Effect of morphine and a low dose of ketamine on the T cells of patients with refractory cancer pain *in vitro*

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Abstract. The combination of morphine and ketamine is considered safe and efficacious in many patients. However, a considerable number of immunomodulatory effects have been reported to be produced by both morphine and ketamine. The aim of the present study was to assess the direct effect of morphine and a low dose of ketamine on the T cells of patients with refractory cancer pain *in vitro*. Venous blood was obtained from patients with refractory cancer pain and peripheral blood mononuclear cells were isolated using the Ficoll-Hypaque density gradient method. Anti-CD3 beads were used to isolate T cells by positive selection. Subsequently, the T cells were treated with vehicle, 200 ng/ml of morphine or 200 ng/ml of morphine + 100 ng/ml ketamine for 24 h, following which the cells were stimulated with anti-CD3 and anti-CD28. Flow cytometric analysis of $CD3^+$ T cells, and interleukin (IL)-2 and interferon (IFN)-γ in the supernatant, reverse transcription-quantitative PCR analysis for the detection of IL-2 and IFN-γ and western blotting for the detection of p65 nuclear factor (NF)-κB were performed. *In vitro*, the $CD4^+$ and $CD8^+$ T cell counts, $CD4^+/CD8^+$ ratio, secretion of IL-2 and IFN-γ in the supernatant, mRNA expression levels of IL-2 and IFN-γ and expression of p65 NF-κB were significantly decreased following treatment with morphine and morphine + ketamine, compared with results in the control group (all $P<0.05$). However, there was no significant difference between treatment with morphine and that with morphine + ketamine. Treatment with morphine + ketamine *in vitro* decreased the immune functions of patients with refractory cancer pain, although the effect of treatment with morphine and a low dose of ketamine did not differ significantly from that with morphine treatment alone.

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Introduction

Pain associated with cancer is a serious problem for patients, significantly affecting their quality of life and is difficult to treat. A considerable proportion of patients suffer from refractory cancer pain. Refractory pain is associated with the growth and metastasis of tumors (1,2), therefore, pain relief may be beneficial for improving the patient's quality of life while also supplementing cancer therapy. The World Health Organization has proposed that a three-step analgesic ladder is effective for treating cancer pain (3). For neuropathic pain in advanced cancer, the first-line therapeutic agent is typically an opioid, such as morphine (4-7), however, pain may not be fully relieved for many patients. In these cases, the combination of pharmacological agents may be necessary to relieve the refractory pain. The combination of morphine and ketamine has been considered safe and efficacious in numerous patients (8-13). Clinically, patients with refractory cancer pain report improved pain relief with fewer side effects when morphine and a low dose of ketamine were used compared with morphine alone.

A number of patients experience cancer pain driven by nociception and neuropathy (14) and neuropathic cancer pain driven by nociception is considered to be resistant to opioids (15-17). For neuropathic pain, peripheral and central sensitization are the primary causes. Ketamine is an N-methyl-D aspartate (NMDA) antagonist and is also a non-opioid anesthetic agent. The primary clinical application of ketamine is for short diagnostic or surgical procedures (17). NMDA receptors are activated by the excitatory neurotransmitter glutamate in the spinal dorsal horn, which results in sensitization of the central nervous system (18-21). Therefore, attenuating this sensitization may underlie the analgesic effects of ketamine (19-21). Similarly, NMDA antagonism may be involved in the ketamine-mediated attenuation of acquired opioid tolerance (20-22).

However, in laboratory animals and humans, morphine has been reported to produce numerous immunomodulatory effects *in vivo* and *in vitro*. As a potent immunomodulator, morphine has inhibitory and stimulatory effects on immune

function, including altering the expression of $CD4^+$ and $CD8^+$ T cells, reducing the activity of natural killer cells (23-25), altering the balance between Th1 and Th2 cells (26,27), increasing the production of interleukin (IL)-12 by peritoneal macrophages (28), increasing the production of IL-6 and tumor necrosis factor when induced by lipopolysaccharides, and increasing the activity of nuclear factor-κB (NF-κB) in macrophages and peripheral blood mononuclear cells (PBMCs) (29,30). In particular, in activated T cells, morphine has been shown to enhance the expression of IL-4 and suppress the expression of interferon (IFN)-γ and IL-2 (31-35). For patients with cancer and an impaired immune system, the immunomodulatory effects of morphine may cause unwanted side effects during pain treatment. However, there are few reports regarding the effects of morphine on T cells in patients who suffer from refractory cancer pain. Similarly, there are few reports regarding the effect of ketamine on immune cells, particularly in T cells. Therefore, the aim of the present study was to assess the direct effect of morphine and a low dose of ketamine on the T cells of patients with refractory cancer pain *in vitro*.

