A Novel Micro-Linear Vector for In Vitro and In Vivo Gene Delivery and Its Application for EBV Positive Tumors

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Abstract

Background: The gene delivery vector for DNA-based therapy should ensure its transfection efficiency and safety for clinical application. The Micro-Linear vector (MiLV) was developed to improve the limitations of traditional vectors such as viral vectors and plasmids.

Methods: The MiLV which contained only the gene expression cassette was amplified by polymerase chain reaction (PCR). Its cytotoxicity, transfection efficiency in vitro and in vivo, duration of expression, pro-inflammatory responses and potential application for Epstein-Barr virus (EBV) positive tumors were evaluated.

Results: Transfection efficiency for exogenous genes transferred by MiLV was at least comparable with or even greater than their corresponding plasmids in eukaryotic cell lines. MiLV elevated the expression and prolonged the duration of genes in vitro and in vivo when compared with that of the plasmid. The in vivo pro-inflammatory response of MiLV group was lower than that of the plasmid group. The MEKK1 gene transferred by MiLV significantly elevated the sensitivity of B95-8 cells and transplanted tumor to the treatment of Ganciclovir (GCV) and sodium butyrate (NaB).

Conclusions: The present study provides a safer, more efficient and stable MiLV gene delivery vector than plasmid. These advantages encourage further development and the preferential use of this novel vector type for clinical gene therapy studies.

Introduction

An effective vector for gene delivery should afford satisfactory transfection efficiency while assuring safety clinical application [1]. Traditionally, plasmid and viral-based vectors are two commonly used vectors. However, limitations such as immunogenicity and cytotoxicity reduce the clinical viability of viral vectors [2]. While plasmid-based gene transfection is considered to be less toxic, the relatively small transfection efficiency and short duration of transgene expression of plasmids have limited the feasibility of this method in clinical applications [3]. Furthermore, the numerous CpG sequences contained in the plasmid backbone can cause immunotoxic effects, including the elimination of transfected cells by the host immune responses [4]. The immune responses caused by unmethylated CpG dinucleotide motifs can further decrease the efficiency of gene transfection [5]. Another significant disadvantage for propagation of plasmids in bacteria is that it contains bacterial remnants such as lipopolysaccharides (LPS) or endotoxins, which can cause adverse clinical effects [6]. Therefore, these traditional vectors should be improved before clinical translation.

Recently, several novel approaches have been used to improve the traditional vectors applied in gene therapy. For example, previous studies have revealed that minicircle DNA was less immunogenic, had greater diffusivity and more stability than conventional plasmids [7–9]. These characteristics are attributed to the smaller size of the molecules and little contamination with DNA sequences that originated in the bacteria. The minimalist
immunologically defined gene expression (MIDGE) vectors created by Witting and his colleagues [10] are linear molecules containing only a promoter, a target gene and an RNA stabilizing sequence, flanked by two short hairpin oligonucleotide sequences. Each MIDGE, particularly when it was conjugated with nuclear localization signal (NLS) peptides, has greater transfection efficiency as compared with its corresponding plasmid, both in vitro and in vivo [11–14]. However, production of MIDGEs are costly and time consuming, particularly for the conjugation of NLS peptides. PCR-amplified DNA fragments, used as a model for double-stranded synthetic genes in gene therapy, have been proven to be efficient for both in vitro and in vivo gene delivery [15,16]. However, transfection efficiency of PCR-amplified DNA fragments is lower than that of plasmid, especially when the DNA fragment is delivered as cationic complexes [16]. This is likely due to the instability and poor rate of transcription of the DNA fragment when incorporated into cells. Therefore, all of these recently developed novel vectors require further modifications before they would be feasible for clinical application.

Here, we report the development of a novel linear DNA delivery vector. Briefly, the gene expression cassette was ligated with hairpin oligodeoxynucleotides (ODNs), amplified by PCR by use of a ligation mixture as the template, and purified by use of PCR cleanup kits. We have named the process the Micro-Linear Vector (MiLV). The capability and pro-inflammatory responses of the MiLV to deliver genes were investigated both in vitro and in vivo. As a proof of concept, the MiLV was evaluated as a vector in gene therapy for Epstein-Barr virus (EBV) positive cancer cells.

