CircAkt3 participates in bone cancer pain progression in rats by modulating MAPK signalling pathway

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Research Article

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Abstract

Background: Cancer-induced bone pain caused by advanced tumor bone metastasis remains a clinical challenge, and the underlying mechanisms of BCP remain unknown. This study aimed to screen the expression profile of circular RNAs in a BCP rat model and provide a new theoretical basis for the role of circular RNA in the occurrence and development of BCP.

Methods: We established a BCP rat model. The top four differentially expressed circRNAs (DECs) in the model were validated by agarose gel electrophoresis and Sanger sequencing between the BCP group and sham group. A circRNA-miRNA-mRNA network was constructed based on the interactions among circRNAs, microRNA (miRNA), and mRNA, which were predicted by TargetScan. mRNA and circRNA expression levels were detected by quantitative RT-PCR. In addition, Western Blot was performed to identify the protein levels of p-ERK, ERK, and Col8a1.

Results: CircRNA parent genes were mainly enriched in MAPK and neurodevelopmental signalling pathways. CircAkt3 and circMap4k1 were significantly up-regulated in the BCP group. CircaAkt3 may increase p-ERK expression by upregulating Col8a1, which may further activate the MAPK pathway.

Conclusions: The circAkt3 pathway may influence the development of bone cancer pain by activating the MAPK signaling pathway. This study provided important targets for the development of therapeutic strategy against BCP.

Introduction

Bone cancer pain (BCP) is one of the most common and painful types of cancer pain in patients with advanced metastatic tumours, caused by primary bone tumours or breast, lung, or prostate cancer metastatic to bone[1]. Bone cancer pain is a common clinical complication of patients with bone metastasis, and it has a negative impact on patients' quality of life and survival rate[2]. Studies have shown that the incidence of cancer pain in patients with advanced malignancies is 75%-95%[3].

After the lungs and liver, bones are the third most common metastasis target sites[4]. Multiple myeloma, as well as cancers of the breast, prostate, lungs, thyroid, kidneys, and ovaries, are the most common causes of bone metastases[5]. Pathological changes in the bones are estimated to occur in 70% of patients at the time of cancer, whilst cancer of the breast, lungs, and prostate are jointly responsible for 80% of cancer metastases to the bones[6]. The treatment options for bone cancer pain are currently limited. The pathological mechanisms of bone cancer pain are multifactorial, including (1) chemical mediators, such as changes in the local microenvironment of bone tissue[7]; and (2) mechanical deformations, such as tumour space-occupying effects and the resulting pressure, which activate stimulatory receptors on the bone endothelium[8]. Despite the World Health Organization's recommendation of a three-step analgesic ladder for the treatment of cancer pain, it has been reported that most cancer-related pain relief is insufficient, and patients experience extreme pain, as well as a risk
of opioid addiction\textsuperscript{[9]}. As a result, further research into the molecular mechanisms of bone cancer pain is required.

