Research Article

An Evaluation on Transfection Efficiency of pHRE-Egr1-EGFP in Hepatocellular Carcinoma Cells Bel-7402 Mediated by PEI-MZF-NPs

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To improve transfection and expression efficiency of target gene, especially under cancer anoxic microenvironment, we have developed pHRE-Egr1-EGFP/PEI-MZF-NPs nanosystem, in which pHRE-Egr1-EGFP, eukaryotic gene expression plasmid, is constructed by combining radiation promoter Egr1 with anoxia induction components (HRE), forming anoxic radiation double sensitive HRE/Egr1 promoter to activate reporter gene EGFP expression. MZF-NPs ($Mn_{0.5} Zn_{0.5} Fe_2O_4$ magnetic nanoparticles), obtained by coprecipitation method, are coated with cation poly(ethylenimine) (PEI). We transferred pHRE-Egr1-EGFP into hepatocellular carcinoma Bel-7402 cells, using PEI-MZF-NPs as the carrier and tested some relevant efficacy. The results show that PEI-MZF-NPs have good DNA-binding ability, protection ability, release ability, little toxicity, and high transfection efficiency, obviously superior to those of the liposome method and electricity perforation method. Moreover, the expression level of EGFP gene induced by anoxia and radiation was significantly higher than that of single radiation activation. It is therefore concluded that HRE/Egr1 can induce and improve target gene expression efficiency in cancer anoxic microenvironment, and that PEI-MZF-NPs can be used as a novel nonviral gene vector which offers a viable approach to the mediated radiation gene therapy of cancer.

1. Introduction

Developed recently to address the problems in gene therapy and radiation therapy, radiation-gene therapy is a promising area of study in cancer treatment. More specifically, it is a cancer therapeutic method that while doing local radiotherapy, the genes with antitumor effect are induced by the radiation to express to kill cancer cells, resulting in dual therapy of ray and gene on cancer, thus working together to their best advantage, and playing a synergistic antitumor role. Egr1, a transcription factor, regulating cell growth early after being radiated, can induce the expression of its downstream genes by ionizing radiation, attaining a spatio-temporal regulation on the target gene expression. Notable achievements in radiation-gene therapy by using radiosensitivity of Egr1 radiation promoter have been obtained [1–3]. However, this method has not overcome the common

problem in gene therapy yet, that is, how to ensure a safe gene delivery into cells with high transfection efficacy. In addition, the anoxic microenvironment in solid tumor is likely to curb the induction activity of radiation promoter.

Hypoxia response element (HRE), an enhancer sensitive to hypoxia, can induce the expression of its downstream genes when combined particularly with HIF-1, hypoxia inducible factor-1. It was reported that HRE/HIF system existed in both mammalian cells and human tissue, and HIF-1α was overexpressed in 68–84% of cancers, which suggested that HRE can be used to regulate expression of target gene in tumor hypoxia environment [4–7]. Shibata et al. [8] has confirmed that 5 copies of HRE connected with a promoter can increase its downstream gene expression by 500 times in hypoxia condition.

In this study, 5HRE was inserted in the front of Egr1 promoter to construct a hypoxia-radiation dual-sensitive

promoter and then connected with EGFP, a reporter gene, to develop an eukaryotic recombinant plasmid–pHRE–Egr1–EGFP to induce and improve EGFP expression in tumor cells using hypoxia and radiation. In this system, radiation can be used as a switch to start the transcription of target gene, regulating its expression in space-time. Because of the radiation dose, range and times can be artificially controlled; the expression of the target gene is likely to occur within a certain range, at an appropriate time, even at the desirable level. Thus, the goal of controlling gene expression can be achieved to some extent, and the level of target gene expression will be improved in hypoxic microenvironment of solid tumors.

The choice of gene transfer vector is another key issue in gene therapy. As two major kinds of gene transfer vectors currently viral vector system and nonviral vector system both have their own advantages and disadvantages. Being the most efficient so far, the former is not regularly used in clinic due to small gene capacity, poor targeting specificity, self-immunogenicity, and serious biosafety risk in particular. In spite of having avoided the major security risk, the latter is greatly inferior to the former in transfection efficiency, and meaningful expression of target gene is hardly available. Currently lipofection and electroporation are the two non-viral vector transfection methods used most. But besides high cytotoxicity, liposome can be quickly cleared by serum in vivo, which greatly limits its utility, in spite of good transfection efficiency [3]. Electroporation method with high efficiency is suitable for transient expression and stable transfection, but it only applies to cells or tissue in vitro, not suitable for transfection in vivo. Moreover, a large number of cells will be killed by electric shocks [9–11].

Encouragingly, nanotechnology developed recently has offered a new means for solving the problem of gene transfer vector [12–19]. The research on the gene transfer vector based on nanoparticle has attracted wide attention—the therapy genes such as DNA or RNA wrapped in the nanoparticles or adsorbed on the surface are taken into cells and released. Compared with traditional carriers, nano-vectors used for gene transfer have a lot of advantages such as nonimmunogenicity, repeated injection, no genetic toxicity and cytotoxicity, no cell transformation or death, slow release of the genes to extend response time effectively, and main-taining effective concentration of the product, improving transfection efficiency and bioavailability of the product, and so on [20, 21]. Such vectors have become a new promising carrier system because they not only integrate advantages of the virus vectors and traditional non-viral vectors, but also avoid their defects [22–28]. Particularly, besides properties of general nanoparticles, the superparamagnetic effect of magnetic nanoparticle gene transfer vector will produce transfection with high efficiency and directional movement in an external magnetic field, which in turn helps to carry out targeted gene therapy.

