

Efficacy of lentivirus-mediated *Drosophila melanogaster* deoxyribonucleoside kinase combined with (E)-5-(2-bromovinyl)-2'-deoxyuridine or 1-β-D-arabinofuranosylthymine therapy in human keloid fibroblasts

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Abstract. Keloid scarring is a type of fibroproliferative disease with a high recurrence rate. However, no effective treatment is currently available. Combined therapy with recombinant lentivirus-mediated *Drosophila melanogaster* deoxyribonucleoside kinase (Dm-dNK) and prodrug has been widely studied and used for cancer treatment. Due to the similarities between keloid scars and tumors, the aim of the present study was to investigate the efficacy of a Dm-dNK/nucleoside analog system for the treatment of keloid scars. Recombinant lentivirus expression of the Dm-dNK suicide gene was assessed. Western blotting was used to examine the protein expression of lentivirus mediated Dm-dNK in keloid fibroblasts. Enzyme activity assays were conducted using [³H]-labeled substrates. Furthermore, cytotoxicity and bystander effects were evaluated using MTT assays. The expression of green fluorescent protein was observed using fluorescence microscope and results indicated that there was no notable difference in lentivirus infectivity between the multiplicity of infection (MOI) of 1 and 10 in cells. Notably, western blotting revealed that Dm-dNK was stably expressed in keloid fibroblasts and the enzymatic activity assays revealed that the enzyme was activated following introduction into the keloid fibroblasts via the lentivirus. The cytotoxicity and bystander effects of Dm-dNK combined with cytotoxic nucleoside analogs

were both observed in Dm-dNK⁺ keloid fibroblasts. These results demonstrated that the lentivirus-mediated Dm-dNK therapy may be effective in treating keloid fibroblasts, which provides some evidence for the use of Dm-dNK/prodrug therapy for keloid treatment *in vivo* in the future.

Introduction

Wound healing includes two processes: Tissue regeneration, which generally occurs in lower organisms and early embryos in response to trauma, and scar healing, which typically occurs in adult wound healing. Scars may be divided into non-pathological scars (normal scars) that are not raised on the skin and pathological scars (abnormal scars), which appear raised on the skin and occur in the majority of wound healing processes; pathological scars also include hypertrophic scars and keloids (1-3).

Keloid scarring is a type of fibroproliferative disease with pathological features that include excessive proliferation of fibroblasts and collagen-based excessive deposition of extracellular matrix components (1). Keloid scars are characterized by persistent hyperplasia that is beyond the boundaries of original lesion and gradually invade the surrounding normal skin tissue (1). Keloid scarring severely affects the appearance and function of skin and is one of the most common clinical orthopedic diseases (1).

Tumors are neoplasms that arise from the abnormal proliferation and differentiation of cells upon stimulation from various tumor initiating and promoting factors. Once formed, neoplasms grow uncontrollably and invade nearby organs and adjacent normal tissues (2). Based on the similarities between the features and biological behaviors of keloid scars and tumors, the strategies applied to tumor therapy may also be effective for keloid treatment. The primary treatment strategy for keloid scarring is surgery with adjuvant physical methods, including chemotherapy and photodynamic treatment (2). However, the relapse rate of keloid scarring is 45-100% via surgical resection only due to a lack of efficacious treatment methods (2,3).

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The principle of suicide gene therapy is the transduction of cells with a gene that encodes an enzyme that can convert an inactive prodrug into a cytotoxic metabolite (4). In addition to affecting cells expressing the enzyme, adjacent untransduced cancer cells are killed by the transfer of the phosphorylated nucleoside analog between cells. This phenomenon, known as the 'bystander effect', results in the killing of a larger portion of cells than is transduced with the suicide gene (5-7). *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase (Dm-dNK) is a type of suicide gene that has been used for the treatment of various cancers. Although four dNKs have been identified in humans, the insect *drosophila melanogaster* has only one known multisubstrate dNK. Dm-dNK has the ability to phosphorylate purine and pyrimidine nucleosides, as well as mediating DNA synthesis by integrating into the genome of cells (5,6). Furthermore, Dm-dNK preferably phosphorylates pyrimidines, which enhances cell sensitivity to several cytotoxic nucleoside analogs, including (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and 1- β -D-arabinofuranosylthymine (araT) (8,9). Zheng *et al* (10) demonstrated that Dm-dNK may be expressed in human cells with enzymatic activity.

