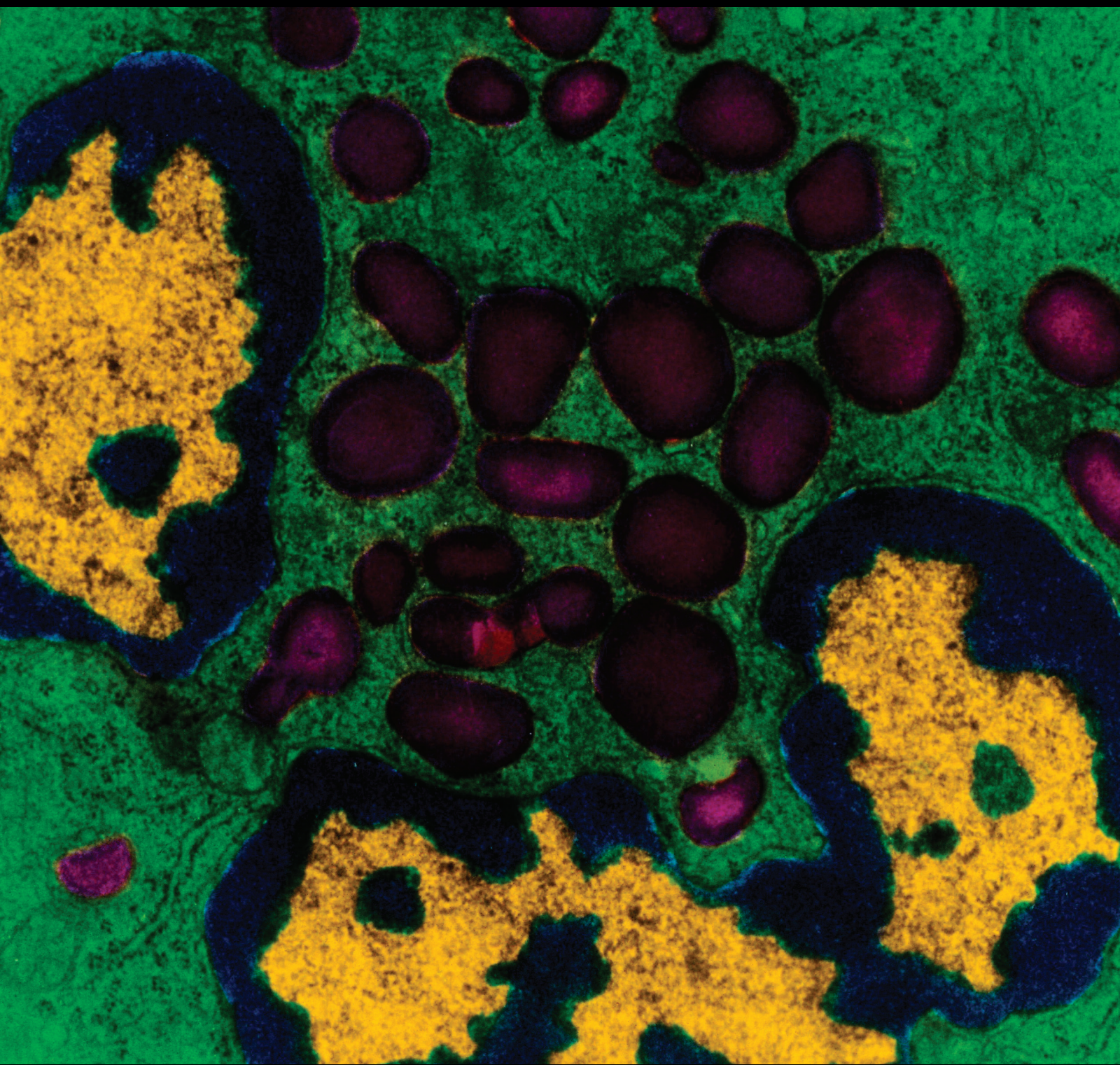
Mediators of Inflammation

Regulation of Cytokines in Cancer Pain

Guest Editors: Xue-Jun Song, Robert H. LaMotte, and Zhong Xie



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Editorial Board

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Editorial

Regulation of Cytokines in Cancer Pain

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Cancer pain possesses a major challenge in clinical treatment, yet its underlying mechanisms remain elusive. Recent advances in understanding the molecular pathways mediating the inflammatory processes accompanied with cancer and cancer pain suggest the importance of proinflammatory cytokines. Further studies in this area will facilitate the discovery and validation of potential therapeutic targets for cancer pain. In this special issue, we present original research articles and clinical studies, as well as review articles, on the role of various cytokines and their regulation in the development and maintenance of cancer pain.

This special issue features two review articles. In the paper entitled "Treatment of Cancer Pain by Targeting Cytokines," I. Vendrell et al. presented a comprehensive overview of the literature on the importance of cytokines in cancer pain and discussed the existing strategies to control the release of cytokines that have an impact on cancer pain. C. Panis and W. R. Pavanelli in "Cytokines as Mediators of Pain-Related Process in Breast Cancer" analyzed the major proinflammatory cytokines produced in breast cancer and discussed the evidences from current research regarding their role in the generation of pain-related clinical features.

The research article by G. Zhu et al. entitled "Radiotherapy Suppresses Bone Cancer Pain through Inhibiting Activation of cAMP Signaling in Rat Dorsal Root Ganglion and Spinal Cord" investigated the mechanisms of a major clinical treatment for bone cancer pain. They reported that, in a rat tumor cell implantation model, X-ray radiation reduced IL-1β and TNF-α concentrations and suppressed bone cancer pain by inhibiting the activation of cAMP-PKA signaling pathway in DRG and the spinal cord.

In the paper "Levo-Tetrahydropalmatine Attenuates Bone Cancer Pain by Inhibiting Microglial Cells Activation," M. Zhang et al. examined the analgesic roles of L-THP in rats with bone cancer pain. This study described a possible clinical utility of L-THP administration in the treatment of bone cancer pain. The analgesic effects of L-THP on cancer pain may result from the inhibition of microglial cells activation and proinflammatory cytokines (TNF-α and IL-18) production.

S. Zhu et al. described the potential role of the spinal sigma-1 receptor in the development of bone cancer pain in a research article titled "Sigma-1 Receptor Antagonist BD1047 Reduces Mechanical Allodynia in a Rat Model of Bone Cancer Pain through the Inhibition of Spinal NR1 Phosphorylation and Microglia Activation." The authors reported that intrathecal injection of sigma-1 receptor antagonist BD1047 attenuated mechanical allodynia and activation of microglial cells and suggested that targeting sigma-1 receptor may be a new strategy for the treatment of cancer pain.

The research paper by R. Pan et al., "Inducible Lentivirus-Mediated siRNA against TLR4 Reduces Nociception in a Rat Model of Bone Cancer Pain," explored the potential of targeting TLR4 as a long-term treatment for bone cancer pain. The authors presented results showing the antinociception effect of a tetracycline inducible lentivirus carrying siRNA targeting TLR4. Proinflammatory cytokines as TNF-α and IL-1β in spinal cord were also decreased.

In the clinical study titled "Efficacy and Safety of Ropivacaine Addition to Intrathecal Morphine for Pain Management in Intractable Cancer," Y. Huang et al. compared the efficacy and safety of an intrathecal continuous infusion of morphine and ropivacaine with intrathecal morphine alone

in cancer patients and showed that combined morphine and ropivacaine administration through intrathecal access ports is efficacious and safe and significantly improved patients' quality of life.

In another clinical study titled "A Traditional Chinese Medicine Xiao-Ai-Tong Suppresses Pain through Modulation of Cytokines and Prevents Adverse Reactions of Morphine Treatment in Bone Cancer Pain Patients," Y. Cong et al. reported that oral administration of Xiao-Ai-Tong alone or together with morphine can effectively suppress pain and reduce adverse reactions following morphine treatment. The study also found that Xiao-Ai-Tong treatment increased the anti-inflammatory cytokine interleukin-10 and decreased the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α in blood.

Together, the reviews, research articles, and clinical studies featured in this special issue expand our knowledge and understanding of the mechanisms of cancer pain, especially the roles of the proinflammatory cytokines. We believe these data may contribute to improved management and treatment of cancer pain.

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Research Article

Radiotherapy Suppresses Bone Cancer Pain through Inhibiting Activation of cAMP Signaling in Rat Dorsal Root Ganglion and Spinal Cord

Guiqin Zhu,[1,2] Yanbin Dong,[1] Xueming He,[1] Ping Zhao,[1] Aixing Yang,[1,2] Rubing Zhou,[1] Jianhua Ma,[1] Zhong Xie,[1] and Xue-Jun Song[1,3,4]

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Radiotherapy is one of the major clinical approaches for treatment of bone cancer pain. Activation of cAMP-PKA signaling pathway plays important roles in bone cancer pain. Here, we examined the effects of radiotherapy on bone cancer pain and accompanying abnormal activation of cAMP-PKA signaling. Female Sprague-Dawley rats were used and received tumor cell implantation (TCI) in rat tibia (TCI cancer pain model). Some of the rats that previously received TCI treatment were treated with X-ray radiation (radiotherapy). Thermal hyperalgesia and mechanical allodynia were measured and used for evaluating level of pain caused by TCI treatment. PKA mRNA expression in dorsal root ganglion (DRG) was detected by RT-PCR. Concentrations of cAMP, IL-1β, and TNF-α as well as PKA activity in DRG and the spinal cord were measured by ELISA. The results showed that radiotherapy significantly suppressed TCI-induced thermal hyperalgesia and mechanical allodynia. The level of PKA mRNA in DRG, cAMP concentration and PKA activity in DRG and in the spinal cord, and concentrations of IL-1β and TNF-α in the spinal cord were significantly reduced by radiotherapy. In addition, radiotherapy also reduced TCI-induced bone loss. These findings suggest that radiotherapy may suppress bone cancer pain through inhibition of activation of cAMP-PKA signaling pathway in DRG and the spinal cord.

1. Introduction

Pain is one of the most prevalent symptoms in patients with primary bone sarcomas and with the distant metastases of nonbone primary tumors [1, 2]. Studies have indicated that bone cancer pain has complex and unique mechanisms that may involve both inflammatory and neuropathic pain [3]. Clinically, most patients with bone cancer have already passed the optimal time for radical surgery and multidisciplinary therapies. However, with radiotherapy, majority of these patients experience pain relief, control of tumor growth, and prolonged survival. Radiotherapy is not only an effective method in the clinical treatment of bone cancer, but also an important approach for treatment of the severe pain associated with bone cancer. However, mechanisms underlying radiotherapy for bone cancer pain have not been well investigated and remain elusive.

Cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) signaling pathway plays important roles in a number of cellular processes, including immune function [4], growth [5], differentiation [6], and metabolism [7], and is essential to the plasticity in neural synapses in CNS [8].

Activation of cAMP-PKA pathway has been reported to enhance presynaptic neurotransmitter synthesis and vesicular transportation probably through phosphorylation of key transcriptional factors (i.e., cAMP response element-binding protein) and synaptic vesicle proteins [9–11]. In addition, recent studies found that cAMP-PKA signaling pathway is involved in both inflammatory pain [12–14] and neuropathic pain [15–17]. The peripheral hyperalgesic actions of inflammatory mediators are mediated by the cAMP-PKA signaling pathway [18]. We have recently demonstrated that the cAMP-PKA pathway is crucial for the maintenance of dorsal root ganglia (DRG) neuronal hyperexcitability and behaviorally expressed hyperalgesia, in an *in vivo* neuropathic pain animal model of chronic compression of the DRG (CCD model), as well as in an *in vitro* model of acute DRG dissociation [15, 16, 18]. Recently, we have further found that activation of the cAMP-PKA signaling pathway plays an important role in both induction and maintenance of bone cancer pain in rats [19]. However, it remains unknown whether and then how cAMP-PKA signaling would contribute toward radiotherapy treatment for bone cancer pain. This study provides evidence supporting an idea that radiotherapy may suppress bone cancer pain through inhibition of abnormal activation of cAMP-PKA signaling pathway in DRG and the spinal cord.

2. Materials and Methods

2.1. Animals and Drugs. Female adult Sprague-Dawley rats (160–180 g at the start of the experiment) were housed in a controlled lighting environment with free access to food and water. All experiments were approved by the Institutional Animal Care and Use Committees in Oriental Hospital and conducted in accordance with the Declaration of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (publication number 85-23, revised 1985). Surgery was performed under anesthesia with intraperitoneal injection of sodium pentobarbital (50 mg/kg, i.p.).

2.2. Animal Model of Bone Cancer Pain. The protocols of the bone cancer pain model were similar to that described previously [19–21]. In brief, following induction of general anesthesia with intraperitoneal injection of sodium pentobarbital, rats were placed abdominal side up. After disinfecting with 75% v/v ethanol, a one-centimeter rostrocaudal incision was made in the skin directly above the top half of the tibia. Tumor cells (1×10^5 cells/μL, 5 μL), extracted from the ascetic fluid of female rats that received Walker 256 mammary gland carcinoma cells, were injected (tumor cell implantation, TCI) into the intramedullary space of the right tibia to induce bone cancer. Injection site was closed with bone wax while the syringe was removed. The incision was then dusted with penicillin powder and closed. Rats in the sham group were injected with the same number of boiled tumor cells.

2.3. Radiotherapy. A single dose of radiation was given 9 days after TCI. Rats were immobilized in an acryl jig, and a dose of 6 Gy was delivered to the right tibia area using a collimator system with 6 MV X-rays [22]. Sham radiotherapy applied in TCI rats was using the same protocol, except that they were not given the real X-ray radiation.

2.4. Radiographic Observation. On the 17th day after TCI treatment, radiographic images were taken (exposure setting: 12 ms, 31 KVp) using a Philips Digital Radiographer System (Digital Diagnost VM; Philips Medical Systems DMC GmbH, Hamburg, Germany). Bone destruction was evaluated on a scale of 0–5 [20, 23]: 0 = normal bone structure without any sign of deterioration; 1 = small radiolucent lesions in the proximal epiphysis (<3); 2 = increased number of radiolucent lesions (>3) indicating loss of medullary bone; 3 = loss of medullary bone, plus erosion of the cortical bone; 4 = full-thickness unicortical bone loss; and 5 = full-thickness bicortical bone loss and displaced fracture. The experimenter was blinded to the treatment of the samples.

2.5. Behavioral Test. Thermal hyperalgesia was indicated by a significantly shortened latency of foot withdrawal in response to heat stimulation. To determine thermal hyperalgesia, a radiant heat source was focused and delivered on a portion of the hind paw; the thermal stimuli shut off automatically when hind paw moved (or after 20 s to prevent tissue damage). Thermal stimuli were delivered 3 times to each hind paw at 5–8-minute intervals. Mechanical allodynia was indicated by a significant decrease in the threshold of paw withdrawal to mechanical indentation of the plantar surface of each hind paw, with a sharp, cylindrical probe. The probe was applied to 6 designated loci distributed over the plantar surface of the foot. The minimal force that induced paw withdrawal was read off the display [19, 24]. The experimenters who performed these behavioral tests were blinded to the treatment condition of the animals.

2.6. mRNA Isolation and RT-PCR. The total RNA isolated with TRIzol Reagent (Invitrogen, USA) was reverse transcribed using M-MLV reverse transcriptase (Takara). Primer sets were synthesized by Integrated DNA Technologies (Sangon Biotech); their sequences are shown in Table 1. The amplification conditions were set as follows: 94°C for 2 min and 30 cycles of 94°C for 30 s and 58°C for 40 s. PCR products were analyzed on agarose gel electrophoresis and were verified by DNA sequencing. The gels were imaged with Tanon 2500 Imaging Systems and analyzed by analysis software ImageJ 1.48u.

2.7. Measurement of cAMP, IL-1β, and TNF-α Levels and PKA Activity. The DRG and the spinal cord at segments of L_4-L_5 ipsilateral to TCI were collected on postoperative days 10 and 14 for further neurochemical analysis. Commercial enzyme-linked immunosorbent assay kits were used to determine the concentrations of cAMP, IL-1β, and TNF-α and activity of PKA, according to the manufacturers' instructions. ELISA kit for cAMP was purchased from Cayman Chemical (Ann Arbor, Michigan, USA); ELISA kits for IL-1β, TNF-α, and activated PKA were purchased from R&D Systems (Minneapolis, Minnesota, USA).

Table 1: Primer sequences of the genes studies for RT-PCR.

Primer name	Forward	Reverse
PKA-RII	5-ACCTCAGACGGCTCCCTTTG-3	5-CGTCTCCAACCGCATAAGCAG-3
PKA-C	5-ACCTTGGGAACGGGTTCCTTCG-3	5-TACACCCAATGCCCACCAGTCC-3
β-actin	5-TCTACAATGAGCTGCGTGTG-3	5-AATGTCACGCACGATTTCCC-3

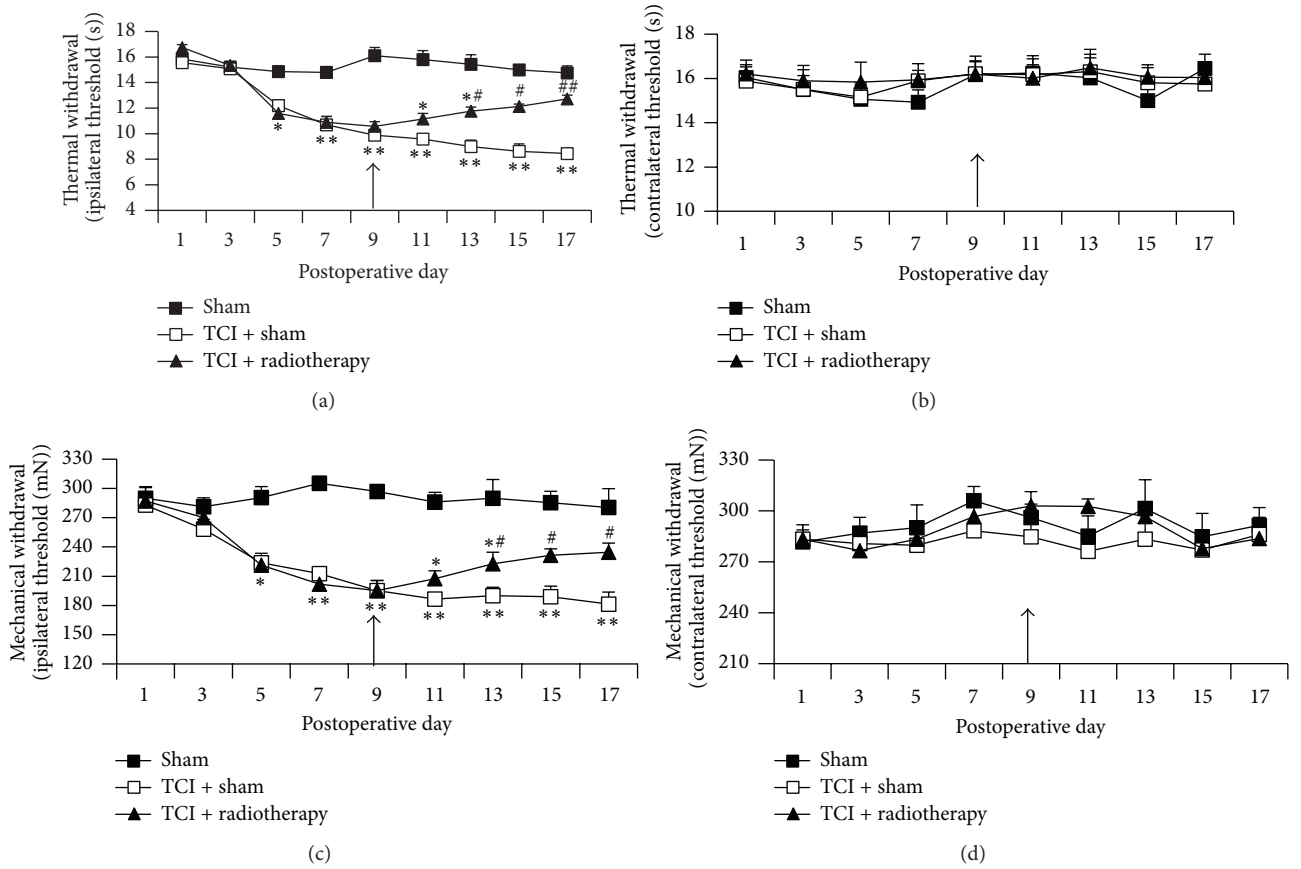

Figure 1: Radiotherapy relieved thermal hyperalgesia and mechanical allodynia in TCI rats. Thermal hyperalgesia of the hind paw ipsilateral (a) and contralateral (b) to TCI. Mechanical allodynia of the hind paw ipsilateral (c) and contralateral (d) to TCI. Arrows indicate administration of X-radiation (6 Gy) on the 9th day after surgery. Eight rats were included in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus TCI + sham radiotherapy.

2.8. Statistical Analysis. All statistical analyses were carried out using Statistical Product and Service Solutions, ver. 15.0 (SPSS Inc., Chicago, Illinois, USA). Alterations in cAMP mRNA and concentration, PKA activity, and the levels of IL-1β and TNF-α were tested using one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests. Two-way repeated-measures ANOVA (days × groups) was used to test the behavioral responses to thermal and mechanical stimuli, followed by Bonferroni *post hoc* tests. The nonparametric statistical method was used for analyzing the bone radiographs. All data were presented as means ± SEM. Statistical results were deemed significant if P value was less than 0.05.

3. Results

3.1. Radiotherapy Attenuated TCI-Induced Thermal Hyperalgesia and Mechanical Allodynia. TCI-treated rats exhibited significant thermal hyperalgesia and mechanical allodynia during postoperative days 5–17. The thresholds of thermal and mechanical withdrawal ipsilateral, but not contralateral, to the TCI treatment decreased approximately by 50% during postoperative days 9–17 compared to sham control. To determine whether radiotherapy treatment affects bone cancer pain, a single dose of X-radiation (6 Gy) was applied on the 9th day after operation. Such radiotherapy produced long-lasting inhibition of TCI-induced thermal hyperalgesia and mechanical allodynia. The inhibition lasted for a week with 1 d delay following the X-radiation application. The peak values of inhibition of the thermal hyperalgesia and mechanical allodynia were approximately 25–50%. The thermal and mechanical withdrawal of the hind paw contralateral to TCI treatment was not altered following the radiotherapy. Data are summarized in Figure 1.

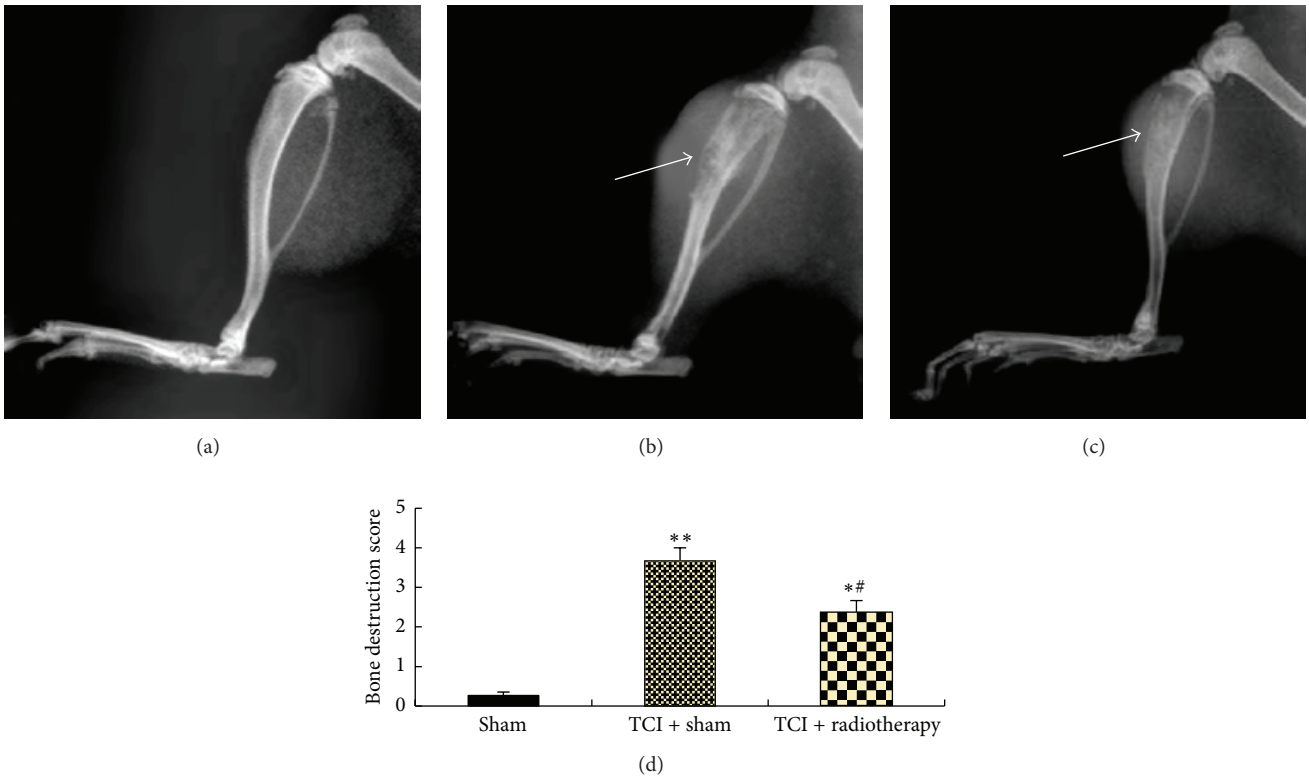

Figure 2: Radiotherapy alleviated TCI-induced tibia bone destruction. Radiographic images were taken on the 17th day after TCI. (a, b, c) Representative bone destruction and tumor growth in groups of sham (a), TCI + sham radiotherapy (b), and TCI + radiotherapy (c). Data are summarized in (d). Eight rats were included in each group. Arrows in (a–c) point to the areas of the bone destruction. $^{**}P < 0.01$ versus sham; $^{\#}P < 0.05$ versus TCI + sham; $^{*}P < 0.05$ versus sham.

3.2. Radiotherapy Alleviated TCI-Induced Bone Destruction. Bone structure integrity was evaluated by radiographic observation on the 17th day after TCI. No radiographic change (score = 0, no bone destruction) was found in the group of sham without radiotherapy. Bone destruction was seen clearly in groups of TCI with and without radiotherapy, respectively. Score of the TCI group with sham therapy was 3.75 ± 0.43 (range: 3–5). Radiotherapy greatly reduced TCI-induced tibia bone destruction. The score in the group of TCI with radiotherapy dropped to 2.38 ± 0.52 (range: l–3), which was significantly less than the score in TCI group with sham therapy. Radiological changes of the tibia are shown in Figure 2.

3.3. Radiotherapy Inhibited TCI-Induced PKA mRNA Expression in DRG. Our previous studies have shown that expression of PKA-RII and PKA-C mRNAs was increased after TCI treatment in a time-dependent manner [19]. To test whether radiotherapy has an effect on this TCI-induced PKA mRNA expression, we measured the levels of PKA-RII and PKA-C mRNAs on day 1 and day 5 after radiotherapy treatment (postoperative days 10 and 14, resp.) using RT-PCR. We found that radiotherapy treatment greatly inhibited TCI-induced increase of expression of PKA-RII and PKA-C mRNA. The expression of PKA-RII and PKA-C mRNA in the TCI + radiotherapy group was significantly reduced compared with the TCI + sham group (Figure 3).

3.4. Radiotherapy Reduced TCI-Induced Increase of cAMP Level and PKA Activity in DRG and the Spinal Cord. We have previously shown that cAMP concentration and PKG activity in DRG and the spinal cord were significantly increased in a time-dependent manner after TCI treatment [19]. To test the hypothesis that cAMP-PKA signaling pathway might be altered by radiotherapy, we measured cAMP level and PKA activity in DRG and the spinal cord following radiotherapy treatment. The results showed that radiotherapy treatment significantly reduced TCI-induced increase of cAMP concentration as well as PKA activity in DRG and the spinal cord (Figure 4).

3.5. Radiotherapy Reduced TCI-Induced Activity of IL-1β and TNF-α in the Spinal Cord. We have recently shown that IL-1β and TNF-α levels in the spinal cord were significantly increased in a time-dependent manner after TCI treatment [25]. To test whether radiotherapy could affect TCI-induced increase of IL-1β and TNF-α, we measured levels of IL-1β and TNF-α in the spinal cord after radiotherapy on postoperative days 10 and 14 (1 and 5 days after radiotherapy). Our results showed that radiotherapy treatment significantly reduced

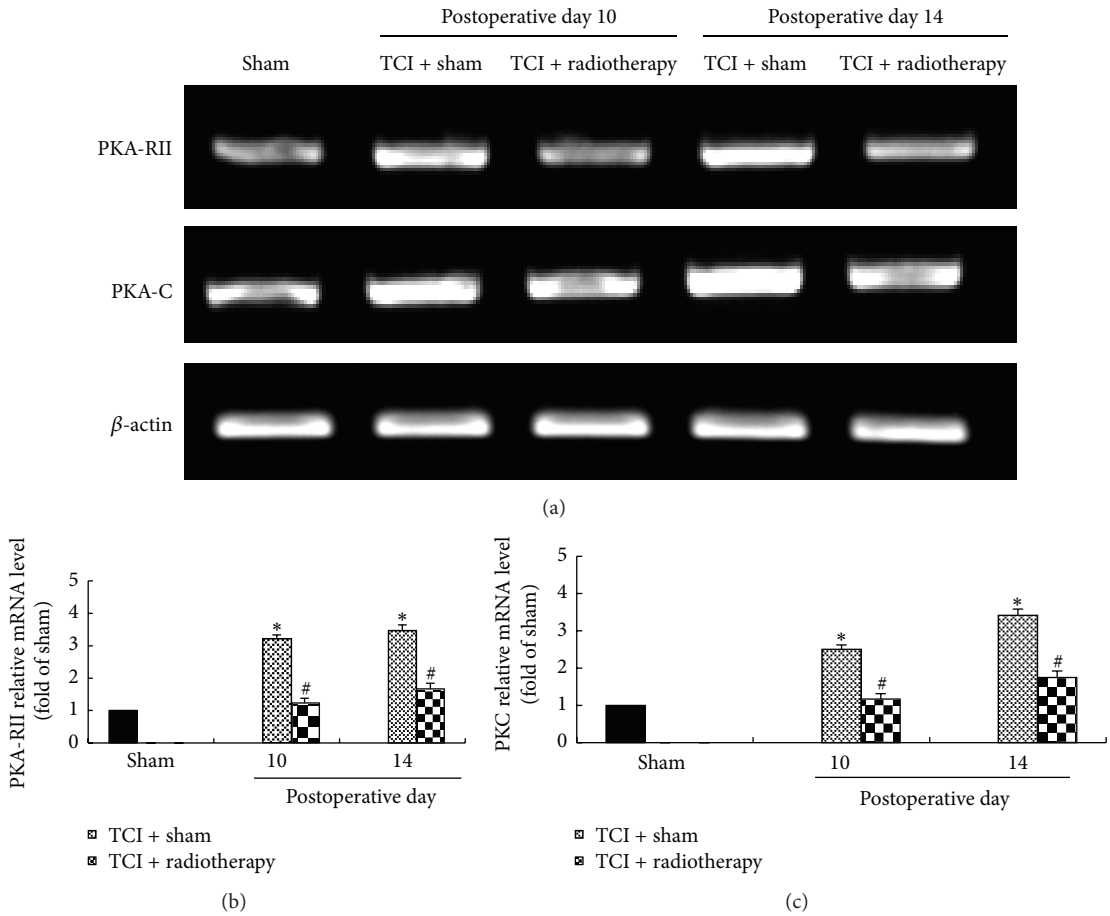

Figure 3: Levels of PKA-RII and PKA-C mRNA in DRG. (a) Representative bands showing levels of PKA-RII and PKA-C mRNA analyzed by RT-PCR. (b and c) Data quantification. Four samples were used for each group with two ganglia in each sample. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus TCI + sham.

