Targeted expression of BikDD eliminates breast cancer with virtually no toxicity in noninvasive imaging models

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Abstract

Breast cancer is a major public health problem all over the world, and the current treatment strategies are not potent enough for some patients, especially those with triple-negative breast cancer. Therefore, novel and more effective treatments are critically needed. Of the current methods, targeted therapy, which not only retains cancer-specific expression but also limits toxicity, is a new strategy for treating cancers. In this study, we have found that the human telomerase reverse transcriptase (hTERT; T) promoter also possesses high target specificity in breast cancer. Moreover, we developed a versatile T-based breast cancer-specific promoter VISA (VP16-Gal4-WPRE integrated systemic amplifier) composite (T-VISA) to target transgene expression in breast tumors, which has stronger activity comparable or higher than that of the cytomegalovirus (CMV) promoter in cancer cells. Thereafter, targeted expression of BikDD (a mutant form of proapoptotic gene Bik) through the T-VISA platform in breast cancer initiated robust antitumor effects and prolonged survival in multiple xenograft and syngeneic orthotopic mouse models of breast tumors with virtually no toxicity in intact mice. Thus, these findings demonstrate our T-VISA-BikDD nanoparticles effectively and safely eradicate breast cancer in vitro and in vivo, and are worthy of development in clinical trials treating breast cancer.
Introduction

As the most common malignancy among women worldwide, breast cancer has the highest mortality rate of all cancers, especially in economically developing countries, with an estimate of 458,400 deaths and 1,383,500 new cases in the world in 2008 (1). Many effective therapy approaches for treating breast cancer are increasingly possible with state of the art diagnostics and treatments (2-4). However, many challenges still exist in choosing the optimal therapeutic strategy for a specific patient population, such as triple-negative and advanced-staged patients who do not respond to current cancer treatment modalities or those who recurred. Therefore, more effective and alternative therapeutic strategies to the treatment of breast cancer are urgently needed. More recently, there is evidence that shows in addition to current chemotherapy and radiotherapy, targeted therapy can also effectively and preferentially treat cancers (5-7). A delivery vector, which can selectively deliver therapeutic genes to target cells efficiently and safely, is critical for successful targeted cancer therapy (8, 9). The development of current viral vectors for targeted treatment is restricted by a number of issues such as immunogenicity, toxicity (10, 11), and potential recombination or complementation (12). Luckily, nonviral promoter-based targeted therapy has gained widespread attention for its cancer specificity, optimal antitumor activities, and less toxicity to surrounding normal cells (13-16). Thus, a promoter that is highly active and specific for breast tumor would be ideal for designing such targeted therapy.

By searching the literature and the Serial Analysis of Gene Expression database, we identified several candidate promoters including the human telomerase reverse transcriptase (hTERT; T) (17, 18), survivin (19, 20), claudin-4 (21-23), fatty acid synthase (FASN) (24, 25) and β-catenin (26). After comparing the activities of these promoters in different breast cancer cell lines, we found that the T promoter has the
highest potential to be used for targeted breast cancer therapy among all other promoters tested. However, the expression levels of most of the identified promoters are much weaker than the CMV promoter, which holds strong activity without tumor specificity. Fortunately, to resolve this issue, we developed a versatile T-based breast cancer-specific promoter VISA (VP16-Gal4-WPRE integrated systemic amplifier) (13, 14) composite (T-VISA) to target transgene expression in breast tumors, the activity of which is stronger than or comparable to that of CMV in cancer cells.

In order to develop a breast cancer nonviral target therapy approach, we incorporated the BikDD (27, 28) (a mutant form of proapoptotic gene Bik from the Bcl-2 family and possesses potent tumor-killing activity) gene into the T-VISA vector, and detected the cell-killing effects in vitro as well as tumor inhibition characteristics in vivo. In this study, systemic delivery of T-VISA-BikDD nanoparticles (29) preferably targeted the breast cancer cells and significantly suppressed the tumor growth as well as prolonged survival in multiple breast tumor xenograft and syngeneic orthotopic mouse models. Moreover, T-VISA-BikDD demonstrated virtually no toxicity in intact mice, suggesting that the VISA-nanoparticles are worthy of moving into clinical trials.
Materials and Methods

Cell lines and culture. The following cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were passaged in our laboratory for less than six months after resuscitation of frozen aliquots: human breast cancer cell lines (MDA-MB-231, MDA-MB-361, MDA-MB-435, MDA-MB-453, MDA-MB-468, MCF-7, T47D, BT474, 4T1, SKBR-3, ZR-75-1), normal mammary epithelial (184A1, MCF-10A, MCF-12A) and normal lung fibroblasts (WI-38). All cells were maintained according to the supplier’s instructions. Before use, all cell lines were authenticated by short tandem repeat DNA profiling, and found to be free of Mycoplasma infection.

