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ORIGINAL PAPER

The Ubiquitination of Spinal MrgC Alleviates Bone Cancer Pain and Reduces Intracellular Calcium Concentration in Spinal Neurons in Mice

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Abstract

Mas-related G-protein-coupled receptor subtype C (MrgC) has been shown to play an important role in the development of bone cancer pain. Ubiquitination is reported to participate in pain. However, whether MrgC ubiquitination plays a role in bone cancer pain remains unclear. To answer this question, we designed and performed this study. Osteosarcoma cells were implanted into the intramedullary space of the right femurs of C3H/HeJ mice to induce progressive bone cancer pain. MrgC agonist bovine adrenal medulla 8–22 (BAM 8–22) or MrgC antagonist anti-MrgC antibody were injected intrathecally on day 14 after bone cancer pain was successfully induced. The pain behaviors, the MrgC ubiquitination levels and intracellular calcium concentration in spinal neurons were measured before and after injection, respectively. With comparison to normal and sham group, mice in tumor group exhibited serious bone cancer pain on day 14, and the level of MrgC ubiquitination and intracellular calcium concentration in spinal neurons was significantly higher. Intrathecal injection of BAM 8–22 significantly alleviated bone cancer pain, increased the MrgC ubiquitination level and decreased intracellular calcium concentration in spinal neurons; however, these effects were reversed by administration of anti-MrgC antibody. Our study reveals that MrgC ubiquitination participates in the production and maintenance of bone cancer pain in mice, possibly through the regulation of intracellular calcium concentration in mice spinal neurons.

Keywords Bone cancer pain · Mas-related G-protein-coupled receptor subtype C (MrgC) · Ubiquitination · Calcium concentration

Introduction

Bone cancer pain is a challenging clinical problem in patients with primary or metastatic bone cancer. It severely impairs the life quality of patients. However, pharmacological managements are still unsatisfactory because of low efficacy and side effects [1]. Currently, drugs that target G protein-coupled receptors (GPCRs) are the primary treatment strategy for patients with pain, however, more effective therapeutics with less side effects are still needed [2, 3].

Mas-related G-protein-coupled receptor subtype C (MrgC), also known as sensory neuron–specific receptors, shares substantial homogeneity with its human homolog, MrgX1. MrgC is exclusively expressed in small-sized neurons in trigeminal and dorsal root ganglia, which are important parts of the pain transmission pathway in mammals [4, 5]. Also, MrgC is involved in the pain process. Additionally, MrgC functions as a receptor for bovine adrenal medulla peptide (BAM), a proteolytically cleaved product of the proenkephalin A [6, 7]. Recent studies [8, 9] have reported that MrgC may contribute to a novel pain inhibitory mechanism. Intrathecal injection of BAM 8–22, an agonist of MrgC that activates endogenous MrgC, can decrease the expression of calcitonin gene related peptide and neuronal nitric oxide synthase (nNOS) as well as the release of excitatory amino acids [10]. Thus, activation of MrgC has been shown to decrease neuronal excitability in spinal dorsal horn

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and to inhibit both inflammation and neuropathic pain in animal models [6, 11, 12]. Furthermore, our previous studies have revealed that the expression of MrgC in the spinal cord increased with the development of bone cancer pain in mice [13, 14]. Intrathecal injection of BAM 8–22 in mice significantly alleviated bone cancer pain, with up-regulation of MrgC and inhibiting G protein α subunits, and down-regulation of spinal 2B subunit of *N*-methyl-D-aspartate receptor (NR2B) and nNOS, while administration of anti-MrgC antibody caused opposite effect [13, 14].

