The potential value of IncRNA-BC050642 in osteosarcoma origination and outcomes

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**ABSTRACT**

**Background:** The purpose of our research was to explore potential value of IncRNA-BC050642 in osteosarcoma origination and prognosis.

**Methods:** In this study, the tissue specimens were collected from 97 osteosarcoma patients and 97 age-matched healthy controls. Besides, human osteosarcoma cell lines U2OS and normal osteoblastic cell line hFOB1.19 were selected for experiments in vitro. IncRNA-BC050642 levels were measured through quantitative real-time polymerase chain reaction (qRT-PCR). Relative expression of the gene c-myc was determined via ELISA analysis. χ² test was implemented to appraise possible relationship between BC050642 level and clinicopathological features. Cell proliferation assay, plate colony formation assay, and cell apoptosis assay were adopted to analyze the influence of BC050642 on tumor development. Besides, prognostic value of BC050642 was estimated using Kaplan–Meier and cox regression analysis.

**Results:** BC050642 levels showed distinctive increases in osteosarcoma tissues and cell lines compared with controls. And c-myc expression was down-regulated in osteosarcoma. There was a negative correlation between the expressions of BC050642 and c-myc. BC050642 expression was proved to be significantly correlated with Ennking and histological type. Up-regulated BC050642 could promote cell proliferation, induce colony formation and meanwhile inhibit cell apoptosis.

**Conclusions:** BC050642 is up-regulated in osteosarcoma and its over-expression promotes tumor development via down-regulating the expression of c-myc. It also may be an independent prognostic biomarker for osteosarcoma.

**Introduction**

Osteosarcoma, a most common primary malignancy in bone, represents a leading cause of cancer-related death among adolescents and young adults [1]. Despite its relatively low incidence, osteosarcoma shows soaring malignancy because of its high propensity of metastasis and local relapse [2]. Main treatments for osteosarcoma are surgical excision and chemotherapy. But, it is frequent for the patients to develop lung metastasize which contributes to the cases’ deaths for the most part, and curative rate of osteosarcoma is low, with a 5-year survival rate of < 30% [3]. Recent years, comprehensive therapies make 5 years survival rate raise to 60–70% [4]. Whereas, main mechanism of osteosarcoma occurrence and development remain to be explored. Moreover, osteosarcoma still shows poor prognosis and serious harm. Therefore, it is necessary to explore its mechanisms and to develop schemes for the improvement of its diagnosis, treatment and prognosis.

Long non-coding RNAs (lncRNAs) refer to transcribed RNA molecules possessing a length of over 200 nt, with limited or no protein coding ability [5,6]. LncRNAs can regulate gene expression at epigenetic, transcriptional, and post-transcriptional levels [7]. It is reported that lncRNAs play an important role in many cell processes such as proliferation, apoptosis, cell migration, and tumor metastases [8–11]. lncRNAs could function as oncogene or tumor suppressor in different cancers [12–16]. Linc0974 as a new biomarker interacts with KRT19 to promote proliferation and metastasis of hepatocellular carcinoma [17]. lncRNA THOR can directly target the middle region of SOX9 3'-UTR, and then increase osteosarcoma cell stemness and migration by enhancing SOX9 mRNA stability [18]. LINC00963 can promote the proliferation and invasion of osteosarcoma by inhibiting miR-204-3p/FN1 axis, and is a potential therapeutic target for osteosarcoma [19]. What’s more, LncRNA BC050642 was found to be associated with c-myc promoter-binding protein-1, while the expression of c-myc protein was correlated with osteosarcoma prognosis [20]. Therefore, we hypothesized that IncRNA-BC050642 might be associated with progression of osteosarcoma. However, the related studies had been rarely reported.

In our study, the expressions of BC050642 and c-myc in osteosarcoma were detected by qRT-PCR and ELISA,
respectively. The role of BC050642 in the progress of osteosarcoma, including in cell proliferation, colony formation and cell apoptosis, was explored. The relationships of BC050642 with clinicopathologic characteristics and overall survival were analyzed to determine prognostic value of BC050642.