But it is well known that the smaller particles, the more agglomerate, and soft agglomeration widely exist in nanoparticles (especially magnetic nanoparticles). Only modified on the surface can nanoparticles get into the cells to play a role of gene transfer vector, and dispersion of
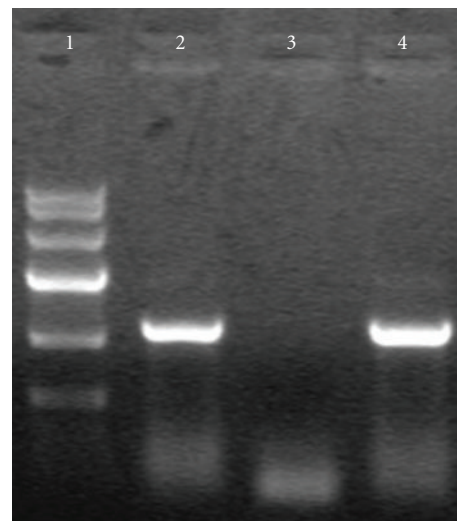

FIGURE 1: PCR gel electrophoresis of pCDNA3.1-Egr1-EGFP transfected (lane 1: Marker IV (each band in turn from top to bottom is 7 K, 5.5 K, 3.5 K, 2 K, 1 K, 500 bp); lane 2: pCDNA3.1-Egr1-EGFP; lane 4: pCDNA3.1-Egr1-EGFP).

the particles and surface functional groups have a direct impact on the further application of particles [13, 17]. As a common powder surface modification agent, PEI, whose monomer ($-CH-CH_2-NH_2-$) has good ability to bind DNA and adhere to cells, has electrostatic repulsion and steric hindrance effect [29–31]. In the previous study in our lab, $Mn_{0.5}Zn_{0.5}Fe_2O_4$, a thermosensitive Mn, and Zn ferrite magnetic nanoparticles, were successfully prepared chemically and modified surfacely by PEI, and good transgenic effect on cos-7 cells was obtained [32]. But whether PEI-MZF-NPs can be used for transfection of pHRE-Egr1-EGFP into cancer cells to perfect radiation-gene therapy for tumors and what the effect will be remain unknown to us.

In this project, attempts were made to develop a pHRE-Egr1-EGFP/PEI-MZF-NPs composite nanosystem, in which pHRE-Egr1-EGFP were transferred into hepatocellular carcinoma Bel-7402 cells by PEI-MZF-NPs as the carrier, and some relevant efficacy was tested. In addition, a comparative study of the level of gene expression by double induction of hypoxia and radiation and separate induction of radiation was made, which was expected to offer a viable approach to the mediated radiation gene therapy of cancer.

2. Materials and Methods

2.1. Main Reagent. Lipofectamine TM2000 purchased from Invitrogen Corporation; PEI (polyethylenimine, PEI) and dimethyl sulfoxide (DMSO) purchased from Sigma; Agarose purchased from MRI; DNaseI from Amercso; DMEM medium and fetal bovine serum from Gibco; Thiazolyl blue (MTT) from AMRESCO; PCDNA3.1-EGFP from Biotech Co.Ltd. Changsha Ying Run. Bel-7402 cells were provided by the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences.

Sequence	0	1		0	
Sequence	1	301	CAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACACC	360	

Sequence 0 1   0
Sequence 1 361 AATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTC 420

Sequence 0 1   0
Sequence 1 421 AATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTGC 480

Sequence 0 1   54
Sequence 1 481 GATCGCGACCCGGAAACGCCATATAAGGAGCAGGAAGGATCCCCCGCCGGAACAGACCTT 540

Sequence 0 55 ATTTGGGCAGCGCCTTATATGGAGTGGCCCAATATGGCCCTGCCGCTTCCGGCTCTGGGA 114
Sequence 1 541 ATTTGGGCAGCGCCTTATATGGAGTGGCCCAATATGGCCCTGCCGCTTCCGGCTCTGGGA 600

Sequence 0 115 GGAGGGGCGAGCGGGGGTTGGGGCGGGGGCAAGCTGGGAACTCCAGGCGCCTGGCCCGGG 174
Sequence 1 601 GGAGGGGCGAGCGGGGGTTGGGGCGGGGGCAAGCTGGGAACTCCAGGCGCCTGGCCCGGG 660

Sequence 0 175 AGGCCACTGCTGCTGTTCCAATACTAGGCTTTCCAGGAGCCTGAGCGCTCGCGATGCCGG 234
Sequence 1 661 AGGCCACTGCTGCTGTTCCAATACTAGGCTTTCCAGGAGCCTGAGCGCTCGCGATGCCGG 720

Sequence 0 235 AGCGGGTCGCAGGGTGGAGGTGCCCACCACTCTTGGATGGGAGGGCTTCACGTCACTCCG 294
Sequence 1 721 AGCGGGTCGCAGGGTGGAGGTGCCCACCACTCTTGGATGGGAGGGCTTCACGTCACTCCG 780

Sequence 0 295 GGTCCTCCCGGCCGGTCCTTCCATATTAGGGGCTTCCTGCTTCCCATATATGGCCATGTAC 354
Sequence 1 781 GGTCCTCCCGGCCGGTCCTTCCATATTAGGGGCTTCCTGCTTCCCATATATGGCCATGTAC 840

Sequence 0 355 GTCACGGCGGAGGCGGGCCCGTGCTGTTCCAGACCCTTGAAAATAGAGGCCGATTCGGGGA 414
Sequence 1 841 GTCACGGCGGAGGCGGGCCCGTGCTGTTCCAGACCCTTGAAAATAGAGGCCGATTCGGGGA 900

Sequence 0 415 GTCGCGAGAGATCCCAGCGCGCAGAACTTGG 445
Sequence 1 901 GTCGCGAGAGATCCCAGCGCGCAGAACTTGGGCTAGCGTTTAAACTTAAGCTTGGTACCG 960

Sequence 0 446   445
Sequence 1 961 AGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCTGCAGTCGACGGTACCGCGGGCCCGG 1020

Sequence 0 446   445
Sequence 1 1021 GATCCACCGGTCGCCACCATGGTGAGCAAGGGCGA 1055

Figure 2: Sequencing and alignment of pCDNA3.1-Egr1-EGFP (sequence 0: Egr1 promoter sequences without CMVE; sequence 1: pCDNA3.1-Egr1-EGFP sequences with ab1 reverse complementary sequences; software: Dnassit 2.0).