Once tumour cells have established themselves in bone, they divide, resulting in progressive bone damage. Tumor cells, stromal cells, and inflammatory cells recruited by tumour cells (macrophages, T cells, neutrophils mast cells) produce and release a variety of mediators, whilst bone-supplying osteoblasts, osteoclasts, and nerve fibres serve as effectors\textsuperscript{[10]}. Among these mediators are endothelin, bradykinin, proteases, interleukin (IL) 6, hydrogen ions (H\textsuperscript{+}), nerve growth factor (NGF), prostaglandin (PG) E\textsubscript{2}, and tumor necrosis factor (TNF)\textsuperscript{[10]}. These mediators sensitize or activate bone-innervating sensory nerve endings. The pathological process and the resulting damage are communicated to the spinal cord and then to the brain, where perception occurs\textsuperscript{[11]}. Activated immune cell mediators, tumour cells and stromal cells activate prostaglandin (PG) receptors, cytokine receptors, endothelin receptors (ETAR), purinergic receptor (P2X3), TrkA receptors, bradykinin receptors, cytokine receptors, chemokine receptors, TRPV1 (transient receptor potential channel, vanilloid subfamily member 1), and acid-sensing ion channel 3 (ASIC3). Endothelin (ET) is released by tumour cells, which stimulates osteoblast proliferation, resulting in the formation of new bone that is weak and prone to pathological fracture. Activated osteoblasts secrete receptor activator of nuclear factor kappa B ligand (RANKL), which signals osteoclast proliferation and maturation\textsuperscript{[12]}. Osteoclasts generate ATP and acidosis-inducing H\textsuperscript{+}, which activate the appropriate receptors (P2X, TRPV1, ASICS3) on bone-supplying neurons, as well as growth factors, collagenases, and proteases, which cause bone demineralization and damage bone matrix protein\textsuperscript{[13]}. Tumor cells also release NGF and brain-derived neurotrophic factor (BDNF), which stimulate macrophages to produce pro-inflammatory cytokines (e.g., TNF, IL1) and prostaglandins, which cause pain by binding to receptors on sensory neurons\textsuperscript{[14]}. Tumour cells do not directly damage bone tissue; rather, they activate the RANKL/RANK (receptor activator for nuclear factor kappa B) system. The binding of RANKL to RANK is thought to play an important role in the proliferation, differentiation, and maturation of osteoclasts, which resorb bone. TRPV1 or ASIC3 channels are activated by resorption-induced acidosis, resulting in cancer-induced bone pain (CIBP)\textsuperscript{[15]}. In the recent years, circular RNA has been shown to be involved in mediating diverse process in organ injury \textsuperscript{[16,17]}. CircRNAs are involved in the progression of multiple cancers\textsuperscript{[16,18,19]}. Although several studies have confirmed that circRNA can regulate bone pain in cancer metastasis\textsuperscript{[20]}, the pathogenesis of bone cancer pain remains unknown. The authors previously discovered significant changes in circRNAs in the bone marrow of a BCP rat model\textsuperscript{[21]}. CircRNAs, which are created by back splicing pre-mRNAs, have been identified as important regulators of cancer initiation and progression, as miRNA sponges, and as transcription modulators\textsuperscript{[22–24]}. Despite the fact that circRNAs have been linked to many aspects of cancer pathogenesis, little is known about their role in the generation and maintenance of severe pain caused by bone metastasis. A previous study found that neuropathic pain caused by nervous system damage was accompanied by significant changes in non-coding RNA expression, including the expression of long non-coding RNAs (lncRNAs), miRNAs, and circRNAs\textsuperscript{[25]}. Furthermore, the circRNA,
circAnks1a, in dorsal horn neurons has been shown to modulate hypersensitivity and neuropathic pain associated with nerve injury by sponging miR-324-3p to regulate vascular endothelial growth factor β expression[26]. In this study, we used the KEGG database to examine the expression of candidate differential circRNA genes in the BCP signalling pathway in order to investigate the possible mechanisms of circRNA involvement in bone pain and to provide new theories and ideas for the molecular mechanisms of bone cancer pain.

Materials And Methods

1. Reagents

Antibodies: p-ERK (9106) and ERK (9106) were purchased from Cell Signaling Technology (CST), Col8a1 primary antibodies (ab192350) were purchased from Abcam, and secondary antibodies (111-035-003) were purchased from Jackson. PCR reagents were purchased from Promega and Vazyme. RPMI 1640 medium was purchased from Invitrogen, USA, and fetal bovine serum was purchased from Invitrogen.

2. Cell culture

Walker 256 cells were purchased from ATCC (USA). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 mg/L penicillin and 100 mg/L streptomycin in an incubator at 37°C with 5% CO₂.

3. Animal model of bone cancer pain

The rat model was established according to our previous methodology[21]. Walker256 cells in the logarithmic phase were collected and washed with PBS 3 times. The cell concentration was adjusted to 1×10⁷ cells/ml and 0.5ml was injected into the peritoneal cavity of SD rats. 9-12 days later, the ascites was centrifuged to collect the Walker 256 cells and 5μl (4×10⁵ cells) of cell suspension was injected into the bone marrow cavity 0.5cm below the tibia, while the sham operation group was injected with Walker 256 cells boiled for 20 minutes and underwent the same surgical procedure as above. The BCP group and the sham control group were each composed of 3 animals, which were kept for 21 days after surgery and subsequently anaesthetized and sacrificed.

4. GO and KEGG analysis

We reanalysed the circRNA sequencing results, analyzed the mRNA parent gene of circRNA origin, and conducted a GO analysis based on KEGG signalling pathway results.