Preparation of plasmids and liposome. Plasmids pGL3-T-Luc, pGL3-Survivin-Luc, pGL3-Clandin-4-Luc, pGL3-FASN-Luc, pGL3-β-catenin-Luc, and pGL3-CMV-Luc containing the T, Survivin, Claudin-4, FASN, β-catenin, and CMV promoter, respectively, to drive the expression of the firefly luciferase gene were previously described (14, 15, 19, 21, 26). The VISA amplification system was incorporated into the pGL3-T-Luc plasmid to create pGL3-T-VISA-Luc.

To construct the therapeutic plasmids, we replaced the luciferase gene with BikDD, which was digested with BglII/NheI, followed by ligation to the vector backbone. In order to avoid ampicillin resistance in the clinic, the pUK21-T-VISA-BikDD containing kanamycin resistance gene was created by inserting the functional part of pGL3-T-VISA-BikDD vector into NotI and SalI sites of the pUK21 vector. Therapeutic plasmids purified by Qiagen Endo-Free Mega Prep kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. The endotoxin level was determined to be <10 endotoxin units/mg of DNA (QCL-1000 kit, BioWhittaker,
Walkersville, MD, USA). Plasmid was incorporated into extruded DOTAP/Cholesterol liposome (29) that was produced in our laboratory according to the previously published protocol (30).

**Stable cell lines expressing firefly luciferase.** MDA-MB-231, MDA-MB-468 and 4T1 cells were transfected with pcDNA-Luc-Neo and selected with G418 (1 mg/ml, Invitrogen). Ten individual G418-resistant clones with a high-level luciferase activity, designated as MDA-MB-231-Luc, MDA-MB-468-Luc and 4T1-luc, were collected, identified, and maintained.

**Luciferase reporter assay.** To evaluate the promoter activities, cells were transiently cotransfected with 1 μg indicated plasmid and 0.1 μg pRL-TK (internal control) in 24-well plates using Lipofectamine (Invitrogen) for cell killing experiment. Forty-eight hours later, cells were subjected to lysis and assayed for luciferase activity using the dual-luciferase reporter assay system (Promega) according to the manufacturer’s protocol.

To evaluate the tissue distribution of luciferase expression, tissue specimens from tumors and major organs, including the lung, heart, liver, spleen, kidney, and GI (gastrointestinal), were harvested and homogenized (13). Luciferase assays were performed 48 h post-transfection using a luminometer (Lumat LB9507; Berthold Technologies, Bad Wildbad, Germany), and the protein concentration was determined as previously described (13).
**Breast cancer animal model and in vivo gene delivery.** Female BALB/c mice and athymic female BALB/c nu/nu mice at 4–6 weeks of age were used for mouse syngeneic and human xenograft tumors, respectively. All mice were maintained in a specific pathogen-free environment at the Animal Experiment Center of Sun Yat-Sen University, and all procedures were approved by the Animal Care and Use Committee of Sun Yat-Sen University and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory animals.

To test the specificity and activity of the promoters in vivo, we established a xenograft tumor model. A suspension of 4T1 cells \(5 \times 10^5\) were inoculated at the left fourth inguinal mammary gland of BALB/c mice. Ten days after inoculation, the mice received a single injection of 100 µl plasmid/liposome complex containing 50 µg of CMV-Luc or T-VISA-Luc through the tail vein. Forty-eight hours later, mice were then noninvasively imaged in vivo with the In Vivo Imaging System (IVIS; Xenogen, Alameda, CA).

