Intrathecal injection of lentivirus-mediated glial cell line-derived neurotrophic factor RNA interference relieves bone cancer-induced pain in rats

Fu-fen Meng,1,8 Yang Xu,2,8 Qi-qin Dan,2,3 La Wei,1 Ying-jie Deng,4 Jia Liu,3 Mu He,5 Wei Liu,2 Qing-jie Xia,5 Fiona H. Zhou,6 Ting-hua Wang2,3 and Xi-yan Wang2

1Department of Anesthesia, Xinjiang Tumor Hospital, Urumqi; 2Department of Anesthesia, Translational Neuroscience Center, West China Hospital, Sichuan University, Chengdu; 3Institute of Neuroscience, Kunming Medical University, Kunming; 4The Second Department of Orthopedics, Xinjiang Traditional Chinese Medicine Hospital, Urumqi; 5Translational Neuroscience Center, West China Hospital, Sichuan University, Chengdu, China; 6Sansom Institute of Health, University of South Australia, Adelaide, South Australia, Australia; 7Department of Hepatopancreatobiliary Surgery, Xinjiang Tumor Hospital, Urumqi, China

Bone cancer pain is a common symptom in cancer patients with bone metastases and the underlying mechanisms are largely unknown. The aim of this study is to explore the endogenous analgesic mechanisms to develop new therapeutic strategies for bone-cancer induced pain (BCIP) as a result of metastases. MRMT-1 tumor cells were injected into bilateral tibia of rats and X-rays showed that the area suffered from bone destruction, accompanied by an increase in osteoclast numbers. In addition, rats with bone cancer showed apparent mechanical and thermal hyperalgesia at day 28 after intratibial MRMT-1 inoculation. However, intrathecal injection of morphine or lentivirus-mediated glial cell line-derived neurotrophic factor RNAi (Lvs-siGDNF) significantly attenuated mechanical and thermal hyperalgesia, as shown by increases in paw withdrawal thresholds and tail-flick latencies, respectively. Furthermore, Lvs-siGDNF interference not only substantially downregulated GDNF protein levels, but also reduced substance P immunoreactivity and downregulated the ratio of pERK/ERK, where its activation is crucial for pain signaling, in the spinal dorsal horn of this model of bone-cancer induced pain. In this study, Lvs-siGDNF gene therapy appeared to be a beneficial method for the treatment of bone cancer pain. As the effect of Lvs-siGDNF to relieve pain was similar to morphine, but it is not a narcotic, the use of GDNF RNA interference may be considered as a new therapeutic strategy for the treatment of bone cancer pain in the future.

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vectors have emerged as powerful tools in many fields like neuroscience, hematology, developmental biology, stem cell biology, and transgenics. Lentiviral vectors used in gene therapy are known as real therapeutic alternatives for many inherited monogenic diseases like Parkinson’s disease. Moreover, LV vectors are currently being explored in human clinical trials. Lentiviral vectors with a self-inactivating architecture enhance their safety properties. In addition, LV vectors have been inserted into host genomes in the gene coding regions rather than in the promoter regions, which also reduces the risk of proto-oncogene upregulation.

In the present study, we used an LV vector containing an artificial GDNF siRNA to further downregulate the expression of GDNF in the lumbar spinal cord of rats with bone cancer. We found that intrathecal injection of LV-mediated GDFN RNAi (Lvs-siGDNF) may be a novel strategy to attenuate pain-related behaviors by inhibiting the level of substance P (SP) and blocking the pain-related ERK signaling pathway.

Materials and Methods

Complete materials and methods, excluding packaging of the lentivirus, intrathecal catheterization, drug administration, and behavioral analysis, are described in Document S1.

Animals. All animal protocols were carried out in accordance with the guidelines of the International Association for the Study of Pain.

Cell cultures. Mammary rat metastasis tumor (MRMT-1) cells were cultured in flasks as previously described.

Bone cancer pain model. The model of rat bone cancer pain induced by cancer was established by intratibial injection of MRMT-1 rat mammary gland carcinoma cells as previously described.

