

Original Article

Quantitative evaluation and comparison of two prodrug-activating suicide gene therapies on oral squamous cell carcinoma

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Abstract: Prodrug-activating suicide gene therapy (PA suicide gene therapy for short) for cancer is to introduce cancer cells with suicide genes. The enzyme encoded by suicide gene is not toxic but is able to kill cancer cells by converting a non-toxic prodrug into a toxic compound. This approach is a promising cancer gene therapy that could reduce non-specific toxicity to normal tissue. However, there is no quantitative method to evaluate efficacy of suicide gene therapy in preclinical study. The aim of this study is to develop a new method to quantitatively evaluate and compare prodrug-activating suicide gene therapies. This study was carried out on an oral squamous cell carcinoma (OSCC) cell line CAL-27. Suicide genes were integrated into ROSA26 locus of CAL-27 by CRISPR-Cas9. CAL-27 cell lines stably expressing herpes simplex virus-thymidine kinase (TK) or yeast cytosine deaminase (CD) were used to evaluate and compare PA suicide gene therapies. The efficacies of PA suicide gene therapies were quantitatively evaluated from three aspects: effective prodrug concentration, prodrug treatment time, and bystander effect. This method also could be used for different types of suicide gene therapies and different types of cancer. When the prodrug concentration, treatment time, and rate of suicide gene-positive cells (related to bystander effect) are fixed, anti-cancer effects could be quantitatively measured. This information is important for suicide gene therapy preclinical development.

Keywords: Suicide gene therapy, CD/5-FC, HSV-TK/GCV, bystander effect, OSCC

Introduction

Gene therapy is the insertion of genes into the cells of patients to treat diseases. Among gene therapy, cancer gene therapy makes up the largest category [1], which introduces new genetic materials into cancer cells that will selectively induce cancer cells death. So far, several gene therapy strategies have been developed and tested for cancer treatment, including anti-angiogenesis therapy, delivery of cytotoxic or suicide genes, immunomodulation through delivery of cytokines, delivery of tumor suppression/repair genes, delivery of small RNAs, delivery of antigens for stimulating antigen-presenting cells, delivery of antibodies that block signaling, and prodrug-activating suicide gene

therapy [2]. The development of prodrug-activating suicide gene therapy is one of the major breakthroughs in cancer gene therapy, which makes effective and safe treatment become a reality [3]. The basic concept of prodrug-activating suicide gene therapy is to introduce cancer cells with a suicide gene. The enzyme encoded by the suicide gene converts a non-toxic prodrug into a cytotoxic compound, which kills cancer cells in targeted tissues. Herpes simplex virus-thymidine kinase/ganciclovir (HSV-TK/GCV) and cytosine deaminase/5-fluorocytosine (CD/5-FC) are the most widely studied systems in prodrug-activating suicide gene therapy [4]. For HSV-TK in combination with GCV, the virus-originated HSV-TK metabolizes the non-toxic prodrug GCV into a monophosphate deriv-

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ative, then further converts to GCV triphosphate, which elicits toxicity through incorporation into DNA without inhibiting progression through the S-phase [5]. CD gene converts the inactive prodrug 5-FC into highly toxic chemotherapeutic 5-FU to kill cancer cells [6].

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck malignant tumor [7]. It is ranked the 8th leading cause of cancer, with around 300,000 new cases worldwide every year [8, 9]. OSCC is an aggressive cancer, which can be potential cured with a long-term survival rates of 80% at an early stage, (or) early stages. However, distant metastasis represents an ominous prognostic factor in OSCC. The two-year survival rate drops to 30% with stage III and IV of the disease [10]. Treatment of patients with locally advanced OSCC remains a challenge. The combination of surgical treatment with radiotherapy or chemotherapy is the principal treatment of OSCC. It often causes severe muscle atrophy, significant functional deficits in speaking and a long-term dysphagia, etc [11]. More recently, dramatic increases in the knowledge of molecular and genetic basis of cancer leads to novel molecular therapies for this disease. Gene therapy, which involves the transfer of genetic materials to cells to produce therapeutic effects, has become a promising alternative treatment for OSCC. The preliminary clinical results concerning gene therapy as well as the combination of these strategies with convention therapy on OSCC are encouraging [12].

The prodrug-activating suicide gene therapy has been tested on different types of cancers [3]. Clinical trials of HSV-TK/GCV system along with CD/5-FC system have been approved by FDA on prostate cancer, recurrent gynecologic cancer, glioblastoma multiform, head and neck cancer and hepatocellular carcinoma [4]. Suicide gene therapy is also a promising approach for OSCC patients. A major advantage of suicide gene therapy for OSCC is the solid tumors of OSCC are easily accessible for direct injections of genetic materials. Thus, suicide gene therapy is considered as a novel approach for OSCC treatment. In this study, we developed and tested a method to evaluate suicide gene therapy for OSCC. The suicide genes were edited into the genome of cancer cells by CRISPR-Cas9. Efficacy of PA suicide gene therapies

were quantitatively evaluated from three aspects: effective prodrug concentration, prodrug treatment time, and bystander effect. Our study demonstrates that this method is an effective and universal approach for PA suicide gene therapy evaluation.

Materials and methods

Plasmids and cloning

1000 bp DNA from human ROSA26 locus was cloned from CAL-27 genomic DNA by using primers: forward primer 5'-ctccgccccgggtccca-cgcctg-3', and reverse primer 5'-acatttaagaac-gtgaactagggagggaataaaagc-3'. This DNA fragment was cloned into home-made vector to generate a pROSA26 vector. Herpes simplex virus-thymidine kinase (HSV-TK) and yeast cytosine deaminase (CD) genes were purchased from Addgene (Addgene# 126677 and 21911). These suicide genes were firstly cloned into a vector with mVenus and then the whole expressing cassette was cloned into the pROSA26 vector (**Figure 2**). sgRNA2 and sgRNA4 were synthesized by IDT and cloned into a pX330 vector [13].

Cell culture and transfection

CAL-27 was purchased from ATCC (CRL-2095). Cells were maintained in DMEM with 10% FBS and 1% antibiotics (ThermoFisher) at 37°C with 5% CO₂. One day before transfection, 1.5 × 10⁵ CAL-27 cells were seeded into one well of 12-well plate with 1 ml culture medium. 1 µg DNA were transfected into cells by using 6 transfection reagents from 3 vendors, including Lipofectamine 2000, Lipofectamine 3000, LTX&PLUS from ThermoFisher, FuGENE HD and ViaFect from Promega, and GenElm from MTI-GlobalStem. Transfection was performed according to vendors' instructions.

Stable cell line generation

Donor vector and sgRNA were co-transfected into CAL-27 cells by using FuGENE (Promega). 48-h after transfection, CAL-27 cells were dissociated by Trypsin and diluted to 0.5 cell/100 µl in DMEM culture medium. 100 µl medium with cell was added into one well of 96-well plate. After 8-10 days' culture, single cell would form a single colony. The fluorescent positive colonies were picked out for the experiment.