5. Heat map

We used differential expression to perform heat map analysis on the differential expression of 20 circRNAs corresponding to the parent mRNA of the KEGG signalling pathway.
6. ceRNA regulatory network construction

According to the ceRNA mechanism, the 10 circRNAs with the largest differences in the above heat map results were selected for mRNA target gene prediction, and the predicted mRNAs were intersected with the differential mRNAs (screening criteria: $|\log_2 (\text{BCP/Control})| > 0.67$ and $P < 0.05$) obtained from the sequencing of the previous article[20], and the predicted miRNAs, and construct a schematic diagram of a circRNA-mRNA-miRNA regulatory network.

Using mRNA sequencing results, a circRNA-miRNA-miRNA network analysis was performed to recreate network diagram 1.

7. Core gene screening and loop validation

The four circRNAs with the most target genes in the spinal cord samples of three pairs of rat models were validated by gel electrophoresis and Sanger sequencing for expression and cyclicity. Real-time fluorescence quantitative PCR was conducted to detect their expression in the 3 BCP samples and the 3 controls.

The details were as follows: Firstly, reverse transcription was performed: M-MLV Reverse Transcriptase, and the reaction system was:

M-MLV Reverse Transcriptase

RNA 2 μg

Primer Mix 1 μl

RNase-free water to 15 μl

70°C 5 min

5 min on icecc

5×buffer 5 μl

2mM dNTP 6.25 μl

M-MLV 1 μl

RNase-free water 12.75 μl

25 μl

42 °C 60min

Fluorescence quantitative PCR: ChamQ SYBR qPCR Master Mix, reaction system:
ChamQ SYBR qPCR Master Mix 10μl
F primer(10 μm) 0.5μl
R primer(10 μm) 0.5μl
cDNA template 1.5μl
Nuclease-Free Water 7.5 μl
20 μl

8. Protein extraction

Rats were deeply anaesthetized with sodium pentobarbital (1% saline, 50 mg/kg, intraperitoneal injection) and killed by decapitation. Spinal cord segments L3-L5 were rapidly stripped and stored in liquid nitrogen. Tissue samples were homogenized in 1 ml of 0.1 M PBS, and the homogenate was transferred to a new tube; the supernatant was removed by centrifugation at 13,000 r/min for 5 min at 4°C. We washed the precipitate twice with pre-cooled PBS, centrifuged it again at 13,000 r/min for 5 min at 4°C, and aspirated the PBS supernatant. We re-suspended the tissue homogenate precipitate with pre-chilled protein lysate containing protease inhibitor and place it on ice for 30 min. The incubated lysate was centrifuged at 13,000 r/min for 20 min at 4°C. The supernatant containing the proteins was collected and stored at -70°C. The bicinchoninic acid assay was used to determine the protein concentration.

9. Western blotting

Equal amounts of protein lysate and protein marker (100 ng) were separated by 8% SDS-PAGE at 120 V for 90 min and electrotransferred onto PVDM membranes with a 200 mA current for 2h. The membranes were closed with 5% skim milk containing 0.1% Tween for 1h at room temperature and subsequently incubated with the primary antibody overnight at 4 °C, washed three times with pre-chilled PBS, and incubated at room temperature with the corresponding secondary antibody for 1h. After three washes of PBS, the membranes were developed with ECL colour-developing solution and exposed to X-ray, and images were acquired.

Results

1. GO and KEGG pathway analysis of circRNA parent gene mRNA

The KEGG results demonstrated that the parent genes of differential circRNAs were mainly enriched in MAPK signalling, Neurotrophin signalling, Wnt signalling, TGF-beta signalling, and other pathways (Figure 1). The results suggested that the parent gene was mainly enriched in cellular processes, cell, binding, and other functions (Figure 2). The heat map in Figure 3 shows the clustering analysis results of the top 20 differential circRNAs enriched according to the KEGG pathway, which contains 16 up-regulated
circRNAs and 4 down-regulated circRNAs according to the screening criteria: $|\log_2 (BCP/\text{Control})| > 0.67$ and $P < 0.05$.

2. ceRNA regulatory network of circRNA

Potential regulatory miRNAs were predicted and a ceRNA regulatory network map was constructed using the top 10 circRNAs of pathway parent gene-related expression differences, their predicted target gene mRNAs, and sequenced mRNA intersections. Red indicates circRNA, pink indicates miRNA, and blue indicates mRNA, as shown in Figure 4.

