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Effect of down−regulation of voltage−gated sodium channel Nav1.7 on activation of astrocytes and microglia in DRG in rats with cancer pain

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ABSTRACT

Objective: To evaluate the effect of down-regulation of Nav1.7 on the activation of astrocytes and microglia in DRG of rats with cancer pain, and explore the transmission of the nociceptive information. **Methods:** Lentiviral vector harboring RNAi sequence targeting the *Nav1.7* gene was constructed, and Walker 256 breast cancer cell and morphine was injected to build the bone cancer pain model and morphine tolerance model in rats. Lentiviral vector was injected. Rats in each model were divided into 4 groups: model group, PBS group, vehicle group and LV-Nav1.7 group. The expression levels of GFAP and OX42 in dorsal root ganglia (DRG) were measured. **Results:** After the animal model was built,the level of Nav1.7, GFAP and OX42 was improved obviously with the time prolonged, which was statistically significant (*P*<0.05). The expression level of GFAP and OX42 in the DRG in the LV-Nav1.7 group declined obviously compared to the model group, PBS group and vehicle group (*P*<0.05). **Conclusions:** Intrathecal injection of Navl.7 shRNA lentiviral vector can reduce the expression of Nav1.7 and inhibit the activation of astrocytes and microglia in DRG. The effort is also effective in morphine tolerance bone cancer pain model rats.

1. Introduction

Bone cancer pain is caused by malignant bone tumor invasion or metastasis to bone. At present, it is mainly treated with opioid analgesic drugs[1,2]. Due to opioid tolerance, currently there are not any good methods to control bone cancer pain, so the quality of life of cancer patients has been seriously affected. After the damage of nervous system, nerve cell excitability is increased, and discharge frequency is added, can cause ectopic discharge. These are closely related with activity of voltage-gated sodium channel in sensory neurons[3−5].

Voltage-gated sodium channel Nav1.7 is mainly expressed in dorsal root ganglia (DRG). Nav1.7 has its unique character of activation and steady-state inactivation, which gives fast activation and inactivation, and slow resurrection and close. When small subliminal stimulus arrives in nerve cell, it can still work, which make the depolarization level of neurons to external stimuli improve, then to expand the impulsion, which in turn improves the sensitivity to external stimuli[6,7]. Human genetic study shows that gain-of-function Nav1.7 mutation will cause hereditary erythromelalgia. When its function becomes inactivating mutation, it will cause congenital insensitivity to pain with anhidrosis[8,9]. Minett *et al*[10] studied that *Nav1.7* gene expression in nerve cell of silent rats can eliminate the reaction of mechanical pain, inflammatory pain and thermal stimulus, but hotplate test and neuropathic pain are not affected by it. Gingras *et al*[11] reported that DRG of Nav1.7 are highly expressed in bone cancer pain in the rat, but it is not clear that if Nav1.7 level will affect the degree of bone cancer pain.

Normally, nerve cells send normal signal to gliocyte through ending release neurotransmitter/tempering. Gliocyte can compound and release neurotrophic factors such as nerve growth factor and brain-derived neurotrophic factor to support the function and survival of nerve cell. According to the different stimuli of gliocyte, it will produce different cytokines like chemotactic factor, and these generations of cytokines are the process of positive feedback, which will cause waterfall reaction, and generate and maintain the pathological state of pain. In the process of bone cancer pain, it is uncertain that whether intervention excitability of nerve cell can interdict and reverse the activation of gliocyte, and produce persistent effective resistance to damage effect. Therefore, in the present study, we built the bone cancer pain model and morphine tolerance model in rats. Through intrathecal injection of Nav1.7 shRNA lentivirus, down-regulation of Nav1.7 to the activation of astrocytes and microglia in DRG of rats with cancer pain was observed, and the transmission of the nociceptive information was explored, which will provide reference and method for the treatment of bone cancer pain, especially morphine tolerance bone cancer pain.