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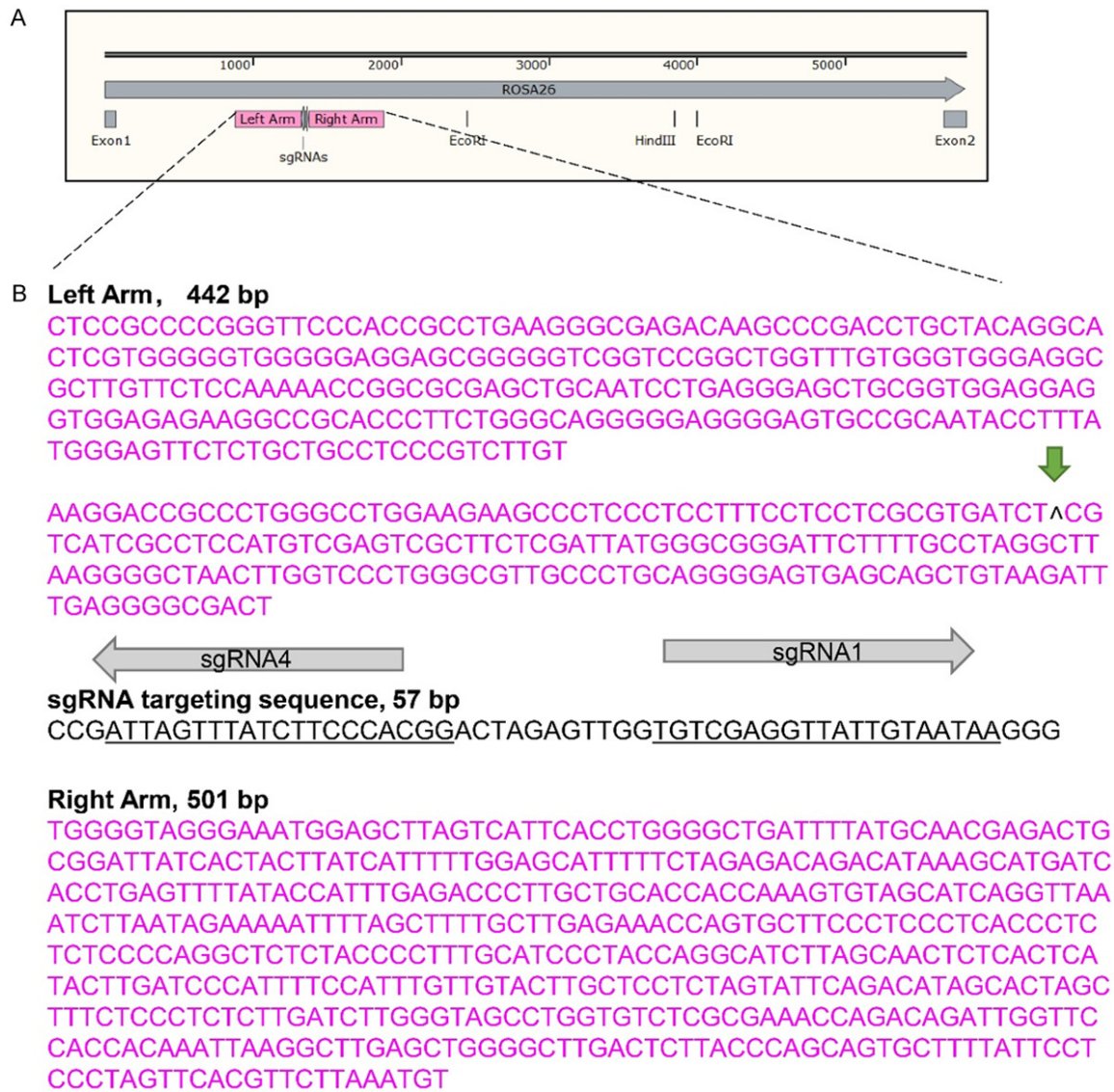


Figure 1. Homology arms and sgRNAs targeting to human *ROSA26* locus. A. Schematic representation showing the location of homology arms and sgRNAs on *ROSA26* locus. Exon1 and Exon2 are putative exons on *ROSA26* locus. B. The sequences of left and right homology arms and sgRNAs. The left and right homology arms are in pink; the sgRNA sequences are underlined and in black. The grey arrows indicate the direction of sgRNA. The green arrow in the left arm indicates *ROSA26* insertion site in a previous study [17], which is 131 bp upstream from the insertion site in this study. The 57 bp sequence in black, which is targeted by sgRNAs, will be deleted from the genome after successful gene integration.

Drug treatment

Ganciclovir (GCV) and 5-fluorocytosine (5-FC) were purchased from Sigma-Aldrich (PHR1593 and F7129). GCV was dissolved in DMSO at the concentration of 30 mM as stock solution. 5-FC was dissolved in water at the concentration of 100 mM. Before treating cells, drugs were diluted to desired concentration by using DMEM culture medium. 15,000 CAL-27 cells were seeded into one well of 96-well plate with 100

μl medium. After overnight culture, old medium was aspirated and fresh medium with the drug was added.

MTT assay

The cell viability was assessed by MTT assay (ThermoFisher) following the manufacturer's instruction. 15,000 cells were seeded into one well of a 96-well plate. At different time points after drug treatment, 10 μL of MTT reagent (5

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mg/ml MTT in PBS) was added to each well and incubated for 1 h. After that, 150 μ l of solubilization solution (10% SDS in 0.01 M HCl) was added to each well and the plate was kept on 37°C overnight. The soluble formazan was measured by using FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) at 570 nm wavelength.

Results

Homology sequences and sgRNAs targeting to human ROSA26 locus

In this study, we chose human ROSA26 locus to stably integrate suicide gene in CAL-27 cancer cells. The reason to use this locus is that mouse homologous ROSA26 locus is commonly targeted for ubiquitous and constitutive gene expression [14, 15] and human ROSA26 locus has been successfully targeted for sustained gene expression [16] and genetically modification of human embryonic stem cells [17]. Here, we designed a CRISPR-Cas9 based knock-in approach to integrate suicide gene into human ROSA26 locus. A 1000 bp genomic DNA sequence between exon1 and exon2 of ROSA26 was chosen for homology directed recombination (**Figure 1A**). The gene insertion site is in the middle of this 1000 bp sequence, which is very close to a previously reported ROSA26 integration site (**Figure 1B**) [17]. The 1000 bp DNA sequence was divided into left homology arm and right homology arm with length of 442 and 501 bp, respectively. The left and right arms will be fused to suicide genes for homology directed repair (HDR). The 57-bp sequence between left and right arm was targeted by two sgRNAs (sgRNA1 and sgRNA4) for double-stranded DNA break (DSB). Once the sgRNAs induce DSB on this 57-bp sequence, suicide genes flanking with right and left arms will be integrated into ROSA26 locus through HDR. After integration, the sgRNA-targeted 57-bp sequence will be deleted from the ROSA26 locus to prevent further DNA cleavage.

