RESEARCH COMMUNICATION

Potent Anticancer Effects of Lentivirus Encoding a Drosophila Melanogaster Deoxyribonucleoside Kinase Mutant Combined with Brivudine

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Abstract

Objective: Deoxyribonucleoside kinase of Drosophila melanogaster (Dm-dNK) mutants have been reported to exert suicide gene effects in combined gene/chemotherapy of cancer. Here, we aimed to further evaluate the capacity of the mutanted enzyme and its potential for inhibiting cancer cell growth. Methods: We altered the sequence of the last 10 amino acids of Dm-dNK to perform site-directed mutagenesis and constructed active site mutant Dm-dNK (Dm-dNKmut). RT-PCR and western blotting studies were used to reveal the expression of lentivirus mediated Dm-dNKmut in a breast cancer cell line (Bcap37), a gastric cancer cell line (SGC7901) and a colorectal cancer cell line (CCL187). [3H]-labeled substrates were used for enzyme activity assays, cell cytotoxicity was assessed by MTT assays, cell proliferation using a hemocytometer and apoptosis induction by thannexin-V-FITC labeled FACS method. In vivo, an animal study was set out in which BALB/C nude mice bearing tumors were treated with lentivirus mediated expression of Dm-dNKmut with the pyrimidine nucleoside analog brivudine (BVDU, (E)-5-(2-bromovinyl)-(2-deoxyuridine). Results: The Dm-dNKmut could be stably expressed in the cancer cell lines and retained its enzymatic activity. Moreover, the cells expressing Dm-dNKmut exhibited increased sensitivity in combination with BVDU, with induction of apoptosis in vitro and in vivo. Conclusion: These findings underlined the importance of BVDU phosphorylated by Dm-dNKmut in transduced cancer cells and the potential role of Dm-dNKmut as a suicide gene, thus providing the basis for future intensive research for cancer therapy.

Keywords: Dm-dNK - lentivirus - mutant - nucleoside - analogue - suicide gene

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Introduction

Differs from the four known deoxyribonucleoside kinases of mammals, insect Drosophila melanogaster has only one kinase, multisubstrate deoxyribonucleoside kinase (Dm-dNK) (Munch-Petersen et al., 1998b). It has the unique ability to phosphorylate with a high catalytic rate all precursor nucleosides and plays a key role in rate-limiting step of nucleoside analogue activation (Zheng et al., 2001a, b). It can phosphorylate both purine and pyrimidine nucleosides and subsequently incorporated into cellular genome to block DNA synthesis (Springer and Niculescu-Duvaz, 2000; Johansson et al., 1999). Furthermore, Dm-dNK has a preference for pyrimidine nucleoside such as brivudine (BVDU, (E)-5-(2-bromovinyl)-2’-deoxyuridine). It has been reported that the catalytic rate of the enzyme for pyrimidines is 100-600 times higher than for purines (Munch-Petersen et al., 1998a; Munch-Petersen et al., 2000). All these data underline the importance of the potential role of Dm-dNK as a suicide gene, and therefore, this gene has been used successfully in suicide gene therapy against a variety of human cancers (Ito et al., 2010; Ma et al., 2011; Zhu et al., 2011b; Ma et al., 2012).

Over the past decade, evidence has accumulated indicating that a limited number of amino acid residues plays a central role for the substrate specificity in nucleoside analogue activation. It has been reported that the substrate specificity of Dm-dNK could be changed by a few amino-acid substitutions (Knecht et al., 2002). Nicola Solaroli et al have laid down a foundation that the mutant enzymes exhibit a relatively increased sensitivity to nucleoside analogs in comparison with the wild type enzyme (Solaroli et al., 2003). Here, we set out to construct the mutanted Dm-dNK (Dm-dNKmut) in which the amino acid sequence was changed randomly at the sites of 244E, 245S, 251S and 252T. From this, we wished to further understand the phosphorylation capacity of mutagenesis and anti-tumor function and to an extent that, if achieved in vivo, the mutanted gene could be identified as a candidate gene associated with solid tumor therapy.