2.2. Construction and Identification of the Eukaryotic Expression Plasmids: Egr1-HRE-EGFP

2.2.1. PCR Amplification of Egr1 Promoter Segments.
Egr1 promoter was inserted into pIRES vector. In front of the Egr1 promoter, there was a CMV fragment to enhance the expression efficiency of the promoter. The primer sequences were designed according to CMVE-Egr1p template, and CMVE-Egr1p fragments were amplified by PCR.

    Egr1-f: 5′-CCG CTCGAG CG ACGCGT GATCTT-CAATATTGGCCATTAGC-3′

    —MluI: CG ACGCGT;

    —XhoI: CCG CTCGAG

    Egr1-r: 5′-CCG CTCGAG CTA GCTAGC CCA-AGTTCTGCGCGCTGGGAT-3′

    —NheI: CTA GCTAGC

    —XhoI: CCG CTCGAG

Product length = 1152 bp.

2.2.2. Egr1p Were Subcloned into pCDNA3.1-EGFP.
The above CMVE-Egr1p fragments amplified were collected and subcloned into pCDNA3.1-EGFP on MluI and NheI sites. After transfection, the plasmids were purified and identified by PCR.

    Egr1-f: 5′-CCG CTCGAG CG ACGCGT GATCTT-CAATATTGGCCATTAGC-3′,

    Egr1-r: 5′-CCG CTCGAG CTA GCTAGC CCAAGT-TCTGCGCGCTGGGAT-3′.

Product length = 1152 bp.

    According to the PCR electrophoresis results, the correct pCDNA3.1-Egr1-EGFP sequences were selected to test, EGFP-N-3 as the sequence primer.

2.2.3. To Synthesize 5HRE.
5HRE was synthesized according to [33]. In order to facilitate subsequent experiment, several restriction sites were added on the two sides of 5HRE. Synthetic products were identified by enzyme cleavage and gene sequence analysis (gene name: 5HRE; gene length: 185 bp; carrier name: pUC57; cloning site: EcoRI, HindIII).

2.2.4. Construction of pcDNA 3.1-5HRE-Egr1p-EGFP.
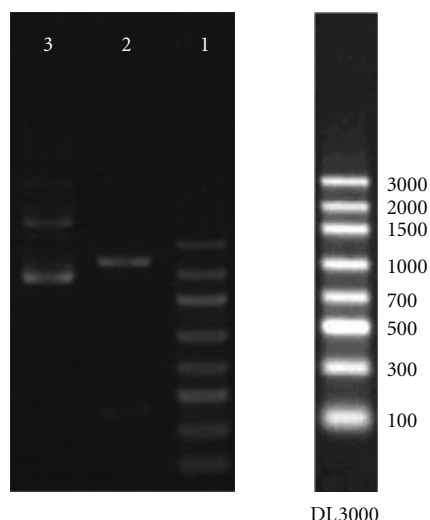PUC57-5HRE and pCDNA3.1-Egr1p-EGFP were connected

Figure 3: pUC57-5HRE was identified by restriction enzyme digestion (Lane 1: DL3000; lane 2: pUC57-5HRE digested by NdeI and HindIII; lane 3: pUC57-5HRE).

after digestion by MluI, respectively. Then the connected products were transfected into DH5α. At last, the plasmids were isolated and purified after enlargement culturing and verified by BglII enzyme cleavage and gene sequence analysis.

2.3. MZF-NPs Preparation and Modification

2.3.1. Preparation. MZF-NPs were obtained by Chemical Coprecipitation Method, referring to [34].

2.3.2. Surface Modification [35]. Firstly, some MZF-NPs were dissolved in deionized water to prepare 4% of the magnetic fluid; supernatant was discarded after ultrasonic dispersion and high-speed centrifugation. Secondly, the precipitate was resuspended in PBS and ultrasonic dispersed. Thirdly, PEI was added slowly to the resuspender. The mixture was blended in a shaker at constant temperature for 24 h. Magnetic particles were separated from the solution by magnetic method and washed repeatedly by distilled water and methanol. After being vacuum dried, nanomagnetic particles modified surfacely by PEI were obtained (namely PEI-MZF-NPs).

2.4. Binding Assay of PEI-MZF-NPs and pHRE-Egr1-EGFP.

PEI-MZF-NPs and pHRE-Egr1-EGFP were mixed together at mass ratios 0 : 1, 5 : 1, 10 : 1, 20 : 1, 40 : 1, and 80 : 1 (the final volume was 500 μL, and DNA concentration was constantly 0.01 μg/μL). 30 minutes later at room temperature, 10 μL of the above composite was taken, respectively, to test DNA binding ability of magnetic nanoparticles using agarose gel electrophoresis. According to the electrophoresis results, the optimal ratio of binging DNA with nano-magnetic particles could be observed.

2.5. DNase-I Digestion Experiment of PEI-MZF-NPs-DNA.

PHRE-Egr1-EGFP and PEI-MZF-NPs were mixed in Tris Buffer, containing 60 mM $MgCl_2$, at 1 : 40 of mass ratio. 30 minutes later at room temperature, DNase I was added to the above mixture, water bathing at 37°C. Stop solution (400 mmol/L NaCI, 100 mmol/L EDTA) was used to terminate the reaction at 10, 20, 40 min of digestion. pHRE-Egr1-EGFP was eluted from the composite by SDS, then extracted by phenol and chloroform, precipitated by absolute ethyl alcohol, and washed by 75% ethanol. The sediment was dissolved in distilled water, and the product was taken to do agarose gel electrophoresis assay to observe the ability of pHRE-Egr1-EGFP/PEI-MZF-NPs to resist nucleic acid enzymatic hydrolysis. As a control, the naked plasmids pHRE-Egr1-EGFP were done by the same approach.