Material and methods

Patients and specimens

Ninety-seven osteosarcoma patients (29 females and 68 males, with a median age of 30.09 ± 11.54 years) were selected in our study. The patients were histopathologically confirmed independently by two pathologists. If there was disagreement, the histological results would be confirmed by the superior physician. All of the patients had undergone no treatments before operation. Besides, 97 healthy people with matched age were taken as controls. The study was approved by the Ethics Committee of Academy of Orthopedics of Guangdong Province, the Third Affiliated Hospital, Southern Medical University in Guangzhou province (approval no. 2017–0120). All patients signed written informed consents in advance.

Osteosarcoma tissues, paired adjacent tissues and healthy tissues were collected and frozen in liquid nitrogen immediately, and then stored at −80°C for RNA extraction. Clinicopathologic profiles such as age, sex, tumor site, Ennking, Histological type, therapies, distant metastasis and recurrence were documented in a database. Follow-up was operated for 5 years and subjects dying from unexpected events or other illnesses were removed from this research.

Cell culture and cell transfection

Human osteosarcoma cell lines U2OS and normal osteoblastic cell line hFOB1.19 were gained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). U2OS cell lines were maintained in Dulbecco’s modified Eagle’s medium while hFOB1.19 in DMEM/F-12 (1:1; HyClone, Logan, UT). All mediums were supplemented with 10% fetal bovine serum (FBS; Gibco, NY), 1% penicillin/streptomycin and 2 mM glutamine, and incubated at 37°C in 5% CO2-humidified atmosphere.

U2OS cell lines were seeded in 96-well plates (6 × 103 cells/well) until the concentration reached to 50%. The cells were transfected with siRNA targeting BC050642 or with non-specific control siRNA (si-NC), using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) abide by relevant guidance overnight. All experiments were repeated three times.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from tissue samples and cell lines adopting TRIzol (Invitrogen). RT-PCR reaction was conducted in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) after synthesizing the first chain of cDNA with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). U6 was chosen as endogenous control for BC050642. Relative quantification of BC050642 was calculated via comparative cycle threshold (CT) method. All samples were in triplicate.

Plasmid construction and oligonucleotides

In the study, we constructed the following vectors: pcDNA3.1/c-myc. The sequences of primers were: forward-5'-CCGGGAATTCCTGGATTTTTTCGGGTAGTG-3' and reverse-5'-CCGGCTCGAGTTACGAACAAGAGTTCCGTAG-3'.

ELISA analysis

Total protein was isolated from tissue samples and cell lines. Then ELISA kit (R&D systems, Minneapolis, MN and Biocheck Inc., Foster City, CA, respectively) was used to measure the expression of the protein encoded by c-myc.

Cell proliferation assay

Osteosarcoma cell lines U2OS and normal osteoblastic cell line hFOB1.19 transfected with siRNAs or si-NC were seeded in 96-well plates (2 × 103/well), and cell viability at different time points (0, 24, 48 and 72 h) was determined at 450 nm adopting an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA). Every trial was rerun thrice.

Plate colony formation assay

Logarithmic growth phase cells were collected, and adjusted to the density of 1 × 105/ml. The cells were seeded in 96-well plates. Medium was changed every 3 days, for 14 days of incubation. The cells were stained using crystal violet and then counted. Colony formation was quantified using colony formation number.

Cell apoptosis assay

Annexin V Apoptosis Detection Kit APC (ebioscience, San Diego, CA) was used to analyze cell apoptosis. First, logarithmic phase cells were selected applying three compound perforations, and adjusted to the density of 1 × 105/ml. Then, they were rinsed utilizing binding buffer, and staining buffer was supplemented to resuspend precipitate. Later, 100 µl cell suspension was employed for dyeing via 5 ml Annexin V-APC. Following 15 min of incubation in dark, the mixture was tested operating FACS Calibur (BD Biosciences, Franklin Lakes, NJ).