Despite progress in the delivery vector, the major
limitation of suicide gene therapy remains the inadequate transduction in the target cells. Several studies have investigated that in vivo the number of cells mediated by retroviral vector contribute less than 10% to the target cell population (Caruso et al., 1993; Kianmanesh et al., 1997). Moreover, the cell cycle distribution plays essential roles in the quantitative efficiency of gene transfer with a retroviral vector (Springett et al., 1989). For this reason, lentiviral vector is expected for its advantage in incorporating into the host genome to allow for efficient and stable transgene expression in both dividing and nondividing cells and therefore, has been considered as a potentially effective vector for gene transfer. To date, no event has been reported that transduction performance of lentivirus mediated the expression of Dm-dNKmut in different types of cancer cells.

In the present study, we selected the pyrimidine analog brivudine (BVDU) to test whether it was a good substrate of lentivirus mediated Dm-dNKmut. It was the first time that we investigated lentivirus mediated Dm-dNKmut gene therapy for three types of cancer cells combined with BVDU both in vitro and in vivo, the results showed that this system was a more effective prodrug activation system and could provide a foothold for potent targeted cancer gene therapy.

Materials and Methods

Cell lines and culture

Human breast cancer cell line Bcap37 was obtained from the Cancer Institute of China Medical University (Shenyang, China) and cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640, Gibco, BRL, Germany). Human gastric carcinoma cell line SGC7901 and human colorectal carcinoma cell line CCL187 were both purchased from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM, Gibco, BRL, Germany) and low glucose Dulbecco’s Modified Eagle Medium (DMEM, Gibco, BRL, Germany), respectively. All the three cell lines were maintained in mediums supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, BRL, Germany), 1% L-glutamine and 1% penicillin-streptomycin in a high-humidity incubator at 37 °C supplied with 95% air/5% CO₂.

Vector construction and virus production

The fragment of Dm-dNKmut was amplified using polymerase chain reaction (PCR) technique from plasmid pLXSN-dNKmutant with the upstream primer 5’-CCG GAA TTC (EcoRI) ACC ATG GCC GAG GCA-3’ and downstream primer 5’-CGG GGA TCC (BamHI) TCA TTA TCT GCC GAC-3’. Synthetic DNA sequence was released with endonucleases EcoRI and BamHI (New England, Biolabs, Beverly, MA, USA). Then the DNA fragment of Dm-dNKmut-3Flag was amplified by PCR and ligated into plasmid PGC-FU consisting of a 5’-long terminal repeat (LTR), cytomegal ovirus (CMV) promoter, multiple clone site, a green fluorescent protein (GFP) sequence and a 3’-LTR (Genechem, Shanghai, China). In order to construct recombinant plasmid PGC-FU-dNKmutant, the GFP sequence was removed with endonucleases AgeI and EcoRI (NEB, England) from the plasmid PGC-FU. Then, the two plasmids PGC-FU and PGC-FU-dNKmutant together with two packaging plasmids PHelp1.0 (gag, poland rev, component) and the plasmid PHelp2.0 (VSVG, component) were packed and co-transfected into the human embryonic kidney cell line (HEK293T) using LipofectamineTM 2000 reagent (Invitrogen, USA) according to the manufacturer’s instructions. Then viral plaques appeared several days after co-transfection and were sublimited three times. The lentiviruses were identified by PCR and named Lenti-GFP and Lenti-CMV-dNKmut, respectively. Lenti-GFP was used to determine the lentivirus infectivity among cell lines. All the cell lines were transduced with the lentivirus containing medium mixed with 6 µg/mL polybrene (Sigma, USA) to increase infection efficiency.

Fluorescent-activated cell sorting analysis.