GPCRs produce different biological pain effects after binding to different G proteins, meanwhile protein ubiquitination plays an important role in biased excitation of GPCRs. It is reported that different subfamilies of G protein α subunits (Gα proteins) could interact with MrgC to mediate signal transduction [15]. However, accurate signal transduction demands that GPCRs selectively regulate a few of available and relevant G proteins expressed within a given cell [16]. Ubiquitination, one of the most common form of the protein post-translational modification, directly regulates many physiological processes by determining the trafficking, abundance, and localization of many transmembrane proteins, including MrgC [17]. Many studies [18, 19] have revealed that ubiquitination plays important role in a series of pathological processes such as cancer, metabolic syndromes, neurodegenerative diseases, autoimmunity, inflammatory disorders, infection and muscle dystrophies [20]. Recently, protein ubiquitination has been shown to be crucial for neuropathic pain development by regulating spinal cord plasticity [21]. At present, there are few studies on MrgC ubiquitination at home and abroad. However, whether MrgC ubiquitination participates in the development of bone cancer pain remains unknown.

In this study, we aim to investigate the role of spinal MrgC ubiquitination in the development of bone cancer pain by using mice model. We conclude that MrgC ubiquitination in the spinal cord alleviates the bone cancer pain and reduces the intracellular calcium concentration in spinal neurons.

Materials and Methods

Experimental Animals

This experiment was approved by the Institution of Animal Care and Usage Committee at the Medical School of Nanjing University. All the procedure were in accordance with the guidelines of the laboratory animals usage [22]. Adult male mice C3H/HeJ (4–6 weeks old, weighing 18–22 g, n = 152) were purchased from Beijing Vital River Experimental Animal Center (China). The mice were kept individually under a 12-h light/dark schedule at constant room temperature (21 ± 1 °C) with free access to food and water. All possible efforts have been done to minimize mice suffering and to reduce the number of animals used in this study.

Cell Culture

Osteosarcoma cells NCTC 2472 (purchased from American Type Culture Collection, Manassas, VA, USA, ATCC 2087787) were cultured in NCTC 135 medium (Sigma Aldrich, St. Louis, MO, USA) with 10% equine serum (Gibco, Carlsbad, CA, USA) at 37 °C. The cells were passaged twice a week.

Establishment of Bone Cancer Pain Model

The mouse model of bone cancer pain was established following the previous method described by Schwei et al. [23]. Briefly, on day 0, the mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), and then a right knee arthrotomy was performed. Later, 20 µl of NCTC 2472 cell suspension (about 2×10^5 cells) was injected into the intramedullary space of the femur to establish bone cancer pain model. For sham control, 20 µl of alpha-MEM medium alone was injected. Subsequently, the injection hole was sealed by using bone wax and the wound was carefully sutured.

Drug Treatments and Animal Grouping

BAM 8–22 (Sigma Aldrich, St. Louis, MO, USA) was completely dissolved in normal saline and diluted to 8.0 nM in accordance with previous description of Jiang et al. [24]. Similarly, anti-MrgC antibody (Biorbyt, San Francisco, CA, USA) was dissolved in saline at a ratio of 1:20. Saline was used for vehicle treatment. The mice were randomly divided into seven groups: Normal group (without treatment, n = 8), S + V group (sham group with vehicle, n = 32), S + B group (sham group with BAM 8–22 treatment, n = 8), S + A group (sham group with anti-MrgC antibody treatment n = 8), B + V group (bone cancer group with vehicle, n = 32), B + B group (bone cancer group with BAM 8–22 treatment, n = 32), and B + A group (bone cancer group with anti-MrgC antibody treatment, n = 32). On day 14 post-operation, 5 µl of BAM 8–22 or anti-MrgC antibody or saline was injected intrathecally.

Pain Behavior Tests

All tests were performed during the light phase by the experimenter who was blind to different treatments. Before test, mice were allowed to adapt to environment for about 30 min. The pain behavior tests were performed at different time points: before injection, and several time points

post-administration of BAM 8–22, anti-MrgC antibody and vehicle (1 h, 2 h, 12 h, and 24 h).

Number of Spontaneous Flinch (NSF)

The mouse was individually placed into plexiglass compartments (10 cm × 10 cm × 15 cm) and the NSF of the right hind paw within 2 min was measured. Every lift that was not for walk or groom was considered to be one flinch. Each mouse was tested five times.