TCI-induced increase of IL-1β and TNF-α in the spinal cord (Figure 5).

4. Discussion

This study demonstrates that radiotherapy is an effective treatment approach for treating bone cancer pain. The cAMP-PKA signaling pathway may be a mechanism that underlies the analgesic effect of radiotherapy in bone cancer pain. Radiotherapy suppresses TCI-induced painful behaviors, thermal hyperalgesia, and mechanical allodynia and alleviates TCI-induced massive bone destruction. Radiotherapy reduces TCI-induced increased expression of PKA mRNAs in DRG as well as the increased level of cAMP concentration and PKA activity in both DRG and the spinal cord. In addition, radiotherapy results in a significant decrease of IL-1β and TNF-α activity in the spinal cord. These findings suggest that radiotherapy treatment may suppress TCI-induced hyperalgesia and allodynia by inhibiting the cAMP-PKA signaling pathway in DRG and the spinal cord.

Pain is one of the most prominent symptoms in clinically advanced cancer patients. Approximately 50% of these patients experience moderate to severe pain [1]. Mechanisms of cancer pain are thought to be complex and may involve a combination of inflammation, nerve injury, and other unique factors [26]. Considerable evidence of cancer pain treatment shows that radiotherapy effectively relieves pain in up to 95% of patients and can maintain the level of analgesia in more than 70% of the patients for up to three months [27–29]. Previous studies have shown that activation of the cAMP-PKA signaling pathway contributes to noxious stimulus-induced peripheral and central sensitization [30–32]. We have found that *in vivo* chronic compression of DRG or *in vitro* acute DRG dissociation resulted in activation of the cAMP-PKA signaling pathway. Continued activation of the cAMP-PKA signaling pathway is required to maintain hyperexcitability of the DRG neurons and behaviorally expressed hyperalgesia in these two different injury-related stress conditions [15, 17]. Recently, we have confirmed that activation of the cAMP-PKA pathway plays an essential role in the induction and maintenance of bone cancer pain [19, 25]. Here, we provide direct evidence that the elevated activity of cAMP-PKA signaling pathway is significantly decreased by radiotherapy in a bone cancer pain model. These results support an idea that radiotherapy may suppress bone cancer pain through inhibition of abnormal activation of cAMP-PKA signaling

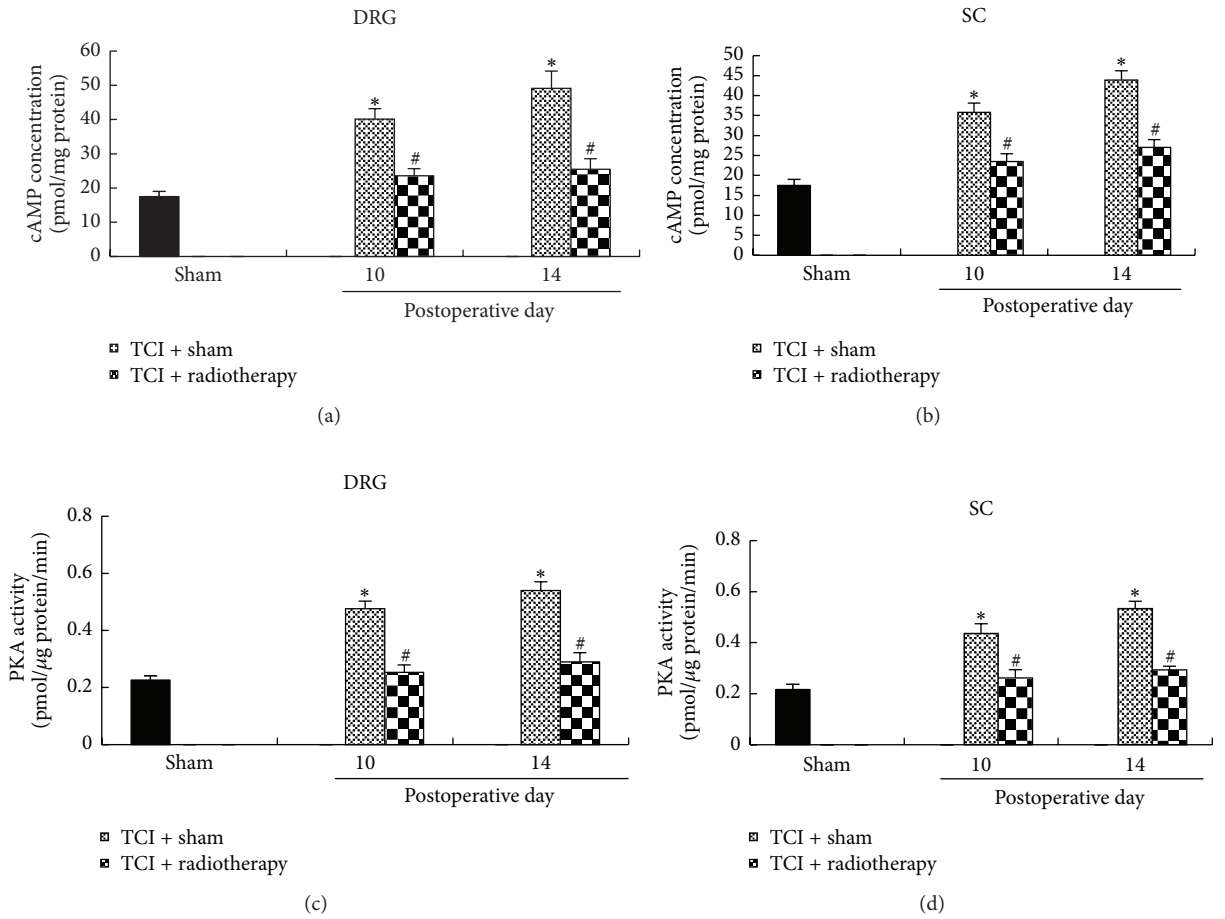

Figure 4: Alterations of cAMP concentration and PKA activity in DRG and the spinal cord after TCI with or without radiotherapy. (a and b) cAMP concentration. (c and d) PKA activity. The tissues were collected on postoperative days 10 and 14, that is, 1 and 5 days after radiotherapy, respectively. Each group included four samples with two ganglia in each sample. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus TCI + sham.

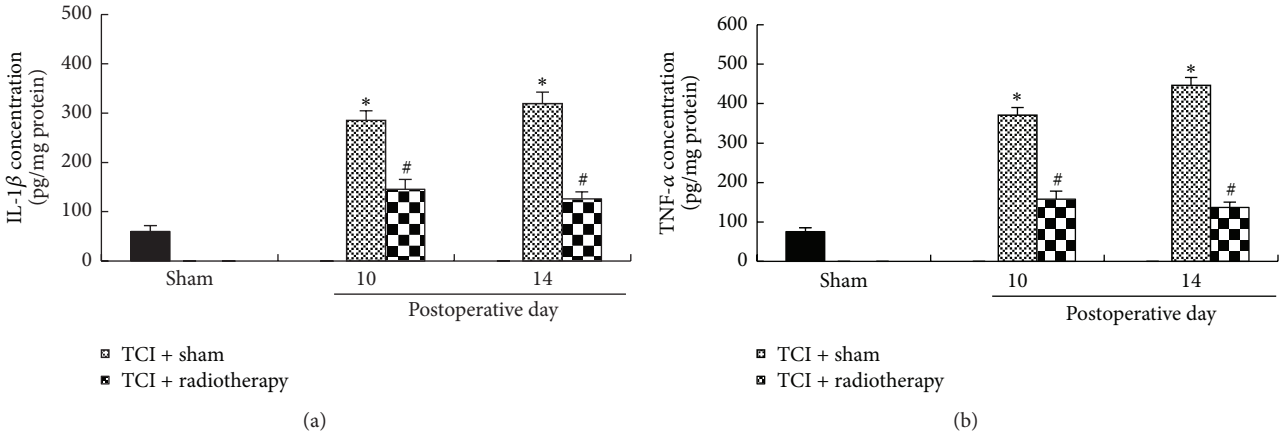

Figure 5: Radiotherapy reduced TCI-induced increase of IL-1β and TNF-α in the spinal cord. IL-1β and TNF-α concentrations were analyzed by ELISA. The tissues were collected on postoperative days 10 and 14, that is, 1 and 5 days after radiotherapy, respectively. Four samples were included in each group and each sample included a segment of the spinal cord at L_4-L_5. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus TCI + sham.

pathway, suggesting a new mechanism for the radiotherapy of bone cancer pain.

The proinflammatory cytokines are activators of the cAMP-PKA pathway in primary afferent neurons [25, 33–35]. During tumor growth and development of bone cancer pain, certain proinflammatory cytokines such as TNF-α and IL-1β are activated and released from the astrocytes and microglial cells and contribute to bone cancer pain [25, 36, 37]. Radiotherapy can reduce these proinflammatory cytokines and inhibit activation of the cAMP-PKA signaling pathway and thus result in relief of bone cancer pain. These findings suggest that the cytokines are also targets that may be responsible for radiotherapy-induced analgesia. In addition, our results showed that radiotherapy resulted in less bone loss in TCI rats. This is consistent with the previous finding that radiotherapy with high dose may kill part of the tumor cells or reduce their activation and thus delay the destruction of the bone structure [38, 39]. These findings indicate that radiotherapy may reduce both pain and loss of bone structure following TCI treatment.

5. Conclusions

Our results demonstrate that radiotherapy can effectively suppress bone cancer pain probably through inhibition of activation of cAMP-PKA signaling pathway in the primary sensory neurons and the spinal cord. This study may suggest a new mechanism underlying radiotherapy-induced analgesia of bone cancer pain and support the clinical use of radiotherapy in treatment of certain cancer pain conditions.

Disclosure

This study should be attributed equally to the institutions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Guiqin Zhu and Yanbin Dong contributed equally to this study.

Acknowledgments

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Research Article

Levo-Tetrahydropalmatine Attenuates Bone Cancer Pain by Inhibiting Microglial Cells Activation

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Objective. The present study is to investigate the analgesic roles of L-THP in rats with bone cancer pain caused by tumor cell implantation (TCI). *Methods*. Thermal hyperalgesia and mechanical allodynia were measured at different time points before and after operation. L-THP (20, 40, and 60 mg/kg) were administrated intragastrically at early phase of postoperation (before pain appearance) and later phase of postoperation (after pain appearance), respectively. The concentrations of TNF-α, IL-1β, and IL-18 in spinal cord were measured by enzyme-linked immunosorbent assay. Western blot was used to test the activation of astrocytes and microglial cells in spinal cord after TCI treatment. *Results*. TCI treatment induced significant thermal hyperalgesia and mechanical allodynia. Administration of L-THP at high doses significantly prevented and/or reversed bone cancer-related pain behaviors. Besides, TCI-induced activation of microglial cells and the increased levels of TNF-α and IL-18 were inhibited by L-THP administration. However, L-THP failed to affect TCI-induced astrocytes activation and IL-1β increase. *Conclusion*. This study suggests the possible clinical utility of L-THP in the treatment of bone cancer pain. The analgesic effects of L-THP on bone cancer pain maybe underlying the inhibition of microglial cells activation and proinflammatory cytokines increase.

1. Introduction

Treatment of bone cancer pain continues to be a major clinical challenge. Over 60% of patients with primary or metastatic bone cancer suffer from moderate or severe pain [1]. New therapeutic strategies for bone cancer pain are urgently needed. Studies have demonstrated that the mechanism of bone cancer pain is of unique characteristics and involve a combination of inflammatory and neuropathic pain [2, 3]. Inflammation at the tumor site and products released from cancer cells and immune cells have been suggested to be the primary trigger of bone cancer pain. In our previous study, we found obvious activation of astrocytes and microglial cells in spinal cord of rats during bone cancer pain [4, 5]. Astrocytes and microglial cells, which act as parts of the innate immune system, could release various substances, including tumor necrosis factor α (TNF-α), IL-1β, and IL-18 which could evoke hyperalgesia and allodynia [6–8].

Levo-tetrahydropalmatine (L-THP), a tetrahydroprotoberberine isoquinoline alkaloid, is a primary active constituent from the genera *Stephania* and *Corydalis*. Studies have shown that L-THP has excellent analgesic effects and has been used clinically in China for more than 40 years as an analgesic with sedative/hypnotic properties [9–11]. Although L-THP was most well known as a traditional analgesic agent, the mechanism of the antinociceptive action of L-THP remains unclear. Whether this traditional analgesic could attenuate cancer pain is still unknown. In our present, we assessed the effects of L-THP on bone cancer pain and its possible mechanisms.

2. Materials and Methods

2.1. Animals, Anesthesia, Drugs, and Administration. All animals were used in accordance with the regulations of the ethics committee of the International Association for the Study of Pain and all protocols were approved by the Institutional Animal Care and Use Committees. Adult, female, Sprague-Dawley rats (160–180 g) were used in this study. Rats were maintained in a controlled lighting environment, with free access to food and water.

All surgery was performed under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally). L-THP (optical purity ≥ 99.5%) was obtained from Nanning Pharmaceuticals (Guangxi, China). L-THP (20, 40, or 60 mg/kg, dissolved in saline with 0.5% DMSO, 2 mL/rat) was administered intragastrically. The doses of L-THP were chosen on the basis of previous studies [12]. Behavioural and neurochemical testing was performed 2 h after L-THP or vehicle administration.

2.2. Model of Bone Cancer Pain. According to previous studies, tumor cells were extracted from ascetic fluid of rats that received Walker 256 rat mammary gland carcinoma cells. Tumor cell implantation (TCI) was mimicked by injecting the cells ($1 * 10^5$ cells/μL, 5 μL) into the intramedullary space of the right tibia to induced bone cancer in rats [13, 14].

2.3. Behavioral Test. Thermal hyperalgesia was determined by significant shortened latency of foot withdrawal in response to heat stimulation. In brief, the heat source was focused on a portion of the hind paw, and a radiant thermal stimulus was delivered to that site. The stimulus shut off automatically when hind paw moved (or after 20 s to prevent tissue damage). Thermal stimuli were delivered 3 times to each hind paw at 5- to 8-minute intervals.

Mechanical allodynia was assessed by measuring incidence of foot withdrawal to mechanical indentation of the plantar surface of each hind paw with a sharp, cylindrical probe with a uniform tip diameter of approximately 0.2 mm provided by an electronic von Frey. The probe was applied to six designated loci distributed over the plantar surface of the foot. The minima force (in grams) that induced paw withdrawal was read off the display. Threshold of mechanical withdrawal in each animal was calculated by averaging the six readings and the force was converted into millinewtons (mN).

2.4. Western Blot. The L4-L5 spinal cord was quickly removed from deeply anesthetized rats and stored at −80°C. Sequential precipitation procedures were used on the tissue samples that were lysed in ice-cold Np-40 or RIPA lysis buffer containing a cocktail of protease inhibitor, phosphatase inhibitors, and phenylmethylsulfonyl fluoride (Sigma-Aldrich). The homogenates were incubated for 20–30 minutes in ice-cold water, vortexed for 10 seconds on the highest setting every 5 minutes, and then centrifuged at 13,000 g for 10 minutes. The supernatants were collected and the protein concentration in supernatants was estimated using the bicinchoninic acid assay.

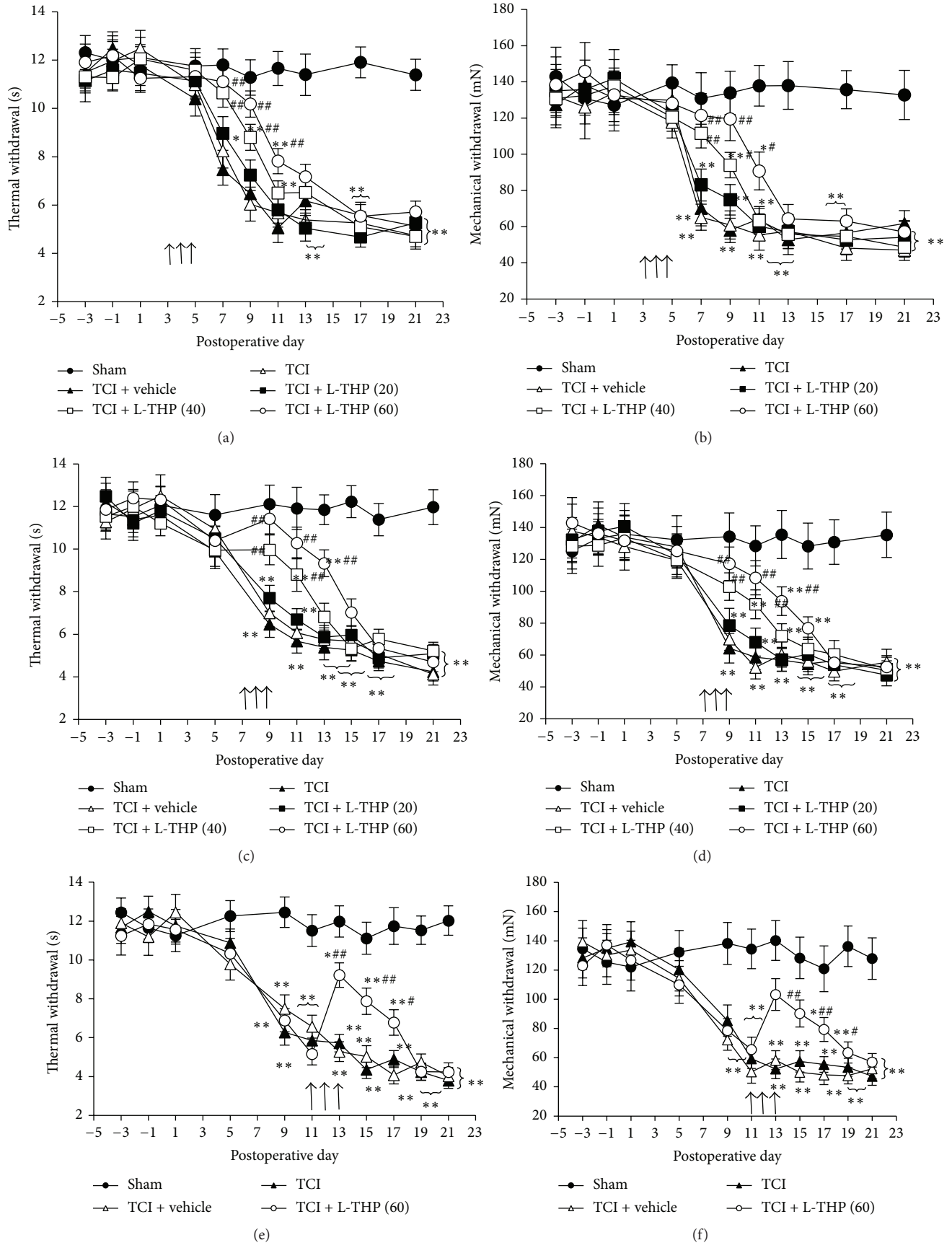
The total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to 0.2-μm nitrocellulose or polyvinylidene fluoride membrane (both from Bio-Rad Laboratories, Hercules, CA, USA). The following primary antibodies were used: anti-GFAP (1 : 500), anti-Iba-1 (1 : 1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), s-100β (1 : 800) (Abcam, Cambridge, UK), and anti-GAPDH (1 : 10000) (Sigma-Aldrich, USA). After washing with Tris-buffered saline with Tween 20 (TBST) for 3 × 5 minutes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 5000) (R&D Systems, Minneapolis, MN) for 2 hours at room temperature and then washed with TBST for 3 × 5 minutes. The membranes were then developed by enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA). Data were analysed with the Molecular Imager (ChemiDoc XRS, Bio-Rad Laboratories) and the associated software Quantity One-4.6.5 (Bio-Rad Laboratories).

2.5. Levels of TNF-α, IL-1β, and IL-18 Determination. The whole spinal cord at the L4-L5 segments was rapidly removed from deeply anesthetized rats ($n = 8$ each group). The tissues were homogenized in ice-cold 100 mM PBS. Protein concentrations were determined by the bicinchoninic acid assay. The levels of TNF-α, IL-1β, and IL-18 were measured using an enzyme-linked immunospecific assay (ELISA) (TNF-α kit: RTA00 and IL-1β kit: RTB00) (R&D Systems) (IL-18 kit: KRC2341; Thermo Fisher Scientific) according to the manufacturer's instructions [14, 15].

2.6. Statistical Analysis. SPSS version 15 (SPSS Inc., Chicago, IL) was used to conduct all the statistical analyses. The significance of differences in the latency of thermal withdrawal and the threshold of mechanical withdrawal over time were tested with two-way repeated measures analysis of variance (RM ANOVA) followed by Bonferroni's post hoc test. Alterations of the concentrations of cytokines and the expression of the proteins detected among groups were tested with one-way ANOVA with repeated measure followed by Bonferroni's post hoc test. All data are presented as mean ± SEM. Statistical results are considered significant if $P < 0.05$.

3. Results

3.1. Dose-Dependent Inhibitory Effects of L-THP on TCI-Induced Hyperalgesia and Allodynia. Consistent with previous studies [13, 14], rats that received TCI exhibited significant thermal hyperalgesia and mechanical allodynia beginning on postoperative day 7 (Figure 1). High doses of L-THP significantly reduced the production and persistence of these pain-related behaviors in all the TCI rats tested. Repetitive administration of L-THP at 40 and 60 mg/kg at postoperative days 3, 4, and 5, respectively, produced significant delay of the induction of TCI-induced hyperalgesia and allodynia (Figures 1(a) and 1(b)). The induction of pain behaviors started at postoperative days 9–11 after L-THP administration.



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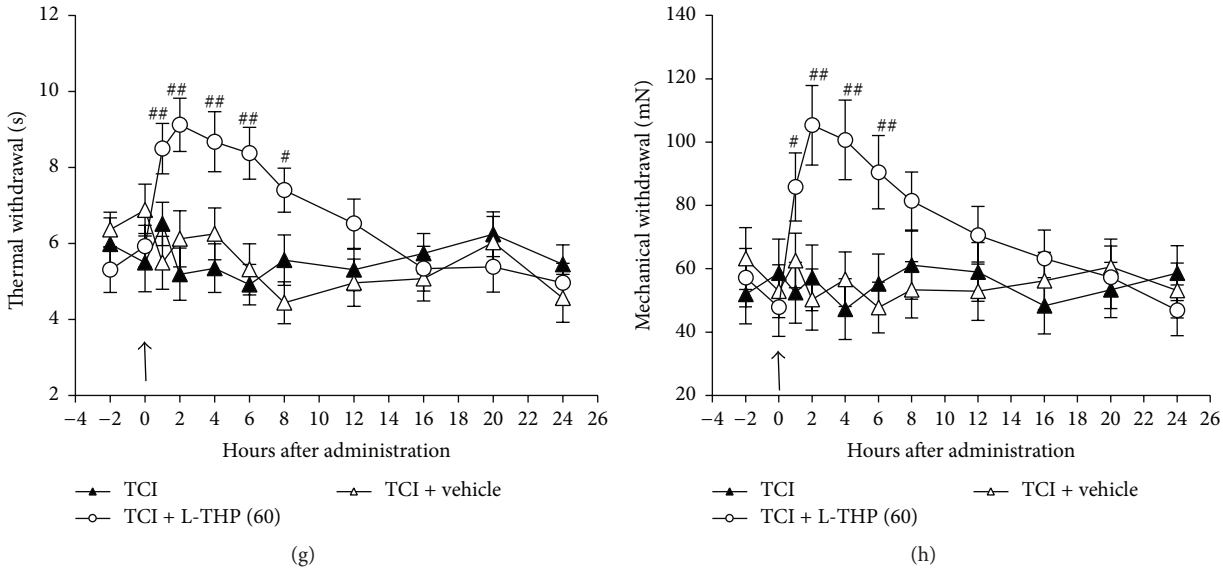

Figure 1: Dose-dependent inhibitory effects of L-THP on TCI-induced thermal hyperalgesia and mechanical allodynia. Thermal hyperalgesia (a, c, and e) and mechanical allodynia (b, d, and f) are shown in the hind paw ipsilateral to TCI. L-THP at different doses (20, 40, and 60 mg/kg, dissolved in saline with 0.5% DMSO, 2 mL/rat) were administered intragastrically on postoperative days 3, 4, and 5 (a and b) or 7, 8, and 9 (c and d), or 11, 12, and 13 (e and f) indicated by arrows, respectively. Single administration of L-THP (60 mg/kg) was applied at postoperative day 11 (g and h). Arrows represent the time points of L-THP administration. Eight rats were included in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences compared with sham group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ indicate significant differences compared with TCI group.

The same doses of L-THP administered at postoperative days 7, 8, and 9 produced a significant, transient attenuation of thermal hyperalgesia and mechanical allodynia (Figures 1(c) and 1(d)). Compared to the pretreatment level, hyperalgesia and allodynia were reduced by 30%–60% and the analgesic effect of L-THP lasted for 2–4 days, depending on the dosage. However, L-THP at 20 mg/kg or vehicle did not affect the hyperalgesia and allodynia induced by TCI ($P > 0.05$). Similarly, L-THP (60 mg/kg) administrated at postoperative days 11, 12, and 13 also produced a significant and transient (3~4 days) reversion of hyperalgesia and allodynia (Figures 1(e) and 1(f)). A single administration of L-THP (60 mg/kg) at postoperative day 11 could also induce a significant analgesic effect (Figures 1(g) and 1(h)). Such analgesic effect appeared from 1 h and peaked at 2 h after L-THP administration, and the analgesic effect of single L-THP (60 mg/kg) administration lasted for 6~8 hours (Figures 1(g) and 1(h)).

3.2. Long-Term Inhibitory Effects of L-THP on TCI-Induced Pain Behaviors. We further investigated long-term effects of repetitive application of L-THP on TCI-induced hyperalgesia and allodynia. Because, in previous study, we found that L-THP at 60 mg/kg showed the most obvious analgesic effect, here we only test the role of L-THP at 60 mg/kg on TCI-induced pain behaviors. As shown in Figure 2, L-THP at 60 mg/kg intragastrically administrated for 7 days, from postoperative days 7 to 19, once every other day, significantly reduced the severity of hyperalgesia (Figure 2(a)) and allodynia (Figure 2(b)) by 50% ~80%. The analgesic effects lasted for an additional 8~10 days after termination of the L-THP application (Figures 2(a) and 2(b)).

3.3. L-THP Administration Suppressed TCI-Induced Increased Levels of TNF-α and IL-18, but Not IL-1β. As we found previously [8, 14], TCI treatment significantly increased the levels of TNF-α, IL-1β, and IL-18 in spinal cord, exhibiting a progressive increase with time during the period from 5 to 21 days after TCI treatment (Figure 3(a)). The increase in TNF-α and IL-18 was significantly inhibited by L-THP administration. Repeated administration of L-THP (60 mg/kg) at early phase (postoperative days 3, 4, and 5) and late phase (postoperative days 7, 8, and 9) significantly suppressed the TCI-induced increase of TNF-α and IL-18 by approximately 60%–70% (Figures 3(b) and 3(c)). However, L-THP administration showed no effect on TCI-induced IL-1β increase (Figure 3(d)). The levels of IL-1β concentration at different time points in L-THP group expressed no differences compared with those in vehicle group ($P > 0.05$).

3.4. L-THP Administration Inhibited TCI-Induced Activation of Microglial Cells, but Not Astrocytes. Given that the increased levels of proinflammatory cytokines are the result of glial cells activation in spinal cord, we examined the role of L-THP in glial cells activation. During bone cancer pain, astrocytes and microglial cells in spinal cord are significantly activated (Figures 4(a) and 4(b)). The expressions of GFAP (specific protein of astrocyte) and Iba-1 (specific protein of microglial cell) significantly increased after TCI treatment

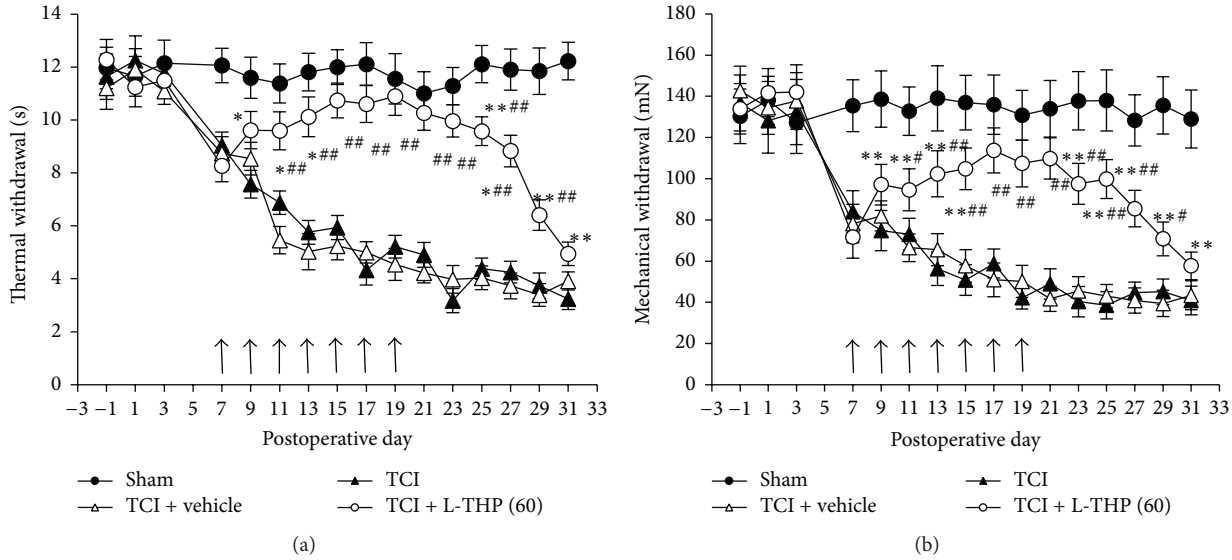

Figure 2: Long-term inhibitory effects of repetitive administrations of L-THP on TCI-induced pain behaviors. L-THP 60 mg/kg was administered intragastrically from postoperative day 7 and then once every other day for repetitive 7 days till postoperative day 19. Thermal hyperalgesia (a) and mechanical allodynia (b) are shown in the hind paw ipsilateral to TCI. Administration is indicated by arrows. Eight rats were tested in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences compared with sham group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ indicate significant differences compared with TCI group.

(Figures 4(a) and 4(b)). Astrocyte began activation on postoperative day 7 and till day 21 (the last test day), and the activation of microglial cells started from postoperative day 5 and peaked at days 14–21. The expression of GFAP at postoperative day 6 after TCI treatment showed no obvious change compared with sham group (Figures 4(c) and 4(e)). L-THP (60 mg/kg) administration at early phase and late phase significantly inhibited TCI-induced activation of microglial cells (Figures 4(c)–4(f)). However, interestingly, L-THP administration had no effect on the activation of astrocyte induced by TCI (Figures 4(d) and 4(f)). To confirm this phenomenon, we used another specific marker of astrocyte, s-100β, the prominent expression of which is the characteristic of astrocyte activation [16, 17]. Same as GFAP, the expression of s-100β in spinal cord significantly increased after TCI treatment (Figure 4(a)). Administration of L-THP (60 mg/kg) at early phase or late phase showed no effect on the expression of s-100β (Figures 4(c)–4(f)), which suggested that L-THP have no effect on TCI-induced astrocyte activation in spinal cord.