Materials and methods

T cell culture, T cell activation and reagents. The entire protocol (SDTHEC201504001) was approved by the Ethics Committee of Shandong Cancer Hospital and Institute (Jinan, China; Chairperson Dr Jinming Yu) and conducted according to the principles of the Helsinki Declaration. Informed, written consent was obtained from all patients with cancer pain, and the patients were involved in the study for >3 months.

Blood was withdrawn following venipuncture. Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Hypaque density gradient method according to the manufacturer's protocol (Cedarlane). The T cells were isolated by positive selection using anti-CD3 beads (Miltenyi Biotec, Inc.) according to the manufacturer's protocol, and confirmed by fluorescence-associated cell sorting (85% purity). A final concentration of $1x10^5$ cells/ml were plated into 96-well plates, and the T cells were cultivated at 37˚C and 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.).

The T cells were treated with either vehicle (normal saline), 200 ng/ml of morphine (Humanwell Healthcare (Group) Co., Ltd.), or 200 ng/ml of morphine + 100 ng/ml ketamine (pure hydrochloride salt without preservatives, Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C. By determining the plasma concentration of morphine and ketamine in patients with refractory cancer pain in the clinic, and referring to other human and animal experiments using morphine and ketamine, a morphine concentration of 200 ng/ml and ketamine concentration of 100 ng/ml were established (36-38). These concentrations are approximately equal to the doses of morphine and ketamine commonly used in patients with refractory cancer pain in the clinic. Subsequently, the cells were stimulated with anti-CD3 and anti-CD28 (R&D Systems, Inc.; mouse-anti-human, 5 mg/ml) for 24 h at 37˚C. The groups were designated as the control group, morphine group and morphine + ketamine group. To activate T cells, it is necessary for the antigen-specific T cell receptor and a second co-receptor, such as CD28, to be sufficiently occupied (39). In the present study, anti-CD3 and anti-CD28 stimulation were used to investigate the effects of morphine and ketamine on the T cells.

Flow cytometric analysis of $CD3^+$ T cells. Following experimental treatment, the T cells ($1x10^5$/ml) were harvested by centrifugation for 5 min at 300 x g room temperature, washed in phosphate-buffered saline and subsequently incubated with trichromatism monoclonal antibody reagent [CD4 fluorescein isothiocyanate/CD8 phycoerythrin/CD3 PerCP (BD Biosciences), 0.5 mg/ml] according to the manufacturer's protocol. Subsequently, the quantity and percentage of CD3/CD4/CD8T cells were measured using a flow cytometer (FACSCalibur; BD Biosciences).

Supernatant cytokine protein analysis. Cytokine-specific ELISA kits (R&D Systems, Inc.) were used to measure the protein concentrations of IL-2 and IFN-γ according to the manufacturer's protocol. The experiments were repeated three times.

Reverse transcription-quantitative PCR analysis. Total RNA was extracted from the T cells by lysis using guanidinium isothiocyanate and phenol acid extraction. A total of 1 μg RNA, 0.5 μl RNase H minus (Promega Corporation) and 1 μl Moloney murine leukemia virus reverse transcriptase were used for cDNA synthesis with the following thermocycling conditions: 95˚C 5 min; 95˚C 15 sec, 60˚C 35 sec, 40 cycles; 72˚C 5 min; 4˚C terminal. For each PCR, 2 μl cDNA was used and the primers were obtained from BD Biosciences. The primer sequences were as follows: IL-2 forward, 5'-GAATGGAAT TAATAATTACAAGAATCCC-3' and reverse, 5'-TGTTTC AGATCCCTTTAGTTCCAG-3'; and IFN-γ forward, 5'-TCG GTAACTGACTTGAATGTCCA-3' and reverse, 5'-TCCTTT TTCGCTTCCCTGTTTT-3'. The Light Cycler-Fast Start DNA Master SYBR Green I kit (Roche Diagnostics) was used for the RT-qPCR analysis of IL-2, and the Revert Aid™ First Strand cDNA Synthesis kit (Roche Diagnostics) was used for IFN-γ, according to the manufacturer protocols. The quantitative analysis of original template was performed by the change of fluorescence of the amplification product.