2.6. DNA Release Assay for DNA-PEI-MZF-NPs. pHRE-Egr1-EGFP and PEI-MZF-NPs were mixed at 1 : 40 of mass proportion to form a composite. Then the composite was stirred in a shaker at 37°C after being dissolved by TE. Respectively, at 1 h, 4 h, 8 h, 12 h, 1 d, 2 d, 3 d, 4 d, the solution was centrifuged at 15000 r/min, and then 20 μL supernate was taken to do agarose gel electrophoresis to observe DNA release ability of PEI-MZF-NPs-DNA. TE was replenished whenever some supernatant was taken, and the stir was continued.

2.7. To Evaluate Transfection Efficiency of PEI-MZF-NPs and the Gene Expression Induced by Anoxia and Radiation

2.7.1. Cell Culture. Bel-7402 cells were cultured in DMEM medium supplemented with 10% heat-inactivated calf serum, penicillin (100 units/mL), and streptomycin (0.1 g/liter) and grew in the presence of 5% CO_2 in air at 37°C.

2.7.2. Evaluation of Transfection Efficiency and Gene Expression Induced by Anoxia and Radiation. pHRE-Egr1-EGFP were transfected into Bel-7402 cells by using PEI-MZF-NPs as carrier, and the transfection efficiency was evaluated, compared with that of lipofection transfection method and that of electroporation transfection method.

PEI-MZF-NPs transfection: (1) the plasmid DNA and PEI-MZF-NPs were diluted with serum-free culture medium, respectively, and then they were mixed together (mass ratio of PEI-MZF-NPs and DNA was 40 : 1). 30 minutes later at room temperature, pHRE-Egr1-EGFP/PEI-MZF-NPs composite was obtained. (2) The Bel-7402 cells were seeded in 6-well plates (5 $\times$ 10^5 cells per well) and incubated in routine conditions. About 18 hours later (80% cells were confluent), the original culture medium was discarded, and the cells were washed twice by PBS and washed once by DMEM with no serum. (3) Serum-free DMEM with pHRE-Egr1-EGFP/PEI-MZF-NPs was added to the wells (Every well contained 3 μg DNA), and then the plates continued to be in the couveuse. 5 hours later, the medium with no serum was replaced by fresh DMEM medium with serum, and then the cells continued to be incubated for 48 h.

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Sequence 0   1                                                                    0
Sequence 1   1   TGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCG  62

Sequence 0   1                                                                    0
Sequence 1   63  GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAAC  124

Sequence 0   1                                                                    0
Sequence 1   125 TATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAG  186

Sequence 0   1                                                                    0
Sequence 1   187 ATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGG  248

Sequence 0   1                                                                    0
Sequence 1   249 AAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCA  310

Sequence 0   1                                                                    0
Sequence 1   311 AGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAG  372

Sequence 0   1       ACGCGTGCTAGCAATTGCCACAGTGCATACGTGGGCTCCAACAGGTCCACAGTGC  55
Sequence 1   373 TGAATTCACGCGTGCTAGCAATTGCCACAGTGCATACGTGGGCTCCAACAGGTCCACAGTGC  434

Sequence 0   56  ATACGTGGGCTCCAACAGGTCCACAGTGCATACGTGGGCTCCAACAGGTCCACAGTGCATAC  117
Sequence 1   435 ATACGTGGGCTCCAACAGGTCCACAGTGCATACGTGGGCTCCAACAGGTCCACAGTGCATAC  496

Sequence 0   118 GTGGGCTCCAACAGGTCCACAGTGCATACGTGGGCTCCAACAGGTGGATCAGATCTACGCGT  179
Sequence 1   497 GTGGGCTCCAACAGGTCCACAGTGCATACGTGGGCTCCAACAGGTGGATCAGATCTACGCGT  558

Sequence 0   180 AAGCTT                                                           185
Sequence 1   559 AAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAA  599
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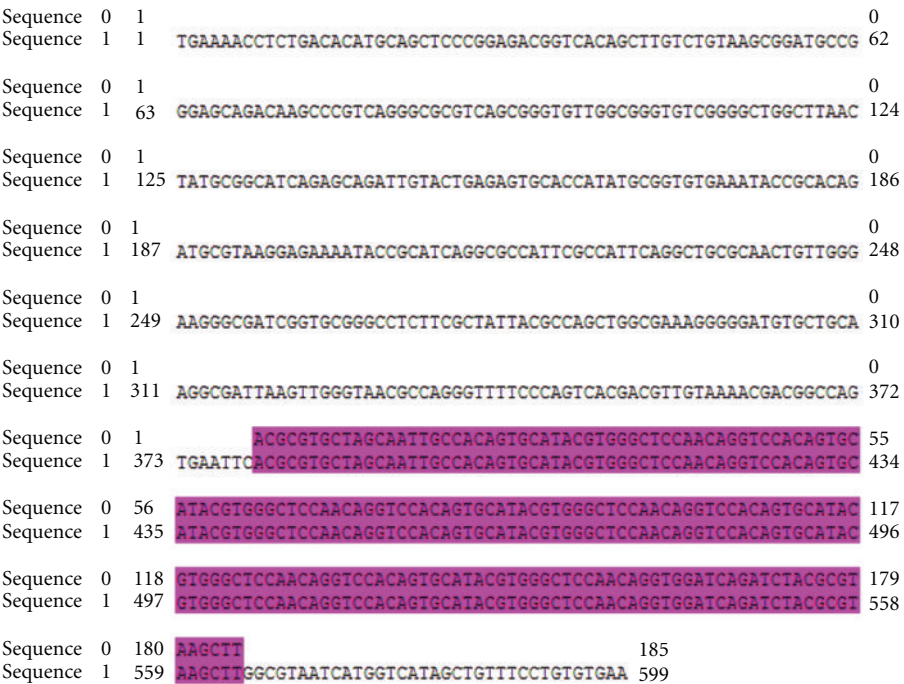
FIGURE 4: Sequencing and alignment of pUC57-5HRE (sequence 0: 5HRE; sequence 1: 5HRE with ab1 reverse complementary sequences; software: Dnassit 2.0).
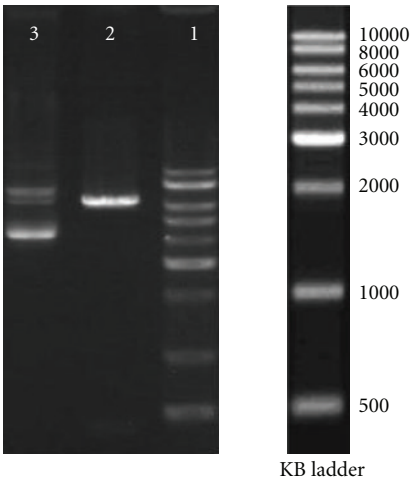

