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Effects of Intraperitoneal and Intrathecal Morphine Analgesia on the Expression of μ-Opioid Receptors in Bone Cancer Pain Rats

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Abstract

Backgrounds: This study compared analgesic effects and μ-opioid receptor expression levels during long-term intraperitoneal and intrathecal treatment in a bone cancer pain rat. **Methods:** Twenty-four female Sprague-Dawley rats were injected Walker 256 tumor cells into the femur to create a bone cancer pain model. The control group was injected with saline intraperitoneally and intrathecally. The intraperitoneal group was injected with morphine intraperitoneally and saline intrathecally. The intrathecal group was injected saline intraperitoneally and morphine intrathecally. Changes in pain threshold, μ-opioid receptor expression levels in spinal cord, and tumor tissue were compared between 3 groups. **Results:** The intrathecal morphine group and the intraperitoneal group showed no difference in analgesia effects ($P > .05$). Western blot and immunohistochemical staining of μ-opioid receptors demonstrated that its level in the intrathecal group was significantly lower than the intraperitoneal group ($P < .05$) and without significant difference with the control group ($P > .05$). The expression levels of μ-opioid receptor in the spinal cord tissue did not reveal a difference among these 3 groups ($P > .05$). **Conclusion:** Intrathecal group and intraperitoneal group showed significant difference in μ-opioid receptor expressions although with no difference in analgesia effects. Long-term intrathecal morphine administration provided similar analgesia compared to systemic morphine.

Keywords

intrathecal injection, morphine, μ-opioid receptor, cancer pain

Introduction

Chronic pain due to cancer directly affects the survival quality of patients with cancer during their survival period.[1] Opioids have many direct and indirect effects on cancer cells and could promote proliferation and metastasis of malignant cells by direct promotion of cell growth and inhibition of cellular immunity.[2] Its molecular mechanisms are still largely unknown.[3,4] Several researchers observed that overexpression of the morphine μ-receptor (MOR) facilitates tumor growth and metastasis.[5-7] In preclinical models, opioids stimulate angiogenesis and tumor progression through the MOR. It was initially reported that opioids at clinically relevant doses were proangiogenic in a model of breast cancer xenografts.[8] Support for the hypothesis that the MOR is involved in cancer progression comes from other work showing a reciprocal transactivation of the vascular endothelial growth factor receptor and potentiation of bevacizumab and 5-fluorouracil and also mammalian target of rapamycin inhibitors in human endothelial cells by the peripheral opiate antagonist methylnaltrexone (MNTX). In MOR knockout mice, there was markedly diminished progression of Lewis lung carcinoma, and MNTX or

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naltrexone infusions blocked tumor growth and metastasis. It was also showed that opioids are more potent in the spinal cord than in the periphery.[9] Intrathecal morphine reduces the dose of overall opioid usage and obtains a better survival rate.[10]

However, the main drawback of current research is that most studies are in vitro and could not replicate biological conditions of cancer cells in vivo. We propose to explore the in vivo MOR expression levels at a variety of metastatic sites with different routes of morphine administration in a rat model. We aim to compare MOR expression levels between long-term intrathecally morphine-treated rats and the intraperitoneally morphine-treated rats.

Material and Methods

Experimental Animals

Twenty-four female Sprague-Dawley SD rats (150-180 g) were purchased from Beijing WeitongLihua Experimental Animal Technology Co Ltd (animal production license number SCXK [Beijing] 2016-0011]. After 14 days of adaptation, the rats observed a steady increase in body weight. Experiments and care of these rats strictly abide by provisions stipulated by the Experimental Animal Ethical Review Committee of the Beijing Institute of Tuberculosis on Breast Cancer.

Main Reagents and Instruments

The cell line Walker 256 rat ascites carcinoma cell line was obtained from American Type Culture Collection (Manassas, Virginia) The μ-opioid receptor rabbit monoclonal antibody (ab134054) was purchased from Abcam (Cambridge, Massachusetts). The GAPDH [5174] was purchased from Cell Signaling Technology (CST, Danvers, Massachusetts). The PE10 catheter was purchased from Smiths Medical (Ashford, Kent, United Kingdom). The Von Frey fiber probe (NC12775-99) was purchased from North Coast Medical (Morgan Hill, California).