Notably, the lentiviral vector has been identified to facilitate the incorporation of target genes into the host genome for efficient and stable expression of target genes in dividing and non-dividing cells (11,12). In the present study, a lentivirus vector was used to express the Dm-dNK suicide gene in keloid fibroblasts (KF). The efficacy of Dm-dNK combined with BVDU or araT as a type of keloid treatment was investigated.

Materials and methods

Cell lines and culture. A total of 30 randomly selected patients (20 females and 10 males) aged from 20 to 50 years old diagnosed as spontaneous keloid from December 2014 to February 2015 in the Plastic Surgery of Aoyang Hospital were included in the present study. All patients underwent surgery and primary keloid fibroblasts were successfully obtained from 13 patients (4 males, 9 females). The present study was approved by the Ethics Committee of Aoyang Hospital (Zhangjiagang, China) and written informed consent was provided by all patients.

Keloid tissues were washed with PBS, cut into small pieces. Finally, they were adhered and cultured on the bottom of tissue culture flasks. After 3-5 d of culture, the keloid fibroblasts were extracted from these tissues and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin in an atmosphere containing 5% CO₂ at 37°C. After 5 days, 1 ml DMEM with 20% FBS were added. On day 8, the medium was first replaced, then every 2-3 days until cells were cultured to 80% confluence and then passaged. The 2-3 generation of cells with ~60% coverage rate were selected for subsequent experiments.

Construction of the lentiviral plasmid and virus production. Dm-dNK cDNA was amplified from PLXSN-dNK plasmid (10) using the following primers: Forward, 5'-CCGGAATTC ACCATGGCGCAGGCA-3' and reverse, 5'-CGCGGATCC

TCATTATCTGGCGAC-3', as previously described (10). The sequences of endonucleases *EcoRI* and *BamHI* (New England Biolabs, Beverly, MA, USA) were designed using the forward and reverse primers. Dm-dNK-3Flag was amplified by polymerase chain reaction (PCR) kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocols. Amplification was performed via quantitative (q)PCR with the following primers: Upstream, 5'-CCGGAATTCACCATGGCGCAGGCA-3' and downstream, 5'-CGCGGATCCTCATTATCTGGCGAC-3'. The gene was amplified by qPCR using following thermocycling conditions: 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min. The PGC-FU plasmid (GeneChem, Inc., Shanghai, China) consisted of a 5'-long terminal repeat (LTR), cytomegalovirus (CMV) promoter, multiple clone sites, green fluorescent protein (GFP) sequences and a 3'-LTR. The endonucleases *AgeI* and *EcoRI* (New England Biolabs) were used to remove GFP of PGC-FU plasmid and then co-cultured with Dm-dNK-3Flag in 293T cells to generate the recombinant plasmid PGC-FU-dNK. Following the manufacturer's protocol PGC-FU-dNK or PGC-FU plasmids together with two packaging plasmids PHelper1.0 (with gag, pol and rev components) and PHelper2.0 (with VSVG component) were co-transfected into 293T cells using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The lentivirus-containing medium (aforementioned) was collected and filtered. Lentiviruses were concentrated and stored at -80°C. The titer of lentiviruses and multiplicity of infection (MOI) was determined through dilution as described (13). Control viruses containing GFP were used to determine the infection efficiency. For infection, keloid fibroblasts were seeded in 6-well plates at a density of 4x10⁵ cells/well. The cells were infected with Lenti-GFP and Lenti-dNKflag viruses at 10 of MOI for 24 h at 37°C with 6 μ g/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); untransduced cells served as the control. Cells were viewed under fluorescence microscopy at 488 nm, 24 h following transfection (magnification, x200).

