

Enhancement of nanoparticle-mediated double suicide gene expression driven by 'E9-hTERT promoter' switch in dedifferentiated thyroid cancer cells

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ABSTRACT

Differentiated thyroid cancer (DTC), such as papillary thyroid cancer, has a good prognosis after routine treatment. However, in the course of treatment, 5% to 20% of cases may dedifferentiate and can be transformed into dedifferentiated DTC (deDTC) or anaplastic thyroid cancer, leading to treatment failure. To date, several drugs have been used effectively for dedifferentiated thyroid cancer, whereas gene therapy may be a potential method. Literature reported that double suicide genes driven by human telomerase reverse transcriptase promoter (hTERTp) can specifically express in cancer cells and kill them. However, the weak activity of hTERTp limits its further research. To overcome this weakness, we constructed a novel chitosan nanocarrier containing double suicide genes driven by a 'gene switch' (a cascade of radiation enhancer E9 and a hTERTp). The vector was labeled with iodine-131 (¹³¹I). On one hand, E9 can significantly enhance the activity of hTERTp under the weak radiation of ¹³¹I, thereby increasing the expression of double suicide genes in deDTC cells. On the other hand, ¹³¹I also plays a certain killing role when it enters host cells. The proposed nanocarrier has good specificity for deDTC cells and thus deserves further study.

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Introduction

Thyroid cancer has been the most common endocrine cancer in the world [1]. Most cases are differentiated thyroid cancers (DTCs), of which papillary thyroid cancer and follicular thyroid carcinoma (FTC) are the major histological types [2]. Although most DTCs are curable by surgery and iodine treatment, 5% to 20% of patients may suffer disease recurrence with a high mortality rate [3].

The dedifferentiation of DTC into poorly or dedifferentiated DTC (deDTC) and anaplastic thyroid cancer (ATC) is responsible for the poor prognosis in patients, possibly due to radioactive iodine (RAI) refractoriness resulting from the decreased expression of sodium iodide symporter (NIS) [4]. Clinically, DeDTC is characterized by aggressive growth, recurrence, distant metastasis, and resistance to RAI therapy, along with poor prognosis and high early mortality [5].

When the treatment (surgery and RAI administration) fails in high-risk patients with deDTC, effective therapy is lacking [6]. Thus, a new therapeutic strategy is needed, with gene therapy being a promising method.

Previously, the transfer of double suicide gene system, thymidine kinase *E. coli* cytosine deaminase (TK/CD), which encodes enzymes that convert nontoxic prodrugs to toxic forms and thus kill the host cells, was suggested to cause cell death after the administration of prodrugs [7]. Two-gene directed enzyme prodrug therapies have been intensively studied in various cancer cells, and they exerted marked effects on host cells [8]. To selectively express TK/CD in cancer cells rather than normal cells, scholars have investigated several promoters, such as human telomerase reverse transcriptase promoter (hTERTp) [9], KDR promoter [10], and survivin promoter [11]. Of these promoters, hTERTp has

been mostly suggested to drive gene expressions specifically in a number of studies.

Evidence indicated that the suicide gene system driven by hTERTp shows specificity toward thyroid cancer cells [12]. However, hTERTp exhibits a weak driving efficiency in cancer cells [13].

On the basis of the above evidence, we hypothesized that specifically enhancing the activity of hTERTp will effectively drive the expression of its downstream suicide gene in cancer cells, effectively killing them. Thus, we aimed to construct a novel carrier containing double suicide genes (TK/CD) driven by hTERTp. To overcome the shortage of hTERTp, we added a radiation enhancer (E9) before hTERTp and wrapped the plasmids in chitosan-based nanoparticles. Then, the particles were labeled with iodine-131 (^{131}I). Hence, under low radiation, the efficiency of hTERTp can be significantly enhanced. Moreover, the chitosan-based nanoparticles can be devoured easily by cancer cells. Thus, the killing efficacy of the plasmids on cancer cells may be significantly enhanced.