2. Materials and methods

2.1 Reagent and instrument

P12 cell and Walker 256 breast cancer cell were purchased from Cell Bank of Chinese Academy of Sciences. Trans1-T1 competent cell was purchased from Beijing TransGen Biotech Co., Ltd. pGLV-U6-GFP vector was purchased from novogen Tech. Co. Ltd. 4 ligase was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. Agarose Gel DNA Fragment recovery kit and High Quality Plasmid Mini Kit were obtained from Shanghai Xinze Bio-Technique Co. Ltd. BCA Protein Quantitative Kit was purchased from Shanghai Haling Biological Technology Co., Ltd. Morphine hydrochloride was obtained from Shenyang No. 1 Pharmaceutical Co., Ltd. Nav1.7, Astrocyte marker GFAP, Microglia markers OX42 and β-actin primary antibody, secondary antibody of goat anti mouse Nav1.7-HRP (1:10 000), secondary antibody of goat anti rabbit GFAP-HRP, secondary antibody of goat anti mouse OX42-HRP, and secondary antibody of goat anti mouse β-actin-HRP were purchased from Abcam. Gel electrophoresis and Vilber Lourmat were purchased from Bio-Rad, CA, USA.

2.2. Experimental method

2.2.1. Establishment of bone cancer pain model in rats

After Walker 256 breast cancer cell grew to exponential growth phase, pancreatic enzymes were trypsinized and centrifuged, and

then culture medium was add to adjust the concentration to 1×10^7 mL until used. 30 SD female rats weighting 200 g were randomly divided into three groups: normal group (n=6), PBS group (n=6) and model group (n=18). Rats were received intraperitoneal anesthesia with 3% pentobarbital sodium, and were fixed limbs in supine position, then left hind leg was shaved and the skin was sterilized with iodine. Skin of upper tibia was cut for 7 mm, and blunt dissection was carried out, with 5 mL syringe for punching in tubercle of tibia, and with 1 mL syringe to inject the dilute Walker 256 cell suspension (control group was injected the equivalent normal saline). Then the wound was washed with normal saline containing gentamicin. Muscle was sutured with 4-0 surgical suture, while skin was sutured with 3-0 surgical suture, then it was sterilized with iodine and rats were feeding carefully.

2.2.2. Construction of lentiviral vector

Primer sequence was designed to connect to linearized shRNA vector according to siRNAi sequence targeting the *Nav1.7* gene and was compounded by Sangon Biotech (Shanghai) Co., Ltd.

After mixtured primer and annealing buffer to anneal to form double strands nucleotide, it was mixed with lentivirus plasmid pGLV-U6-GFP vector, T4 ligation buffer and T4 ligase at 4 ℃ for the night to reaction. 50 mL competent cell Trans1-T1 was placed on ice for 5 min, then 3 μL reaction product was added on ice for incubation of 30 min. After that heat shock was performed at 42 ℃ for 1 min, then it was placed on ice for 2 min, and added Luria-Bertani broth at 37 ℃ for 45 min of thermostatic incubation. After centrifugation, supernatant was abandoned, and then the resuspended fluid was smeared in the LB culture plate with Ammonia benzyl resistance. After incubation of 17 h, recombinant monoclonal was chosen to inoculate LB culture medium with Ammonia benzyl resistance liquid for 17 h incubation, then plasmid was extracted by High Quality Plasmid Mini Kit and enzyme validation was carried out.

PC12 cell was inoculated in DMEM medium containing 10% FBS and was to cultivate to 80% of the fusion in 5% CO_2 incubator at 37 ℃. Discarded the culture medium, it was washed with PBS, trypsinized, centrifuged, resuspended and inoculated to 6 well plate with 10×10^5 cells per well. After 24 h of cultivation, 1 mL entivirus liquid was added and 1ml DMEM culture medium was added in control group. After 24 h of cultivation, culture medium containing lentivirus was discarded, and fresh complete medium was added for 96 h continued cultivation. The complete medium of 100 ng/mL nerve growth factor was changed, and after cultivation of 12 h, cells were collected.

Cell lysis solution was added to 6-well plate for fully cracking cells. Lysis solution was collected to centrifuge tube, added isometric SDS-PAGE loading buffer and boiled at 100 ℃ for 5

min. After cooling on ice and centrifugation, 10% SDS-PAGE was used for supernate electrophoresis, and then gel was transferred to nitrocellulose membrane under 60V constant pressure. After sealing and washing membrane, Nav1.7 (1:1 000) primary antibody or β-actin (1:10 000) was added for incubation for the night at 4 ℃. After washing membrane again, HRP marker GaMIgG (1:10 000) was added to incubate at room temperature for 1 h. Then it was incubated in reaction liquid at room temperature for 1 h after washed with PBS. Exposure to X-ray film, quantity one analysis software was used to analyze each specificity of stripe of gray after image scanning.

