High expression of small nucleolar RNA host gene 3 predicts poor prognosis and promotes bone metastasis in prostate cancer by activating transforming growth factor-beta signaling

Xinhua Xi\textsuperscript{a,4}, Zhengbo Hu\textsuperscript{a,8}, Qiang Wu\textsuperscript{a}, Konghe Hu\textsuperscript{a}, Zhengguo Cao\textsuperscript{c}, Jun Zhou\textsuperscript{a}, Junjian Liao\textsuperscript{a}, Zhipeng Zhang\textsuperscript{a}, Yongyu Hu\textsuperscript{a}, Xueren Zhong\textsuperscript{a}, and Yongzheng Bao\textsuperscript{a}

\textsuperscript{a}Department of Orthopaedics, Yuebei People’s Hospital Affiliated to Shantou University Medical College, Shaoguan, Guangdong, China; \textsuperscript{b}Department of Orthopedics, Shaoguan First People’s Hospital Affiliated Southern Medical University, Shaoguan, Guangdong, China; \textsuperscript{c}Department of Urology, Yuebei People’s Hospital Affiliated to Shantou University Medical College, Shaoguan, Guangdong, China

ABSTRACT

Bone metastasis is closely related to tumor death in prostate cancer (PC). Long noncoding RNA small nucleolar RNA host gene 3 (SNHG3) has been implicated in the initiation and progression of multiple human cancers. Nevertheless, the biological function of SNHG3 in PC has not been elucidated. Our results indicated that SNHG3 was upregulated in bone metastasis-positive PC tissues compared to bone metastasis-negative PC tissues and adjacent normal tissues. High expression of SNHG3 indicates advanced clinicopathological features and predicts poor prognosis in patients with PC. Meanwhile, SNHG3 knockdown suppressed the proliferation, migration, and invasion abilities of PC cells and inhibited PC cell metastasis to the bone. Mechanistically, SNHG3 enhanced the expression of transforming growth factor beta receptor 1 (TGFBR1) and activated transforming growth factor-Beta (TGF-β) signaling by targeting miR-214-3p. Our study demonstrated the novel role of the SNHG3/miR-214-3p/TGF-β axis in tumor growth and bone metastasis in PC, indicating that SNHG3 may act as a biomarker and promising therapeutic target against PC.

1. Introduction

Prostate cancer (PC) is the main cause of cancer-related deaths among men in Western countries [1,2]. Its propensity to metastasize to the bone may be mainly responsible for PC-related deaths [3]. Approximately 70% of the patients with advanced PC develop bone metastases after diagnosis [4]. Bone tumors formed by metastatic PC cells lead to poor quality of life and ultimately death of the patients due to limited treatments and skeletal complications, such as instability of the spine, spinal cord injury, and hypercalcemia [5]. Currently, metastatic bone tumors are not curative, and related treatment cannot stop cancer progression [4,6,7]. Therefore, there is an urgent need to investigate the pathogenic mechanisms of tumor initiation and bone metastasis in PC and develop novel therapeutic strategies.

Numerous studies have revealed that long noncoding RNAs (lncRNAs) play pivotal roles in a majority of cell processes, including growth, invasion, migration and stemness [8]. Dysregulated expression of lncRNA has been observed in many human cancers in association with tumor initiation and metastasis [9–12]. LncRNA glycolysis-associated lncRNA of colorectal cancer (GLCC1) prevents the classic oncogenic transcription factor MYC proto-oncogene (MYC) from ubiquitination by directly interacting with heat shock 90kD protein (HSP90) protein and mediates the transcriptional regulation of downstream target genes induced by MYC to enhance cell glycolysis, which further promotes...
cell proliferation in colorectal cancer [13]. LncRNA differentiation antagonizing non-protein coding RNA (DANCR) promotes tumor progression in bladder cancer by activating the signal transducer and activator of transcription 3 (STAT3) pathway and upregulating cyclin D1 (CCND1) expression by interacting with leucine rich pentatricopeptide repeat containing (LRPPRC) protein to stabilize related mRNA [11].

SNHG3, small nucleolar RNA host gene 3, has been revealed to be oncogenic in multiple human cancers, including colorectal, ovarian, gastric, and liver cancer [14–19]. SNHG3 competitively targets several miRNAs to derepress the degradation of mRNA and binds to proteins to maintain the stability of mRNA, through which SNHG3 enhances tumor development. However, the biological function of SNHG3 in bone metastatic PC remains unclear.