Induction of Bone Cancer

On the day 14, all rats (about 180 g) were anesthetized with pentobarbital (40 mg/kg) in the prone position. The right hind limb was shaved and the skin disinfected with 70% (vol/vol) ethanol. A 1 cm incision was made in the skin over upper femur area, and tissue was dissected to expose the femur with minimal damage to surrounding muscles or blood vessels. A cavity was created inside the femur with a 23G needle by rotating and punching; 3 mL of Dulbecco modified Eagle medium containing 1×10^5 Walker 256 rat ascites carcinoma cells were injected, and the needle hole was sealed with bone wax. Skin was closed, and rats were placed in transparent cages until they have regained consciousness before returning to their home cages.

Intrathecal Catheterization

On the 21st day after successful establishment of cancer in our rat model, rats were anesthetized with intraperitoneal injection of pentobarbital (40 mg/kg). After shaving and disinfection of the skin, a longitudinal incision of about 1 cm was made between L6 and S1 spinous processes. Muscles were bluntly separated to expose spinous processes. A PE-10 catheter was slowly inserted between the spinous processes until clear cerebrospinal fluid was seen on the catheter indicating successful intrathecal placement. The catheter was placed intrathecally about 3 cm and sutured in place proximally. It was then tunneled under the skin to the back of the neck, sealed with needle, and sutured onto the skin.

We further confirmed the intrathecal placement by injecting 20 μL of lidocaine through the catheter, and paraplegia was observed within 30 seconds and recovered within 30 minutes. After 24 hours of continuous observation, none of these rats showed abnormal behavioral activities, paralysis, lameness, and severe weight loss.

Grouping and Processing

After all rats were placed with intrathecal catheters, 24 rats were randomly divided into 3 groups ($n = 8$): cancer pain control group (group N), intraperitoneal group (group IP) with intraperitoneal morphine analgesia, and intrathecal group (group IT) with intrathecal morphine analgesia. The rats grouped and obtained MOR after injected with cancer cells 21 days later. On the 21st day after cancer cell injections (D35), group N received intraperitoneal injection of saline 1 mL, intrathecal injection saline 20 μL once a day; group IP received intraperitoneal injection of morphine 1.25 mg in 1 mL, intrathecal injection saline 20 μL once a day; and group IT received intrathecal injection 0.025 mg morphine in 20 μL, which has an analgesic efficacy equivalent dose with the intraperitoneal group, through the intrathecal catheter, intraperitoneal injection saline 1 mL, once a day; for a total of 16 days.

Bone Cancer Pain Model Confirmations

On the 7th (D21), 14th (D28), and 21st (D35) days after cancer cell injection, radiographs were taken to evaluate the extent of tumor-induced bone destruction. After sacrifices (D51), rat femurs were taken, and tumor growth and invasion were observed directly with naked eyes. Bone tumor tissues were fixed with paraffin, then hematoxylin and eosin staining was performed for pathological analysis.

Behavioral Determination Index of Bone Cancer Pain Rats

Mechanical allodynia was measured by the hind paw withdrawal response to stimulation with von Frey filaments.[11] The pain threshold of each group was measured every 3 days after cancer cell injections (from D14). Rats were placed in cages with metal nets at the bottom and allowed to settle for 5 to 10