To test the antitumor effect of CMV-BikDD and T-VISA-BikDD in vivo, both xenograft and syngeneic orthotopic mouse models were established. Luciferase-expressing MDA-MB-231 cells \(5 \times 10^6\) or 4T1 cells \(5 \times 10^5\) were injected into the left fourth inguinal mammary gland of mice. After inoculation, mice were noninvasively imaged by In vivo Imaging System (Xenogen) to assess tumor growth and then randomly assigned to one of three groups for every tumor model. Each group of mice received 100 µl of DNA:liposome complexes that contained 15 µg of pUK21-T-VISA (control), pUK21-CMV-BikDD, or pUK21-T-VISA-BikDD administered through tail-vein injection, twice per week for 3 consecutive weeks. Tumor volumes were calculated from the equation: \(0.5 \times a \times b^2\) in millimeters, where a is the length and b is the width.
**IVIS system and quantification.** The growth and metastasis of tumors were imaged once per week for a total of 6 weeks before each treatment. Before each imaging, mice were anesthetized with a mixture of oxygen and isoflurane and intraperitoneally injected with 100 μl of D-luciferin (Xenogen; 15 mg/ml in phosphate-buffered saline). Ten minutes later, images of mice were taken by the IVIS imaging system, and Living Imaging software (both from Xenogen) was used to quantify the signals.

**In vivo apoptosis assays.** TUNEL staining was carried out to detect fluorescence of apoptotic cells using the *In situ* Cell Death Detection Kit (Roche Applied Science) in tumor tissues according to the manufacturer’s protocol. Briefly, slides were incubated with 50 μl of TUNEL reaction mixture containing terminal deoxynucleotidyl transferase in a humidified atmosphere for 1 h at 37 °C in the dark. Rinsed slides were then stained with 1 μg/ul propidium iodide (Sigma) in phosphate-buffered saline for counterstaining and then were washed again with phosphate-buffered saline. The images were captured under an Olympus BX 71 fluorescence microscope using Ce2001 Cell Explorer software (BioSciTec, Frankfurt, Germany). The percentage of TUNEL-apoptosis cells were analysed by randomly selecting four independent fields for each sample.

**Analysis of acute toxicity.** Intact BLAB/c mice were used for acute toxic effects induced by high doses of plasmid injection (50 or 100 μg). Blood was collected by retro-orbital bleeding using a heparinized microcapillary tube at indicated time after plasmid injection. The concentration of serum AST, ALT, BUN and Cr were
measured by automatic analyzer (Roche Cobas Mira Plus, Roche, Mannheim, Germany).

**Statistical analysis.** The results are given as mean ± SD. Student’s t-test was used to compare the differential expression or cytotoxicity effect in cancer and normal groups. All statistical tests were two-sided. Survival curves were obtained by the Kaplan–Meier method. The difference in survival time between two groups was analysed with the log-rank test. The significance level was set at \( P < 0.05 \).

**Results**

**The T promoter is active specifically in breast cancer cells**

To explore a breast cancer-specific promoter, we searched for the literature and the Serial Analysis of Gene Expression database and found five candidates: T, Survivin, Claudin-4, FASN and \( \beta \)-catenin (Fig. 1A). By detecting the activities of these promoters in different breast cancer cell lines and normal cell lines, the T promoter was shown to be highly specific in breast cancer cells with less activity in normal cell lines (Fig. 1B). These results indicate that the T promoter would be feasible as a targeted therapy for breast cancer.

**T-VISA is highly and specifically expressed in breast cancer cells**

As described above, the expression level of T promoter in breast tumor was much weaker than the CMV promoter. Thus, to resolve this issue, we integrated the T promoter into our VISA amplification system (15) and measured the relative activity of the T-VISA to the CMV promoter in 11 breast tumor cell lines (Figs. 1C, D). The T-VISA system demonstrated robust amplification effect, yet had lower activity in
normal cell lines compared with CMV-Luc ($P<0.001$) (Fig. 1E). We also evaluated the kinetics of the luciferase activity in MDA-MB-231, MDA-MB-435 and 4T1 breast cancer cells after transient transfection and showed the total expression index (TEI) of T-VISA (in comparison with values of the luciferase activity in Fig. 1F, with CMV activity set as 1) was 2.76 in MDA-MB-231, 3.22 in MDA-MB-435 and 2.82 in 4T1 cells, respectively. The increase in luciferase activity in T-VISA is likely attributed to the presence of the woodchuck hepatitis post-transcriptional regulatory element (WPRE; an RNA stabilizing element) in the VISA system as described before.