Materials and methods

Cell culture

The human DTC cell line FTC-133 and Nthy-ori3-1, a thyroid follicular epithelial cell line, were obtained from *American Type Culture Collection*, cultured in the mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, and grown at 37°C and 5% CO_2 .

The dedifferentiated FTC-133 (dFTC-133) cell line was established by limiting dilution analysis after ^{131}I radiation in accordance with the literature [14]. The cells with the stable and lowest RAI uptake (RAIU) were set as dFTC-133, and their iodide-metabolizing molecules were detected.

RAIU assay

RAIU assay was conducted in accordance with the literature to evaluate the ability of cells to uptake iodine [14]. The radioactivity was normalized to

the number of cells present at the time of the assay as cpm every 10^6 cells.

Synthesis of chitosan nanoparticles

pCDNA3.1-yCDglyTK was provided by Chonqing Sijun Biotech company. The cDNA product of hTERTp was synthesized in accordance with the literature [15]. The primers for hTERTp: F: GGCGGCATTAATGGCCCCCTCCCTCGGGT TACCC; R: TTATTAGCTAGC CGCGGGGGTG GCCGGGGCCAG.

The radiation response element of CARg originating from early growth response 1 was synthesized by Shanghai Qinglan Biotech Company. Each fragment was digested by tool enzymes and linked to the plasmid pCDNA3.1 in the following sequence: pCDNA3.1 E9-hTERTp- yCDglyTK (pE9-hTERTp-yCDglyTK).

The plasmids were wrapped by chitosan to construct nanoparticles in accordance with our previous study [16]. Chloramine-T method was used to label the nanoparticles with ^{131}I [17].

As a result, the following vectors were constructed and prepared for experiments: 1) vector 1: ^{131}I -labeled+chitosan+pE9-hTERTp-yCDglyTK; 2) vector 2: chitosan+phTERTp-yCDglyTK; 3) vector 3: ^{131}I -labeled+chitosan; 4) chitosan.

The morphological characteristics of nanoparticles were observed by using transmission electron microscopy.

Cell viability and apoptosis assays

Cells were plated in 96-well plates (1×10^4 cells/well). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were used to assess cell viability, and apoptosis was evaluated by using the Annexin V-FITC apoptosis detection kit (Beytime, China), as described in our previous reports [16].

Quantitative polymerase chain reaction (PCR) assay

Real-time PCR was conducted with a PikoReal Real-Time PCR system (Thermo Fisher Scientific, Vantaa, Finland). The total RNA of cells was extracted and then reversely transcribed to yield cDNA. PCR reactions were conducted. The

expression levels of the genes were determined by a threshold cycle number (Ct), which was normalized against the internal reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using the $\Delta\Delta C_t$ method. The primer pairs are listed as follows [14,18]:

GAPDH: F: ATTCCACCCATGGCAAATTC, R: GCATCGCCCCACTTGATT; NIS: F: GACA AACCTCTGAGGACAGGG, R: ATACTGGGGA CGTGAAGC; TPO: F: ACACAGGCAAATCG GAAATC, R: GCAATGTTTACAAGAAAAGGC C; Tg: F: GGGCATGTTACTGCATGTC, R: TTTG AACACAGGTCTGCCA; TK/CD: F: CGTGGCG GCAAGGTGAT, R: TGCTATGGCCGCGAGAA.

Statistical analysis

Data were expressed as mean value \pm standard deviation (SD). Differences between groups were analyzed with one-way analysis of variance (ANOVA) or *t*-test. The *t*-test was used for the comparison between two groups. ANOVA was used for the comparison of three or more groups. When the result was positive, Student–Newman–Keuls test was used between groups. The analyses were performed by utilizing SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL). A *P* value of less than 0.05 was considered statistically significant.

Results

Dedifferentiated thyroid cancer is a serious threat to human health. deDTC cells were established. The double suicide gene driven by hTERTp showed its specificity toward cancer cells. However, the hTERTp exhibited a weak activity. Thus, we constructed a novel chitosan nanocarrier containing double suicide genes driven by a ‘gene switch’ (a cascade of radiation enhancer E9 and hTERTp). The vector was labeled with ^{131}I . The cytotoxic efficacy of the nanocarrier was also evaluated.