Cancer cells Bcap37, SGC7901 and CCL187 were seeded in six-well dishes at a density of 4x10⁵ cells/well and cultured for 24 h. Subsequently, Lenti-GFP at MOIs of 1 and 10 was added to the media. Polybrene (6 µg/mL) was added to all cultures. Three days later, all cells were harvested and suspended in PBS with 0.5% bovine serum albumin at the concentration of 1x10⁶ cells/mL. The samples were quantified for green fluorescent protein expression profile by a FACScan flow cytometer [equipped with CellQuest and ModFITLT for Mac V1.01 software (Becton-Dickinson, San Jose, CA)].

RNA extraction and reverse transcriptional-PCR

Bcap37, SGC-7901 and CCL187 cells were seeded in six-well dishes at a density of 4x10⁵ cells/well and cultured for 24 h, followed by infection with Lenti-GFP and Lenti-CMV-dNKmut at a MOI of 10. Polybrene (6µg/mL) was added to all cultures. Three days later, total cellular RNA of cell line samples was isolated using Trizol Reagent (Sigma, USA) and reverse transcribed from 2 ng of total RNA into cDNA for Dm-dNKmut expression using the RT-PCR kit (Takara, Japan) following the manufacturer’s protocols. Cycling conditions for amplification of Dm-dNKmut gene was initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1.5 min. PCR amplification of GAPDH gene was initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, followed by a final elongation at 72 °C for 1.5 min. The intensity of each gene was normalized against GAPDH expression. Amplification was performed using PCR with following primers: Dm-dNKmut (upstream 5’-CCG GAA TTC ACC ATG GCC GAG GCA-3’ and downstream 5’-CGG GGA TCC TCA TTA TCT GCC GAC-3’). Synthetic DNA sequence was released with endonucleases EcoRI and BamHI (New England, Biolabs, Beverly, MA, USA).

When the reaction product were run on 2% agarose gel at 60V and the intensity of the bands in the gels was sequentially visualized by SYBY green staining.
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Western blot analysis

Cellular extracts of Bcap37, SGC7901 and CCL187 were isolated at 72 h after infection with lentivirus. After harvest with lysis buffer (50 mmol/L Herpes at pH 7.4, 250 mmol/L NaCl, 1 mmol/L NaF, 1 mmol/L EDTA, 1% Triton X-100, 1 mmol/L DTT) containing protease inhibitors, the samples were boiled in sample buffer (Invitrogen) for 5 min. Then equal amounts of the proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Millipore). The membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 and 5% nonfat milk for 2 h at room temperature followed by overnight incubation with primary antibodies against Flag (Abcam, CA, USA) or β-actin (Santa Cruz Biotechnology, CA, USA) at 4 °C which diluted 1000 folds and 500 folds, respectively. After extensive washing in TBS with 0.1% Tween 20, membranes were incubated with a secondary horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, CA, USA) as appropriate for 2 h at room temperature. The detection of protein bands was visualized with a chemiluminescence reagent (Thermol Biotech Inc, Rockford, IL, USA) and the signal was obtained after exposing to autoradiography (BioMaxfilm, Kodak). β-actin was performed as an internal control for equal protein loading.

Enzyme activity assays

Cell protein extracts were prepared as previously described (Sandrini et al., 2007) from cells at 72 h post-infection with Lenti-GFP or Lenti-CMV-dNKmut respectively at a MOI of 10. A 35ml reaction mixture containing 50 mM Tris-HCl at pH 7.6, 100 mM KCl, 2 mM dithiothreitol, 15 mM NaF, 5mM MgCl2, 5mM ATP, 0.5 mg/mL of bovine serum albumin, and 0.6mg of protein extract was used to detect the activity of Dm-dNKmut. In this assay, aliquots of the samples in which 2.5 mM [methyl-3H]dTd (Moravek Biochem) mixed with equivalent amounts of unlabeled substrates were spotted on Whatman DE-81 filter paper disks after 10, 20 and 30 min incubations at 37 °C. After drying for 1 h, washing 3 times with 5 mM ammonium formate, the nucleoside monophosphates were then eluted with 0.5M of KCl and the radioactivity was sequentially quantified by scintillation counting.

