incorporated plasmid DNA into extruded DOTAP:cholesterol liposome (Templeton *et al.*, 1997) to form nanoparticle complexes, which were then intravenously (i.v.) injected into mice bearing Huh7 (Figure 2) or HepG2 (Figure 3) liver tumors. Bioluminescent imaging of mice treated with eAFP-VISA-Luc showed that signals were detected in the abdomen, but nearly undetectable in the thoracic area (Figures 2a and 3a). Unlike eAFP-VISA-Luc, mice treated with CMV-Luc showed strong signals in the thoracic area (lungs and heart) (Figures 2a and 3a). These findings suggest that after systemic delivery, the CMV-Luc:liposome complexes flowed through the circulatory system, remained mostly in the lungs and heart, and expressed luciferase because of non-specificity of the CMV promoter, and this data is consistent with the previous studies (Xie *et al.*, 2007, 2009; Sher *et al.*, 2009).

Ex vivo imaging (Figures 2b and 3b) showed that the lungs and hearts of mice treated with CMV-Luc produced strong photon signals, which corresponded to the signals observed in the thoracic area shown in Figures 2a and 3a. Luciferase expression as indicated by photon signals generated from eAFP-VISA was stronger than that of the CMV promoter in the tumor. In addition, quantitative biodistribution analysis demonstrated that unlike CMV-Luc, eAFP-VISA has higher promoter activity in liver tumor, but is relatively inactive in normal organs (Figures 2b and 3b, bottom panels). Specifically, luciferase activity in the tumors of mice treated with eAFP-VISA-Luc was 4.5- or 1.4-fold higher in Huh7 or Hep2G, respectively, than tumors from mice treated with CMV-Luc. Not surprisingly, luciferase activity in the lungs and hearts of mice treated with CMV-Luc was higher than eAFP-VISA-Luc by 23- and 63-fold in Huh7 or Hep2G, respectively, compared with mice treated with eAFP-VISA-Luc ($P < 0.005$). We calculated the cancer-specific index (Chen *et al.*, 2004) by comparing luciferase activity in the tumor versus that in the lungs on the basis of the above quantitated biodistribution and determined it to be 4.3–4.5 for eAFP-VISA-Luc and 0.04 for CMV-Luc. These findings indicate that compared with the CMV promoter, systemically administered eAFP-VISA resulted in a 108- to 113-fold improvement in the selectivity of

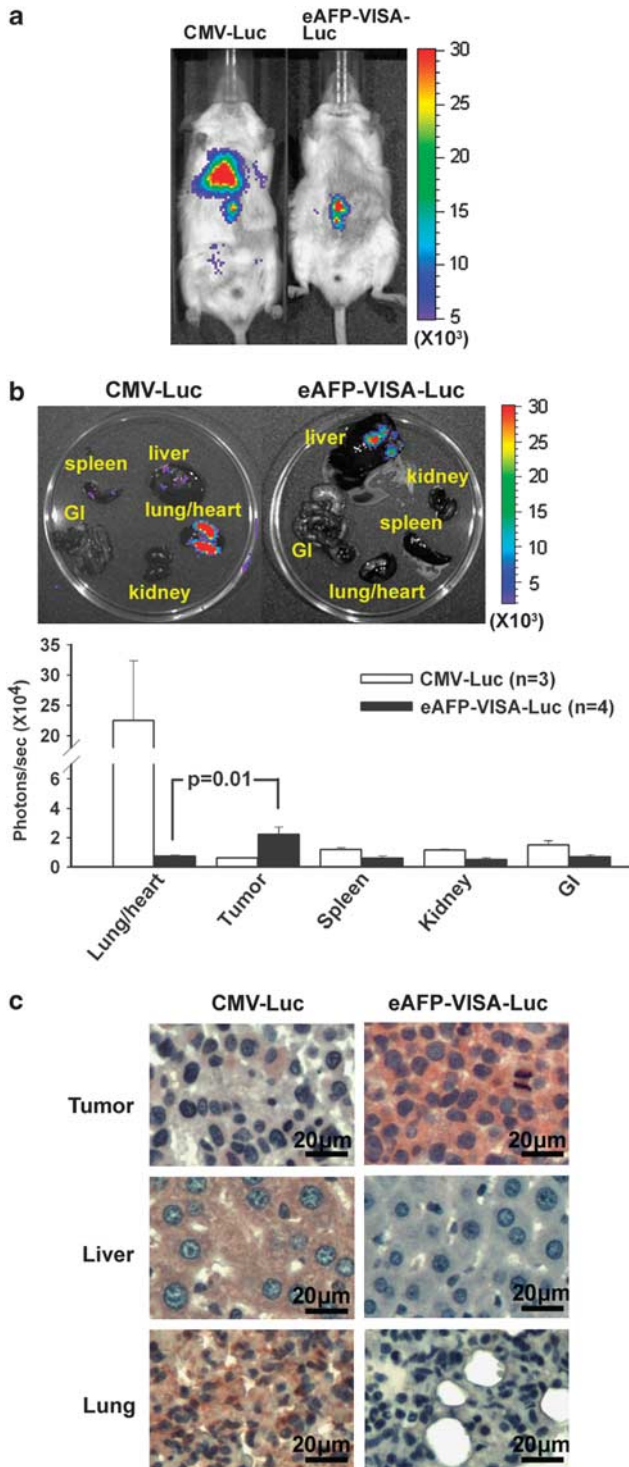
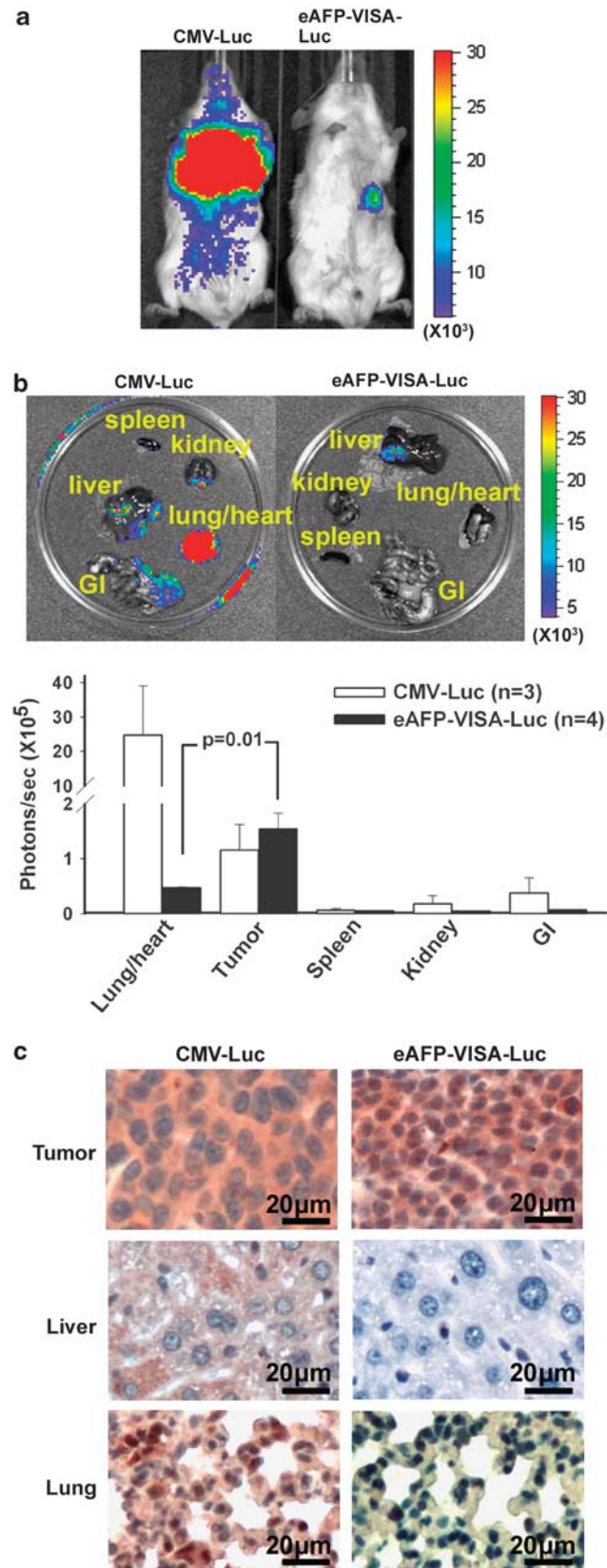