3. circRNA loop validation and expression analysis

To validate our results, we selected the four circRNAs with the largest differential expression (circAkt3, circMap4k1, circNf1, circTgfbr2) for validation. The qRT-PCR results demonstrated that the expression of circAkt3 and circMap4k1 were significantly up-regulated in the bone pain group (Figure 5, B), which was consistent with the previous sequencing results. As a result, these two genes were selected for the follow-up study. The PCR products were analyzed by agarose gel electrophoresis and Sanger sequencing. The band sizes of the PCR products of the four circRNAs of the bone pain group cDNA were as expected, while no amplified product was found in the PCR experiment with gDNA as the template (Figure 5, A). Meanwhile, Sanger sequencing was used to confirm the linkage sites for reverse splicing.

4. circAkt3 and circMap4k1 target gene validation

To investigate the potential mechanisms of circAkt3 and circMap4k1 in bone pain, we used qRT-PCR to confirm changes in their target genes in the ceRNA network. The findings revealed that miR-146a-5p, a network-interacting target gene of circcMap4k1, was significantly upregulated. Still, the change of its target gene Nfkb1 was not statistically significant, and the other target gene, mir-138-5p, and its corresponding target gene vim did not change (Figure 6, B). MiR-29b-3p, a target gene of circAkt3, was significantly decreased, and the expression of its target gene Col8a1 was also significantly decreased. The expression of its target gene Col8a1 was significantly increased (Figure 6, A), and the rest were unchanged. Therefore, we speculate that circAkt3 may be involved in bone pain regulation through Col8a1.

5. Activation of ERK signalling pathway regulates BCP

Col8a1 was found to be involved in the ERK pathway of MAPK in a previous KEGG analysis, so we hypothesized that Col8a1 might regulate the ERK pathway of MAPK by regulating Col8a1 expression. We examined the expression levels of p-ERK, ERK, Col8a1, and GAPDH in the bone pain group by protein blotting. WB results revealed that p-ERK and Col8a1 expression levels were significantly higher in the bone pain group (BCP) (Figure 6, C). The statistical analysis showed that expression of Col8a1 was significantly increased and the ERK pathway was activated (Figure 6, D). As a result, we hypothesize that
circAkt3 plays a role in bone pain regulation by increasing Col8a1 expression and thus activating the ERK signalling pathway.

Discussion

Numerous studies have found that circRNAs play a role in the development and pathology of various diseases. circRNAs are formed by back-splicing in a non-classical splicing manner. circRNAs can interact with RNA-binding proteins to affect the expression of parental gene mRNAs or interplay with paired linear RNAs with competitive complementation among introns in the formation process, involving the expression of mRNA, and further affecting protein translation. Furthermore, circRNAs can adsorb microRNAs by competing for endogenous RNA (ceRNAs). Due to their covalently closed circular structure, circRNAs are more stable in vivo and could be used as biological markers of many conditions and disease progression.

In a previous study, we sequenced BCP group rats and control rats. Based on the functions of the parent genes, we reselected the corresponding circRNAs of mRNAs involved in neural cell development, pain receptors, osteoblast differentiation, and inflammatory cytokine pathways. The four circRNAs with the greatest expression differences in the newly constructed model rats were then verified in this experiment. The results revealed that circAkt3 and circMap4k1 expression was significantly increased. High expression of blood exosome circAkt3 in hepatocellular carcinoma has been linked to a high recurrence and mortality rate[27]. In addition, in human dental pulp stromal cells (hDPSCs), circAkt3 positively regulates the osteogenesis of hDPSCs through adsorption of miR-206, which also corroborates the possible involvement of circAkt3 in the mechanistic regulation of bone pain[28]. Another study discovered that circAkt3 expression was significantly increased in cisplatin-resistant gastric cancer tissues[29] and that it adjusted cancer cell sensitivity to cisplatin in lung cancer[30]. Therefore, the trend of circAkt3 and prognosis in these malignancy studies is consistent with our findings.