Paw Withdrawal Mechanical Threshold (PWMT)

For mechanical allodynia test, PWMT was assessed by using Von Frey filaments (Stoelting, Wood Dale, IL) [25]. The mouse was individually placed into a transparent plexiglass compartment (10 cm × 10 cm × 15 cm) with a metal mesh floor (graticule: 0.5 cm × 0.5 cm). Von Frey filaments (0.16 g–2.0 g) were applied to the right hind paws. The filaments were pressed vertically against the plantar surface with sufficient force until causing a slight bending against the paw. The force was held for 6–8 s. Paw withdrawal or flinching was regarded as a positive response, and each mouse was tested five times. The lowest von Frey filament stimulus strength that caused at least three positive responses was regarded as PWMT.

Co-Immunoprecipitation

At day 14 after transplantation, the mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and sacrificed. The L3–L5 segments of the lumbar spinal cord were quickly isolated and stored in liquid nitrogen. The collected tissue was homogenized in lysis buffer and centrifuged at 4 °C. The supernatant was collected. 500 µl of supernatants were incubated with 1 µl anti-MrgC antibody (Biobyt) or 10 µl anti-ubiquitin antibodies (Santa Cruz Biotechnology) overnight at 4 °C. The protein A agarose beads were washed triplicates with lysis buffer, and then incubated with supernatants and antibodies for 2–4 h at 4 °C. After centrifugation, the supernatant was carefully removed. Subsequently, the protein A agarose beads were washed with lysis buffer and the bond proteins were eluted. Protein precipitated by antibodies was analyzed by western blot.

Western blot Analysis

Tissue samples were obtained from mice of each group at 2 h after administration. Mice were sacrificed rapidly by cervical dislocation after deep anesthesia. The spinal cord L3–L5 segments were removed rapidly and were homogenized in lysis buffer. The homogenate was centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant was collected. BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine protein concentrations. Then, protein samples were separated on SDS-PAGE gel and transferred to polyvinylidene difluoride filters (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk and then incubated with different primary antibodies, including rabbit anti-MrgC antibody (1:500; Biorbyt, San Francisco, CA, USA) and mouse monoclonal anti-ubiquitin antibody (1:250; Santa Cruz Biotechnology). The membranes were washed with TBST and then incubated with the corresponding secondary antibodies: polyclonal goat anti-rabbit IgG (1:5000; Abcam, Cambridge, UK) or polyclonal goat anti-mouse IgG (1:5000; Abcam, Cambridge, UK). Immunoblots were visualized in electro-chemi-luminescence solution (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 min and exposed to hyper-films (Amersham Biosciences, Piscataway, NJ, USA) for 1–10 min. β-actin (1:1000; Abcam, Cambridge, UK) was used as a loading control for total protein. The gray value of each band was quantified with a computer-assisted imaging analysis system (IPLab software, Scanalytics, Fairfax, VA, USA).

Neuron Isolation and Intracellular Calcium Measurement

The mice were sacrificed under deep anesthesia with pentobarbital sodium (50 mg/kg) 2 h after administration. The spinal cord was bluntly separated, washed with cold PBS triplicates and digested with 0.25% papain enzyme (Sigma-Aldrich, St. Louis, MO, USA) for 3 min. The digested cells were harvested, filtered through a 200-mesh filter, and then were used to measure the intracellular calcium concentration with the Fura 3-AM probe (Solarbio, Beijing, China) and on the Till Vision Imaging System (T.I.L.L. Photonics, Kaufbeuren, Germany).

Statistical Analysis

All statistical analyses were performed by IBM SPSS statistics version 21.0 (SPSS Inc., Chicago, USA). All data were shown as the mean ± SD. One-way ANOVA was used for multiple group comparison followed by post hoc test (Bonferroni test). A value of $p < 0.05$ was defined as statistically significant.