Figure 2 eAFP-VISA specifically targets the luciferase expression to the liver tumor in the orthotopic animal model bearing the Huh7 tumor. (a) SCID mice bearing the orthotopic Huh7 tumor were intravenously (i.v.) injected with 50 µg of liposomal DNA CMV-Luc and eAFP-VISA-Luc, respectively. After 48 h, mice were intraperitoneally injected with D-luciferin and subjected to *in vivo* imaging. (b) The organs from mice were further dissected for *ex vivo* imaging. The representative images are shown on the top panel. The intensity of images was quantified by the living image software and illustrated as mean with s.e.m. in the bottom panel. The data shown here are representative of two independent experiments. (c) Tissue samples from (b) were fixed in 4% formalin immediately. After fixation, the specimens were processed for immunohistochemical staining of firefly luciferase expression.

transgene expression in tumor versus lung (the usual site of vector sequestration). Immunohistochemical staining for luciferase from tissues also showed that eAFP-VISA

was tumor specific and not expressed in normal liver or in lungs (Figures 2c and 3c).



Targeted expression of therapeutic gene *BikDD* by *eAFP-VISA* preferentially kills AFP-producing liver cancer cells, but not normal cells *in vitro*

To investigate whether the eAFP-VISA vector can deliver a therapeutic gene to selectively kill liver cancer cells, we replaced the luciferase reporter gene with *BikDD* (eAFP-VISA-*BikDD*; Figure 4a) with its expression that has previously been shown to promote cancer cell death by inducing apoptosis in various preclinical models (Xie *et al.*, 2007, 2009; Sher *et al.*, 2009). The expression of *BikDD* driven by CMV promoter or eAFP-VISA was detected using western blot analysis after 24-h transient transfection (Supplementary Figure S1). We then performed experiments in a panel of liver cancer and normal cell lines as mentioned above. Using pUK21 (control plasmid), CMV-*BikDD* and eAFP-VISA-*BikDD*, we separately cotransfected each with CMV-Luc (the index of cell viabilities) into the cells as indicated. The cell killing activity was determined as measured by the loss of luciferase activity 48 h later and showed that expression of *BikDD* under the eAFP promoter in the VISA backbone more effectively killed AFP⁺ liver cancer cells than AFP⁻ liver cancer cells or normal cells (Figure 4b). As expected, CMV-*BikDD* has more general killing effect in both cancer and normal cells. We further demonstrated that eAFP-VISA-*BikDD* efficiently killed AFP⁺ primary liver cancer cells obtained from three individual patients (P1, P2 and P3) compared with the control vector (Figure 4c). Thus, eAFP-VISA-*BikDD* has the ability to preferentially kill both AFP⁺ liver cancer cell lines and primary liver cancer cells.

eAFP-VISA-BikDD inhibits tumor growth and enhances survival more effectively than CMV-*BikDD* in orthotopic xenograft mouse models

To evaluate the therapeutic effects of eAFP-VISA-*BikDD* on liver cancer *in vivo*, Severe-combined immune deficient (SCID) mice bearing the orthotopic luciferase-expression Huh7 (Huh7-Luc) liver tumors were *i.v.* injected with 10, 20 or 30 μg of eAFP-VISA-*BikDD* or 30 μg of eAFP-VISA (vector control) in liposomal complexes. Huh7-Luc is a luciferase-expressing stable

Figure 3 The eAFP-VISA specifically targets the luciferase expression to the liver tumor in the orthotopic animal model bearing the HepG2 tumor. (a) SCID mice bearing the orthotopic HepG2 tumor were *i.v.* injected with 50 μg of liposomal DNA CMV-Luc and eAFP-VISA-Luc, respectively. After 48 h, mice were intraperitoneally injected with D-luciferin and subjected to *in vivo* imaging. (b) The organs from mice were further dissected for *ex vivo* imaging. The representative images are shown on the top panel. The intensity of images was quantified by the living image software and illustrated as mean with s.e.m. in the bottom panel. The data shown here are representative of two independent experiments. (c) Tissue samples from (b) were fixed in 4% formalin immediately. After fixation, the specimens were processed for immunohistochemistry staining of firefly luciferase expression.

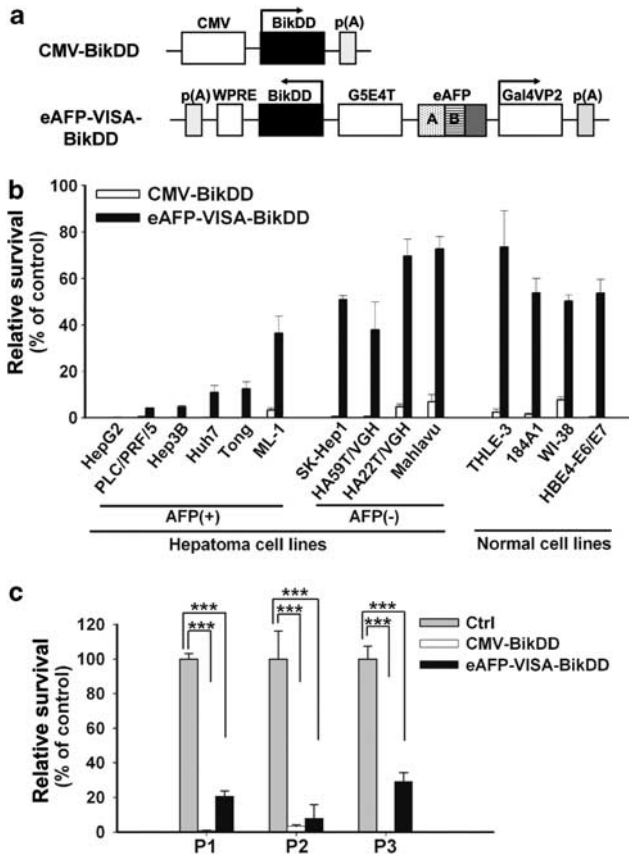


Figure 4 BikDD driven by eAFP-VISA effectively and preferentially kills AFP-producing liver cancer cells *in vitro*. (a) Schematic sketch of engineered α -fetoprotein (AFP) promoter-based VISA-BikDD constructs. CMV, cytomegalovirus promoter; eAFP, human AFP promoter combined with domain A and B of the AFP enhancer; Gal4VP2, Gal4 DNA-binding domain fused with two copies of transcriptional activation domain of HSV-1 VP16; G5E4T, five copies of Gal4 responsive element in a minimal promoter; WPRE, the posttranscriptional regulatory element of the woodchuck hepatitis virus. (b) The killing activities of therapeutic BikDD driven by CMV and eAFP-VISA promoters were carried out in HCC cells and normal cell lines. The vector control, CMV-BikDD and eAFP-VISA-BikDD were separately cotransfected with CMV-Luc (the index of cell viabilities) into the cells as indicated. After 48 h, the luciferase activities were measured in liver cancer cell lines and normal cell lines. The data were normalized with CMV-Luc activity in cells transfected with vector control, which was set as 100%. AFP (+): AFP-producing; AFP (-): non-AFP-producing. (c) BikDD expression driven by eAFP-VISA also kills AFP (+) primary human liver cancer cells effectively. *** indicates $P < 0.001$.