Lentivirus package containing artificial siGDNF-specific gene (Lvs-siGDNF) or empty psiHIV-U6 vector (Lvs-NC). The vector containing the GDNF siRNA was provided by GeneCopoeia gene company (Rockville, MD, USA): vector size, 9027 bp (backbone only, insert not counted); stable selection marker, puromycin; reporter gene, mcherryFP; hairpin loop sequence, TCAAGAG. The GDNF gene expression cassette vector and Lenti-Pac HIV Expression Packaging Kits (GeneCopoeia, Rockville, MD, USA), containing an HIV packaging mix of the three packaging plasmids, were cotransfected using EndoFectin Lentis transfecnt reagent into 293Ta cells to package capable lentiviruses, and were defined as pseudovirion siGDNF. Then the viral supernatant was collected into sterile capped tubes 48 h post-transfection and centrifuged at 10,000 g for 10 min to get rid of cell debris. Following centrifugation, LV supernatants were harvested and mixed with Lenti-Pac Concentration Solution (GeneCopoeia) at the ratio of 5:1, then centrifuged at 3500 g for 25 min at 4°C after incubate at 4°C for 2 h. The supernatant was then discarded carefully to avoid disturbing the virus pellet. The virus pellet at the bottom of the tube was resuspended using PBS at 1/100 of the original sample volume by gently pipetting up and down. The concentrated viral samples were titrated by infecting HT-1080 cells, and they were stored at −80°C in single-use aliquots until use. The average titer was $3 \times 10^7$ infectious units/mL. Lvs-NC was packaged in the same way.

Application of immunosuppressant. Cyclosporine was injected i.p.

Intrathecal catheterization. The operation procedures were modified based on Fang et al. Briefly, the fourth coccygeal spinous process was removed and the dura mater exposed after each rat was anesthetized. The dura was punctured by a needle, resulting in some leakage of cerebrospinal fluid. A polyethylene catheter (PE-10, 15 cm) filled with normal saline prior to the procedure was immediately inserted 2 cm through the dura slit into the subarachnoid space. Subsequently, a segment of the catheter near the dura opening was fixed with the surrounding tissues. The rest of the catheter was buried under the skin and the tip of the catheter was punctured through the skin, at the nape of the neck and tightened with silk threads. The catheter orifice was connected to the needle of a microsyringe (50 μL) that was used for intrathecal injection. The dead space of the catheter lumen was approximately 10 μL.

All animals were checked the next day for any neurological abnormalities. Lidocaine, 2% (10 μL) was injected through the catheter to temporarily paralyze the rats’ hind limbs to confirm the correct intrathecal localization.

Drug administration. The Lvs-siGDNF and Lvs-NC aliquots were dissolved in PBS (0.01 M) for subarachnoid administration (injection dose, 20 μL). Salt morphine (10 mg/mL) was dissolved in sterile normal saline at the final concentration (1 μg/mL) for subarachnoid administration in the same volume.

Behavioral analysis. For each rat, the behavioral tests were carried out pre-surgery to determine the baseline and on days 7, 14, 21, and 28 after tumor injection. After subarachnoid catheter and gene treatment, the tests were measured on days 7 and 14. For the morphine-treated group, the tests carried out pre-injection and 10 min post-morphine application at each time point. For both tests, mean data were established by averaging the records of four tests with a 10-min interval between each animal.

Tail-flick latency (TL) test. Thermal hyperalgesia tests were determined by measuring the time of the rats’ tail responded to a radiating thermal stimulus. An automatic tail-flick analgesiometer (7360; Ugo Basile, Varese, Italy) was used in this test. Parameter settings were: intensity, 80; cut-off time, 20 s, which was used to prevent tissue damage. The TL was not recorded automatically from the onset of the test, when the tail was withdrawn, until an abrupt flick of the tail was sensed.

Paw withdrawal threshold (PWT) test. Mechanical nociceptive withdrawal responses were measured using the Randall–Selitto paw pressure device (Bioseb, Chaillie, France). The mechanical hyperalgesic threshold was recorded when a rat withdrew its paw. The data were recorded in grams × 20 g.

Statistical analyses. Data were recorded as mean ± SD; the mean difference was significant at the 0.05 level.

