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Enhanced EJ Cell Killing of ^{125}I Radiation by Combining with Cytosine Deaminase Gene Therapy Regulated by Synthetic Radio-Responsive Promoter

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Abstract

Aim: To investigate the enhancing effect of radionuclide therapy by the therapeutic gene placed under the control of radio-responsive promoter.
Methods: The recombinant lentivirus *E8-codA-GFP*, including a synthetic radiation-sensitive promoter *E8*, cytosine deaminase *(CD)* gene, and green fluorescent protein gene, was constructed. The gene expression activated by ^{125}I radiation was assessed by observation of green fluorescence. The ability of converting 5-fluorocytosine (5-FC) to 5-fluorourial (5-FU) by *CD* enzyme was assessed by high-performance liquid chromatography. The viability of the infected cells exposed to ^{125}I in the presence of 5-FC was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the infected cells exposed to ^{125}I alone served as negative control and 5-FU as positive control.
Results: The recombinant lentiviral vector was constructed successfully. On exposure of infected cells to ^{125}I, green fluorescence can be observed and 5-FU can be detected. MTT assay showed that the survival rate for infected cells treated with ^{125}I was lower compared with the ^{125}I control group, but higher than the positive control group.
Conclusion: The synthetic promoter *E8* can induce the expression of downstream *CD* gene under ^{125}I radiation, and the tumor killing effect of ^{125}I can be enhanced by combining *CD* gene therapy with radiosensitive promoter.

Key words: 5-fluorocytosine, ^{125}I, cytosine deaminase, radiation-sensitive promoter, recombinant lentivirus

Introduction

Radionuclide based therapy plays an increasingly important role in the treatment of cancer. However, its efficacy is limited for many tumors because the delivery of the curative dose of radionuclide to the tumor is frequently limited by the normal tissue tolerance, especially for myelotoxicity. Targeting therapy, including radioimmunotherapy,[1,2] radio-receptor therapy,[3,4] and the therapy targeting specific tumor genes,[5] which is designed to irradiate tumor cells yet minimize the radiation dose received by normal cells, is a theoretically effective therapeutic approach and has achieved considerable success in hematological malignancy (such as radiolabeled anti-CD20 in the treatment of CD20-positive non-Hodgkin's lymphomas). However, curative therapy for most of solid tumors still remains challenging.[6] Although described as a ''targeted'' treatment, it does not specifically target the tumor and the distribution in the normal organ is also frequently beyond a safety margin. Therefore, the innovative approaches with an improved therapeutic ratio are required.

One of the limiting factors to the success of radionuclide-based therapy is the radiosensitivity of the target organ. Contemporary molecular biological techniques made it possible to increase the tumor radiosensitivity through altering the genetic profile of a tumor. By transducing therapeutic genes with a radiation-sensitive promoter on the upstream into tumor cells, the tumor cells will be sensitized

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to radiation and the killing effect is expected to be enhanced. The concept of using a radio-responsive gene to enhance therapeutic efficacy has already been used in radiotherapy. In 1994, Boothman proposed that promoter sequences of radiation-inducible genes could be used for the controlled expression of therapeutic genes.[7] Recently, more attention focused on the combination of radiotherapy with gene therapy using the cytosine deaminase *(CD)* gene,[8] herpes simplex virus thymidine kinase *(HSV-tk)* gene,[9] and tumor necrosis factor *(TNF)*-α gene[10] as the therapeutic gene placed downstream of early growth response gene *(Egr-1)* sequences used as the radiation-responsive promoter. Tumor cells containing these genes were sensitized to the radiation at a lower dose. The results showed that the novel approach can lead to more effective tumor cell killing, induce tumor growth delay, enhance tumor growth control with relative sparing of normal tissue, and thus increase the therapeutic ratio. The Phase I/II clinical trials have been conducted with *Ad.Egr.TNF*-α plasmid (referred to as *TNFerade*) in combination with radiotherapy.[10,11] A significant tumor response was observed with no server local or systemic toxicity. *Egr-1* can also be activated by the radiation of radionuclide.[12,13] Therefore, it is to be expected that the efficacy of radionuclide-based therapy will be increased by introducing the therapeutic gene with radio-responsive promoter into tumor cells. Besides *Egr-1*, the synthetic radio-responsive promoter consisting of continuous repeated CC(A/T)6GG sequences (*CArG* elements) was also reported and shown to be more radiation sensitive than the native *Egr-1* enhancer.[14]