Morphological characteristics of the nanoparticles

The nanoparticles of the plasmid were nearly 80–120 nm in size and approximately round shaped

with a smooth surface, implying that the nanoparticles were successfully constructed (Figure 1a).

Establishment of dFTC-133 cells

dFTC-133 cells were cultured. The RAIU of the cells was evaluated. The relative RAIU of the dFTC-133 cells was 0.21 relative to the primary FTC-133 cells (Figure 1b). Moreover, the mRNA expression levels of NIS, TPO, and Tg in dFTC-133 cells were significantly lower than those in FTC-133 cells (Figure 1c).

CD-TK expression

CD/TK expression was studied by reverse-transcription PCR (RT-PCR) in cells after transfection to test the driven efficacy of the promoters in the plasmids.

dFTC-133 and Nthy-ori3-1 cells were cultured and divided into six groups: dFTC-133, dFTC-133 + vector1, dFTC-133+ vector2, Nthy-ori3-1, Nthy-ori3-1+ vector1, and Nthy-ori3-1+ vector2. The cells were treated with/without relevant vectors at a dose of 10 $\mu\text{g}/\text{ml}$. After 48 h, the mRNA expression of CD/TK was detected by RT-PCR. The results showed that the mRNA expression of CD/TK was only detected in dFTC-133+ vector1 and dFTC-133+ vector2 groups, suggesting the specificity of hTERTp in cancer cells. Moreover, the mRNA expression level of CD/TK was higher in dFTC-133+ vector1 group than in the dFTC-133 + vector2 group (Figure 1d).

Cytotoxicity analysis

Cytotoxicity experiments were conducted to test the effect of nanoparticle transfection on the cells. The dFTC-133 cells were divided into four groups: 1) dFTC-133+ vector1; 2) dFTC-133+ vector2 3) dFTC-133+ vector3; 4) dFTC-133+ vector4. Each group was subdivided into two groups based on prodrug content.

Cell proliferation was detected by MTT assays. As shown in Figure 1e–f significantly decreased cell viability and increased cell apoptosis were observed in cells treated with prodrugs in groups 1 and 2 ($P < 0.05$), in which cell apoptosis markedly increased in group 1 relative to that in group