Results

Induction of MRMT-1-induced cancer bone cancer pain model. Two parameters for hyperalgesia, relative PWT (× 20 g) and TL (s), of survived rats were used to detect the pain induced by bone cancer (Fig. 1a). Reduced bone mineral density was confirmed by X-ray 28 days after MRMT-1 injection into the tibia (Fig. 1b), and increased tumor cells and osteoclasts at the carcinoma injection site stained with H&E were observed 14 days after treatment (Fig. 1c). Consequently, implantation of MRMT-1 cells into the tibia of bilateral hind limbs of rats induced mechanical and thermal hyperalgesia as indicated by decreased PWT and TL (Fig. 1a). X-ray films showed engrafted MRMT-1 cells invaded into the tibia medullary canal (Fig. 1b1, black arrow). Bone destruction characterized as decreased bone mineral density irregularly could be seen in the injection area (Fig. 1b2) 28 days later. A large number of...
GDNF knockdown relieves bone cancer pain in rat

**Mechanical and thermal hyperalgesia significantly improved after virus injection.** For analysis of the mechanical and thermal hyperalgesia, we used PWT and TL tests (Fig. 4a,b). Bone cancer pain model treated with Lvs-siGDNF showed a significant extension in TL (Lvs-siGDNF vs normal, control, Nor+Lvs-siGDNF, saline, and NC, P = 0.003, 0.000, 0.000, 0.005, and 0.000, respectively) 7 days after intrathecal injection. In this study, morphine, an opioid agonist commonly used to ease pain, was used as a positive control. The effect of Lvs-siGDNF to relieve thermal hyperalgesia was similar to morphine (morphine vs control group, P = 0.002). Although Lvs-siGDNF treatment showed a trend in the increase in mechanical PWT similar to the significant effect of the morphine-treated group (morphine vs normal, Nor+Lvs-siGDNF, control, and saline group, P = 0.026, 0.000, 0.000, and 0.000, respectively), no statistical significance between Lvs-siGDNF and Nor+Lvs-siGDNF was found on day 7 after treatment. However, by day 14 after intrathecal injection, treatment with Lvs-siGDNF significantly abolished both thermal and mechanical hyperalgesia (TL: Lvs-siGDNF vs normal, control, Nor+Lvs-siGDNF, saline, and NC, P = 0.009, 0.000, 0.000, 0.006, and 0.000, respectively; PWT: Lvs-siGDNF vs control, saline, and NC, P = 0.003, 0.016, and 0.046, respectively; Fig. 4b). In addition, treatment with morphine continued to extend the TLs and PWTs 14 days after treatment (TL: morphine vs normal, control, Nor+Lvs-siGDNF, and saline, P = 0.019, 0.000, 0.013, and 0.013, respectively; PWT: morphine vs control, Nor+Lvs-siGDNF, and saline, P = 0.002, 0.000, and 0.000, respectively). Treatment with saline or nothing resulted in significant reduction in PWT compared to normal rats without bone cancer 7 and 14 days after treatment: PWT: morphine (morphine vs control group, P = 0.000) 7 days and 14 days after treatment: PWT: Lvs-siGDNF vs control, saline, and NC, P = 0.001, 0.006 at 7 days, respectively. Furthermore, we assessed the translation level of GDNF in lumbar spinal cords of the BCIP model (Fig. 4c). Treatment with Lvs-siGDNF significantly reduced the translation of GDNF (0.07 ± 0.003) compared to saline (0.43 ± 0.08), morphine (0.38 ± 0.11) and vehicle (0.28 ± 0.10) treatment groups (P = 0.000, 0.002, 0.005, and 0.036, respectively).

**Lentivirus-mediated siGDNF downregulated expression of SP.** Before investigating the chemical effect of Lvs-siGDNF treatment in our bone cancer model, we examined the expression of SP, a nociceptor in the dorsal horn of rat’s spinal cord. Our finding showed that SP staining existed in laminae I-II of the dorsal horn of normal rats’ spinal cord (Fig. 5a). However, rats with bone cancer showed significant enhanced levels of SP (Fig. 5b–e). Notably, 14 days after intrathecal injection, Lvs-siGDNF treatment significantly reduced SP (P = 0.026, 0.015, respectively) (Fig. 5f). Although a similar trend in reduction of SP staining was found in the morphine treatment group (66.4 ± 15.2-positive spots), no statistical significance was found (P > 0.05) (Fig. 5f). These data indicated that Lvs-siGDNF can further exert analgesic effects by reducing the expression of SP, whereas morphine treatment did not.