2.2.3. Establishment of animal model

First of all, subarachnoid catheter of lumbosacral portion in rats was carried out. After injection of pentobarbital sodium for anesthesia, back of the rat was sterilized, and spine clearance was exposed after the skin was cut. L3 vertebral spines were picked up with haemostatic forceps, and ligamentum flavum and endorhachis were pricked with curved needle. PE-10 catheters were put from crevasse into subarachnoid space of about 2 cm. After suture of muscle fascia, the other side of catheter was led from the neck and back of the rats, which was exposed 2 cm, and the catheter was sealed with haemostatic forceps. Rats were feed separately after the skin was sutured. After one week of rat model of bone cancer pain, NS 10 μL and 10 μg morphine were injected respectively twice a day with continuous dosing for one week.

2.2.4. Grouping and operation

After modeling, 90 rats with bone cancer pain were randomly divided into three groups: cancer pain+PBS group, cancer pain+ empty virus group, cancer pain+Nav1.7 shRNA lentivirus vector group. 45 rats with cancer pain morphine tolerance were randomly divided into three groups: cancer pain morphine tolerance+PBS group, cancer pain morphine tolerance+ empty virus group, and cancer pain morphine tolerance+ Nav1.7 shRNA lentivirus vector group. After 4 days of modeling, PBS group, empty virus group and LV-Nav1.7 group were injected with 10 μL PBS, 10 μL empty virus and 10 μL LV-Nav1.7 respectively by intrathecal injection. The virus solution was taken out at -80 ℃, put in to water bath at 37 ℃ for rewarming, adjusted the titer to 1×10^9 TU/mL, and then it was put on ice for injection.

2.2.5. Detection of Nav1.7 in DRG and effect of protein level of GFAP and OX42 in spinal cord

Rats were deeply anesthetized with 3% pentobarbital sodium and prone to the flat board. Rats with bone cancer pain were sacrificed with injection of excessive sodium pentobarbital on Day 8, 12 and 18, while rats with morphine tolerance were sacrificed on Day 24.

After shaving, the skin of back was cut, spinal muscles on both sides were dealt with blunt dissection, and spinal spines and transverse process were exposed. After cutting spinous process, vertebral plate and transverse process of both sides were removed. L4-6 on the left side of the DRG and spinal cord were removed and washed with PBS. After weighting, 0.5 mL cell lysis buffer and PMSF were added, shaked in homogenizer, and centrifuged at 14 000 r/min for 10 min. Then working solution was prepared according to instructions of BCA Protein Quantitative Kit, and standard curve was drawn. After incubation at 37 ℃ for half an hour, OD_{570} was identified with ELIASA, and protein concentration was calculated according to standard curve. Protein supernate and loading buffer were mixed to boil for 10 min, cooled and centrifuged. 12% SDS-PAGE was used for electrophoresis after collecting protein sample, and then gel was transferred to nitrocellulose membrane under 15 V constant pressure. After 60 min, it was sealed with 5% skim milk powder, washed the membrane with PBS, and Nav1.7 (1:1 000), GFAP (1:500), OX42 (1:500) or β-actin (1:10 000) primary antibody were added and incubated for the night at 4 ℃. After washing with PBS for three times, secondary antibody of Nav1.7 (1:10 000) IgG (H+L) (HRP-labeled Goat Anti-Mouse IgG (H+L), IgG-HRP (H+L) GFAP secondary antibody (1:10 000), secondary antibody Goat Anti-Mouse OX42 and secondary antibody Goat anti Mouse β-actin (1:10 000) were added and incubated for 1 h at room temperature, and them washed the membrane again. Exposure to X-ray film, Image J was used to analyze the specificity of stripe of gray after image scanning.

2.3. Statistical methods

Data obtained from the experiment were analyzed with SPSSl3.0 statistical software. t test was used to analyze the protein expression level of Nav1.7 protein, GFAP and OX42 in different time points among different groups and MWT value in the same time point among different groups. Differences are statistically significant at $P<0.05$.