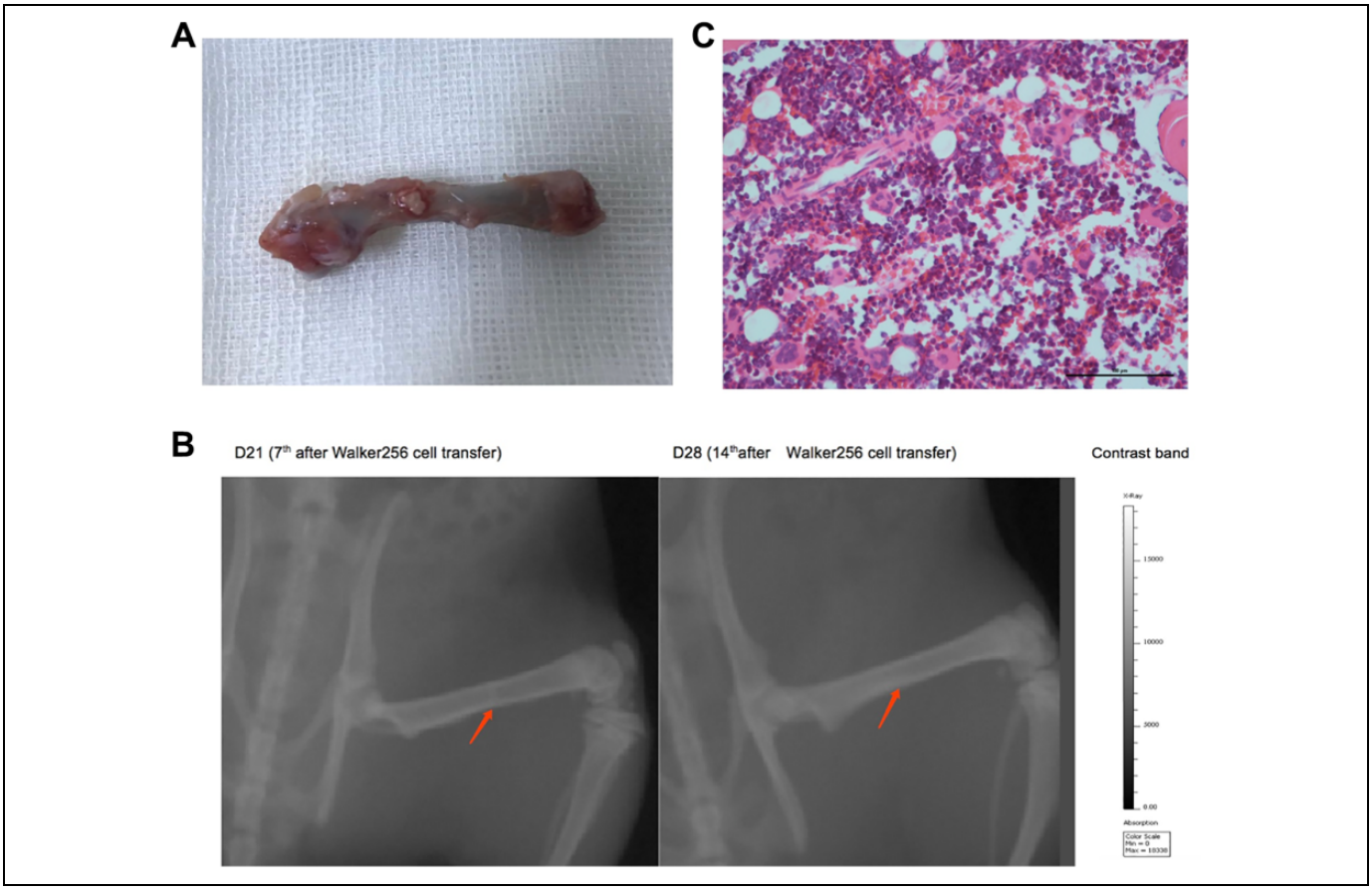

Figure 1. Bone cancer pain model establishment. A, Gross anatomy of Walker 256 carcinoma cells injected femur in rats. B, X-ray image of cancer cell transferred femur in rats. C, Femoral mass photographs of the Walker 256 carcinoma cell transfer side.

minutes. The middle part of the hind foot was stimulated with a 2 g Von Frey fiber probe initially and increased until the rat showed foot lifting or positive cowardly foot reaction. The minimum intensity when a positive reaction occurs was recorded and at least 5 minutes was given between tests. The mean value of 4 consecutive measurements was used as the mechanical pain threshold.