cell line generated to facilitate real-time imaging of tumors using IVIS (*in vivo* Imaging System, Xenogen, Alameda, CA, USA). As shown in Figure 5a, eAFP-VISA-BikDD significantly inhibited tumor growth in a dose-dependent manner compared with the control vector. We further compared the therapeutic efficacy between eAFP-VISA-BikDD and CMV-BikDD in SCID mice bearing the orthotopic Huh7-Luc tumors by i.v. injection of DNA:liposome nanoparticles containing 20 μ g of CMV-BikDD, eAFP-VISA-BikDD or eAFP-VISA DNA thrice weekly for 3 weeks. The results indicated that eAFP-VISA-BikDD inhibited tumor growth (Figure 5b) and prolonged mouse survival

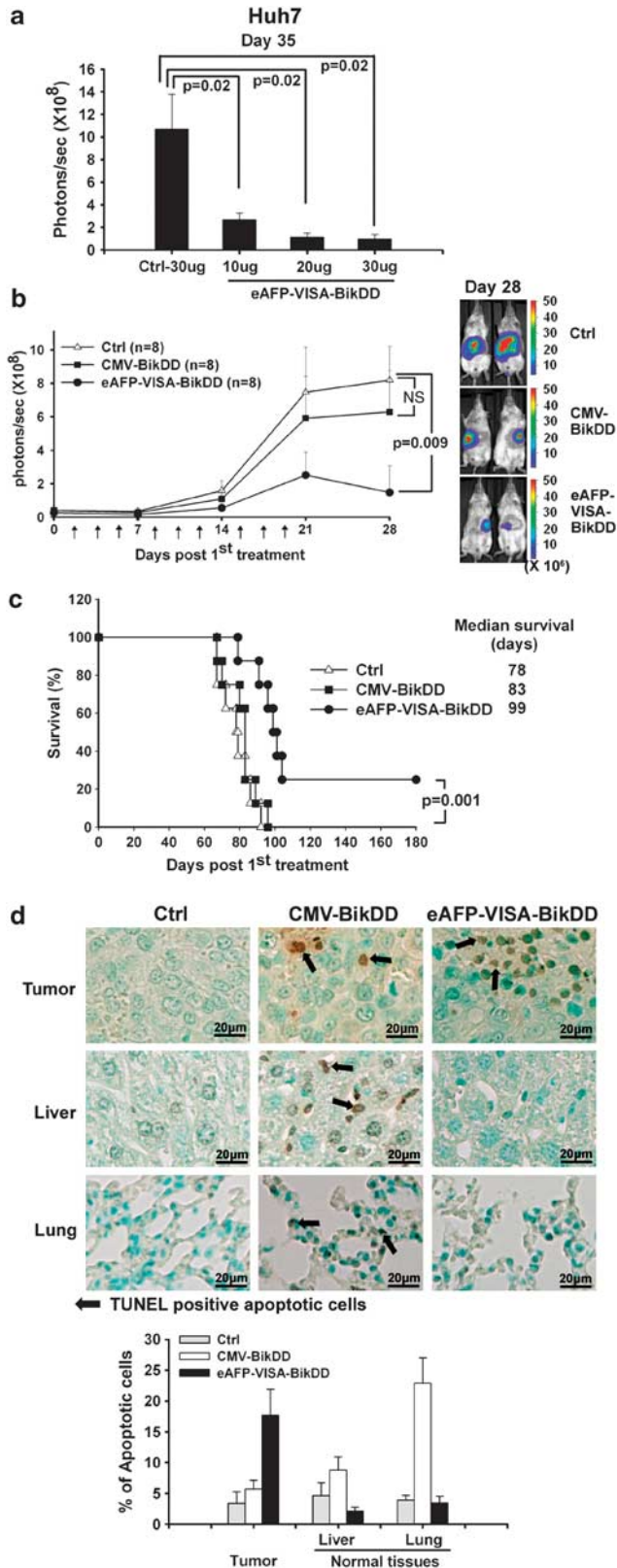
(Figure 5c) more effectively than CMV-BikDD after systemic delivery of nanoparticle complexes. We also carried out *in vivo* apoptosis detection (Figure 5d) and immunohistochemical staining of BikDD expression (Supplementary Figure S2a) in the tissues from mice bearing orthotopic Huh7-Luc tumors 2 days after the sixth gene therapy. Indeed, consistent with the expression of BikDD in liver tumor tissues and normal tissues (Supplementary Figure S2a), analyzes of apoptosis using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays showed that although eAFP-VISA-BikDD only induced apoptosis in tumor tissue, apoptosis was observed in normal liver and lung in addition to tumor tissues from mice treated with CMV-BikDD (Figure 5d). In tumor tissues, the percentage of apoptosis from eAFP-VISA-BikDD treatment was 3.1- and 5.2-fold higher than CMV-BikDD and control, respectively (Figure 5d, bottom panel). In contrast, the percentage of apoptosis in normal liver and lung of eAFP-VISA-BikDD-treated mice was relatively low and similar to that of the control although CMV-BikDD treatment produced high level of apoptosis even in the normal liver and lung tissues.

We also generated stable clone of HepG2 cell line expressing luciferase (HepG2-Luc) and carried out similar experiments as described above for Huh7. In the HepG2 model, we found that both eAFP-VISA-BikDD and CMV-BikDD inhibited tumor growth (Figure 6a), prolonged mice survival (Figure 6b), and induced approximately similar percentage of apoptosis in tumor cells (Figure 6c) albeit shorter median survival for mice treated with CMV-BikDD. Although these results indicated a differential tumor growth inhibition between Huh7 and HepG2 animal models under eAFP-VISA-BikDD and CMV-BikDD treatment, they seemed to be not only correlated with the expression of BikDD driven by eAFP-VISA or CMV in HepG2-induced tumors (Supplementary Figure S2b), but also consistent with the *in vivo* luciferase targeting assays shown in Figure 3 in which eAFP-VISA-Luc and CMV-Luc demonstrated similar activity in HepG2-induced tumors. Nonetheless, our data demonstrated that eAFP-VISA-BikDD consistently shows better therapeutic efficacy *in vivo*.

eAFP-VISA-BikDD inhibits tumor growth and metastasis and prolongs survival in orthotopic syngeneic mouse model