**Materials and Methods**

**Cell Culture**

Embryo kidney cell line 293 (HEK 293), mouse embryonic fibroblast cell line NIH 3T3, human nasopharyngeal carcinoma line CNE2 and EBV-positive monkey (tamarin) lymphocyte cell line B95-8 were purchased from the American Type Culture Collection (ATCC) (Rockville, MD) and maintained in our laboratory. The HEK 293, NIH 3T3 and CNE2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and B95-8 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% CO2 incubator. The medium was replaced until cells became 80% confluent and then passaged using 0.25% trypsin/EDTA.

**Construction of eGFP-MiLV**

Procedures for constructing the eGFP-MiLV are illustrated in Figure 1. Briefly, the eGFP expression cassette, including CMV promoter (pCMV), eGFP gene and RNA-stabilizing sequences (polyadenylic acid, SV40), was bi-digested by AseI and Afl II from pEGFP-N3 plasmid (BD Biosciences Clontech, NJ) at 37°C for 4 h. Two ODN caps containing AseI and Afl II restriction enzyme site respectively were designed as structural analogues of tRNA’s D- and TCA GCG TGT CCG 3’.

**Flow Cytometry**

The green fluorescence in transfected cells was quantified by flow cytometry by use of an Epics XL (Coulter Immunotech, Hamburg, Germany). Cells were washed twice, resuspended with ice-cold PBS, and fixed with 70% ethanol. The fluorescence was measured by use of a 530-nm/30-nm band pass filter after illumination with an argon ion laser tuned at 488 nm. Cells transfected with transfection reagent served as the control. Magnitude of expression of GFP was reported as the percentage of GFP+ cells [10].
Cell Proliferation and Cytotoxicity Assay

For cell proliferation assays, HEK 293 cells were inoculated into 6-well plates and incubated for 24 h before exposure to 1 μM plasmid or MiLV. Cells were harvested every 24 h, and then cell density was calculated by use of a hemacytometer. Cytotoxicity was determined by use of the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO) assay. Briefly, after being transfected by the MEKK1-MiLV or pCMV/MEKK1 plasmid for 24 h, B95-8 cells were treated with 1 mM NaB for 18 h followed by treatment with 100 μg/ml ganciclovir (GCV) for 3 or 6 days. MTT was then added to each well to make a final concentration of 0.5 g/L, and then cells were incubated for a further 4 h. Supernatant solutions were then aspirated, and the cells solubilized in 200 μL dimethyl sulfoxide (DMSO). Optical density was measured at 570 nm.

In vivo Gene Transfer

BALB/c male mice (6–8 weeks, Experiment Animal Center of Sun Yat-sen University, Guangzhou, China) were reared and maintained under conventional breeding conditions with food and water ad libitum, on a 12:12 h light: dark cycle. The experimental protocol was approved by the Ethics Committee for Animal Research at Sun Yat-sen University. Twenty micrograms eGFP-MiLV and 60 μg pEGFP-N3 plasmid (equal molar) were packaged with Lipofectamine 3000 Reagent (1:1.5, g/ml) in a total volume of 100 μL. The mixtures were injected intramuscularly within 5 s. The control group was injected with PBS. To investigate expression of GFP, in each group, at least one mouse was euthanized each week for a total of 8 weeks. Mice were euthanized by use of standard surgical procedures. Muscle was sectioned transversely (5 μm) with a Leica CM 1850 cryostat (Leica, Nussloch, Germany) maintained at −20°C. Sections were examined for expression of GFP by use of a laser scanning confocal microscope. The calculation of fluorescence intensity was processed with previously published method [17] with slight modification. Briefly, both the normalized photon counts and the area of eGFP signals were quantified. Then we subtracted the photon counts/second/mm² of region of interest (ROI) by the photon counts/second/mm² of the eGFP− area and calculated the total photon counts of generated by eGFP+ cells by timing the normalized intensity with the area of eGFP+ region.