In the previous study, the authors have investigated the potential of using the ^{131}I-labeled anti-bladder cancer monoclonal antibody BDI-1 for radioimmunoimaging and radioimmunotherapy of bladder cancer and obtained promising results.[15,16] To further enhance the therapeutic efficacy and minimize the damage to normal tissues, in this study, the authors construct the vector containing *CD* gene ligated to the synthetic radiosensitive promoter containing eight repeated *CArG* elements on the upstream and green fluorescent protein *(GFP)* coding sequence as the report gene to monitor the expression of the therapeutic gene and compare the killing efficacy of ^{125}I radiation on bladder cancer EJ cells infected with the vector in the presence of prodrug 5-fluorocytosine (5-FC) with ^{125}I radiation alone.

Materials and Methods

Cell lines and reagents

The human bladder cancer EJ cell line was purchased from the Pathology Department of Peking University and was cultured at 37°C, 5% CO_2 in monolayer culture in a complete medium comprising the RPMI 1640 culture medium supplemented with 10% fetal calf serum (FCS). All cell culture reagents were purchased from Hyclone Corporation. The pCD_2 vector was purchased from ATCC (American Type Culture Collection). 5-FC and 5-Fluorourial (5-FU) were purchased from Sigma Corporation. Liquid $Na^{125}I$ (2.22 GBq/mL, radiochemical purity >99%) was purchased from Beijing Atom HighTech Co. Ltd.

Construction of the plasmid

Construction of *pGC-FU-codA-GFP* plasmids. The pCD_2 plasmid was used as the basis for the vector construction. The primer was designed according to the *CD* sequence from GenBank, containing Age I sites at 5′ end and 3′ end, respectively. The *CD* gene sequence—Age I-Forward: 5′ GAGGATCCCCGGGTACCGGTCGCCACCATGTCGAA TAACGCTTTACAAAC3′. The CD gene sequence—Age I-Reward: 5′TCACCATGGTGGCGACCGGACGTTTGTAA TCGATGGCTTC3′. The cDNA of *CD* gene was obtained from the template of pCD_2 using polymerase chain reaction (PCR), and the PCR products were separated by 1.0% agarose gel electrophoresis. The purified PCR products were inserted into the lentivirus vector containing the *GFP* gene (*pGC-FU* vector; from Genechem Company) at the site of Age I. The vector was transformed into *Escherichia coli* strain DH5α cells preprocessed by $CaCl_2$, and the transformants were selected for apramycin resistance. The positive clones were implanted into the culture media after being determined by PCR. The plasmids were extracted from the strains and named as *pGC-FU-codA-GFP*. The sequence of the plasmid was confirmed by sequencing.

Synthesis of the radio-responsive promoter E8 and construction of *pGC-FU-E8-codA-GFP* plasmid. The radio-responsive promoter E8 containing eight repeated *CArG* elements was engineered to contain Pac I/BamH I sites at 5′ end and 3′ end, respectively. The gene sequence was 5′TTAATTAACCGCGGCCTTATTTGGCCTTATTTGGC CTTATTTGGCCTTATTTGGCCTTATTTGGCCTTATTT GGCCTTATTTGGCCTTATTTGGCCGCGGGGATCC3′. The synthetic *E8* and *pGC-FU-codA-GFP* were digested with Pac I/BamH I, purified, and ligated to generate the *pGC-FU-E8-codA-GFP* vector. The vector was transformed into *E. coli* strain DH5α cells preprocessed by $CaCl_2$, and the transformants were selected for apramycin resistance. The positive clones were determined by PCR and implanted into the culture media. The plasmids were extracted from the strains. The sequence of the plasmid was confirmed by automatic sequencing.

The package of recombinant lentivirus. The purified *pGC-FU-E8-codA-GFP* reconstructed vector or empty vector *pGC-FU* was cotransfected with *pHelper 1.0* and *pHelper 2.0* vector into 293T cells using Lipofectamine 2000 for 8 hours. The supernatant of 293T cells was collected, centrifuged at 4000 *g*, 4°C for 10 minutes, and filtrated with a 0.45-μm filter. The recombinant lentivirus *E8-codA-GFP LV* or the control lentivirus *GC-FU-GFP LV* containing empty vector was obtained and stored at −80°C.