As SCID mice are immunodeficient, they are used to produce human xenograft to test tumor suppression activity for human cancer cell. To determine whether the therapeutic effect can also be observed in the immunocompetent status, we also examined tumor suppression activity using an intact immunocompetent mouse model. BALB/c mice bearing the ML-1 tumor were i.v. injected with nanoparticle complexes containing 20 μ g of CMV-BikDD, eAFP-VISA-BikDD or eAFP-VISA DNA thrice weekly for 3 weeks. A week after the last treatment, mice were killed and the tumors were dissected and weighed. Liver harvested from mice treated with both CMV-BikDD and eAFP-VISA-

BikDD significantly inhibited tumor growth compared with the control vector in orthotopic syngeneic mouse bearing ML-1 tumor after systemic delivery of



nanoparticle complexes (Figure 7a). However, eAFP-VISA-BikDD-treated mice demonstrated significantly longer survival than CMV-BikDD- or control vector-treated mice (Figure 7b). Consistent with the results from our xenograft model described above (Figure 6), we detected a significant increase in the percentage of apoptotic cells in tumor tissues from both CMV-BikDD- and eAFP-VISA-BikDD-treated mice compared with the control. Importantly, however, eAFP-VISA-BikDD did not induce apoptosis in normal liver tissues compared with CMV-BikDD (Figure 7c, bottom panel). We also investigated whether eAFP-VISA-BikDD could inhibit metastasis of ML-1 tumors, which are highly aggressive with metastatic ability. As shown in Table 1, five out of eight (63%) mice in control group developed lung metastasis. However, only two of ten (20%) mice after treatment with eAFP-VISA-BikDD showed lung metastasis, which was lower than that of CMV-BikDD-treated mice (30%), suggesting that systemic treatment with eAFP-VISA-BikDD also inhibited HCC metastasis.

eAFP-VISA-BikDD exerts no systemically acute toxicity in mice compared with CMV-BikDD

An ideal gene therapy system not only provides therapeutic efficacy, but also retains safety, that is, exerts very low or no toxicity. With this notion, we further evaluated systemic toxic effect of eAFP-VISA-BikDD in BALB/c mice by analyzing various blood tests including liver (aspartate transaminase; and alanine transaminase assays) and kidney functions (blood urea nitrogen assay). In Figure 8a, compared with CMV-BikDD, systemic administration of eAFP-VISA-BikDD produced virtually no acute toxicity in BALB/c mice based on the aspartate transaminase and alanine transaminase assays. Moreover, we did not observe

Figure 5 Expression of BikDD driven by eAFP-VISA promoter inhibits tumor growth and enhances survival more effectively than that driven by CMV promoter in orthotopic xenograft mouse models bearing Huh7-Luc liver cancer. (a) SCID mice bearing the orthotopic Huh7-expressing luciferase (Huh7-Luc) tumor were i.v. injected with liposomal DNA at indicated doses. The growth of tumors was monitored 2 weeks after the last gene therapy (day 35) by the real-time *in vivo* imaging IVIS system. The photon signals were quantified using Xenogen's living imaging software and illustrated as mean with s.e.m. (b) SCID mice bearing the orthotopic Huh7-Luc tumor were i.v. injected with 20 μ g of liposomal DNA CMV-BikDD, eAFP-VISA-BikDD or eAFP-VISA (vector control, Ctrl), thrice weekly for 3 weeks. The growth of tumors was monitored as in (a). The photon signals (left panel) were quantified using Xenogen's living imaging software and illustrated as mean with s.e.m.. The representative images are shown (right panel). NS, not significant (CMV-BikDD versus Ctrl); $P=0.009$ (eAFP-VISA-BikDD versus Ctrl). (c) The mice survival was analyzed. $P=0.001$ (eAFP-VISA-BikDD versus Ctrl). The data shown here are representative of two independent experiments. (d) Detection of *in vivo* apoptosis of tissue samples using TUNEL assays. SCID mice bearing orthotopic Huh7-Luc tumors were systemically injected with 20 μ g of plasmid DNA/liposome complex through tail vein. Two days after the sixth gene therapy, mice were killed and removed for detecting *in situ* apoptosis by TUNEL assay. The TUNEL-positive cells were stained brown and counted from randomly selected four fields per section. The data are represented as mean with s.d. Arrows indicate TUNEL-positive apoptotic cells.

an increase in blood urea nitrogen at any assayed time points in mice treated with control, CMV-BikDD or eAFP-VISA-BikDD (data not shown). In addition, eAFP-VISA-BikDD in DNA:liposome complexes showed 100% event-free survival compared with 60% in CMV-BikDD-treated mice at a dose of 100 μ g DNA (Figure 8b). Together, we demonstrated that eAFP-VISA-BikDD nanoparticle-mediated systemic gene

therapy provides an effective therapeutic approach in multiple animal models with relatively safe profile compared with the commonly used CMV promoter-driven vector as well as scientific basis for consideration of future clinical trials.

Discussion

The fetal serum protein, AFP, is highly expressed in liver cancer and has been used as a tumor biomarker. HCC, which is a highly aggressive and metastatic disease, requires a systemic treatment to achieve an effective therapeutic outcome. Unfortunately, until recently there has been no promising systemic therapy for HCC. In our study, we developed a non-viral AFP promoter-driven expression vector for systemic liver cancer gene therapy. Using both *in vitro* and *in vivo* approaches, we tested this vector extensively for activity and cancer specificity/tumor targeting as well as toxicity. Our data demonstrated the superiority of our engineered eAFP-VISA system that harbored desirable features of both liver cancer-specificity and high basal activity features to allow higher expression of target gene only in liver tumors, but not normal liver tissues or other organs such as the lungs/heart. First demonstrated in pancreatic cancer, the VISA system expressing a potent proapoptotic protein, BikDD, dramatically reduced pancreatic tumor in animal model. As the success of the first VISA-mediated gene therapy, we have repeatedly demonstrated therapeutic benefit in other cancer types including lung (Sher *et al.*, 2009), ovarian (Xie *et al.*, 2009) and liver as described here. Numerous groups have also exploited the AFP promoter for transcriptional targeting of therapeutic gene, such as tBid (Ma *et al.*, 2010), TRAIL (Ren *et al.*, 2006), interleukin-2 (Bui *et al.*, 1997) and herpes simplex thymidine kinase (Mawatari *et al.*, 1998; Ido *et al.*, 2001) in HCC. However, most of the studies utilized viral vector for