**T-VISA nanoparticles are specifically activated in breast tumors**

To further investigate whether the specificity and activity of T-VISA expression is still retained *in vivo*, we treated the 4T1 tumor-bearing mice with the plasmid DNA/nanoparticle complexes carrying either CMV-Luc or T-VISA-Luc via the tail vein. The T-VISA-Luc exhibited significant luciferase activity primarily at the tumor region (Fig. 2A). In contrast, the majority of the luciferase activity of CMV-Luc was found in the lungs and heart with weak signals in the tumor area. To more precisely evaluate the source of signal, we sacrificed the mice immediately after *in vivo* imaging and harvested the corresponding organs for *ex vivo* imaging (Fig. 2B). In addition, we also measured the luciferase activity of the dissected organs by a luminometer (Fig. 2C). The *in vivo* results demonstrated that T-VISA is specific in breast tumor mouse model.

**Targeted expression of therapeutic gene BikDD driven by the T-VISA promoter preferentially inhibits the breast cancer cells growth *in vitro* with very limited toxicity in normal cells**
To test whether the T-VISA vector can target a therapeutic gene to breast cancer cells, we constructed CMV-BikDD and T-VISA-BikDD plasmids (Fig. 3A). The expression of BikDD driven by CMV or T-VISA was detected by Western blot 24-h post transient transfection in MDA-MB-468 breast tumor cells (Fig. 3B). Moreover, we evaluated the killing effects of T-VISA-BikDD in a panel of breast cancer cell lines and normal cell lines. The results showed that the T-VISA-BikDD inhibited cell growth at least as effectively as CMV-BikDD *in vitro* and in a dose-dependent manner (Figs. 3C and D). However, in normal cells, the cell killing activity of CMV-BikDD is more potent than T-VISA-BikDD. Thus, by expressing BikDD under T-VISA vector, we the cytotoxic effect of BikDD is potent in cancer cells but limited in normal cells, indicating that T-VISA-BikDD is tumor specific *in vitro* and can be a potential therapeutic agent to treat breast cancer.

**T-VISA-BikDD nanoparticles suppress tumor growth and prolong mouse survival time more effectively than CMV-BikDD in syngeneic orthotopic mouse model**

To investigate the therapeutic effects of T-VISA-BikDD *in vivo*, we established an syngeneic orthotopic animal model with breast cancer cell line, 4T1, and then separately delivered 5, 15 or 45 μg of T-VISA-BikDD or 45 μg of T-VISA (vector control) in liposomal complexes via the tail vein. As shown in Figure 4A, twenty-eight days after the first treatment, T-VISA-BikDD significantly inhibited tumor growth in a dose-dependent manner compared with the control vector. We further compared the therapeutic efficacy between T-VISA-BikDD and CMV-BikDD administering DNA-liposome nanoparticles containing 15 μg of CMV-BikDD, T-VISA-BikDD, or T-VISA DNA via intravenous injection to 4T1-Luc tumor bearing
mice twice weekly for 3 consecutive weeks. The results indicated that both CMV-BikDD and T-VISA-BikDD nanoparticle complexes inhibited tumor growth (P = 0.028 and P = 0.016, respectively) (Fig. 4B) and prolonged mouse survival (P = 0.013 and P = 0.007, respectively) (Fig. 4C) after systemic treatment, but there was no significant difference in tumor suppression between CMV-BikDD and T-VISA-BikDD treatment groups (P = 0.072). To further investigate apoptosis in vivo, 2 days after the second injection of the BikDD complexes, two mice per group were sacrificed and their tumors, lungs, and livers were sectioned for apoptosis detection by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay. Our data showed that T-VISA-BikDD induced remarkable tumor cells apoptosis in tumor cells but not in the normal tissues such as the lungs and the liver, while CMV-BikDD led to substantial apoptosis both in tumors and in surrounding organs (Fig. 4D). Quantitative analysis of apoptosis suggested that the levels of apoptosis CMV-BikDD treatment group were relatively high in normal tissues but the relatively low in both T-VISA-BikDD and control groups.