FIGURE 5: PCDNA3.1-5HRE-Egr1-EGFP was identified by restriction enzyme digestion (lane 1: KB Ladder; lane 2: pCDNA3.1-5HRE-Egr1p-EGFP digested by BglII; lane 3: pCDNA3.1-5HRE-Egr1p-EGFP).

with no serum was replaced by fresh DMEM medium with serum, and then the cells continued to be incubated for 48 h.

Electroporation transfection: (1) the cells, digested by trypsin, were collected and centrifuged for 5 minutes (1000 g) after the trypsin reaction was terminated with culture medium with serum. (2) After the supernatant was discarded, the cells were washed twice by 1.25% E buffer (containing 1.25% DMSO in medium with no serum). (3) The cells were diluted with concentration of 5×10^6/mL by E buffer. (4) 3 μg DNA was added to every 400 mL of the cell suspension. (5) When being blended, the above mixture was transferred to electrode cup. (6) Electrode parameters were 250 v/950 uf, and the thickness of the electrode cup was 4 mm. (7) The electrode cup containing cells and DNA was placed in the electric box and received the electric shock. Then it was kept at room temperature for 10 minutes, and the cell suspension was transferred into full culture medium and then cultured in routine conditions. 24 h later, fresh full DMEM medium was used to replace the old medium to abandon dead cells, and then the cells continued to be incubated for 48 h.

The cells, transfected by the above three methods, were exposed to X-ray at 4 Gy (6 Mev) and then cultured for 24 h at 37°C in hypoxia environment (0.1% O_2, 5% CO_2 and N_2 balance gas). Fluorescent protein expressed in the cells was observed under the fluorescence microscope, and fluorescence intensity and transfection ratio were tested quantitatively by flow cytometric analysis. As a control, a group transfected by PEI-PEI-MZF-NPs and induced by radiation alone was designed, and the untransfected cells were used as a negative control.

Liposome method: (1) the plasmid DNA and liposome were diluted with serum-free culture medium, respectively, and then they were mixed together (mass ratio of PEI-MZF-NPs and DNA was 40 : 1). 30 minutes later at room temperature, DNA-liposome composite was obtained. (2) The Bel-7402 cells before transfection were done by the same way as PEI-MZF-NPs group. (3) Serum-free DMEM with DNA-liposome was added to the wells (every well contained 3 μg DNA, mass ratio of liposome and DNA was 5 : 3). After plates continued to be incubated for 5 hours, the medium

Sequence 0 1
Sequence 1 1   ACCGACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACG 0
                                                                              62

Sequence 0 1   ACGCGTGCTAGCAATTGCCACAGTGCATACGTGGGCTCCAACAGGTCCACAG 52
Sequence 1 63   GGCCAGATAT ACGCGTGCTAGCAATTGCCACAGTGCATACGTGGGCTCCAACAGGTCCACAG 124

Sequence 0 53   TGCATACGTGGGCTCCAACAGGTCCACAGTGCATACGTGGGCTCCAACAGGTCCACAGTGCA 114
Sequence 1 125 TGCATACGTGGGCTCCAACAGGTCCACAGTGCATACGTGGGCTCCAACAGGTCCACAGTGCA 186

Sequence 0 115 TACGTGGGCTCCAACAGGTCCACAGTGCATACGTGGGCTCCAACAGGTGGATCAGATCTACG 176
Sequence 1 187 TACGTGGGCTCCAACAGGTCCACAGTGCATACGTGGGCTCCAACAGGTGGATCAGATCTACG 248

Sequence 0 177 CGT 179
Sequence 1 249 CGT GATCTTCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATAT 310

Sequence 0 180 179
Sequence 1 311 TGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATTGGCTCAT 372

Sequence 0 180 179
Sequence 1 373 GTCCAATATGACCGCCATGTTGGCATTGATTATTGACTAGTTATTAATAGTAATCAATTACG 434

Sequence 0 180 179
Sequence 1 425 GGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCC 496

Sequence 0 180 179
Sequence 1 497 GCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAG 558

Sequence 0 180   179
Sequence 1 559 TAACGCCAA 567

Figure 6: Sequencing and alignment of pCDNA3.1-5HRE-Egr1-EGFP (sequence 0: 5HRE, sequence 1: pCDNA3.1-5HRE-Egr1p-EGFP with ab1 reverse complementary sequences; software: Dnassit 2.0).
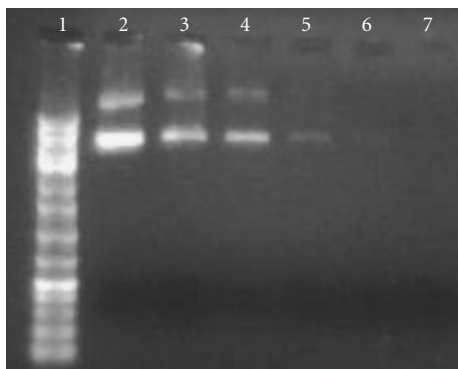