4. Discussion

The present study investigated the analgesic effect of L-THP in rats for bone cancer pain. Our results show that systematic application of high doses of L-THP transiently and dose-dependently delayed and reversed TCI-induced thermal hyperalgesia and mechanical allodynia. The analgesic effects of L-THP were mediated by inhibition of microglial cells activation, as well as TNF-α and IL-18 increase. To our knowledge, this study demonstrates, for the first time, that L-THP can significantly alleviate TCI-induced hyperalgesia and allodynia and provide new experimental evidence that support the utility of L-THP in treatment of chronic pain and expanding the use of L-THP in clinical treatment.

The analgesic and antinociceptive effects of L-THP have been proved by clinical and experimental evidences [10, 18–22]. L-THP has been found to alleviate headache, chest pain, hypochondriac pain, and abdominal pain in human [18, 19] and inflammatory and neuropathic pain in experimental animals [12, 20–22]. However, the mechanism underlying L-THP analgesic effect remains poorly understood. It has been shown that the analgesic action of L-THP is mediated by blocking D2 dopamine receptors in striatum and the arcuate nucleus of the hypothalamus, leading to activation of the descending antinociceptive system from the midbrain periaqueductal gray to the spinal dorsal horn and suppression of nociceptive signaling transduction [10, 23, 24]. However, there was less evidence for the role of L-THP in spinal cord, the primary central system of nociception.

As we mentioned above, bone cancer pain is complex and may involve a combination of inflammatory and neuropathic pain [2, 3]. Considering several studies having demonstrated that L-THP could relieve inflammatory and neuropathic pain, we hypothesized that L-THP may be used to treat bone cancer pain. At the present study, by behavioral test, we found that oral administration of L-THP significantly inhibited and reversed TCI-induced pain-related behavioral in a dose-dependent manner. And this analgesic effect of L-THP was associated with inhibiting proinflammatory cytokines TNF-α and IL-18 release in spinal cord. The cytokines have been proved to be important factors contributing to central and peripheral sensitization [25] during chronic pain and are known to be upregulated after nerve injury. L-THP could

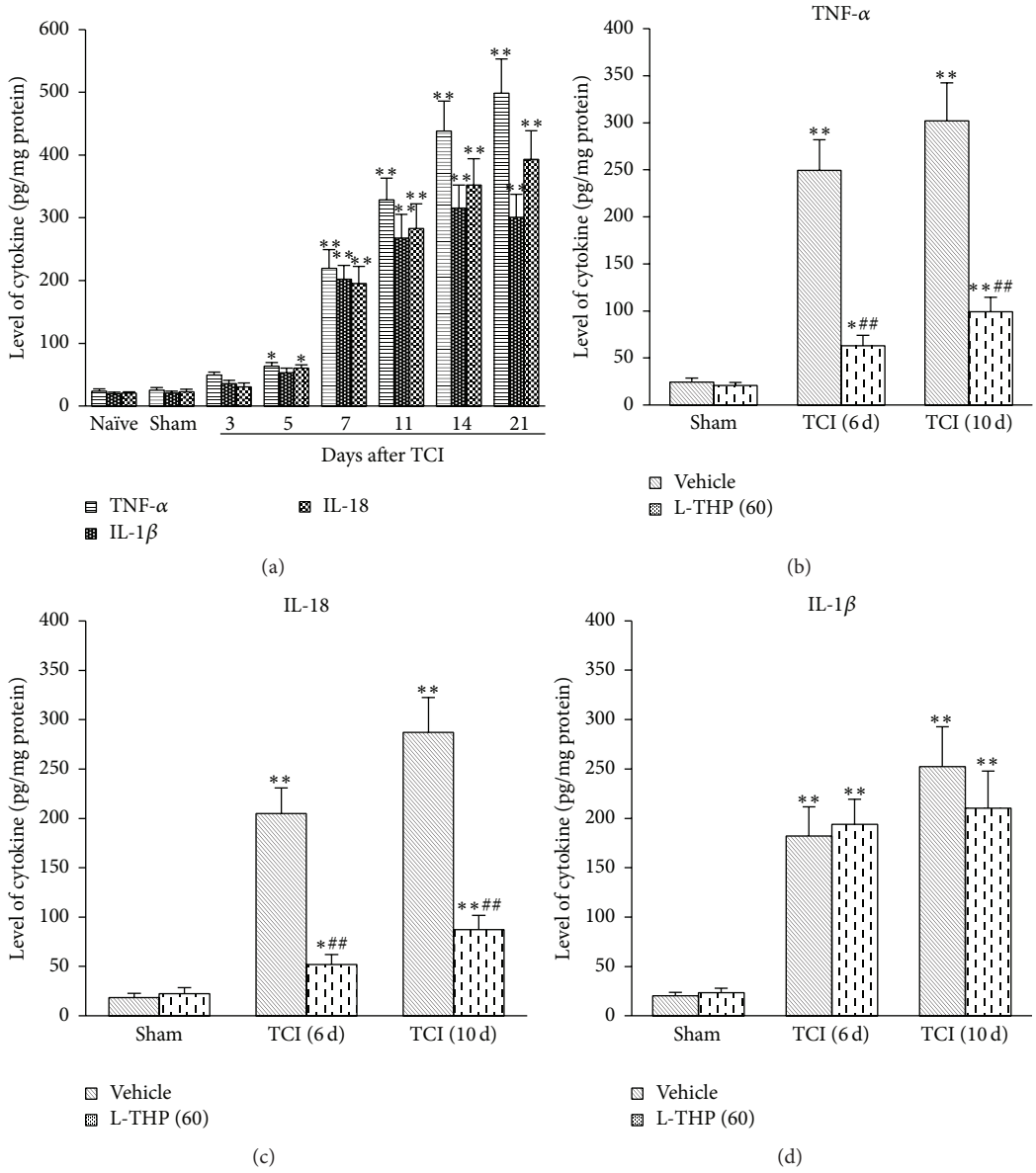

Figure 3: Effects of L-THP administration on the increased levels of TNF-α, IL-1β, and IL-18 in spinal cord after TCI treatment. (a) Time course of changes in TNF-α, IL-1β, and IL-18 in spinal cord after TCI treatment. (b and c) Repetitive administration of L-THP suppressed TCI-induced increased levels of TNF-α and IL-18. (d) Repetitive administration of L-THP showed no effect on TCI-induced increased levels of IL-1β. L-THP (60 mg/kg) was administrated on postoperative days 3, 4, and 5 (early phase) or postoperative days 7, 8, and 9 (late phase) after TCI. Tissues were collected one day after the last administration, that is, day 6 for the early phase treatment and day 10 for the late phase treatment. Eight samples were included in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences compared with naïve group (a) or sham + vehicle group (b–d). $^{\#\#}P < 0.01$ indicates significant differences compared with relevant TCI + vehicle group.

inhibit proinflammatory mediators [26]. It was reported that L-THP could inhibit TNF-α-induced monocyte-endothelial cell adhesion and NF-kappa B nuclear translocation [27]. During myocardial ischaemia-reperfusion injury, L-THP administration significantly decreased the accumulation of inflammatory factors, including TNF-α and MPO [28]. These reports indicate that L-THP may be involved in regulating proinflammatory cytokines. However, Beyond the consideration, at present study, L-THP administration showed no effect on IL-1β expression. The concentration of IL-1β in spinal cord after repetitive administration of L-THP remained at high level. In our pervious study, we found the same phenomenon [8]. Wnt/β-catenin signaling contributed to neuropathic pain by regulating TNF-α and IL-18, but not IL-1β. So is there any relationship between L-THP and Wnt signaling? What is the mechanism underlying L-THP regulating proinflammatory cytokines? Further studies are needed.

Given most proinflammatory cytokines in spinal cord coming from activated glial cells, we further investigated the role of l-THP on spinal glial cell activation. Our result

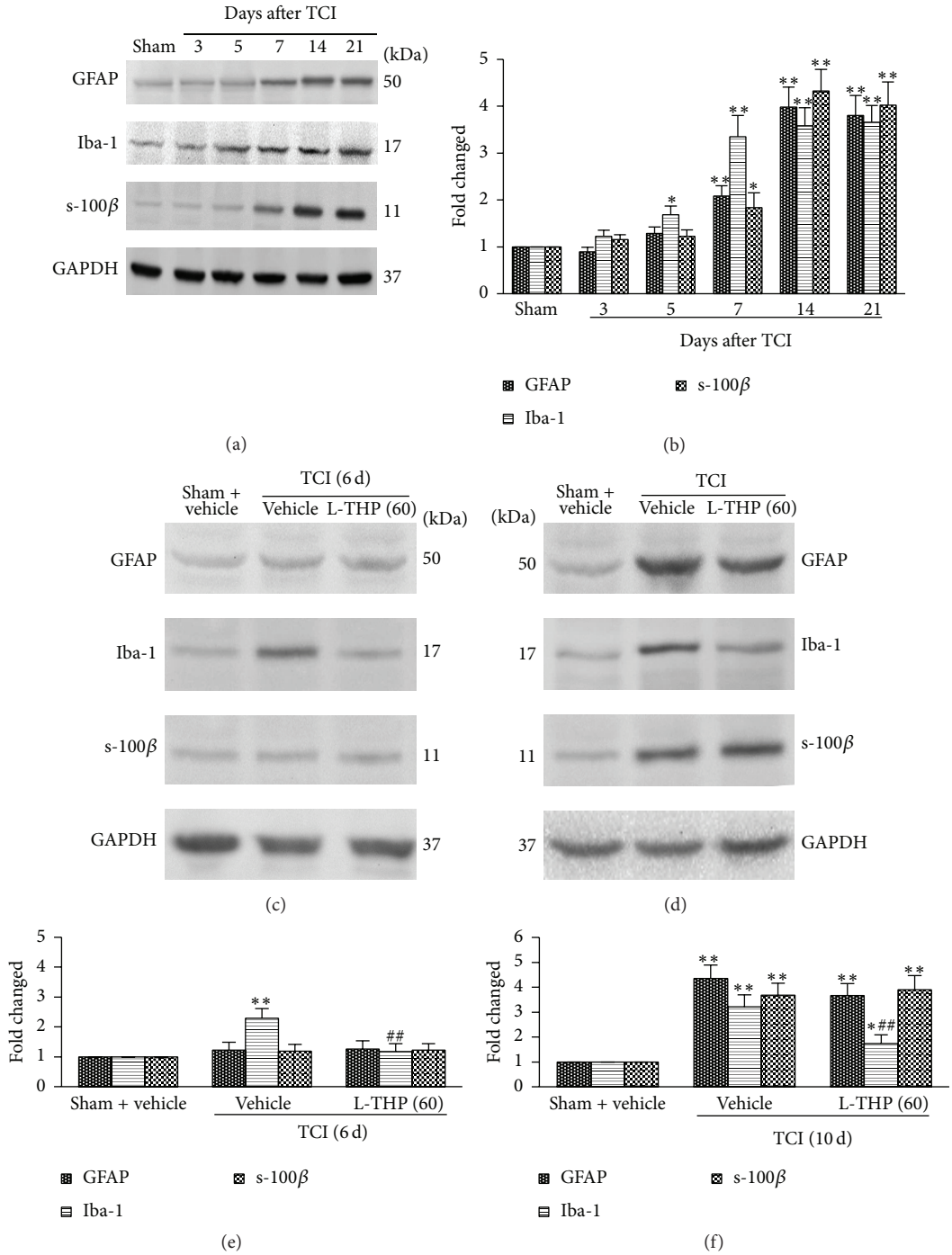

Figure 4: Intragastric administration of L-THP suppressed TCI-induced activation of microglia cells but had no effect on activation of astrocytes. (a and b) Time courses for astrocytes and microglial cells activation after TCI treatment. (c and e) Repetitive administration of L-THP at early phase (postoperative days 3, 4, and 5) significantly suppressed TCI-induced microglial cells activation. (d and f) Repetitive administration of L-THP at late phase (postoperative days 7, 8, and 9) suppressed TCI-induced microglial cells activation but had no effect on TCI-induced astrocytes activation. GFAP and s-100β are the specific protein markers for astrocyte. Iba-1 is the specific protein marker for microglial cell. Tissues were collected one day after the last administration of L-THP. Four samples were included in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences compared with sham group (b) or sham + vehicle group (e and f). $^{\#\#}P < 0.01$ indicates significant differences compared with relevant TCI + vehicle group.

showed that L-THP administration significantly suppressed TCI-induced microglial cells activation. However, L-THP showed no effect on TCI-induced astrocyte activation. To confirm the effect of L-THP on astrocyte, we chose another protein marker, s-100β, to represent the astrocyte activation. Consistent with GFAP, the expression of s-100β in spinal cord after TCI treatment significantly increased. However, L-THP administration also showed no effect on the upregulation of s-100β. This interesting finding suggested that the mechanisms of activation between astrocyte and microglial cell are different [29–31]. And this phenomenon maybe partially explained why L-THP administration showed no effect on IL-1β release. It was reported that the generation and release of IL-1β mainly occurred on astrocytes [32, 33]. According to the present study, L-THP had no effect on astrocyte activation, so it showed no effect on IL-1β expression and release.

In bone cancer pain models, there is a large variation in spinal microglial reaction. At present study, we found that microglial activation started at the early stage (from day 5 after TCI) and remained at a high level till day 21 after operation. These findings are consistent with previous studies [13, 14, 34]. However, recently, Yang et al. [35] reported an inconsistent finding that bone cancer elicited a delayed activation of microglial cells. They found that microglial marker Iba-1 did not upregulate until postoperative day 14, and microglia showed no effect on the induction of bone cancer pain. On the contrary, Wang et al. [36] found that intrathecal administration of minocycline (microglia inhibitor) at early stage (from day 4 to day 6) could significantly prevent cancer-induced bone pain, while at late stage (from day 10 to day 12) it showed no effect. These variations may be due to the differences in animal strains, sexes, and the origins of tumor cells. At present study, we chose Sprague-Dawley rats while Yang et al. used Wistar rats in their study. There may be another possible reason to explain the differences. In our studies the obvious bone destruction appeared from postoperative days 5 to 7, while in Yang et al.'s study [35], the evident bone destruction did not appear until postoperative day 14. With the growing, tumor cells contacted with, injured, and then destroyed the distal processes of sensory fibers that innervate the bone marrow and mineralized bone. Microglial cells are well known as the early responding cells of the CNS after injury. So tumor cell-induced bone destruction resulted in microglial cells activation during the early phase.

In summary, L-THP at high doses can effectively reduce TCI-induced thermal hyperalgesia and mechanical allodynia. Mechanisms underlying L-THP-induced attenuation of bone cancer pain may be partially through inhibiting microglial cells activation, as well as proinflammatory cytokines TNF-α and IL-18 releasing, in spinal cord. However, at present study, we could not exclude other possible mechanisms of L-THP for treating bone cancer pain, such as the sedative effect. The present study provides experimental evidence that demonstrates the analgesic roles of L-THP in bone cancer pain and suggests the clinical utility of L-THP in treatment of bone cancer pain.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Mao-yin Zhang and Yue-peng Liu contributed equally to this study.

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Research Article

Sigma-1 Receptor Antagonist BD1047 Reduces Mechanical Allodynia in a Rat Model of Bone Cancer Pain through the Inhibition of Spinal NR1 Phosphorylation and Microglia Activation

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Previous studies have demonstrated that sigma-1 receptor plays important roles in the induction phase of rodent neuropathic pain; however, whether it is involved in bone cancer pain (BCP) and the underlying mechanisms remain elusive. The aim of this study was to examine the potential role of the spinal sigma-1 receptor in the development of bone cancer pain. Walker 256 mammary gland carcinoma cells were implanted into the intramedullary space of the right tibia of Sprague-Dawley rats to induce ongoing bone cancer-related pain behaviors; our findings indicated that, on days 7, 10, 14, and 21 after operation, the expression of sigma-1 receptor in the spinal cord was higher in BCP rats compared to the sham rats. Furthermore, intrathecal injection of 120 nmol of sigma-1 receptor antagonist BD1047 on days 5, 6, and 7 after operation attenuated mechanical allodynia as well as the associated induction of c-Fos and activation of microglial cells, NR1, and the subsequent Ca^{2+}-dependent signals of BCP rats. These results suggest that sigma-1 receptor is involved in the development of bone cancer pain and that targeting sigma-1 receptor may be a new strategy for the treatment of bone cancer pain.

1. Backgrounds

Bone cancer pain (BCP), which is the most common complication when tumors metastasize to the bone, can cause depression, anxiety, and other complications in patients and even be highly debilitating to the patients' functional status and quality of life [1]. Due to the limitations of the existing treatment, many patients with bone cancer pain suffer limited pain relief and adverse side effects. Thus, understanding the potential cellular and molecular mechanisms underlying bone cancer pain is important for effectively treating these patients.

As a subtype of sigma receptor, the sigma-1 receptor is highly expressed in both neurons and glia of multiple regions within the central nervous system [2]. Extensive literature on molecular weight of sigma-1 receptor that is composed of 223 amino acids indicated a value ranging from 25 to 30 kDa. Sigma-1 receptor anchoring at the endoplasmic reticulum has been implicated in regulating inositol trisphosphate receptor- (IP3R-) mediated Ca^{2+} signaling and could translocate to

the plasma or nuclear membrane once activated by the ligands [3, 4]. At the plasma membrane, the sigma-1 receptor can modulate the activation of various ion channels and receptors, such as K^+ channels, N-methyl-D-aspartate (NMDA), dopamine, and γ-aminobutyric acid (GABA) receptors [5–8].

Since the discovery of the sigma-1 receptor, many preclinical studies have implicated the receptor in many diseases such as depression and neurodegenerative disease and addition [9–11]. Recently, some reports also demonstrated that sigma-1 receptor was involved in the regulation of neuropathic pain by enhancing NMDA receptors activity [12, 13]. In view of the critical role of NMDA receptors in spinal nociceptive processing of bone cancer pain, we hypothesized that the sigma-1 receptor may play an important yet unknown role in development of bone cancer pain.

In the present study, Walker 256 mammary gland carcinoma cells were implanted into the intramedullary space of the right tibia of Sprague-Dawley rats. We examined the sigma-1 receptor expression changes in the spinal cord of BCP rats. Furthermore, we tested whether intrathecal administration of the selective sigma-1 receptor antagonist BD1047 could suppress mechanical allodynia and the activation of spinal microglia as well as NR1 and the subsequent Ca^{2+}-dependent signals of BCP rats.

2. Materials and Methods

2.1. Animals. Female Sprague-Dawley rats (Experimental Animal Center of Xuzhou Medical College, China) weighing 180 to 220 g were used. Animals were housed under controlled temperature (21 ± 2)°C and relative humidity (50% ± 10%), under a 12 h light-dark cycle (light on from 08:00 to 20:00), with ad libitum access to food and water. All experimental protocols were approved by the Animal Care and Use Committee of Xuzhou Medical College. Animal treatments were performed according to the Guidelines of the International Association for the Study of Pain [14].

2.2. Bone Cancer Model. The Walker 256 mammary gland carcinoma cell line was prepared as previously described [15, 16]. The cells were collected and diluted to a concentration of 2×10^7 cells/mL. A rat model of bone cancer pain was utilized as described previously [16]. In brief, under anesthesia with chloral hydrate (350 mg/kg, i.p.), rats were fixed, and the right tibia was prepared for surgery. The tibial plateau was exposed with minimal damage to the muscle and nerves. Walker 256 cells (1×10^5 cells in 5 μL of normal saline) were injected into the medullary canal through a hole drilled on the right tibia, whereas sham rats were injected with 5 μL media alone. The syringe was left in place for an additional 2 min and then the injection site was closed using bone wax. The wound was sutured and treated with penicillin.

2.3. Drug Administration. The intrathecal injection procedure followed the method of Xu et al. in 2006 [17]. Briefly, the rats were anesthetized with sevoflurane. The lumbar region was disinfected with 75% (v/v) ethanol after hair shaving, and the intervertebral spaces were widened by placing the animal on a plexiglass tube. Next, a 29-gauge microinjection syringe needle filled with the drug was inserted in the L5-6 interspace. A brisk tail flick could be observed immediately after the needle entry into subarachnoid space. Motor function was evaluated by the observation of placing or stepping reflexes and righting reflexes at 2 minutes before a nociceptive test. Animals with signs of motor dysfunction were excluded from the experiments.

2.4. Mechanical Allodynia. Mechanical allodynia was assessed with Von Frey filaments (Stoelting, Wood Dale, IL) as described previously [15]. Animals were placed in separate plastic box (20 cm × 25 cm × 15 cm) on a metal mesh floor and allowed to acclimate for 30 min. The filaments were applied sequentially in an ascending order of force. Each rat was tested bilaterally. The duration of each stimulus was approximately 5 seconds with a 5-minute interval between applications. The filaments were presented perpendicularly to the plantar surface, and brisk withdrawal or paw flinching was considered as positive responses. The paw withdrawal threshold (PWT) was assessed by the "up-and-down" method [18]. All the tests were performed between 9:00 am and 12:00 am and the behavioral tests on days 5, 6, and 7 after inoculation were performed before the intrathecal injection.

2.5. Western Blot. For the results of Section 3.2, rats were killed on days 3, 7, 10, 14, and 21 after inoculation with cancer cells or normal saline (sham and naïve rats were killed on day 10); for the results of Sections 3.4 and 3.5, rats were killed at 2 h after BD1047 intrathecal administration on day 7. The L4-5 spinal cords of the rats were quickly extracted and stored in liquid nitrogen. All tissue samples were homogenized in lysis buffer containing PMSF and 0.02% protease inhibitor cocktail. The supernatant, after centrifugation at 12,000 revolutions per minute for 15 minutes at 4°C, was used for western blot. Equivalent amounts of protein (50 μg) were separated using SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with 5% bovine serum albumin (BSA) for 2 hours at room temperature (RT) and then washed in Tris-buffered saline with Tween 3 times for 10 minutes each. After that, membranes were incubated with primary antibodies for rabbit anti-p-NR1 (Ser896) (1 : 1000; Cell Signaling, USA), rabbit anti-p-ERK1/2 (Thr202/Tyr204) (1 : 1000; Cell Signaling, USA), rabbit anti-p-p38 (1 : 1000; Cell Signaling, USA), rabbit anti-Iba-1 (1 : 1500; Wako, Japan), rabbit anti-TNF-α (1 : 1000; Cell Signaling, USA), rabbit anti-GAPDH (1 : 1000; Sigma, USA), or rabbit anti-sigma-1 (1 : 200; Abcam, UK) overnight at 4°C. The membranes were incubated for 2 h with HRP-conjugated anti-rabbit secondary antibody (1 : 1500; R&D, USA). Bands were visualized using an ECL system. Data were analyzed with a Molecular Imager (ChemiDoc XRS; Bio-Rad, USA) and the associated software Quantity One-4.6.5 (Bio-Rad, USA).

2.6. Immunohistochemistry. At 2 h after BD1047 intrathecal administration on day 7, rats from all groups were deeply

anesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused intracardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. L4-5 spinal cords were removed, postfixed in 4% paraformaldehyde overnight at 4°C, and then placed in a 30% sucrose solution overnight at 4°C. 25 μm transverse sections were cut on a cryostat. After elimination of endogenous peroxidase activity with hydrogen peroxide and preblocking with 10% normal donkey serum and 0.3% Triton X-100 at room temperature for 2 h, the sections were incubated in primary polyclonal rabbit anti-c-Fos antibody (1 : 200; Abcam, UK) overnight at 4°C and then incubated in polymer helper (ZSGB-BIO, CN) at 37°C for 30 min and in poly-HRP anti-rabbit IgG (ZSGB-BIO, CN) at 37°C for 30 min. Finally, the sections were treated with 0.05% diaminobenzidine for 5–10 min and rinsed with PBS to end the reaction, mounted on gelatin-coated slides, air-dried, dehydrated with 30%–100% alcohol, cleared with xylene, and cover-slipped for microscopic observation (Nikon Eclipse E600, Japan). For the Iba-1 protein assay, the sections were incubated in 10% normal donkey serum and 0.3% Triton X-100 at room temperature for 2 h and then in primary polyclonal goat anti-Iba-1 antibody (1 : 200; Abcam, UK) at 4°C for 16 h. After three washes with PBS, the sections were further incubated with Alexa Fluor 488 anti-goat IgG (1 : 200; Invitrogen, USA) for 2 h at room temperature, mounted on gelatin-coated slides, and cover-slipped with a mixture of 50% glycerin in 0.01 M PBS. Images were captured with Olympus confocal microscope (Olympus FV1000, Japan) and analyzed by Image Pro-Plus 6.0 (Image Pro-Plus Kodak, USA).

2.7. Statistical Analysis. Statistical analysis of data was generated using GraphPad Prism 5 (GraphPad Software Inc.). All data are shown as mean ± SEM. Data from immunohistochemical analysis and western blot studies were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett post hoc testing. Data of paw withdrawal threshold for mechanical allodynia testing were analyzed by two-way ANOVA followed by Bonferroni post hoc test for mechanical allodynia testing. A value of $P < 0.05$ was considered statistically significant. All the experimental testing was performed blind.

3. Results

3.1. Mechanical Allodynia Induced by Bone Cancer. All rat groups exhibited similar baseline hind paw withdrawal threshold (PWT) to mechanical stimulation ($n = 10$, $P > 0.05$). BCP rats displayed a significant decrease in PWT of the ipsilateral hind paw compared with sham rats on day 5 ($P < 0.01$; Figure 1). With the progression of bone cancer, the PWT progressively decreased in the inoculated hind paw from days 5 to 21 ($P < 0.01$; Figure 1).

3.2. Sigma-1 Receptor Expression Is Increased in the Spinal Cord of BCP Rats. Western blot analysis revealed that the expression of the sigma-1 receptor significantly increased in the spinal cord on day 7 following inoculation with Walker 256 cells compared with sham rats ($n = 4$, $P < 0.01$; Figures 2(a) and 2(b)). The protein levels further peaked on day 10

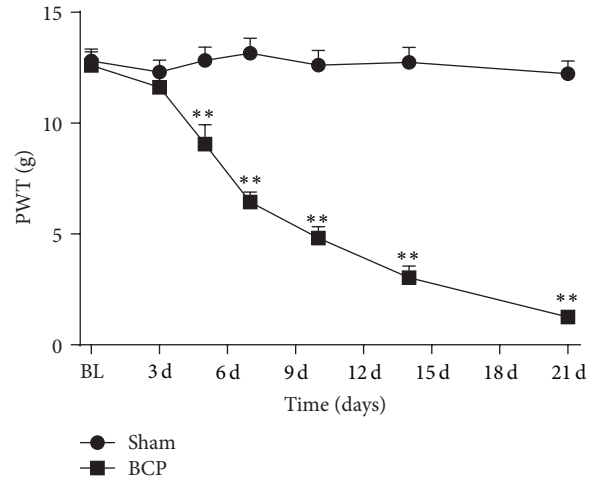

Figure 1: Rats with tibia tumors after Walker 256 cells inoculation displayed mechanical allodynia. The PWT progressively decreased on days 5, 7, 10, 14, and 21 ($n = 10$) after inoculation in BCP group compared with sham group. Results are given as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group.

($P < 0.01$; Figures 2(a) and 2(b)) and declined slowly from days 14 to 21 ($P < 0.01$, $P < 0.05$; Figures 2(a) and 2(b)) in BCP rats. In the spinal cord of sham rats, the expression of the sigma-1 receptor on days 3, 7, 10, 14, and 21 after surgery did not increase when compared with naïve rats ($n = 4$, $P > 0.05$; Figures 2(c) and 2(d)).

3.3. Blocking Sigma-1 Receptor Activation Delays Initiation of Mechanical Allodynia of BCP. To investigate the role of sigma-1 receptor in initiation of BCP, we measured mechanical allodynia in BCP rats after a continuous administration of the selective sigma-1 receptor antagonist BD1047 injection. We used a continuous drug administration of BD1047 (120 nmol/20 μL, once a day for 3 consecutive days) from day 5 to day 7 after inoculation with Walker 256 cells. There were no significant differences in baseline PWT among all groups ($n = 10$, $P > 0.05$). Compared with normal saline-treated (NS-treated) sham group, there were no remarkable changes of PWT in BD1047-treated sham group ($P > 0.05$; Figure 3). BCP group exhibited a decrease of PWT compared with sham group on day 5. Intrathecal administration of BD1047 significantly alleviated bone cancer induced mechanical allodynia compared with NS-treated BCP group ($P < 0.01$; Figure 3).

3.4. Blocking Sigma-1 Receptor Activation Suppresses the Upregulation of c-Fos and p-NR1 and p-ERK in the Spinal Cord of BCP Rats. Immunohistochemistry data demonstrated that, compared with sham rats, the expression of c-Fos was strikingly increased in BCP rats in the ipsilateral spinal cord on day 7 ($n = 8$, $P < 0.01$; Figures 4(a) and 4(b)). Intrathecal administration of BD1047 from day 5 to day 7 significantly reduced the level of c-Fos protein expression in the ipsilateral spinal cord of BCP rats compared with NS-treated BCP group ($P < 0.01$; Figures 4(a) and 4(b)). There were no changes of c-Fos protein expression in NS-treated sham group and BD1047-treated sham group ($P > 0.05$; Figures 4(a) and 4(b)).

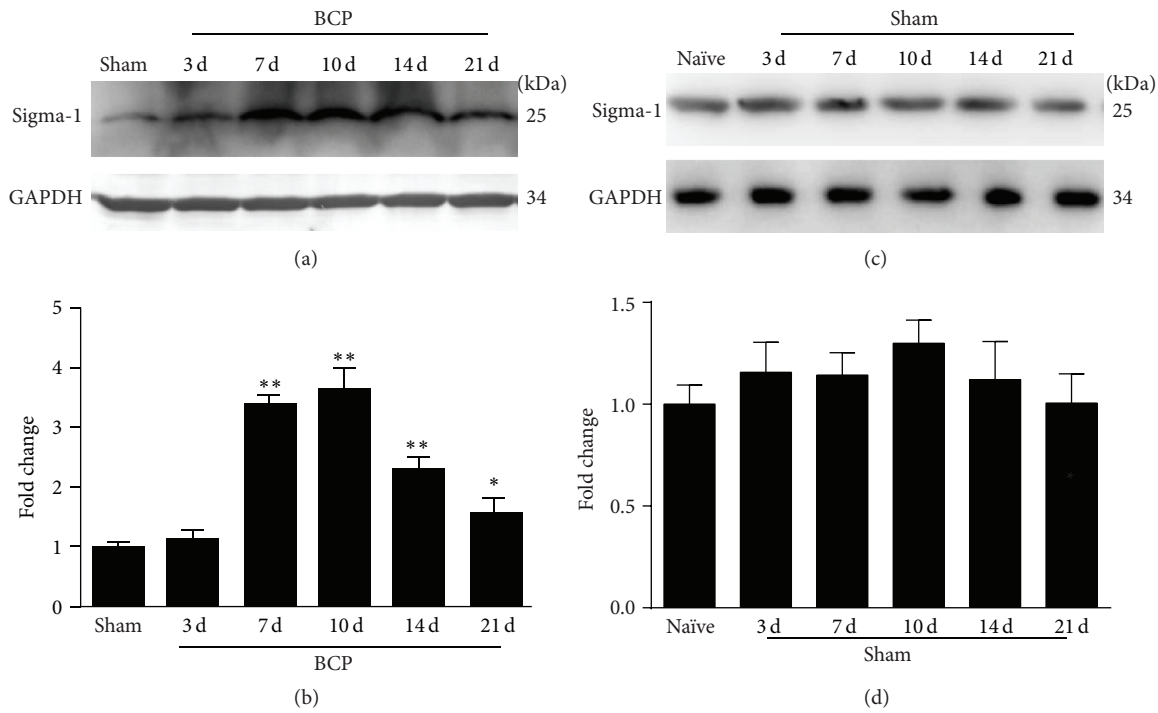

Figure 2: Walker 256 cells inoculation-induced sigma-1 receptor expression increased in the spinal cord. (a) Western blot analysis showed a significant upregulation of sigma-1 protein level in the spinal cord of BCP rats on days 7, 10, 14, and 21. GAPDH was used as a loading control. (b) Quantification of sigma-1 protein level in the spinal cord. (c) Western blot analysis showed no significant change of sigma-1 protein level in the spinal cord of sham rats on days 3, 7, 10, 14, and 21. GAPDH was used as a loading control. (d) Quantification of sigma-1 protein level in the spinal cord. Sigma-1 receptor levels were normalized against GAPDH ($n = 4$). Results are given as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group or naïve group.