Statistical analysis

All quantified data were introduced as mean ± standard deviation (SD). Shapiro–Wilks and Levene tests were used to detect the normality and homogeneity of variance of the continuous variables. If the normality and homogeneity of variance were satisfied, Student’s t-test was performed for
their comparison between two groups while one-way analysis of variance (ANOVA) analysis was applied for the analyses among three groups, otherwise, non-parametric test (Mann-Whitney U-test) was used. Chi-square test was conducted to assess possible link of clinicopathologic characteristics with BC050642 expression. The survival curves of the included patients based on their expression of BC050642 were plotted using Kaplan-Meier method with log rank test. In addition, cox regression analysis was performed to estimate the clinical significance of clinical parameters and BC050642 expression for the patients with osteosarcoma. Difference had statistical significance if $p < .05$. Statistical analysis was performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL).

**Result**

**BC050642 expression was increased in osteosarcoma tissues and cell lines**

In this study, we detected BC050642 levels in osteosarcoma, adjacent and healthy tissues as well as in tumor cell lines U2OS and normal osteoblastic cell line hFOB1.19 through qRT-PCR. All the continuous data obtained in our study conformed to the normality and homogeneity of variance. Student's $t$-test results showed that BC050642 expression was significantly higher in osteosarcoma tissues than in adjacent and healthy controls (Figure 1(A), $p < .001$). Compared with normal osteoblastic cell line hFOB1.19, BC050642 expression was significantly up-regulated in osteosarcoma cell lines U2OS (Figure 1(B), $p < .001$). Taken together, these results confirmed that BC050642 expression showed increased tendency in osteosarcoma.

**The expression of c-myc in osteosarcoma patients**

C-myc was considered to be related to the expression of BC050642 and to the prognosis of osteosarcoma, we measured its expression using ELISA analysis. The outcome proved that c-myc expression was decreased in cancer tissues compared to adjacent and healthy ones (Figure 2(A), $p < .05$). As for in cell lines, c-myc expression was also lower in U2OS than in hFOB1.19 (Figure 2(B), $p < .05$). These findings suggested that the expressions of C-myc were decreased in osteosarcoma.

**BC050642 promoted osteosarcoma cell proliferation, increased colony formation and inhibited cell apoptosis**

To determine whether BC050642 functioned as an oncogene in osteosarcoma, we conducted MTT assay, colony formation assay and FACS assay. MTT assay showed that the proliferation of cells transfected with si-BC050642 was significantly
decreased than those transfected with si-NC (Figure 3(A)). The clone number of cells transfected with si-BC050642 was reduced when compared with those transfected with si-NC (Figure 3(B,C), \( p < .05 \)). Besides, apoptosis percentage of U2OS cells was significantly increased in si-BC050642 group compared to si-NC group (Figure 3(D,E), \( p < .05 \)). These findings suggested that the knockdown of BC050642 resulted in inhibition on cell proliferation and colony formation, and promoting effects on cell apoptosis of osteosarcoma cell lines U2OS, revealing the oncogenic potential of BC050642 in osteosarcoma.

C-myc-inhibited osteosarcoma cell proliferation, reduced colony formation and induced cell apoptosis

To examine the effect of c-myc on osteosarcoma, pcDNA 3.1-c-myc was designed to enhance the expression of c-myc in osteosarcoma cell lines. The efficiency of the transfection was detected through qRT-PCR. As displayed in Figure 4, the transfection of pcDNA 3.1-c-myc obviously promote the expression of c-myc in osteosarcoma cells. Following, the cell experiments were designed to explore the function of c-myc in osteosarcoma. The restored expression of c-myc inhibited cell proliferation (Figure 5(A)) and suppressed colony formation number (Figure 5(B,C)). In addition, cell apoptosis assay showed that the over-expression of c-myc promoted cell apoptosis (Figure 5(D,E)). These results demonstrated that the anti-tumor of c-myc on cell proliferation, colony formation and apoptosis.

The relationship between clinicopathologic characteristics and BC050642

To investigate whether the expression of BC050642 was related to the progression of osteosarcoma, we estimated its relationship with clinicopathologic characteristics. According to the expression of BC050642, osteosarcoma cases were sorted into high and low BC050642 expression groups, based on a median expression of 3.958 ± 0.504. As displayed in Table 1, Ennking (\( p = .000 \)) and histological...
type ($p = .022$) were significantly related to the expression of BC050642. Whereas, BC050642 had no relationship with age, gender, tumor site, therapies, distant metastasis or recurrence.