Integration of suicide genes into human ROSA26 locus

Two most studied prodrug activating suicide gene therapies (PA suicide gene therapies) were evaluated on oral squamous cell carcinoma (OSCC) in this study: cytosine deaminase

(CD) coupled with 5-fluorocytosine (5-FC) and thymidine kinase (TK) couple with ganciclovir (GCV). In this study, the CD is from yeast and TK is from herpes simplex virus. To facilitate downstream stable cell line selection, a fluorescent protein, mVenus, was fused to the N-terminal of suicide genes (**Figure 2A**). After that, the expression cassette, which contains SV40 promoter, chimeric intron, and SV40 polyA signal, was fused with ROSA26 left and right arms (**Figure 2B**). The resultant plasmids are served as donor vectors for suicide gene integration. After co-transfection donor vectors with sgRNA1 or sgRNA4, sgRNAs will guide Cas9 complex to generate double-stranded DNA break (DSB) at ROSA26 locus. In this case, donor vector containing left and right homology arms will guide DNA repair by homology directed repair (HDR). Suicide gene will be integrated into ROSA26 locus after DNA repair. As donor vectors do not contain sgRNA-targeting sequence (**Figures 1B** and **2B**, middle 57 bp sequence), Cas9-sgRNA complex will not cleave ROSA26 locus again after successful suicide gene insertion.

Selection of appropriate transfection reagent for CAL-27 cell line

CAL-27 cell line was chosen as a cell model of OSCC [18]. It was found that it is hard to achieve satisfactory transfection efficiency for this cell line. Thus, we screened 6 transfection reagents from 3 vendors for an appropriate transfection reagent for CAL-27, including Lipofectamine 2000, Lipofectamine 3000, and LTX&PLUS from ThermoFisher, FuGENE HD and ViaFect from Promega, and GenIn from MTI-GlobalStem. Plasmid encoding mVenus was transfected into CAL-27 cells by using those transfection reagents. Fluorescent and differential interference contrast (DIC) images were taken to evaluate transfection effect. Two values were measured to evaluate transfection performance: transfection efficiency and toxicity to the cell line. As shown in [Supplementary Figure 3A](#), although GenIn and ViaFect mediated high transfection efficiency, they caused a lot of CAL-27 cell death. Among the reagents that had medium transfection efficiency, FuGENE HD achieved a balance between transfection efficiency and toxicity ([Supplementary Figure 3A](#)). Thus, FuGENE HD was chosen for the transfection reagent for the CAL-27 cell line in this study ([Supplementary Figure 3B](#)).

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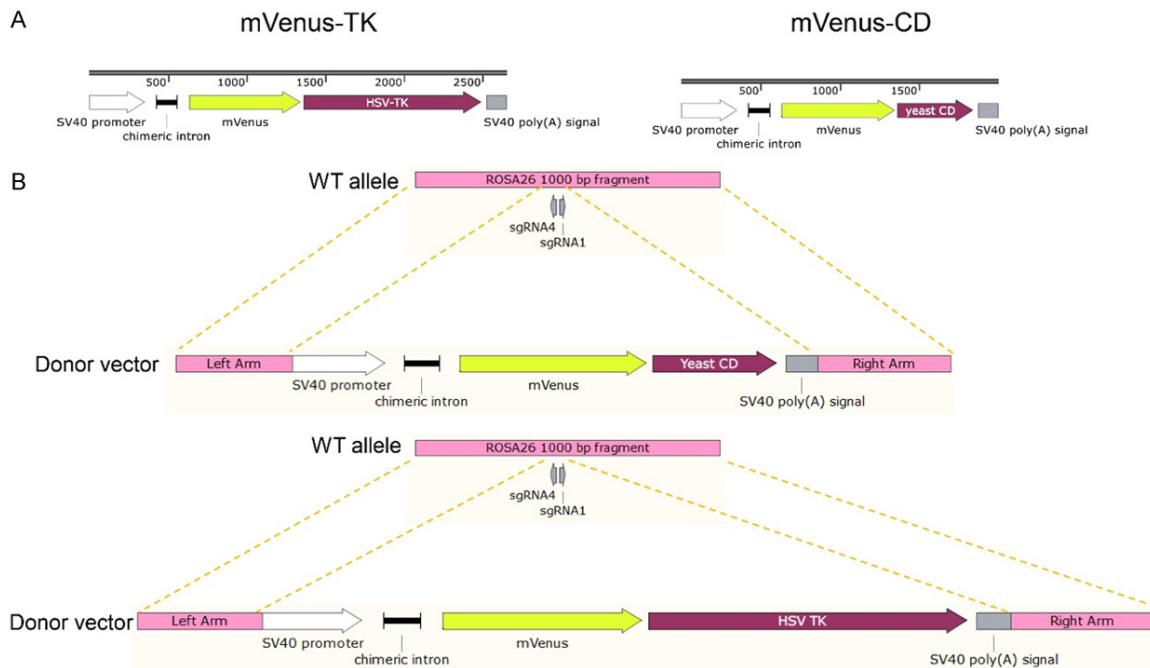


Figure 2. Expression cassette of suicide genes and donor vector for CRISPR-Cas9-based knock-in. **A.** Expression cassette of CD and TK. mVenus, which is a monomeric version of yellow fluorescent protein Venus, is fused to the N-terminal of suicide genes. Chimeric intron after SV40 promoter will enhance gene expression. **B.** Donor vectors for gene integration. The left and right arms are fused to expression cassette of suicide genes for homology directed repair (HDR) after sgRNA directed double-stranded DNA break (DSB).

Generation of CAL-27 cell lines stably expressing CD or TK

FuGENE HD was used to transfect sgRNA (either sgRNA1 or sgRNA4) and donor vector into CAL-27 cells. After culturing for 2 passages post transfection, CAL-27 cells were limited diluted into 96-well plate with density of 0.5 cell/well. Single cells will form colonies in 10-14 days. After examining under fluorescent microscope, fluorescent-positive cell colonies were picked up for downstream experiments. As shown in **Figure 3A**, stable cell lines expressing suicide genes (mVenus-CD and mVenus-TK) have similar morphology as its parental cell (No transfection CAL-27 cell) and control cell (mVenus only: CAL-27 stably expressing mVenus). Almost all cells were fluorescent positive (**Figure 3A**). Sequencing results show that CD and TK are correctly inserted into ROSA26 locus as design (data not shown). The growth curve of each cell line is the same as parental cell line (**Supplementary Figure 1**). Importantly, CAL-27 mVenus-TK and CAL-27 mVenus-CD cell lines are sensitive to GCV or 5-FC treatment (**Figure 3B** and **3C**). All these results indicate

the CAL-27 mVenus-TK and CAL-27 mVenus-CD cell lines are suitable to evaluate suicide gene therapy.