Western Blot Analysis

Sixteen days after continuous analgesia (from D35 to D51), 5 rats were randomly selected from each group and killed by pentobarbital anesthesia. The lumbar spinal cord L4 to L6 and the tumor tissues were lysed with radioimmunoprecipitation assay (P001; Ukzybiotech Ltd) containing protease inhibitors and phosphatase inhibitors (04693116001; Roche, Basel, Switzerland), incubated on ice, centrifuged, and the supernatant was removed. Next, the lysate protein concentrations were determined with a bicinchoninic acid protein assay kit (02912E, CWbiotech), mixed with 5 × sodium dodecyl sulfate (SDS) sample buffer, and boiled for 10 minutes. Equal samples of protein from animals were electrophoresed by SDS-polyacrylamide gel electrophoresis in 12% polyacrylamide gel and transferred to polyvinylidene fluoride (Millipore) membrane. The membranes were stained with Ponceau staining reagent after the transfer was completed. The membrane was well immersed in 5% bovine serum albumin–tris-buffered saline with Tween 20 (BSA-TBST) and incubated on a horizontal shaker for 2 hours. A 5% BSA-TBST diluted with primary anti-μ-opioid receptor rabbit antibody (1:500, ab134054; Abcam, Cambridge, United Kingdom) was added and immersed overnight at 4°C. Secondary antibodies diluted in 5% BSA-TBST: goat antirabbit, goat antimouse immunoglobulin G (H + L) horseradish peroxidase (1:10000; 111-035-003, 115-035-003, Jackson Medical Supply, Vacaville, California) were then added and incubated for 40 minutes at room temperature. After TBST was washed 3 times, the electrochemiluminescence (WBKLS0500; Millipore) solution was added dropwise to the protein surface of the membrane to react, expose, develop, and fix. After the image was scanned, the image was subjected to gray analysis using the software Gel Image system version 4.00 (Tanon, Shanghai, China), and the results were statistically compared. GAPDH (5174, CST) was used as an internal reference.

Immunochemistry

Immunohistochemistry was performed according to the standard protocols. The sample that had been fixed and sliced was soaked in 4% paraformaldehyde (phosphate-buffered saline [PBS]) for 24 hours. In order to eliminate enzymatic activity,

tissues were incubated in 3% H_2O_2 for 10 minutes and washed 3 times for 5 minutes using PBS. The samples were blocked using 5% bovine serum albumin (A8020, Solarbio, diluted in PBS) and incubated at room temperature for 30 minutes. The blocking serum was removed, primary antibody was added (anti-MOR antibody, 1:50, ab134054, Abcam, Cambridge, United Kingdom), and then incubated at 4°C overnight. After washing with PBS twice and followed by reacting with the secondary immunoglobulin at 37°C for 30 minutes, direct observation with an inverted microscope (Nikon Ci-S) and analysis using a Nikon imaging system (Nikon DS-U3) were performed.

Immunohistochemical images of tumor tissue were collected, and 200-fold images were taken for semiquantitative analysis. The cells with opioid receptor peptides staining specificity of the secondary antibody, the color of positive cells was deeper than others. Five sections of each group were randomly selected, and each of the slides was taken from the top left, right top, middle, bottom left, and right bottom fields for counting. Each field was counted by 2 different researchers.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 7 statistical analysis software, and $P < .05$ was established as statistically significant. The mechanical pain threshold results were expressed as mean (standard deviation). Pain thresholds were compared using continuous repeated measures, and 1-way analysis of variance (ANOVA) was used to compare the mechanical pain threshold of different time points and difference between groups. The differences of gray levels of MOR protein in spinal cord and tumor tissues of each group were tested by normality and then analyzed by 1-way ANOVA. When significant differences were found, statistical analysis between groups was made by Student *t* test with the Bonferroni adjustment for multiple comparisons.

Results

Bone Cancer Pain Model Establishment

Anatomical change in the pathology of the femur after cancer injection was showed in Figure 1A. Femur X-ray exhibited cortical bone reactions in the tumor cells injected femur of rats on the 14th day (D28) after injection (Figure 1B). Hematoxylin and eosin staining revealed densely packed heterogeneous clusters of nuclei in the rat femur tissue section, which was consistent with the pathological changes of bone tumor cells (Figure 1C). The rat bone cancer pain model was successfully established.

Two rats died during the period of continuous analgesia, including 1 in the control group (D38) and 1 in the intrathecal group (D47). Another 5 rats detached the intrathecal catheter and the analgesia could not be continued, including 1 in the control group, 2 in the intraperitoneal group, and 2 in the intrathecal group. Both of the 2 rats' death may be due to an intracranial infection resulting from an intrathecal administration procedure.