We hypothesized that SNHG3 play an important role in the PC bone metastasis. This study aimed to explore the role of SNHG3 in bone metastasis of PC. Our data indicated that SNHG3 expression was elevated in PC tissues with bone metastasis compared with PC tissues without bone metastasis. Significantly, high SNHG3 expression was correlated with advanced clinical features, bone metastasis status, and poor survival in patients with PC. SNHG3 silencing inhibited the proliferation and metastatic behavior of PC cells in vitro. In addition, SNHG3 knockdown suppressed PC cell metastasis to the bone in vivo. Mechanistic investigation revealed that SNHG3 served as an miR-214-3p sponge to increase TGFBR1 expression, which enhanced tumor growth and bone metastasis in PC by activating TGF-β signaling.

2. Materials and Methods

2.1 Cell culture, transfection and Clinical samples

All human cell lines (REPW-1, DU145, VCaP, LNCaP, C4-2B, 22RV1, PC3) in this study were purchased from ATCC. The specific information about cell culture and transfection was shown in Supplemental Material. All PC tissues were collected from our hospital from March 2010 to March 2017. The informed consent was signed by all patients. This study was approved by the ethical committee of Shantou University.

2.2 Real-time Quantitative PCR (RT-qPCR)

The detailed procedure of RT-qPCR was according to the previous study [20]. RNA extraction was performed using Trizol reagent. The primers sequences were as follow: SNHG3, forward: 5′-CTTGGCTGTTGTCACCTCTGA-3′, reverse: 5′- ACACACAGTTGGGTTCAACCA -3′; TGFBR1, forward: 5′- GACAAACGTACAGGTCTTGCTCA-3′, reverse: 5′C CGCCACTTTCTCTCTCCAAACT -3′; GAPDH, forward: 5′- ACGCTTCACAGCTTGCGGATGAG -3′, reverse: 5′-TGACAGGTGGTCACTCCTCATG -3′.

2.3 Western blotting

Western blotting was conducted as previously described [21]. The detailed information about Western blotting was presented in Supplemental material. Antibodies for TGFBR1 were purchased from Sigma (USA), α-tubulin (Proteintech, China) was utilized as the loading control.

2.4 Transwell assay

The transwell assays were performed to investigate the migratory and invasive abilities of PC cells according to previous study [22]. The specific protocol was shown in Supplemental material.

2.5. Proliferation assays

Colonie formation assays, 5-Ethynyl-2′-deoxyuridine (EdU), and Cell Counting Kit-8 (CKK8) assays were conducted to examine the proliferative ability of PC cells. The detailed protocols about colony formation assays, EdU and CKK8 assays were consistent with the previous studies [23–25].

2.6 Luciferase reporter assay

SNHG3-wt, SNHG3-mut, TGFBR1-wt or TGFBR1-mut sequences were cloned into pGL3-reporter. The luciferase intensity was determined by Dual-Luciferase Reporter Assay Kit (Promega, USA). The detailed method was presented in
Supplemental material, and luciferase reporter assay was carried out according to previous study [22].

2.7 Bone metastasis model

All animal experiments were approved by the Ethics Committee of Shantou University and performed as previously described [26]. Every group included 5 male mice. The detailed information about animal study was described in the previous study [22].

2.8 Statistical analysis

Data were analyzed by SPSS 20.0 (USA). All values were expressed as the mean ± standard deviation. The difference analysis between two groups was investigated by the student’s t-test. \( P < 0.05 \) was considered statistically significant.

3. Results

In this study, we investigated the function of SNHG3 in PC cell metastasis to the bone. Our results revealed that SNHG3 expression was increased in bone metastasis-positive PC and that the upregulation of SNHG3 was related to advanced clinicopathological features. Moreover, increased expression of SNHG3 predicted poor overall survival (OS) and bone metastasis-free survival (BMFS) in PC patients. In vitro experiments demonstrated that the knockdown of SNHG3 significantly inhibited PC cell proliferation, migration and invasion. In vivo experiments indicated that depletion of SNHG3 suppressed bone metastasis of PC cells. Through bioinformatic analysis, SNHG3 and TGFBR1 were found to bind to miR-214-3p. The interaction between them was confirmed using a luciferase reporter assay. SNHG3 upregulated TGFBR1 expression by competitively binding to miR-214-3p. Further assays indicated that the SNHG3/miR-214-3p/TGFBR1 axis activates TGF-\( \beta \) pathway to promote bone metastasis in PC.