Evaluation and comparison of effective prodrugs concentration

To find out effective prodrug concentration that can kill all suicide gene-expressing cells, a series of two-fold dilutions of GCV or 5-FC were used to treat CAL-27 stable cell lines for 4 days. MTT assay, which evaluates cell metabolism level, was used to measure cell viability. The result shows that GCV at 0.39 μM killed ~93% of cells, while 50 μM 5-FC only killed ~84% cells, indicating CAL-27 is more sensitive to GCV treatment than to 5-FC treatment (**Figure 4A**). Increasing 5-FC to 100 μM only killed 87% cells, showing 13% of cell cannot be killed by 5-FC. This resistant phenomenon was also found for GCV treatment. GCV as high as 100 μM could only kill 97% of cells (**Supplementary Figure 2**), showing around 3% of cells were resistant to GCV treatment. In terms of overall cell sensitivity to prodrugs, GCV (97% at 100 μM) is better than 5-FC (84% at 100 μM).

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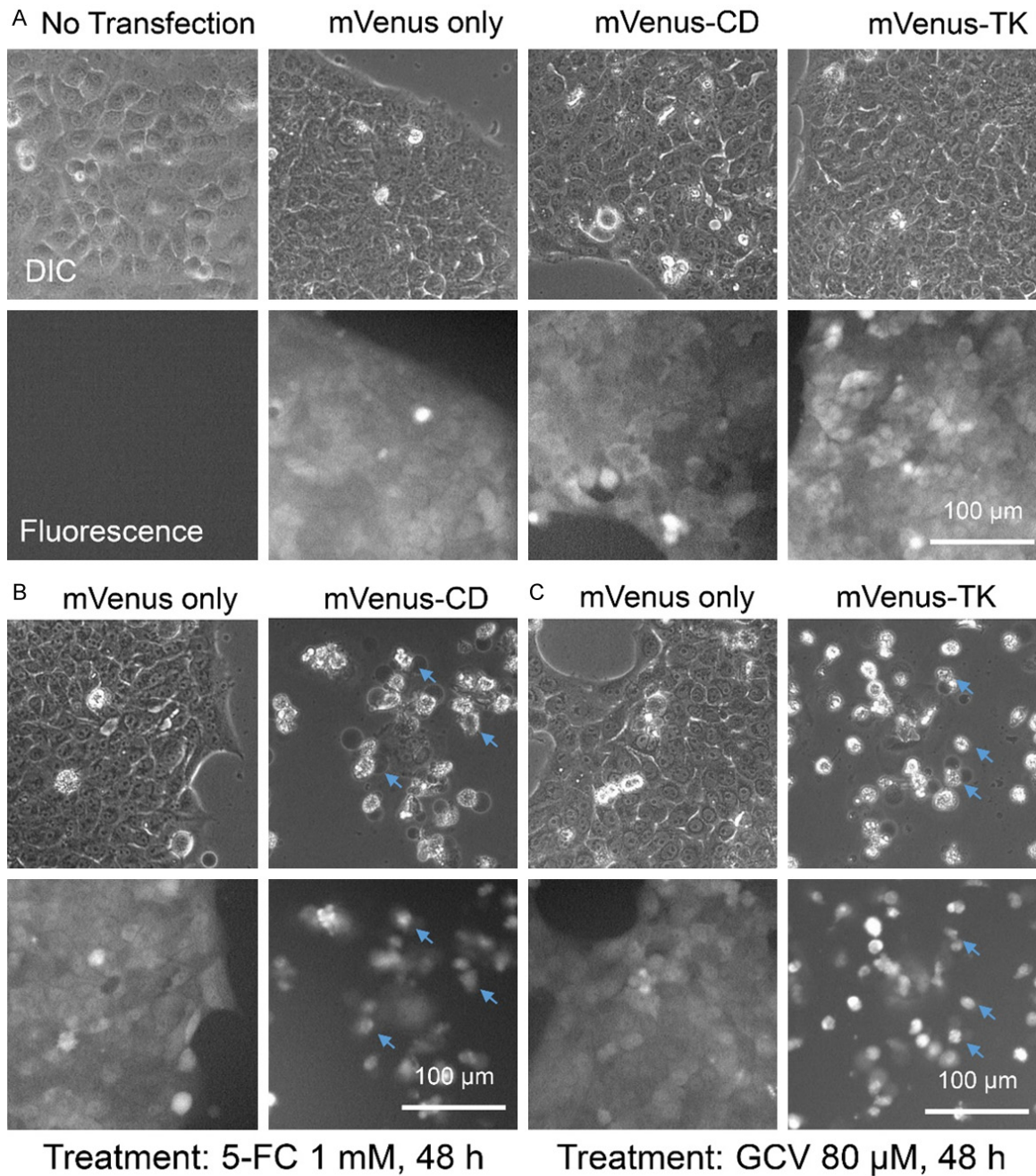


Figure 3. CAL-27 cell lines stably expressing mVenus-CD or mVenus-TK and their responses to prodrug treatment. A. Microscope pictures showing fluorescent and corresponding Differential Interference Contrast (DIC) images of CAL-27 cells stably expressing mVenus-CD or mVenus-TK. CAL-27 expressing mVenus only served or serves as a negative control in experiments. B. Both CAL-27 Venus and mVenus-CD cell lines were treated with 1 mM 5-FC for 48 h. 5-FC induced cell death of CAL-27 expressing mVenus-CD, which is indicated by arrows. C. Both CAL-27 mVenus and mVenus-TK cell lines were treated with 80 μM GCV for 48 h. GCV induced cell death of CAL-27 expressing mVenus-TK as indicated by the blue arrows. All scale bars are 100 μm.

Evaluation and comparison of killing time of prodrugs

To find out when prodrug will kill CAL-27 cancer cells after prodrug treatment, CAL-27 mVenus-

CD cells was treated with 50 μM 5-FC and CAL-27 mVenus-TK was treated with 0.39 μM GCV, respectively. Two prodrugs at these concentrations could kill all drug-sensitive cells (**Figure 4A**). At various time points after prodrug treat-