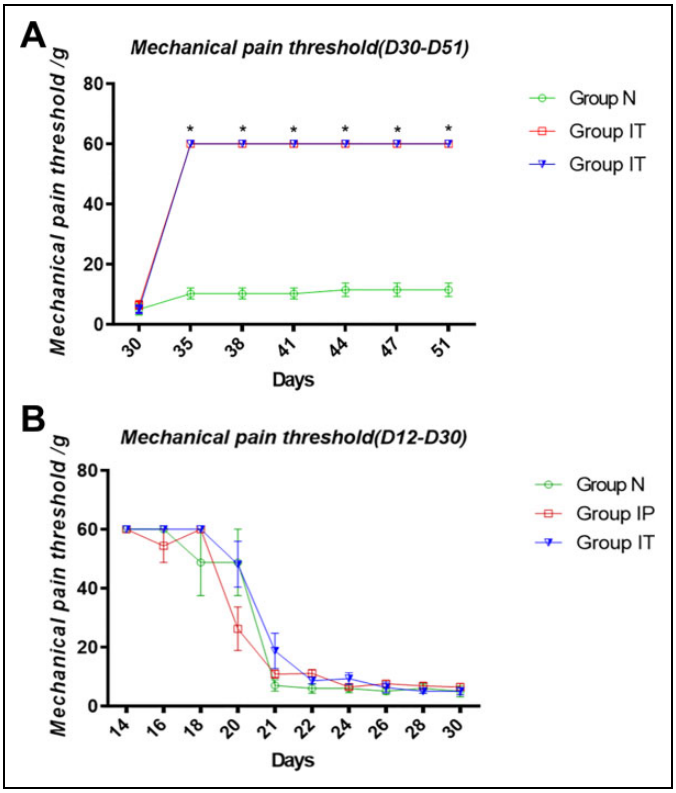

Figure 2. Mechanical pain threshold. A, Mechanical pain threshold of bone cancer pain rats from 14th to 30th day. On D14, Walker256 cells was injected into the femur and the innocent pain threshold was 60 g (cutoff intensity). Since D20, it began to decline. On day 30, there was no significant difference between 3 groups (group IT vs group N: $P = .999$; group IP vs group N: $P = .769$; group IT vs group IP: $P = .676$). B, Pain thresholds of group IP and group IT increased after analgesia were provided and maintained at baseline (60 g). Pain threshold in group N was unchanged. Since D35, group IT and group IP were significantly lower than group N ($P < .0001$).

Mechanical Pain Threshold

Mechanical pain threshold of the intrathecal group (group IT, n = 5), intraperitoneal group (group IP, n = 5), and control group (group N, n = 5) significantly declined on the sixth day after cancer cells injection and maintained at stable low values. There was no significant difference among 3 groups before analgesia was provided (Figure 2A).

During analgesia period (from the D35 to the end of the experiment, Figure 2B), the mechanical pain threshold was unchanged in the control group. In both the intrathecal group and the intraperitoneal group, pain threshold increased dramatically and returned to baseline without differences between the 2 groups. However, both group IT and group IP pain thresholds were significantly higher than the control group.

Expression Levels of MOR in the Spinal Cord and Tumor Tissues

After 16 days of continuous analgesia (D35-D51), MOR protein levels among the 3 groups of spinal cord tissue (n = 5)