Cell viability and proliferation assays

Cell viability was evaluated using MTT assay. In brief, cells were seeded in 96-well plates and treated with graded concentrations of BVDU from 0.001 to 10 µM after infecting with Lenti-GFP or Lenti-CMV-dNKmut. Then the medium was replaced with fresh medium and cells were incubated with 20 µL of MTT (Promega, USA) (5 mg/mL) in each well. Four hour later, cells were lysed by addition of 200 µL of dimethylsulphoxide (DMSO). The solubilized formazan products were quantified for absorbance at a wavelength of 570 nm. To detect cell proliferation, cells transfected with lentivirus combined with pre-drug of BVDU (1 µM) were trypsinized and suspended in serum-free medium. Subsequently cell counting was performed with a hemocytometer. Each experiment was conducted in triplicate and repeated three times.

Flow cytometry apoptosis assays

Induction of apoptosis was performed to clarify the mechanism of this suicide gene therapy using an Annexin V-FITC/PI double staining kit (Genmed Bioscience, China) following the manufacturer’s protocols. In brief, Bcap37, SGC-7901 and CCL187 were plated in six-wells, allowed to attach by overnight incubation and transfected with Lenti-GFP or Lenti-CMV-dNKmut and polybrene (6 µg/mL) was added to all cultures. 72 h post-transfection, 1 µM of BVDU was treated. Cells were continuously cultured for 48 h and harvested. Before reading on the flow cytometer, cell suspensions were washed in PBS, resuspended with a 1×binding buffer and exposed to 5 µL of Annexin V-FITC (20 µg/mL) and 10 µL of propidium iodide (PI; 50 µg/mL). After a incubation of 20 min in the dark, the samples were subjected to a FACScan flow cytometer [equipped with CellQuest and ModFITLT for Mac V1.01 software (Becton-Dickinson, San Jose, CA)].

In vivo studies

To explore the therapeutic efficacy of Lenti-CMV-dNKmut/BVDU system in vivo, eighteen female BALB/C nude mice 6 to 7 weeks old were purchased from the Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). All studies involving mice were followed the Guide for the Care and Use of Laboratory Animals (National Research Council 1996). A total of 1.0×105 Bcap37 cells suspended in 100 µL PBS were implanted in the flanks of nude mice. When the tumors grew to 100-120 mm3, the mice were randomly assigned to three groups (6 mice per group). Tumors were injected with 1×105 PFU (plaque forming units) of Lenti-CMV-dNKmut, Lenti-GFP or control suspended in 100 µL PBS, respectively. Treatments were given three times at 2 days intervals. Subsequently, 5 mg/kg of BVDU was administered into the peritoneal cavity daily over 7 consecutive days. Injections were distributed equally into each of four tumor quadrants. Xenograft tumor burdens were inspected every 5 days and measured with a calipers in two dimensions for up to 30 days. Tumor volume was determined by the following formula [1/2 ×length×width2].

Statistical analysis

All data were expressed as means±standard error or SD from three independent experiments. Differences between groups were examined for statistical analysis using the statistical software SPSS (version 10.1, Chicago, IL, USA). Values of P < 0.05 were considered significant.

Results

Expression of Dm-dNKmut

We performed site-directed mutagenesis with the sites of amino acid residues 244E, 245S, 251S and 252T changed to determine the properties of the enzyme (Figure 1A). As shown in Figure 1B, the GFP expression cassette in plasmid PGC-FU was replaced by an expression cassette...
respectively. As shown in Figure 2A, the three cancer cell lines (Bcap37, SGC7901 and CCL187) were transduced by lentiviral vector to cells, Lenti-GFP at a MOI of 1 or 10 titers were $2 \times 10^9$ TU/ml. To investigate the infectivity of lentiviral vector to cells, Lenti-GFP at a MOI of 10 and strong green fluorescence was detected under fluorescence microscopy.