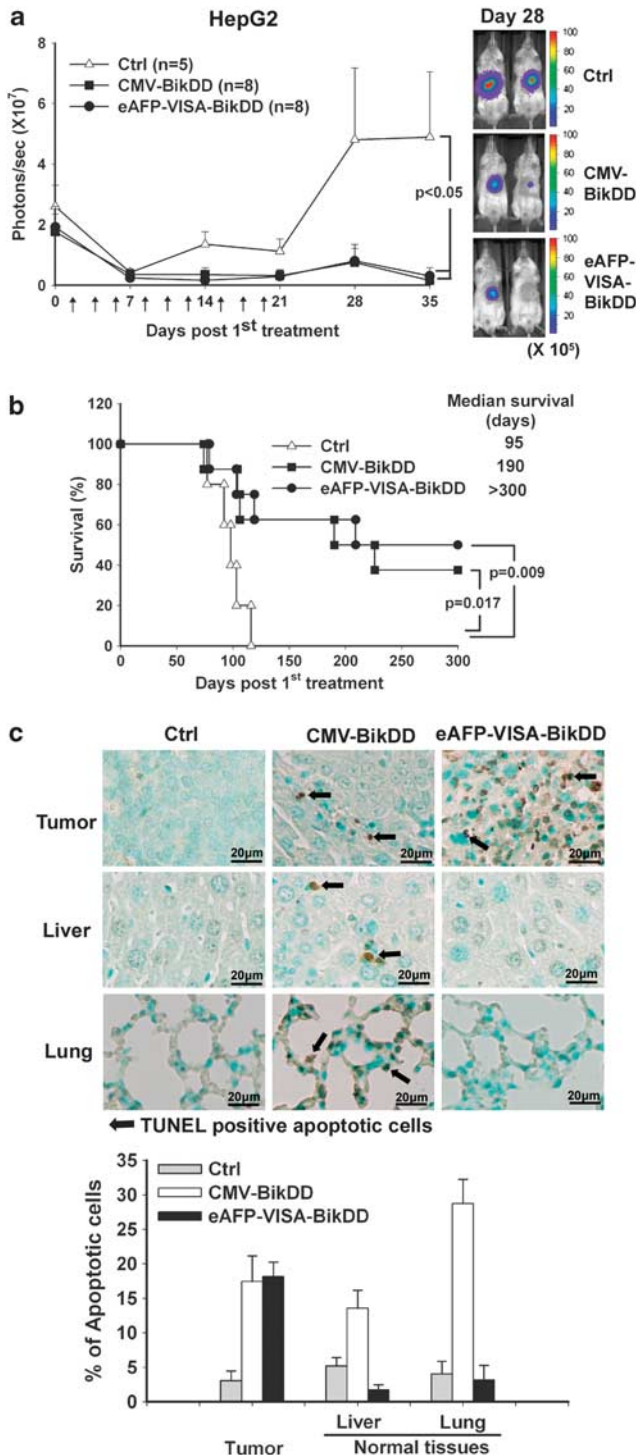


Figure 6 Expression of BikDD driven by eAFP-VISA promoter inhibits tumor growth and enhances survival more effectively than that driven by CMV promoter in orthotopic xenograft mouse models bearing HepG2-Luc liver cancer. **(a)** SCID mice bearing the orthotopic HepG2-expressing luciferase (HepG2-Luc) were i.v. injected with 20 μ g of liposomal DNA CMV-BikDD, eAFP-VISA-BikDD or eAFP-VISA (vector control, Ctrl), thrice weekly for 3 weeks. The growth of tumors was monitored by the real time *in vivo* imaging IVIS system. The photon signals (left panel) were quantified using Xenogen's living imaging software and illustrated as mean with s.e.m. The representative images are shown (right panel). $P < 0.05$ (both CMV-BikDD or eAFP-VISA-BikDD versus Ctrl). **(b)** The mice survival was analyzed. $P = 0.009$ (eAFP-VISA-BikDD versus Ctrl). $P = 0.017$ (CMV-BikDD versus Ctrl). The data shown here are representative of two independent experiments. **(c)** Detection of *in vivo* apoptosis of tissue samples using TUNEL assays. SCID mice bearing orthotopic HepG2-Luc tumors were systemically injected with 20 μ g of plasmid DNA/liposome complex through tail vein. Two days after the sixth gene therapy, mice were killed and organs were removed for detecting *in situ* apoptosis by TUNEL assay. The TUNEL-positive cells were stained brown and counted from randomly selected four fields per section. The data are represented as mean with s.d. Arrows indicate TUNEL-positive apoptotic cells.

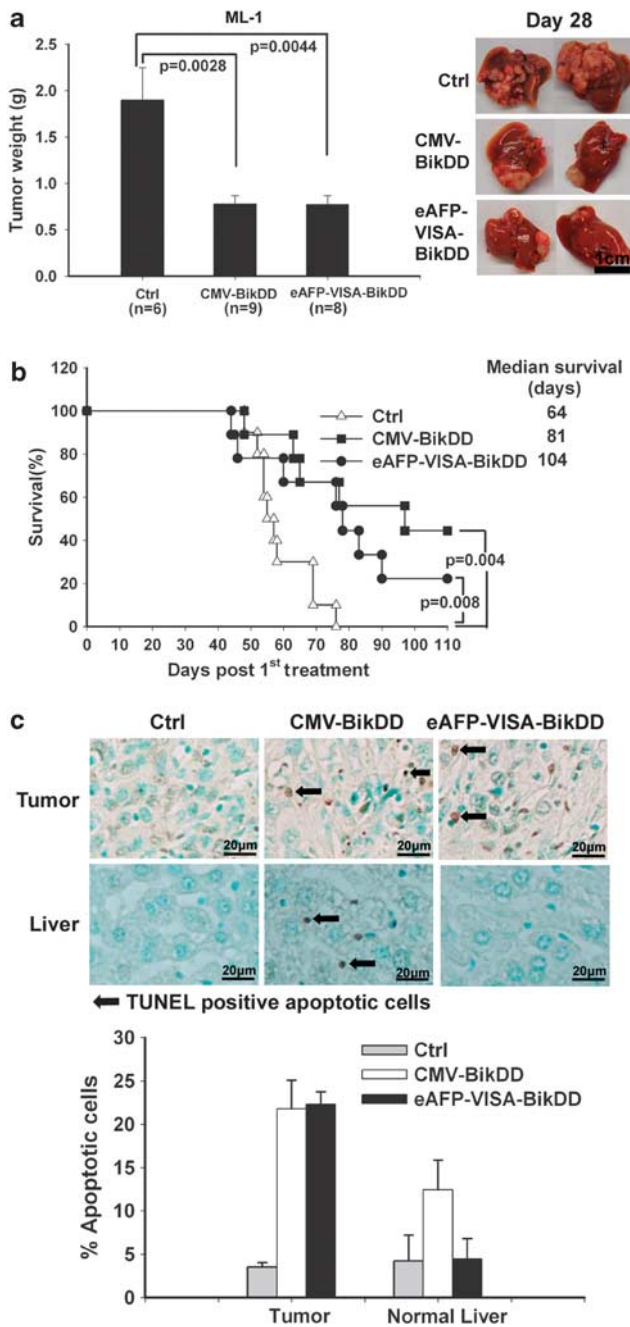


Figure 7 eAFP-VISA-BikDD inhibits tumor growth and prolongs survival in orthotopic syngeneic mouse model *in vivo*. (a) BALB/c mice bearing the ML-1 tumor were i.v. injected with 20 μ g of liposomal DNA CMV-BikDD, eAFP-VISA-BikDD or eAFP-VISA (vector control, Ctrl), thrice weekly for 3 weeks. One week after the last treatment, the mice were killed, and the tumors were dissected and weighed. Representative images from each group were shown on the right panel. The data are represented as mean with s.e.m. Bar = 1 cm. (b) BALB/c mice bearing the ML-1 tumor were treated as in (a) and the mouse survival was analyzed. (c) Detection of *in vivo* apoptosis of tissue samples using TUNEL assays. Tissue specimens from (a) were fixed and processed for detecting *in situ* apoptosis by TUNEL assay. The TUNEL-positive cells were stained brown and counted from randomly selected four fields per section. The data are represented as mean with s.d. Arrows indicate TUNEL-positive apoptotic cells. Error bars indicate s.d.