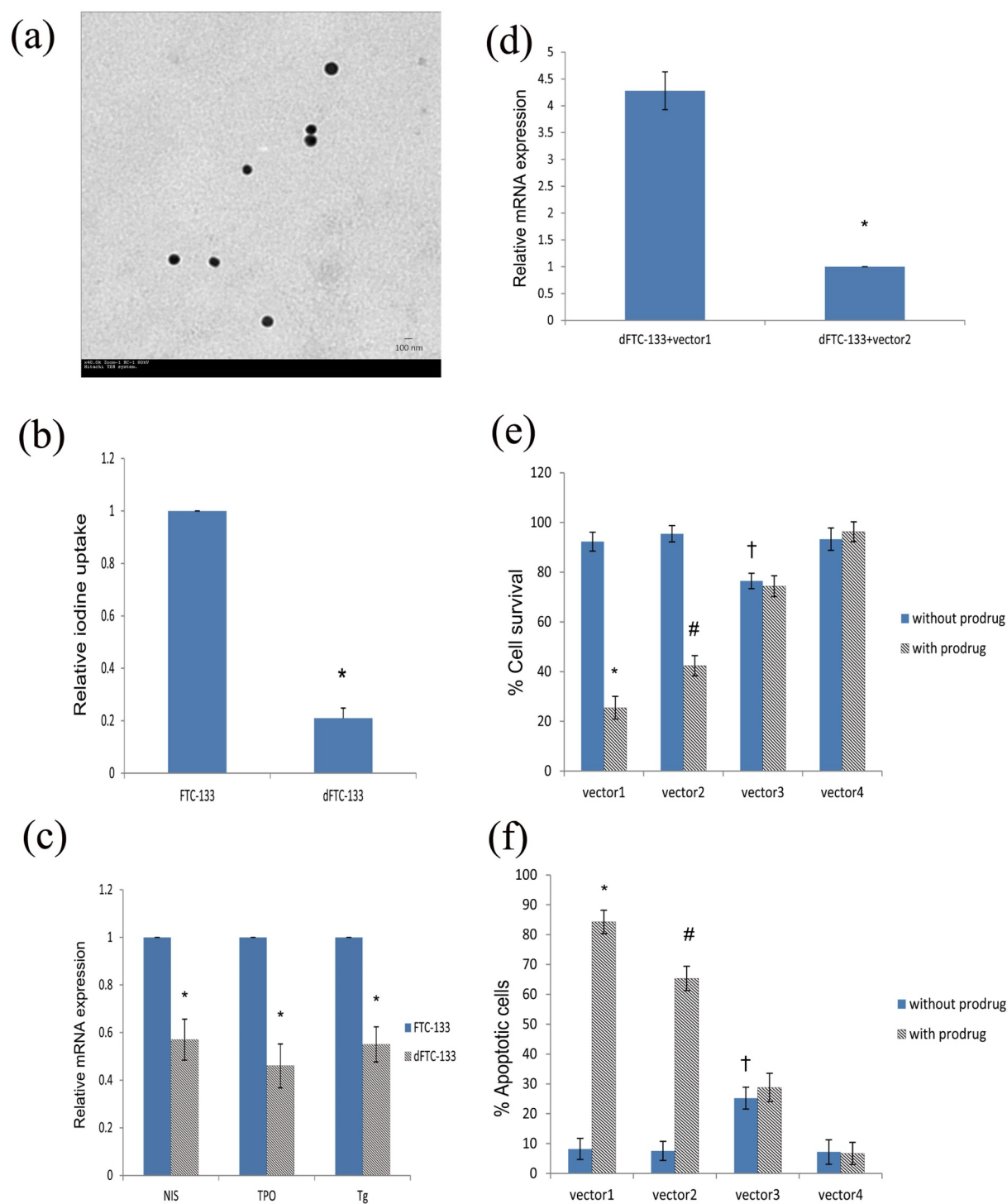


Figure 1. (a) Transmission electron microscopic image of the constructed nanoparticles (vector1). (b) The radioiodine uptake was lower in dFTC-133 cells than that in FTC-133 ones (* $P < 0.05$, FTC-133 vs dFTC-133). (c) Relative mRNA expression of the thyroid specific markers were decreased in dFTC-133 cells relative to FTC-133 cells, respectively (* $P < 0.05$, FTC-133 vs dFTC-133 in each comparison). (d) The expression level of CD/TK mRNA was significantly higher in dFTC-133+ vector1 than that in dFTC-133+ vector2 (* $P < 0.05$, 'dFTC-133+ vector1' vs 'dFTC-133+ vector2'). (e-f) Marked difference between 'with prodrug' and 'without prodrug' groups can be shown in vector1 and vector2 group, respectively (* $P < 0.05$, # $P < 0.05$, 'with prodrug' vs 'without prodrug' in each subgroup). Nevertheless, increased cell apoptosis and decreased cell viability can be observed in cells transfected with vector1 compared with those with vector2 (* vs #, $P < 0.05$, 'with prodrug' in 'vector1' vs 'vector2'). Increased cell apoptosis and decreased cell viability were observed in vector3 group compared with vector4, regardless of prodrug treatment († $P < 0.05$, 'vector3' vs 'vector4' in each subgroup).

2 ($P < 0.05$), indicating that the combination of double suicide gene with the prodrug can result in cell damage, whereas the combination of E9 enhancer and low radiation from ^{131}I may significantly enhance the driving efficiency of the downstream promoter and thus increase cell apoptosis. Notably, in group 3, increased cell apoptosis was observed in cells regardless of the presence of prodrugs, suggesting that the labeled ^{131}I may help exert cytotoxic effects on host cells.