Immunization of BALB/c Mice and Cell-mediated Immune Response

Forty micrograms eGFP-MiLV and pEGFP-N3 plasmid were precipitated on 20 ml of 1 μm gold beads, respectively, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA). A suspension of gold beads carrying the DNA was made in 99.5% ethanol and water 1:1.5, g/ml, according to the instructions of manufacturer (BioRad, USA).

Measurement of Pro-inflammatory Cytokines in the Blood

At 2 h after injection of 40 μg eGFP-MiLV and pEGFP-N3 in lipoplex form (1:1.5, g/ml) into the tail vein, the blood was collected by saphenous venepuncture. Blood samples were allowed to coagulate at 4°C for 4 h and then centrifuged at 4000 xg for 10 min. Serum was collected, diluted with PBS and kept at −80°C until analysis. The concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-12 were determined using enzyme-linked immunosorbent assay (ELISA) kits.

Immunoblot Analysis

The immunoblot assays were performed as described previously [18]. In brief, cells were lysed in buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM ethylene glycol tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 mg/ml aprotinin, and 5 mg/ml leupeptin). The lysates were purified initially by centrifugation and denatured by boiling in Laemmli buffer, separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to a nitrocellulose membrane. Following blocking with 5% non-fat milk at room temperature for 2 h, the membrane was incubated with the patient's EBV-TK serum at 1:1000 dilution overnight at 4°C. Membranes were then incubated with a 1:5000 dilution of horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature, and detected with the Western Lightning Chemiluminescent detection reagent (Perkin-Elmer Life Sciences, Wellesley, MA).

In vivo Treatment Efficacy of MiLV

To evaluate the in vivo treatment efficacy of MiLV, 1 x 10⁷ B95-8 cells transfected with equal molar of MEKK1-MiLV or pCMV/MEKK1 plasmid were inoculated subcutaneously into both flanks of 10-week-old male BALB/c nude mice (8 mice for each treatment group). When tumors had become palpable (7–10 days later), they were treated with a single intraperitoneal injection of NaB (500 μL of 50 mM sodium butyrate in PBS) and intraperitoneal injection of GCV (100 mg/kg twice a day for 5 days). Tumor size was monitored by measuring the length and width with calipers, and volumes were calculated with the formula: \((L \times W^2) \times 0.5\), where \(L\) is length and \(W\) is width of each tumor. When tumors became extremely large (greater than 1 cm³) or the mice appeared ill, mice were sacrificed by cervical dislocation, and the tumors were excised and weighed.

Statistical Analysis

Statistical comparisons of differences between treatments were made by use of the paired t test. A p-value of <0.05 was considered to be statistically significant. The statistical analyses were performed using SPSS 17.0 for Windows.

Results

Characteristics of MiLV

Compared with its progenitor pEGFP-N3 plasmid (4.7 kb) which contains antibiotic makers and other bacterial originated genes, the eGFP-MiLV (1.7 kb) is only about one third the size (Figure 1). MiLV and PCR fragment were incubated with exonuclease III to investigate the resistance of MiLV to in vitro degradation. The results indicated that the half-life of eGFP-MiLV was 10 to 15 folds greater than the DNA fragment alone (data not shown). More than 85% of MiLV were resistant against exonuclease digestion.
after 2 h. MTT assay showed that the viability of HEK 293 cells transfected with eGFP-MiLV (95.8 ± 0.70%) were significantly (p < 0.05) higher than that transfected with the pEGFP-N3 plasmid (92.8 ± 0.67%). There was no significant (p > 0.05) difference between cytostatic effects of MiLV or plasmid (data not shown).