Table 1 eAFP-VISA-BikDD inhibits metastasis of HCC

Gene therapy	Total mice	Lung metastasis	Lung metastasis (%)
Control	8	5	63
CMV-BikDD	10	3	30
eAFP-VISA-BikDD	10	2	20

Abbreviations: CMV, cytomegalovirus; eAFP, α -fetoprotein enhancers; HCC, hepatocellular carcinoma; VISA, VP16-GAL4-WPRE4 Integrated Systemic Amplifier.

target gene expression. Our gene therapy approach, in contrast, is unique because it does not rely on the use of viral vectors, which could induce side effects. In addition, the high AFP⁺ liver-cancer specificity of the eAFP-VISA-BikDD vector produced strong tumor suppression activity and prolonged survival in multiple orthotopic tumor mouse models yet retained a relatively safe profile.

It is interesting to note that the relative activities between CMV promoter and eAFP-VISA are opposite when measured by expression levels of BikDD protein and luciferase activity (Supplementary Figure S1 and Figure 1b). As shown in Supplementary Figure S1, the BikDD expression driven by CMV was higher than that driven by eAFP-VISA, which is correlated with the killing effects of CMV-BikDD and eAFP-VISA-BikDD on AFP⁺ human HCC cell lines (HepG2, PLC/PRF/5, Hep3B, Huh7 and Tong) shown in Figure 4b. However, in Figure 1b, eAFP-VISA directed stronger luciferase activity, which is comparable with or even higher than that of CMV promoter, in AFP⁺ human HCC cell lines indicated above. It is conceivable that luciferase and BikDD complementary DNA or their corresponding messenger RNA might respond differently in the two constructs. In this regard, the posttranscriptional regulatory element of the woodchuck hepatitis virus (WPRE), which is known to affect messenger RNA stability and translational rate (Zufferey *et al.*, 1999), in the VISA construct might have differential effects on two messenger RNAs or other mechanisms might contribute to the differential effects. This issue could be interesting to further pursue in a systematical manner in the future. Nevertheless, the important point in the current study is that targeted expression of therapeutic *BikDD* gene by eAFP-VISA more effectively killed AFP⁺ human HCC cell lines (about 90–100% killing effect comparable with the CMV-BikDD's) and mouse ML-1 cells (about 65% killing effect) than AFP⁻ liver cancer cells or normal cells (Figure 4b).

In addition, in *in vivo* orthotopic liver tumor mouse models, we observed that the expression of BikDD driven by eAFP-VISA was highly and specifically expressed in liver tumors, but not in normal livers or lungs of both eAFP-VISA-BikDD-treated Huh7 (Supplementary Figure S2a) and HepG2 (Supplementary Figure S2b) mouse models. However, CMV-BikDD exhibited relatively low BikDD expression in liver tumor of CMV-BikDD-treated Huh7 mouse model compared with that of CMV-BikDD-treated HepG2 mouse model while BikDD was non-specifically expressed in normal

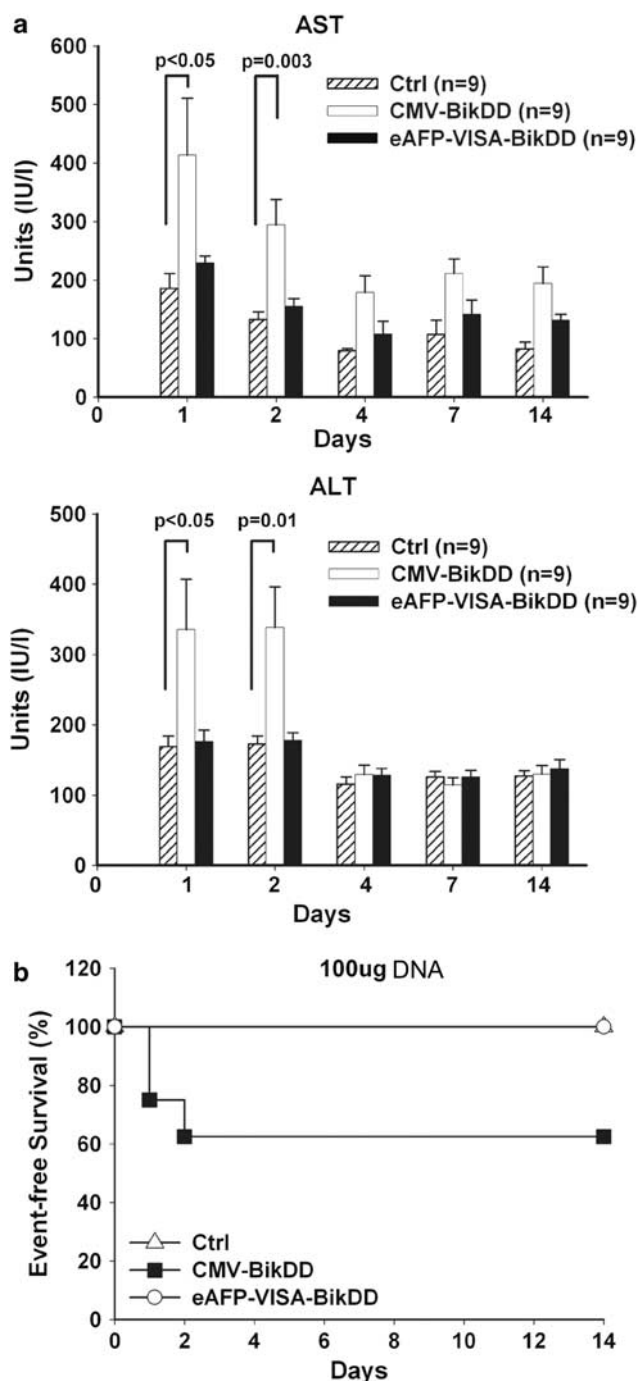


Figure 8 eAFP-VISA-BikDD administration exerts no systemically acute toxicity compared with CMV-BikDD in immunocompetent mice. (a) The 12-week old of BALB/c mice were received a single dose of 50 μ g of liposomal DNA eAFP-VISA (vector control, Ctrl), CMV-BikDD or eAFP-VISA-BikDD by tail vein injection. Blood was collected at indicated time and serum was separated for further analysis of the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The data are represented as mean with s.e.m. (b) Survival curves for mice with intravenous injection of 100 μ g of liposomal DNA complex were analyzed.

liver or lungs of both CMV-BikDD-treated Huh7 and HepG2 mouse models. These results may explain why CMV-BikDD is less effective in tumor cell killing

in the Huh7 mouse model (Figure 5b) although eAFP-VISA-BikDD's tumor cell killing is effective in both tumor mouse models (Figures 5b and 6a). It should be mentioned that these differential BikDD expression and tumor growth inhibition between Huh7 and HepG2 animal models under eAFP-VISA-BikDD and CMV-BikDD treatment are consistent with the *in vivo* luciferase targeting assays shown in Figures 2 and 3 in which eAFP-VISA-Luc and CMV-Luc exerted differential luciferase activity and expression in Huh7-induced tumors (Figure 2), but showed similar luciferase activity and expression in HepG2-induced tumors (Figure 3). Notwithstanding, these results further manifest liver cancer-specificity/targeting feature of eAFP-VISA-BikDD, that is, eAFP-VISA-BikDD consistently and repeatedly showed liver cancer-specificity/targeting and better liver cancer-killing effect in multiple orthotopic mouse models of liver cancer that we have tested, further highlighting the importance of cancer specificity/targeting of gene therapy vector, in particular, when the therapeutic gene is delivered systemically such as using i.v. injection, which can be a crucial factor to enhance therapeutic efficacy *in vivo*. Taken together, our data suggest that eAFP-VISA-BikDD is an excellent candidate to be tested in future clinical trials and might serve as an alternative option for systemic liver cancer treatment.