Results

Intrathecal Injection of BAM 8–22 Attenuates Bone Cancer Pain, While Anti-MrgC Enhances Bone Cancer Pain

Pain behaviors were used to assess the development of bone cancer pain after osteosarcoma cell implantation. Before

injection of BAM 8–22 (the agonist of MrgC) (pre-injection), NSF (Fig. 1a) and PWMT (Fig. 1b) had no significant difference among group S + V, group S + B and group S + A. However, tumor-bearing mice in group B + V, group B + B and group B + A showed significantly increased NSF and significantly decreased PWMT than group S + V, group S + B and group S + A ($p < 0.05$, respectively), which was consistent with our previous studies [13, 14]. After intrathecal injection of BAM 8–22, B + B group displayed decreased NSF ($F_{N1} = 4.12$, $F_{N2} = 6.39$, $F_{N12} = 4.07$, $F_{N24} = 3.37$; $p < 0.05$) and increased PWMT with comparison to B + V group ($F_{p1} = 3.99$, $F_{p2} = 6.46$, $F_{p12} = 4.51$, $F_{p24} = 2.94$; $p < 0.05$). On the contrary, intrathecal injection of anti-MrgC significantly increased NSF ($F_{N1} = 3.91$, $F_{N2} = 5.44$, $F_{N12} = 4.02$, $F_{N24} = 3.63$; $p < 0.05$) and decreased PWMT in B + A group, compared with the B + V group ($F_{p1} = 4.28$, $F_{p2} = 5.36$, $F_{p12} = 4.35$, $F_{p24} = 3.14$; $p < 0.05$). Additionally, both BAM 8–22 and anti-MrgC antibody took effect at 1 h, peaked at 2 h, attenuated after 12 h, and disappeared at 24 h post-administration. No significant change in PWMT and NSF was observed in mice among the S + V, S + B, and S + A group (Fig. 1). These data suggest that bone cancer pain is attenuated by intrathecal administration of BAM 8–22 but aggravated by anti-MrgC antibody injection.

The Level of MrgC Ubiquitination is Increased in the Spinal Cord in Mice with Bone Cancer Pain

To assess the ubiquitination of MrgC during the development of bone cancer pain, co-immunoprecipitation assay was conducted. Compared with the normal and S + V groups, the level of MrgC ubiquitination was significantly up-regulated in B + V group on day 14 after osteosarcoma cell implantation ($p < 0.05$, respectively) (Fig. 2a, b), indicating that MrgC ubiquitination may participate in the development of bone cancer pain.

Intrathecal Injection of BAM 8–22 Increases Whereas Anti-MrgC Antibody Decreases the Level of MrgC Ubiquitination

To verify whether BAM 8–22 or anti-MrgC antibody injection could regulate the level of MrgC ubiquitination, the expression of MrgC or MrC ubiquitination on day 14 post-operation was detected. Results showed that the level of MrgC ubiquitination in the spinal cord was significantly up-regulated at 2 h after BAM 8–22 administration (B + B group, $p < 0.05$), while significantly down-regulated at 2 h after anti-MrgC antibody injection (B + A group, $p < 0.05$), compared with the B + V group (Fig. 3a, b). Meanwhile, no significant alteration in the ratio of ubiquitin/MrgC was observed in S + V, S + B, and S + A group after BAM 8–22 or anti-MrgC antibody administration (Fig. 3a, b). In addition, these changes in the ratio of ubiquitin/MrgC were consistent with those of the pain behavior after BAM 8–22 or anti-MrgC antibody injection. Intrathecal injection of BAM8–22, a highly selective agonist of MrgC, could significantly up-regulate the ratio of spinal ubiquitin/MrgC.

Intrathecal Injection of BAM 8–22 Decreases Intracellular Calcium Concentration in Spinal Neurons, While Intrathecal Injection of Anti-MrgC Increases It

Intracellular calcium concentration in spinal neurons was detected by using calcium imaging. Compared with mice in normal and sham groups (S + V, S + B, S + A), the fluorescence intensity of calcium was obviously higher in mice with bone cancer pain (B + V, B + A, B + B) on