Measurement of the lentivirus titer. The lentivirus titer was measured by a serial dilution method. 293T cells at exponential growth phase were implanted into 24-well plates at the concentration of 1×10^5 cells per well. The cells were infected with the lentivirus with 10-fold dilution series from 1:10 to $1:10^6$ in each well (triplicate). After cultured for 48 hours, the cells were cultured in a regular medium. Four days later, the titer of *GC-FU-GFP LV* was measured by evaluating the fluorescence expressed by the GFP gene. The titer of *E8-codA-GFP LV* was measured by real-time quantitative PCR. In brief, total RNA was extracted from the cells infected with *E8-codA-GFP LV* with TRIzol (Invitrogen). After synthesized by reverse transcriptase, cDNA was used as a template in PCRs. Serially diluted plasmid was

utilized to draw a standard curve. The cutoff point (Ct) of each sample was plotted on the standard curve and the mRNA copy numbers were calculated. Actin was used as an internal control.

Cell infection

The condition of lentivirus infection on EJ cells was tested using *GC-FU-GFP LV* in 96-well plates. The best condition was found in ENi.s (enhanced infection solution; Genechem provided) supplemented with 5-μg/mL polybrene (Genechem provided) when MOI (multiplicity of infection) was 60. EJ cells of 3×10^3 were plated in each well of a 96-well plate. After EJ cells were infected by *E8-codA-GFP LV* for 12 hours, the medium was changed to RPMI 1640 containing 10% FCS. Infected cells were cultured for 48 hours for the assessment of gene expression.

Assessment of the gene expression and 5-FC conversion under ^{125}I radiation

Green fluorescence observation. Infected cells were irradiated as monolayers in 96-well plates by adding 18.5, 37.0, 55.5, 74.0 kBq of Na^{125}I, respectively, and supplemented with the RPMI 1640 medium containing 10% FCS to 100 μL each well ($n=3$). Infected cells without Na^{125}I were used as controls ($n=3$). After 48 hours of incubation, the radiation-induced gene expression was assessed by the observation of green fluorescence under a fluorescence microscope (Nikon eclipse TE2000-s).

Conversion of 5-FC to 5-FU. The infected cells in 96-well plates were divided into three groups (three wells each group) according to the added ^{125}I at the dose of 37, 74, 148 kBq, respectively. Four hundred micrograms per milliliter of 5-FC was added in each well meanwhile. As control, uninfected cells were also divided into three groups ($n=3$) and added the same dose of ^{125}I and 5-FC. After 48 hours of radiation, the cell supernatants of the three wells in each group were mixed, and the concentration of 5-FC and 5-FU was analyzed by HPLC (high-performance liquid chromatography, Prominence LC-20A and Venusil MP C18 HPLC column, 10×250 mm, 5 μm) with the developing solvent consisted of acetonitrile: water = 1:9 (v/v) at the flow rate of 1 mL/min. The retain time for 5-FC and 5-FU was about 4.97 and 6.03 minutes, respectively. The absorption peak area ratio of 5-FU over 5-FC was used to estimate the conversion efficiency.

Measurement of cell survival rate

The infected cells in 96-well plates were divided into four groups, including an experimental group, a ^{125}I control group, a positive control group, and a negative control group. For the experimental group and ^{125}I control group, the cells were divided into three subgroups (three wells each group) according to the ^{125}I dose added. In the experimental group, 37, 74, 148 kBq of Na^{125}I aq. and 400 μg/mL 5-FC were added to the above subgroups, respectively. In the ^{125}I control group, only 37, 74, and 148 kBq of Na^{125}I aq. were added. Infected cells (three wells) with added 400 μg/mL 5-FU served as the positive control group and uninfected cells (three wells) without ^{125}I and 5-FC were used as the negative control group. All the cells were incubated in 5% CO_2, 37°C, for 48 hours.