Western blot analysis for activated p65 NF-κB. The T cells were lysed with RIPA protein lysis buffer (100 μg/ml Nonidet P-40, 150 mol/l NaCl and 50 mmol/l Tris-HCl supplemented with a protease inhibitor mixture). Following one freeze/thaw cycle, the lysates were centrifuged for 15 min at 12,000 g (2-8˚C). A BCA protein concentration kit was used to determine protein concentration. Following electrophoresis using 10% SDS-PAGE, the resolved protein samples were transferred onto an ECL nitrocellulose membrane (EMD Millipore) by electroblotting. 5% (w/v) of skimmed milk powder was used for blocking for 1 h at room temperature. Subsequently, the nitrocellulose membranes were incubated overnight at 4˚C with primary antibody [rabbit anti-NF-κB p65 antibody (cat. no., ab16502); dilution, 1:2,000; Abcam/rabbit anti-β-actin (cat. no., ab8227); dilution, 1:2,000; Abcam], and then incubated with the

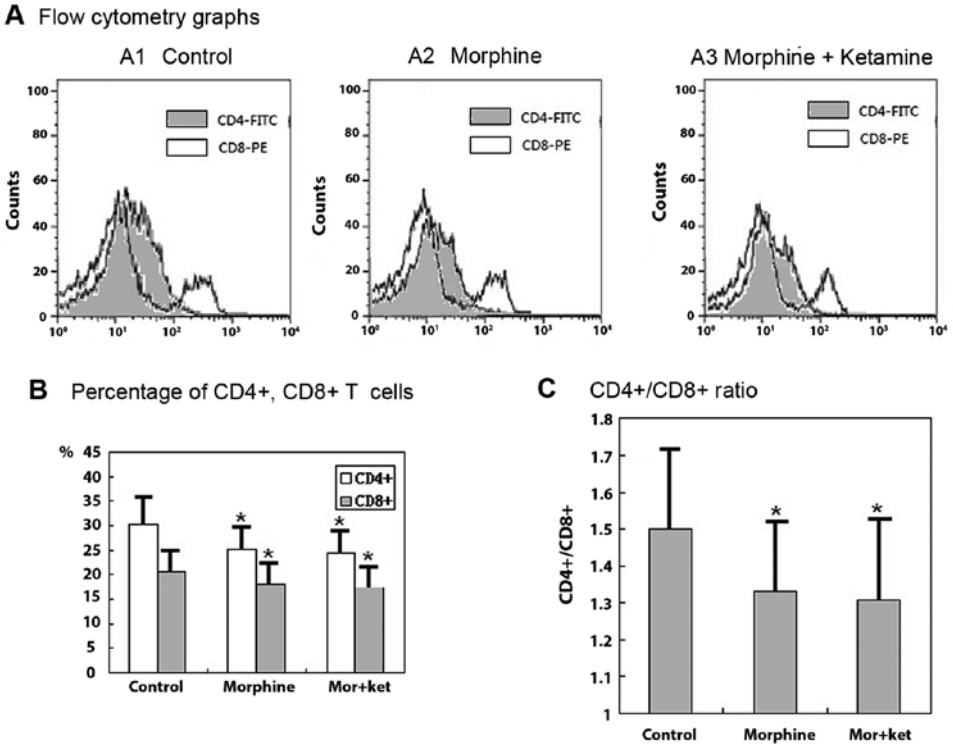

Figure 1. Effects of morphine and low-dose ketamine on the quantity and percentage of $CD3^+/CD4^+/CD8^+$T cells from patients with refractory cancer pain. Flow cytometry was performed to examine cells *in vitro*. (A) Flow cytometry graphs of the (A1) control group, (A2) morphine group and (A3) morphine + ketamine group. (B) Percentages of $CD4^+$ and $CD8^+$ T cells. (C) Ratios of $CD4^+/CD8^+$ T cells. *P<0.05, vs. Control. Mor + ket, morphine + ketamine.

secondary goat anti-Rabbit horseradish peroxidase antibody (cat. no., ab205718; 1:2,000; Abcam) at room temperature for 2 h. Protein concentration was determined by ECL western blotting detection kit (cat. no., ab65623; Abcam). Image J version 1.8.0 (National Institutes of Health) was used for quantification of blots.