**Changes in pERK/ERK ratio in each group.** The phosphorylation state of MAPK (ERK1/2) was evaluated using...
Fig. 2. Changes in glial cell line-derived neurotrophic factor (GDNF) expression in the lumbar spinal cords of rats with bone cancer. (a) Rats with bone cancer-induced hyperalgesia significantly reduced the transcriptional expression of GDNF mRNA. (b) This pain model also reduced GDNF translational protein expression compared with untreated normal rats. (c) Photomicrographs show the location of GDNF (red) in the dorsal horn of the spinal cord. DAPI indicates cell nucleus marker (blue). *P < 0.05, **P < 0.001, one-way ANOVA, Dunnett’s T3 post hoc test. BCIP, Bone Cancer-induced pain.

Fig. 3. Construction of recombinant lentivirus-mediated glial cell line-derived neurotrophic factor RNAi (Lvs-siGDNF). (a) Recombinant information for Lvs-siGDNF. (b) Recombinant 3 was selected as the most effective interference plasmid from four GDNF shRNA recombinants. Left panel, electrophoresis gel picture. Unlabeled lanes from left to right: DL2000 marker; normal; transfection buffer; negative control (lentivirus control); fragment 1 (F1); F2; F3; and F4. Right panel, quantitative analysis. (c) Pseudovirion containing the siGDNF-F3 vector was produced by virus packaging with 293Tα cells as indicated by transfected cells (red). (d) The pseudovirion was transfected into HT1080 cells as indicated by transected cells (red). Red fluorescent protein is a marker protein encoded by a gene segment named mcherryFP (a). *P < 0.05, one-way ANOVA, least significant difference post hoc test.
Immunofluorescence and Western blot analysis at day 14 after intrathecal injection. There was a significant increase in the ratio of pERK/ERK of 7.82 ± 2.23 in our bone cancer model (P = 0.035, compared to normal rats). While Lvs-siGDNF treatment showed a trend in reduced pERK and ERK immunoreactive cells (Fig. 6a–j), Western blot analysis showed that Lvs-siGDNF treatment significantly decreased the ratio of pERK/ERK in spinal cord segment L4–6 compared to the vehicle (NC)-treated group (8.02 ± 2.41 in vehicle treated groups; 0.94 ± 0.48 in Lvs-siGDNF treated group; P = 0.044) (Fig. 6k). Furthermore, treatment with morphine also significantly reduced the ratio of pERK/ERK compared to saline and vehicle (NC)-treated groups (1.10 ± 0.43 in morphine treated group; P = 0.041, 0.048, respectively) (Fig. 6k).

Discussion

Many previous researchers showed that carcinoma-evoked osteoclast activity is involved in bone destruction, which leads to bone cancer pain.22,26,27,30 Currently there are few effective therapies with zero adverse effects available to fight against the severity or frequency of intermittent episodes of incapacitating pain caused by bone destruction.22,26,27,30 Over the past decades, genetic engineering technology has been widely used in pain research, based on a biological understanding of the mechanisms involved in the generation and maintenance of pain states.30 However, its use as a tool for treatment of pain is still a novelty. As increased expression of GDNF was found in the spinal cord of some pain models,9 afferent pain pathways could become a treatment target in our therapeutic approach using LV vector technology. Due to the superiority of LV vectors as previously described, we constructed a recombinant lentivirus vector (Lvs) containing siRNA of GDNF (siGDNF) and administered the Lvs-siGDNF by intrathecal catheterization of the spinal cord. This is because the lumbar enlargement of the spinal cord (segments L4–6) is the most important transfer station between the encephalon and the peripheral nerves that control the hind limbs.

Although our bone cancer model induced bone destruction accompanied by both thermal and mechanical hyperalgesia systemically, the levels of GDNF gene and protein expression were downregulated in the spinal cord. This observation is similar to a previous study showing that levels of aquaporin (AQP)-4, a molecule involved in edema, was also downregulated at the early stage of edema in the brain following ischemia.31 The decrease observed in both cases might be due to an internal defense mechanism to control the pain or edema. While our bone cancer model induced bone destruction without any treatment; Nor+Lvs-siGDNF, normal rats treated with lentiviral vector containing the GDNF interference RNA; P+Lvs-siGDNF, bone cancer pain rats treated with lentiviral vector containing the GDNF interference RNA; P+Mor, bone cancer pain rats treated with morphine; P+NC, bone cancer pain rats treated with negative control virus; P+5a, bone cancer pain rats treated with saline.