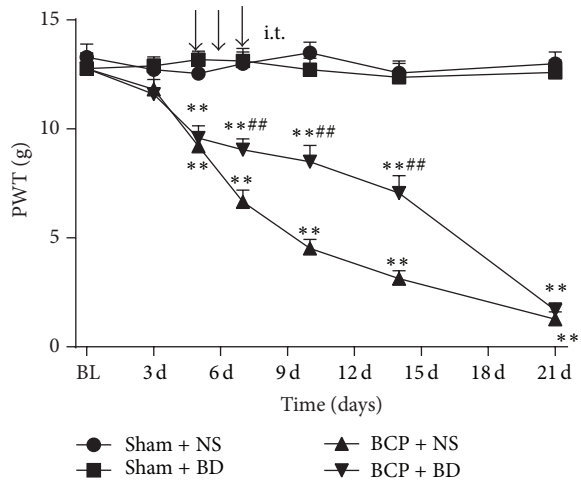

Figure 3: Intrathecal administration of BD1047 delayed bone cancer induced mechanical allodynia. Sigma-1 receptor antagonist BD1047 or normal saline (NS) was intrathecally injected on days 5, 6, and 7 after inoculation ($n = 10$). The PWT of BCP rats was increased after injection of BD1047 on day 7. Results are given as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham + NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus BCP + NS group.

In addition, western blot analysis indicated that the expression of p-NR1 and p-ERK significantly increased in the spinal cord of BCP rats on day 7 compared with sham rats ($n = 4$, $P < 0.01$, $P < 0.05$, $P < 0.01$, and $P < 0.01$; Figures 5(a) and 5(b)). Repetitive treatment with BD1047 from day 5 to day 7 robustly suppressed the upregulation of these molecules in the spinal cord of BCP rats ($P < 0.05$, $P < 0.01$; Figures 5(a) and 5(b)). There were no significant

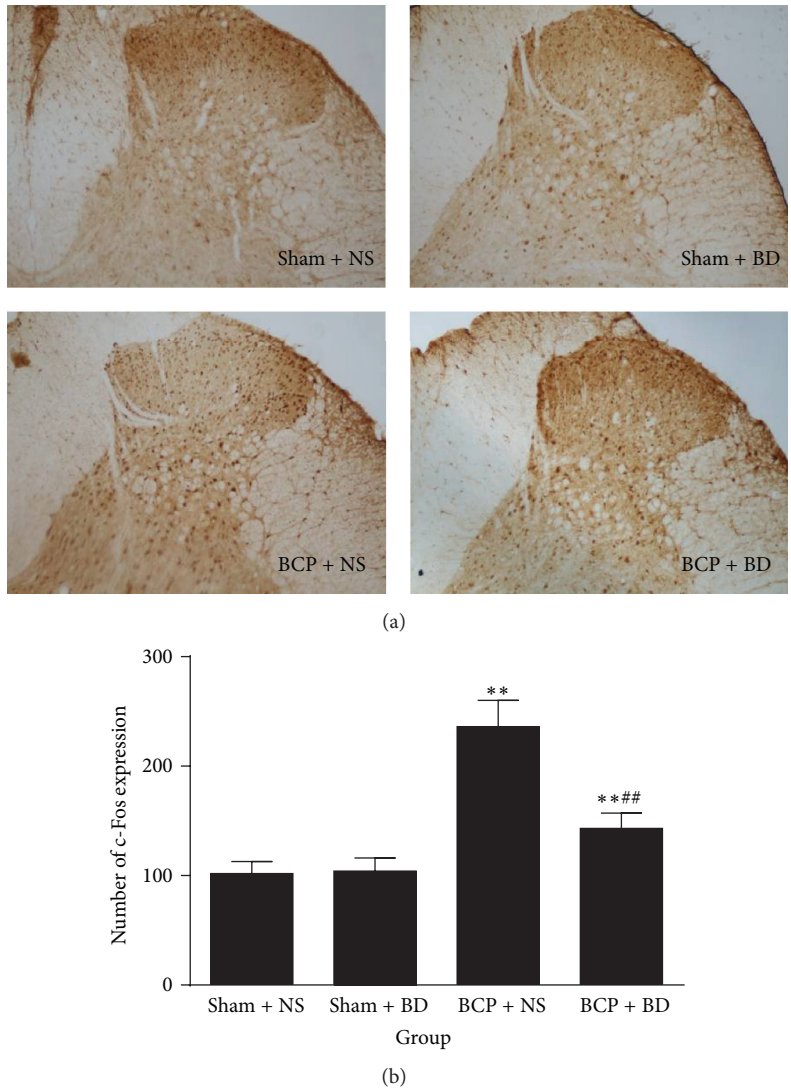

Figure 4: Intrathecal administration of BD1047 suppressed the upregulation of c-Fos protein expression. (a) Compared with sham rats, the c-Fos expression was strikingly increased in BCP rats in the ipsilateral spinal cord on day 7. Intrathecal injection of BD1047 provided a significant decrease of the c-Fos in BCP rats compared with NS-treated BCP group. (b) Quantification of c-Fos level in the dorsal horn ($n = 4$). Results are given as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham + NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus BCP + NS group. Magnification: 100x.

differences of p-NR1 and p-ERK protein expression between NS-treated sham group and BD1047-treated sham group ($P > 0.05$; Figures 5(a) and 5(b)).

3.5. Blocking Sigma-1 Receptor Activation Suppresses the Upregulation of Iba-1, p-p38, and TNF-α in the Spinal Cord of BCP Rats. Immunohistochemistry data revealed that the expression of Iba-1 was significantly higher in BCP rats compared with sham rats in the ipsilateral spinal cord on day 7. Activated microglial cells exhibited hypertrophic morphological changes such as cell body enlargement and retraction of processes (Figures 6(a) and 6(b)). BD1047-treated BCP group showed a striking decrease in the number of Iba-1 immunoreactive (IR) cells in the ipsilateral spinal cord compared with NS-treated BCP group ($n = 8$, $P < 0.01$; Figures 6(a) and 6(b)). Consistent with immunofluorescence staining results, western blot analysis using anti-Iba-1 antibody showed that the expression level of Iba-1 was markedly increased in the spinal cord of BCP rats on day 7 compared with sham rat ($n = 4$, $P < 0.01$, $P < 0.05$; Figures 7(a) and 7(b)). Repetitive treatment with BD1047 from day 5 to day 7 robustly suppressed the upregulation of Iba-1 in the spinal cord of BCP rats ($P < 0.05$; Figures 7(a) and 7(b)).

Western blot analysis also showed that Walker 256 cells implantation induced the upregulation of p-p38 and TNF-α expression in the spinal cord of BCP rats compared with sham rats on day 7 ($n = 4$, $P < 0.01$, $P < 0.05$, $P < 0.01$, and $P < 0.01$; Figures 7(a) and 7(b)). Compared with NS-treated BCP group, there was a significant decrease in the level of these molecules in BD1047-treated BCP group ($P < 0.01$; Figures

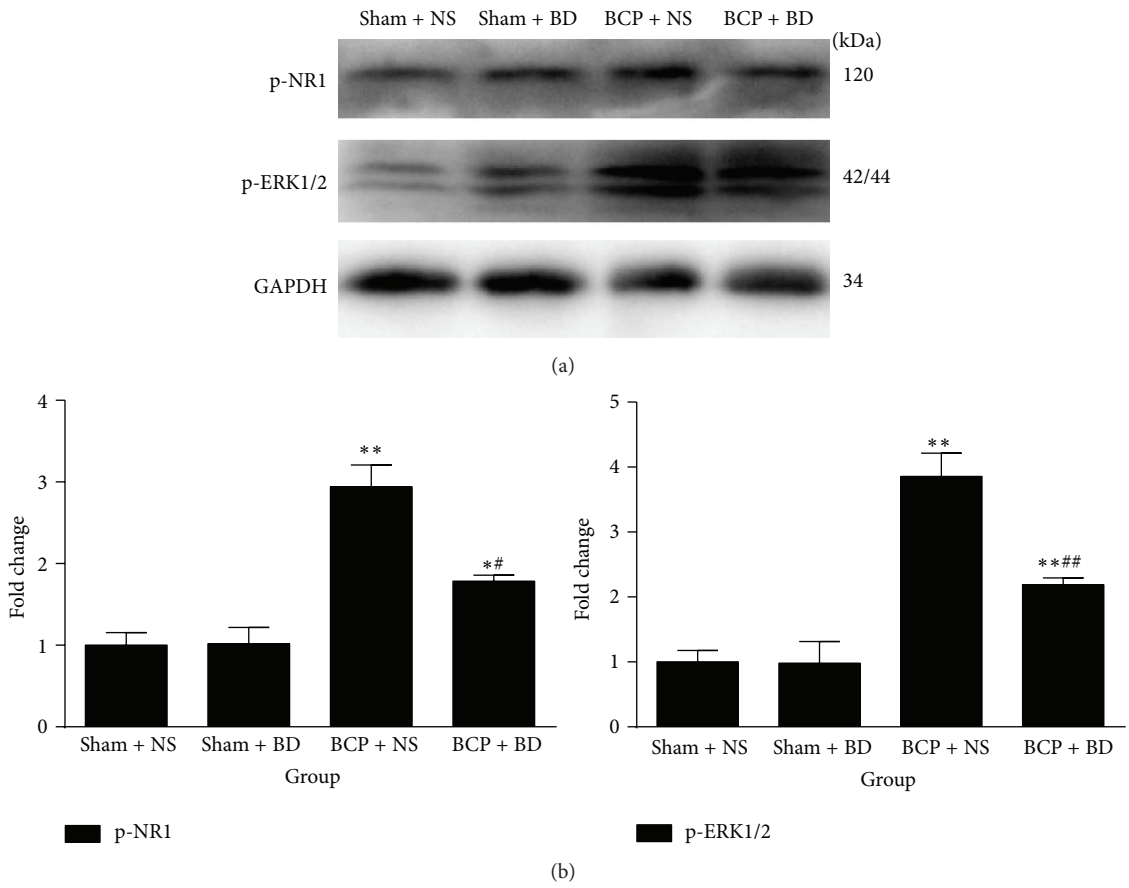

Figure 5: Blocking sigma-1 receptor activation suppressed BCP-induced upregulation of p-NR1 and p-ERK1/2 in the spinal cord. (a) Western blot analysis indicated a significant increase of p-NR1 and p-ERK expression in the spinal cord of BCP rats on day 7. Repetitive treatment with BD1047 significantly decreased these molecules in BCP rats compared with NS-treated BCP group. (b) Quantification of p-NR1 and p-ERK expression level in the spinal cord ($n = 4$). Results are given as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham + NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus BCP + NS group.

7(a) and 7(b)). Iba-1, p-p38, and TNF-α protein expression in NS-treated sham group and BD1047-treated sham group showed no significant differences ($P > 0.05$; Figures 7(a) and 7(b)).

4. Discussion

The results of our study demonstrated that spinal sigma-1 receptor expression was upregulated in BCP rats and declined slowly from days 14 to 21 after surgery. Intrathecal administration of BD1047 significantly suppressed the initiation of mechanical allodynia and the spinal c-Fos expression of BCP rats. Moreover, blockade of sigma-1 receptor prevented the spinal upregulation of p-NR1 and p-ERK. In addition, our results indicated that there may be a potential relationship between the activation of sigma-1 receptor and microglia. The upregulation of Iba-1, p-p38 MAPK, and TNF-α expression were affected by BD1047 administration.

Previous studies have demonstrated that spinal cord sigma-1 receptor expression is upregulated under conditions of neuropathic pain [13, 19]. Our western blot data indicated that sigma-1 receptor expression was upregulated from days 7 to 21 in the spinal cord following Walker 256 cells inoculation, peaked on day 10, and declined slowly from days 14 to 21. The time course of the upregulated spinal sigma-1 receptor was not completely consistent with the development of BCP behavioral response. Thus we guess that spinal sigma-1 receptor plays a pivotal role only in the early stage of BCP.

It has been suggested that neurosteroids including pregnenolone and dehydroepiandrosterone sulfate (DHEAS) are endogenous ligands for sigma-1 receptor [20]. Several studies have reported that the concentration of spinal neurosteroids was significantly increased in neuropathic pain rats [21, 22]. Administration of DHEAS facilitated the induction of mechanical allodynia in neuropathic pain rats and leaded to pain hypersensitivity in naïve rats; both the pronociceptive effects of DHEAS could be blocked by BD1047 [23, 24]. Based on these findings, we believe that the increasing endogenous neurosteroids in the spinal cord induce pain hypersensitivity by activating sigma-1 receptor.

NMDARs are composed of three related families of subunits: NR1, NR2, and NR3 [25]. All functional NMDARs include at least one NR1 subunit and NR1 is required for receptor activity [26]. NMDARs are phosphorylated and

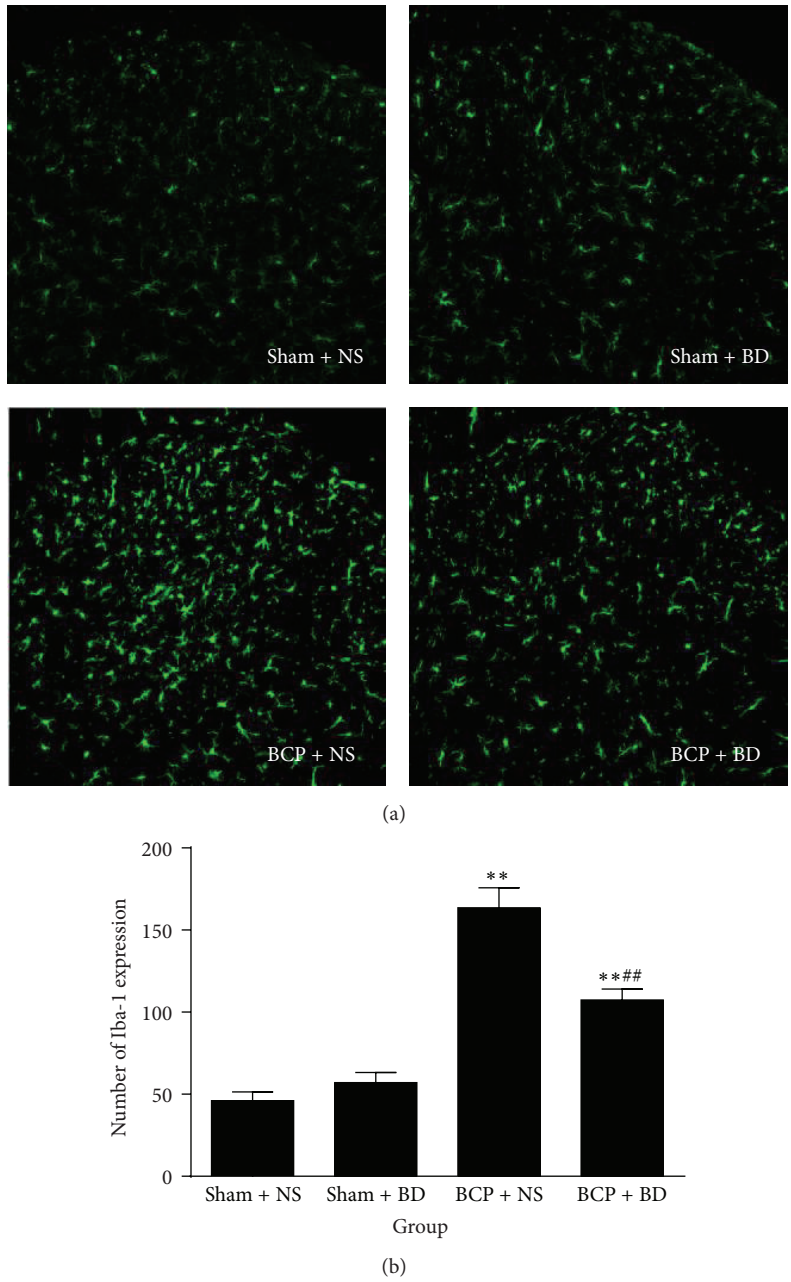

Figure 6: Spinal administration of BD1047 attenuated Iba-1 expression. (a) Immunohistochemistry data revealed that the expression of Iba-1 was significantly higher in BCP rats compared with sham rats in the ipsilateral spinal cord on day 7. Compared with NS-treated BCP group, BD1047-treated BCP group showed a striking decrease in the number of Iba-1 immunoreactive (IR) cells in the ipsilateral spinal cord. (b) Quantification of Iba-1 level in the dorsal horn ($n = 4$). Results are given as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham + NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus BCP + NS group. Magnification: 200x.

dephosphorylated by a variety of kinases [25]. Once activated, NMDARs produce influx of Ca^{2+} and thus increase cytosolic concentration of Ca^{2+} in dorsal horn neurons. In turn, intracellular Ca^{2+} activates Ca^{2+}-dependent second messengers including extracellular signal-regulated kinase (ERK) and calcium/calmodulin-dependent kinase II (CaMKII) that ultimately contribute to pain hypersensitivity [27, 28]. In the pain research field, c-Fos and p-ERK have been extensively used as the marker for the activation of nociceptive neurons and both of them are implicated in pain facilitation [29]. Previous studies reported that intrathecal injection of BD1047 blocked both mechanical allodynia and the increase in spinal NR1 expression [13, 30], which is in concordance with our data. We demonstrated that BD1047 not only attenuated behavioral hypersensitivity but also decreased BCP-induced p-NR1, p-ERK, and c-Fos expression in the spinal cord.

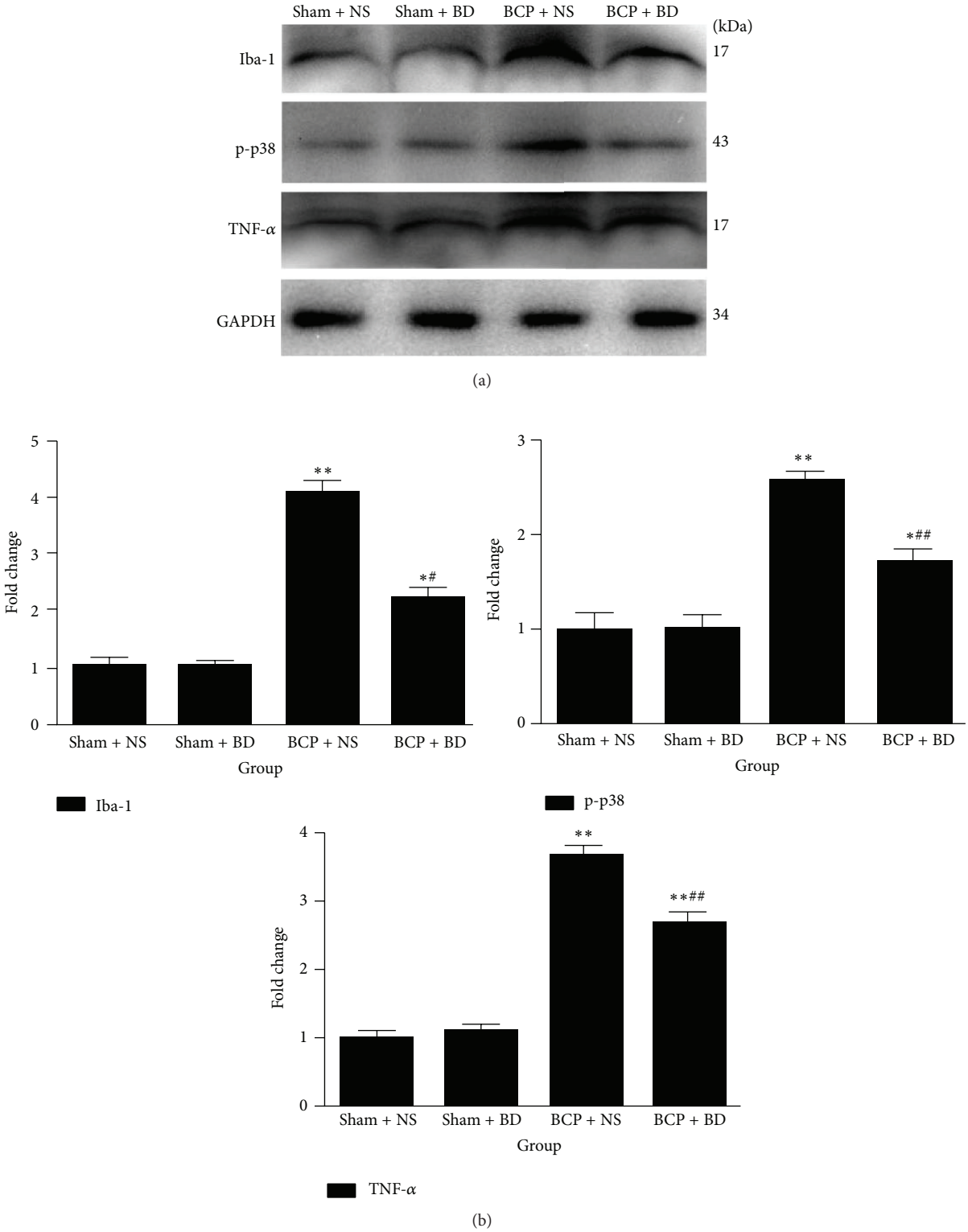

Figure 7: Iba-1, p38 activation. and proinflammatory cytokine expression were reduced by intrathecal administration of BD1047. (a) Western blot analysis indicated that Iba-1, p-p38, and TNF-α expression were higher in the spinal cord of BCP rats on day 7. Intrathecal BD1047 significantly reduced the expression of these molecules in BCP rats compared with NS-treated BCP group. (b) Quantification of Iba-1, p-p38, and TNF-α expression level in the spinal cord ($n = 4$). Results are given as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham + NS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus BCP + NS group.

Therefore, it is assumed that direct blockage of spinal neurons may be, at least in part, a potential target for pharmacological intervention with systemic injection of BD1047.

After Walker 256 cells implantation microglia in the spinal cord become activated [31], p-p38 expression which is exclusively expressed by microglia is markedly increased [32, 33]. The activation of p38 in spinal microglia results in increased synthesis and release of the IL-1β, TNF-α, and BDNF [34–38]. Both p-p38 MAPK and microglia-released mediators contribute to pain hypersensitivity in the spinal cord of BCP rats [38–41]. It has been reported that microglia in the central nervous system express high levels of sigma-1 receptor [42]. Pretreatment with BD1047 attenuated MAPKs and MCP-1 production induced by sigma-1 receptor agonist cocaine in BV-2 cells [43]. Moreover, intrathecal injection of BD1047 reduced the CCI-induced increase in p-p38 MAPK [19]. Our studies also demonstrated that BD1047 could significantly reduce the upregulation of spinal Iba-1, p-p38, and TNF-α induced by BCP. These results indicate that sigma-1 receptor is implicated in pain facilitation through the activation of spinal microglia. Recently, Hall et al. reported that administration of the nonselective sigma-1 receptor agonist DTG significantly decreased the production of TNF-α evoked by LPS in microglia [44]. Ruscher et al. concluded that treatment with the specific sigma-1 receptor agonist SA4503 after MCAO did not affect the increased levels of TNF-α [45]. These discrepancies might not be contradictory and could be explained by different modes of the compounds actions and animal models. DTG is an unspecific sigma receptor agonist with similar binding affinity to the sigma-1 receptor and the sigma-2 receptor. Therefore, further studies are needed to investigate whether the decreased expression of TNF-α is mediated by activation of the sigma-2 receptor. In addition, the treatment windows and mechanisms between models of middle cerebral artery occlusion (MCAO) and bone cancer pain (BCP) are different.

5. Conclusions

This study demonstrates that pharmacological blockade of sigma-1 receptor by i.t. administration of sigma-1 antagonist BD1047 significantly attenuates nociceptive responses to mechanical stimulation induced by Walker 256 cells implantation via the synergistic inhibition of neuronal NMDARs and microglia activation. Thus, these results suggest a potential therapeutic use of sigma-1 receptor antagonists for the clinical management of bone cancer pain.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Shanshan Zhu conceived the experiments and interpreted results; Chenchen Wang performed the experiments and wrote the paper; Yuan Han contributed significantly to paper preparation; Chao Song helped perform the analysis with constructive discussions; Xueming Hu participated in animal models; Yannan Liu supplied experimental materials. Shanshan Zhu and Chenchen Wang contributed equally to this work. Shanshan Zhu and Chenchen Wang are co-first authors.

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Review Article

Cytokines as Mediators of Pain-Related Process in Breast Cancer

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Pain is a clinical sign of inflammation found in a wide variety of chronic pathologies, including cancer. The occurrence of pain in patients carrying breast tumors is reported and is associated with aspects concerning disease spreading, treatment, and surgical intervention. The persistence of pain in patients submitted to breast surgery is estimated in a range from 21% to 55% and may affect patients before and after surgery. Beyond the physical compression exerted by the metastatic mass expansion and tissue injury found in breast cancer, inflammatory components that are significantly produced by the host-tumor interaction can significantly contribute to the generation of pain. In this context, cytokines have been studied aiming to establish a cause-effect relationship in cancer pain-related syndromes, especially the proinflammatory ones. Few reports have investigated the relationship between pain and cytokines in women carrying advanced breast cancer. In this scenario, the present review analyzes the main cytokines produced in breast cancer and discusses the evidences from literature regarding its role in specific clinical features related with this pathology.

1. The Cytokine Status in Breast Cancer: An Overview

Malignant solid tumors can metastasize and infiltrate important organs, damaging tissue and nervous structures, which causes pain. Breast cancer is a malignant cancer that frequently spreads and infiltrates distant sites in the body as the liver, lungs, brain, and bones. Therefore, organ infiltration by breast metastasis causes severe pain by multiple mechanisms [1, 2].

Host-tumor interactions favor the arising of several soluble mediators, including cytokines and chemokines. Although such molecules are not exclusive in breast cancer (as well its clinically related processes), there is a massive production of circulating cytokines during disease development that are likely enrolled in the systemic pain experienced by patients. Depending on the staging of disease, women with breast cancer exhibit distinct patterns of circulating cytokines. In the early stages, when breast cancer is localized, patients display reduced TNF-α and IL-12 in serum [3], and concomitantly there are few reports of pain. On the other hand, patients presenting advanced disease have high systemic levels of TNF-α and IL-1β [3], which are often accompanied by pain. These facts suggest a possible connection between cytokine patterns and pain in breast cancer.

Breast cancer is a heterogeneous disease and is divided into 4 main subtypes according to its clinical molecular characteristics as luminal A and luminal B and HER-2 amplified and triple negative tumors. Each subtype harbors specific clinical behavior and aggressiveness, which affect disease prognosis [4]. Luminal tumors present positivity to estrogen and/or progesterone receptors (luminal A) and can further present the amplification of the receptor of the human epidermal growth factor 2 (HER2). The latter is categorized as luminal B and is more aggressive than luminal A cancers. Some tumors present only the overexpression of HER2 and are named as HER2-amplified. Finally, tumors that do not exhibit any of these receptors are classified as triple negative [5]. This status is a determining factor not only for the clinical outcome of the patient presents but also for the type of circulating cytokines presented.

In this way, women bearing luminal tumors display higher TNF-α and TGF-β1 plasmatic levels than healthy women, and concomitantly reduced levels of IL-12. In relation

to the other subtypes, patients with luminal tumors exhibit less TGF-β1 than those with HER2-amplified tumors, showing no further differences regarding TNF-α, IL-12, IL-1β, and IL-10. Patients diagnosed with HER2-amplified tumors present augmented TNF-α and TGF-β1 when compared to healthy individuals and sustain significantly higher levels of IL-12 in relation to the other subtypes. On contrary, triple negative patients present reduced circulating levels of TNF-α and TGF-β1 in relation to the other subtypes, as well as when compared to healthy volunteers [6]. Therefore, the systemic cytokine profile is closely related with tumor subtype and may affect disease outcome in some instance.

Chemotherapy can also modulate cytokine patterns during breast cancer treatment. A comparative analysis of cytokine levels between untreated patients and women ongoing adjuvant regimen (anthracycline-taxane based) shows that doxorubicin infusion can acutely downregulate TNF-α and IL-1β in plasma, while paclitaxel upregulates the circulating IL-10 after its 1-hour infusion. Moreover, direct exposure of blood cells to chemotherapy can lead to cytokine deregulation, as observed in healthy volunteers. Paclitaxel can reduce TNF-α levels after 1-hour direct contact with whole blood, and doxorubicin promotes reduction of IL-10 and simultaneous augment of IL-1β [7]. The fast capacity of chemotherapy to modulate cytokine availability suggests that such drugs may affect the cellular capability of releasing preformed cytokines, as well as regulating the dynamic of cytokine consumption by cells.

2. Cytokines and Pain in Human Breast Cancer

Although a wide variety of cytokines has been reported in breast cancer aspects, few studies have focused on understanding its relationship with pain. The persistent inflammatory status of cancer results in the production of a wide of cytokines and chemokines that act on the nociceptors causing hypernociception. In general, proinflammatory cytokines are directly related with pain generation, while the anti-inflammatory set constitutes a negative modulator of hypernociception [8, 9]. The role of cytokines in breast cancer is far to be understood; however, some evidences have emerged in the past years. Here we present information regarding the role of cytokines in specific clinical aspects of human breast cancer, as summarized in Table 1.

2.1. Bone Metastasis and Tumor Osteopathy. About 70% of women with advanced breast cancer may develop bone metastasis, a late complication of disease [10]. Such metastases are osteolytic and cause bone destruction, pain, and nerve compression syndromes. Bone metastasis accounts for most of breast-cancer derived metastases and involves a wide of inflammatory mediators, including cytokines and chemokines.

Among cytokines, TGF-β is one of the most abundant growth factors physiologically present in the bone matrix and is released during bone resorption. This mediator is an important regulator of osteoclast activity in homeostasis [11] and possesses functional effects related with the generation of pain in some models of bone-related diseases. Studies have pointed that during bone damage TGF-β is released by cartilage cells and mediates the expression of nerve growth factor (NGF) in chondrocytes, which could potentially be a source of pain under noninflammatory conditions [12]. Further, the functioning of TGF-β axis in bones can be suppressed by proinflammatory cytokines as IL-1β and TNF-α [13], commonly found in breast cancer. Therefore, TGF-β may act as a noninflammatory mediator of pain in bone-related pathologies.

In breast cancer, bone metastasis involves a tight interaction between tumor metastasis and host cells. It is estimated that this event starts when metastatic cells detach from the primary tumor, enter the bloodstream, and infiltrate the distal bone sinusoids and bone marrow. The adaptation of infiltrating metastatic cells to the bone environment allows its growing and invasiveness [14], resulting eventually in bone breaks and pain.

There is a feed-forward vicious cycle for TGF-β in breast cancer metastases to bones [15]. It has been proposed that breast metastatisation to the bones includes a sequential pattern of gene expression that mainly includes TGF-β signaling pathway [16], where tumors cells stimulate osteoclasts to bone matrix resorption, which releases growth factors that cyclically activates tumor cells [17] (Chirgwin and Guise, 2000).

TGF-β is further implicated in the pathogenesis of the osteolytic bone metastasis by inducing the production of parathyroid hormone-related protein, a major mediator of the osteolytic process [18]. Tumor-specific prometastatic T cells also contribute to bone lesions by activating the RANKL pathway after bone colonization [19].