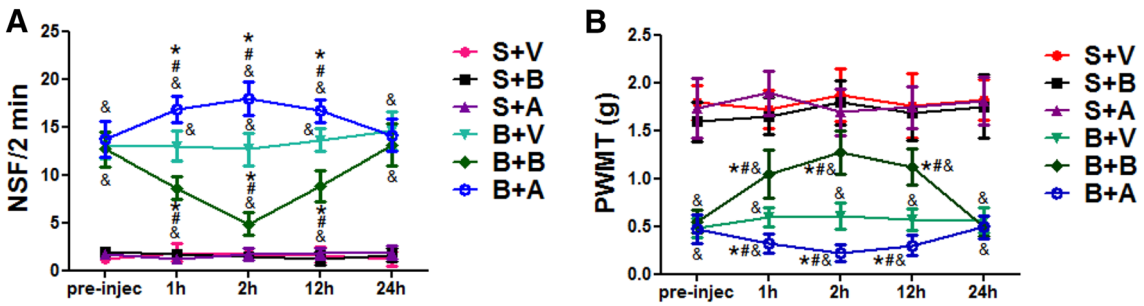

Fig. 1 Analysis of pain behaviors. **a** The number of spontaneous flinch (NSF) (within 2 min) and **b** paw withdraw mechanical threshold (PWMT) were tested before BAM 8–22 injection (pre-injection) and at 1 h, 2 h, 12 h, and 24 h after intrathecal injection of BAM 8–22, anti-MrgC antibody, and vehicle on day 14 post-operation. S + V, sham group mice + vehicle; S + B, sham group mice + BAM 8–22; S + A, sham group mice + anti-MrgC antibody; B + V, bone cancer pain mice + vehicle; B + B, bone cancer pain mice + BAM 8–22; B + A, bone cancer pain mice + anti-MrgC antibody. All data are presented as the mean ± SD. *$p < 0.05$, compared with pre-injection mice; #$p < 0.05$, compared with the B + V group mice; &$p < 0.05$, compared with the S + V group mice; n = 8 mice per group. NSF, number of spontaneous flinches; PWMT, paw withdraw mechanical threshold

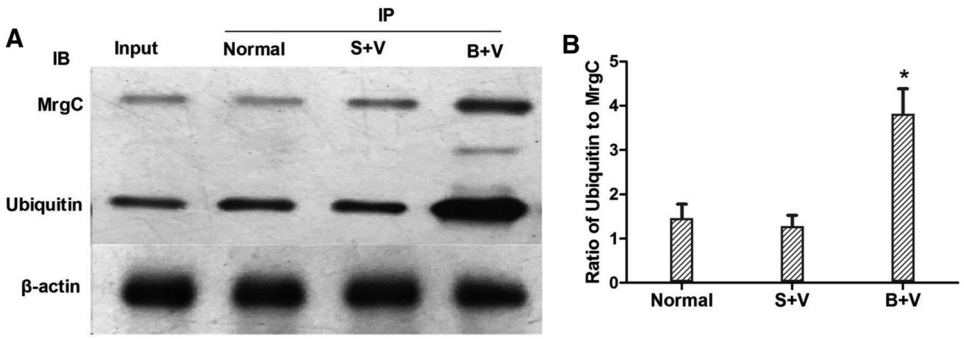

Fig. 2 Analysis of MrgC ubiquitination. Western blot was performed. **a** Representative blots of MrgC and MrgC ubiquitination, and **b** quantification of the ratio (ubiquitin/MrgC) in the spinal cord of the normal, sham, and tumor group mice. All data are presented as the mean ± SD (n = 6). ANOVA, *p < 0.05, compared with the normal group mice. Input: normal spinal lysate as a positive control; *IP* immunoprecipitation, *IB* immunoblotting; S + V, sham group mice + vehicle; B + V, bone cancer pain mice + vehicle