Discussion

In the present study, we first established deDTC cells (dFTC-133), constructed chitosan-encapsulated double suicide gene vectors, and successfully increased CD/TK expression in cultured dFTC-133 cells.

Several effective treatment methods can be used for patients with deDTC. Previously, doxorubicin was the only approved drug for ATC treatment. Nevertheless, the response rate was very low, and numerous side effects may be caused [19]. Tyrosine kinase inhibitors (TKIs), namely, sorafenib and lenvatinib, have also been used for deDTC treatment. However, little evidence indicated that use of TKI can prolong the overall survival time of patients [20]. Hence, novel methods that can effectively treat patients with deDTC are needed.

The suicide gene system can kill cancer cells because it can use an enzyme to convert nontoxic prodrugs into cytotoxic compounds. Ganciclovir (GCV) is often used as an antiviral compound, and the HSV-TK gene encodes thymidine kinase that can phosphorylate GCV to GCV-toxic triphosphate. Hence, the activity of DNA polymerase may be inhibited [21]. The CD gene encodes cytosine deaminase which can be metabolized to 5-Fc and then transformed into the cytotoxic agent 5-Fu [22]. The prodrug (5-FC or GCV) is nontoxic to normal human cells when administered alone. However, the presence of enzymes (CD or TK) may catalyze the prodrug into its cytotoxic form. Then, the generated metabolites will exert antitumor activity. Evidence showed that the combination of the two suicide gene systems (CD/TK) can enhance antitumor activity without damaging normal cells [23]. Therefore, the double suicide gene

system was used in the present study, and the results confirmed its antitumor activity.

A previous study used suicide gene driven by hTERTp to selectively kill thyroid cancer cells [12]. Nevertheless, the intense research and application have been limited due to the weak efficiency of the promoter, although the use of the plasmids has achieved encouraging results. Enhancers can significantly intensify the driving efficiency of promoters [24], and radio-sensitive promoters can greatly improve the driving efficiency under radiations [25]. Thus, to overcome the shortage of plasmids, radiation enhancer 'E9' was added prior to hTERTp to construct a 'gene switch' that can selectively control the CD/TK expression in cancer cells. The driving efficiency of the promoter may be enhanced by low radiation. Then, the plasmid was wrapped by chitosan to construct nanoparticles that can be easily taken up by thyroid carcinoma cells. Afterward, the nanoparticles were labeled by RAI 131. Chitosan is a nanocarrier with numerous advantages, such as good biocompatibility, nontoxicity, and ease of preparation; its absorption-enhancing properties make it an ideal carrier for the delivery of genes [26]. Therefore, the vector we constructed can achieve the anti-tumor effect from two aspects. On the one hand, the labeled ^{131}I can act as low-dose radiation source to drive the expression of CD/TK, and on the other hand, the ^{131}I that entered may directly kill the host cells to a certain extent. Hence, the constructed nanoparticles may increase the efficiency and specificity of suicide gene expression in cancer cells.

Conclusion

In conclusion, we constructed a nanoparticle that may selectively and effectively express double suicide gene in cultured dFTC-133 cells, which may be a potential effective strategy for DeDTC treatment.

Abbreviations

DTCs: differentiated thyroid cancers; FTC: follicular thyroid carcinoma; deDTC: dedifferentiated DTC; ATC: Anaplastic thyroid cancer; TK/CD: Thymidinekinase/E.colicytocytedeaminase; ^{131}I : Iodine-131; DMEM: Dulbecco's modified

Eagle's medium; dFTC-133: dedifferentiated FTC-133; TEM: transmission electron microscopy; Ct: cycle number; ANOVA: Analysis of Variances; RAIU: radioiodine uptake; TKIs: Tyrosine kinase inhibitors; GCV: Ganciclovir; hTERTp: human telomerase reverse transcriptase promoter; RAI: radioactive iodine; NIS: sodium iodide symporter; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation

Data availability statement

All data generated or analyzed during this study are included in this published article.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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