Biphosphonates are a pharmacological class used for treating breast cancer patients presenting with bone metastasis, aiming to reduce the pathological osteolysis [20]. The mechanism of action of biphosphonates consists in inhibiting bone resorption and osteoclasts activity but also involves the induction of inflammation. In this way, biphosphonates are important to control the bone-related pain in advanced breast cancer, as well as the interaction of metastatic cells with bone components.

The relationship between pain and cytokines in the context of biphosphonates is not clear in human studies, but there are some evidences pointing a role for cytokines in the mechanism of action of these drugs. Kaiser et al. [21] demonstrated that biphosphonates can reduce the capacity of breast cancer metastasis to interact with extracellular matrix components and affects the secretion of the chemokine CCL2 by osteoblasts. Moreover, the proinflammatory status induced by biphosphonates is mediated by innate response components, as neutrophils, monocytes, MyD88-TLR4 signaling, and IL-1 [22]. These mediators may support in part the antiosteolytic effects of biphosphonates, which results in reduction of pain.

Therefore, immune-related processes that are linked to the bone destruction may contribute to the generation of pain symptoms in metastatic breast cancer; thus bone-driven

Table 1

Cytokine/chemokine	Role in breast-cancer-related pain	Reference
TGF-β	Major regulator of osteoclasts, affecting bone resorption and osteolysis	[15] [18] [17]
IL1R1 polymorphism	Patients carrying the minor allele for a single nucleotide polymorphism (SNP) in IL1R reported less pain before breast surgery	[23]
IL1R2 polymorphism	The SNP rs11674595 is associated with the occurrence of persistent breast pain after surgery	[24]
IL13 polymorphism	Women carrying the minor allele for a SNP in IL13 reported significant pain prior to breast surgery	[23]
IL10 gene haplotype	The haplotype A8 is associated with the occurrence of persistent breast pain after surgery	[24]
CCL3	Paclitaxel-mediated pain	[25]
CCR2	Paclitaxel-mediated pain	[26]
CX3CL1	Paclitaxel-mediated pain	[27]
IL10 (serum)	Paclitaxel-induced joint pain	[28]
G-CSF	Muscle pain	[29]

blocking of TGF-β may be an interesting approach to avoid pain syndromes in advanced breast cancer.

2.2. Persistent Pain and Breast Surgery. Chronic pain is a common feature in patients with breast cancer [30] and is mainly related with the releasing of inflammatory mediators from the tumor, nerve involvement, and tissue injury. This process results from a network formed by proinflammatory cytokines, genes from the inflammatory pathways and cytokine genes polymorphisms [23].

In this context, a role for polymorphisms in cytokine-related genes and the individual susceptibility to pain in cancer has been discussed [31]. McCann and coworkers [23] investigated a group of patients with breast cancer prior to breast cancer surgery to evaluate the profile of genetic polymorphisms in inflammatory genes and its relation with the occurrence of pain. The authors found that about 25% of women experienced pain, reaching a significant pain score that affected their quality of life. Further, genetic variations enrolling IL1R1 (rs2110726) and IL13 genes were correlated with preoperative pain in breast cancer. Patients carrying the minor allele for a single nucleotide polymorphism (SNP) in IL1R reported less pain before breast surgery, while those with the minor allele for a SNP in IL13 reported significant pain prior to breast surgery. This study reinforces the relevance of cytokines in breast cancer pain independent on mechanical features or tissue damage. Experimental IL1 silencing decreases pain-related behaviors [32], while IL13 has anti-inflammatory activity and affects IL1 releasing [33]. Thus, these cytokines may represent important regulatory points in cancer pain controlling, independent of surgical intervention.

The persistence of pain after breast tumor removal is a problem of clinical relevance that includes mechanical and inflammatory components. The chronic postsurgical pain is a consequence of sustained nociceptors activation and nerve damage [34]. The surgical manipulation of the site of surgery releases innumerous inflammatory mediators that induce peripheral sensitization; therefore, sustained stimulation may result in persistent pain.

The maintenance of pain after breast surgery involves immune mechanisms recently identified by a cytokine signature study [24]. In such study, one single nucleotide polymorphism for interleukin 1 receptor (ILR2, rs11674595) and one haplotype for IL-10 (haplotype A8) were associated with the occurrence of persistent breast pain after surgery [24]. ILR2 gene encodes the IL-1 type II receptor that possesses anti-inflammatory effects, while IL-10 down regulates the proinflammatory cascade. The results from this study suggest that the IL-10 haplotype A8 diminishes the risk for development of severe chronic pain, while the polymorphism of ILR2 could be associated with increased risk for the occurrence of severe persistent breast pain.

2.3. Chemotherapy and Peripheral Neuropathy. Chemotherapy-induced peripheral neuropathy (CIPN) is a neurotoxic adverse effect of chemotherapy which is frequently related with the interruption of cancer treatment and therapeutic failure [35]. According to the National Cancer Institute (NCI) it constitutes the main reason for interrupting cancer treatment, which strongly affects patient outcome during treatment [36].

Evidences have suggested a role for cytokine-axis in regulating the chemotherapy-related pain. Studies concerning the quality of life in patients with breast cancer ongoing chemotherapy-based clinical trials have demonstrated that the combined treatments for metastatic breast cancer may induce short-lived episodes of pain [37, 38]. Early studies have established that doxorubicin-paclitaxel-based chemotherapy is a causative agent of pain in breast cancer and include

mild peripheral neuropathy and mild myalgia/arthralgia episodes [39] (Amadori et al., 1996), but paclitaxel seems to be the main responsible for pain-related episodes in cancer chemotherapy.

Paclitaxel is one of the main drugs used for treating breast cancer. Its mechanism of action consists in affecting microtubule dynamics, inducing cell death. The same mechanism responsible for its antineoplasic effect is associated with its neurotoxic property. Paclitaxel may affect the peripheral nervous system by damaging microtubules and interfering in microtubule-based axonal transport, leading to peripheral nerve degeneration [35].

The direct effects of paclitaxel that cause peripheral neuropathy can be associated with the cumulative effect of subsequent doses of this drug. However, patients also experience acute episodes of neuropathy, which suggest that additional mechanisms may be included in the chemotherapy-induced neurotoxicity. Since cancer is an inflammatory disease, disruption of cytokines and inflammatory mediators may be enrolled in this process.

Experimental models of neuropathy suggest that blocking proinflammatory molecules can suppress the painful sensation [40], implicating cytokines as modulators of pain. Indeed, proinflammatory cytokines can modulate nociceptors [35].

Paclitaxel infusion can activate macrophages to infiltrate peripheral nerves and cause injury to sensory neurons [41]. The mechanisms of pain mediated by paclitaxel include the upregulation of chemokines as CCL3 [25], monocyte chemoattractant protein-1 (MCP-1), its cognate receptor CCR2 [26], and CX3CL1 [27]. Molecules related with innate immune response, as the toll-like receptor TLR4 and MyD88 signaling pathway, are also implicated [42]. In addition, joint pain has been reported in women with breast cancer undergoing paclitaxel weekly and correlates with increased IL-10 in plasma [28].

Thereby, cytokines have been pointed as putative targets for intervention in the chemotherapy-induced painful peripheral neuropathy. TNF-α, IL-1β, IL-6, and chemokines are highlighted as putative candidates for therapeutics against chemotherapy-induced peripheral neuropathy (CIPN), since they constitute a primary mechanism that allows the neuron-immune interfacing and are enrolled in pain and hyperalgesia mechanisms [43].

2.4. Estrogen Deprivation and Pain Syndromes. Postmenopausal women undergoing hormone replacement therapy possess reduced pain thresholds when compared to those without hormone replacement or men [44]. This fact suggests that ovarian-derived hormones may affect the individual susceptibility to pain. Estrogen deprivation can result in bone demineralization, osteoporosis, and bone fracture. Studies have reported that the estrogen-dependent bone resorption is regulated by upregulation cytokines as TNF-α [45] and other proinflammatory components as IL-1, IL-6, IL-17, and IFN-γ [46]. Younger age has been pointed as a risk factor for developing pain following breast cancer surgery [47], indicating a role for female hormones in breast cancer related-pain.

The use inhibition of estrogen production by using aromatase inhibitors has been associated with musculoskeletal pain in a mechanism enrolling proinflammatory cytokines and NFκB modulation [48]. In spite of this, information regarding the direct relationship between cytokines and pain in cancer models of estrogen deprivation is unknown.

2.5. Pain and the Use of Granulocyte Colony-Stimulating Factor (G-CSF). Several studies have reported the systemic effects of the granulocyte colony-stimulating factor (G-CSF) analog in breast cancer patients. G-CSF is a biologic response modifier, and its analog is widely employed in association with other antineoplastic drugs to revert the neutropenia resulting from chemotherapy. This association minimizes myelotoxicity, enhances neutrophil counts, and prevents delays in treatment [49].

In spite of its positive modulating effects on immune response of cancer patients, G-CSF-based treatments are known by causing significant pain-related symptoms. Bone, muscle, and joint pain are common side effects reported by breast cancer patients after G-CSF treatment, even after the modification of this drug to its pegylated form [50, 51]. The association of G-CSF with epirubicin for treating advanced breast cancer revealed that an important set of patients (55%) report myoskeletal pain [29].

These data point out the fact that the stimulation of granulopoiesis affects the peripheral sensitivity in patients with breast cancer and draw attention to the fact that the activation of immune response may be directly associated with triggering/enhancing pain sensation in patients undergoing chemotherapy.

3. Perspectives and Conclusions

Cytokines are clearly enrolled as mediators of pain in advanced breast cancer. TGF-β is the major mediator of the osteolytic lesions and bone-related pain. Some gene polymorphisms and haplotypes also appear to affect the individual susceptibility to pain sensitivity and involve IL1, IL10 and IL13 genes. Chemokines are mainly implicated in paclitaxel-mediated pain, while the G-CSF is a stimulator of muscular pain. Although few cytokines have been proved as direct effectors of pain in breast cancer, it is expected that the proinflammatory components as TNF-α and IL-6 may be enrolled in such process in some extent. Therefore, such mediators may constitute putative targets for pharmacological intervention aiming to control pain in women bearing advanced breast cancer.

Conflict of Interests

The authors declare no conflict of interests.

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Clinical Study

A Traditional Chinese Medicine Xiao-Ai-Tong Suppresses Pain through Modulation of Cytokines and Prevents Adverse Reactions of Morphine Treatment in Bone Cancer Pain Patients

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Treating cancer pain continues to possess a major challenge. Here, we report that a traditional Chinese medicine Xiao-Ai-Tong (XAT) can effectively suppress pain and adverse reactions following morphine treatment in patients with bone cancer pain. Visual Analogue Scale (VAS) and Quality of Life Questionnaire (EORTC QLQ-C30) were used for patient's self-evaluation of pain intensity and evaluating changes of adverse reactions including constipation, nausea, fatigue, and anorexia, respectively, before and after treatment prescriptions. The clinical trials showed that repetitive oral administration of XAT (200 mL, bid, for 7 consecutive days) alone greatly reduced cancer pain. Repetitive treatment with a combination of XAT and morphine (20 mg and 30 mg, resp.) produced significant synergistic analgesic effects. Meanwhile, XAT greatly reduced the adverse reactions associated with cancer and/or morphine treatment. In addition, XAT treatment significantly reduced the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α and increased the endogenous anti-inflammatory cytokine interleukin-10 in blood. These findings demonstrate that XAT can effectively reduce bone cancer pain probably mediated by the cytokine mechanisms, facilitate analgesic effect of morphine, and prevent or reduce the associated adverse reactions, supporting a use of XAT, alone or with morphine, in treating bone cancer pain in clinic.

1. Introduction

Pain is a common symptom in patients with advanced malignant cancer and greatly increases physical and mental suffering. There are approximately ten million new cancer patients annually worldwide. Cancer pain is observed in 30%–50% of all these new-onset patients. Most of the patients (60%–90%) with advanced cancer present different degrees of pain, of which 30% are with unbearable pain symptoms [1, 2]. The pathophysiology of cancer pain remains elusive and therapeutic approaches are very limited. Surgery, radiotherapy, and chemotherapy have been used as effective approaches to treating certain cancer pain, but pain in more than half of the cancer patients remains unrelieved [3]. Relieving cancer pain is critical to improving quality of life of the cancer patients. The "Three-Step Therapy" for treating cancer pain proposed by World Health Organization (WHO) thirty years ago has been widely implemented clinically and accepted by a majority of cancer patients and medical professionals, but the clinical effectiveness is still limited. Continuous efforts are needed to realize the goal of "relieving pain of all cancer patients." To effectively, safely, and economically control pain and to improve the quality of life for the patients with cancer pain are important issues of global concern.

Traditional Chinese medicine is an alternative, effective therapeutic measure for treating cancer pain and has potential to overcome the shortcomings of the "Three-Step Therapy" by enhancing treatment effect and reducing toxicity

of morphine, the core drug used in the WHO "Three-Step Therapy" [4]. A traditional Chinese medicine named Xiao-Ai-Tong (XAT), a prescription medication (decoctio) in Chinese, consists of Corydalis Tuber (tuber of *Corydalis* turtschaninovii, *yanhusuo*), wild ginger (*Asarum*), *Arisaema consanguineum*, Venenum Bufonis, and Arisaema Tuber (Tian-Nan-Xing, Rhizoma Arisaematis) and has been used in clinic for years for reducing chronic pain. However, there is no evidence-based research demonstrating analgesic effect of XAT on cancer pain patients. This clinical study aimed to evaluate the analgesic effect of XAT and its impact on morphine treatment-induced adverse reactions including abstraction, nausea/vomiting, fatigue, and anorexia and quality of life in patients with moderate-to-severe cancer pain. Our results indicate that XAT decoction can effectively alleviate bone cancer pain, suppress morphine-induced adverse actions, reduce the proinflammatory cytokines IL-1β and TNF-α, and increase the endogenous anti-inflammatory cytokine IL-10. This study supports a clinical use of XAT, alone or with morphine, in treating bone cancer pain.

2. Materials and Methods

2.1. Patient General Information. Patients with painful bone metastasis received treatment at Lianyungang City Oriental Hospital. The clinical trials were conducted according to Declaration of Helsinki principles, approved by the ethics committee of Lianyungang Oriental Hospital (number 20130910088) (2013). A total of 60 patients with painful bone metastasis were included in this study including 32 males (53.33%) and 28 females (46.67%), aged from 44 to 70 years (mean ± SD, 58.36 ± 7.31). These patients were randomly divided into four groups: (1) firstly, based on the time sequence, the 1st, 2nd, 3rd, and 4th patient recruited were included in groups 1, 2, 3, and 4, respectively. Subsequently, the 5th to 8th patients recruited were included in groups 1, 2, 3, and 4 again, respectively. Then this method of grouping was repeated for all the patients that were recruited later for this study. However, considering balance and similar proportion of male and female patients in each of the groups, the male and female patients were grouped in a separate time sequence in the same way as in the different groups. Four groups received four different treatments (see treatment procedures in Section 2.2). Each of the four groups included 15 patients with 8 males and 7 females.

Inclusion criteria for these patients were as follows: (1) pathologically diagnosed as cancer accompanied by painful osseous metastasis, requiring drug treatment; (2) with normal pain sensation and judgment (without intellectual or mental disorders); (3) the patient's pain self-evaluation Visual Analogue Scales (VAS) [5, 6] test score reaching 4 or higher; and (4) expected survival being longer than one month.

Exclusion criteria for these patients were as follows: (1) with dysfunctions of heart, liver, kidney, and other severe physical disorders; (2) with histories of psychiatric or analgesic drug abuse; (3) with history of significant respiratory depression, airway obstruction, hypoxia, or bronchial asthma; or (4) received radiotherapy and/or chemotherapy, and so forth.

2.2. Treatment Prescriptions and Procedures. Patients that received morphine treatment were taking morphine sulfate controlled-release tablets (each 10 mg tablet provided by Beijing Mundipharma Pharmaceutical Co., Ltd.), 20 or 30 mg, q12h, by oral administration for 7 consecutive days. Patients who received XAT treatment were taking XAT decoction containing Corydalis Tuber, wild ginger, *Arisaema consanguineum*, Venenum Bufonis, and Arisaema Tuber, 200 mL, q12h, by oral administration for 7 consecutive days. Two groups of patients received treatment of XAT (200 mL) + morphine (20 mg and 30 mg, resp., q12h, by oral administration for 7 consecutive days). The seven treatments in each group were applied daily during days 0–6.

2.3. Evaluation of Pain Intensity. Changes in pain intensity were evaluated by calculating changes of VAS scores [5, 6], which was used for patient's self-evaluation. VAS scores were ranging from 0 to 10 marked on a horizontal line, where 0 represented painless condition, 1–3 referred to mild pain, 4–6 denoted moderate pain, and 7–10 suggested severe pain. At the testing moment, the patients were asked to circle a number that best represented their pain. VAS test was given at 3 and 1 days prior to the treatment and once a day during 1–7 postoperative days following the 7 treatments during days 0–6. Two more tests were given on the 10th and 14th day after termination of the treatments.

2.4. Adverse Reactions due to Morphine Treatment. The in-patients sometimes showed adverse reactions such as expiratory dyspnea, changes in blood pressure, pale complexion, cold sweat, palpitation, headache, dizziness, lethargy, delirium, blurred vision, instability of gait, dryness, nausea, vomiting, constipation, bellyache, diarrhea, pruritus of skin, skin rashes, and dysuria. Morphine treatment resulted in or facilitated some of these adverse reactions. These signs were honestly recorded in each of the in-patient's profiles, but most of them were not included in the data show in this study. The data we monitored and showed here included four symptoms, nausea with or without vomiting, constipation, anorexia, and fatigue, which are expressed based on the analysis using the Quality of Life Questionnaire (EORTC QLQ-C30) [7]. This EORTC's scores were used for evaluating the changes of these adverse reactions and was given on day 7 and day 10, that is, 1–4 days after termination of the treatment.

2.5. Statistical Analysis. SPSS Rel 17 was used to conduct all the statistical analyses. Alterations of pain intensity following treatment over time among groups were tested with two-way ANOVA with repeated measures followed by Bonferroni post hoc tests, respectively. Differences in changes of the aversive reactions among groups were tested with one-way ANOVA. Data are presented as mean ± SEM with VAS tests or EORTC QLQ-C30 scores, except when indicated separately. Statistical results are considered significant if $P < 0.05$.

3. Results

The total of 60 patients with painful bone metastasis were included in this study including 32 males (53.33%) and 28 females (46.67%), aged from 44 to 70 years (mean ± SD, 58.65 ± 7.38). These patients were randomly divided into four groups (male and female patients were kept at similar proportion in each group). Each of the groups received different treatments and included 15 patients with 8 males and 7 females. Patients who received morphine treatment (morphine group) included 15 patients with 8 males aged 58.10 ± 7.14 and 7 females aged 58.04 ± 8.26. Patients who received XAT treatment (XAT group) included 15 patients with 8 males aged 57.60 ± 8.14 and 7 females aged 59.40 ± 7.09. Two groups of patients who received treatment of combination of XAT and morphine (in two different doses) included, in each group, 15 patients with 8 males aged 58.22 ± 8.45 and 57.15 ± 7.62 and 7 females aged 59.33 ± 6.53 and 58.87 ± 6.76, respectively. The data included here were from these 60 patients.

3.1. Repetitive Oral Administration of XAT Reduced Cancer Pain and Facilitated Analgesic Effect of Morphine. Patients in this study received treatment of morphine, XAT, and morphine + XAT, respectively. As expected, morphine treatment alone (30 mg, every 12 h, and oral administration for 7 consecutive days) greatly reduced cancer pain evidenced by the significantly decreased VAS scores. The analgesic effect quickly reached the peak level after the second dose and was maintained at this level for up to 14 days without obvious tolerance. Interestingly, repetitive oral administration of the Chinese medicine XAT (200 mL, every 12 h, and oral administration for 7 consecutive days) produced significant, time-dependent, gradually increased analgesic effect on the cancer pain. The significant analgesic effect was starting from the second day (XAT) and reached peak 5-6 days after the daily treatment. The peak analgesic effect was maintained for up to at least 14 days, that is, 8 days after termination of the last treatment. Analgesic effect of XAT was significantly less than that of morphine during the period of 1–5 days. However, during 6–14 days, the analgesic effect of XAT alone was much closer to that of morphine alone. Furthermore, administration of a combination of XAT with morphine at 20 mg produced significant analgesic effect, which was at the similar level of analgesia produced by morphine alone at 30 mg. Administration of a combination of XAT with morphine at 30 mg produced significantly greater analgesic effect than that produced by morphine alone at 30 mg, XAT with morphine at 20 mg, and XAT alone. These analgesic effects were maintained as the best level of analgesia for at least one week after termination of the last treatment. Data are summarized in Figure 1(a).

We also analyzed the possible sexual difference of analgesic effects of XAT and morphine on these patients (Figures 1(b)–1(d)). The data was from the same group of the patients in Figure 1(a). The data point at day 1 was as follows: the VAS scores for female patients in the groups of morphine treatment (30 mg) (Figure 1(b)) and morphine (30 mg) with XAT treatment (Figure 1(d)) were significantly lower than that of male patients, suggesting that these females had better response to the first-time analgesic treatment. However, the analgesic effects of XAT and morphine and their combinations were overall not significantly different between male and female patients (Figures 1(b)–1(d)).

3.2. Repetitive Oral Administration of XAT Reduced or Prevented Morphine-Induced Adverse Reactions. Given that repetitive oral administration of XAT reduced cancer pain and facilitated analgesic effect of morphine, we continued to analyze the possible effects of the Chinese medicine XAT on the adverse reactions accompanied with cancer pain and/or with morphine treatment. We used the Quality of Life Questionnaire (EORTC QLQ-C30) to score the adverse reactions in these patients. The higher the score, the worse the adverse reaction. The data showed that the scores of the gastrointestinal adverse reactions constipation and nausea were greatly increased after repetitive morphine treatment (morphine 30 mg, daily for 7 consecutive days). These morphine treatment-related adverse reactions were significantly prevented or reduced by the treatment of a combination of XAT with morphine (Figures 2(a) and 2(b)). Another two adverse symptoms, fatigue and anorexia, were found in all of these cancer pain patients before application of our treatment prescription, indicating that these two symptoms were because of the cancer and/or cancer pain. The scores of fatigue and anorexia were greatly reduced by repetitive XAT treatment, but not by morphine (Figures 2(c) and 2(d)).

3.3. Repetitive Oral Administration of XAT Inhibited Proinflammatory Cytokines IL-1β and TNF-α and Activated IL-10. We continued to examine whether the Chinese medicine XAT might affect the activity of the proinflammatory cytokines IL-1β and TNF-α, which are important in the development of inflammation and cancer pain, as well as the anti-inflammatory cytokine IL-10, which may inhibit inflammation and pain. Levels of these three cytokines in blood in these patients before application of our treatment prescriptions were 174 ± 25 pg/mL (IL-1β, Figure 3(a)), 297 ± 35 pg/mL (TNF-α, Figure 3(b)), and 4.70 ± 0.79 pg/mL (IL-10, Figure 3(c)), respectively. Repetitive treatment of XAT, morphine, or combinations of XAT and morphine, in the same protocols described above, greatly reduced the levels of IL-1β and TNF-α. Meanwhile, level of IL-10 was greatly increased following the treatments. Among these four different therapies, the combination of XAT and morphine produced the best modulatory effects on the three cytokines. Data are summarized in Figure 3.

4. Discussion

This study reveals that repetitive oral administration of the Chinese traditional medicine XAT decoction can suppress pain probably through modulation of cytokines and prevents adverse reactions of morphine treatment in bone cancer pain patients. The major findings are fourfold: (1) repetitive oral administration of XAT for 7 consecutive days greatly reduces bone cancer pain; (2) treatment with combination of XAT

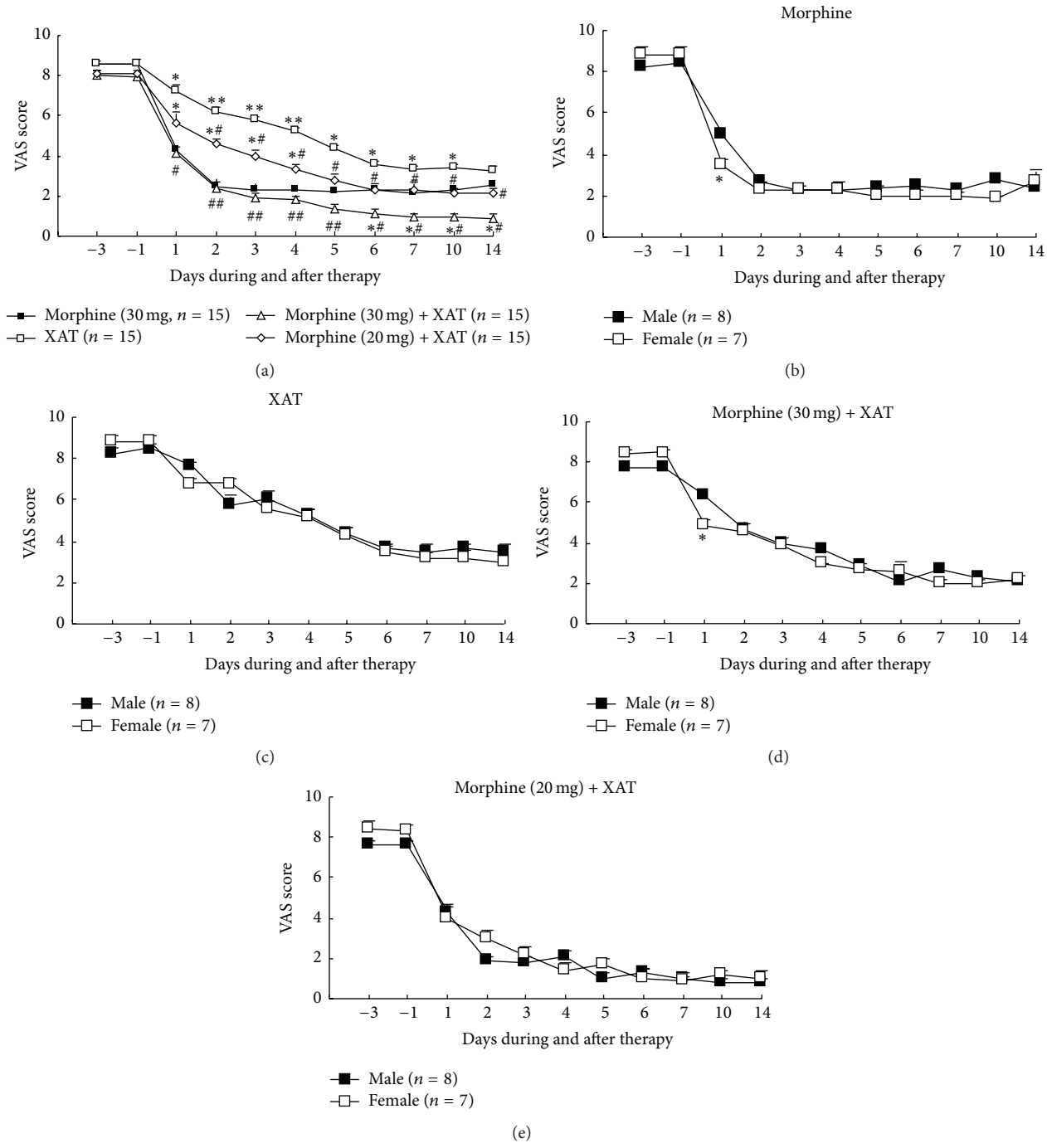

Figure 1: Repetitive oral administration of XAT reduced cancer pain and facilitated analgesic effect of morphine on patients with bone cancer pain. VAS test was used for evaluating the pain intensity. (a) Comparison of analgesic effects (VAS scores) of morphine, XAT, and combination of XAT and morphine. (b-c) Comparison of analgesic effects of morphine, XAT, and combination of XAT and morphine between males and females. Data are expressed as mean ± SEM. Number of patients in each group is indicated in the parentheses. Note that all of the patients indicated in (b-c) were from the same male and female patients' group in (a). The treatments in each group were applied daily for 7 consecutive days during days 0–6. Two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus morphine (a) or versus male (b, d). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus XAT (a).

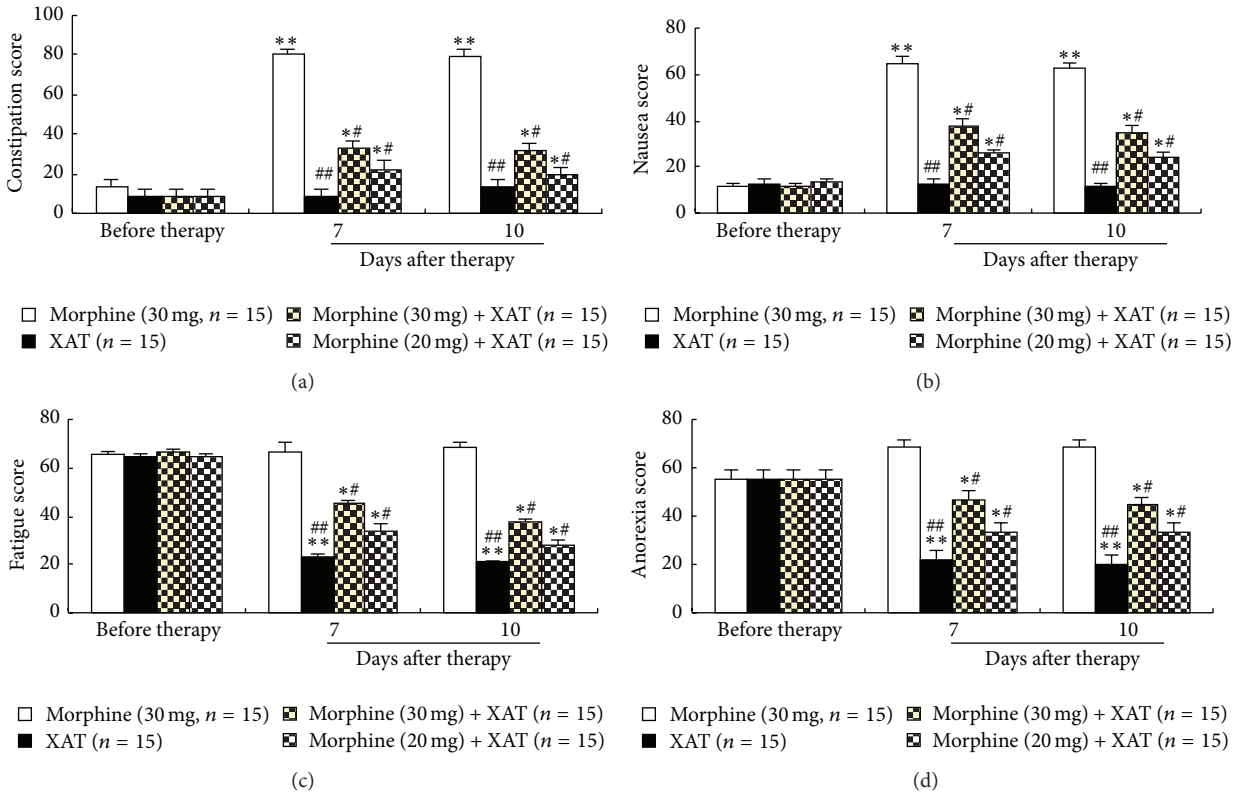

Figure 2: Effects of repetitive oral administration of XAT on the adverse reactions accompanied with cancer pain and/or morphine treatment. The Quality of Life Questionnaire (EORTC QLQ-C30) was used to score the adverse reactions. The higher the score, the worse the adverse reaction. These adverse reactions included constipation (a), nausea (b), fatigue (c), and anorexia (d). Comparisons were made among groups of morphine, XAT, and combination of XAT and morphine at two different doses. Data are expressed as mean ± SEM. Number of patients in each group is indicated in the parentheses and all of the patients indicated in (a–d) were the same patients included in Figure 1(a) and they received the same treatments in the same protocols in the corresponding groups. The evaluation was made on day 7 and day 10, that is, 1 and 4 days after termination of the last treatment. One-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the same group before therapy. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus morphine after therapy in the corresponding group.

and morphine produces synergistic analgesic effects; (3) XAT can prevent or alleviate the gastrointestinal adverse reactions constipation, nausea, fatigue, and anorexia accompanied with cancer pain and/or caused by morphine treatment; and (4) XAT treatment significantly reduces the proinflammatory cytokines IL-1β and TBF-α and increases the endogenous anti-inflammatory cytokine IL-10 in blood. These results demonstrate that XAT has a strong capacity of relieving bone cancer pain probably at least partly through its modulation of cytokines in patients as well as a capacity of antigastrointestinal adverse reactions, caused by cancer/cancer pain and/or morphine treatment. This study supports a use of XAT decoction in treating bone cancer pain, alone or with morphine, in clinic.