Statistical analysis. All variables were assessed in triplicate and all experiments were repeated at least three times. All data are expressed as the mean ± standard deviation. Statistical comparisons between experimental groups were performed, where appropriate, with SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was analyzed using a paired t-test or one-way ANOVA followed by a non-parametric Student-Newman-Keuls test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Flow cytometric analysis of $CD3^+$ T cells. The counts of T cells expressing CD4 and CD8 were significantly decreased when the cells were treated with morphine and when treated with morphine + ketamine. The decrease in the count of $CD4^+$ T cells was larger than that of $CD8^+$ T cells, therefore, the ratio of $CD4^+/CD8^+$ was also significantly decreased by morphine and morphine + ketamine treatments (Fig. 1A-C; all P<0.05). However, the decreases in the $CD4^+$ and $CD8^+$ T cell counts and the $CD4^+/CD8^+$ ratio did not differ significantly when the cells were treated with morphine compared with those when the cells were treated with morphine + ketamine.

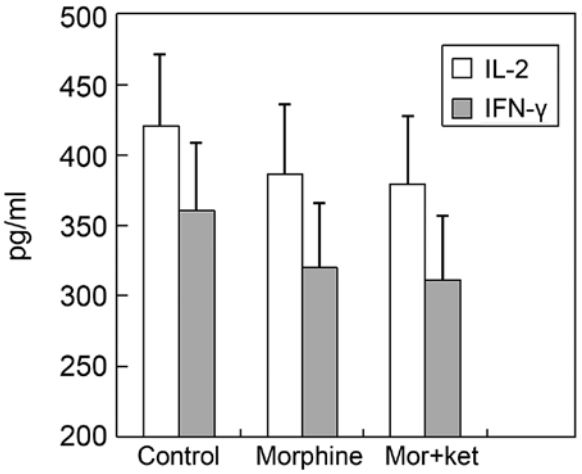

Figure 2. Supernatant protein concentrations of IL-2 and IFN-γ of T cells from patients with refractory cancer pain treated with morphine and low-dose ketamine *in vitro*. IL-2, interleukin-2; IFN-γ, interferon-γ; Mor + ket, morphine + ketamine.

Supernatant cytokine protein analysis and RT-qPCR analysis. There were no significant differences in the concentrations of IL-2 and IFN-γ in the supernatants of the morphine group and the morphine + ketamine group compared with those in the control group (Fig. 2). However, the mRNA expression levels of IL-2 and IFN-γ were significantly decreased in the morphine group and the morphine + ketamine group compared with those in the control group (Fig. 3; P<0.05). The results of the morphine group did not differ significantly from those of the morphine + ketamine group.

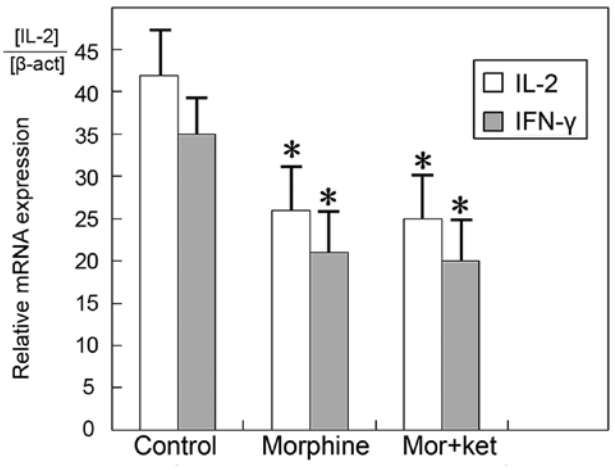

Figure 3. Reverse transcription-quantitative polymerase chain reaction analysis of IL-2 and IFN-γ of T cells from patients with refractory cancer pain treated with morphine and low-dose ketamine *in vitro*. *P<0.05, vs. Control. IL-2, interleukin-2; IFN-γ, interferon-γ; Mor + ket, morphine + ketamine.