The cell survival rate was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, aforementioned cells were incubated with 20 μL of MTT (5 mg/mL, in pH7.4 phosphate-buffered saline) for 3 hours at 37°C and 200 μL of dimethyl sulfoxide was added to solubilize the crystals for 20 minutes at room temperature. The plates were agitated to ensure complete mixing, and optical density (OD) was measured on a multiplate reader (microplate reader model 680) at a wavelength of 490 nm. Blank wells containing water added the same volumes of MTT solution were used as blank. The cell survival rate is expressed as (OD value of the experimental group or ^{125}I control group or the positive control group/OD value of the negative control group $\times100\%$). The cell survival rate is taken as 100% when the measured OD value of the experimental group or ^{125}I control group or the positive control group is greater than the OD value of the negative control group.

Results

Analysis of the E8 and CD gene

The *CD* gene obtained by PCR using *pCD*$_2$ as template was analyzed by gel electrophoresis and found to have a length of 1327 bp (Fig. 1). For analysis of the *E8* promoter in *pGC-FU-E8-codA-GFP* plasmid, the plasmid was digested with Pac I/BamH I and then electrophoresed. The result showed a fragment of 96 bp corresponding to the *E8* gene (Fig. 2). Plasmid *pGC-FU-E8-codA-GFP* was transformed into *E. coli* DH5α strains and the transformants were selected for apramycin resistance, and positive clones were picked up and verified by PCR. DNA sequence of the positive transformant showed that the plasmid contains eight $CC(A/T)_6GG$ elements and the *CD* gene code, which is conformed with the X63656.1 sequence in the GenBank. The first base A in the *CD* gene of *pGC-FU-E8-codA-GFP* plasmid is different from that in the GenBank (its first base is G) because the former is obtained from the prokaryote *E. coli* with its start codon of GUG,[17] but in the latter, the start codon is AUG for eukaryota.

The sequence of *E8* promoter is as follows: 5′TTAAT TAACCGCGGCCTTATTTGGCCTTATTTGGCCTTATT

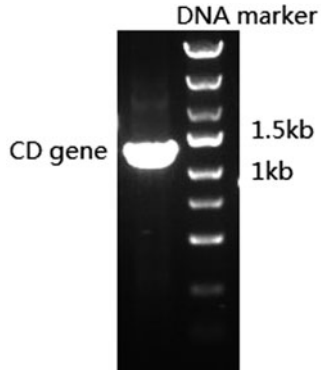

FIG. 1. Gel electrophoresis of cytosine deaminase *(CD)* gene in *pGC-FU-E8-codA-GFP* plasmid. The *right lane*: DNA marker, the *left lane*: *CD* gene (1327 bp).

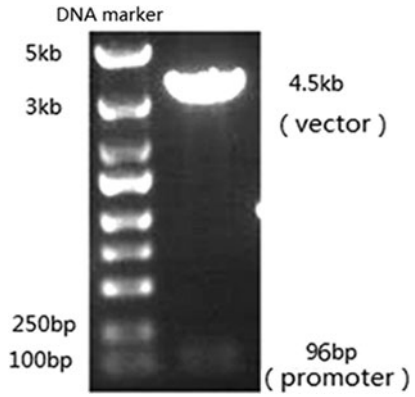

FIG. 2. Gel electrophoresis of *E8* promoter in *pGC-FU-E8-codA-GFP* plasmid. The *right lane*: molecular marker, the *left lane*: *E8* gene (96 bp).

TGGCCTTATTTGGCCTTATTTGGCCTTATTTGGCCT TATTTGGCCTTATTTGGCCGCGGGGATCC3′.

The package of recombinant lentivirus

The recombinant lentivirus *E8-codA-GFP LV* and the lentivirus containing parental vector *GC-FU-GFP LV* were successfully obtained by transfecting the plasmids into 293T cells cotransfected with *pHelper 1.0* and *pHelper 2.0* vectors in the presence of Lipofectamine 2000. The titer of *GC-FU-GFP LV* and *E8-codA-GFP LV* was 2×10^9 and 2×10^8 TU/mL, respectively.

GFP gene expression

The infected cells at the presence of 18.5, 37.0, 55.5, and 74.0 kBq Na^{125}I for 48 hours and the controls without ^{125}I were observed under the fluorescence microscope. The expression of fluorescence can be seen at ^{125}I dose as low as 18.5 kBq and was clearer with the increased dose of ^{125}I in the dose range from 18.5 to 74.0 kBq (Fig. 3). This showed that synthetic radiation-responsive promoter E8 could activate downstream *GFP* gene expression with the induction of low dose of ^{125}I. The cells with no ^{125}I added almost did not express the green fluorescence.