Figure 7: Image of agarose gel electrophoresis of pHRE-Egr1-EGFP, free and mixed with PEI-MZF-NPs at different mass ratios (PEI-MZF-NPs: pHRE-Egr1-EGFP) (lane 1: Mark; lane 2: 0 : 1; lanes 3 to 5: 1; lane 4: 10 : 1; lane 5: 20 : 1; lane 6: 40 : 1; lane 7: 80 : 1).
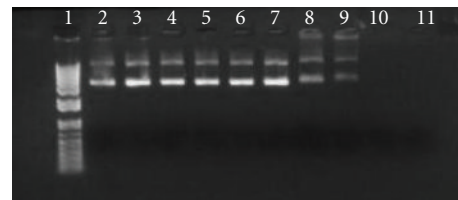

Figure 8: DNase-I resistance of pHRE-Egr1-EGFP-PEI-MZF-NPs (lane 1: Mark; lane 2: DNA-PEI-MZF-NPs with DNase-I for 10 min; lane 3: DNA-PEI-MZF-NPs with DNase-I for 20 min; lane 4: DNA-PEI-MZF-NPs with DNase-I for 30 min; lane 5: DNA -PEI-MZF-NPs with DNase-I for 40 min; lane 6: DNA -PEI-MZF-NPs with DNase-I for 60 min; lane 7: DNA without DNase-I; lane 8: DNA with DNase-I for 10 min; lane 9: DNA with DNase-I for 20 min; lane 10: DNA with DNase-I for 30 min; lane 11: DNA with DNase-I for 40 min).

2.7.3. MTT Assay for DNA-PEI-MZF-NPs Cytotoxicity. Bel-7402 cells, transfected by the above three methods, were subcultured in 96-well plates (2×10^3/well) for 72 h in air 5% CO_2 at 37°C, respectively. The same number of cells seeded without transfection were taken as the negative control. Then 20 μL (5 g/liter) of MTT was added to the cells in every well and incubated for 4 h at 37°C. The culture medium was replaced by 150 μL of DMSO and vibrated for 10 min. Then the optical density (OD) values were measured at a wavelength of 493 nm using a microplate reader (Multiskan MK3-353, USA). The cell survival ratio was counted with the following formula: survival ratio (%) = (OD of the experimental group)/(OD of the negative control group) × 100%.

2.8. Statistical Analysis. Values were expressed as mean ± SD. The data were analyzed with the SPSS 14.0 program, with $P < 0.05$ taken as the criterion for statistical significance.

3. Results and Discussion

3.1. Construction and Identification of pHRE-Egr1-EGFP Plasmid. Recombinant plasmid of pCDNA3.1-Egr1-EGFP was confirmed by PCR amplification and gene sequence analysis, which can be seen from Figure 1 with Egr1 specific banding in lane 2 and lane 4 and Figure 2 with the same sequence as that of Egr1 template.
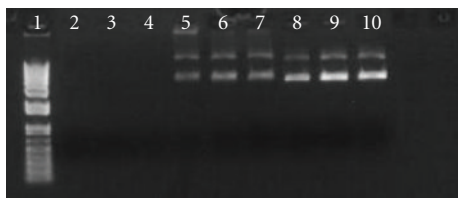
Figure 9: In vitro DNA release of PEI-MZF-NPs (lane 1: Mark; lane 2: 1 h; lane 3: 4 h; lane 4: 8 h; lane 5: 12 h; lane 6: 1 d; lane 7: 2 d; lane 8: 3 d; lane 9: 4 d; lane 10: 5 d).

If 5HRE fragment was successfully inserted, the fragment length between NdeI and HindII should be about 480 bp, because the length between NdeI and HindIII in empty pUC57 vector is about 290 bp. As shown in Figure 3 of restriction enzyme digestion and Figure 4 of sequencing, PUC57-5HRE was cloned correctly. Figure 5 was the restriction pattern of pCDNA3.1-5HRE-Egr1-EGFP, and Figure 6 was the HRE sequencing of pCDNA3.1-5HRE-Egr1-EGFP. Theoretically, there was only one BglII restriction site in the original pCDNA3.1-Egr1p-EGFP, if 5HRE was successfully inserted into the plasmid, another BglII restriction site would be brought in, and the fragment length between the two sites of BglII should be about 380 bp. Seen from Figure 5, 5HRE has been successfully inserted into pCDNA3.1-Egr1p-EGFP. As shown in Figure 6, DNA sequencing is quite correct. So it can be seen that the eukaryotic recombinant plasmid pCDNA3.1-HRE-Egr1p-EGFP was successfully constructed.

3.2. pHRE-Egr1-EGFP Packaging of PEI-MZF-NPs. Figure 7 represents the results of agarose gel electrophoresis of the composite with different proportion of pHRE-Egr1-EGFP and PEI-MZF-NPs. A clear DNA band could be seen in each lane with 1 : 0, 1 : 5 and 1 : 10 of DNA and PEI-MZF-NPs, and the band in the lane with 1 : 20 was significantly weakening, and there was no band to be seen in either the lane with 1 : 40 or over. These results suggested that PEI-MZF-NPs could combine all the plasmids in the system at the ratio of 1 : 40 and beyond, and 1 : 40 was the optimal proportion.

