1 INTRODUCTION

Osteosarcoma is common in children and teenagers (Anderson, 2016), whose incidence is second only to lymphoma among malignant tumors in teenagers. Osteosarcoma accounts for about 3%–4% of all pediatric tumors and 30% of malignant bone tumors (Moore & Luu, 2014; Wycislo & Fan, 2015), and it often occurs in the epiphysis and other sites near the knee, proximal tibia, and distal femur (Isakoff, Bielack, Meltzer, & Gorlick, 2015). Many patients with osteosarcoma have varying degrees of lung metastasis when they are first diagnosed (Zhou et al., 2014). If lung metastasis occurs, the 5-year-survival rate of these patients will be less than 20% (Friebele, Peck, Pan, Abdel-Rasoul, & Mayerson, 2015).

Currently, the focus of research on osteosarcoma has been extensively and gradually extended to genetic, molecular, and protein levels. For example, Li, Dou, Liu, & He (2017) published a paper Application of Long Noncoding RNAs in Osteosarcoma: Biomarkers and Therapeutic Targets. Zhang et al. (2016) found that the level of long noncoding RNA

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**Abstract**

**Background:** Long noncoding RNA (lncRNA) exerts a potential regulatory role in tumorigenesis. LncRNA NEAT1 expression remains high in osteosarcoma tissues. However, its biological mechanism in osteosarcoma remains unknown.

**Methods:** In this study, NEAT1 expression in osteosarcoma cells was detected by qRT-PCR. Proliferative and apoptosis potentials of osteosarcoma cells were determined by CCK-8 assay and Flow Cytometry, respectively. We identified the potential target of NEAT1 through bioinformatics and dual-luciferase reporter gene assay. Furthermore, their interaction and functions in regulating the development of osteosarcoma were clarified by Western blot and RIP assay.

**Results:** Our results demonstrated a high expression of NEAT1 in osteosarcoma tissues and cells. Overexpression of NEAT1 markedly accelerated proliferative and reduced apoptosis potentials of osteosarcoma cells. Besides, NEAT1 could positively regulate the expression of HOXA13 by competing with miR-34a-5p.

**Conclusion:** These results indicated that NEAT1 participated in the development of osteosarcoma as a ceRNA to competitively bind to miR-34a-5p and thus mediate HOXA13 expression.

**Keywords**

apoptosis, long noncoding RNA, osteosarcoma, proliferation
(lncRNA) *MALAT1* in osteosarcoma tissues was higher than that in paired adjacent normal tissues, and it affects the invasion and metastasis abilities of osteosarcoma cells. To further explore the pathogenesis of osteosarcoma, the mechanisms of the occurrence, progression and apoptosis of osteosarcoma were elucidated at the epigenetic level. Our results are of great significance to improve the diagnostic and therapeutic approaches of osteosarcoma.

LncRNAs are noncoding RNAs with more than 200 nucleotides that are capable of regulating gene expressions (Lorenzen & Thum, 2016; Sun, Yang, Xu, & Guo, 2017). They have been widely concerned in recent years due to their complex biological functions. It is reported that certain lncRNAs exert their crucial effects on proliferation, apoptosis, invasion, and infiltration of many types of tumor cells (Chen, Xu, & Zhang, 2017; Mao et al., 2017; Min et al., 2016; Wang et al., 2017). LncRNA Nuclear Enriched Abundant Transcript 1 (*NEAT1*, NCBI Gene ID: 283131) is an lncRNA with diverse functions in tumorigenesis (Dong et al., 2018; Ghafouri-Fard & Taheri, 2019; Yang et al., 2017). Previous studies have indicated that *NEAT1* participates in the pathogenesis of various diseases, such as nervous system diseases, cardiovascular diseases, and various tumors (Ahmed et al., 2018; Fujimoto et al., 2016; Sunwoo et al., 2017). The promotive role of *NEAT1* has been identified in hepatocellular carcinoma (Fang et al., 2017) and ovarian cancer (An, Lv, & Zhang, 2017).

In this study, we examined *NEAT1* in osteosarcoma tissues and adjacent noncancerous tissues. Our results verified that *NEAT1* was highly expressed in osteosarcoma tissues. The biological role of *NEAT1* in the pathological process of osteosarcoma has been pointed out (Li et al., 2018). However, the specific mechanism of *NEAT1* involvement in osteosarcoma development still needs to be explored.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethical compliance

The research was approved by the Ethics Committee of The Third Hospital of Hebei Medical University.

### 2.2 | Specimen collection and processing

In this study, 72 pairs of osteosarcoma tissues and matched adjacent normal tissues were collected through surgery and osteosarcoma was confirmed by postoperative pathological examination. Immediately after the fresh tissues from the lesion and the normal tissue adjacent to the tumor were removed by professional physicians using special forceps, these tissues were washed with DEPC, put into the refrigerated tube, and placed in the labeled liquid nitrogen tank for freezing. Subsequently, the clinical data were collected in the department of pathology. Tissue sample collection was approved by the patients and the Ethics Committee. Among these patients, there were 26 females and 46 males aged between 12 and 31, with an average age of 18.3 ± 5.7.