3.1 SNHG3 is upregulated in PC with bone metastasis

To reveal the clinical significance of SNHG3 in PC, we first analyzed the expression pattern of SNHG3 in The Cancer Genome Atlas-Prostate Adeno carcinoma (TCGA-PRAD) database. The analysis results indicated that SNHG3 expression was increased in matched PC tissues relative to that in normal prostate tissues (Figure 1a). We also found that SNHG3 expression was higher in PC tissues than in normal prostate tissues (Figure 1b). Notably, high expression of SNHG3 was prevalent in PC with bone metastasis (PC/BM) relative to PC without bone metastasis (PC/nBM)(Figure 1c). We then investigated the expression of SNHG3 in our clinical samples. The RT-qPCR assay demonstrated that SNHG3 was elevated in PC tissues relative to that in adjacent normal tissues (ANT, Figure 1d). Moreover, SNHG3 expression was increased in PC/BM relative to that in PC/nBM (Figure 1e). Meanwhile, compared with primary PC tissues (P-PC), upregulated SNHG3 expression was observed in metastatic PC tissues from bone (B-PC), which indicated that SNHG3 might be implicated in bone metastasis in PC (Figure 1f). Further results showed that SNHG3 expression was increased in PC cell lines compared to the normal prostate cell line (RWPE1) (Figure 1f). These results indicated the upregulation of SNHG3 in PC, especially in the PC/BM.

3.2 Upregulation of SNHG3 increases the risk of advanced clinicopathological features in PC patients

We explored the relationship between SNHG3 levels and clinicopathological features in patients with PC. The findings suggested that high expression of SNHG3 was positively correlated with advanced stages of PC in TCGA-PRAD and our cohort (Gleason score >7, Figure 2a and b). In addition, we found that SNHG3 was upregulated in PC with progression compared to PC without progression (Figure 2c). Moreover, in the receiver operating characteristic (ROC) curve analysis, SNHG3 expression showed a significant predictive value for the diagnosis of PC (Figure 2d), which indicated the importance of
SNHG3 as a potential diagnostic biomarker. Furthermore, multivariate Cox regression analysis demonstrated that high levels of SNHG3 may act as an independent risk factor for death and progression in PC (Figure 2e and f). These findings indicated that high expression of SNHG3 increased the risk of advanced clinicopathological features in PC.

### 3.3 High SNHG3 expression predicts poor prognosis in PC patients

We subsequently investigated the relationship between SNHG3 levels and the prognostic status of patients with PC in TCGA-PRAD and our cohort. The findings from TCGA database indicated that high levels of SNHG3 predicted poor overall, disease-specific, and progression-free survival (Figure 3a-c). In our cohort, we also found that upregulation of SNHG3 was associated with shorter OS and BMFS (Figure 3d and e). The above results demonstrated that SNHG3 might serve as an important prognostic factor in patients with PC.

### 3.4 SNHG3 knockdown inhibits PC cell proliferation, migration, and invasion

To explore the biological function of SNHG3 in PC, SNHG3 was exogenously silenced in PC3 and C4-2B cells (Figure 4a). CCK8 assays showed that silencing SNHG3 reduced the viability of PC cells significantly (Figure 4b-c). EdU assays indicated that SNHG3 knockdown decreased the proportion of EdU-positive cells in the S phase (Figure 4d). Colony formation assays demonstrated that downregulation of SNHG3 inhibited colony formation ability of PC cells (Figure 4e). These results revealed that SNHG3 knockdown suppressed the proliferation ability of PC3 and C4-2B cells. To further determine the influence of SNHG3 on the metastatic behavior of PC cells, a transwell assay was conducted in vitro. These findings suggested that SNHG3 knockdown...
markedly decreased the migratory and invasive ability of PC3 and C4-2B cells (Figure 4f). The above findings revealed that SNHG3 knockdown inhibited the metastatic behaviors of PC cells \textit{in vitro}.

### 3.5 SNHG3 knockdown inhibits bone metastasis in PC

Since bone metastasis is a significant prognostic factor for PC patients, we investigated the role of SNHG3 in PC bone metastasis. Our results manifested that silencing SNHG3 potentially inhibited PC cell metastasis to the bone, indicated by decreased luciferase signal in the hind legs of mice and reduced incidence of bone metastasis (Figure 5a-c). Survival analysis suggested that SNHG3 knockdown prolonged BMFS and OS in mice (Figure 5d and e). Thus, SNHG3 knockdown inhibited PC cell metastasis to the bone \textit{in vivo}.