3. Results

3.1. Protein level of Nav1.7 in DRG and GFAP and OX42 in spinal cord

Figure 1 shows the results of Western blot that Nav1.7 was not significantly different in control and PBS groups ($P>0.05$). Compared with control and PBS groups, Nav1.7 in model group was significantly increased in 8 d. Nav1.7 expression in model group was significantly higher in 12 d than that of in the 8 d ($P<0.05$),

and significantly higher in 18 d than that of in 12 d ($P<0.05$). GFAP had no significant difference in control and PBS groups ($P>0.05$). Compared with control and PBS groups, GFAP in model group was significantly increased in 12 d ($P<0.05$). GFAP in model group was significantly higher in 18 d than that of in 12 d ($P<0.05$). OX42 had no significant difference in control and PBS groups ($P>0.05$). Compared with control and PBS groups, OX42 in model group was significantly increased in 8 d ($P<0.05$). OX42 in model group was significantly higher in 12 d than that of in 8 d ($P<0.05$). There was no significant difference compared with 12 d and 18 d ($P>0.05$).
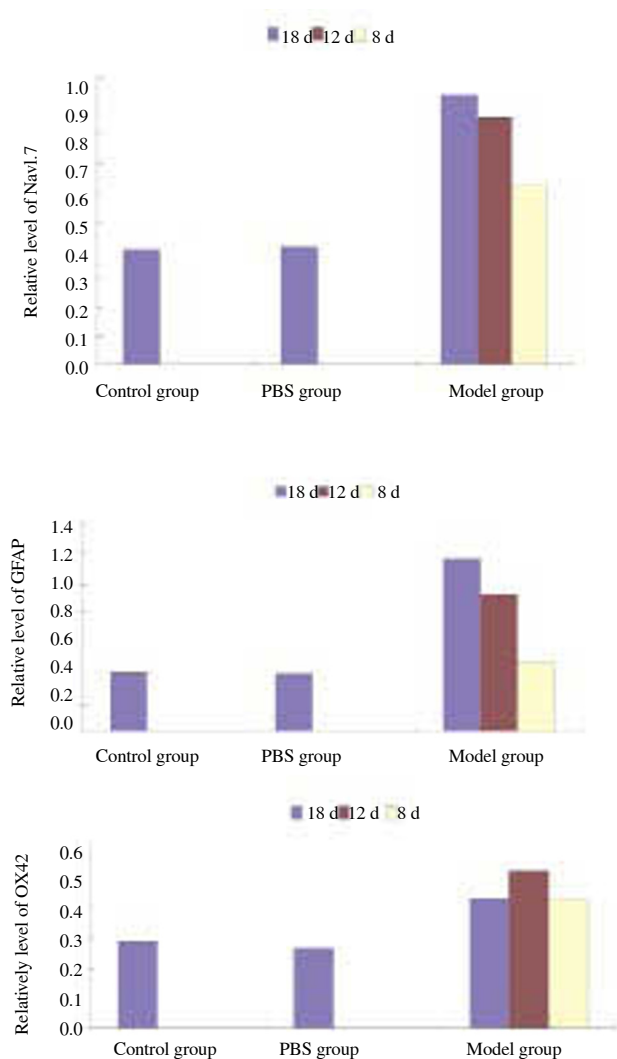
3.2. Effect of shRNA lentiviral vector on Nav1.7 expression level of PC12 cells

After 96 h of infection, compared with control group, protein expression level of Nav1.7 in empty virus vector group was slightly decreased, but had no significant difference. Protein expression level of Nav1.7 in c378 group is significantly decreased, which had significant difference ($P<0.05$) (Figure 2).
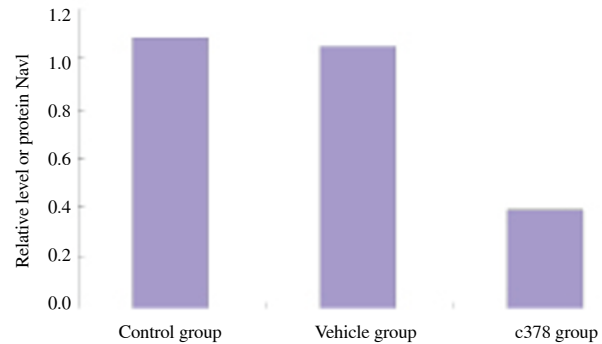