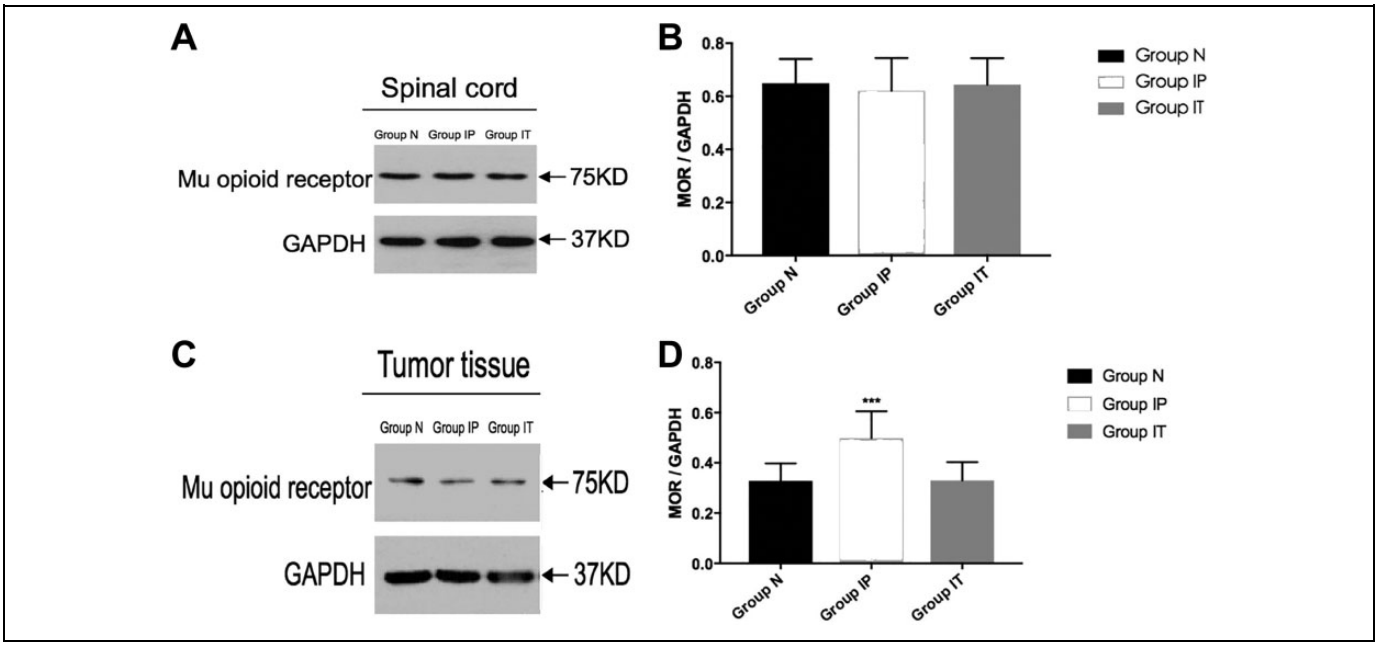

Figure 3. Expression levels of MOR; μ-opioid receptor expression levels in the spinal cords of cancer pain rats after 16 days treatment with saline (group N), intraperitoneal morphine (group IP), or intrathecal morphine (group IT; D51). A, Western blot detection of MOR expression in spinal cord. B, The ratio of MOR to GAPDH levels in spinal cord. C, Western blot detection of MOR expression in tumor tissue. D, The ratio of MOR to GAPDH levels in tumor tissue. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase; MOR, morphine μ-receptor.

showed no statistical difference ($P = .9334$). The MOR protein levels of bone tumors were significantly higher in the intraperitoneal group than in the control group ($P = .020$). However, there was no significant difference between the intrathecal group and the control group ($P = .999$, Figure 3). The MOR level of the intraperitoneal group was obviously higher than that of the intrathecal group ($P = .021$, Figure 3).

Immunohistochemistry of MOR Expression and MOR Positive Cells in Tumor Tissues and Spinal Cord

For tumor tissues, the percentage of MOR-immunopositive cells of the control group compared with the intrathecal group showed no statistical difference ($P = .761$). However, the percentage of MOR-immunopositive cells was statistically higher in the intraperitoneal group ($P < .0001$, Figure 4). For the spinal cord tissue, there was no statistical significance among these 3 groups in terms of the percentage of MOR-immunopositive cells (group N vs group IT: $P = .806$; group N vs group IP: $P = .973$; group IT vs group IP: $P = .912$, Figure 4).

Discussion

With emphasis on the control of cancer pain and the prolonged survival of patients with cancer, short-term cancer pain control has been gradually transformed to long-term analgesic therapy. Long-term systemic medications can relieve pain, but often carry serious side effects, including sedation, confusion, constipation, and fatigue.[12] Therefore, it is important to search for alternative administration routes which can reduce side effects, improve analgesia, and increase patient satisfaction.