In vitro GFP Transfection of MiLV and Plasmid

Three eukaryotic cell lines (HEK 293, NIH 3T3 and CNE2) were selected to compare the in vitro transfection efficiency of eGFP-MiLV and its corresponding plasmid pEGFP-N3. Green fluorescent protein was monitored 48 h later after transfection. When cells were transfected with equal molar concentrations of plasmid or eGFP-MiLV, transfection efficiencies were comparable (Figure 2). This result was confirmed by flow cytometry (Figure 3 A). In HEK 293 cells, eGFP-MiLV resulted in significantly (p < 0.05) greater efficiency of transfection of GFP than the pEGFP-N3. The efficiency of transfection of eGFP-MiLV was as great as 30%. The efficiency of transfection of GFP of the two vectors was comparable in NIH 3T3 and CNE2 cell line.

Figure 1. Construction of eGFP-MiLV. The eGFP expression cassette was digested using Asel and Afl II from pEGFP-N3 plasmid. After the ligation of DNA fragment and ODN caps, the mixture was used as temple for PCR amplification. The eGFP-MiLV was purified by PCR cleanup kits and used for further in vitro and in vivo experiments.

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of expression of GFP in cells transfected with eGFP-MiLV was significantly \(p<0.05\) greater than the duration of expression of GFP in both HEK 293 and CNE2 cells transfected with the pEGFP-N3 plasmid, while comparable in NIH 3T3 cells (Figure 3 B). Expression of GFP lasted for nearly one month in HEK 293 cells when transfected with eGFP-MiLV, while the pEGFP-N3 plasmid lasted for only about 20 days (Figure 3 C). This result suggests that the eGFP gene was more stable in eukaryotic cells when transfected by MiLV than cells transfected by use of a plasmid.

**In vivo GFP Expression of MiLV and Plasmid**

To assess *in vivo* expression of the GFP gene, 20 µg eGFP-MiLV or 60 µg pEGFP-N3 plasmid [equal molar] was injected into a hind leg of mice. After durations of 1, 2, 4 or 8 weeks after intramuscular injection, at least one mouse of each group was euthanized. Green fluorescence was detected in muscle of mice more than two months after injection of eGFP-MiLV. Maximum fluorescence was observed 2–4 weeks after injection (Figure 4). However, there was just limited fluorescence observed 4 weeks after injection with the pEGFP-N3 plasmid. The results suggested that MiLV is a more stable vector than plasmid for *in vivo* gene transfection. Transfection efficiency was quantified by measuring the fluorescence in muscle 2 weeks after injection. Fluorescence intensity in leg muscle of mice injected with eGFP-MiLV was significantly greater (3.2 fold, \(p<0.05\), \(n=3\) for each group) than in the muscle of mice injected with the pEGFP-N3 plasmid.

To compare the immune responses to encoded antigen of plasmid and MiLV, the eGFP-MiLV and pEGFP-N3 plasmid were precipitated on gold particles and coated onto tefzel-tubings before gene gun immunization. Antibodies directly against the encoded antigen (GFP) were examined by ELISA in mouse sera taken at the indicated time-points after the primary immunization.
the MiLV and plasmid group showed a positive antibody response towards the GFP antigen in serum samples taken 2 weeks after the first immunization. Mice immunized with MiLV showed a significantly ($p < 0.05$) higher antibody response after the second immunization compared with the plasmid group (Figure 5). After four times immunization, the immune response of plasmid group is only about 38% of the MiLV group. It suggested that the gene delivery and expression efficiency of equal weight MiLV is significantly greater than that of plasmid.

In vivo Inflammatory Response

The immunostimulatory activities of the eGFP-MiLV and pEGFP-N3 formulations were tested by determining serum TNF-α, IL-6 and IL-12 levels after injection (40 μg DNA) for 2 h. Figure 6 shows the levels of TNF-α, IL-6 and IL-12 levels in blood at 2 h after injection of eGFP-MiLV and pEGFP-N3. The levels of TNF-α, IL-6 and IL-12 in pEGFP-N3 group were 1.5-, 1.4-, and 1.2-fold higher than that in eGFP-MiLV group, respectively. The results revealed that MiLV, which contains much less CpG motifs than plasmid, reduces the in vivo inflammatory responses to gene delivery vector.