Figure 1. Alignment of Dm-dNKmut with Dm-dNK, Schematic Structure, Infectivity of Lentivirus and GFP Expression in Cancer Cell Lines. (A) Sequence of alignment of Dm-dNK and Dm-dNKmut. Grey boxes indicate conserved amino acid residues compared with Dm-dNK. Numbers on top indicate amino acid numbering based on the Dm-dNK sequence. (B) Schematic representation of PGC-FU and PGC-FU-dNKmutant-3Flag. To construct plasmid PGC-FU, dNKmutant-3Flag, GFP cassette in plasmid PGC-FU was replaced with cDNA encoding dNKmutant-3Flag. LTR, long terminal repeat; PCMV, CMV promoter. (C) Cancer cell lines (Bcap37, SGC7901 and CCL187) were infected with Lenti-GFP at MOIs of 1 and 10 and the percentages of GFP-positive cells were determined by FACS analysis. About 85-95% of the cells could be infected with Lenti-GFP at a MOI of 10. (D) Cancer cell lines (Bcap37, SGC7901 and CCL187) was transduced by Lenti-GFP at a MOI of 10 and strong green fluorescence was detected under fluorescence microscopy encoding dNKmut to generate plasmid PGC-FU-dNKmut-3Flag. In our report, lentiviral vectors were used to express the green fluorescence (Lenti-GFP) and Dm-dNKmut (Lenti-CMV-dNKmut), respectively. After co-infection of 293T cells, lentiviruses vectors were produced and the virus titers were $2 \times 10^9$ TU/ml. To investigate the infectivity of lentiviral vector to cells, Lenti-GFP at a MOI of 1 or 10 were added to the cells. Compared to lentivirus at a MOI of 1, significant higher percentages of the GFP-positive cell population (about 85-95%) were demonstrated at a MOI of 10 (Figure 1C). The fluorescence photograph in Figure 1D showed the expression of green fluorescence of the Lenti-GFP transduced cells at a MOI of 10.

To compare gene expression of Dm-dNKmut at the mRNA and protein levels, Bcap37, SGC7901 and CCL187 cells were initially infected with lentivirus at a MOI of 10. RT-PCR and western blot assay were performed, respectively. As shown in Figure 2A, the three cancer cells transduced with Lenti-CMV-dNKmut all displayed apparent Dm-dNKmut expression, whereas the cells transduced with Lenti-GFP and control group both showed absent Dm-dNKmut expression at the RNA level. Consistently, similar results were obtained by western blotting analysis of Dm-dNKmut protein for the three cancer cells. Noticeable expression of Dm-dNKmut protein were shown in the Lenti-CMV-dNKmut group, with a negligible presence of protein in the Lenti-GFP or control group (Figure 2B).

To further probe whether the Dm-dNKmut retained enzymatic activity when imported into the three cancer cells, we assessed the phosphorylation of dThd in cell extracts. At 72 h, the dThd kinase activity in the SGC7901 cells was increased about 5-7 folds in the Lenti-CMV-dNKmut group compared with cells transduced with Lenti-GFP or control group. As for Bcap37 and CCL187 cells, the dThd kinase activity in cell extracts...
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was increased about 3-5 folds in the Lenti-CMV-dNKmut group (Figure 3). Taken together, these data in three different cancer cells illustrated that the enzyme kept its activity when imported into cells via lentivirus, which is consistent with its expression at the RNA and protein level.

**In vitro cytotoxicity, proliferation and induction of apoptosis**

To assess the cytotoxicity of lentivirus mediated Dm-dNKmut in vitro, Bcap37, SGC7901 and CCL187 cells were infected with lentivirus at a MOI of 10. Subsequently increasing concentrations of BVDU (0-10 µM) was added respectively within 5 days and followed by MTT cytotoxicity assay. As shown in Figure 4A, for the Bcap37 and SGC7901 cell lines, more than 50% of cells were killed in the Lenti-CMV-dNKmut group by treating of 1 µM of BVDU, respectively. Similarly, a cytotoxic effect was observed in the CCL187 cell line and a significant reduction of about 60% cells was detected in the Lenti-CMV-dNKmut group.