Western blotting. Following 72 h of transfection, cells were harvested and lysed in lysis buffer [20 mM Tris (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM ethylenediaminetetraacetic acid, 1% sodium carbonate, 0.5 μ g/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride] with protease inhibitors. The protein concentrations were determined via a Bicinchoninic Acid protein assay and then boiled in a sample buffer (Invitrogen; Thermo Fisher Scientific, Inc.) at 100°C for 5 min. Equal amounts of protein (~20 μ g per lane) were separated using 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequently, samples were blocked in 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 2 h at room temperature. Membranes were incubated with antibodies against Flag (ab213519, 1:1,000, Abcam, Cambridge, MA, USA) or β -actin (sc130300, 1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. Subsequently, membranes were washed in TBST five times each for 10 min and incubated with a secondary horseradish peroxidase-conjugated antibody (sc-516180; Santa Cruz Biotechnology, Inc.) at 1:5,000 dilution, at room temperature for 2 h. Protein bands were detected using a chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.)

and autoradiography (BioMaxfilm, Kodak, Rochester, NY, USA). β -actin served as an internal control.

Enzyme activity assay. Following 72 h of infection, cell proteins were extracted as previously described (14). To detect the activity of Dm-dNK, a 35-ml reaction mixture containing 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 2 mM dithiothreitol, 15 mM NaF, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mg/ml bovine serum albumin and 0.6 mg protein extract was prepared. A total of 2.5 mM [methyl- 3H] thymidine (dThd; Moravsek, Inc., Brea, CA, USA) was mixed with equal amounts of unlabeled substrates. Aliquots of the samples were spotted on Whatman Grade DE-81 filter paper. Following 10, 20 or 30 min incubation at 37°C, samples were dried for 1 h and washed three times with 5 mM ammonium formate. Subsequently, nucleoside monophosphates were isolated using 0.5 M KCl. Radioactivity was sequentially quantified through scintillation counting.

Cell viability assay. The cell viability and lethal bystander effects were evaluated using the MTT assay. Briefly, 3×10^3 cells from untransduced, Lenti-GFP and Lenti-Dm-dNK groups were cultured in 96-well plates overnight at 37°C. Following infection with Lenti-GFP or Lenti-Dm-dNK for 3 days, cells were treated with graded concentrations of BVDU or araT from 0.00001 to 100 mM for 4 days. The medium was replaced, as aforementioned and cells were incubated with 20 μ l MTT (Promega Corp., Madison, WI, USA; 5 mg/ml) for 4 h at room temperature. Following this, the medium was replaced with 200 μ l dimethyl sulfoxide. The solubilized formazan product was quantified according to the absorbance at the wavelength of 570 nm. The assay was conducted in triplicate and repeated in triplicate.

The assay for lethal and bystander effects was performed as described (15). KF cells expressing Dm-dNK were mixed at different ratios with their respective parental cell lines. To promote cell contacts, the mixed cells were plated in 24-well plates at 3×10^5 cells/well. After 24-h incubation, cells were trypsinized and a 1:100 dilution of the cells was distributed into 96-well plates in five replicates. Cells were cultured subsequently in the presence of BVDU or araT for 2 to 3 days until cells without prodrugs reached confluency. The proliferation of the cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. We calculated the inhibition of bystander cells proliferation using a method described previously (16). Cells were divided into seven groups: Groups A and B were controls with 100% Dm-dNK⁺ transduced KF without drugs and nontransduced KF cells, respectively whereas groups C-G were experimental groups constituting Dm-dNK gene-transduced KF cells of various percentages (0, 25, 50, 75 and 100% Dm-dNK gene-transduced cells), incubated in the presence of the 1 mM BVDU or araT.