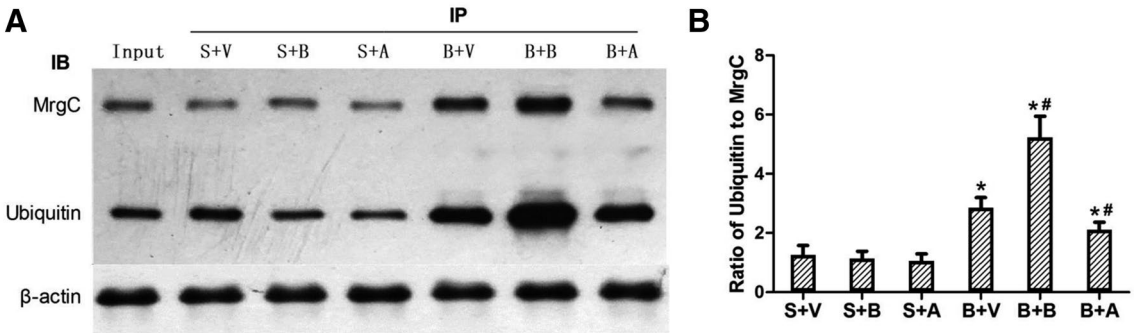

Fig. 3 Analysis of MrgC ubiquitination after intrathecal injection of BAM 8–22 or anti-MrgC antibody. Western blot was performed. **a** Representative blots of MrgC and ubiquitin, and **b** quantification of the ratio (ubiquitin/MrgC) in the spinal cord at 2 h after intrathecal injection of BAM 8–22, anti-MrgC antibody, and vehicle on day 14 post-operation. S + V, sham group mice + vehicle; S + B, sham group mice + BAM 8–22; S + A, sham group mice + anti-MrgC antibody; B + V, bone cancer pain mice + vehicle; B + B, bone cancer pain mice + BAM 8–22; B + A, bone cancer pain mice + anti-MrgC antibody. All data are presented as the mean ± SD (n = 6). ANOVA, *p < 0.05, compared with S + V group; #p < 0.05, compared with the B + V group mice. Input: normal spinal lysate as a positive control; *IP* immunoprecipitation, *IB* immunoblotting

day 14 after osteosarcoma cell implantation (Fig. 4a, b), which indicates that the intracellular calcium concentration is increased in spinal neurons with the development of bone cancer pain. Additionally, administration of BAM 8–22 significantly decreased the intracellular calcium concentration in spinal neurons of mice in B + B group post-2 h injection with comparison to mice in the B + V group ($p < 0.05$). However, after 2 h of anti-MrgC antibody administration, the intracellular calcium concentration in spinal neurons showed an up-regulation in mice of B + A group than that in mice of the B + V group ($p < 0.05$) (Fig. 4a, b). These results confirmed that the intracellular calcium concentration in spinal neurons was significantly decreased after BAM 8–22 injection but increased with administration of anti-MrgC antibody.

Discussion

Currently, the management of bone cancer pain, which severely influence the life quality of patients with primary or metastatic bone cancer, remains far from satisfactory [26]. Our study provides primary insight into the physiologic roles of MrgC ubiquitination in bone cancer pain. Intrathecal injection of BAM 8–22, an agonist of MrgC, significantly attenuated bone cancer pain, with up-regulated MrgC ubiquitination level in the spinal cord and down-regulated intracellular calcium concentration in spinal neurons. On the contrary, intrathecal injection of anti-MrgC antibody aggravated bone cancer pain, accompanied with MrgC ubiquitination decrease and the intracellular