**Prognostic value for BC050642 in osteosarcoma**

Kaplan–Meier analysis revealed shorter overall survival for the subjects harboring high BC050642 levels when compared with low ones (log-rank test, $p < .001$, Figure 6). Cox regression analysis was used to further inspect the clinical significance of BC050642 in the cancer prognosis. As a result, Ennking ($HR = 1.535$, 95% CI = 0.355–6.641, $p = .012$), distant metastasis ($HR = 4.241$, 95% CI = 1.431–12.564, $p = .009$) and BC050642 ($HR = 13.846$, 95% CI = 2.362–91.153, $p = .004$) acted as influential factors for osteosarcoma prognosis (Table 2). Furthermore, BC050642 might be an independent biomarker for cancer prognosis.

**Discussion**

Osteosarcoma stands for a differentiation disease which was caused by genetic changes that interrupt osteoblast differentiation from mesenchymal stem cells [21]. It generally stems from the metaphysis of long bones in children and juveniles, such as distal femur, proximal tibia and proximal humerus.
Table 1. The relationship between clinicopathologic characteristics and BC050642 in patients with osteosarcoma.

| Clinicopathologic characteristics | LncBC050642 expression | \( \text{High} \) | \( \text{Low} \) | \( p \) |
|----------------------------------|------------------------|-------------|-------------|------|
| Age (<30)                        |                        | 50          | 55          | .486 |
| Age (≥30)                        |                        | 47          | 45          | .436 |
| Gender Female                    |                        | 29          | 22          | .000 |
| Gender Male                      |                        | 68          | 45          | .000 |
| Tumor site Femur                 |                        | 20          | 18          | .473 |
| Tumor site Tibia                 |                        | 35          | 16          | .022 |
| Tumor site Humeral bone          |                        | 31          | 16          | .022 |
| Tumor site Others                |                        | 11          | 8           | .022 |
| Enneking I                       |                        | 23          | 21          | .022 |
| Enneking II                      |                        | 40          | 20          | .022 |
| Enneking III                     |                        | 34          | 10          | .022 |
| Histological type Osteoblastic   |                        | 20          | 7           | .147 |
| Histological type Chondroblastic |                        | 36          | 15          | .147 |
| Histological type Fibroblastic   |                        | 30          | 21          | .147 |
| Histological type Telangiectatic |                      | 11          | 8           | .147 |
| Therapies Neoadjuvant chemotherapy|                      | 28          | 19          | .837 |
| Therapies Resection              |                        | 48          | 23          | .837 |
| Therapies Postoperative chemotherapy|                     | 21          | 9           | .837 |
| Distant metastasis Absent        |                        | 39          | 21          | .837 |
| Distant metastasis Present       |                        | 58          | 30          | .837 |
| Recurrence Absent                |                        | 36          | 18          | .696 |
| Recurrence Present               |                        | 61          | 33          | .696 |

Figure 6. Kaplan–Meier analysis for osteosarcoma patients according to their expression of BC050642. Patients with high BC050642 expression had shorter overall survival than those with low BC050642 expression (log-rank test, \( p < .001 \)).

Table 2. Multivariate analysis adjusting clinical factors for prognostic value of BC050642 in patients with osteosarcoma.

| Parameter                        | Risk ratio | 95% confidence interval | \( p \) |
|----------------------------------|------------|-------------------------|------|
| Enneking                         | 1.535      | 0.355–6.641             | .012 |
| Distant metastasis               | 2.421      | 1.431–12.564            | .009 |
| Low-LncRNA-BC050642              |            | 0.000                   | \( p \) |
| High-LncRNA-BC050642             | 13.846     | 2.362–81.153            | .004 |