**Antitumor efficacy of Lenti-CMV-dNKmut/BVDU in vivo**

In this study, Bcap37 cells and a BALB/C nude mouse tumor model were used to explore the antitumor potential of Lenti-CMV-dNKmut/BVDU. Results showed that the Bcap37 tumor growth inhibition treated with Lenti-CMV-dNKmut/BVDU was much stronger. At 30 days, data showed a significant arrest of tumor growth in Lenti-CMV-dNKmut treated group (475.9835 mm³) compared with control group (992.9824 mm³) or Lenti-GFP treated group (1156.766 mm³) (P<0.05, Figure 6).

**Discussion**

Over the past decade, suicide gene therapy has emerged as a promising strategy and offered a number of exciting treatment in different types of cancer cells. This strategy involves the introduction of an enzyme into transfected cells followed by a prodrug treatment. As a modality, herpes simplex virus thymidine kinase (HSV-TK) combined with...
ganciclovir (GCV) has been widely used, in which HSV-TK converts a nontoxic GCV into a toxic compound within target cells causing DNA chain termination and thereby leading to cell death. Recently virus transduction exhibited attractive features in suicide gene delivery. Xinyu Zheng et al has showed that retrovirus-mediated Dm-dNK have therapeutic effects in osteosarcoma cells and pancreatic adenocarcinoma cells (Zheng et al., 2000). Studies by Zhi Zhu has reported that adenovirus-mediated Dm-dNK mutants have a simultaneous positive therapeutic effect in breast and gastric cancer cells (Zhu et al., 2011a). However, it fail significantly to improve the survival of the patient because of high cytotoxic side effect. More recently, lentiviral vectors now have emerged as safe tools for clinical trials because they can mediate stable integration and long term expression of transgenes into non-mitotic cells with high efficiency. Thus this vector provide several hints and show promise for the suicide gene delivery with the treatment of cancer. This study was based on the lentivirus-mediated Dm-dNKmut gene expression and our data indicated that the activation of CMV transcription was significantly expressed in different type of cancer cells.

Due to the fantastic efficacy, targeted cancer therapies have been considered as a potential therapeutic strategy in various types of cancer (Gerber, 2008). It was reported that using the tissue-specific promoter, such as the carcinomaembryonic antigen (Okabe et al., 2003), surviving (Kamizono et al., 2005), tyrosinase (Nettelbeck et al., 2002), could be a desirable strategy. Although most of the promoters are weaker than commonly used promoter such as CMV promoter and the transcriptional activities of these promoters vary in different types of cancer cells, other attempts such as double suicide gene driven by these promoters, radiation-induced regulation of gene expression will make up for this obstacle. Recently there has been an increasing interest in the development of new targets associated with angiogenesis, which provides novel molecular insight into tumor suppression (Eichholz et al., 2010). Thus strategies to utilize suicide gene combined with anti-angiogenic factors therapy can be designed to enhance the wide target spectrums and have a potent advantage to selectively treat cancer cells. More research is needed to better understand the role of this combined therapy in targeted cancer gene therapy.

On the other hand, substantial strides have been made in dealing with nucleoside analogs. Recently a number of nucleoside analogs such as BVDU, dFdC, araT are used in cancer gene therapy and show some promise. Wolfgang knecht et al report from the structure basis that gemcitabine is a good substrate for Dm-dNK (Knecht et al., 2009). In addition, N Solaroli et al find an evidence for the association between the mutant Dm-dNK and nucleoside analogs, suggesting that a increased cytotoxic potential for pyrimidine nucleoside analogs (Solaroli et al., 2007). However, in many cases their detailed mechanism remains poorly understood. Clearly, more work is needed in the future.

In summary, our findings demonstrated that lentivirus mediated Dm-dNKmut combind with BVDU was a promising strategy. Therefore, further development of target controlling of the Dm-dNKmut could constitute a potential therapeutic strategy in clinical cancer trials.

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References


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