Figure 1. Effect of bore cancer pain on Nav1.7, GFAP, and OX42 in rats.
A: Nav1.7 expression level in model group was significantly higher in 8 d than that of in PBS group, $t=14.117$, $P=0.000$, 12 d higher than 8 d, $t=13.223$, $P=0.018$, 18 d higher than 12 d, $t=10.125$, $P=0.001$. B: GFAP expression level in model group was significantly higher in 12 d than that of in PBS group, $t=12.162$, $P=0.000$, 18 d higher than 12 d, $t=10.114$, $P=0.023$. C: OX42 in model group was significant increased in 8 d, $t=13.963$, $P=0.000$, 12 d significant higher than 8 d, $t=12.193$, $P=0.000$, There was no significant difference compared with 12 d and 18 d, $t=9.660$, $P=0.079$).

Figure 2. Effect of shRNA lentiviral vector on Nav1.7 expression level of PC12 cells.
* indicated statistical significance compared with control group. Compared with control group, vehicle group $t=9.653$, $P=0.624$; c378 group $t=14.629$, $P=0.000$.

3.3. Effect of intrathecal injection of Nav1.7 shRNA lentiviral vector on protein level of Nav1.7 in DRG and GFAP and OX42 in spinal cord in rats

Figure 3 showed the results of Western blot that after modeling in different time point, expression level of Nav1.7 in vehicle group had no significant difference compared with that of in PBS group ($P>0.05$). After modeling of 12 d, 18 d, expression level of Nav1.7 in LV-Nav1.7 group was significant lower than that of in PBS group, which had significant difference ($P<0.05$). After modeling, expression level of Nav1.7 in PBS and vehicle groups were increased with the time prolonged, and both had significant difference ($P<0.05$), while expression level of Nav1.7 in LV-Nav1.7 group had no significant difference in different time point ($P>0.05$).

After modeling, compared with model group, protein level of OX42 in PBS and vehicle groups in different time point had no significant difference ($P>0.05$), while OX42 in LV-Nav1.7 group was significant decreased ($P<0.05$). In addition, along with time, OX42 level in LV-Nav1.7 group had a downward trend, but compared with normal rats, it was still significantly increased in different time point ($P<0.05$).
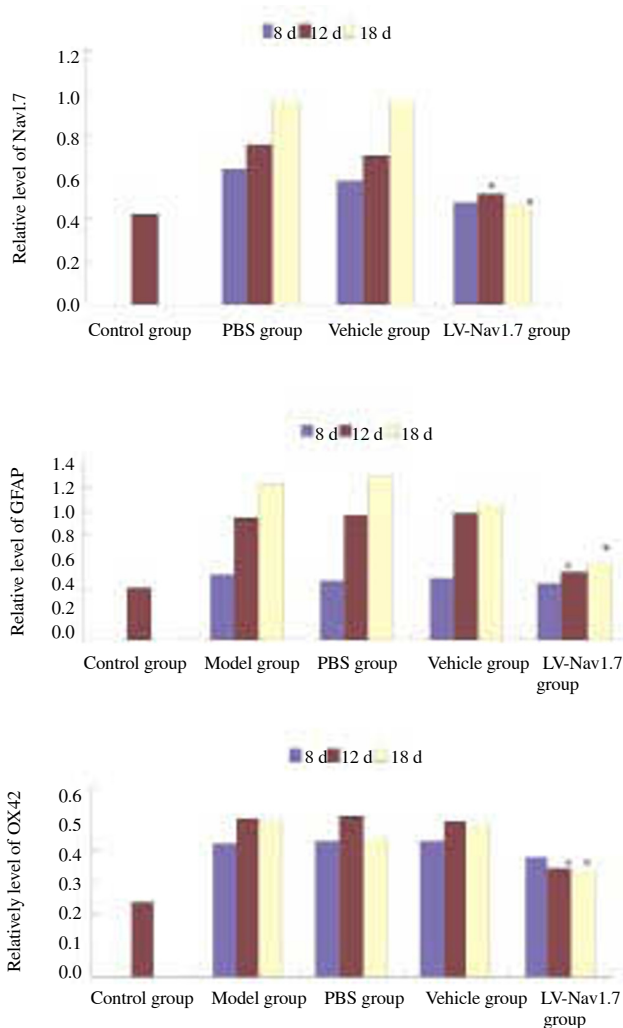
Figure 3. Effect of intrathecal injection of Nav1.7 shRNA lentiviral vector on protein level of Nav1.7 in DRG and GFAP and OX42 in spinal cord in rats.