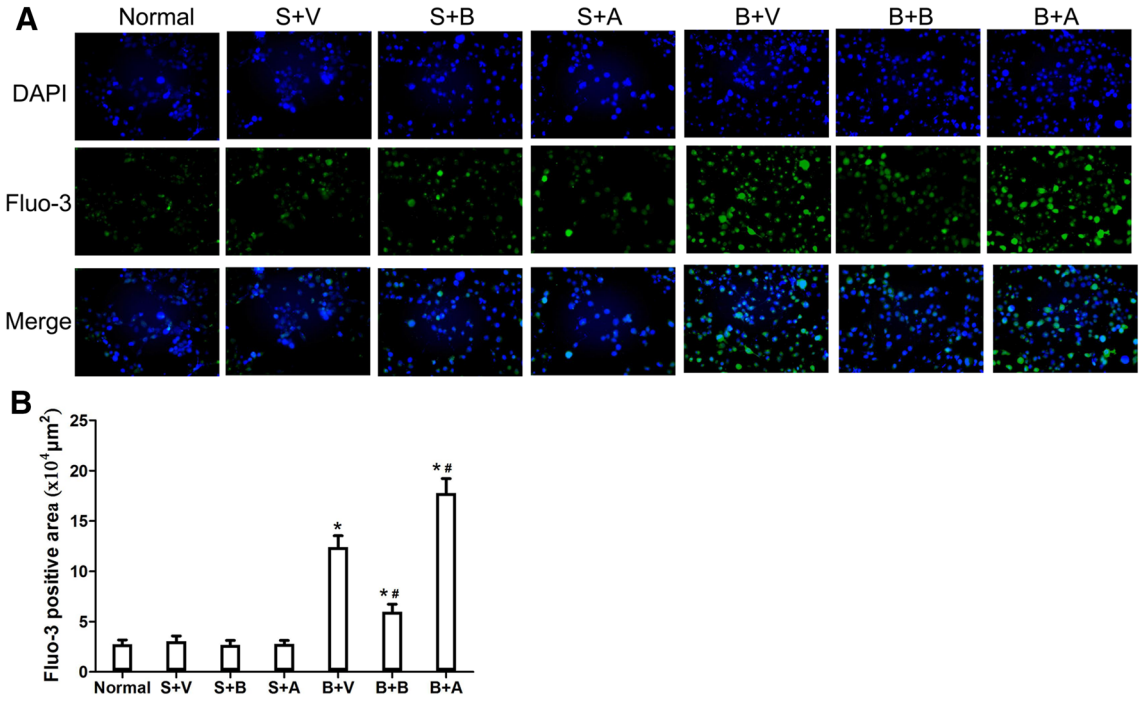

Fig. 4 Analysis of intracellular calcium concentration. **a** Representative images of calcium imaging and **b** Quantification of the fluorescence intensity of intracellular calcium in the spinal cord at 2 h after intrathecal injection of BAM 8–22, anti-MrgC antibody, and vehicle on day 14 post-operation. DAPI (blue) was used to stain nucleus; Fluo-3 fluorescent probe (green) indicated the expression of Ca^{2+} in neurons. S + V, sham group mice + vehicle; S + B, sham group mice + BAM 8–22; S + A, sham group mice + anti-MrgC antibody; B + V, bone cancer pain mice + vehicle; B + B, bone cancer pain mice + BAM 8–22; B + A, bone cancer pain mice + anti-MrgC antibody. All data are presented as the mean ± SD (n = 12). ANOVA, *$p < 0.05$, compared with the S + V group; $^{\#}p < 0.05$, compared with the B + V group

calcium concentration increase. Our results reveal that the ubiquitination of MrgC might contribute to the development of bone cancer pain, which indicates a promising way for bone cancer pain treatment.

In this study, osteosarcoma NCTC 2472 cells were injected into the intramedullary space of the femur to establish the mice model of bone cancer pain, as previously described [23]. Our previous studies [27, 28] confirm that tumor-bearing mice displayed increased NSF and decreased PWMT on day 7 post-operation with comparison to mice in normal and sham group, which indicated that the model of mice with bone cancer pain was successfully established. With the growth of tumor, the bone cancer pain gradually developed and became serious on day 14, which was consisted with our previous studies [27, 28]. Here, according to the previous studies and our preliminary experiment [11, 24], BAM 8–22 (an agonist of MrgC) and anti-MrgC antibody were injected intrathecally on day 14 post-operation to investigate the role of MrgC in bone cancer pain development. And data showed that activated MrgC played an important role in the development of bone cancer pain.