Statistical analysis. All data were presented as means \pm standard error or standard deviation where appropriate, from three independent experiments. SPSS software (version 10.1, SPSS, Inc., Chicago, IL, USA) was used for all analyses. Comparisons amongst groups were performed using one-way analysis of variance and the Student-Newman-Keuls post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

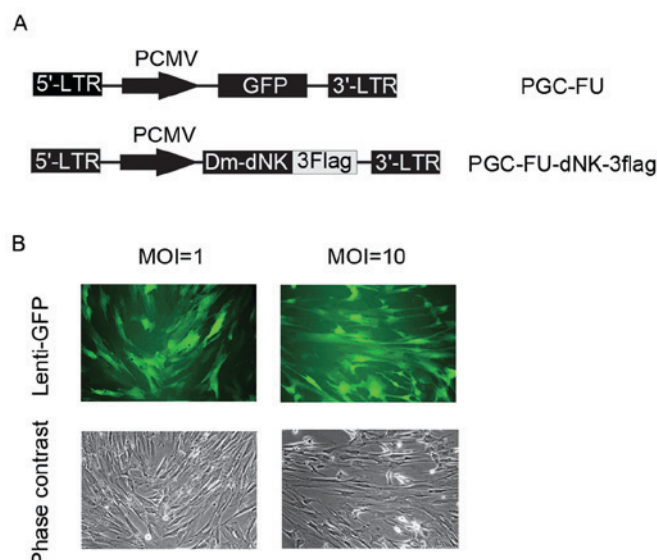


Figure 1. Generation of PGC-FU-dNK plasmid and its expression in the keloid fibroblasts. (A) Schematic representation of PGC-FU and PGC-FU-dNK plasmids. The GFP cassette in the PGC-FU plasmid was replaced with Dm-dNK to form PGC-FU-dNK plasmid. (B) The infection efficiency of lenti-GFP viruses at a MOI of 1 and 10 in the keloid fibroblasts. GFP was observed under fluorescence microscopy (magnification, $\times 200$). GFP, green fluorescent protein; Dm-dNK, *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase; LTR, long terminal repeat; PCMV, cytomegalovirus promoter; MOI, multiplicity of infection.

Results

Expression of Dm-dNK in keloid fibroblasts. The expression of GFP (Lenti-GFP) and Dm-dNK (Lenti-CMV-dNK) was driven by lentiviral plasmids. In PGC-FU-dNK plasmid, the GFP fragment in the PGC-FU plasmid was replaced by Dm-dNK-3flag (Fig. 1A). To examine infection efficiency, lentiviruses with GFP at a MOI of 1 or 10 were used to infect cells. As indicated in Fig. 1B, the expression of GFP was observed in both infections, however, the percentage of GFP-positive cells infected with lentiviruses at a MOI of 10 was not markedly different from that infected with lentiviruses at a MOI of 1.

Keloid fibroblasts were initially infected with lentivirus at a MOI of 10. The expression level of Dm-dNK protein was assessed using western blotting. Dm-dNK protein expression was observed in the keloid fibroblasts following Lenti-dNKflag transfection, whereas the cells transfected with Lenti-GFP and untransduced cells demonstrated low or no Dm-dNK protein expression, respectively (Fig. 2A).

To evaluate enzymatic activity of transfected Dm-dNK in human keloid fibroblasts, phosphorylation of dThd in cell extracts was assessed. dThd kinase activity in keloid fibroblasts with Lenti-Dm-dNK was significantly increased by 10-15-fold following 72 h infection compared with untransduced cells or following infection with Lenti-GFP ($P < 0.05$; Fig. 2B).

In summary, these experiments revealed that human KF cells transduced with the Lenti-CMV-dNK may express Dm-dNK protein, which resulted in an increase of nucleoside and nucleoside analog phosphorylation.