A: expression level of Nav1.7 in LV-Nav1.7 group was significant lower in 12 d and 18 d compared with PBS group, 12 d, $t=10.088$, $P=0.021$, 18 d, $t=13.723$, $P=0.000$; B: expression level of GFAP in LV-Nav1.7 group was significant lower in 12 d and 18 d compared with PBS group, 12 d, $t=12.088$, $P=0.007$, 18 d, $t=13.533$, $P=0.000$. Compared with control group, 12 d, $t=10.332$, $P=0.020$, 18 d, $t=11.360$, $P=0.009$. C: expression level of OX42 in LV-Nav1.7 group was significant lower in 12 d and 18 d compared with PBS group, 12 d, $t=11.673$, $P=0.008$, 18 d, $t=12.179$, $P=0.003$. Compared with control group, 8 d, $t=13.780$, $t=0.000$, 12 d, $t=13.582$, $P=0.000$ 8, 18 d, $t=11.652$, $P=0.013$).

3.4. Effect of intrathecal injection of Nav1.7 shRNA lentiviral vector on protein level of Nav1.7 in DRG and GFAP and OX42 in spinal cord in morphine tolerance bone cancer pain model rats

After modeling for 24 h, expression level of Nav1.7, GFAP and OX42 in vehicle group had no significant difference compared with PBS group ($P>0.05$). Expression level of Nav1.7 and GFAP in LV-Nav1.7 group was significantly lower than PBS group, which had

significant difference ($P<0.05$). Expression level of OX42 in LV-Nav1.7 group had no significant difference compared with PBS group and vehicle group ($P>0.05$) (Figure 4).
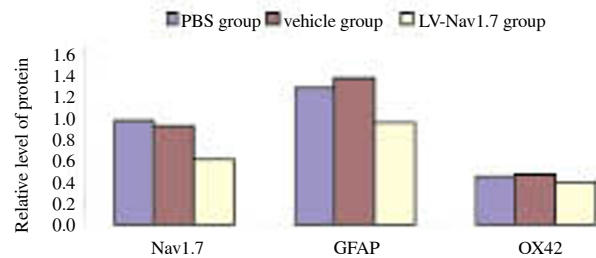

Figure 4. Protein expression level of Nav1.7 in DRG of morphine tolerance bone cancer pain model rats and GFAP and OX42 in spinal cord after the decrease of Nav1.7 expression.

Compared with LV-Nav1.7 group, expression level of Nav1.7 in PBS group $t=14.664$, $P=0.001$; expression level of GFAP $t=13.116$, $P=0.000$.

4. Discussion

Nav1.7 is more slowly in closed-state inactivation and resurrection and has specific expression character. Currently, studies have shown that Nav1.7 has closely relation with mechanical pain and inflammatory pain[12–14]. However, there is still no conclusion that if it involves in and affect neuropathic pain. Inflammatory pain and neuropathic pain are coexisted in cancer pain. It is still not clear that whether the expression level of Nav1.7 influence cancer pain. The present study detected the change of pain behavior in DRG of rats with cancer pain and morphine tolerance bone cancer pain before and after down-regulation of Nav1.7, and verified that down-regulation of Nav1.7 expression level in DRG can significantly relieve the bone cancer pain of the rats.