### 3.3 SNHG3 promotes PC progression via sponging miR-214-3p

To explore the underlying mechanism of SNHG3-mediated progression in PC, we investigated the localization of SNHG3. We found that SNHG3 was mainly distributed in the cytoplasm of PC3 and C4-2B cells (Figure 6a-b). Previous studies have indicated that cytoplasmic lncRNAs regulate mRNA expression by sponging miRNAs, suggesting that SNHG3 may participate in bone metastasis of PC by acting as a competitive endogenous RNA (ceRNA). The results predicted by StarBase and Lncbase websites suggested that SNHG3 may bind to eight miRNAs, including miR-6884-5p, miR-519e-5p, miR-510-5p, miR-515-5p, miR-650, miR-4662a-5p, miR-3612, and miR-214-3p (Figure 6c). Subsequent RT-qPCR assay demonstrated that SNHG3 upregulated miR-214-3p expression, but did not alter the expression of other miRNAs in PC3 and C4-2B cells.
The direct binding between SNHG3 and miR-214-3p was examined using a luciferase reporter assay. The results indicated that the SNHG3-wt reporter’s luciferase activity was decreased by miR-214-3p, whereas the luciferase activity of the SNHG3-mut reporter remained unchanged (Figure 6f and g). Further RIP experiments revealed that miR-214-3p enhanced SNHG3 enrichment in Ago2 (Figure 6h). These findings suggested that SNHG3 directly binds to miR-214-3p. Functionally, the miR-214-3p inhibitor reversed the suppressive effect of silencing SNHG3 on the proliferation, migration, and invasion of PC3 and C4-2B cells (Figure 6i-l). Taken together, SNHG3 enhanced tumor progression by targeting miR-214-3p in PC.

3.7 SNHG3 activates TGF-β signaling via sponging miR-214-3p

Emerging evidence indicates that lncRNAs and miRNAs can participate in tumor progression by activating related pathways [4,7,10,22,25,27,28]. Hence, we screened the signaling pathways regulated by SNHG3 and miR-214-3p. As shown in Figure 7a, TGF-β signaling was significantly inhibited by SNHG3 downregulation and miR-214-3p mimics. Gene Set Enrichment Analysis also indicated that high expression of SNHG3 was notably enriched in the TGF-β pathway (Figure 7b). We revealed that the miR-214-3p inhibitor reversed the inhibition of TGF-β signaling induced by SNHG3 knockdown (Figure 7c).

3.8 TGFB1 mediates the regulation of TGF-β signaling induced by SNHG3 and miR-214-3p

The potential target mRNAs of miR-214-3p were predicted by Starbase to determine the reason contributing to the activation of TGF-β signaling induced by SNHG3 and TGFB1 was selected as the candidate mRNA. TGFB1, being the core component of TGF-β signaling, is responsible for the regulation of TGF-β signaling. A luciferase reporter assay was conducted to explore whether...
Figure 4. SNHG3 promotes proliferation, migration, invasion of PC cells. (a) Real-time PCR analysis of SNHG3 expression in the indicated cells. (b-e) CCK8 (b and c), EdU (d) incorporation assay and (e) colony formation assay was conducted to test cell proliferation in PC3 and C4-2B cells. (f) Transwell assay was performed to analyze migration and invasion of PC3 and C4-2B cells in the indicated groups. Scale bar, 50 um. *P < 0.05.

Figure 5. SNHG3 promotes bone metastasis in PCa. (a) Representative BLIs signal of bone metastasis of a mouse from the indicated groups of mice on day 60 (n = 5 per group). (b) Luciferase signal in hind legs of mice from the indicated group on day 60 (n = 5 per group). *P < 0.05 (c) The incidence of BM in the indicated group. *P < 0.05 (d and e) Kaplan–Meier analysis of overall and bone metastasis-free survival curves of the mice in the indicated group.
miR-214-3p directly targets TGFBR1. The results demonstrated that luciferase activity of TGFBR1-wt reporter was downregulated by miR-214-3p, while TGFBR1-mut reporter’s luciferase activity was unaltered in PC3 and C4-2B cells (Figure 8a-c). RT-qPCR assays indicated that compared with the control group, TGFBR1 expression was reduced by miR-214-3p mimics in PC cells (Figure 8d). Since SNHG3 serves as a ceRNA, it may upregulate TGFBR1 expression by targeting miR-214-3p. Further experiments showed that silencing SNHG3 reduced the level of TGFBR1, whereas the miR-214-3p inhibitor partially abolished the downregulation of TGFBR1 mediated by SNHG3 knockdown (Figure 8e and f). Collectively, SNHG3 activates TGF-β signaling by targeting miR-214-3p to increase TGFBR1 expression.