The conversion efficiency of 5-FC to 5-FU

Different doses of ^{125}I and 5-FC were added in the infected cells. After 48 hours of incubation, cell supernatants were analyzed by HPLC. The authors could see ultraviolet absorption peaks of 5-FC and 5-FU at the retain time of about 4.97 and 6.03 minutes, respectively, at a wavelength of 254 nm. The absorption peak area ratios of 5-FU over 5-FC were 11.86, 19.14, and 54.77 at 37 kBq, 74, and 148 kBq of ^{125}I radiation, respectively. It can be seen that the conversion efficiency of 5-FC to 5-FU increased with the increased dosage of ^{125}I and was at the highest when the dose of ^{125}I was 148 kBq in the dose range from 37 to 148 kBq. No absorption peak of 5-FU was observed with the uninfected cell supernatants when added the same dose of ^{125}I and 5-FC. This showed that infected EJ cells could express cytosine deaminase, which did not exist in mammalian cells, and then convert 5-FC to cytotoxic agent 5-FU and the expression of *CD* gene was increasing with increased dosage of ^{125}I.

TABLE 1. CELL SURVIVAL RATE OF DIFFERENT GROUPS (%)

Groups	*Survival rate (%)*	*Groups*	*Survival rate (%)*
148 kBq ^{125}I + 5-FC	65.43 ± 16.20	148 kBq ^{125}I	99.33 ± 15.64
74 kBq ^{125}I + 5-FC	68.29 ± 14.59	74 kBq ^{125}I	97.46 ± 6.31
37 kBq ^{125}I + 5-FC	72.57 ± 9.29	37 kBq ^{125}I	100
		5-FU	29.14 ± 1.10

5-FC, 5-fluorocytosine; 5-FU, 5-fluorourial.

The cell killing effect

After incubation of the infected EJ cells in the medium containing different doses of ^{125}I-NaI with or without 400 μg/mL 5-FC for 48 hours, MTT assay was performed. From the results it could be seen that ^{125}I in the dose range of 37–148 kBq could not kill the EJ cells. The combination of ^{125}I radiation and the *CD* gene therapy with 5-FC resulted in, obviously, the decrease in cell survival rate compared with ^{125}I radiation alone (Table 1).

Discussion

When exposed to ionizing radiation (IR), radiation-sensitive promoter can induce the expression of downstream genes, leading to increased gene expression. Introducing suicide genes with radiation-sensitive promoter on the upstream into tumor cells proposes an attractive approach for tumor gene therapy combined with radionuclide therapy. The *CD* gene encoding for cytosine deaminase and existing in yeasts and bacteria can convert nontoxic prodrug 5-FC

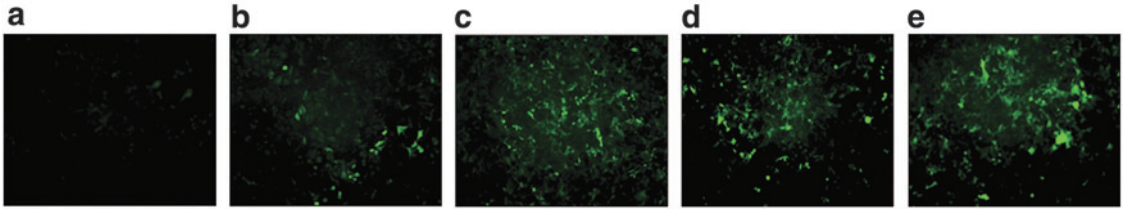

FIG. 3. Infected EJ cells green fluorescence expressions induced by different doses of ^{125}I GFP-positive cells were seen in *dark green*. Group **(a–e)** performed cells induced by 0, 18.5, 37.0, 55.5, and 74.0 kBq ^{125}I. The expression of fluorescence can be seen at the dose as low as 18.5 kBq and was clearer with the increased dose of ^{125}I.

into cytotoxic 5-FU, which plays an important role in tumor therapy.[18] Combination of radiotherapy with *CD* gene containing radio-responsive promoter in the presence of 5-FC has been proved to be an effective strategy in tumor cell killing.[19] Radionuclide can also activate radiation-sensitive promoter and then regulate the expression of suicide genes in space and time to play killing effects on tumor cells. It has been reported that ^{125}I-UdR can activate *Egr-1* promoter and increase the antitumor effect in combination with Egr-1 promoter-based *IFNγ* gene therapy.[12,20]