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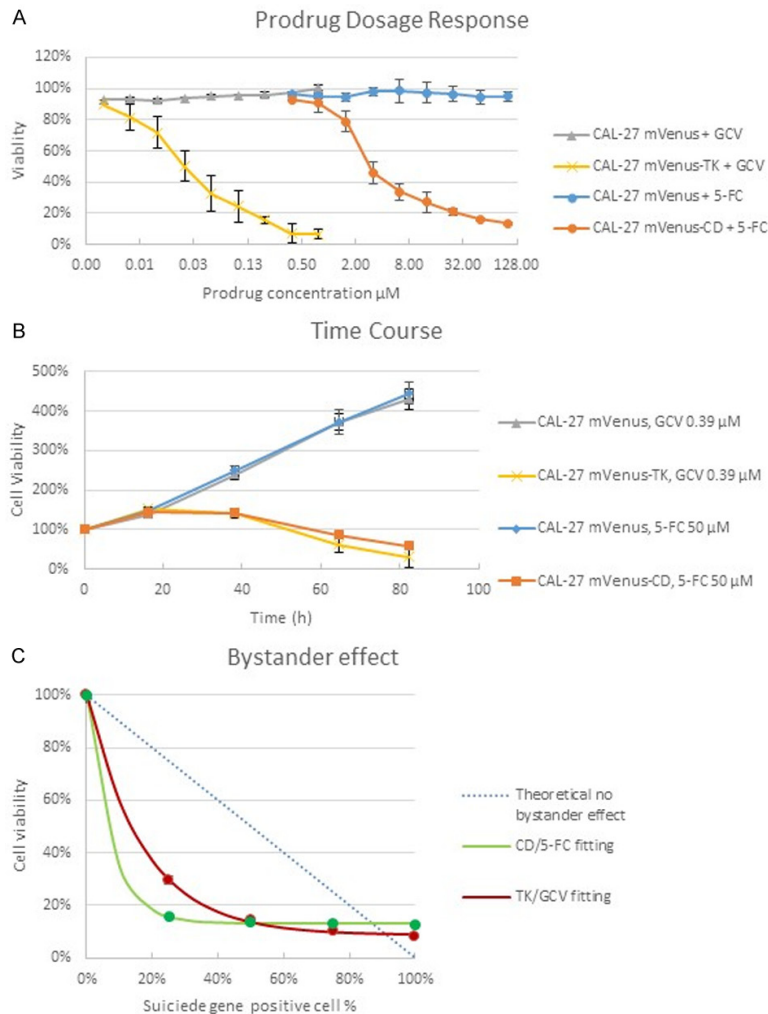


Figure 4. Evaluation and comparison of suicide gene therapies. A. Prodrug dosage test for suicide gene therapy. CAL-27 mVenus-CD and mVenus-TK cell lines were treated with a series of two-fold dilutions of 5-FC or GCV respectively for 4 days. MTT assay was used to measure cell viability. CAL-27 expressing mVenus only was served as negative control. B. Prodrug killing time course test for suicide gene therapy. CAL-27 mVenus-CD and mVenus-TK cells were treated with 50 μM 5-FC or 0.39 μM GCV. At various time points, cell viability was measured by MTT assay. C. Evaluation and comparison of suicide gene therapies' bystander effect. CAL-27 cells expressing mVenus-CD or mVenus-TK were mixed with CAL-27 cells expressing mVenus only at ratios of 100%, 75%, 50%, 25%, and 0%. Cell mixtures were then treated with 50 μM 5-FC or 0.39 μM GCV for 4 days. Cell viability was measured by MTT assay. Cell viability was plotted against percentage of suicide gene positive cell. Red dots and green dots are cell viability of GCV or CD treated cells. Blue dashed line represents theoretical viability curve when suicide gene doesn't have bystander effect. Red and green lines are fitted line by exponential equation. Experiments were repeated twice. Scale bars: standard deviation.

ment, cell viability was measured by MTT assay. The result shows that both GCV and 5-FC did not inhibit cell growth after prodrug treatment for 16 hours (Figure 4B). Inhibition of cancer cell growth was found after prodrug treatment

for 38 hours. After that, CAL-27 cells were gradually killed by prodrug. At 80 h, almost all drug-sensitive cells were killed. In terms of killing time, GCV and 5-FC have the same profile.

Quantitative evaluation and comparison of bystander effect of suicide gene therapies

Bystander effect is a phenomenon describing prodrug not only kills suicide gene-expressing cancer cells but also kills contacting suicide gene negative cancer cells. To evaluate bystander effect, CAL-27 cells expressing mVenus-CD or mVenus-TK were mixed with suicide gene negative cells (CAL-27 cells expressing Venus only) at the ratios of 100%, 75%, 50%, 25%, and 0%. Then cell mixtures were treated with 50 μM 5-FC or 0.39 μM GCV for 4 days. Cell viability was measured by MTT assay after 4-day treatment. As shown in Figure 4C, cell viability was plotted with percentage of suicide gene positive cells. The blue dashed line in Figure 4C represents theoretical cell viability curve when suicide gene does not have bystander effect. If suicide gene has bystander effect, viability curve will be under the theoretical non-bystander effect curve. As expected, the viability curves of CD/5-FC and TK/GCV are under the theoretical non-bystander effect curve, indicating CD/5-FC and TK/GCV have bystander effects which is consistent with the previous reports [19, 20]. Notably, when suicide gene positive cells constituted 25% of total cells, the CD/5-FC therapy killed more cancer cells than the TK/GCV therapy at the same ratio (Viability: CD/5-FC 15.6% versus TK/GCV

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Table 1. Quantitative evaluation and comparison of suicide gene therapies

Pair	Prodrug concentration	Treatment time	a	b	c	% of area
CD/5-FU	50 μ M	4 days	0.870	14.0	0.130	63.2%
TK/GCV	0.39 μ M	4 days	0.913	5.85	0.087	52.3%

29.4%), indicating the CD/5-FU pair has a better bystander effect than TK/GCV pair at 25% of positive cell ratio.

To quantitatively evaluate this bystander effects, we developed a method. First, the data in **Figure 4C** were fitted to an exponential equation: $y = a \cdot \text{EXP}(-b \cdot x) + c$, where y is cell viability and x is percentage of suicide gene positive cells. The fittings were good for both CD/5-FU and TK/GCV with R-square of 0.9999 and 0.9997, respectively. The a , b , and c values for CD/5-FU and TK/GCV were listed in **Table 1**. In the exponential equation, b is decay constant, which represents how fast the cell viability decrease with increase of percentage of suicide gene positive cell. The faster the decrease is, the better bystander effect is, because prodrug will kill more cells with the same ratio of suicide gene positive cells. Thus, the value of b can be used to compare bystander effects at low positive cell ratio. A bigger b value will represent a better bystander effect. As shown in **Table 1**, the b value of CD/5-FU is bigger than TK/GCV (CD/5-FU, 14.0; TK/GCV, 5.85), which is consistent with the observation that CD/5-FU has a better bystander effect than TK/GCV at 25% of positive cell ratio.

As the b value only reflects the decrease speed, it cannot show the overall bystander effect. To quantitatively measure the overall bystander effect, the % of area was calculated based on curves in **Figure 4C**. First, the area between fitted exponential curve and theoretical no bystander effect curve was calculated. This area is directly related to by-stander effect of suicide gene. Then the percentage of this area to the area of triangle surrounded by x -axis, y -axis, and the theoretical no bystander effect curve was calculated. As shown in **Table 1**, CD/5-FU still has a larger % of area than TK/GCV (63.2% vs 52.3%), indicating CD/5-FU has a better overall bystander effect than TK/GCV.