Materials and methods

Cell lines

The HCC cell lines HepG2, Hep3B, Huh7, HA22T/VGH, HA59T/VGH, PLC/PRF/5, Tong/HCC, Mahlavu and SK-Hep1 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD, USA), antibiotics (penicillin 100 U/ml and streptomycin 100 μ g/ml) and 1% non-essential amino acids (Life Technologies, Carlsbad, CA, USA) in a 37 $^{\circ}$ C humidified incubator containing 5% CO₂. The immortalized normal liver cells (THLE-3), normal lung fibroblasts (WI-38), immortalized normal mammary epithelial (184A1) and immortalized normal bronchial epithelial (HBE4-E6/E7) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained according to the manufacturer's instructions. The mouse liver cancer cell line ML-1, derived from BALB/c mice (Chen *et al.*, 1992), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37 $^{\circ}$ C under 5% CO₂. All of these cell lines were free of mycoplasma before start of experiments.

Primary human liver cancer cells were obtained from patients with liver cancers who received curative hepatic resection. The study was approved by the Institutional Review Board of China Medical University. Tissues were collected under patients' consent.

Plasmid construction and preparation

To construct the AFP-VISA-Luc, the AFP promoter was excised from pGL3-AFP-Luc using *SpeI* and *EcoRV* and cloned into pGL3-C-VISA-Luc plasmid (Xie *et al.*, 2007) to replace CCKAR promoter. For construction of eAFP-VISA-Luc plasmid, domains A and B of eAFP and the AFP

promoter were separately amplified by PCR with linker *EcoRV* and *SpeII* restriction sites (Nakabayashi *et al.*, 1991; Takahashi *et al.*, 2002) and inserted into pGL3-C-VISA-Luc plasmid. To construct the pUK21-eAFP-VISA-BikDD plasmid, the eAFP was released from pGL3-eAFP-VISA-Luc using *EcoRV* and *SpeII* and inserted into pUK-Survivin-VISA-BikDD (Sher *et al.*, 2009).

The plasmid DNA used in animal study was purified by an Endo-free Mega Prep Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The amount of endotoxin in plasmids was determined by a chromogenic Limulus amoebocyte clotting assay (QCL-1000 kit, BioWhittaker, Walkersville, MD, USA), and the level of endotoxin was <10 EU per mg of DNA.

Evaluation of promoter activity and cytotoxicity

The extruded DOTAP:cholesterol liposomes were produced in our lab according to Dr Nancy Templeton's protocol (Templeton *et al.*, 1997). Cells cultured in 24-well plates were cotransfected with 0.8 µg of CMV-Luc, AFP-VISA-Luc or eAFP-VISA-Luc and 50 ng of internal control plasmid pRL-TK using extruding DOTAP:cholesterol liposomes. After 48 h, cells lysates were harvested for detecting luciferase activity of firefly and renilla by dual luciferase assay system (Promega, Madison, WI, USA) using the FB12 Luminometer (Berthold Technologies, Bad Wildbad, Germany).

To determine the *in vitro* killing effect of various BikDD constructs, cells were cotransfected with 0.8 µg of control vector, CMV-BikDD, or eAFP-VISA-BikDD and 50 ng of reporter plasmid CMV-Luc (the index of cell viabilities). After 48 h, the cells were harvested for detecting luciferase activity. The luciferase activity in cells transfected with CMV-BikDD or eAFP-VISA-BikDD was compared with that in cells transfected with control plasmid (set as 100%).

Stable cell lines expressing firefly luciferase

HepG2 and Huh7 cells were transfected with pcDNA3.1-Luc-Neo and selected with G418 (Amresco, Solon, OH, USA). Independent transfectants were cloned, screened for high-level activity of firefly luciferase and designed as HepG2-Luc and Huh7-Luc, respectively.

Animal models of liver cancer and in vivo gene delivery

SCID mice (BioLASCO, Yilan, Taiwan) were used for orthotopic xenograft mouse model. After anesthetization, mice were undergone medial laparotomy. SCID mice were inoculated with HepG2-Luc or Huh7-Luc cells into the subcapsular parenchymal of the left liver lobe. One week after inoculation, mice were imaged by the IVIS system and randomly grouped into three groups. For orthotopic syngenic animal model, BALB/c mice were inoculated with ML-1 cells as described above. For *in vivo* gene targeting, mice were *i.v.* injected with 50 µg of CMV- or eAFP-VISA-Luc mixing with liposomes. After 2 days, the organ/tissue distribution of targeted plasmids in living mice was observed by IVIS imaging system. For *in vivo* gene therapy, 20 µg of DNA/extruded DOTAP:cholesterol liposome complex was *i.v.* injected into the HCC-bearing mice in a total volume of 100 µl each time, thrice weekly for 3 weeks.

IVIS imaging system and quantification

Tumor growth was measured by IVIS imaging system (Xenogen). Briefly, mice received intraperitoneal injections with D-luciferin (30 mg/ml; Xenogen). After 10 min, mice were anesthetized with a mixture of isoflurane and oxygen and then

imaged with IVIS imaging system. Quantitative analysis of imaging signals (photons/s) was analyzed by Living Imaging software.

Immunohistochemistry

Tissue specimens were fixed in 4% neutral-buffered formaldehyde and embedded in paraffin. Tissue sections of 5 µm thickness were stained with hematoxylin and eosin. Immunostaining of firefly luciferase was performed as previously described (Xie *et al.*, 2007) by using the goat anti-firefly luciferase antibody (Abcam, Cambridge Science Park, Cambridge, UK), horseradish peroxidase-conjugated avidin biotin complex from the Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) and AEC chromogen (Vector Laboratories). After hematoxylin counter-staining, sections were mounted and examined under microscope.

In vivo apoptosis assays

To investigate cell apoptosis induced by BikDD expression, an *in situ* TUNEL assay (BioVision, Mountain View, CA, USA) was performed according to the manufacturer's instructions with some modification. Briefly, after deparaffinization and rehydration, tissue sections were permeabilized by proteinase K, washed by PBS and then immersed in 3% H₂O₂/methanol to quench the endogenous peroxidase activity. After terminal deoxynucleotidyl transferase-end labeling, the sections were incubated with antibody solution, developed in substrate 3,3'-diaminobenzidine (DAB) and counterstained with methyl green. The percentage of TUNEL-positive apoptotic cells were counted by randomly selecting four fields for each sample.

Analysis of acute toxicity

Mice were injected with high dose (50 or 100 µg) of plasmid/liposomes complex through tail vein administration. Blood was collected at indicated time after plasmid injection. The concentration of serum aspartate transaminase, alanine transaminase and blood urea nitrogen was measured by automatic analyzer (Roche Cobas Mira Plus, Roche, Mannheim, Germany). The BALB/c mice were used for 50 µg plasmid injection and ICR mice were used for high-dose (100 µg) plasmid injection.