In vitro cytotoxicity and lethal bystander effects of Dm-dNK/prodrug system in keloid fibroblasts. To evaluate

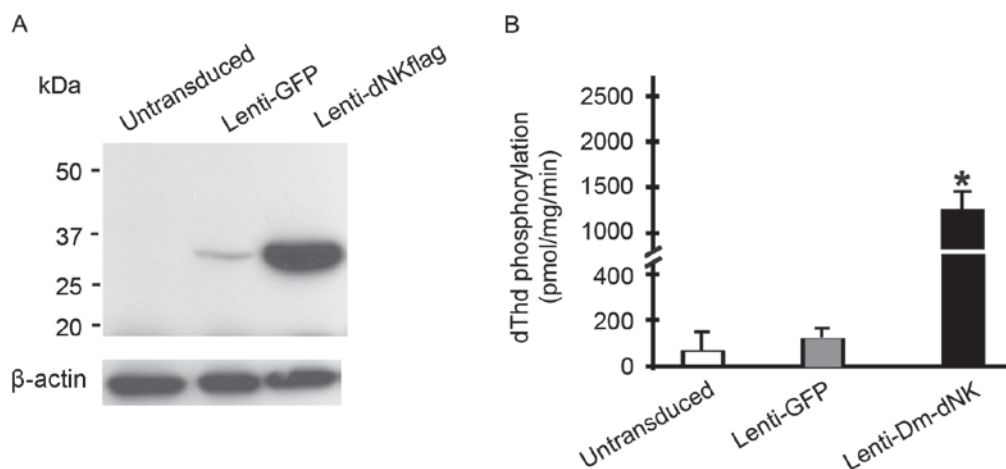


Figure 2. Expression and enzymatic activity of Dm-dNK in keloid fibroblasts. (A) Protein expression of Dm-dNK was examined using western blotting. The keloid fibroblasts were infected with Lenti-GFP or Lenti-CMV-dNK viruses at a MOI of 10 for 72 h. β -actin was used as an internal control. (B) Enzymatic activity of Dm-dNK was examined by measuring phosphorylated $[^3H]$ thymidine in cell extracts. Data are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ vs. untransduced or Lenti-GFP. GFP, green fluorescent protein; Dm-dNK, *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase; CMV, cytomegalovirus; MOI, multiplicity of infection.

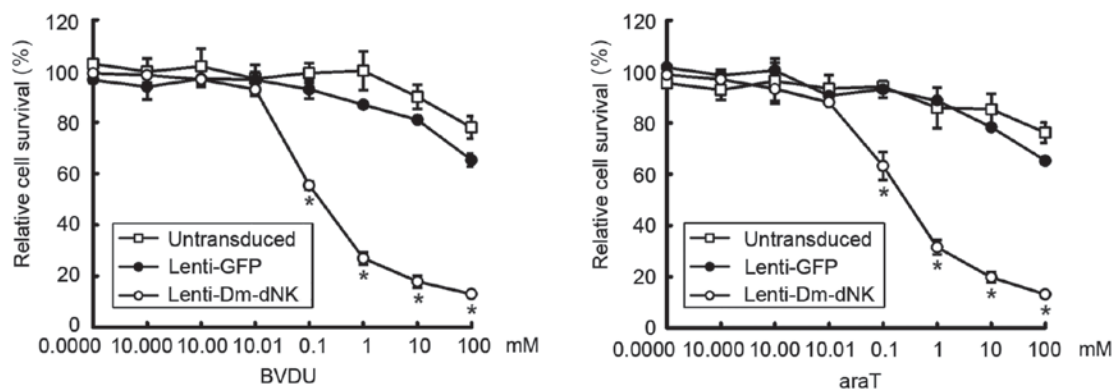


Figure 3. Cell viability of keloid fibroblasts infected with Lenti-GFP or Lenti-CMV-dNK viruses. Keloid fibroblasts were infected with Lenti-GFP or Lenti-CMV-dNK viruses at a MOI of 10 combined with various doses of BVDU or araT treatment. Following 4 days of treatment, the surviving keloid fibroblasts were detected by MTT analysis. Data were presented as the means \pm standard deviation of three independent experiments. * $P < 0.05$ vs. Lenti-GFP. GFP, green fluorescent protein; Dm-dNK, *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase; CMV, cytomegalovirus; MOI, multiplicity of infection; BVDU, E-5-(2-bromovinyl)-2'-deoxyuridine; araT, 1- β -D-arabinofuranosylthymine.