Interactions between GPCRs and their cognate G proteins are known to be involved in processes of many diseases. Currently, the primary treatments for acute or chronic pain are pharmacological agents that can promote analgesia, such as opioids, cannabinergics, and antidepressants, which mainly targeted GPCRs. Activated G protein produces different biological effects, they can induce excitation or inhibition of nerve, immune, and glial cells, which are important for the onset and maintenance of pain [2]. G proteins must distinguish between selected groups of GPCRs, because each member of the four different subfamilies of G protein α subunits (Gα proteins) is capable of interacting with multiple GPCRs [16]. The GPCRs can be activated by two or more different ligands (like different subtypes of Gα protein) to produce "biased agonism" [29]. As a member of GPCRs, MrgC has been reported to exclusively locate in primary nociceptive neurons, which are critical for treatment of pathological pain [6, 7, 10]. Moreover, MrgC has been reported to be up-regulated with the development of bone cancer pain [30]. Also, our previous study has confirmed that MrgC was involved in the regulation of NMDA receptor activity in NR2B subunits [13, 14]. Phosphorylation of NR2B has been reported to increase NMDA (*N*-methyl-D-aspartic acid) receptor activity and Ca^{2+} influx as well as nNOS (neuronal nitric oxide synthase) production [31–33],

which are crucial in acceleration of bone cancer pain development [13, 27, 34]. Thus, functional selectivity of MrgC may be critical in pain process.

Ubiquitin is an evolutionarily conserved protein that post-translationally marks proteins for degradation [20, 35]. Ubiquitination is a multistep enzymatic process that results in the attachment of ubiquitin or chains of ubiquitin to the target protein. The protein quality and quantity on the membrane are often regulated by ubiquitination. Recent studies have revealed that ubiquitination is important for protein localization, trafficking, and recognition by signaling or regulatory complexes, which further affects all aspects of cellular signaling and homeostasis [17, 36]. The protein–protein interactions regulated by ubiquitination may be highly selective, which lead to biased agonism. Protein ubiquitination could be an innovative therapeutic approach for many diseases. However, it remains unknown whether ubiquitination of MrgC contributes to bone cancer pain.

In our present study, the level of ubiquitination of MrgC was obviously enhanced on day 14 after osteosarcoma cell implantation. Moreover, intrathecal administration of BAM 8–22, significantly attenuated bone cancer pain, with up-regulation of the MrgC ubiquitination at 2 h. On the contrary, anti-MrgC antibody injection accelerated bone cancer pain and down-regulated the ubiquitination of MrgC. It has been proposed that physical interactions between an agonist and a receptor can result in a preferential or "biased" interaction with certain signaling components over others [29, 37]. These results together indicate that BAM 8–22 could increase the level of MrgC ubiquitination, and it might lead to the biased agonism and participate in the pathological process of bone cancer pain. In addition, we also detected the intracellular calcium concentration in spinal neurons via calcium imaging. It was observed that the intracellular calcium concentration in spinal neurons decreased in mice with bone cancer pain after BAM 8–22 administration. Collectively, our data suggest that the ubiquitination of MrgC may be correlated with the regulation of calcium concentration in neurons.

Besides, recent studies [38, 39] have also indicated that protein ubiquitination is critical in mediating spinal plasticity as well as regulating pain process. It was found that the ubiquitin ligase Nedd 4-2 (E3 ubiquitin protein ligase NEDD4 like protein) mediated ubiquitination of Na(v)s led to excessive excitation of dorsal root ganglion neurons, contributing to the development of pathology pain [40]. Additionally, spinal Fbxo3-dependent Fbxl2 ubiquitination of active zone protein Rab3-interactive molecule-1α was confirmed to promote spinal plasticity through $Ca_V2.2$ activation [21]. Furthermore, ubiquitin C-terminal hydrolase L1 (UCHL1) and ubiquitin was observed to be distributed and increased in the spinal cord of rats with bone cancer pain. Inhibition of spinal UCHL1 attenuated bone cancer pain by down-regulating ubiquitin expression and glial activation [41]. Our results showed that the ubiquitination of MrgC contributed to the development of bone cancer pain, which may be one of the potential mechanisms underlying the analgesic effects of BAM 8–22.

Collectively, our findings are the first to indicate that the ubiquitination of spinal MrgC involved in regulation of bone cancer pain and decreases intracellular calcium concentration in spinal neurons in mice. Targeting MrgC ubiquitination may be a promising therapy for relieving bone cancer pain, with limited side effects.

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Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical Approval This experiment was approved by the Institution of Animal Care and Usage Committee at the Medical School of Nanjing University.

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