Case Study: MEKK1-MiLV to EBV Positive Cells and Tumor

Because the results of the in vitro and in vivo proof of concept studies suggested that MiLV had good potential application prospects at gene therapy, the MEKK1 gene, which can elevate the sensitivity of B95-8 cells to NaB/GCV and the expression of thymidine kinase (TK) gene [18], was chosen to construct MEKK1-MiLV (Figure 7 A). The pLMP1 promoter was included to ensure that MEKK1 would only be expressed in cells that are EBV positive. The immunoblot results revealed that MEKK1 delivered by MiLV or plasmid could significantly enhance the TK expression in B95-8 cells (Figure 7 B), which was consistent with our previous study [18]. The results indicated that MEKK1 transfection efficiency of MEKK1-MiLV was at least comparable with pCMV/MEKK1 plasmid.

After being transfected with either MEKK1-MiLV or MEKK1 plasmid for 24 h, B95-8 cells were treated with 1 mM NaB for 18 h followed by exposure to 100 μg/ml GCV for 3 days. Nuclei of cells transfected with the MEKK1 plasmid or MEKK1-MiLV became smaller, elongated and fusiform (Figure 8 A). The relative viability of MEKK1-MiLV transfected cells (Figure 8 A, MiLV group ) was less than MEKK1 plasmid.
transfected cells (Figure 8 A, Plasmid group). This result was confirmed by the results of the MTT assay. B95-8 cells transfected with MEKK1-MiLV were significantly (p<0.05, paired t test) more sensitive than cells transfected with the plasmid to co-exposure to GCV/NaB for 3 or 6 days (Figure 8 B). Thus, it was concluded that transfection with MEKK1 could enhance the sensitivity of EBV positive cells to GCV/NaB, particularly when MEKK1 was transferred by MiLV.

We tested the effects of MEKK1 gene delivered by MiLV or plasmid on established nasopharyngeal carcinoma growth in mice. As shown in Figure 9, tumor cells transfected with MEKK1-MiLV or pCMV/MEKK1 plasmid both significantly suppressed the growth of B95-8 subcutaneous tumors when compared with that of control (not transfected) (p<0.01), suggesting that MEKK1 gene could enhance the in vivo sensitivity of EBV positive tumor cells to GCV/NaB. Furthermore, the tumor volumes were significantly (p<0.05) reduced in the group treated with MEKK1-MiLV compared with the group treated with pCMV/MEKK1 plasmid. These results suggested that the MEKK1-MiLV has a more favorable antitumor effect than plasmid in vivo.

**Discussion**

The design and optimization of the expression system is a major part of the development of successful gene therapy [19]. One reasonable approach to enhance the efficiency of transfection is to remove the non therapeutic genes from the plasmid, especially immunostimulatory CpG motifs that originate in bacteria [20]. In the present study, the ligation product was used directly as the template for PCR amplification. The vector was duplicated at the end of each PCR cycle. After the vector was purified using a PCR cleanup kit and ensured by DNA sequencing, it could be used for in vitro and in vivo experiments. A possible concern about using
Figure 5. Mean ELISA values of GFP from equal weight (40 μg) eGFP-MiLV and pEGFP-N3 plasmid immunized mice. The ELISA was performed with mouse sera from individual animals of different vaccination groups. Blood samples were taken and prepared at the indicated time-points before each eGFP-MiLV or pEGFP-N3 plasmid vaccination, and analyzed for reactivity against the GFP (the antigen). * p<0.05; ** p<0.01.

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Figure 6. Effect of DNA-induced pro-inflammatory cytokines on GFP in the blood after intravenous injection eGFP-MiLV and pEGFP-N3 plasmid. Mice received an intravenous injection of 40 μg eGFP-MiLV and pEGFP-N3 plasmid. At 2 h after injection, the levels of TNF-α, IL-6 and IL-12 in blood were measured. The results are expressed at the mean ± SD of three mice. * p<0.05 compared to the pEGFP-N3 group.

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NaB for 18 h, followed by treatment with 100 μg/ml GCV for 3 days. The TK expression was detected by immunoblot analysis with a patient’s EBV-TK serum. The β-actin protein levels served as the loading controls.