Small dose of intrathecal morphine can achieve the same analgesic effect as oral or parenteral administration. Due to the much smaller dosage and limited action site at the level of spinal cord, side effects are much less.[13-15] Interestingly, a randomized controlled clinical trial found that patients received intrathecal morphine obtained better survival compared to systemic morphine patients.[10] Studies have linked μ-opioid receptor in the improved survival with intrathecal morphine.[16] A μ-opioid receptor is the main receptor for opioid analgesic drugs. Enhancement of the potency of μ-opioid receptor agonists could arise from the changes in the affinity and/or the number of μ-opioid receptors.[17,18] In lung[5] and prostate cancer,[6] it was observed an increase in the MOR levels in tumor tissues. In addition, overexpression of MOR in human non-small cell lung cancer cells increased tumor growth and metastasis both in vitro and in vivo.[7] Furthermore, it was showed that knocking out the MOR gene from lung cancer mice or using specific MOR blocker naltrexone (MNTX) could inhibit the growth and metastasis of lung cancer cells.[19] Controlling the activation of opioid receptors might have important implications during cancer progression and metastasis. Research also found that the group with lower MOR expression level is more likely to have a better prognosis.[16,20]

Previous research of intrathecal morphine was short term in nature via either direct lumbar puncture or a single use intrathecal drug delivery system.[21,22] The challenges of long-term indwelling intrathecal catheters are large invalid medication volume and the fact that rats will bite these catheters. We designed a tunneled intrathecal delivery system with a short exiting segment at the neck, making biting difficult for rats.