Egr-1 is the most studied radio-sensitive promoter in radiation-induced gene therapy. Weichselbaum et al. reported a 3.2-fold increase in chloramphenicol acetyltransferase (CAT) reporter gene expression in human HL252 cells *in vitro* and a 2.7-fold increase in SQ-20B tumor xenografts after a single 20 Gy treatment using the *Egr-1* enhancer.[21] The DNA sequences that drive the IR response of *Egr-1* are located in the enhancer region of the promoter.[22] These were identified as 10 nucleotide motifs of the consensus sequence $CC(A/T)_6GG$, also known as CArG elements. The murine and human *Egr-1* promoters contain five and four CArG elements, respectively.

To create gene therapy vectors that can be activated by lower doses of IR, synthetic enhancers containing isolated CArG elements were manufactured.[14] These promoters consist of an enhancer region containing tandemly arranged $CArG_2$ elements, and therefore lacked binding sites for the potentially antagonistic transcription factors that appeared in the native Egr-1 promoter, which may deregulate the expression (could restrict radio-responsiveness) under radiation. The results showed that the synthetic enhancers were more responsive to IR than the wild-type *Egr-1* gene.[14,23] An enhancer containing four isolated copies of the $CArG_2$ element (*E4*) showed 3.1-fold (±0.25) increase after IR compared with 1.86-fold (±0.2) for the native human *Egr-1* counterpart after 3 Gy activation dose, and the synthetic promoters responded to radiation doses as low as 1 Gy.[14] Scott et al. tested the promoters carrying 4, 6, 9, and 12 tandem-repeat copies of the prototype CArG sequence (*E4, E6, E9,* and *E12* enhancers) and found that increasing the number of $CArG_2$ elements from 4 to 6 and then 9 improved the specific IR, but the *E12* promoter was less inducible than *E9*.[23] In this study, the promoters carrying eight tandem-repeat copies of the *CArG* sequence were synthesized, and the result showed that it is sensitive to low dose of ^{125}I.

The efficient delivery of transgene to tumor sites is a key factor for successful gene therapy. It remains a formidable task, but progress has been made in recent years. Viral vectors and nonviral vectors are usually used in gene delivery systems. Major viral-based gene delivery systems are adenoviral vectors, adeno-associated viral vectors, and retroviral/lentiviral vectors. Nonviral-based delivery systems include cationic liposome, PEGylated system, HESylated system, and nanoparticle-based delivery system.[24] Viral vectors have a high transduction efficiency, but are safety concerned due to systemic toxicity and the immunogenicity induced in the host. Nonviral vectors have the advantages of nonimmunogenic, available to production on large scale and easy modification, but the transduction efficiency is relatively low. Lentivectors have been under development for use in gene therapy since the mid-1990s. Substantial effort has led to the development of the third-generation lentivectors now in use. The new generation of lentivectors has the advantages of long-term expression, cell- and tissue-specific tropism, high transduction efficiency, and large packaging capacity for the delivery of therapeutic genes.[25–27] It is reported that lentivectors were 2–10 times more effective in transducing human dendritic cells (DCs) and mouse DCs than adenovectors.[26]

Recently, targeting vectors, which could be injected intravenously and bind selectively to the tumor, have been studied. Adding nonviral vectors, such as incorporating targeting peptides (EGF, RGD, APRPG) into polyethylenimine (PEI),[28] polylysine (PLL),[29] and viral vectors, such as insertion of RGD peptide into the H1 loop of the fiber knob domain in adenovirus,[30] genetically incorporates targeting peptides into adeno-associated virus capsids.[31] Each technique has advantages and disadvantages. However, it will become more and more mature with the progress of transgene technology. By combining with gene therapy containing radiosensitive promoter, the radionuclide-based therapy shows promise and a bright hopeful future in the therapy of systemic tumors.