4. Discussion

The current study uncovered the significant function of SNHG3 in PC cell metastasis to the bone. Our results showed that SNHG3 is elevated in PC tissues that develop bone metastasis. In addition, high expression of SNHG3 was positively correlated with advanced clinicopathological features and poor prognosis in patients with PC. Mechanistic investigation revealed SNHG3 increases TGFBR1 levels by targeting miR-214-3p, further activating the TGF-β pathway, leading to bone metastasis in PC. Therefore, this study suggests that SNHG3 can be a prognostic biomarker and a promising target against bone metastases in PC.

LncRNAs have been extensively reported to be dysregulated in PC, including nuclear paraspeckle...
assembly transcript 1 (NEAT1), LINC00844, zinc finger homeodomain enhancer-binding protein antisense RNA 1 (ZEB-AS1) and HOX transcript antisense RNA (HOTAIR), which are involved in the progression of PC via multiple mechanisms [29–38]. For example, NEAT1 knockdown suppressed the metastatic ability of glioma by upregulating SRY-Box transcription factor 2 (SOX2) repression by miR-132 [29]. Overexpression of ZEB-AS1 promoted tumor development in prostate cancer by epigenetically upregulating zinc finger homeodomain enhancer-binding protein (ZEB1) expression, further activating the downstream target gene of ZEB1 [33]. Lang et al. revealed that lncRNA prostate cancer associated transcript 7 (PCAT7) was increased in PC/BM compared with that in PC/nBM and promoted PC cell metastasis to the bone by upregulating the activity of TGF-β signaling [35]. In return, the TGF-β-induced SMAD family member 3 (SMAD3)/Sp1 transcription factor (SP1) complex transcriptionally upregulated PCAT7 levels, further forming a feedback loop between PCAT7 and the TGF-β pathway. Additionally, Lang et al. found that m6A modification of prostate cancer associated transcript 6 (PCAT6) promoted the formation of bone metastasis in PC [25]. Upregulation of PCAT6 enhanced the stability of insulin like growth factor 1 receptor (IGF1R) mRNA by interacting with insulin like growth factor 2 mRNA binding protein 2 (IGF2BP2), further activating IGF/IGF1R signaling and downstream pathways in PC [25]. These findings suggest that targeting lncRNA may be a promising therapeutic strategy to block PC cells from forming metastatic lesions in the bone microenvironment. In this study, we found that SNHG3 expression was gradually increased from normal prostate tissues, PC/nBM to PC/BM, which implied that SNHG3 might be involved in tumor 

**Figure 7.** SNHG3 activates TGF-β signaling by sponging miR-214-3p in PC. (a) Relative luciferase activities of different pathways in the indicated groups. *P < 0.05 (b) GSEA analysis shows that High expression of SNHG3 is enriched in TGF-β signaling. (c) Relative luciferase activities in the indicated groups. *P < 0.05.
Figure 8. TGFBR1 is the target of miR-214-3p. (a) The binding sites between miR-214-3p and TGFBR predicted by Starbase. (b and c) Relative luciferase activities of TGFBR1-wt or -mut reporter in the indicated groups. (d) Real-time PCR analysis of TGFBR1 expression in the indicated groups. *P < 0.05 (e) Relative expression of TGFBR1 in the indicated groups. (f) Western blot assay showed the TGFBR1 proteins expression in the indicated groups.

initiation and bone metastasis in PC. Functional experiments indicated that upregulation of SNHG3 promoted the proliferation, invasion, and migration abilities of PC cells in vitro, while enhancing bone metastasis in vivo. Collectively, these findings reveal the pro-bone metastatic role of SNHG3 in PC, indicating that targeting SNHG3 may have promising prospects to decrease bone metastasis in PC.