the cytotoxicity of lentiviral vectors with Dm-dNK *in vitro*, keloid fibroblasts were transfected with lentivirus at a MOI of 10. Prodrug BVDU or araT was added at concentrations of 0.00001-100 mM for 4 days and cytotoxicity was examined using an MTT assay. Results indicated that the viability of keloid fibroblasts was reduced by 70% following transfection with Lenti-DM-dNK combined with 1 mM bromovinyldeoxyuridine (BVDU) or araT (Fig. 3).

No significant difference in lethality (cell death) lethal effect was observed among the three control groups (groups A-C). However, the lethal effects in the experimental group (groups D-G) were markedly higher compared with those in the control group (groups A-C). Furthermore, 50% Dm-dNK-expressing KF cells (group E) induced cell death to a degree similar to that obtained with 25% Dm-dNK-expressing cells (group F; Fig. 4).

In summary, the results of the present study demonstrated that Dm-dNK-expressing KF cells exhibited increased sensitivity of cell death in combination with BVDU and araT and may have also induced bystander cell death in KF cells.

Discussion

In past decades, several types of prodrugs that are activated and sensitized by suicide genes have been reported to serve roles in the treatment of cancers (17). For example, the herpes simplex virus thymidine kinase gene and the *Escherichia coli* cytosine deaminase gene may combine with ganciclovir and 5-fluorocytosine, respectively (18). Xu *et al* (19) demonstrated that the combination of recombinant adenovirus-mediated-double suicide genes *E. coli* cytosine deaminase and herpes simplex virus type 1 thymidine kinase, via a polyglycine spacer, and prodrug therapy is effective for the treatment of keloid fibroblasts. Additionally, our previous studies suggested that the Dm-dNK/nucleoside analog system may be a novel effective treatment for various types of cancer, including breast cancer and gastric carcinoma, and may improve antitumor effects (20,21). Furthermore, retrovirus-mediated Dm-dNK may be used to treat osteosarcoma cells and pancreatic adenocarcinoma cells (10). A replicative adenovirus, ZD55-dNK,

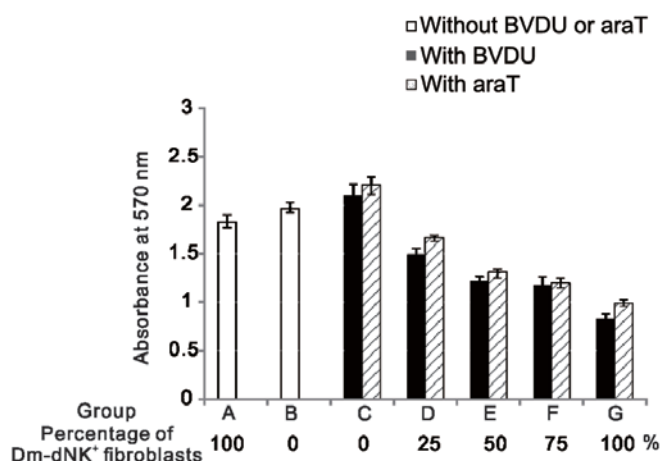


Figure 4. *In vitro* bystander effects of Dm-dNK/prodrug in keloid fibroblasts. Y axis represented the results of MTT analysis under different conditions as indicated in X axis: Nontransduced and Dm-dNK gene-transduced cells were mixed in various percentages (0, 25, 50, 75, and 100% Dm-dNK gene-transduced cells), incubated in the presence of the 1 mM BVDU or araT. Data are presented as the mean \pm standard deviation of three independent experiments.