Discussion

In this study, we evaluated two most widely studied PA suicide gene therapies on OSCC cell lines CAL-27. To our knowledge, this is the first study to quantitatively evalu-

ate and compare two leading PA suicide gene therapies for OSCC. The dosage study shows that the minimum concentration of 5-FU to kill all drug-sensitive cell is around 50 μ M, while this concentration for GCV is 0.39 μ M, indicating CAL-27 cells is more sensitive to GCV than 5-FU. The pharmacokinetics studies in human show that GCV can achieve peak serum concentration >9 mg/L (35.3 μ M), while the peak serum concentration of 5-FU is ~ 20 mg/L (155 μ M) [21-23]. Compared with GCV killing concentration to CAL-27 cells (0.39 μ M), the GCV peak serum concentration is about 90-fold higher than GCV killing concentration for CAL-27 (0.39 μ M). In contrast, the peak serum concentration of 5-FU is only about 3-fold higher than killing concentration of 5-FU (50 μ M). Thus, it would be easier for GCV to reach effective drug concentration in patients. This may explain why GCV/TK therapy is more extensively tested in clinical trials than CD/5-FU [3]. If the minimum killing concentration of 5-FU could be reduced, such as by improving CD catalytic efficiency [24], the *in vivo* efficacy of CD/5-FU therapy may be improved.

The time course study showed GCV or CD only killed cancer cells after about 2-day treatment. This killing time is consistent with previous study [25]. This long killing time course may be related to cancer killing mechanism of these two drugs.

Our results demonstrate that the bystander effect can be quantitatively evaluated and compared (**Figure 4C**). Two values were calculated to quantify the bystander effect: the b value and overall bystander effect. The b value is the decay constant of fitted exponential equation. Bigger b value means suicide gene therapy could kill more cancer cells at the same ratio of suicide gene positive cells, especially at the low ratio of positive cells. CD/5-FU has a bigger b value than TK/GCV (**Table 1**). As shown in **Figure 4C**, at the 25% of positive cells, CD/5-FU killed more cancer cells than TK/GCV.

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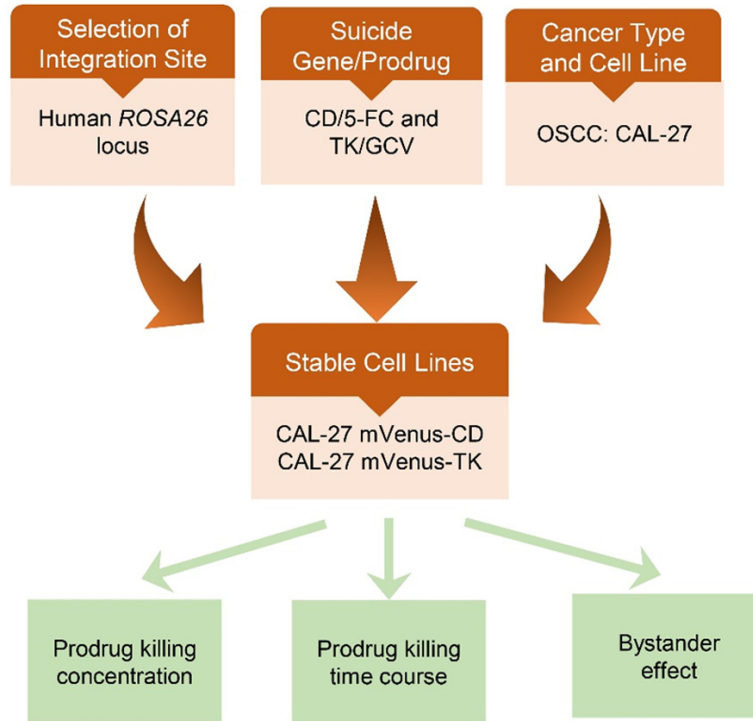


Figure 5. The universal method to evaluate and compare suicide gene therapy.

However, at the 50% of positive cells, CD/5-FU killed the same cancer cells as TK/GCV. In this regard, the *b* value cannot reflect bystander effect on a whole. To avoid bias of the *b* value, the % of area was calculated to measure the overall bystander effect across all ratio of suicide gene positive cells. As shown in **Table 1**, CD/5-FU still has a bigger % of area than TK/GCV, although the difference is smaller than the difference of *b* value. In summary, if transfection/infection efficiency is not a bottleneck of suicide gene therapy, the % of area will be a good indicator for bystander effect. In contrast, if high transfection/infection efficiency is hard to achieve, the *b* value will be a useful value to evaluate bystander effect.

The evaluation and comparison approach in this study (**Figure 5**) is a general method to evaluate PA suicide gene therapy. It can be used in new therapy development in preclinical study. Other types of cancer can be tested by changing the cancer cell lines. For example, to study breast cancers, we could use MCF-7 cells. To study leukemia, we could use HL-60 cell line. We could even use primary cancer tissue. This method could also be used to evaluate other prodrug-activating suicide gene thera-

py pair, such as NTR/CB19-54, CPG2/Nitrogen Mustard, and P450/oxazaphosphorine [4]. For example, when new version of CD or TK has been developed [24, 26]. In addition, this method could also be developed into an *in vivo* model to evaluate PA suicide gene therapy on animal models. Taking CAL-27 cell line as an example, this cell line is tumorigenic after inoculating in immunocompromised nude mice. By injection of the CAL-27 cell lines expressing suicide gene in this study into nude mice, the dosage response, killing time course, as well as bystander effect could be evaluated and compared *in vivo*.

When we tested for effective killing concentration, it was found that a portion of CAL-27 cells were resistant to either

5-FU (~13%) or GCV (~3%) treatment. This phenomenon is also found in other study [25]. Currently, the underlying mechanism is not clear. One possibility is that these drug-resistant cells may go into cell quiescence to stay alive. Mechanism study on drug resistant cells may lead to improvement on therapy efficacy. In clinical trial, there are attempts to express CD and TK simultaneously in cancer to increase anticancer efficacy [27]. Our results imply that this combination approach could greatly reduce population of drug-resistant cancer cells and improve anti-cancer efficacy. In theory, for CAL-27 cell lines, combinational therapy with CD and TK may reduce resistant cells below 1% ($13\% \times 3\% = 0.39\%$).

For OSCC therapy, TK/GCV therapy could be a good approach, but its bystander effect is not as good as CD/5-FC. Less efficient bystander effect means TK/GCV needs high transfection/infection rate to effectively kill cancer cells in patients. However, considering low transfection efficiency of OSCC cell lines, it may not be possible to achieve satisfactory suicide gene transfection/infection efficiency in patients. CD/5-FC has a better bystander effect, but it is hard to achieve effective kill concentration *in vivo*. In

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our opinion, both CD/5-FC and TK/GCV therapies tested in this study may not be appropriate for OSCC treatment. Bystander effect should be improved for TK/GCV and prodrug kill concentration should be improved for CD/5-FC.