Our clinical trials indicate that the Chinese medicine XAT decoction can be used as an effective analgesic for treating bone cancer pain and the associated adverse reactions such as fatigue and anorexia in cancer patients. Further, if used together with morphine, XAT can facilitate the analgesic effect and reduce the side effects including constipation and nausea caused by morphine treatment. This study supports that XAT may be a good candidate for being used alone or with morphine in treating cancer pain in clinic and suggests a general idea that an integrated treatment measure combining certain Chinese herbs or other therapeutic measures such as acupuncture with opioids such as morphine may be a great strategy for treating cancer pain and thus improve the treatment effect of WHO "Three-Step Therapy" for cancer pain. It is an ideal strategy to have effective pain relief with minimal side effects, which greatly enhances quality of life of the patients, particularly for the severe terminal cancer patients.

Cancer pain is a severe, lifelong, intractable chronic pain [1–3]. Pathophysiology of cancer pain is very complicated and involved at least in two complex mechanisms, that is, neuropathic and inflammatory pain mechanisms [3, 8, 9]. Studies have confirmed that bone cancer pain is associated with the tumor itself, primary or metastatic bone cancer [8–10], and changes in the local bone tissues [11, 12]. Bone cancer pain may be initiated by compression and stimulation of the neighboring sensory nerves by tumor tissues, tumor cell, and related immune cell released cytokines, which sensitize the nociceptors, bone deconstruction, and local ischemia as well as hypoxic microenvironment caused by the tumor [13, 14].

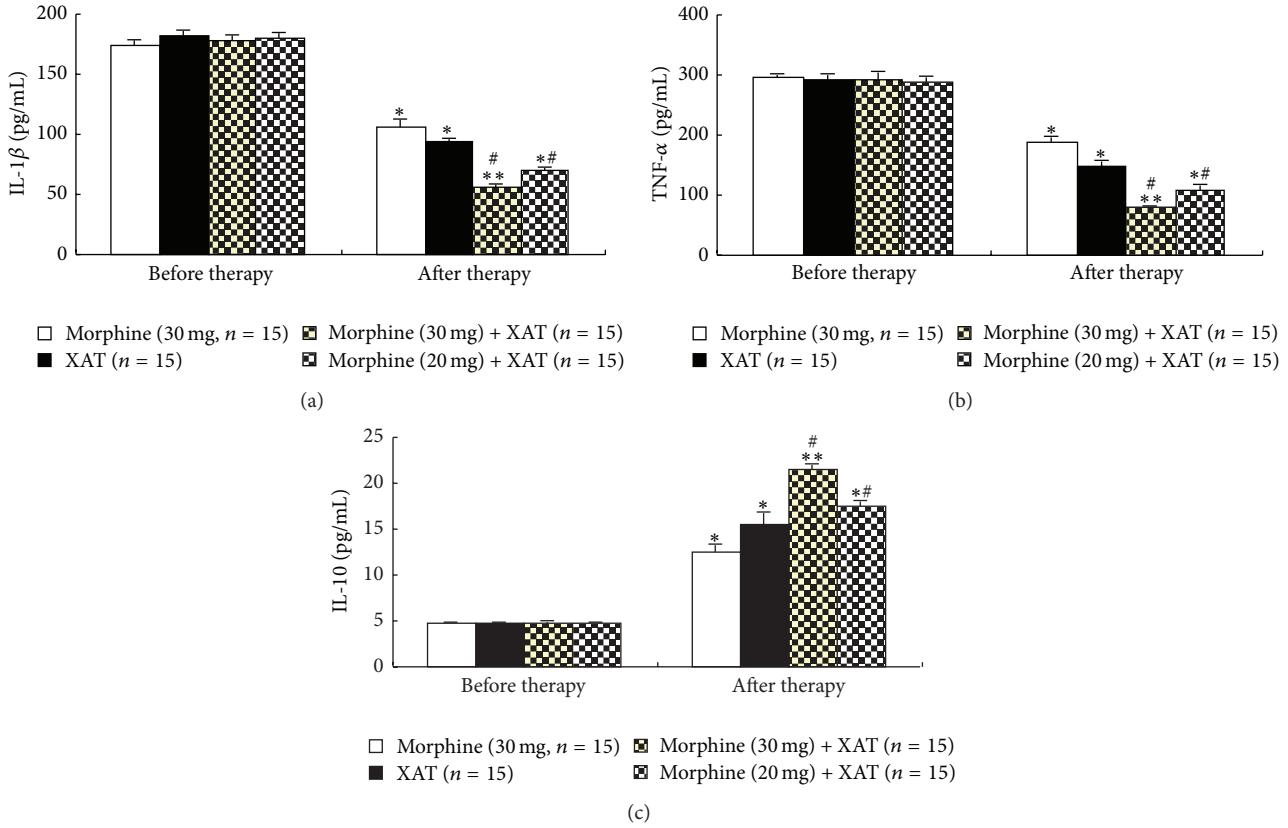

Figure 3: Effects of repetitive oral administration of XAT, morphine, and their combinations on the blood cytokines IL-1β, TNF-α, and IL-10. ELISA was used to measure these cytokines in blood from the cancer pain patients. Number of patients in each group is indicated in the parentheses and all of these patients were the same patients included in Figure 1(a) and they received the same treatments in the same protocols in the corresponding groups. The blood samples were collected on day 7, that is, 1 day after termination of the last treatment. One-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the same group before therapy. $^{\#}P < 0.05$ versus morphine after therapy in the corresponding group.

All of these elements contribute greatly to the development of cancer pain. The complex mechanisms of cancer pain result in a big trouble to well control the cancer pain in clinic.

When we are pursuing better strategy to treat cancer pain, some of the Chinese medicines may provide us with a different view. According to traditional Chinese medicine, the primary pathogenesis of cancer pain involves "stagnation of pneuma and blood" or "malnutrition." "Malnutrition" is primarily attributed to renal deficiency unlikely to promote bone marrow generation. "Stagnation of pneuma and blood" is mainly associated with the Qi stagnation due to severe cold and blood stasis [2]. Cancer pain has been brilliantly described in ancient medical books of traditional Chinese medicine. It was described in *The Medicine of The Yellow Emperor* as "bones becoming dry and brittle, muscles becoming emaciated, thoracic fullness, asthmatic inconvenience, inner pain inducing shoulder and neck pain." Based on the idea of the traditional Chinese medicine, we believe that the cancer pain may be caused by accumulation of toxicity, blood stasis and vital energy retardation, and accumulation of phlegm-dampness in the meridians, and thus resulting in pneuma and blood stasis, which contribute to development of cancer pain. The therapeutic effects of traditional Chinese medicines, in a concept, have been confirmed, as persistent, nonadditive and nonresistant drugs. Some analgesic herbs can be used alone to treat cancer pain in the first and second steps of the WHO "Three-Step Therapy" and considered as a preferred combination in the third step of the "Three-Step Therapy" to reduce the dose of opioid analgesics and the toxicity. In this study, we recommend that XAT decoction, which contains Corydalis Tuber, wild ginger, *Arisaema consanguineum*, Venenum Bufonis, and Arisaema Tuber, may be used in clinic in treating cancer pain. Actually, recent studies have shown that certain Chinese herbs such as the Corydalis Rhizoma, named Yuanhu in Chinese, have been proved to have analgesic effect on patients and on small animals with neuropathic pain as well as other treatment effects [15–19]. Our clinical observations here demonstrate that XAT, containing Corydalis Rhizoma and so forth, used alone and with combination of morphine, has a great value for treating bone cancer pain in patients. XAT may reduce cancer pain, reduce doses of morphine, and diminish the adverse reactions caused by morphine.

Studies have demonstrated that astrocytes and microglial cells, which act as parts of the innate immune system, become active in the status of bone cancer and release various

substances, including the proinflammatory cytokines IL-1β and TNF-α, which could evoke hyperalgesia and allodynia [20–25]. In this study, we show that repetitive oral administration of XAT decoction for a week can greatly suppress the increased IL-1β and TNF-α associated with the cancer pain. Meanwhile, XAT therapy significantly increases the anti-inflammatory cytokines IL-10. These findings may provide an explanation for the analgesic effect of XAT. Further large-scale clinical studies as well as studies in small animals for underlying the possible mechanisms of analgesia of XAT are urgently needed.

5. Conclusions

This clinical trial indicates that the Chinese medicine XAT decoction is an effective analgesic for treating chronic pain and can alleviate certain adverse reactions such as fatigue and anorexia associated with bone cancer pain and, if used with morphine, can prevent or reduce repetitive morphine treatment-induced constipation and nausea. This study supports a clinical use of XAT decoction in patients with bone cancer pain and proposes a general idea that a combination of certain Chinese herbs with opioids may be a better strategy for treating cancer pain and the morphine-treatment associated adverse reactions, thus improving the treatment effect by the WHO "Three-Step Therapy" for cancer pain. It is an ideal strategy to have effective pain relief with minimal side effects that greatly enhances quality of life of the patients, particularly for the terminal cancer patients with severe pain.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yan Cong, Kefu Sun, and Xueming He contributed equally to this study.

Acknowledgments

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Research Article

Inducible Lentivirus-Mediated siRNA against TLR4 Reduces Nociception in a Rat Model of Bone Cancer Pain

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Although bone cancer pain is still not fully understood by scientists and clinicians alike, studies suggest that toll like receptor 4 (TLR4) plays an important role in the initiation and/or maintenance of pathological pain state in bone cancer pain. A promising treatment for bone cancer pain is the downregulation of TLR4 by RNA interference; however, naked siRNA (small interference RNA) is not effective in long-term treatments. In order to concoct a viable prolonged treatment for bone cancer pain, an inducible lentivirus LvOn-siTLR4 (tetracycline inducible lentivirus carrying siRNA targeting TLR4) was prepared and the antinociception effects were observed in bone cancer pain rats induced by Walker 256 cells injection in left leg. Results showed that LvOn-siTLR4 intrathecal injection with doxycycline (Dox) oral administration effectively reduced the nociception induced by Walker 256 cells while inhibiting the mRNA and protein expression of TLR4. Proinflammatory cytokines as TNF-α and IL-1β in spinal cord were also decreased. These findings suggest that TLR4 could be a target for bone cancer pain treatment and tetracycline inducible lentivirus LvOn-siTLR4 represents a new potential option for long-term treatment of bone cancer pain.

1. Introduction

Cancer-induced bone pain, characterized by spontaneous pain, hyperalgesia, and allodynia, is estimated to affect about 36%–50% of cancer patients [1]. Severe and long-lasting pain brings agonies to people's daily life, especially for those terminal cancer patients [2]. However, the treatment of bone cancer pain remains a clinical challenge. New methods are urgently needed for this worldwide problem.

Bone cancer pain is considered to be mechanistically unique compared with inflammatory or neuropathic pain states [3]. The activation and upregulation of glial cells in the spinal cord play an important role in initiation and/or maintenance of pathological pain state in bone cancer pain [4, 5]. One of the neuron-to-glial activation signals has proposed that proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, were released via the microglial TLR4 receptor in a rat model of bone cancer pain [6]. Administration of a potent TLR4 antagonist (FP-1) reduced both thermal hyperalgesia and mechanical allodynia in the chronic constrictive injury (CCI) models of mice [7]. Thus, blocking the TLR4 signaling pathway might be a useful way of treating bone cancer pain.

RNA interference (RNAi) technique, a promising and potent gene-silencing method, has demonstrated a clinical potential of treating chronic pain by synthetic small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) [8]. The key point of this technique is to obtain small interfering RNAs with high "knockdown" efficiency. In the previous work, we screened siRNA sequence with reporter vector and obtained a siRNA against TLR4 with high efficiency [9]. Intrathecal injection of this TLR4 siRNA reduced nociception in a neuropathic pain model of rats [9]. However, the antinociception only lasted for 4 days with naked siRNA. For prolonged antinociception effect, a lentiviral system was addressed in the present study. Meanwhile, for controlling of the targeted gene expression, a tetracycline-regulated gene expression (Tet-on) system was addressed to regulate the expression of small RNA of TLR4. In this system,

the targeted gene expression is turned on under the control of doxycycline or tetracycline (Tet). Thus, the antinociception of siRNA expressed by inducible lentivirus was detected in a bone cancer pain model of rats and the inducible effect of doxycycline was observed.

2. Materials and Methods

2.1. Production and Identification of Inducible Lentivirus LvOn-siTLR4. The siRNA (GUCUCAGAUAUCUAGAUCU) against TLR4 gene (GenBank accession NM_019178) was screened and tested as described in our previous study [9]. Based on the "Tuschl" principle and the sequences of the inducible lentiviral vector, target sequences were designed and chemically synthesized in United Gene Company (Shanghai, China). The target sequences were then cloned into plasmid pLenR-TRIP and named pLenR-TRIP-TLR4. To produce recombinant inducible lentivirus LvOn-siTLR4 (lentivirus expressing siRNA of TLR4), pRsv-REV (20 μg), pMDlg-pRRE (15 μg), and pMD2G (10 μg) were cotransfected into HEK 293T cells with Lipofectamine 2000 [10, 11]. About 48 h after transfection, the lentivirus was harvested. The final titer of recombinant lentivirus was adjusted to 1×10^9 TU/mL.

2.2. Induction of Bone Cancer. Bone cancer was induced by Walker 256 cells (breast tumor cells) as we previously described [12]. The left leg was shaved and the skin was disinfected with 70% (v/v) ethanol, after rats were anesthetized by intraperitoneal administration of sodium pentobarbital (40 mg/kg). A rostrocaudal incision of 1 cm was made in the skin over the lower one-third of the tibia for clear exposure with minimal damage to nerves and muscles. The medullary canal was approached by inserting a 23-gauge needle proximally through a hole drilled in the tibia. The needle was then replaced with a 20 μL microinjection syringe. A 10 μL volume of Walker 256 cells (2×10^5 cells) or boiled cells (sham group) was injected into the bone cavity. The syringe was removed after a 2 min delay allowing cells to fill the cavity. The drill hole was sealed with bone wax and the wound was closed using 1-0 silk threads. The rats were allowed unrestricted movement in the cages after recovery and the general condition of rats was monitored during the experiment.

2.3. Lumbar Subarachnoid Catheterization. Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). A PE-10 catheter (Becton Dickinson, Sparks, MD, USA) was inserted into the lumbar subarachnoid space between lumbar vertebrae 5 (L5) and L6 [13]. The catheter was chronically implanted and the external part of the indwelling catheter was protected according to Milligan's method [14]. A lidocaine test was given to determine the functionality and position of the catheter tip in the subarachnoid space.

2.4. Intrathecal Delivery of Lentivirus. Rats were randomly divided into 6 groups ($n = 60$ per group): a sham group (sham surgery + normal saline), a normal saline (NS) group (cancer + NS), an Lv-MM group (cancer + Lv-MM), an LvOn-siTLR4 group (cancer + LvOn-siTLR4 + NS), a Dox group (cancer + Dox), and an LvOn-siTLR4 with Dox group (cancer + LvOn-siTLR4 + Dox). Lentivirus Lv-MM expressing scrambled siRNA (TTCTCCGAACGTGTCACGT) was used as a control. Four days after cancer cells injection, rats in the LvOn-siTLR4 group and LvOn-siTLR4 with Dox group were given the virus LvOn-siTLR4 (1×10^7 TU/10 μL), respectively. In the Lv-MM group, the same titer of the lentivirus Lv-MM was given intrathecally as a control. The normal saline of equal volume was administered intrathecally in rats of remaining 3 groups. In the Dox group and LvOn-siTLR4 with Dox group, doxycycline was given orally.

2.5. Mechanical Allodynia Test. To assess mechanical hyperalgesia, animals were acclimated daily for 10 min/day to the test environment during 3 days, which was a Plexiglass box on a metal grid surface. On test days, rats were allowed to acclimate for 5–10 min. The nociceptive stimulus, a single rigid filament attached to a hand-held transducer (Electronic von Frey Anesthesiometer; IITC, Woodland Hills, CA), was applied perpendicularly to the medial surface of the hind paw with increasing force. The endpoint was taken as nocifensive paw withdrawal accompanied by head turning, biting, and/or licking. As soon as this reaction occurred, the required pressure was indicated in grams, and this value was considered to be the individual paw withdrawal threshold (PWT) value. Each rat was tested in triplicate per time point and the average for the three measurements was then calculated.

2.6. Spinal Cord RNA Extraction and Real-Time PCR. The real-time PCR was performed on the 1st, 3rd, 7th, 14th, and 21st days after intrathecal injection of the virus. Total RNA (6 samples of each group) was extracted from L4-L5 spinal cord. Extracted RNA was treated with DNase I at 37°C for 30 min before reverse transcription was performed using a kit (TaKaRa, Japan). The PCR primers were as follows: 5′-CGGGAG CTC TGA ATG CTC TCT TGC ATC TGG CTG GC-3′ (forward) and 5′-CGG GTC GAC GCC ATA CAA TTC GACCTG CTG-3′ (reverse). The Real-Time PCR Detection System (Roche, Switzerland) continually monitors the increase in fluorescence, which is directly proportional to the PCR product [15].

2.7. Western Blot Assay. The proteins of tissues from lumbar spinal cord (L4-L5) were prepared on the 7th day after injection as previously described [16]. Proteins were separated by 8% polyacrylamide SDS-PAGE and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blotted with a primary antibody against TLR4 (1 : 100, Santa Cruz, USA) and then with secondary antibody conjugated with horseradish peroxidase. Protein signals were detected with an ECL system (Amersham Pharmacia, Uppsala, Sweden). GAPDH (Sigma Chemical Co., MO, USA, 1 : 500) was used as a loading control. The images were captured and analyzed by ImageJ software.

2.8. Enzyme Linked Immunosorbent Assay (ELISA). To detect TNF-α and IL-1β proteins, samples from the spinal cord (L4-L5) were analyzed by ELISAs specific for these cytokines. The samples were prepared on the 1st, 3rd, 7th, 14th, and 21st days after intrathecal injection of the virus as previously described [17]. The ELISAs for TNF-α and IL-1β in the spinal tissue were performed according to the manufacturer's instructions (Peprotech, UK). Total protein concentrations of TNF-α and IL-1β were determined by the Bradford assay and used to adjust results for sample size [18].

2.9. Statistical Analysis. All data were expressed as mean ± standard error (SEM). Statistical analysis was carried out using two-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism software (Version 5. GraphPad Software Inc., CA, USA). The data from western blotting was compared using one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LvOn-siTLR4 with Dox Attenuated Bone Cancer Pain. To examine the impact of inducible lentivirus LvOn-siTLR4 on pain response *in vivo*, modulation of pain perception in the bone cancer pain model was investigated. PWT was used to measure the mechanical allodynia. After surgery, mechanical allodynia was induced, in correspondence with the reduced PWT. Compared with that in the sham group, mechanical allodynia significantly increased in the rats receiving Walker 256 cells injection ($P < 0.01$, $N = 10$, Figure 1). In contrast to the Lv-MM group and NS group, mechanical allodynia was decreased in the LvOn-siTLR4 with DOX group on the 3rd, 7th, 14th, and 21st days after viral injection ($P < 0.01$), which suggested that the small RNA expressed by the lentivirus was effective. Meanwhile, in the LvOn-siTLR4 group, rats without Dox had no effect in mechanical allodynia, which illustrated that the expression of small RNA was induced by Dox. Moreover, PWT did not change in the Dox group compared to that in the NS group, suggesting that Dox did not contribute to the mechanical allodynia. The process lasted for about 21 days, which indicated that the antinociception effect of LvOn-siTLR4 was long-lasting.

3.2. LvOn-siTLR4 with DOX Decreased TLR4 Expression. LvOn-siTLR4 with DOX was intrathecally delivered into the rats with bone cancer pain and protein expressions of TLR4 and its mRNA were detected. As shown in Figure 2, TLR4 mRNA expression was increased significantly in the rats which received Walker 256 cells injection compared with that in the sham group ($P < 0.01$, $N = 6$). Similar results were shown in the protein expression, which suggested that TLR4 increased in the bone cancer pain models (Figure 3). TLR4 and its mRNA expressions were decreased in the LvOn-siTLR4 with DOX group compared with that in other four groups of bone cancer pain, suggesting that lentivirus expressed small RNA of TLR4 interfered TLR4 expression.

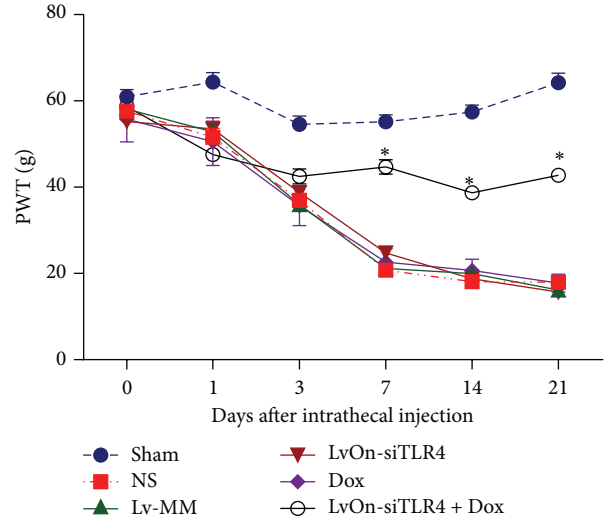

FIGURE 1: Impact of LvOn-siTLR4 with Dox on PWT in bone cancer pain rats. The bone cancer pain was set up by the injection of Walker 256 cells into the legs of rats. After cancer cells injection, rats received intrathecal administration of the virus on the 4th day. On the 7th, 14th, and 21st days after intrathecal administration of the virus, bone cancer pain rats receiving intrathecal LvOn-siTLR4 with oral administration of doxycycline showed significantly attenuated mechanical allodynia compared to the rats treated with Lv-MM, normal saline, doxycycline, and LvOn-siTLR4 on PWT ($^*P < 0.01$ versus Lv-siTLR4 group, Lv-MM group, NS group, and Dox group, two-way ANOVO analysis followed by Tukey's multiple comparisons, $N = 10$).

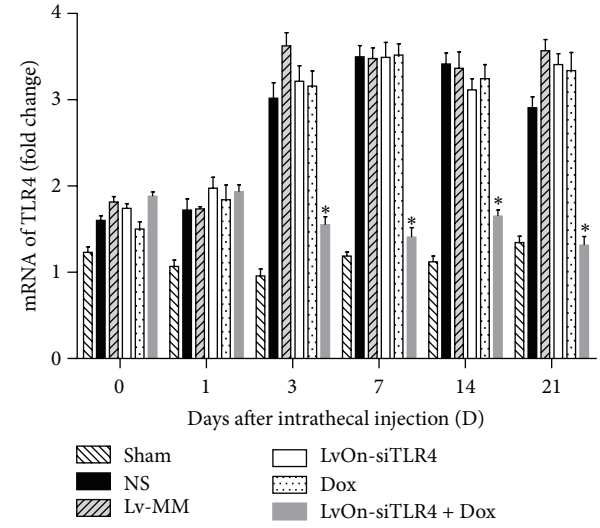

FIGURE 2: mRNA expression of TLR4 detected by real-time PCR. As shown in Figure 2, bone cancer in legs increased the TLR4 mRNA expression in the spinal cord in the five groups receiving Walker 256 cells injection. On the 3rd, 7th, 14th, and 21st days after delivery of Lv-siTLR4 with oral administration of doxycycline, the TLR4 mRNA expression decreased significantly in the Lv-siTLR4 with Dox group compared the other four groups receiving Walker 256 cells injection ($^*P < 0.01$ versus Lv-siTLR4 group, Lv-MM group, NS group, and Dox group, two-way ANOVO analysis followed by Tukey's multiple comparisons, $N = 6$). No differences were observed on the 1st day after injection.

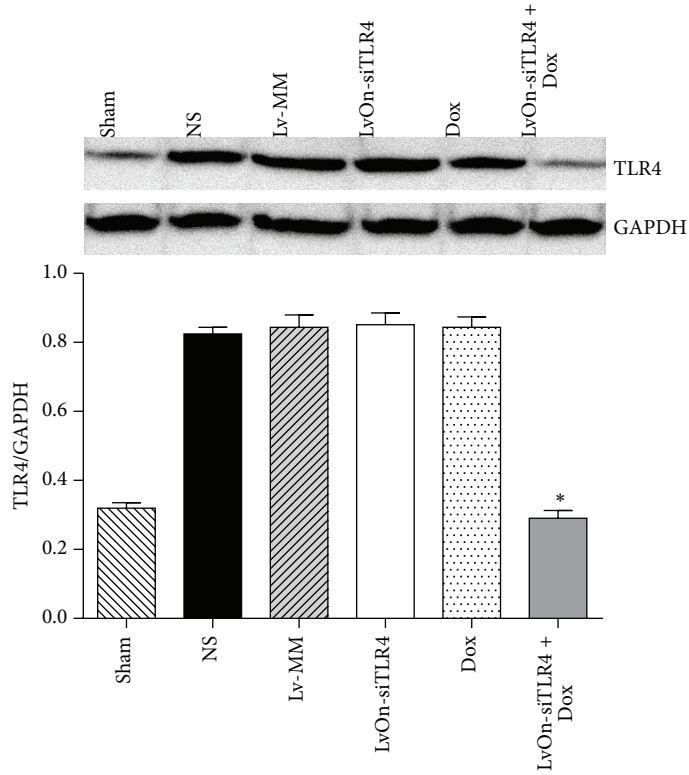

Figure 3: Western blot assay of TLR4 expression. On the 7th day after viral injection, the protein of lumbar spinal cord was prepared and the expression of TLR4 was detected. The protein expression of TLR4 in the Lv-siTLR4 with Dox group was also markedly downregulated compared to the other four groups receiving Walker 256 cells injection ($^{*}P < 0.01$ versus Lv-siTLR4 group, Lv-MM group, NS group, and Dox group, one-way ANOVA analysis, $N = 6$).

3.3. LvOn-siTLR4 with DOX Decreased TNF-α and IL-1β. To investigate whether the antiallodynia effects of the inducible lentivirus were associated with decreased production or release of proinflammatory cytokines, protein levels of TNF-α and IL-1β were assessed in the spinal cord. As shown in Figures 4(a) and 4(b), rats with bone cancer pain markedly induced upregulation of TNF-α and IL-1β ($P < 0.01$ versus sham group). In the LvOn-siTLR4 with Dox group, small RNA expressed by the LvOn-siTLR4 significantly inhibited the release of IL-1β and TNF-α in the spinal cord ($P < 0.01$ versus other four groups). These results indicated that TLR4 small RNA expressed by the virus LvOn-siTLR4 with DOX could disrupt the release of TNF-α and IL-1β.

4. Discussion

In this study, the effects of nociception on bone cancer pain models were investigated by inducible lentivirus expressing siRNA against TLR4. Based on our results, not only could LvOn-siTLR4 attenuate mechanical allodynia by intrathecal injection, but also its induction significantly downregulated mRNA and protein expression of TLR4 in the spinal cord. IL-1β and TNF-α release were significantly inhibited by small RNA expressed by LvOn-siTLR4 as well. In addition, the small RNA expression by LvOn-siTLR4 could be induced by oral administration of doxycycline. Our findings suggest that the lentivirus-mediated siRNA against TLR4 may be used for gene therapy of bone cancer pain in an experimental setting. If this is successful, the downregulation of TLR4 expression by inducible lentivirus LvOn-siTLR4 may be used to treat bone cancer pain in clinic.

It has been reported that the glial activation in the spinal cord is involved in bone cancer pain [19]. The activated glia and subsequent release of proinflammatory mediators have been implicated in initiating and maintaining pain response. TLR4 has shown mechanistic links between glial activation, innate immunity, and the initiation of behavioral hypersensitivity [20]. Through the activation of TLR4-MyD88-dependent or -independent pathways [21, 22], TLR4 activates intracellular signal molecules including TNF receptor-associated factor 6 and IKKα, β (IκB kinase) to form a complex and phosphorylated IκB [23]. The phosphorylation leads to the degradation of IκB and subsequent translocation of NF-κB, which controls the releasing of proinflammatory cytokines including TNF-α and IL-1β [24]. The downregulation of TLR4 by small RNA leading to decrease in the TNF-α and IL-1β was confirmed in the present study, which was consistent with the former research where blocking the TLR4 decreased the expression of TNF-α and IL-1β [15]. Activation of the TLR4 pathway in the spinal cord is proven to contribute to the neuropathic pain [7, 25, 26] and the suppression of TLR4 protein expression by siRNA could

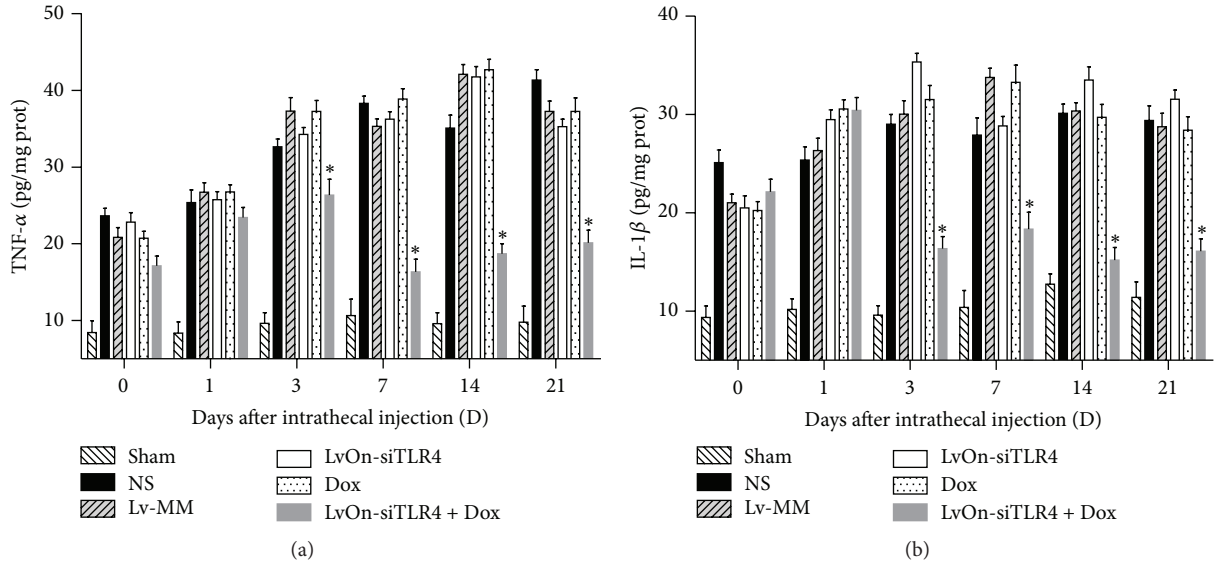

Figure 4: Effects of LvOn-siTLR4 with Dox on proinflammatory cytokine in spinal cord. The dorsal spinal cord tissue samples were prepared on the 1st, 3rd, 7th, 14th, and 21st days after intrathecal injection of the virus for the detection of TNF-α and IL-1β expression. A significant increase of TNF-α (a) and IL-1β (b) in the spinal cord was shown in the five groups receiving Walker 256 cells injection. On the 3rd, 7th, 14th, and 21st days after delivery of Lv-siTLR4 with oral administration of doxycycline, the TNF-α (a) and IL-1β (b) expression decreased markedly in the Lv-siTLR4 with Dox group compared to the other four groups ($^*P < 0.01$ versus Lv-siTLR4 group, Lv-MM group, NS group, and Dox group, two-way ANOVA analysis followed by Tukey's multiple comparisons, $N = 6$). No differences were observed on the 1st day after injection.

alleviate hyperalgesia and allodynia in CCI model of rats [9]. The present study showed the reduction of TLR4 and attenuation of allodynia after intrathecal injection of the lentivirus expressing small RNA. It is consistent with the former report that tactile allodynia in rats with bone cancer could be attenuated by the blocking TLR4 [6]. All these suggested that TLR4 signal pathway may involve bone cancer pain and that attenuation of TLR4 may probably be a new treatment of bone cancer pain.