Our findings also confirmed that circAkt3 expression was increased in the BCP group, as was its corresponding target gene in the network, Col8a1. We further investigated its signalling pathway, and KEGG results revealed that Col8a1 was associated with the MAPK signalling pathway ERK. Meanwhile, we used protein blotting to examine changes in protein expression levels of p-ERK, ERK, and Col8a1 in both groups. The results demonstrated that the BCP group had significantly higher levels of p-ERK and Col8a1 protein expression. In contrast, there was no significant change in total ERK expression. Col8a1 may be involved in the MAPK signaling pathway that regulates bone pain via p-ERK activation. A previous study found that after establishing a bone pain model with Walker 256 cells on SD rats, p-ERK expression increased in spinal cord neurons, microglia, and astrocytes. Furthermore, ERK phosphorylation (p-ERK) and transcription factor cAMP response element-binding protein (p-CREB) expression were increased in the spinal cords of BCP rats, and the expression was reduced after intrathecal injection of inhibitors. It has been proposed that inhibiting the ERK pathway could be a potential therapeutic approach for relieving bone cancer pain. As a result, circAkt3 could be a potential target for relieving bone cancer pain if it is indeed involved in mediating bone cancer pain by activating p-ERK.
However, our study has some limitations. This experiment only demonstrated that circAkt3 follows the same pattern as Col8a1 and p-ERK; more functional experiments and mechanism studies are required to determine the relationship between them.

In conclusion, we screened differential circRNAs of bone cancer pain-related pathways in this study and verified that circAkt3 and circMap4k1 expression was elevated in a rat model of bone cancer pain. Furthermore, circAkt3 was consistent with the trend of corresponding mRNA Col8a1 changes in the ceRNA regulatory network. P-ERK, which is activated in the bone pain group, may be a downstream pathway regulated by circAkt3 and Col8a1.

Declarations

Ethics approval and consent to participate

The animal studies and experiments were approved by Animal Ethical and Welfare Committee (AEWC), China (Approval No: IACUC-AEWC-F). All experiments were performed in accordance with relevant guidelines and regulations. The manuscript reporting adheres to the ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments.

Consent for publication

Not Applicable

Availability of data and materials

The data and materials are available to be obtained from Dr Yiwen Zhang, Department of Anesthesiology, Shunde Hospital of Southern Medical University (The First People’s Hospital of Shunde Foshan), China upon request. The datasets generated and analysed during the current study are available in the Gene Expression Omnibus(GEO),


If you are a reviewer, enter secure token as: elwlkcimhxqlfaz

In addition, the database information has been uploaded as PDF as supplement data.

Competing interests

The authors declare that they have no competing interests.

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**Authors’ contributions**

LC carried out the molecular genetic studies, participated in the sequence alignment, and drafted the manuscript. XZ carried out the immunoassays. YS participated in the sequence alignment. TT carried out animal model experiments. HC participated in the design of the study and performed the statistical analysis. YZ conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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**References**


Figures

Figure 1

GO and KEGG analysis of bone marrow differential circRNA parent gene mRNA. (A) According to the results of circRNA sequencing in PMID:32395748[21], the above BCP-related signal pathways enriched by circRNA mother gene were analyzed, in which MAPK and neurodevelopmental signal pathways were significantly enhanced.
Figure 2

GO analysis based on the enhanced signaling pathways from Figure 1.
Figure 3

According to the enhanced signaling pathways from figure 1, the top 20 differential circRNA was selected for heat mapping, of which 16 were up-regulated and 4 down-regulated (screening criteria: $\log_2 (\text{BCP/Control}) > 0.67$ and $P < 0.05$.)
Construction of the circRNA-mRNA-miRNA regulatory network. Red indicates circRNA, pink indicates miRNA, and blue indicates mRNA.
Figure 5

Critical circRNA screening. (A) The 4 circRNAs with the largest differences were selected for loop validation, and the gel map and Sanger sequencing indicated that the 4 circRNAs were looped. (B) QPCR was validated in sequenced samples from 6 tissues (3 sham-operated groups, 3 model tissues), in which circMap4k1 and circAkt3 were significantly up-regulated, and these 2 circRNAs were selected for follow-up studies.
CircAkt3 acts through Col8a1 in the ERK signaling pathway to regulate BCP. (A) CircAkt3 (rno-miR-29b-3p, col18a1, Mmp, Col8a1, Col3a1, Col1a1) results show a significant upregulation trend of Col8a1, which is consistent with the trend of circAkt3. (B) CircMap4k1 (rno-miR-146a-5p-Nfkb1, rno-mir-138-5p-vim) showed significant upregulation of miR146a-5p, but no significant change in its handle gene. (C, D) Based on the KEGG signaling pathway, col18a1 was found to be involved in the ERK signaling pathway, a branch of MAPK, and we used WB to detect the activation of this signaling pathway in sequenced samples. The results revealed that pERK was significantly upregulated in tissues from BCP rats.

**Supplementary Files**

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