According to our experience, cell lines derived from OSCC are hard to be transfected. In this study, we screened 6 commercially available transfection reagents and found FuGENE HD achieved the best balance between transfection efficiency and cell viability for CAL-27 cell line. However, in terms of transfection efficiency, FuGENE HD is not the best one. GenelIn and ViaFect are much better than FuGENE HD. But these two reagents caused drastic cell death. Further research and development on GenelIn or ViaFect to reduce transfection-related cell death may facilitate research in OSCC field.

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Disclosure of conflict of interest

None.

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References

[1] Ginn SL, Amaya AK, Alexander IE, Edelstein M and Abedi MR. Gene therapy clinical trials

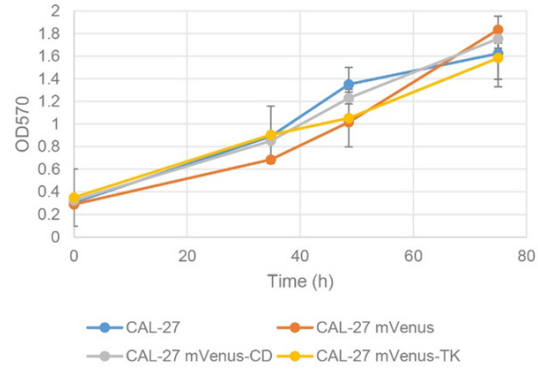
worldwide to 2017: an update. *J Gene Med* 2018; 20: e3015.

- [2] Santiago-Ortiz JL and Schaffer DV. Adeno-associated virus (AAV) vectors in cancer gene therapy. *J Control Release* 2016; 240: 287-301.
- [3] Zarogoulidis P, Darwiche K, Sakkas A, Yarmus L, Huang H, Li Q, Freitag L, Zarogoulidis K and Malecki M. Suicide gene therapy for cancer-current strategies. *J Genet Syndr Gene Ther* 2013; 4: 16849.
- [4] Karjoo Z, Chen X and Hatefi A. Progress and problems with the use of suicide genes for targeted cancer therapy. *Adv Drug Deliv Rev* 2016; 99: 113-128.
- [5] Wang J, Lu XX, Chen DZ, Li SF and Zhang LS. Herpes simplex virus thymidine kinase and ganciclovir suicide gene therapy for human pancreatic cancer. *World J Gastroenterol* 2004; 10: 400-403.
- [6] Miller CR, Williams CR, Buchsbaum DJ and Gillespie GY. Intratumoral 5-fluorouracil produced by cytosine deaminase/5-fluorocytosine gene therapy is effective for experimental human glioblastomas. *Cancer Res* 2002; 62: 773-780.
- [7] Pires FR, Ramos AB, Oliveira JB, Tavares AS, Luz PS and Santos TC. Oral squamous cell carcinoma: clinicopathological features from 346 cases from a single oral pathology service during an 8-year period. *J Appl Oral Sci* 2013; 21: 460-467.
- [8] Jiang X, Wu J, Wang J and Huang R. Tobacco and oral squamous cell carcinoma: a review of carcinogenic pathways. *Tob Induc Dis* 2019; 17: 29.
- [9] Markopoulos AK. Current aspects on oral squamous cell carcinoma. *Open Dent J* 2012; 6: 126-130.
- [10] Jadhav KB and Gupta N. Clinicopathological prognostic implicators of oral squamous cell carcinoma: need to understand and revise. *N Am J Med Sci* 2013; 5: 671-679.
- [11] Abbas S, Tariq MUU, Raheem A, Saeed J, Hashmi SS, Karim M and Nizam M. Assessment of factors affecting quality of life in oral squamous cell carcinoma patients using university of washington quality of life questionnaire. *Cureus* 2019; 11: e3904.
- [12] Vattemi E and Claudio PP. The feasibility of gene therapy in the treatment of head and neck cancer. *Head Neck Oncol* 2009; 1: 3.
- [13] Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA and Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013; 339: 819-823.
- [14] Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG and Soriano P. Disruption of

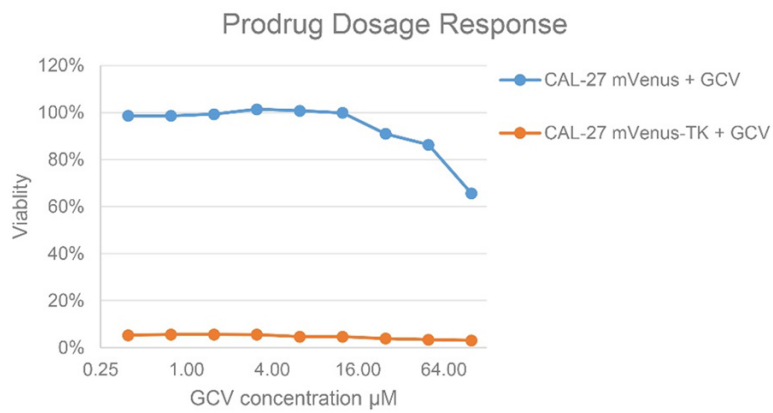
The comparison of suicide gene therapies on OSCC

- overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A* 1997; 94: 3789-3794.
- [15] Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; 21: 70-71.
- [16] Perez-Pinera P, Ousterout DG, Brown MT and Gersbach CA. Gene targeting to the ROSA26 locus directed by engineered zinc finger nucleases. *Nucleic Acids Res* 2012; 40: 3741-3752.
- [17] Irion S, Luche H, Gadue P, Fehling HJ, Kennedy M and Keller G. Identification and targeting of the ROSA26 locus in human embryonic stem cells. *Nat Biotechnol* 2007; 25: 1477-1482.
- [18] Zhao M, Sano D, Pickering CR, Jasser SA, Henderson YC, Clayman GL, Sturgis EM, Ow TJ, Lotan R, Carey TE, Sacks PG, Grandis JR, Sidransky D, Heldin NE and Myers JN. Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites. *Clin Cancer Res* 2011; 17: 7248-7264.
- [19] Freeman SM, Abboud CN, Whartenby KA, Packman CH, Koeplin DS, Moolten FL and Abraham GN. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res* 1993; 53: 5274-5283.
- [20] Erbs P, Regulier E, Kintz J, Leroy P, Poitevin Y, Exinger F, Jund R and Mehtali M. In vivo cancer gene therapy by adenovirus-mediated transfer of a bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion gene. *Cancer Res* 2000; 60: 3813-3822.
- [21] Pescovitz MD, Ettenger RB, Strife CF, Sherbotie JR, Thomas SE, McDiarmid S, Bartosh S, Ives J, Bouw MR and Bucuvalas J. Pharmacokinetics of oral valganciclovir solution and intravenous ganciclovir in pediatric renal and liver transplant recipients. *Transpl Infect Dis* 2010; 12: 195-203.
- [22] Vermes A, Guchelaar HJ and Dankert J. Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. *J Antimicrob Chemother* 2000; 46: 171-179.
- [23] Pai MP, Bruce H and Felton LA. Clinical pharmacokinetics of oral controlled-release 5-fluorocytosine. *Antimicrob Agents Chemother* 2010; 54: 1237-1241.
- [24] Stolworthy TS, Korkegian AM, Willmon CL, Ardiani A, Cundiff J, Stoddard BL and Black ME. Yeast cytosine deaminase mutants with increased thermostability impart sensitivity to 5-fluorocytosine. *J Mol Biol* 2008; 377: 854-869.
- [25] Lee M, Ha J, Son YS, Ahn H, Jung KB, Son MY and Kim J. Efficient exogenous DNA-free reprogramming with suicide gene vectors. *Exp Mol Med* 2019; 51: 82.
- [26] Black ME, Kokoris MS and Sabo P. Herpes simplex virus-1 thymidine kinase mutants created by semi-random sequence mutagenesis improve prodrug-mediated tumor cell killing. *Cancer Res* 2001; 61: 3022-3026.
- [27] Moriuchi S, Wolfe D, Tamura M, Yoshimine T, Miura F, Cohen JB and Glorioso JC. Double suicide gene therapy using a replication defective herpes simplex virus vector reveals reciprocal interference in a malignant glioma model. *Gene Ther* 2002; 9: 584-591.

The comparison of suicide gene therapies on OSCC

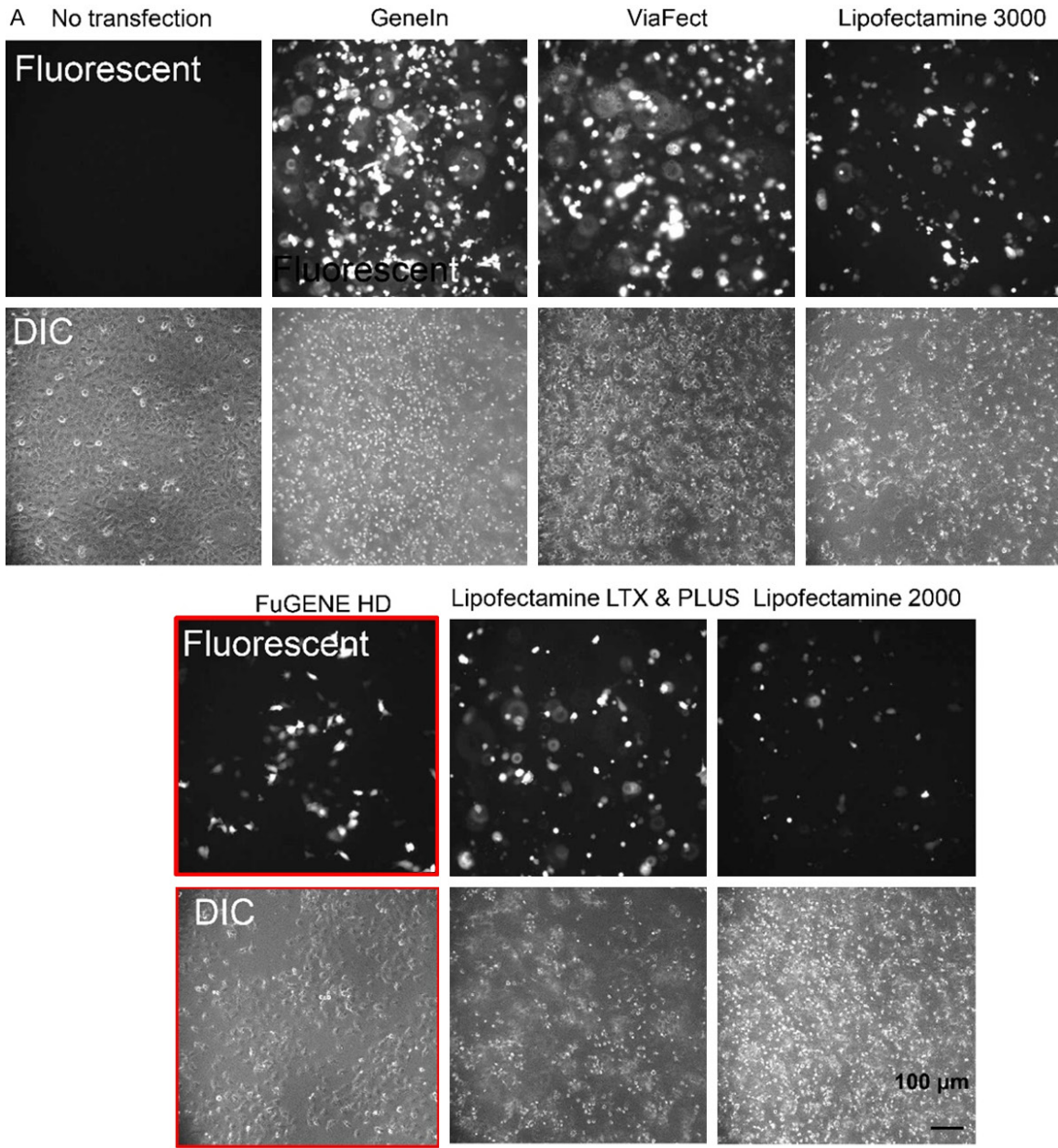


Supplementary Figure 1. Growth curve of CAL-27, CAL-27 mVenus, CAL-27 mVenus-CD, and CAL-27 mVenus-TK.



Supplementary Figure 2. CAL-27 mVenus-TK cell line was treated with a series of two-fold dilutions of GCV from 0.39 µM to 100 µM for 4 days. MTT assay was used to measure cell viability. CAL-27 expressing mVenus only serves as negative control.

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B

Reagents	Transfection Efficiency	Toxicity
Geneln	High	Toxic
ViaFect	High	Toxic
Lipofectamine 3000	Medium	Toxic
FuGENE HD	Medium	Not Toxic
Lipofectamine LTX & PLUS	Medium	Toxic
Lipofectamine 2000	Low	Toxic

Supplementary Figure 3. Screen for appropriate transfection reagent for CAL-27 cell line. A. Microscope pictures showing fluorescent and corresponding Differential Interference Contrast (DIC) images of CAL-27 cells after transfection by various transfection reagents. Bright dots in fluorescent pictures are successfully transfected cells. Live cells in DIC pictures show attached, spreading and darker morphology as shown for non-transfected cells. In contrast, dead cells are round, bright and floating in DIC image. The best transfection is outlined by red line. Scale bar, 100 μ m. B. Summary of screening result. The best transfection reagent for CAL-27 cells is highlighted in red.