Conclusion

In the context, the authors constructed the plasmid containing *CD* gene and the *GFP* coding sequence under the control of the synthetic promoter *E8*. The *E8* promoter and *CD* gene sequence were confirmed by DNA sequencing. To monitor the gene expression, the authors used the *GFP* as a reporter. The cells without ^{125}I inducing showed no notable fluorescence, which indicated that the basal expression can be neglected. The expression of fluorescence can be seen with ^{125}I dose as low as 18.5 kBq, which indicated that the plasmid was very sensitive to radiation and low dose of ^{125}I can induce the gene expression. Similar results were reported by Zhao et al.[32] who used ^{125}I-UdR to irradiate Egr-1 radio-responsive promoter-controlled *IFNγ* gene to study the *in vitro* radiation inducible gene expression and found that *IFNγ* protein levels with 5, 10, 20, 50 kBq/mL doses of ^{125}I -UdR were obviously higher than no ^{125}I -UdR added group, and the gene expression levels are both dose dependent and time dependent.

The activity of *CD* protein can be evaluated by the conversion of 5-FC to 5-FU. The conversion efficiency can be measured either by the ^{3}H-5-FU tracer method[33] or by the HPLC method.[19] The authors used the HPLC method to measure the 5-FU level in the infected cell supernatants irradiated with ^{125}I at the dose of 37–148 kBq. The 5-FU peak can be detected even at the ^{125}I dose of 37 kBq and the peak area increased at the higher doses. The area ratio of 5-FU to 5-FC absorption peak was used to evaluate the conversion efficiency. The result showed that the conversion efficiency has the tendency of increasing with the increased dosage of ^{125}I and was at the highest when the dose of ^{125}I was 148 kBq at the dose range from 37 to 148 kBq. This indicated that infected EJ cells could express cytosine deaminase, which did not exist in mammalian cells, and then convert 5-FC to cytotoxic agent 5-FU and the level of expression was in a dose-dependent manner.

To detect the cell killing effect of the *pGC-FU-E8-codA-GFP* plasmid combined with different doses of ^{125}I radiation, MTT assay was performed. The result showed that the survival rates of EJ cells delivered the purpose gene irradiated

with ^{125}I given the prodrugs 5-FC were lower than that of by being radiated with ^{125}I alone (Table 1). The cell survival rates were in the range of 97.46%–100% with the ^{125}I radiation at the dose of 37–148 kBq, which showed ^{125}I radiation at aforementioned dose had little killing effect to the EJ cells. In combination with *CD* gene affection, the cell survival rates were decreased to a certain extent. The result showed that the cell killing effect of ^{125}I radiation can be enhanced by the combined treatment with *CD* gene-ligated synthetic radioresponsive promoter and the corresponding prodrug 5-FC.

In these cell survival studies, the lowest cell survival rate was 65.43%, which was not strong enough to justify the translation of this approach to animal models and clinical studies. The main possible reason for this was that the ^{125}I and 5-FC dose did not achieve the optimized level. In radionuclide therapy, ^{125}I with a half-life of 59.4 days and average energy of 27.4–35.5 Kev provides a continuous low-dose rate irradiation, which has little killing effect at low dosage in the short time period. ^{125}I brachytherapy using ^{125}I seed is the most clinically used modality for ^{125}I therapy. The results by Ma et al.[34] and Takabayashi et al.[35] showed the radiation dose up to 2 Gy (generated by the in-house model containing 9 ^{125}I seeds with the surface activity of 15.3–22.2 MBq and irradiated for 44–96 hours) can reach the therapeutic level to the SW-1990 pancreatic cancer cells and gastric cancer cells. The therapeutic effect of ^{125}I can be significantly increased by being labeled to the targeting molecules such as ITdU, which can incorporate into DNA of the cells[36] or monoclonal antibodies binding to tumor cell surface.[37] In the dose range of 37–148 kBq in this study, ^{125}I radiation alone had almost no killing effect to the EJ cells. However, the therapeutic efficacy may be increased by increasing the ^{125}I dosage or conjugating the radionuclide to the targeting molecules. From the cell survival rate results, one can also see that the cell survival rate in the 5-FU-positive control group was lower than in the experimental group, which indicated that there was a room to increase the killing effect by increasing the conversion rate of 5-FC to 5-FU, which is also expected to be achieved by increasing the ^{125}I dosage and optimizing the dose of 5-FC. Thus, although the killing effect is not strong enough to reach the very effective tumor therapeutic level in this study, this result was promising since one can expect the cell survival rates will be further decreased by increasing ^{125}I dose to the therapeutic level and optimizing the 5-FC dose. Further research is undergoing.

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Disclosure Statement

No competing financial interests exist.

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