3.3. Nuclease Resistance of the Composite. When exposed to DNase I for 60 minutes, no change in strip brightness could be observed in the case of compound nanoparticles, whereas, with the digestion time extended, gradual degradation of naked DNA was apparent, and almost all was digested 40 minutes later (Figure 8). These results indicated that the DNA was stably incorporated within the nanoparticle matrix and therefore effectively protected from nuclease degradation. The reason for this protection against enzymatic digestion may be due to (1) repulsion of Mg^{2+} ions (which are necessary for the enzymatic reaction) by the amino groups, (2) a hindered access of the enzymes to the DNA that was immobilized on the nanoparticle surface, or (3) both (1) and (2) [36].
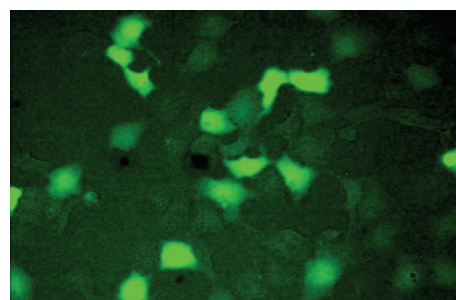
3.4. PEI-MZF-NPs Release. To be a candidate as a DNA carrier, the plasmid should be released from the particles in
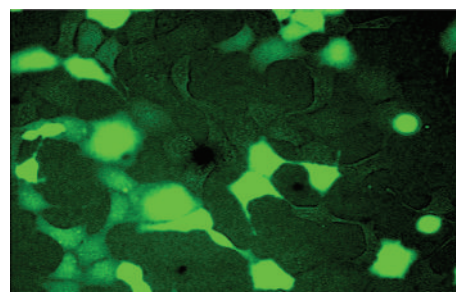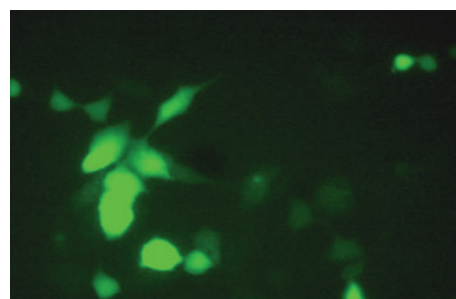

(a)
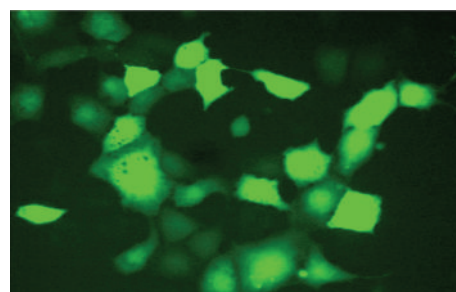
(b)

(c)

(d)

Figure 10: Bel-7402 cells transfected with pHRE-Egr1-EGFP delivered with PEI-MZF-NPs, electroporation, and liposome and induced by double hypoxia-radiation or radiation alone. The observed green cellular fluorescence is from the expressed EGFP, which has been confirmed by fluorescence microscopy ((a) magnetic nanoparticle group induced by hypoxia-radiation; (b) electroporation group induced by hypoxia radiation; (c), liposome group induced by radiation-hypoxia; (d) magnetic nanoparticle group induced by radiation alone).

2.001

Quad	Gated (%)	Y mean	Y geo mean
UL	0	***	***
UR	64.65	193.89	82.99
LL	0	***	***
LR	35.35	4.87	4.58

(a)

4.001

Quad	Gated (%)	Y mean	Y geo mean
UL	0	***	***
UR	67.6	215.12	87.98
LL	0	***	***
LR	32.4	5.22	4.92

(b)

1.002

Quad	Gated (%)	Y mean	Y geo mean
UL	0	***	***
UR	45.77	132.69	64.79
LL	0	***	***
LR	54.23	3.94	3.65

(c)

5.001

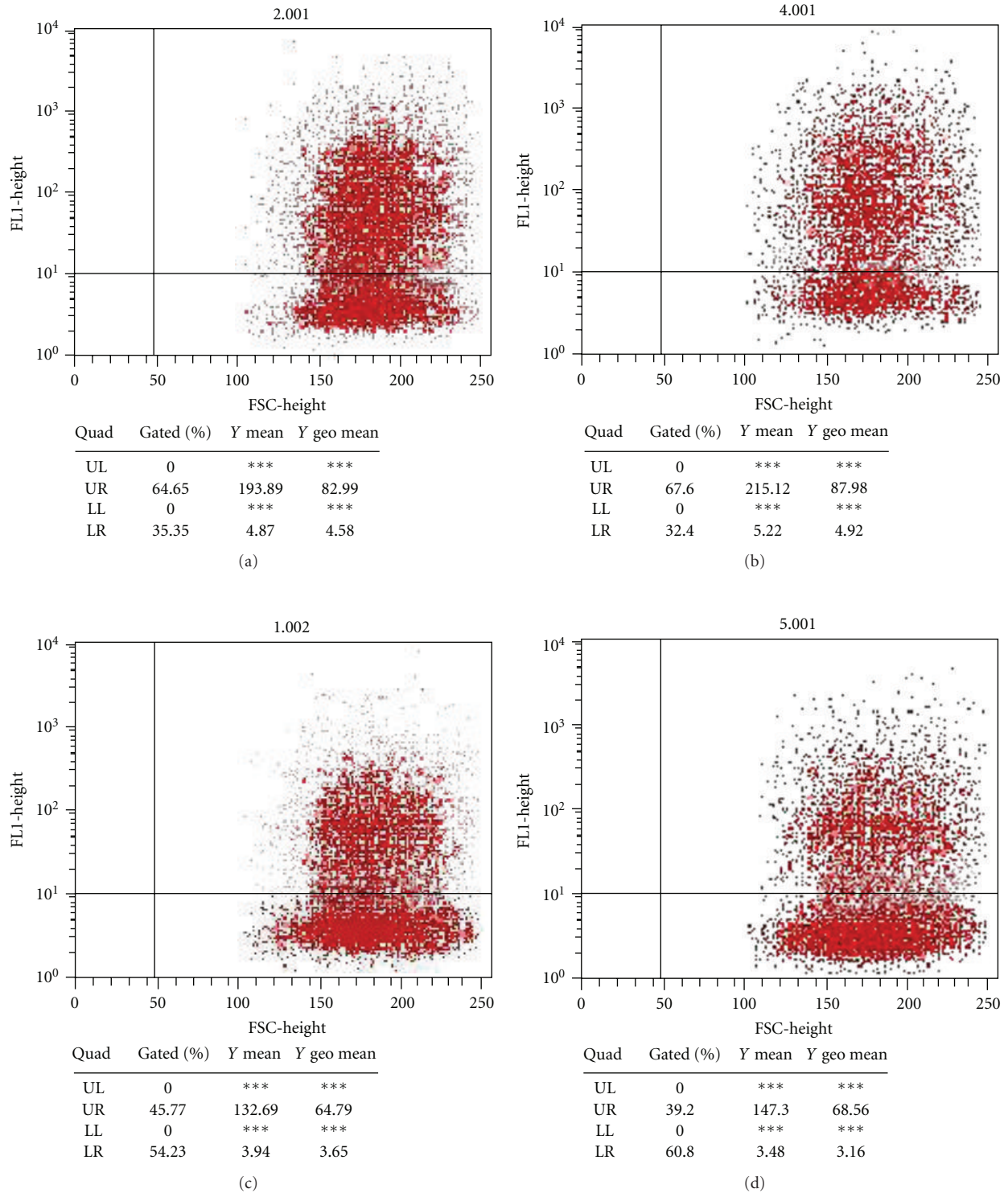| Quad	Gated (%)	Y mean	Y geo mean
UL	0	***	***
UR	39.2	147.3	68.56
LL	0	***	***
LR	60.8	3.48	3.16