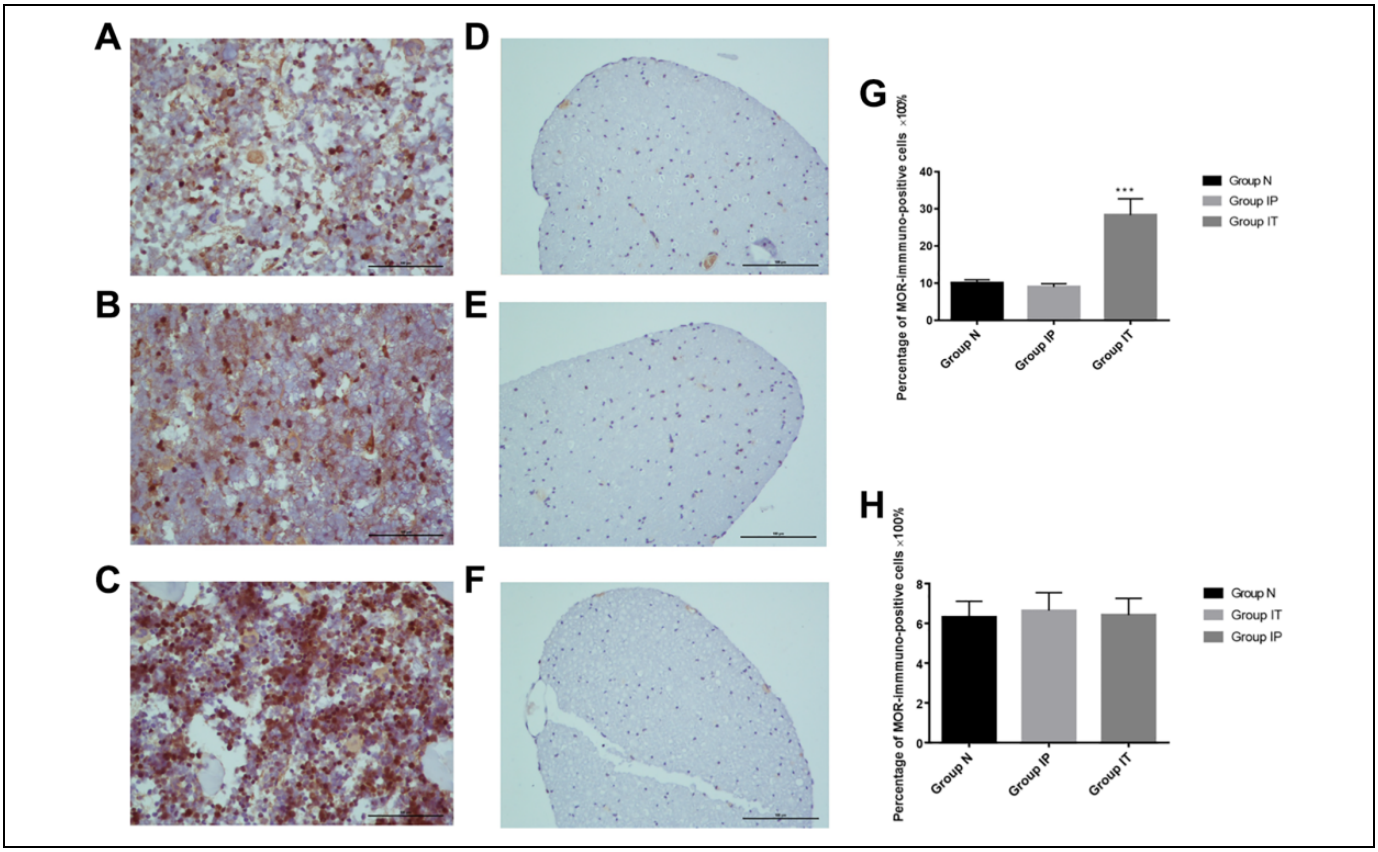

Figure 4. Immunohistochemistry of MOR expression and MOR positive cells in tumor tissues and spinal cord. Representative image of μ-opioid receptor expression in tumor tissues in control group (A), intrathecal group (group IT, B), intraperitoneal group (group IP, C), and in spinal cord tissues of group N (D), group IT (E), and group IP (F). The number of MOR-immunopositive cells in tumor tissue (G) and spinal cord (H) of rats in control group (group N), intrathecal group (group IT), and intraperitoneal group (group IP). Data are shown as mean (SD); n = 5 experiments per observation/data shown. ***$P < .0001$, significantly different vs all other measurements. Magnification 200×. Scale bars = 100 μm. MOR indicates morphine μ-receptor; SD, standard deviation.

This system is suitable for long-term and repeated intrathecal administration of medication in rat models.

To investigate the molecular mechanisms of intrathecal opioids on pain control and cancer progression, we developed a bone cancer pain rat model to reflect the in vivo environment of patients with cancer. Intraperitoneal morphine obviously upregulated MOR expression and the percentage of MOR immune positive cancer cells in the tumor tissues. Preclinical data from several laboratories have suggested that μ-opioids can promote cancer progression[23-25]; emerging literature involving epidemiologic, cellular, and animal data suggests that μ-opioids influence cancer progression and recurrence. There also appear to be effects mediated by the MOR, even in the absence of exogenous opiates.[7,19] Other data have suggested that MOR agonists can enhance the metastatic potential of a cancer by increasing vascular permeability.[6] Another approach was to examine MOR expression in tumors. Several laboratories have demonstrated that the MOR is overexpressed in both malignant lung and prostate tissue.[3,26] The increased MOR could potentially lead to more tumor growth, metastasis, and death. The most interesting finding of our study is that long-term intrathecal administration of morphine did not increase the MOR expression and the percentage of MOR-immune positive cancer cells in the tumor tissues, which may lead to less tumor growth, less metastasis, and better survival.

The expression of μ-opioid receptors in the tumor tissue could be related to local morphine concentrations. With a much lower concentration of morphine at the peripheral tumor cells in the intrathecal group, MOR expression might be less stimulated and thus maintained at a low level comparable with the control group. The lack of difference in the MOR levels between the intrathecal group and control group, coupled with the fact that intrathecal group has significantly less pain, might suggest that pain is not related to the expression level of MOR in tumor tissues.

We found that intrathecal long-term morphine analgesia has similar analgesic effect at a much lower dose. There was no significant difference in MOR expression levels in the spinal cord among all 3 groups, indicating that neither intrathecal nor intraperitoneal application of morphine affected MOR expression in the spinal cord. The enhanced analgesic effects in the intrathecal group might be from higher local concentration of morphine or stronger affinity with receptors.

There are several limitations of our study. First, our sample size was small. Second, we only observed rat model for 16 days and did not study the patterns of metastasis with intrathecal and intraperitoneal morphine administration. We plan to observe these 3 groups for a much longer time in our future study to elucidate the effects on tumor metastasis. Third, immunosuppression caused by opioids was also an important factor that may influence the growth and metastasis of tumors during long-term cancer pain treatment. We plan to study the cytokine levels in our rat model under different routes of opioid administration. In summary, long-term intrathecal morphine administration provided similar analgesia compared to systemic morphine with much lower MOR expression levels of tumor tissues in a bone cancer pain rat model.

Declaration of Conflicting Interests

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