### 2.3 | Cell culture and transfection

Human normal osteoblast cell line hFOB1.19 and osteosarcoma cell lines Saos2, MG63, U2OS, SJSA1, and HOS were purchased from ATCC (Manassas VA). Cells were cultured in DMEM containing 10% FBS (Beyotime, Nantong, China), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Invitrogen, USA), and maintained under 5% CO₂ at 37°C. *NEAT1* overexpression plasmid, *NEAT1* siRNA, miR-34a-5p mimics and miR-34a-5p inhibitor were all constructed by GenePharma (Shanghai, China). Transfection was performed using Lipofectamine 2000 (Invitrogen, CA).

### 2.4 | RNA extraction and qRT-PCR

Total RNA was isolated using the TRIzol Reagent (Invitrogen, USA). Using the Reverse Transcription Kit (Takara, Tokyo, Japan), cDNA was obtained from reverse transcription of RNA. Subsequently, an ABI 7900 system (Applied Biosystems, CA) and SYBR Green PCR kit (TaKaRa Biotechnology, Dalian, China) were adopted for qRT-PCR. With GAPDH as an internal control, the lncRNA expression level was normalized, and 2^−ΔΔCt method was adopted to assess the fold change in lncRNA expression. Primer sequences are displayed below.

**NEAT1**:
- F: 5′ TCTGTGTGTCAAAGCAAGGC 3′,
- R: 5′ AGATGCCACTGATATCACC 3′,
**HOXA13**:
- F: 5′ CTGCCCTATGCTACTCTCAG 3′,
- R: 5′ CCGGGCTATCCGTACTTG 3′,
**GAPDH**:
- F: 5′ CCGGAAACCTGTGGCCTCAG 3′,
- R: 5′ AGGTGGAGGGTGGGTGTCGCTG 3′.

### 2.5 | Cell proliferation assays

Cells were cultured in 96-well plates and incubated with CCK-8 reagent (Beyotime, Nantong, China) for 1 hr. The absorbance at 450 nm was recorded using a TECAN infinite M200 Multimode microplate reader (Tecan, Mechelen, Belgium).

### 2.6 | Cell apoptosis assays

Cells were dyed with Annexin V-FITC and propidium iodide (PI), and apoptosis was determined by flow cytometry (BD Biosciences, Franklin Lakes, NJ). Apoptosis was assayed through dual-staining with annexin V-FITC and propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ). In brief, transfected cells for 48 hr were incubated with Annexin
V-FITC and PI in dark, and subjected to analysis by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

2.7 | Subcellular distribution

RNAs in cytoplasm and nucleus were extracted with the PARIS Kit (Life Technologies, USA). Total RNA in each fraction was quantified by qRT-PCR. GAPDH and U6 were utilized as cytoplasm and nucleus internal references, respectively.

2.8 | Dual-luciferase reporter gene assay

Wild-type plasmids NEAT1-WT and HOXA13-WT, as well as mutant-type plasmids NEAT1-MUT and HOXA13-MUT were constructed. HOS and Saos2 cells seeded into 24-well plates were co-transfected with 50 nmol/L miR-34a-5p mimics or a negative control and wild-type or mutant-type plasmid using Lipofectamine 2000. 5 ng of pRL-SV40 was added per 80 ng of plasmid. Dual-luciferase reporter assay kit (Promega, Madison, WI) was used for determining luciferase intensity on a microplate reader.

2.9 | RNA-binding protein immunoprecipitation (RIP)

The Magna Nuclear RIP™ (Native) Nuclear RIP Kit (Millipore, Bedford, MA) was used for RIP assay. Cells were lysed in complete RIPA buffer containing protease inhibitor cocktail and an RNase inhibitor. Cell extract was incubated with RIP buffer containing magnetic beads conjugated to human anti-AGO2 antibody (Millipore) or IgG control. Immunoprecipitated RNA was obtained from protein digestion. Finally, the purified RNA was quantified by qRT-PCR. Anti-NEAT1 used for RIP assay was purchased from Abcam (Cambridge, MA).

2.10 | Western blot

Protein samples were extracted and quantified by BCA, separated by SDS-PAGE gel electrophoresis, and blocked with 5% skim milk. Membranes were then incubated with the primary antibodies (rabbit anti-human IgG antibodies against HOXA13 and GAPDH) and corresponding secondary antibodies. Bands were exposed, and the color was developed by chemiluminescence.