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Figure 7. Expression of TK in B95-8 cell transfected by use of MEKK1-MiLV or pCMV/MEKK1 plasmid. A: The structure of MEKK1-MiLV. The pLMP1 resulted in MEKK1 being expressed only in EBV positive cells. B: B95-8 cells were transfected by equal molar of MEKK1-MiLV or pCMV/MEKK1 plasmid for 24 h, and then treated with 1 mM GCV/NaB for 18 h, followed by treatment with 100 μg/ml GCV for 3 days. The TK expression was detected by immunoblot analysis with a patient’s EBV-TK serum. The β-actin protein levels served as the loading controls.

PCR amplification for MiLV is that errors occur during amplification. Two or more polymerases (such as Taq and Pyrobest polymerases) with different fidelities can reduce these errors. Furthermore, DNA sequencing after PCR amplification with different fidelities can reduce these PCR amplification errors. The PCR-amplified MiLV has several significant advantages over plasmid DNA and other similar gene delivery vectors.

The MiLV is safer than plasmids and other vectors. The MiLV reduces numbers of inflammatory unmethylated CpG motifs which are contained in the skeleton of plasmid. Unmethylated CpG dinucleotides, or CpG motifs, which are uncommon in mammalian DNA, stimulate immune cells through Toll-like receptor 9 (TLR9). Unmethylated CpG dinucleotides have been reported to reduce the transgene expression in eukaryotic cells [33]. One recent study revealed that the deletion of CpG motifs in plasmid improved the duration of in vivo transgene expression when administered as a DNA/polymer complex [34]. Collectively, the greater in vitro and in vivo gene delivery efficiency of the MiLV than plasmid in the present study might be due to the reduction of bacterial CpG motifs as well as its smaller size.

Stability is another important factor affecting expression of transgenes. In the present study, new ODN caps were designed according to the D loop of tRNA in eukaryotic cells. The results of in vitro digestion experiments revealed that the cap could protect the vector from exonuclease effectively (85% of MiLV were resistant against exonuclease digestion for 2 h). Furthermore, the duration of GFP expression in vitro or in vivo that had been transfected by use of MiLV was significantly greater than that transfected by the plasmid. Fluorescence was quenched after 4 weeks in mice transfected by using the plasmid; while in mice transfected with MiLV, fluorescence lasted for more than 2 months. In addition to small molecules enhancing the transfection efficiency of the MiLV, the continuous expression of the foreign genes might be due to the fact that almost all nontherapeutic sequences have been removed. Therefore, mice immunized with MiLV showed a significantly (p<0.05) lower pro-inflammatory response compared with the plasmid group. Further studies will be needed to demonstrate more detailed reasons for the prolonged transgene expression of MiLV.

EBV is a ubiquitous human herpes virus that is associated with variety of human malignancies, including nasopharyngeal carcinoma (NPC), Burkitt’s lymphoma (BLs), T cell lymphoma and gastric carcinoma [36]. Nearly 100% of NPCs and 90% of BLs contain EBV episomes [37]. Our previous study indicated that the constitutive activation of MEKK1 can increase the sensitivity of EBV positive cells to GCV/NaB via a TK-dependent mechanism [18]. In the present study, the cells transfected with MEKK1-MiLV were more sensitive to GCV/NaB than that transfected with...
In summary, this study provides proof of the efficacy of a safer gene delivery vector with satisfactory transfection efficiency both in vitro and in vivo. This PCR generated vector does not require bacteria for production. Therefore, it removes the possibility of LPS contamination during plasmid preparation. These advantages further support the use of MEKK1-MiLV as a therapeutic strategy to enhance the sensitivity of EBV-positive tumor cells to GCV/NaB.
combined with the optimized biological safety encourage further development and the preferential use of this new vector type in clinical gene therapy studies.

**Author Contributions**
Conceived and designed the experiments: JD SYC HSW. Performed the experiments: HSW ZJC GZ XLO. Analyzed the data: CKCW JPG XLY. Wrote the paper: HSW.

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