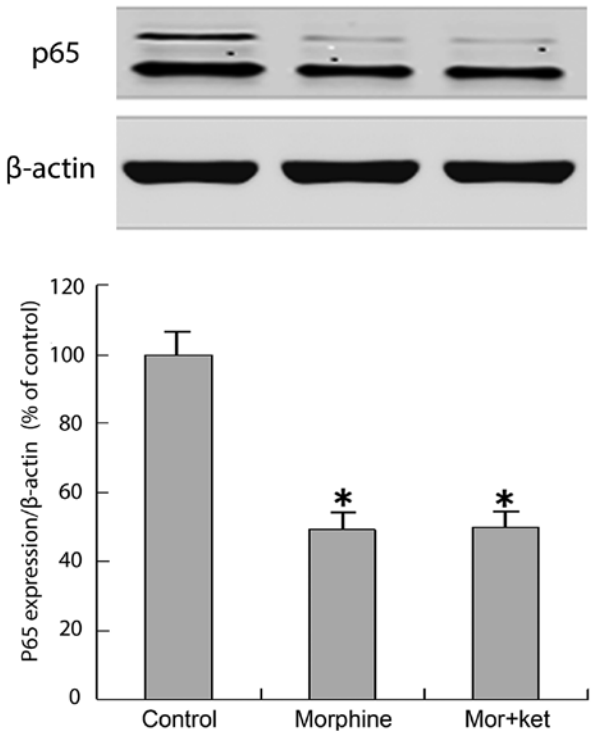

Figure 4. Western immunoblot analysis of activated p65 nuclear factor-κB of T cells from patients with refractory cancer pain treated with morphine and low-dose ketamine *in vitro*. *P<0.05, vs. Control. Mor + ket, morphine + ketamine.

Western blot analysis of activated p65 NF-κB. The morphine and morphine + ketamine treatments decreased the quantity of activated p65 NF-κB entering the nuclei of T cells compared with that in the control group (Fig. 4; P<0.05). The results in the morphine group did not differ significantly from those in the morphine + ketamine group.

Discussion

Patients with refractory cancer pain suffer from pain and consequent stress on a daily basis. Those individuals experiencing severe pain, stress or who become addicted to opioids are generally immunosuppressed resulting from activation of the neuroendocrine-immune network and the potent effect of neuroactive ligands on cells of the immune system (40-45). Opioids have been demonstrated to modulate immune responses, and opioid receptors are expressed on immune cells (41). *In vivo* and *in vitro*, the therapeutic and chronic use of morphine affects the physiological function of the immune system, including innate and adaptive immunity (46,47).

T cells and the various subpopulations serve important roles in cell-mediated immunity. The ratios of the subpopulations of T cells are stably maintained to ensure an effective immune response. In the present study, $CD4^+$ and $CD8^+$ T cells, and the ratio of $CD4^+/CD8^+$ T cells were all decreased by morphine, which suggested an attenuation of the immune response.

IL-2 is a pivotal cytokine for T cell function, and a previous study demonstrated that chronic treatment with morphine can inhibit the mRNA expression and secretion of IL-2 (48). Similarly, in the present study on patients with refractory cancer pain, the mRNA expression levels of IL-2 and IL-2 were downregulated by morphine treatment. A previous study showed that the expression of IL-2 was regulated almost entirely at the transcriptional level and that the essential elements lie within 300 bp upstream of the start codon (49), which can be bound by several inducible transcription factors including NF-κB. It has been hypothesized that the production of IL-2 is inhibited with chronic morphine treatment at the transcriptional level and through epigenetic mechanisms (31,34,35,50,51).