Emerging studies have found that many lncRNAs regulate tumor progression by binding to specific miRNAs and abolishing miRNA-mediated repression off mRNA [39–43]. In this study, SNHG3 was mainly detected in the cytoplasm of PC3 and C4-2B cells, indicating that SNHG3 may promote the development of PC by interacting with miRNA, which is also supported by previous findings on osteosarcoma, acute myeloid leukemia, breast cancer, liver cancer and gastric cancer [15–18,44–48]. For example, in acute myeloid leukemia, high expression of SNHG3 enhances cell proliferation and inhibits cell apoptosis by acting as a miRNA sponge for miR-758-3p to enhance serglycin (SRGN) levels [44]. A study by Ma et al. showed that upregulation of SNHG3 in breast cancer promoted the abilities of tumor growth and invasiveness via miR-384/heparin binding growth factor (HDGF) signaling axis [15]. Zheng et al. reported the pro-proliferation and -metastasis role of SNHG3 in osteosarcoma through the activation of the miR-151a-3p/Ras-related protein Rab-22A (RAB22A) axis [16]. Further experiments revealed that SNHG3 increased TGFBR1 levels by interacting with miR-214-3p, constitutively activating the TGF-β pathway and promoting PC cell metastasis to the bone. In other studies, SNHG3 was reported to participate in tumor progression in multiple human cancers through other mechanisms [14,19,45,49]. For example, E2F transcription factor 1 (E2F1)-mediated transcriptional upregulation of SNHG3 in lung cancer enhanced the abilities of cell proliferation and migration by activating the TGF-β pathway and interleukin 6 (IL-6) signaling [14]. Xuan et al. revealed that SNHG3 recruited enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) to the promoter region of mediator complex subunit 18 (MED18), which significantly decreased the expression of MED18 via methylation regulation, further increasing the metastatic properties of gastric cancer cells [45]. These studies uncovered the oncogenic capability of SNHG3 in a variety of human cancers.
depending on multiple molecular mechanisms. In addition, other lncRNAs can exert their function in an ceRNA manner [50,51]. LncRNA neuroblastoma highly expressed 1 (NHEG1) increased high mobility group box 1 (HMGB1) expression by sponging miR-655 to promote neuroblastoma progression [50]. In breast cancer, lncRNA cancer susceptibility candidate 7 (CASC7) upregulated levels of tumor necrosis factor ligand superfamily member 6 (FASLG) by targeting miR-21-5p to inhibit proliferation, invasion, and migration of cancer cells [51].

miRNAs have been implicated in human tumor progression by targeting the 3′-untranslated region (UTR) of mRNA [52–55]. In this study, miR-214-3p mediated the activation of the TGF-β pathway induced by SNHG3. miR-214-3p has been shown to play vital roles in many human cancers, including renal cell carcinoma, cervical cancer, hepatocellular carcinoma and osteosarcoma [56–59]. In these studies, miR-214-3p exerts its function by sponging lncRNAs and circRNAs. For example, in cervical cancer, lncRNA HOTAIR enhances proliferation and suppresses apoptosis by binding to miR-214-3p [56]. LINC00882 is upregulated by activating transcription factor (ATF) in hepatocellular carcinoma and promotes tumor progression by abolishing miR-214-3p-mediated degradation of centromere protein M mRNA [57]. In liver cancer, circ_0008450 enhanced tumor development by sponging miR-214-3p and increasing EZH2 expression [60,61]. Our study uncovered the pro-bone metastatic function of miR-214-3p in PC.

This study has several limitations. The mechanism of SNHG3 upregulation remains unclear, and further studies should focus on it. Meanwhile, we should investigate whether targeting SNHG3, such as antisense oligonucleotide drugs, can inhibit bone metastasis in vivo. This study provides only the theoretical basis for SNHG3 as a potential target against bone metastasis.

Conclusion

In summary, this study demonstrated the pro-bone metastatic role of SNHG3, indicating that SNHG3 can act as a promising therapeutic target by disrupting the SNHG3-miR-214-3p-TGFB1-TGF-β signaling axis.

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Availability of Data and Materials
All data generated or analyzed during this study are included in this published article.

Abbreviations
PC, prostate cancer; BM, bone metastasis; lncRNA, long noncoding RNA; ceRNA, competing endogenous RNA; RIP, RNA immunoprecipitation; RT-qPCR: Real-time quantitative PCR; SNHG3, Small nucleolar RNA host gene 3; TGFB1, Transforming Growth Factor Beta Receptor 1; TGF-β, Transforming Growth Factor-Beta

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