In the application of RNAi technique, the efficiency, specificity, and stability of siRNA in target cells should be considered [27, 28]. Our previous work has showed the specificity and efficiency of small RNA in targeting of TLR4 [15]. However, naked siRNA mediated downregulation of gene expression is not stable and only lasts for 3 to 5 days [9]. Hence, the lentivirus, capable of expressing the target gene for several months and suitable for bone cancer pain treatment [29, 30], was introduced as a tool for siRNA. As hypothesized, results showed that the antinociception effect lasted for about 21 days, which provided a suitable tool for the treatment of bone cancer pain as a chronic state. Our previous work has shown that the lentivirus was successfully transfected into dorsal horn of rats after intrathecal injection [31], which was consistent with the location of TLR4 [32]. The results showed that LvOn-siTLR4 intrathecal injection along with oral administration of doxycycline attenuated allodynia of rats, which suggested that the lentivirus LvOn-siTLR4 could suppress nociception in the bone cancer models through decreasing the expression of TLR4.

For further clinical usage, the levels and timing of TLR4 expression need to be regulated to prevent overreduction of target gene. In the current research, tetracycline-regulated gene expression (Tet-on) system was introduced into the lentivirus. The tetracycline (tet) repressor was fused to a herpes simplex virus (HSV) VP 16 transactivation domain in this system to form a reverse tet-controlled transcriptional activator (rtTA) [33, 34]. In this case, in the presence of Dox or Tet, rtTA is able to bind operator sequences to activate transcription, turning on the target gene expression [35, 36]. Our results showed that TLR4 and its mRNA expressions were decreased after injection of the virus LvOn-siTLR4 with oral administration of Dox, while rats without Dox had no effect on TLR4 and its mRNA. The results illustrated that the expression of small RNA by LvOn-siTLR4 was under the control of Dox. In this case, the timing and levels of TLR4 could be controlled precisely by oral administration of doxycycline to maintain protein concentrations within a therapeutic window for further clinical practice.

However, TLR4 expression regulated by oral administration of doxycycline in a dose-dependent manner is needed. Further researches are warranted.

5. Conclusion

In the present study, we showed that downregulation of TLR4 by intrathecal injection of inducible lentivirus LvOn-siTLR4 could prevent bone cancer-induced tactile allodynia and that TLR4 expression could be controlled by doxycycline in regulation of the lentivirus. Our study provided a new approach of bone cancer pain treatment by downregulation of TLR4 expression using an inducible lentivirus LvOn-siTLR4.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ruirui Pan and Huiting Di contributed equally to this work.

Acknowledgment

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Clinical Study

Efficacy and Safety of Ropivacaine Addition to Intrathecal Morphine for Pain Management in Intractable Cancer

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Objective. Although intrathecal drug infusion has been commonly adopted for terminal cancer pain relief, its adverse effects have made many clinicians reluctant to employ it for intractable cancer pain. The objective of this study is to compare the efficacy and security of an intrathecal continuous infusion of morphine and ropivacaine versus intrathecal morphine alone for cancer pain. *Methods*. Thirty-six cancer patients received either a continuous morphine ($n = 19$) or morphine and ropivacaine ($n = 17$) infusion using an intrathecal catheter through a subcutaneous port. Numerical Rating Scale (NRS) scores and the Barthel Index were analyzed. Adverse effects and complications on postoperative days 1, 3, 7, and 15 were also analyzed. *Results*. All patients experienced pain relief. Compared to those who received morphine alone, patients receiving morphine and ropivacaine had significantly lower postoperative morphine requirements and higher Barthel Index scores on the 15th postsurgical day ($P < 0.05$). Patients receiving morphine and ropivacaine had lower NRS scores than patients receiving morphine alone on postoperative days 1, 3, 7, and 15 ($P < 0.05$). Negative postsurgical effects were similar in both groups. *Conclusions*. Morphine and ropivacaine administration through intrathecal access ports is efficacious and safe and significantly improves quality of life.

1. Introduction

Despite guidelines and recommendations, many patients with cancer still have inadequate pain control [1]. Many patients experience immense pain prior to their death. However, the intravenous or oral dosage of opioids for pain control results in unacceptable sedation [2]. Intrathecal therapy is advocated for patients with severe cancer pain [3].

Intrathecal injection of opioids has already been successfully utilized as a front-line treatment for cancer pain refractory to traditional treatment [4]. Intrathecal morphine has been found to be nonsedative or minimally sedative in multiple studies without the negative effects of parenteral or oral opioids [5]. Cancer pain refers to symptoms resulting from inflammation, compression, and neurological and ischemic damage at various sites [6]. According to the clinical symptoms reported, many patients who have somatic pain also have neuropathic pain. Although opioids appear to be effective in overall pain control, neuropathic pain resulting from major dysfunction of the somatosensory system [7] may be less likely to respond to opioid therapy [8]. In these conditions, opioid doses are continually increased [9]. However, increased opioid doses are associated with unsatisfactory negative effects. Therefore, the combination of opioids with other drugs, such as local anesthetics, must be considered.

Morphine exerts effects against pain by binding to the μ, δ, and κ opiate receptors [10], which stimulates potassium influx, giving rise to postsynaptic neuron membrane hyperpolarization in the dorsal horn of the spinal cord [11]. Voltage-sensitive calcium influx is decreased, thereby decreasing the neurotransmitter release from presynaptic terminals [12]. Ropivacaine, an amino-amide local anesthetic, blocks the generation and conduction of nerve impulses through blockade of sodium influx [13]. Studies have indicated that ropivacaine promotes the effects of intrathecal opioids [14]. Despite existing documentation and studies for the usage of intrathecal ropivacaine mixed with morphine [15], this treatment is still controversial.

Table 1: Demographics and clinical data*.

	Morphine alone	Morphine + ropivacaine
Age (years)	64.0 ± 13.4	62.6 ± 9.92
Sex (male/female)	11/8	14/3
Cancer type (*N*)		
Bladder	2	1
Bile duct	1	0
Colon	2	1
Duodenum	1	1
Esophagus	1	2
Kidney	0	1
Liver	1	2
Lung	3	4
Pancreas	1	0
Penis	1	0
Sarcoma	1	0
Stomach	2	3
Ureter	2	1
Uterus	1	1
Location of pain (*N*)		
Chest	2	3
Abdomen	7	6
Lower limb		
Preoperative systemic opioid type (*N*)	10	8
Morphine	4	2
Oxycodone	10	11
Fentanyl	5	4

*Data from 36 patients.

Few studies have utilized an intrathecal continuous injection of ropivacaine with morphine to treat cancer pain [16]. The objective of this study was to determine the efficacy and safety of continuous ropivacaine and morphine injection using intrathecal access ports in patients suffering from cancer pain refractory to traditional treatment modalities.

2. Materials and Methods

2.1. Patient Selection. This was a double-blind, random clinical study conducted at our hospital. The institutional ethics committee was used to assign patients and provide advice regarding ethical issues. The study was approved by the institutional review board, which also confirmed informed consent by the patients. This study included thirty-six terminal cancer patients between November 2010 and September 2013 (Table 1). Eligible patients were randomly allocated to receive 1 of 2 intrathecal treatments. Randomization numbers were generated using an automated and validated system to assign treatment arms, and the assignments were concealed from patients and investigators. Constant morphine injection was given to group M patients through intrathecal access for pain administration, whereas constant morphine and ropivacaine injection was given to group R patients. All 36 patients of the study had received treatment for pain that had proven refractory to traditional therapies such as transcutaneous electrical nerve stimulation, physiotherapy, pharmacotherapy (anti-inflammatory drugs, nonsteroidal drugs, tricyclic antidepressants, oral/transdermal opioids, anticonvulsants, and antispasmodics), and psychotherapy. Patients were chosen prior to catheter placement according to the following inclusion criteria: intractable cancer pain unmanaged by high dosage of oral or parenteral analgesics, intolerability to surgery for pain, or general failure to relieve pain; emotional stability of the patient and family members; and presence of an accountable and competent care provider [2]. The patients were psychiatrically evaluated before the initial port implant and no progressive psychiatric disease was found.

2.2. Procedure. All 36 patients were at the hospital receiving systemic treatment. They were told about the availability of an intrathecal catheter for pain management (with dosages administered by a responsible and capable caregiver) days ahead of the catheter implantation. In addition to verbal instruction, they were presented with the intrathecal catheter and port system and informed of the implantation treatment in written form, particularly with regard to the advantages and potential side effects of intrathecal therapy. They were also advised in writing of complications and related blood tests, particularly coagulation screening, C-reactive protein and white cell count. All instructions were performed before catheter placement. Clindamycin (600 mg) was administered intravenously 2 h before catheter insertion for antibiotic prophylaxis.

Intrathecal catheterization combined with implantation of a subcutaneous infusion port (Celsite, B. Braun, France) was conducted in the sterile condition in an operating room. The patient was put in the lateral decubitus position and then draped in a sterile fashion. Lumbar puncture was performed at the interspace between L2 and S1 using an 18-gauge Tuohy needle. A 20-gauge intrathecal catheter was introduced through the Tuohy needle. The catheter was then inserted into the subarachnoid space. The catheter location was confirmed by ensuring that aspiration of cerebrospinal fluid was possible. Approximately 15–30 cm of the catheter was introduced intrathecally according to the location of the pain under fluoroscopic guidance. The catheter was moved through the subcutaneous tissue between the lumbar incisions and port pocket and then attached to the port. The port pocket was established through a bone structure (the base of the ribs was usually chosen). The port was placed into a subcutaneous pocket organized in the chosen area and attached to the fascia. Every patient underwent the procedure with no complications. Access to the port was via percutaneous injection using a special noncoring needle that was connected to a patient-controlled analgesia (PCA) device with a total volume of 100 mL and a rate of 0.5 mL/h. The original intrathecal morphine dosage was obtained from the preoperative morphine injection using an oral-intrathecal ratio of 300 : 1 [17]. The starting dose of ropivacaine was 0.375 mg/mL, and the total dose per day was 4.5 mg. The dose

was titrated every 24 h until pain was reduced or therapy-limiting side effects were identified. One day before leaving hospital, the constant intrathecal dosage was selected to be the optimal dosage for placing intrathecal port. The drug container was changed on a weekly basis. The port needle was exchanged on a monthly basis.

2.3. Statistical Analysis. We analyzed the demographic statistics (e.g., sex and age) of the patients, cancer categories, location of pain, morphine dosage history, use of oral medications, and pain intensity before implantation of the port as reported using the Numerical Rating Scale (NRS) in which 0 means no pain and 10 means the greatest pain. The NRS score and intrathecal morphine and ropivacaine consumption on postsurgical days 1, 3, 7, and 15 were evaluated. The Barthel Index, a disability scale with scores from 0 (completely dependent) to 100 (completely independent), was used to evaluate the functional status of the patients. Data regarding technical aspects of the procedure (such as catheter tip location, injection interspace, and device-related complications) and nonpharmacological methods used to reduce cancer pain were obtained. The day before hospital discharge, the use of extrasystemic opioids and adjuvants was also recorded.

The data were analyzed using SPSS 17.0 software. The paired-sample t-test and two-way ANOVA were used for data comparisons. $P < 0.05$ was defined as significant.

3. Results

Thirty-six patients completed the trial: 17 in group R and 19 in group M. In group M, 11 male and 8 female patients were studied, with an average age of 64.0 years (40–85 years). In group R, 14 male and 3 female patients were studied, with an average age of 62.6 years (49–85 years). In group M, nociceptive pain was present in 11 patients, whereas a mixture of neuropathic-nociceptive pain was present in 8 patients. In group R, nociceptive pain was present in 7 patients, whereas mixed neuropathic-nociceptive pain was present in 10 patients. Physicians were asked to rate the pain characteristics based upon complaints from patients, with nociceptive pain defined as "aching, squeezing, or pressure-like sensations"; neuropathic pain was characterized as "tingling, burning, or electrical" sensations. The mean duration of hospital care was 12 days for patients in group M and 15 days for those in group R.

The doses of preoperative systemic opioids (intravenous and oral oxycodone, intravenous morphine, and transdermal fentanyl) were summarized as the oral morphine equivalent dose. Additional usage of opioid treatment was also documented to summarize gross opioid usage on a daily basis and presented as the variation in preoperative opioid dosage. The preoperative oral morphine consumption was between 45 and 600 mg/day (mean: 200.8 mg/day) in group M. The preoperative oral analgesic consumption was between 6 and 750 mg/day (mean: 223.7 mg/day) in group R. The mean preoperative doses of oral morphine in groups M and R were similar ($P = 0.688$).

The daily dosage of intrathecal morphine and ropivacaine was adjusted at a certain time based on the pain degree and the bolus dosage required in the last 24 h. A significant increase was observed in intrathecal morphine administration on postoperative days 3, 7, and 15 in comparison with day 1. In group M, the mean doses of intrathecal morphine on postoperative days 1, 3, 7, and 15 were 0.67 ± 0.48 mg, 1.02 ± 0.64 mg, 1.44 ± 0.86 mg, and 2.36 ± 1.56 mg per day, respectively. In group R, the mean doses on postoperative days 1, 3, 7, and 15 were 0.75 ± 0.66 mg, 0.93 ± 0.80 mg, 1.09 ± 0.99 mg, and 1.23 ± 1.10 mg per day, respectively. The mean doses of intrathecal morphine on postoperative days 1, 3, and 7 in groups M and R were similar ($P = 0.688$, $P = 0.697$, and $P = 0.207$, resp.). However, the mean dose of intrathecal morphine on postoperative day 15 in group R was significantly lower than that in group M ($P = 0.005$). In group R, the mean doses of intrathecal ropivacaine on postoperative days 1, 3, 7, and 15 were 4.5 mg, 6.10 ± 2.10 mg, 6.96 ± 2.48 mg, and 8.71 ± 6.54 mg per day, respectively. Dose escalation was guided by the clinical response. No patient received oral opioid supplementation for 15 days after the surgery.

The average NRS scores for the 19 patients in group M on the preoperative day and postoperative days 1, 3, 7, and 15 were 8.17 ± 0.51, 4.78 ± 1.0, 3.78 ± 0.80, 3.06 ± 0.94, and 2.50 ± 1.04, respectively. The average NRS scores for the 17 patients in group R on the preoperative day and postoperative days 1, 3, 7, and 15 were 7.78 ± 0.73, 3.67 ± 1.37, 2.56 ± 1.34, 2.06 ± 1.16, and 1.33 ± 0.77, respectively. The average NRS scores on the preoperative days were similar in groups M and R ($P = 0.069$). The average NRS scores on the postoperative days 1, 3, 7, and 15 decreased gradually in both groups M and R ($F = 40.26$, $P < 0.001$ and $F = 30.62$, $P < 0.001$). However, the average NRS scores on postoperative days 1, 3, 7, and 15 were statistically lower in group R than in group M ($F = 37.38$, $P < 0.001$) (Figure 1).

All patients in group M and group R reported an improved quality of life. The Barthel Index scores on the preoperative day and the 15th postoperative day were 53.61 ± 6.82 and 63.06 ± 7.70, respectively, in group M. The Barthel Index scores on the preoperative day and the 15th postoperative day were 55.0 ± 6.86 and 68.33 ± 6.64, respectively, in group R. The Barthel Index score on the preoperative day was similar in groups M and R ($P = 0.472$). However, the Barthel Index score on the 15th postoperative day was higher in group R than in group M ($P = 0.017$) (Figure 2).

Most intrathecal catheters were implanted at the L2-3 interspace in both group M (72.2%) and group R (61.1%), and most of the catheters reached T10 in both group M (55.6%) and group R (44.4%). Intrathecal morphine and ropivacaine presented few complications or adverse effects. No patient experienced respiratory depression. In group M, one patient experienced transient urinary retention and three experienced nausea and vomiting. In group R, one patient experienced transient urinary retention, one constipation, and one nausea and vomiting. The adverse effects were managed with conservative therapies. Urinary retention was controlled through transient urinary catheterization. No complications of intrathecal drug delivery were noted. No surgical complications were found, and meningitis and

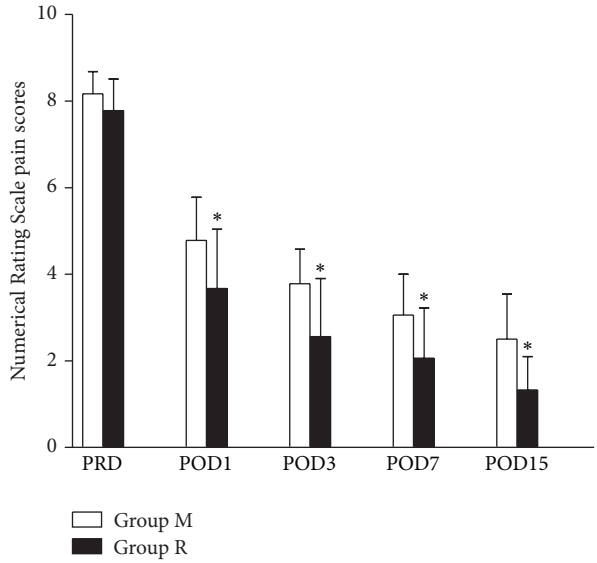

Figure 1: Numerical Rating Scale pain scores on pre- and postoperative days. Data are expressed as mean ± SD. $^{*}P < 0.01$ compared to the group treated with morphine alone. PRD, preoperative day; POD, postoperative day.

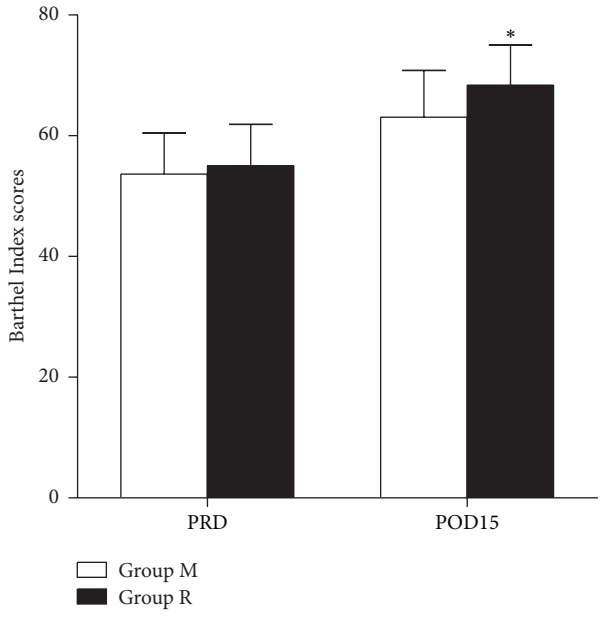

Figure 2: Barthel Index scores on pre- and postoperative days. Data are expressed as mean ± SD. $^{*}P < 0.05$ compared to the group treated with morphine alone. PRD, preoperative day. POD, postoperative day.

infection were not observed. Regional headaches were also not observed. Ropivacaine was added to the opioid injection without any significant dermal numbness or decreased sensation. There were no complications or adverse effects serious enough to require intrathecal port removal or treatment changes.

4. Discussion

The first study of intrathecal morphine in a cancer patient was performed by Tung et al. in 1980 [18]. The first study of intrathecal ropivacaine in a cancer patient was reported in 1998 [19]. The present findings are in agreement with existing studies demonstrating a decrease in pain upon intrathecal drug delivery. Our findings illustrate that administration of ropivacaine and intrathecal morphine through an indwell injection port is both efficacious and safe for severe cancer pain. Simultaneously, this study demonstrated a larger decrease in NRS rates using intrathecal morphine and ropivacaine compared to intrathecal morphine alone. Our findings demonstrate that intrathecal ropivacaine combined with morphine is safe; adverse effects were mild and rare.

The mechanism of action of intrathecal opiates is under debate. As an opiate receptor agonist, morphine blocks the activity of some cells in the substantia gelatinosa of the medullary dorsal horn [20]. As a result, intrathecal narcotic management decreases cutaneous pain transmission at the dorsal horn and transmission of nociceptive impulses through ascending pathways [21]. Several studies have found that intrathecal morphine management is an efficient approach for analgesia in humans and animals [22]. However, studies have large increases in the required dosage of intrathecal morphine with increasing cancer pain on postoperative days [23]. Among patients with insufficient pain management on intrathecal morphine, it is common to increase the opioid dosage for optimization of pain management. However, such increases are associated with adverse effects. Our study showed that the mean dose of intrathecal morphine on postoperative day 15 in patients receiving both morphine and ropivacaine was significantly lower than that in patients treated with morphine alone.

The combined intrathecal treatment is useful when insufficient pain reduction is realized with intrathecal monotherapy, especially in neuropathic pain patients. Different pain statuses have various potential mechanisms; as a result, a medication with a particular mechanism tends to only be efficient for one or two specific pain conditions. Thus, combining agents tends to be useful to treat multiple aspects of the pain, generating synergistic efficacy for pain management. Intrathecal morphine targets medullary opioid receptors, whereas intrathecal ropivacaine functions at the dorsal root and the nerve roots to promote pain management. Ropivacaine reversibly blocks sodium influx, thereby hindering pain signal transmission through Aδ and C fibers [6]. Ropivacaine may increase opioid efficacy synergetically: (1) decreasing voltage-sensitive calcium influx, thus promoting the opioid-mediated presynaptic inhibition of neurotransmitter release from terminals of Aδ and C fibers [2, 14] by decreasing the conformational transformation of medullary opioid receptors (μ, δ, and κ receptors) [24]. Our findings show higher benefit for pain management and quality of life for inpatients with cancer pain when using the combined treatment. Based on our analysis, the recommended daily dosage of intrathecal ropivacaine to realize pain control was 4.5–15.3 mg. Because we could reduce pain with a low dose

of ropivacaine, the observed pain control may reflect synergy between ropivacaine and opioids. To our knowledge, this is the only study with a standard, quantitative assessment of changes in the NRS score and quality of life when the combination of intrathecal morphine and ropivacaine is used to reduce cancer pain. In our study, the average NRS scores on postoperative days 1, 3, 7, and 15 were significantly lower in patients treated with ropivacaine and morphine than in those treated with morphine alone. The Barthel Index ratings indicate that the quality of life on the 15th postoperative day was significantly higher in patients treated with morphine and ropivacaine when compared with those injected with morphine alone.

The adverse effects observed in this study were similar to those previously reported. Nausea and vomiting tended to be the most common and were observed in 15.7% (3 of 19) of morphine-only patients and 5.8% (1 of 17) of patients treated with ropivacaine and morphine; these symptoms are frequently reversible with ondansetron. Urinary retention is also relatively common with intrathecal opioids. In our patients, urinary retention often resolved in a few days or weeks, and prolonged bladder dysfunction was not common. Both treatment groups had 1 patient with transient urinary retention. Constipation was not observed, as most patients treated with systemic opioids in this research were treated with a bowel stimulant, a stool softener, or laxatives prior to intrathecal management. Constipation occurred in 1 patient in group R. Somnolence and sedation are rare adverse effects for medullary opioids [25]. We did not observe these effects in our series. Infection, respiratory distress, motor dysfunction, seizures, weight increase, reduced sexual impulses, and port malfunction are also possible risks but were not observed in our series.

Because of the above risks and system implantation costs, proper selection of patients prior to subcutaneous intrathecal port transplantation is critical. The following selection criteria are recommended: (1) great pain despite oral narcotic management or unsatisfactory narcotic side effects at the dosage required to manage pain; (2) medication for the underlying disease, for example, radiation tumor treatment or surgical tumor treatment, that has not caused the pain; and (3) neuroablative processes for pain reduction that were refused by patients or deemed as unsatisfactory by physicians. In addition, the outcome with the intrathecal port is greatly determined by the attitude of the patient and family; a peaceful and friendly environment is important. The implantation of intrathecal catheters connected to subcutaneous injection ports was appropriately indicated for patients in this study on the grounds of life expectancy, expense, and drug/dosage requests.

Our study had several limitations. First, the effective sample size was small, as the study included only 36 patients. Second, long-term complications, such as formation of granulomas and intrathecal infection, were not assessed because of the short follow-up period. Third, mild negative effects, such as sedation or pruritus, may have been underestimated, as the patients showed pharmacological adverse effects in a passive manner in the present study. Fourth, the preoperative pharmacological adverse effects of systemic opioids were not available. Thus, it is not clear whether opioid-mediated side effects were decreased by the intrathecal treatment.

5. Conclusions

Intrathecal morphine and ropivacaine management in connection with a transplantable subcutaneous port is a safe and efficient approach to provide treatment of intractable cancer pain. The extra intrathecal ropivacaine enhances pain management and increases the quality of life. The usage of intrathecal ropivacaine is therefore deemed to be safe and acceptable. Large-scale prospective random trials are necessary to assess the advantages and safety of intrathecal ropivacaine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ying Huang devised the research, conducted the statistical analysis, wrote the paper, and checked and submitted the final paper. Xihan Li conducted the statistical analysis, modified the paper, and checked and proofed the final paper. Tong Zhu collected the clinical data and checked and proofed the final paper. Jian Lin planned the research, revised the paper, and read and wrote the final paper. Gaojian Tao designed the research, obtained the paper, and checked and wrote the final version. All authors participated in the discussion of findings and left comments on the paper. Ying Huang and Xihan Li contributed equally to this work.

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Review Article

Treatment of Cancer Pain by Targeting Cytokines

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Inflammation is one of the most important causes of the majority of cancer symptoms, including pain, fatigue, cachexia, and anorexia. Cancer pain affects 17 million people worldwide and can be caused by different mediators which act in primary efferent neurons directly or indirectly. Cytokines can be aberrantly produced by cancer and immune system cells and are of particular relevance in pain. Currently, there are very few strategies to control the release of cytokines that seems to be related to cancer pain. Nevertheless, in some cases, targeted drugs are available and in use for other diseases. In this paper, we aim to review the importance of cytokines in cancer pain and targeted strategies that can have an impact on controlling this symptom.

1. Introduction

Pain is a common symptom of inflammatory-related conditions. In cancer, pain is more frequent in advanced stages with great impact on the quality of life. Cancer pain affects 17 million people worldwide, and its prevalence ranges from 33% in patients after curative treatment to 64% in the metastatic setting and 75–90% will experience moderate to severe pain [1].

Pain can be caused by several different mechanisms, usually by compression, ischemia, and invasion of structures such as nerves. Inflammation is one of the causes of the majority of cancer symptoms, including pain, fatigue, cachexia, and anorexia [2]. Nevertheless, the actual etiology of cancer pain is to this day unknown and cancer seems to generate a specific neurochemical pain state, distinct from inflammatory and neuropathic pain [3, 4].

One of the most accepted hypothesis is that, within a malignant tumor, not only cancer cells but also lymphocytes, fibroblasts, endothelial cells, and neurons produce mediators that are released in the microenvironment and might activate nociceptors [3].

Among the released substances are neurotrophic factors [5], endothelin [6], and formaldehyde [7]. Cytokines are also potential mediators produced by cancer cells and cells recruited to the microenvironment.

Cytokines are small (5 kDa–140 kDa) released secreted proteins by the cells in response to different stimuli that have a specific effect on the interactions and communications between cells [8]. Interferons were the first cytokines discovered in 1957 and soon after the concept was expanded to include chemokines, interleukins, lymphokines, and tumor necrosis factor. The release of different cytokines in a controlled sequence is responsible for the production of different final mediators involved in the induction of inflammatory signs. They are released primarily by antigen presenting cells and can have both pro- and anti-inflammatory functions that will vary according to the microenvironment [9, 10]. They can act on the cells that secrete them, autocrine action, on nearby cells, paracrine action, or, sometimes, on distant cells [11, 12].

The first cytokines described as participating in the development of inflammatory pain were interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), and IL-6. These cytokines are considered proinflammatory [11].

Cancer pain can be caused by different mediators: immune system cells infiltrating the tumor: neutrophils, T cells, and macrophages secrete prostaglandins, endothelin, TNF-α, Transforming Growth Factor (TGF), IL-1 and IL-6, Epidermal Growth Factor (EGF), and Platelet Derived Growth Factor (PDGF) [1, 9]. These mediators act in primary efferent neurons directly or indirectly, causing pain.

Table 1: Drugs targeting cytokines that have been tested in pain control.

Drug class	Mechanism of action	Cytokines targeted	Indication in cancer pain treatment
Steroids	Inhibit NF-κB	Downregulate TNF-α and IL-1	Adjuvants for cancer pain treatment CNS lesions
NSAIDs	COX-1 and COX-2 inhibition	Downregulate IL-1β, TNF-α, and IL-6.	Adjuvants for cancer pain treatment bone pain
Opioids	Release of endogenous opioid peptides	Possible inhibition of TNF-α and IFN-γ release	Moderate to severe pain
Statins	Inhibition of HMG-CoA reductase	FGF, EGF, TGF, and PDGF	Not in use
Anti-TNF agents	Multiple mechanisms of TNF blocking (antibody)	TNF	Not in use
IFN-γ blocking agents	Interaction with CB2 receptor	IFN-γ	Not in use
IL-6 blocking agents	Antibodies	IL-6	Not in use
Targeting chemokines	Antibodies	Chemokines	Not in use
Endothelin antagonists	ET_A receptor antagonists	Endothelin	Not in use

Cytokines have an additional role in pain. It has been suggested that the plasma concentrations of cytokines such as IL-8, IL-12 (p40 and p70), eotaxin, and macrophage inflammatory protein- (MIP-) 1α and MIP-1β are associated with the effectivity of morphine treatment. In one study, patients resistant to morphine treatment had lower concentrations of these plasma cytokines [13]. Although the underlying mechanism is still unknown, it is possible that this results from a cross talk between cytokine signaling and opioid receptor signaling: on one hand, the activation of chemokine receptor 1 (CCR1) by cytokines results in the internalization of μ opioid receptors and inhibition of their function [14]. On the other hand, the prolonged activation of opioid receptors might itself inhibit the function of chemokine receptors on leukocytes [15].

Spinal proinflammatory cytokines are known pain-enhancing signals. In animal models, chronic intrathecal opioid administration induces activation of spinal cord glia and also the release of spinal IL-1β which are mechanisms implicated in the development of hyperalgesia, allodynia, and analgesic tolerance [16]. The administration of morphine to lumbar spinal dorsal cord cells causes an increase in the release of proinflammatory cytokines and chemokines which is detectable in less than 5 minutes after the exposure [14]. This favors the hypothesis that proinflammatory cytokines are correlated with opioid tolerance and opioid induced hyperalgesia [14].