**T-VISA-BikDD nanoparticles have significant antitumor effect against breast cancer in xenograft orthotopic mouse model**

Based on our results above, we then examined whether the T-VISA-BikDD-liposome nanoparticles also elicit robust therapeutic effects in xenograft orthotopic mouse model. We established an orthotopic animal model with human breast cancer cell line, MDA-MB-231-Luc, and delivered the indicated plasmid DNA in liposome complexes when tumors were palpable. Tumor growth was monitored by noninvasive IVIS imaging in real time and the survival information of mice were also recorded. In MDA-MB-231-Luc model, expression of BikDD driven
by T-VISA or CMV demonstrated a strong reduction in tumor growth ($P = 0.0039$ and $P = 0.0092$, respectively), but tumor inhibition by T-VISA-BikDD was more substantial than CMV-BikDD (Fig. 5A). In addition, T-VISA-BikDD significantly prolonged the survival time of mice more effectively than CMV-BikDD ($P = 0.034$), although the survival time of mice in treatment groups had all been extended compared to control group ($P < 0.0001$ in both treatment groups) (Fig. 5B). These findings were further supported by the increase in apoptosis in the tumors of the T-VISA-BikDD treated groups (Figs. 5C and D). Notably, although both treatment groups showed a statistically significant trend of increase in TUNEL-positive cells compared with control, T-VISA-BikDD showed significantly higher activities than CMV-BikDD, suggesting that T-VISA-BikDD has higher targeting power over CMV-BikDD (Figs. 5C and D). Collectively, these results demonstrate that T-VISA-BikDD consistently shows strong antitumor effects on breast tumor in vivo and induces apoptosis with high tumor specificity.

**T-VISA-BikDD nanoparticles exert virtually no acute toxicity in normal mice compared with CMV-BikDD**

To evaluate whether treatment with T-VISA-BikDD is safer than CMV-BikDD, single dose of 50 or 100 μg plasmid DNA was given by tail vein injection in BALB/c mice (Figs. 6A and B). Blood samples were collected from living mice at different time points, and serum levels of liver alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) were analyzed for mice that received 50 μg plasmid DNA. Treatment with CMV-BikDD increased the serum level of ALT and AST on day 2 after injection, while they were within the normal range for the control and T-VISA-BikDD treatment groups at any measured time points (Figs.
6C, D). These results indicate that administration of T-VISA-BikDD is much safer than CMV-BikDD. We did not, however, observe any increase in the BUN level at any assayed time points in mice treated with control, CMV-BikDD or T-VISA-BikDD (Fig. 6E). In addition, T-VISA-BikDD-liposome complexes showed 100% event-free survival compared with 80% or 60% in CMV-BikDD treated mice at a dose of 50 or 100 μg DNA (Fig. 6A and B). To further investigate apoptosis in vivo, tissues from mice per group were sectioned and detected by TUNEL assay (Fig. 6F). Analyzes of apoptosis showed that CMV-BikDD treatment induced a remarkable amount of apoptosis in both lung and liver tissues compared with control and T-VISA-BikDD groups. The percentage of apoptosis cells in CMV-BikDD treatment group was significantly higher than control or T-VISA-BikDD groups ($P < 0.001$). Taken together, our findings suggest that T-VISA-BikDD-liposome-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, thereby acting as a potent anticancer agent for breast cancer.

**Discussion**

The human telomerase reverse transcriptase (T) is highly expressed in a majority of cancer types but not in normal cells, which allows it to be used as a tumor biomarker (15, 17, 18). Previously, we identified and validated the specificity of the T promoter in ovarian cancer and showed that its activity was enhanced by the VISA amplification system (15). In this study, our results further demonstrated that the T promoter is specifically activated in breast cancer cells but had much weaker activity than the CMV promoter. Using the VISA system that was initially developed for pancreatic cancer (13), we have since then demonstrated that this system is highly...
active in ovarian (15), lung (16) and liver (30) cancers. With the goal of further developing a robust and breast tumor-specific vector, we incorporated the T promoter into our VISA system and constructed T-VISA expression carrier. Our engineered T-VISA is a composite that contains three basic elements: the T promoter, the TSTA system, and the WPRE sequence. T-VISA specifically controls expression of BikDD in breast cancer, involving five steps: 1) The T promoter drives the GAL4-VP2 (two copies of VP16) fusion protein specifically in breast cancer cells, not in normal cells; 2) GAL4-VP2 unlocks target gene BikDD mRNA transcripts; 3) the BikDD mRNA transcripts involves the WPRE element, which can stabilize RNA transcripts; 4) the BikDD mRNA transcripts then improve and extend duration of BikDD protein expression, and 5) BikDD binds to Bcl-2, and releases Bax and Bid, leading to apoptosis of breast cancer cells. For normal cells, the activity of T promoter is much lower, resulting in very limited gene expression and, thereafter, cell survival.