LncRNAs have been recently considered to be oncogene or tumor suppressor in different cancers. Without protein-coding capacity, they can regulate gene expression and is involved in the development of human diseases, including tumors [25,26]. More and more lncRNAs have been confirmed to act as diagnostic and prognostic markers or regulators in tumor development. However, studies about lncRNAs in osteosarcoma were rare. Ivan Pasic et al. [27], found the deletion of lncRNA-LC285194 and BC040587 might be a reason of osteosarcoma onset, and predicted poor prognosis, via exploring their functions in vitro. Zhang et al. [28] reported that lncRNA-TUG1 could not only inhibit cell proliferation but promote apoptosis, which might provide a therapeutic strategy for osteosarcoma. LncRNA loc285194 was considered as a p-53 regulated tumor suppressor and inhibited tumor cell growth in osteosarcoma both in vitro and in vivo [29]. Reportedly, 25,733 lncRNAs including 403 lncRNAs over-regulated and 798 lncRNA under-regulated have been discovered in osteosarcoma by Li et al. [30]. In our study, we detected BC050642 levels in osteosarcoma tissues and cell lines. The results revealed that the expression of BC050642 in osteosarcoma tissues or cell lines was higher than that in controls. The analysis of the relationship between clinicopathologic characteristics and BC050642 manifested that BC050642 was related to the progress of osteosarcoma. Biological functions of BC050642 in osteosarcoma cell lines were also studied in vitro. And, we accomplished MTT assay, colony formation assay and FACS assay. According to relevant results, the up-regulation of BC050642 could promote cell proliferation, increase colony formation and inhibit apoptosis in osteosarcoma. Taken together, BC050642 played a critical role in the development and progression of osteosarcoma, being an oncogene in the malignancy.

The \( c\text{-}\text{myc} \) gene, located on the long arm of chromosome-8 (8q24), is a member of myc family decreased in a various of cancers such as lung cancer, breast cancer, prostate cancer, leukemias and lymphomas [31,32]. \( c\text{-}\text{myc} \) can not only induce histone acetyltransferase (HAT) activity but also promote RNA polymerase II (RNAPII) clearance [33]. \( c\text{-}\text{myc} \) has been reported to be involved in several cancers through interacting with lncRNAs. For instance, \( c\text{-}\text{myc} \) increased the expression of lncRNA-CCAT1 and promoted cell proliferation in vitro [29]. Zhang et al. [28] reported that lncRNA-TUG1 could not only inhibit cell proliferation but promote apoptosis, which might provide a therapeutic strategy for osteosarcoma. LncRNA loc285194 was considered as a p-53 regulated tumor suppressor and inhibited tumor cell growth in osteosarcoma both in vitro and in vivo [29]. Reportedly, 25,733 lncRNAs including 403 lncRNAs over-regulated and 798 lncRNA under-regulated have been discovered in osteosarcoma by Li et al. [30]. In our study, we detected BC050642 levels in osteosarcoma tissues and cell lines. The results revealed that the expression of BC050642 in osteosarcoma tissues or cell lines was higher than that in controls. The analysis of the relationship between clinicopathologic characteristics and BC050642 manifested that BC050642 was related to the progress of osteosarcoma.
over-expression of BC050642 [40]. Therefore, we transfected c-myc overexpression vector pcDNA 3.1-c-myc into osteosarcoma cell lines and investigated the effects of c-myc on osteosarcoma development. The result indicated that c-myc over-expression could inhibit cell proliferation, decrease colony formation and induce cell apoptosis.

To investigate BC050642 merit in osteosarcoma prognosis, we conducted Kaplan–Meier and cox regression analysis. Kaplan–Meier showed osteosarcoma cases enjoying low BC050642 levels lived longer than high subjects. Besides, cox regression analysis demonstrated that high BC050642 expression as well as Ennking and distant metastasis significantly influenced osteosarcoma outcomes, and they had the potential of independently predicting the patients’ prognosis.

**Conclusion**

In conclusion, the expression of BC050642 is increased in osteosarcoma and closely correlated with Ennking and histological type. The over-expression of BC050642 promotes cell proliferation, induces colony formation and inhibits cell apoptosis via down-regulating the expression of c-myc. High BC050642 may predict dismal clinical outcomes for the patients with osteosarcoma.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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