Moreover, variants in genes encoding for cytokines have been suggested as candidates of risk of a variety of cancers and both genetic and nongenetic factors are relevant in the severity of pain. For instance, considering persistent breast pain following breast cancer surgery, there are known associations between previously identified extreme persistent breast pain phenotypes (i.e., no pain versus severe pain) and single nucleotide polymorphisms (SNPs) that suggest a role for cytokine gene polymorphisms [17]. In non-small-cell lung cancer, there also seems to be an influence of SNP in IL-8 and severe pain in white patients [18].

Currently, the treatment of cancer pain relies mostly on the severity and type of pain and does not include cytokine targeted treatments. In fact, the World Health Organization (WHO) proposes a model for the treatment of cancer pain that uses severity as the main modulator of analgesic treatment. It includes both opioid and nonopioid drugs (Figure 1).

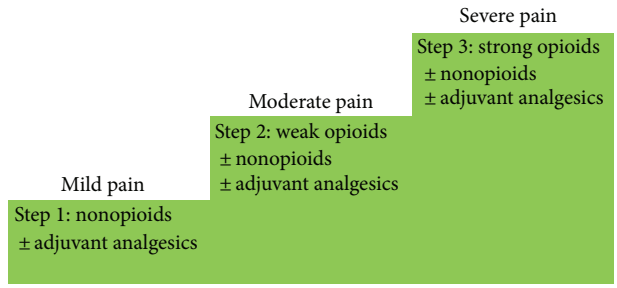

Figure 1: WHO's cancer pain relief ladder for adults.

Nonetheless, cancer pain treatment has different options targeting inflammation. Nonsteroid anti-inflammatory drugs (NSAIDs) and corticosteroids [19] are widely used examples of such drugs. Statins might also be helpful in controlling this symptom, by acting as anti-inflammatory agents [20]. Several other agents have been reported, although their actual efficacy remains to be proven (Table 1).

Knowing the mechanisms behind cancer pain and consequently how to specifically target it with the minimal side effects will help physicians to manage this symptom and will consequently have great impact on quality of life.

2. General Mechanisms of Cancer Pain

One of the most challenging features in treating cancer pain is understanding the underlying mechanisms. The use of animal models helped clarifying the molecular, biochemical, and neurobiological pathways that are involved in cancer pain. There are several cancer pain experimental models currently used to understand these mechanisms [21]. The first models were mice with primary or metastatic bone cancer [1]. It has been observed that in this case a nonpainful level of mechanical stress can induce the release of substance P from primary afferent fibers that terminate in the spinal cord.

Substance P, in turn, binds to and activates the neurokinin-1 receptor and also induces expression of the transcription factor c-Fos on spinal cord neurons [22]. Usually, this condition only occurs with noxious stimuli. Therefore, peripheral sensitization of nociceptors is involved in the generation and maintenance of bone cancer pain [1, 23].

The nociceptor stimulation by tumors occurs due to the release of various factors by immune cells that constitute the tumor. The factors that sensitize or directly excite primary afferent neurons include prostaglandins, TNF-α, endothelins, IL-1 and IL-6, EGF, TGF-β, and PDGF [9]. Their receptors are expressed by primary afferent neurons [1]. Prostaglandins and endothelins are also thought to be involved in the regulation of angiogenesis and tumor growth and can therefore be important in reducing tumor growth and metastasis [24].

There are other characteristics related to the tumor that contribute to cancer pain such as tumor-induced acidosis [1]. The intra- and extracellular environment of solid tumors is characterized by lower pH. It is known that local acidosis is a hallmark of tissue injury and induces excitation of sensory neurons. The acidic microenvironment adjacent to the richly innervated periosteum is a mechanism associated with pain in metastatic bone cancer [25]. Tumor-induced release of protons and acidosis might be particularly important in the generation of bone cancer pain [26]. The osteoclasts reabsorb bone by maintaining an extracellular microenvironment of low pH at the osteoblast-mineralized bone interface. Most sensory neurons that innervate bone express transient receptor potential vanilloid-1 (TRPV1) and/or acid-sensing ion channels (ASICs) [26]. The TRPV1 is a Ca2+ permeable ionotropic receptor and his antagonism in a mouse bone cancer pain model attenuates the movement-evoked nociceptive behaviors. (B) These findings contribute to the unveiling of the etiology of bone cancer pain, with no target drugs already in practice. However, this effect was already seen in a soft tissue cancer model with the injection of SCC [27].

Despite the fact that tumors are not highly innervated by sensory neurons, their rapid growth can cause mechanical injury, compression, and ischemia, producing pain. The proteolytic activity is critical to carcinogenesis and cancer pain. Cancer associated trypsin has been identified in cancers such as ovarian carcinoma, pancreatic cancer, hepatocellular and cholangiocarcinomas, lung neoplasms, colorectal cancers, fibrosarcoma, gastric cancer, and oral cancer (D). Proteases activate cell surface receptors on primary afferent nociceptors within cancer microenvironment, either directly or via peptide products. Proteolytic enzymes that are produced by the tumor cells can also cause injury to sensory and sympathetic fibers, causing neuropathic pain [1]. Protease activated receptors (PARs) are activated by proteolytic (enzymes) cleavage or by a short peptides ligand with similar sequence. There is release of substance P and calcitonin gene related protein from C-fibers in peripheral tissues with multiple second messenger pathways activated, like TRPV1-dependent thermal and TRPV4-dependent mechanical hyperalgesia [28, 29].

Animal studies demonstrated that cancer pain is in part maintained by a state of central sensitization in which there is an increased transmission of nociceptive information, promoted by neurochemical changes (such as increased levels of glutamate and other excitatory neurotransmitters, with astrocyte hypertrophy) in the spinal cord and forebrain. The upregulation of the prohyperalgesic peptide dynorphin was observed in these models [1].

Additionally, not only is cancer pain transmitted by the classically described spinothalamic tract, but also there is evidence that other pathways such as those in the dorsal column might be implicated [1]. As cancer pain, more specifically visceral pain, is frequently bilateral, it might be difficult to manage by surgical approaches since bilateral cordotomies can have relevant complications [1].

3. The Role of Cytokines in Cancer Pain

It is well accepted that cytokines constitute a link between cellular injuries or immunological recognition and the local or systemic signs of inflammation [12]. In this way, cytokine activation and dysregulation are implied in a variety of inflammatory diseases and also in cancer [30]. In fact, they are produced at high levels in cancer with effects that are directly neuronal or immunological. (A) Actually, there is evidence that these mediators are involved in the initiation and persistence of pathological pain by direct and indirect action on nociceptive sensory neurons. Figure 2 schematizes the role of cytokines in cancer pain. Some cytokines are also related to nerve injury/inflammation-induced central sensitization and are associated with hyperalgesia and allodynia phenomena [11, 30]. It has been demonstrated that inflammatory stimuli do not directly induce the release of prostaglandins and sympathetic amines. Instead, a well-defined sequential cytokine cascade precedes the release of these final hypernociceptive mediators. This cytokine cascade is modulated by a parallel release of anti-inflammatory cytokines [12].

Proinflammatory cytokines are produced by activated macrophages. There is a strong evidence of their association with pathological pain, especially IL-1β, IL-6, and TNF-α [11, 12, 30].

Production and secretion of IL-1β are associated with pain in pathological conditions like tumor growth. It is released by monocytes and macrophages and by nonimmune cells, during processes of cell injury, infection, invasion, and inflammation. It is expressed in nociceptive dorsal root ganglion (DRG) neurons and in Schwann cells on peripheral nerves and plays a central role in the generation of hyperalgesia [11]. IL-1β was the first cytokine reported to mediate inflammatory nociception in experimental animals [1]. Corroborating these findings, IL-1β was demonstrated to stimulate the expression of cyclooxygenase 2 (COX-2) and the subsequent release of its products, prostaglandins [11, 12]. Indeed, in some studies, small doses of IL-1β produce a severe mechanical hypernociception that depends on the release of prostanoids [30]. This situation can be prevented by experimental administration of endogenous IL-1 receptor antagonist (IL-1ra) or a COX-2 inhibitor, suggesting that induced hypernociception is mediated by the activation of specific membrane receptors and the subsequent synthesis of prostaglandins [12]. Hence, the peripheral pronociceptive action of IL-1β is mediated by a complex signaling cascade. Besides the sensitization of nociceptor by prostanoids, direct

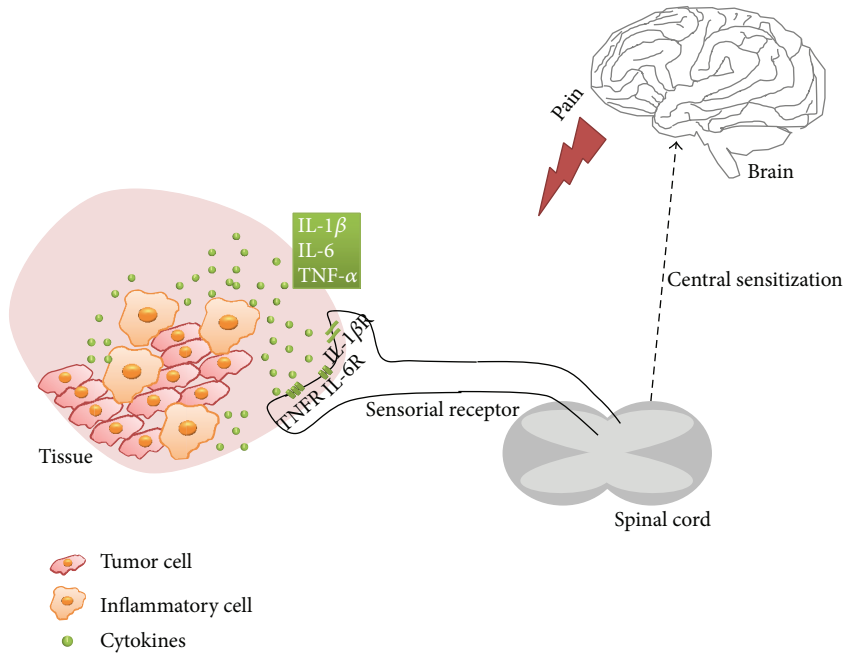

Figure 2: Cytokines in cancer pain. The tumor is composed not only by cancer cells but also by inflammatory cells, among others. The inflammatory cells present in tumor tissue release cytokines that sensitize the sensorial receptor. The painful stimuli, mediated by cytokines, are detected by the sensorial receptor and are transmitted to neurons in spinal cord. The signal is then transmitted to the brain. Adapted from Molecular Mechanisms of Cancer Pain, Nature Reviews Cancer.

nociceptor activation by inflammatory mediators such as serotonin, histamine, or ATP might occur in models of inflammatory pain. In these models, the participation of IL-1β was also demonstrated [31–33]. In addition, there is evidence that IL-1β participates with other cytokines in the genesis of neuropathic pain: it was observed that chronic constriction injury of peripheral nerves induces lumbar spinal IL-1β expression. Similarly, IL-1β or IL-1R1 gene disruption also impairs the neuropathic hypernociception, while transgenic overexpressing IL-1R1 mice have increased neuropathic pain [12].

The serum levels of pleiotropic cytokine IL-6 are increased in malignant tumors. It is markedly upregulated during various pathological situations and has both anti- and proinflammatory effects. IL-6 is generally associated with hyperalgesia and has been shown to play a central role in neuronal reaction to nerve injury. Suppression of IL-6 receptor (IL-6R) *in vivo* by application of anti-IL-6R antibodies led to reduced regenerative effects. IL-6 is also involved in microglial and astrocytic activation and in regulation of neuronal neuropeptides expression. So, IL-6 contributes to the development of neuropathic pain behavior following a peripheral injury [11]. Some data suggest that the inflammatory environment determines the capacity of IL-6 to induce IL-1β production with the release of prostanoids, causing hypernociception in an animal model [34]. However, when IL-1ra was administered, there was no inhibition of hypernociception [12].

TNF-α, also known as cachectin, is considered the prototypic proinflammatory cytokine due to its principal role in initiating the cascade of activation of other cytokines and growth factors in the inflammatory response. After injury or during inflammation, TNF-α is synthesized and released by various cell types, mainly monocytes and macrophages. It has also been demonstrated that Schwann cells can produce TNF-α after injury, suggesting a possible role in neuropathic pain. Studies with intraplantar injection of TNF-α in mice have been shown to induce mechanical allodynia and thermal hyperalgesia. Indeed, subcutaneously injected TNF-α lowers mechanical activation threshold in C nociceptors in the sural nerves of mice [12]. These data demonstrated that blocking TNF-α reduces hyperalgesia in models of painful neuropathy. It interacts with target cells through high-affinity membrane receptors: tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor type 2 (TNFR2). The effects associated with experimental hyperalgesia have been shown to be dependent on TNFR1 following experimental nerve lesion. Downstream of TNFR activation hyperalgesia induced by inflammation or nerve injury is mediated via p38 MAPK. But TNF-α also acts by nuclear factor kB (NF-kB) activation of inflammation and by stress-activated protein kinases (SPAKs) [11, 12]. The TNF-α hypernociceptive effect was partially inhibited by COX inhibitor and β adrenergic receptor antagonists (like atenolol) and eliminated by cotreatment with these drugs, suggesting that this function is mediated by prostanoids and sympathetic amines [12]. In fact, it is known that TNF-α induces the production of IL-1β, IL-6, and IL-8. The cytokine cascade results in the activation of COX-2 dependent prostanoid release and release of catecholamines from sympathetic fibers [35]. On the other hand, TNF-α also participates in bone destruction, which is a major source of tumor related pain. TNF-α can

regulate differentiation and activation of osteoclasts by its receptor, independent of complex receptor activator of NF-kB (RANK) and RANK ligand (RANKL) [12]. Moreover, there is evidence that the latter and TNF-α cooperate in osteoclastogenesis [35]. Despite the evidences described above that the hypernociceptive effects of cytokines are indirect, it has been reported that sensory neurons express TNF-α and IL-1β receptors, suggesting that these cytokines might also directly sensitize the nociceptor during inflammation. It was demonstrated that TNF-α evokes action potentials in nociceptive neurons when applied topically to peripheral axons *in vivo* and increases the sensitivity to mechanical and chemical stimuli. On the other hand, IL-1β acts on sensory neurons increasing their susceptibility to noxious heat.

4. The Role of Cytokines in Chemotherapy Induced Pain

Cancer therapies such as cytotoxic agents can, themselves, cause pain in cancer patients. They usually generate neuropathic pain [36]. Chemotherapeutic agents that are more often associated with neuropathy are not only platinum derived agents (oxaliplatin, cisplatin, and carboplatin) but also taxanes (paclitaxel and docetaxel), vincristine, and others such as bortezomib, thalidomide, lenalidomide, and epothilones [36].

Pain secondary to chemotherapy is probably due to multiple mechanisms. Most chemotherapy agents are able to penetrate the blood-nerve-barrier and bind to the dorsal root ganglia and peripheral axons thus increasing their potential neurotoxicity [36].

Some chemotherapeutic agents have the ability to disrupt tubulin function. Tubulin polymerization is necessary for axonal transport of trophic factors, and drugs that interfere with this process can cause degeneration of sensory neurons and release of proinflammatory cytokines that directly sensitize primary afferent nociceptors [1].

Likewise, cytotoxic agents might also induce neuropathic pain by activating ion channels in the plasma membrane on dorsal root ganglia and dorsal horn neurons. The inflammatory process that is triggered in the immune system cells by chemotherapeutic agents, with the subsequent release of proinflammatory cytokines, can also trigger changes in the sensory neurons and ultimately alter nociceptive processing [36, 37].

Although different chemotherapeutic agents generate neurotoxicity by different mechanisms, overall proinflammatory cytokines not only can contribute, in fact, to axonal damage by activating the inflammatory process but also can modulate spontaneous nociceptor sensitivity and activity, a mechanism involved in allodynia and hyperalgesia after nerve injury [36].

5. Chemokines

Another set of molecules with particular relevance in pain development is chemokines. Their most important role is the recruitment of white blood cells to the site of inflammation, but they also play other parts such as promoting angiogenesis and modulating the immune response and they are involved in fever as well.

There are approximately 50 chemokine ligands, which can be divided into four families based on the sequences of their cysteine residues: CC-chemokine ligands (CCL), CXC-chemokine ligands, CX_3C-chemokine ligands, and XC-chemokine ligands [38]. Chemokine receptors are widely spread in white blood cells, neurons, and glial cells [39]. The relationship between chemokine ligands and their receptors is complicated, due to the fact that each chemokine ligand has multiple receptors. Since some chemokine receptors, such as CCR2, CCR5, CXCR4, and CX3CR1, are located in primary afferent neurons or secondary neurons of the DRG, chemokines (such as CCL2 and CCL3) can potentially alter pain transmission and produce pain behaviors [40]. On the other hand, some of these chemokines, such as CCL2, participate in pain regulation through direct action on sensory neurons and indirect action via leukocyte activation [41]. The chemokine effects on pain sensation are complex, and additional effort is required to clarify the role of these molecules in cancer derived analgesia.

In the complex process of pain in cancer, there are many phenomena difficult to manage. One of the important sensations is the hypernociception. When tissue is damaged, various chemical mediators are released such as prostaglandins, histamine, serotonin, bradykinin, nerve growth factor, cytokines, and chemokines. The chemokines seem to contribute to pain hypersensitivity and spontaneous pain, either by a direct action or by modulation of the activity of nociceptors [42].

The mechanisms underlying the hypernociceptive effects of chemokines remain unclear. Chemokine IL-8/CXCL8 mediates the participation of sympathetic components of the inflammatory hypernociception [11, 12]. IL-8/CXCL8 induces a dose and time-dependent mechanical hypernociception, which is inhibited by β adrenergic receptor antagonists, but not by COX inhibitor. There is also evidence that chemokines participate in neuropathic nociceptive response by inducing the monocyte chemoattractant protein-1 (MCP-1) expression in DRG. MCP-1 acts through its receptor CCR2 which is directly involved in neuronal hyperexcitability and neuropathic pain after chronic compression injury [12]. The CX3CL1 chemokine also participates in the pathophysiology of experimental neuropathic pain. It induces microglia IL-1 and IL-6 production, which mediate mechanical and thermal hypernociception [12].

More recently, studies have shown that CXCR3 might be of pivotal importance in bone cancer pain in mice, acting through Akt and extracellular signal-regulated kinase (ERK) signaling pathways [43]. Nevertheless, there are three splicing variants of the CXCR3 receptor, with opposite biological activities still not extensively understood, which limit the use of therapies targeting this axis [44].

Further studies are needed in order to understand the actual relevance of cytokines in cancer pain and eventually develop targeted therapies that will help to provide a better cancer care.

6. Targeting Cytokines for the Treatment of Cancer Pain

There are currently several different options targeting inflammation for the treatment of cancer pain (Table 1). Nonsteroid anti-inflammatory drugs (NSAIDs), COX-2 inhibitors, opioids, and corticosteroids [19] are widely used. Several other agents have been reported, although their actual efficacy, in most cases, remains to be proven.

6.1. Corticosteroids. Corticosteroids are widely used in cancer treatment and are recommended as adjuvants for cancer pain, as they tend to reduce adverse effects associated with opioid therapy [30, 45]. They exert their anti-inflammatory action through glucocorticoid effects.

Glucocorticoids have two major roles in cancer pain: they reduce the swelling, which is particularly important in central nervous system (CNS) lesions [46] and also reduce the inflammation by diminishing the proinflammatory cytokines.

Corticosteroids might have an effect in different stages of pain (transduction, transmission, modulation, and pain perception) [45]. After synthesis and release resulting from tissue injury, cytokines appear to increase pain perception. By inhibiting the expression of collagenase, responsible for tissue breakdown during inflammatory mechanisms, corticosteroids diminish proinflammatory cytokines and therefore modulate the activation of nociceptive mechanisms [47].

Steroids also seem to have an important role in pain perception at a different level, since there is evidence showing that androgens have analgesic effects in humans while estrogens might have both hyperalgesic and analgesic effects [47, 48].

Steroids bind to specific homodimeric glucocorticoid receptors (GRs) which are expressed in most tissues, forming a complex that will interact with many cytoplasmic molecules and also by direct interaction with DNA sequences known as glucocorticoid response elements (GREs). In particular, NF-κB transcription seems to be antagonized by the GR-ligand complex, by interaction with the p65 subunit of NF-κB, in a protein-protein interaction [49]. Glucocorticoids are reported to have another less important role in repressing NF-κB activity, mainly through the induction of its inhibitor, IκB [49]. Given the important role of NF-κB in activating transcription of proinflammatory cytokines, glucocorticoids will indirectly contribute to the downregulation of cytokines such as TNF-α and IL-1β, with important anti-inflammatory effects [50].

Despite the rational for administering corticosteroids in cancer patients, several studies do not show a significant improve in analgesia in patients already receiving opioids [51]. There is still a lack of studies and further evidence is still needed. Nevertheless, they seem to have an effect on other symptoms such as fatigue, appetite loss, and patient satisfaction and therefore might continue playing an important role in palliative care [52].

6.2. NSAIDs and COX-2 Inhibitors. NSAIDs are commonly used anti-inflammatory agents, recommended as a monotherapy or combined with opioids for improvement of cancer analgesia by increasing pain relief and by reducing opioid dosing and therefore side effects [53]. These drugs act mainly by inhibiting COX-1 and COX-2 and therefore diminishing the conversion of arachidonic acid to prostaglandins and thromboxane. These two isoforms have different roles in pain, as COX-1 is constitutively expressed in most tissues and organs, accounting for most of the adverse effects of NSAIDs, while COX-2 is an inducible enzyme, localized mainly in inflammatory cells and tissues [54]. COX-2 is induced by both mitogens and proinflammatory mediators such as IL-1β, IFN-γ, and TNF-α. COX-2 inhibitors were developed in order to decrease the secondary effects that would result from inhibiting COX-1.

Some NSAIDs can, additionally, inhibit the activation of NF-κB, thus contributing to diminish the production and release of cytokines such as IL-1β, TNF-α, and IL-6 [55, 56].

Moreover, NSAIDs seem to have another target as they act on hydrogen-gated ion channels directly responsible for lowering the pH causing tissue acidic environment that contributes to pain by sensitizing nociceptors. NSAIDs are also inhibitors of ASIC mRNA transcription (usually induced by inflammation), and these two actions together contribute to NSAID reduction of low-pH induced pain [57].

6.3. Opioids. Opioids are the mainstay of cancer pain treatment. Opioid analgesics refer to drugs that include alkaloids extracted from poppy seeds such as morphine and codeine, semisynthetic derivatives of these drugs (oxycodone, hydromorphone, and oxymorphone), synthetic phenylpiperidines (meperidine, fentanyl), and synthetic pseudopiperidines (methadone) [58].

Three main types of opioid receptors were classically described: μ opioid receptor (MOP), δ opioid receptor (DOP), and κ opioid receptor (KOP). More recently, the nociception/orphanin FQ (NOP) has been discovered [58–60].

The analgesia results from the activation of these receptors which will directly inhibit neurons, thus inhibiting spinal cord pain transmission [61]. However, opioids also have diverse immunomodulatory effects through various mechanisms. This was first realized in animal models, when the administration of morphine reduced swelling and peripheral inflammation, an effect not reversed by naloxone administration [62]. These effects seem to result from the influence of opioids on the expression of cell adhesion molecules, which impairs the adhesion of the immune cells to the endothelium [63].

On the other hand, there is also evidence that opioids might interact with TNF-α [64]. Data *in vitro* showed that tramadol and ketobemidone and morphine but not fentanyl inhibited the production of TNF and IL-8 mRNA. This was an effect with a significant dose-response relationship (maximum inhibition occurred at millimolar levels) and was not always reversible by naloxone [65]. Therefore, opioids might have a role in the treatment of peripheral inflammatory pain, by interfering with cytokines.

6.4. Statins. Statins are well-known cholesterol-lowering agents, with a major role in cardiovascular prevention.

They act by inhibiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, with reduced mevalonate synthesis and therefore lowering cholesterol levels [20]. Along with this effect, many other substances are decreased such as inflammatory cytokines and proadhesive molecules, therefore also exhibiting anti-inflammatory properties that could be useful for the treatment of cancer pain [66]. Nevertheless, the use of statins in elderly cancer patients resulted in the increase in pain, mainly due to myositis which is thought to be associated with diminished synthesis of Coenzyme Q [67]. Currently, statins have no role in cancer pain control and to our knowledge there are no clinical trials testing this hypothesis.

6.5. Anti-TNF Agents. There is solid evidence supporting TNF-α as a potential therapeutic target to control inflammatory pain states. Different drugs have been developed such as infliximab (a chimeric anti-TNF-α antibody), etanercept (p75 TNFα receptor/immunoglobulin G fusion protein which reduces p38 MAPK phosphorylation and allodynia) [11], and adalimumab (human monoclonal anti-TNF-α antibody). Despite the data concerning the antinociceptive role of TNF-α and the beneficial effect of these drugs on inflammatory diseases (uveitis, psoriasis, and rheumatoid arthritis), there are few studies suggesting their analgesic effect especially in cancer patients [12]. There is a report of two cases concerning the targeted administration of etanercept in an anatomic site proximal to bone metastasis in patients with refractory pain. This leads to rapid, substantial, and prolonged relieve of the complaints [68]. In this case, the improvement might be to the role of TNF-α in the vicious cycle of bone metastasis, by inhibiting osteoclast mediated bone reabsorption [68].

Theoretically, anti-TNF agents seem to be natural targeted agents for cancer pain and have in fact been studied for supportive care as TNF seems to be implicated in cachexia, fatigue, and cancer associated depression. Nonetheless, more studies are necessary to validate their analgesic effect, and the risk of opportunistic infections, particularly relevant in cancer patients, should be taken into account while using these immune modulators.

6.6. IFN-γ Blocking Agents. Interferon gamma (IFN-γ) is a dimerized soluble cytokine released along with other proinflammatory cytokines upon stimulation of immune system cells. It has long been reported in leukemia patients that treatment with IFN-γ might spontaneously cause pain symptoms [69].

This cytokine appears to be a key modulator of CB2 cannabinoid receptor signaling during neuropathic pain. In response to nerve injury, CB2 receptors modulate glial activation and contribute to the containment of neuropathic pain [70]. IFN-γ is a mediator of CB2 signaling and upon release from astrocytes and neurons will contribute to the perpetuation and progression of neuropathic pain [70]. On the other hand, CB1 receptor has different role in neuropathic pain induced by IFN-γ. In fact, there are reports showing that despite its high expression in the CNS, disrupting the expression of the CB1 receptor does not seem to have a great impact on the development of neuropathic pain [71]. However, the CB1 receptors expressed in peripheral nociceptors do seem to be involved in this type of pain and its activation actually reduces pain sensitivity [70, 72].

Blocking INF-γ is not currently used as a strategy in cancer pain treatment and to our knowledge there are no current trials addressing this question. Similar to anti-TNF agents, by compromising the immune system inhibiting, IFN might carry important risks.

6.7. IL-6 Blocking Agents. Given the known increased levels of IL-6 in many malignant tumors and its association with hyperalgesia, targeting IL-6 might have a role in cancer pain [11].

Tocilizumab is a humanized IL-6 receptor monoclonal antibody [73], approved by the FDA and EMA for use in moderately to severely active rheumatoid arthritis, for children with polyarticular juvenile idiopathic arthritis and for the treatment of systemic juvenile idiopathic arthritis (SJIA).

Tocilizumab has been sporadically administered for the treatment of cancer cachexia with success [74, 75]. Nevertheless, there are no reports of its use in cancer pain.

6.8. Targeting Chemokines. Chemokines and particularly IL-8/CXCL8 have been implicated in inflammatory hypernociception and are suspected to have a role in perineural invasion associated with pancreatic cancer [11, 76]. Therefore, targeting these molecules might also have a role in the treatment of cancer pain.

A fully humanized anti-IL-8 antibody, ABX-IL8, has been developed and proved to inhibit tumor growth, angiogenesis, and metastasis of human melanoma, when compared with control IgG-treated animals [77]. It was also effective in inhibiting tumor growth in orthotopic bladder xenografts [78]. The preclinical efficacy was never translated into clinical benefit in humans since the clinical trials were abandoned after disappointing results for ABX-IL8 in autoimmune diseases. Therefore, although targeting chemokines in cancer pain might be of use, there is still no available approach for IL-8. Targeting other chemokines or even a combined strategy could be possible approaches.

6.9. Endothelin-1 Antagonists. Endothelin-1 (ET-1) is a 21-amino acid peptide with a well-known role in angiogenesis and tumor growth [24]. Several malignant tumors secrete endothelin, such as prostate cancer, pancreatic cancer, colon cancer, ovarian cancer, and renal cell carcinoma [6].

More recently, endothelin has been shown to be implicated in pain, both in humans and animal models [79]. ET-1 is thought to be released by immune system cells, along with cytokines, in response to different stimuli such as tissue damage. Its concentration is variable among different tumors and some malignancies such as oral squamous cell carcinoma produce greater amounts of ET-1 than melanoma. This suggested an important role of the cancer cells as the source of ET-1.

ET-1 is intimately related to cytokines and its implication in pain would occur through ET receptors, believed to be important in inflammatory, neuropathic, and tumoral

pain [6]. In fact, regarding cancer pain, ET-1 concentration seems to be more relevant than tumor volume [80].

Selective endothelin receptor antagonists have been tested in animal models; they were able to reduce pain respond and might be an option to explore concerning cancer pain, particularly in tumors known to secrete ET-1 [81].

In clinical trials, zibotentan or atrasentan, two selective ET_A receptor antagonists, did not seem to have an impact on overall survival or progression-free survival in patients with hormone refractory prostate cancer [82, 83]. However, atrasentan did seem to have an effect on cancer related bone pain and skeletal related events in prostate cancer patients [82].

6.10. Other Agents. Other agents that target inflammation and cytokines are being tested in cancer pain.

Lenalidomide is a second generation analogue of thalidomide, developed in order to maximize the antiangiogenic, antitumorigenic, and immunomodulating activity while reducing side effects such as sedation and neuropathy [84, 85]. It is approved by the FDA and EMA for the treatment of myelodysplastic syndromes and multiple myeloma.

Lenalidomide exerts its anti-inflammatory function by inhibiting production of proinflammatory cytokines such as TNFα, IL-1β, IL-6, and IL-12 and increasing anti-inflammatory cytokines such as IL-10. It also inhibits the expression of COX-2, therefore diminishing the production of prostaglandins [85].

This drug has been tested in a phase II randomized controlled trial that aimed to access the efficacy and safety of lenalidomide in patients with complex regional pain syndrome type 1. The results showed no increase in toxicity. Yet, there is no difference in pain control as well, not confirming the results of a previous pilot study [86].

Yet, one study by MD Anderson Cancer Center tried to evaluate the efficacy of lenalidomide for the treatment of refractory cancer pain (NCT00684242). Only three patients were recruited from May 2008 to November 2010, with one patient excluded before assignment to groups, and the study was terminated for low accrual. The study aimed to assess the changes in cancer pain from baseline to day 15 using Edmonton Symptom Assessment Scale (ESAS) to measure participant responses to 10 common symptoms (pain, fatigue, nausea, depression, anxiety, drowsiness, shortness of breath, appetite, sleep problems, and feeling of well-being). One participant had no change in pain, while the other had an improvement in two points (symptoms rated on a 0 to 10 scale from 0 "no symptom" to 10 "worst possible symptom"). There is no record of other studies with this drug in cancer pain.

7. Conclusion

Inflammation plays an important role in cancer pain and cytokines are key molecules for this process. Although known to be involved in cancer pain, cytokines are seldom targeted. In fact, although already available and in use in other diseases such as rheumatological conditions for their anti-inflammatory properties, most of these drugs have not been tested as analgesics in the setting of cancer pain. Many have not yet been proved relevant in the setting of inflammatory pain. Nevertheless, early therapeutic results, in both animal models and humans, favor the importance of these drugs in cancer pain and warrant further testing.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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