Statistical analysis

All *P*-values were analyzed by student's *t*-test except for evaluation of survival time. The survival curves were obtained by the Kaplan–Meier analysis in SPSS software (SPSS Inc., Chicago, IL, USA). Comparing survival time of two groups was carried out with the log-rank test. The statistical significance of *P*-value is below 0.05.

Conflict of interest

The corresponding author, Dr Mien-Chie Hung, is an inventor on patents covering BikDD as a therapeutic agent filed with the University of Texas MD Anderson Cancer. The other authors declare no conflict of interest.

Acknowledgements

This work was supported by grants from DOH97-TD-I-111-TM003, DOH98-TD-I-111-TM002, NHRI-EX98-9603BC (to L-YL), DOH98-TD-G-111-030 (to M-CH), NSC96-3111-B-039, NSC97-3111-B-039 and DOH99-TD-C-111-005 (to M-CH and L-YL).

References

- Abelev GI, Eraiser TL. (1999). Cellular aspects of alpha-fetoprotein reexpression in tumors. *Semin Cancer Biol* **9**: 95–107.
- Avila MA, Berasain C, Sangro B, Prieto J. (2006). New therapies for hepatocellular carcinoma. *Oncogene* **25**: 3866–3884.
- Bui LA, Butterfield LH, Kim JY, Ribas A, Seu P, Lau R *et al.* (1997). *In vivo* therapy of hepatocellular carcinoma with a tumor-specific adenoviral vector expressing interleukin-2. *Hum Gene Ther* **8**: 2173–2182.
- Chen JS, Liu JC, Shen L, Rau KM, Kuo HP, Li YM *et al.* (2004). Cancer-specific activation of the survivin promoter and its potential use in gene therapy. *Cancer Gene Ther* **11**: 740–747.
- Chen SH, Hu CP, Chang CM. (1992). Hepatitis B virus replication in well differentiated mouse hepatocyte cell lines immortalized by plasmid DNA. *Cancer Res* **52**: 1329–1335.
- El-Serag HB, Rudolph KL. (2007). Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* **132**: 2557–2576.
- Guan M, Rodriguez-Madoz JR, Alzuguren P, Gomar C, Kramer MG, Kochanek S *et al.* (2006). Increased efficacy and safety in the treatment of experimental liver cancer with a novel adenovirus-alphavirus hybrid vector. *Cancer Res* **66**: 1620–1629.
- Hoofnagle JH. (2004). Hepatocellular carcinoma: summary and recommendations. *Gastroenterology* **127**(Suppl 1): S319–S323.
- Ido A, Uto H, Moriuchi A, Nagata K, Onaga Y, Onaga M *et al.* (2001). Gene therapy targeting for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by a hypoxia-inducible enhancer linked to a human alpha-fetoprotein promoter. *Cancer Res* **61**: 3016–3021.
- Izumi R, Shimizu K, Kiriya M, Hashimoto T, Urade M, Yagi M *et al.* (1992). Alpha-fetoprotein production by hepatocellular carcinoma is prognostic of poor patient survival. *J Surg Oncol* **49**: 151–155.
- Kim JW, Wang XW. (2003). Gene expression profiling of preneoplastic liver disease and liver cancer: a new era for improved early detection and treatment of these deadly diseases? *Carcinogenesis* **24**: 363–369.
- Lemken ML, Wybranietz WA, Schmidt U, Graepler F, Armeanu S, Bitzer M *et al.* (2005). Expression liver-directed genes by employing synthetic transcriptional control units. *World J Gastroenterol* **11**: 5295–5302.
- Llovet JM, Burroughs A, Bruix J. (2003). Hepatocellular carcinoma. *Lancet* **362**: 1907–1917.
- Lu SY, Sui YF, Li ZS, Pan CE, Ye J, Wang WY. (2003). Construction of a regulable gene therapy vector targeting for hepatocellular carcinoma. *World J Gastroenterol* **9**: 688–691.
- Ma SH, Chen GG, Yip J, Lai PB. (2010). Therapeutic effect of alpha-fetoprotein promoter-mediated tBid and chemotherapeutic agents on orthotopic liver tumor in mice. *Gene Ther* **17**: 905–912.
- Mawatari F, Tsuruta S, Ido A, Ueki T, Nakao K, Kato Y *et al.* (1998). Retrovirus-mediated gene therapy for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by human alpha-fetoprotein enhancer directly linked to its promoter. *Cancer Gene Ther* **5**: 301–306.
- Nakabayashi H, Hashimoto T, Miyao Y, Tjong KK, Chan J, Tamaoki T. (1991). A position-dependent silencer plays a major role in repressing alpha-fetoprotein expression in human hepatoma. *Mol Cell Biol* **11**: 5885–5893.
- Parkin DM, Bray F, Ferlay J, Pisani P. (2005). Global cancer statistics, 2002. *CA Cancer J Clin* **55**: 74–108.
- Ren XW, Liang M, Meng X, Ye X, Ma H, Zhao Y *et al.* (2006). A tumor-specific conditionally replicative adenovirus vector expressing TRAIL for gene therapy of hepatocellular carcinoma. *Cancer Gene Ther* **13**: 159–168.
- Schafer DF, Sorrell MF. (1999). Hepatocellular carcinoma. *Lancet* **353**: 1253–1257.
- Sher YP, Tzeng TF, Kan SF, Hsu J, Xie X, Han Z *et al.* (2009). Cancer targeted gene therapy of BikDD inhibits orthotopic lung cancer growth and improves long-term survival. *Oncogene* **28**: 3286–3295.
- Shi YJ, Gong JP, Liu CA, Li XH, Mei Y, Mi C *et al.* (2004). Construction of a targeting adenoviral vector carrying AFP promoter for expressing EGFP gene in AFP-producing hepatocarcinoma cell. *World J Gastroenterol* **10**: 186–189.
- Stillwagon GB, Order SE, Guse C, Leibel SA, Asbell SO, Klein JL *et al.* (1991). Prognostic factors in unresectable hepatocellular cancer: Radiation Therapy Oncology Group Study 83-01. *Int J Radiat Oncol Biol Phys* **20**: 65–71.
- Suriawinata A, Xu R. (2004). An update on the molecular genetics of hepatocellular carcinoma. *Semin Liver Dis* **24**: 77–88.
- Takahashi M, Sato T, Sagawa T, Lu Y, Sato Y, Iyama S *et al.* (2002). E1B-55K-deleted adenovirus expressing E1A-13S by AFP-enhancer/promoter is capable of highly specific replication in AFP-producing hepatocellular carcinoma and eradication of established tumor. *Mol Ther* **5**: 627–634.
- Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN. (1997). Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat Biotechnol* **15**: 647–652.
- Xie X, Hsu JL, Choi MG, Xia W, Yamaguchi H, Chen CT *et al.* (2009). A novel hTERT promoter-driven E1A therapeutic for ovarian cancer. *Mol Cancer Ther* **8**: 2375–2382.
- Xie X, Xia W, Li Z, Kuo HP, Liu Y, Ding Q *et al.* (2007). Targeted expression of BikDD eradicates pancreatic tumors in noninvasive imaging models. *Cancer Cell* **12**: 52–65.
- Zufferey R, Donello JE, Trono D, Hope TJ. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* **73**: 2886–2892.

Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)