4.1. Research of Nav1.7

Nav1.7 is the gene SCN9A coding in the distribution of transmembrane protein of human peripheral nervous system. After mutations, Nav1.7 will cause some diseases associated with pain. Primary Erythermalgia (PE) is a kind of autosomal dominant disease, which is caused by function enhancement of Nav1.7 through *SCN9A* gene mutation. Black *et al*[16] found that patients with chronic pain disease were caused by the polymorphism of SCN9A, and healthy women who have this genetic characteristic are more sensitive to pain. Through animal experiment, Shields *et al*[17] confirmed that Nav1.7 current has relationship with superexcitation of esthesioneure and communication of postsynaptic spinal pain pathways. Yang *et al*[18] reported that polypeptide μ-SLPTX-Ssm6a extracted from

centipede can inhibit Navl.7 well, and can relieve pains caused by chemicals and temperature in rodent models. Currently, it is reported that Nav1.8 and Nav1.9, which belong to the same family of Nav1.7, are closely related with bone cancer pain in the expression level of DRG[19,20], but it is unknown that whether the level of Nav1.7 will affect the degree of bone cancer pain.

4.2. Relief of bone cancer pain in rats by intrathecal injection of Nav1.7 shRNA slow virus vector

Tumor in the bone growth stimulates osteoclast to lead to bone destruction and pull of periosteum, causing ischemia and peripheral nerve compression, and then induced microglia activation. It is found that in rat model of bone cancer pain, the spinal cord and carcinoma ipsilateral limb has large astrocyte proliferation, and gliocyte can be activated by morphine and involved in morphine tolerance, which showed that activation of astrocyte may be related to the maintaining of pain[21,22]. General studies detect astrocytes and microglia activation marker glial fibrillary acidic protein (GFAP) and OX42 to determine the degree of activation of two cells[23, 24]. Resuts showed that expression level of Nav1.7 and the two gliocyte markers in bone cancer pain models in rats were significantly increased along with the prolonged time. Although the expression level of GFAP in rats with intrathecal injection of Nav1.7 was increased, it was still significantly lower than bone cancer pain models in rats. Nav1.7 and OX42 were decreased along with the prolonged time. This showed that intrathecal injection of Nav1.7 shRNA lentiviral vector inhibited the expression of Navl.7. Although the variation trend of expression of GFAP and OX42 was not changed, it obviously slowed down the increasing speed of GFAP, accelerated the decreasing speed of OX42, and reduced the activation of astrocyte and gitter cell. The increase of expression of Nav1.7 in rats with bone cancer pain is probable due to the oppression and damage of tumor on local nerve fibers transmits traumatic information to DGR nerve cell repeatedly and stimulates continuous depolarization and activation of DGR nerve cell to promote the increase of dependency of Nav1.7 expression time.

4.3. Relief of morphine tolerance bone cancer pain model rats by intrathecal injection of Nav1.7 shRNA slow virus vector

Opioid drugs are the main way for the drug treatment of controlling cancer pain. As the final section of three stage treatments of cancer pain, opioid drugs will bring great pain to body and mind of the cancer pain patients once they produce morphine tolerance[25]. In the present study, we established morphine tolerance bone cancer pain model rats to explore whether down-regulation of Nav1.7 expression level can relieve the bone cancer pain of morphine tolerance rats

through intrathecal injection of Nav1.7 shRNA lentiviral vector. The results showed that after 24 d of injecting Nav1.7 shRNA lentiviral vector, the expression level of Navl.7, GFAP and OX42 were significantly decreased, which indicated that the decrease of Nav1.7 expression can inhibit the activation of gliocyte. This may be because of the directly abirritation of Nav1.7 shRNA lentiviral vector and the reversion of astrocyte activation, which relieve the morphine tolerance to ease the pain.

4.4. Insufficient and prospect

Our work is not perfect. We only detected the expression of glial cell activation of related proteins in rats with bone cancer pain in 24 d of Nav1.7 silence. But it is unknown how long the anti-nociceptive effect of an intrathecal injection of Navl.7 shRNA lentiviral vector will maintain. Therefore, in the later study, we will further detect the timeliness. We will also further validate the effect of voltage gated sodium channel NaV 1.7 on bone cancer pain rats by detecting the behavioral change of the rats.

In conclusion, a single intrathecal injection of Navl. 7 shRNA lentiviral vectors can reduce rats bone cancer pain, and voltage gated sodium channel NaV 1.7 may become the drug target of selective treatment of pain.

Conflict of interest statement

We declare that we have no conflict of interest.

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