As a cytokine modulating all phases of immune processes, IFN-γ is produced in large quantities in activated T cells (52) and serves an important role in host resistance to infection (53). Previous studies have demonstrated that chronic morphine treatment inhibited IFN-γ protein synthesis (32,54), similar to the results of the present study. Furthermore, the results of the present study demonstrated that the mRNA expression levels of IFN-γ were downregulated by morphine, suggesting that morphine modulated IFN-γ by altering the gene expression and protein synthesis of IFN-γ. It is hypothesized that transcription of the IFN-γ gene is coordinated by the transcription factor NF-κB (55-57).

As a central regulator of the immune response (58), NF-κB regulates the transcription of various genes, including a number of cytokines and genes encoding immunoreceptors (59,60), and it is sequestered in the cytoplasm in the majority of cell types (61). NF-κB is also vital for regulating the activation, proliferation and differentiation of T cells (62). Roy *et al* (30) demonstrated that morphine prevented the binding of NF-κB to its consensus DNA sequence in macrophages and activated T cells of patients who had received chronic morphine treatment.

In vivo, the pathways regulating the immunomodulatory effects of opioids transduced to the immune effector cells primarily include the following: i) Indirectly via the central nervous system, for example, by activation of the hypothalamic-pituitary-adrenal axis (63,64); ii) directly via atypical opioid receptors (25); and iii) directly via the classic μ- (preferential), δ- and κ-opioid receptor subtypes present on immune cells (65,66) that belong to the G_i protein-coupled receptor superfamily (67). Additionally, it has been shown that the activation of NF-κB was inhibited by elevated cAMP in T cells at the molecular level (68-70). Therefore, morphine

may bind to μ-opioid receptors and activate G_i protein-coupled receptors, resulting in the intracellular accumulation of cAMP, and thus leading to decreased NF-κB activation and the inhibition of IL-2 and IFN-γ transcription.

Ketamine is often administered epidurally or intravenously in combination with an opioid for the treatment of chronic cancer pain (71,72). For the clinical treatment of refractory cancer pain, ketamine is often used as an adjuvant analgesic; as ketamine alone is not used for refractory cancer pain, a group treated with ketamine only was not included in the present study. However, ketamine also affects immune cells. It has been suggested that ketamine inhibits the production and function of dendritic cells (73). Treatment with ketamine alone has also been shown to increase the ratios of Th1/Th2 and IFN-γ/IL-4 (74). However, in the present study, ketamine did not induce significant effects in any of the measurements, including $CD4^+$ T cells, $CD8^+$ T cells, the $CD4^+/CD8^+$ ratio, concentrations of IL-2 and IFN-γ in the supernatant, mRNA expression levels of IL-2 and IFN-γ and the activation of NF-κB. A possible explanation for the lack of effect of ketamine was the dose used, which may have been too low. In clinical use, the dose of ketamine used is variable. The epidural administration of ketamine is typically ~0.4 mg/ml (~1.5 mM) (75-80), whereas intrathecal administration is typically <25 mg/ml (~93 mM) (81-83). Another study demonstrated that the infusion dose of ketamine for cancer-associated treatment was 0.084-0.6 mg/kg/h (20). In the present study, a dose of 100 ng/ml of ketamine was considered to be ineffective.

In conclusion, *in vitro* treatment with morphine alone and in conjunction with ketamine inhibited the immune functions of patients with refractory cancer pain. Treatment decreased the counts of $CD4^+$ and $CD8^+$ T cells, $CD4^+/CD8^+$ ratio, concentrations of IL-2 and IFN-γ in the supernatant, mRNA expression levels of IL-2 and IFN-γ and the activation of NF-κB, but the effect of morphine in conjunction with ketamine did not differ significantly from the effect of morphine alone. The present study may provide evidence for clinicians to offer more relief of pain and less suppression of immune function to patients with refractory cancer pain.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NBZ, KGW, ZJF designed the study and completed the organization and writing. NBZ conducted the experiments and analyzed the data.

Ethics approval and consent to participate

The entire protocol (SDTHEC201504001) was approved by the Ethics Committee of Shandong Cancer Hospital and Institute (Jinan, China; chairperson Dr Jinming Yu) and was conducted according to the principles of the Helsinki Declaration. Informed, written consent was obtained from patients with cancer pain.

Patient consent for publication

The patients consented to publication.

Competing interests

The authors declare that they have no competing interests.

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