has been indicated to enhance cancer-specific destruction through prodrug administration by synergistically inducing apoptosis of human gastroduodenal cells and suppressing replication of the adenovirus *in vitro* (22). Similarly, lentivirus-mediated Dm-dNK expression combined with gemcitabine 2',2'-difluoro-deoxycytidine is an effective form of cancer therapy (11). Notably, expressing suicide genes via replication-defective adenoviral and lentiviral vectors may be an effective way to treat human breast cancer (11,12). A previous study demonstrated that the Dm-dNK/BVDU system may provide a safe treatment for breast cancer with the aid of lentiviral vector (12). Considering the similarities between keloids and tumors, the efficacy of Dm-dNK combined with prodrug BVDU or araT for the treatment of keloids was investigated in the present study. The results revealed that lentivirus-mediated Dm-dNK was successfully expressed in keloid fibroblasts and presented with high enzymatic activity. Furthermore, the Dm-dNK/nucleoside analog system had significant efficacy in destroying keloid fibroblasts.

The bystander effect, which may result in the destruction of more cells than those transduced with a suicide gene, serves an important role in keloid fibroblast therapy (23). There are two possible mechanisms by which the bystander effect may be induced by a suicide gene/prodrug system (23). Notably, the bystander effect may be caused by the transfer of phosphorylated prodrug through intercellular gap junctions and the phagocytosis of apoptotic vesicles containing the prodrug metabolites from suicide gene-expressing cells (7,15,24-26). In the present study, the lethal effect was observed when 25% of fibroblasts were transduced with Lenti-CMV-dNK in the presence of BVDU or araT; however, no marked difference was observed in the lethal effect among the groups with 50, 75 or 100% Dm-dNK⁺ fibroblasts. Furthermore, when only 25% of fibroblasts were Dm-dNK⁺, the bystander effect was notable. Thus, the lethal bystander effects may effectively enhance the inhibition the growth of keloid fibroblasts and might destroy more keloid fibroblasts cells.

To date, various treatment methods, including assisted-physical treatment, chemotherapy and photodynamic treatment for keloid fibroblasts have emerged (27). Arno *et al* (27) indicated that some members of the transforming growth factor (TGF)- β superfamily, including Smads, Ski, SnoN, Fuzsels, endoglin, DS-Sily, Cav-1p, AZX100 and thymosin- β 4 and other associated molecules, may be targets for preventing and treating keloid and hypertrophic scars. Fan *et al* (2) suggested that oxymatrine (OMT) is associated with the TGF- β /Smad signaling pathway and inhibits collagen synthesis. This indicates that OMT may prevent keloid and other fibrotic diseases. Furthermore, a previous study reported that Wharton's jelly-derived mesenchymal stem cells from human umbilical cords exhibited antifibrotic properties through paracrine signaling (3). Differently, the principle of suicide gene therapy is the transduction of cells with a gene that encodes an enzyme that can convert an inactive prodrug into a cytotoxic metabolite, regardless of pathogenic mechanism, and this strategy was always used for cancer treatment. The present study demonstrated that Dm-dNK can be expressed in human KF cell; the enzyme retained its enzymatic activity and the cells expressing Dm-dNK exhibited increased sensitivity to some cytotoxic nucleoside analogs, including BVDU and araT. Additionally, the bystander effect may enhance KF cell death. The findings of the present study indicated that Dm-dNK/prodrugs may exhibit a therapeutic potential to treat keloids and may become a novel treatment method of clinical keloids; however, *in vivo* experimental models of this suicide gene strategy require further evaluation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YS analyzed and interpreted the patient data and performed parts of experiments. HJ was a major contributor in writing the manuscript and performed parts of experiments. MG performed majority of experiments. XZ designed the whole project and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Aoyang Hospital (Zhangjiagang, China) and written informed consent was provided by all patients.

Consent for publication

The patient, or parent, guardian or next of kin (in case of deceased patients) provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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