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Morphine, a classical opioid agonist is defined as one of the main analgesics used to alleviate pain of patients with bone cancer.\(^{(32)}\) However, the tolerance to this narcotic analgesia and the number of adverse effects it produces may limit its effectiveness during long-term use.\(^{(33)}\) Morphine was used as a positive control to evaluate the efficiency of Lvs-siGDNF gene therapy. Tail-flick latency of thermal hyperalgesia was a well-characterized and simple model with suitable parameters for predicting analgesia in humans.\(^{(33)}\) so we recorded the value of TL instead of paw withdrawal latency. From our results, intrathecal injection of Lvs-siGDNF further suppressed translation of GDNF protein and subsequently attenuated the

Fig. 5. Lentivirus-mediated glial cell line-derived neurotrophic factor RNAi (Lvs-siGDNF) treatment attenuated the expression of substance P (SP) in the dorsal horn of spinal cord of bone cancer rats. Immunofluorescence for SP in the dorsal horn of normal (a) and bone cancer model (b–e) spinal cords in the intumescentia lumbalis is depicted. (f) At 14 days after gene therapy, quantification of SP immunointensity in laminae I-II revealed an increased level in all bone cancer rats (P) regardless of their treatment compared with normal rats, but only the increase in SP staining from saline (Sa) and vehicle (NC) treatments of bone cancer groups were statistically significant. Moreover, treatment with Lvs-siGDNF significantly attenuated the level of SP immunointensity compared to saline and NC among the treated groups. *P < 0.05, one-way ANOVA, Dunnett’s T3 post hoc test. Mor, morphine; Normal, rats without bone cancer.

Fig. 6. Treatment with lentivirus-mediated glial cell line-derived neurotrophic factor RNAi (Lvs-siGDNF) or morphine reduced the ratio of pERK/ERK at 14 days post injection. (a–j) Representative pERK and ERK immunofluorescence images of lumbar enlargement spinal cord dorsal horn sections from normal rats and saline (Sa), morphine (Mor), vehicle (NC), and Lvs-siGDNF treated bone cancer rats (P) on day 14. Insets 1–10 at the left bottom of (a–j) are magnified views of an area in laminae I-II within the white box. (k) Representative Western blot bands of pERK and ERK and quantification of changes of the ratio of pERK/ERK normalized to β-actin in lumbar enlargement from normal and all treatment groups. Data represent mean ± SEM. *P < 0.05, one-way ANOVA, least significant difference post hoc test for two isoforms of pERK. Normal, rats without bone cancer.
mechanical and thermal hyperalgesia of rats induced by MRMT-1 cells in their tibial bone, whereas the reduction in hyperalgesia by morphine treatment did not involve the down-regulation of GDNF protein expression. This suggests that the analgesic properties of morphine may not be through down-regulation of GDNF, whereas directly silencing GDNF expression played an analgesic role in this bone cancer model. Noticeably, we also investigated the effects of Lvs-siGDNF in normal rats. Consequently, it looks like there is no positive effect on sensory behavior in normal rats after treatment with Lvs-siGDNF. This indicated that normal animals may be different from animals subjected to pathological lesions. Moreover, siGDNF LV vector injected into the spinal cord could reverse hyperalgesia in bone cancer rats compared to controls, but this reversion surpassed the level of normal and Lvs-siGDNF treated normal animals, indicated by the TL test. These results confirmed that administration of Lvs-siGDNF RNA in bone cancer patients is effective and it may be made available for the treatment of bone cancer patients in future clinic practice.

In addition, along with pain behaviors, increase in the expression of SP immunoreactive varicosities in the dorsal horn was observed in our bone cancer model. The stimulation of this nociceptor may indicate that central sensitization within the spinal cord appeared following persistent pain states, whereas the downregulation of GDNF expression using Lvs-siGDNF treatment in normal animals, indicated by the TL test. These results confirmed that administration of Lvs-siGDNF RNA in bone cancer rats is effective and it may be made available for the treatment of bone cancer patients in future clinic practice.

The authors have no conflict of interest.

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Disclosure Statement
The authors have no conflict of interest.
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Supporting Information

Additional supporting information may be found in the online version of this article:

**Doc. S1.** Complete materials and methods excluding packaging of lentivirus, intrathecal catheterization, drug administration, and behavioral analysis.