(d)

FIGURE 11: Flow cytometry showed the expression of EGFP in Bel-7402 cells with pHRE-Egr1-EGFP delivered by PEI-MZF-NPs, electroporation, and liposome and induced by double hypoxia radiation or radiation alone ((a) magnetic nanoparticle group induced by hypoxia-radiation; (b) electroporation group induced by hypoxia radiation; (c) liposome group induced by radiation hypoxia; (d) magnetic nanoparticle group induced by radiation alone).

TABLE 1: Comparison of toxicity on Bel-7402 cells transfected by means of PEI-MZF-NPs, electric perforation, and liposome over a period of 72 h ($\bar{x} \pm s$, $n = 5$).

Group	Optical density (OD)	Relative survival rate (%)
Negative control group	0.599 ± 0.012	
PEI-MZF-NPS group	$0.581 \pm 0.009^{b,c}$	97.1
Electricity perforation group	0.394 ± 0.009^{a}	65.8
Liposomes group	0.249 ± 0.073^{a}	41.5

[a] $P < 0.001$ versus negative control group; [b] $P < 0.001$ versus electricity perforation group. [c] $P < 0.001$ versus liposomes group.

order to allow its expression in cells. The release increased over the first 3 d, showing gradual increase in brightness of electrophoretic bands, reaching the maximum on the 3rd day and then leveled off (Figure 9). It was confirmed, thus, that besides good DNA-binding ability and protection from degradation, PEI-MZF-NPs have a good DNA release ability in appropriate conditions.

3.5. Carrying Efficiency and Cytotoxicity In Vitro. Under fluorescence microscope, green fluorescence in the three transfected groups was visible, and the cells with green fluorescence in electroporation group were more and brighter than those in the other two groups, and the PEI-MZF-NPs group came next, the liposome group the worst, and no green fluorescence in the untransfected group (Figure 10).

Tested by flow cytometry (Figure 11), transfection ratio and mean fluorescence intensity of magnetic nanoparticle group were 64.65% and 193.89, respectively, slightly lower than 67.60% and 215.12 of electroporation group, but obviously higher than 45.77% and 132.69 of liposome group. Moreover, in the magnetic nanoparticle groups, transfection ratio and fluorescence intensity induced by radiation and hypoxia both were significantly higher than 48.94% and 169.15 induced by radiation alone, which was consistent with the results observed by fluorescence microscope.

MTT assay is commonly used as a cell proliferation assay. Bel 7402 cells transfected with pHRE-Egr1-EGFP by PEI-MZF-NPs, liposome, electroporation, respectively, were incubated over a period of 5 d, and the cell viability was determined as described above by MTT. As shown in Table 1, optical density (OD) of the negative control was close to that of PEI-MZF-NPs group ($P > 0.05$), without statistically significant differences, but significantly higher than that of electroporation group ($P < 0.001$) and liposome group ($P < 0.001$), with statistically significant differences. The relative survival rate (97.1%) of cells in the group transfected by PEI-MZF-NPs was significantly higher than that in electroporation group (65.8%) and liposome group (41.5%), with electroporation group lower and liposome group lowest, indicating that transfection by electroporation and lipofection method equally has much toxicity on Bel-7402 cells, whereas almost no toxicity was detected by means of PEI-MZF-NPs.

4. Conclusions

In this study, we developed pHRE-Egr1-EGFP/PEI-MZF-NPs composite nano-system in which pHRE-Egr1-EGFP, eukaryotic gene expression plasmid, was constructed by combining radiation promoter Egr1 with HRE, forming anoxia radiation double sensitive promoter of HRE/Egr1 to activate reporter gene EGFP expression. MZF-NPs, obtained by coprecipitation method, were coated with PEI. The in vitro experimental results demonstrated that PEI-MZF-NPs have good DNA-binding ability, protection ability, release ability, little toxicity, and high transfection efficiency on Bel-7402 cells, obviously superior to those of the liposome and electricity perforation. Moreover, the expression level of EGFP gene induced by anoxia and radiation was significantly higher than that of single radiation activation. It is therefore concluded that HRE/Egr1 can induce and improve target gene expression efficiency in cancer anoxic microenvironment, and that PEI-MZF-NPs can be used as a novel non-viral gene vector which offers a viable approach to the mediated radiation-gene therapy of cancer.

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