Thus, the activity of T-VISA is higher than or comparable with CMV promoter in breast cancer cells but low in normal cells. In addition, the duration of transgene expression induced by T-VISA was also prolonged as well, which can be explained by the presence of WPRE in the VISA system (13, 14). To further examine the targeted function of T-VISA in vivo, we detected the photo signals of luciferase in 4T1-bearing mouse models. These observations reinforce the specificity of T-VISA vector in breast tumors with high expression activity. A better therapeutic effect of targeted gene therapy induced by T-VISA vector would be a potential treatment option to combat breast cancer.

Next, considering that T-VISA possesses strong transgene expression characteristics, we examined the killing effects of proapoptosis gene BikDD under the
control of T-VISA promoter in vitro. Consistent with the targeted trait noted above, there was a significant therapeutic effect on breast cancer cells but a low therapeutic effect on normal cells with T-VISA-BikDD treatment, while both breast cancer cells and normal cells were killed with CMV-BikDD treatment. Moreover, on the basis of experiment results in vitro, we further established multiple animal models and determined the antitumor effects of BikDD driven by T-VISA in plasmid DNA-liposome complexes administrated by the tail vein in xenograft and syngeneic orthotopic mouse models by using imaging of live animals (31) and traditional methods. Some treatment strategies are not potent enough for some specific breast cancer patients who are at high risk of recurrence and metastasis, such as those with the triple-negative subtype of breast cancer. Our in vivo MDA-MB-231 animal model is triple-negative, and 4T1 tumors are at high risk of metastasis. Moreover, our data revealed that T-VISA-BikDD promoted breast tumor cells apoptosis, suppressed tumor growth, and prolonged mouse survival time more effectively than CMV-BikDD in the above models. Although there was no significant difference in inhibition of tumor growth between CMV-BikDD and T-VISA-BikDD in 4T1 animal model above, the survival time was significantly prolonged in T-VISA-BikDD treatment group compared with the CMV-BikDD treatment group. The reason may be attributed to the duaration of transgene expression induced by T-VISA vector (Fig. 1F). As the expression ratio of luciferase from T-VISA-Luc to CMV-Luc at day 6 was consistently higher than at day 2 in the observed three cell lines tested, which suggested that our T-VISA vector prolonged duration of gene expression, we can retain the robust killing effects of VISA nanoparticles without adding the treatment frequencies and courses, then reducing the management cost and improving the quality of life. The acute toxity in vivo of T-VISA-BikDD nanoparticles was further
assessed and the results were consistent with the above study (Fig. 6). Thus, our T-VISA-BikDD system has the following advantages over the CMV-BikDD system: 1) has prolonging-expression of therapeutic gene BikDD, which can improve the treatment efficacy and decrease the dosage and frequency of treatment; and 2) exhibits cancer-targeted expression of BikDD, which can reduce the in vivo toxicity.

In conclusion, our present data, derived from both in vitro and in vivo studies suggest that VISA-BikDD nanoparticles not only retain high specificity but also strong activity in breast cancer. With VISA-BikDD exerting robust tumor inhibition effects, target specificity, and less side effects, there would be no hesitation to test it in future clinical trial with the hope that it might become a novel therapeutic option for breast cancer patients.

Conflicts of interest

The authors declare no conflicts of interest.
References


Figure legends

Fig. 1 Molecularly engineered hTERT-based promoter T-VISA is highly and specifically active, and prolongs duration of gene expression in breast cancer cells. (A) Schematic representation of different promoter-driven luciferase constructs. (B) The hTERT promoter was relatively highly expressed in human breast cancer cell lines as compared in normal cells. (C) Schematic representation of expression constructs in the pGL3 vector backbone. (D, E) The activities and specificities ($P < 0.001$ in normal cells) of T-VISA and CMV promoters. (F) The kinetics of luciferase activity driven by T-VISA promoter compared to CMV promoter.

Fig. 2 The T-VISA selectively targets transgene expression to breast cancer in vivo. (A) 4T1 tumor bearing syngeneic BALB/c mice were given 100 μl plasmid/liposome complex containing 50 μg CMV-Luc or T-VISA-Luc vial tail vein injection. Forty-eight hours later, mice were subjected to luciferase imaging, 5 mice per group. (B) The tumors and organs from mice in (A) were subjected to ex vivo imaging. The photon signals were quantified, and the percentage of the photon signals was shown on the right. The CMV-Luc signal photos were setted as 100%. (C) Tissue specimens from tumors and other specified organs in above mice were detected for luciferase activity with a luminometer. The data are expressed as relative light units (RLU) per milligram of total protein. Error bars indicate SD; GI, gastrointestinal. Asterisk, $P < 0.05$, T-VISA-Luc vs CMV-Luc.
Fig. 3 Expression of BikDD driven by T-VISA effectively and preferentially kills breast cancer cells in vitro. (A) Schematic sketch of expression constructs in the pUK21 backbone. (B) BikDD driven by CMV and T-VISA was detected in MDA-MB-468 cell lysate after 48 hour-transient transfection by Western blot analysis. (C) MDA-MB-231, 435, 453, 4T1 cells were cotransfected with 100 ng of CMV-Luc and increasing amount (0, 0.25, 0.5, 1.0, or 2 μg/well) of T-VISA-BikDD. (D) The in vitro cell killing activities of therapeutic BikDD driven by CMV and T-VISA promoter in breast cancer cells and normal cells (2 μg/well). The relative luciferase activity was measured by setting control (Ctrl) as 100%. The data represent the means of at least three independent experiments. The error bars indicate SD. The P values between breast cancer cells and normal cells treated with CMV-BikDD and T-VISA-BikDD were 0.34 and 0.013, respectively.

Fig. 4 T-VISA-BikDD nanoparticles inhibited tumor growth and prolonged survival time in a syngeneic mouse model of breast cancer. (A) BALB/c mice bearing 4T1 orthotopic tumors were intravenously injected with indicated doses of plasmid in a liposomal complex via the tail vein. The growths of tumor were monitored twice a week. The tumor volumes were calculated and illustrated as mean with SD. (B and C) Expression of BikDD driven by T-VISA promoter inhibited tumor growth and prolonged mouse survival more effectively than BikDD driven by CMV promoter. BALB/c mice bearing 4T1-Luc tumors were treated with 15 μg of liposomal plasmid DNA CMV-BikDD, T-VISA-BikDD or control (ctrl), twice weekly for three consecutive weeks (n = 10 mice/group). (B) The growth photon signals were quantified.
(left panel) with Xenogen’s software and representative images are presented (right panel). Error bars indicate SEM. Arrows indicate the therapy time points. (C) Kaplan-Meier survival analysis from (B) was determined. (D) Detection of in vivo apoptosis of tumor, lung and liver by TUNEL assay and the percentages of apoptotic cells from five fields per section are counted. The data are shown as mean with SD. TUNEL-positive apoptotic cells stained with green (original magnification, × 200). Asterisk, $P < 0.05$ vs control.

**Fig. 5** Targeted expression of T-VISA-BikDD nanoparticles inhibits tumor growth and prolongs mouse survival more effectively than CMV-BikDD in a xenograft mouse model of breast cancer. (A) T-VISA-BikDD greatly suppressed tumor growth of MDA-MB-231 human breast cancer orthotopic xenograft in nude mice. The established tumor-bearing mice were intravenously injected with 15 μg of treatment plasmid DNA as above. (B) Kaplan-Meier survival of mice in different groups was analyzed. Mice survival was monitored after 130 days after tumor transplantation. (C and D) Detection of in vivo apoptosis of tumor, lung and liver by TUNEL assay and the percentages of apoptotic cells from five fields per section are presented (lower panel) (original magnification, × 200). Asterisk, $P < 0.05$ vs control.

**Fig. 6** T-VISA-BikDD nanoparticles have no systemically acute toxicity in normal compared with CMV-BikDD in immunocompetent mice. (A and B) Kaplan-Meier survival analysis. Female BALB/c mice were received single doses of 100 μg or 50 μg plasmid in a liposomal complex via the tail vein. (C, D, and E) Kinetics serum
level of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) in mice after a single dose of 50 μg plasmid injection. (F) Detection of *in vivo* apoptosis of tissue specimens by TUNEL assay and the TUNEL-positive cells were quantified % of apoptotic cells from five fields per section (original magnification, × 200). Asterisk, *P* < 0.05.
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