2.11 | Statistical processing

SPSS 20.0 software and GraphPad Prism 6.0 were utilized for statistical analysis. Quantitative data were represented as mean ± SD. Measurement data were analyzed by the t test, whereas data not conformed to the normal distribution were subjected to nonparametric test. The value of p < 0.05 indicated statistical significance.

3 | RESULTS

3.1 | NEAT1 expression and function in osteosarcoma

NEAT1 expression in osteosarcoma tissues and paired paracancerous tissues was detected by qRT-PCR. The results showed that NEAT1 was highly expressed in osteosarcoma tissues (Figure 1a). We then examined the relative expression of NEAT1 in osteosarcoma cells (Saos2, MG63, U2OS, SJSA1, and HOS) and human normal osteoblast cells hFOB1.19 by qRT-PCR. The above results indicate that NEAT1 is highly expressed in osteosarcoma cells, which is consistent with the results found by Wang, Yu, Fan, & Luo (2017). In particular, HOS cells expressed the highest level, while Saos2 cells expressed the lowest level of NEAT1 among the selected osteosarcoma cell lines, which were chosen for the subsequent experiments (Figure 1b). Additionally, the prognostic value of NEAT1 expression was determined for overall survival in osteosarcoma patients by the Kaplan–Meier analysis, suggesting that high expression level of NEAT1 is significantly correlated with shorter overall survival (Figure 1c and d).

3.2 | NEAT1 facilitates cell proliferation and inhibits apoptosis signaling

After knock-down of NEAT1 in HOS cell line and overexpression of NEAT1 in Saos2 cell line, we calculate their transfection efficiency (Figure 2a). Then, CCK-8 assay indicated that NEAT1 downregulation markedly decreased the proliferative ability of osteosarcoma cells. NEAT1 overexpression accelerated the proliferative rate of osteosarcoma cells (Figure 2b). In addition, cell apoptosis experiment showed that overexpression of NEAT1 reduced the apoptosis capacity of osteosarcoma cells, and NEAT1 downregulation induced the apoptosis capacity of osteosarcoma cells (Figure 2c and d). Taken together, these results indicate that NEAT1 may exert regulatory effects on apoptosis and proliferative potentials of osteosarcoma cells.

3.3 | Subcellular distribution of NEAT1

Subcellular distribution of IncRNA determines its biological function. To confirm the cellular localization of NEAT1, we isolated osteosarcoma cells into cytoplasmic and nuclear fractions, with GAPDH and U6 as controls, respectively. QRT-PCR results showed that 69.5% and 71.05% of NEAT1 were distributed in the cytoplasmic fraction of HOS and Saos2 cells, respectively (Figure 3a). We may conclude that NEAT1 participates in the development of osteosarcoma through post-transcriptional regulation.
3.4 | NEAT1 is targeted by miR-34a-5p

Given that NEAT1 was primarily located in the cytoplasmic fraction, we hypothesized that NEAT1 may act as a ceRNA in the development of osteosarcoma. QRT-PCR data revealed that miR-34a-5p expression was lower in osteosarcoma tissues, which was contrary to the expression trend of NEAT1 (Figure 3b). Through RegRNA, Starbase...
**FIGURE 3** *NEAT1* directly interacts with miR-34a-5p. (a) Cytoplasmic and nuclear levels of *NEAT1* in HOS and Saos2 cells analyzed by qRT-PCR. (b) MiR-34a-5p expression in osteosarcoma tissues. (c) Bioinformatics evidence of binding of miR-34a-5p onto 3′-UTR of *NEAT1*. (d) Dual-luciferase reporter gene assay in HOS and Saos2 cells after transfection with negative control or miR-34a-5p mimics, renilla luciferase vector pRL-SV40 and the reporter constructs. (e) RIP experiments for the amount of *NEAT1* and miR-34a-5p in HOS and Saos2 cells. Data are presented as mean ± SD. *p < 0.05

**FIGURE 4** *HOXA13* is the direct target of miR-34a-5p. (a) The putative miRNA-binding sites in the *HOXA13* sequence. (b) Direct target sites confirmed by dual-luciferase reporter gene assay. (c) *HOXA13* expression in osteosarcoma tissues. (d) Protein level of *HOXA13* in hFOB1.19, HOS and Saos2 cell lines detected by Western Blot. Data are presented as mean ± SD. *p < 0.05
prediction, we found that sequences in miR-34a-5p that were highly matched to NEAT1 3’UTR. Based on these binding sequences, we constructed pGL3-NEAT1-WT and pGL3-NEAT1-MUT (Figure 3c). Luciferase activity was obviously downregulated in HOS and Saos2 cells co-transfected with NEAT1 WT and miR-34a-5p mimics, while it did not change after transfection with NEAT1 MUT (Figure 3d). RIP analysis was carried out to elucidate whether NEAT1 was involved in RNA-containing ribonucleoprotein complex. QRT-PCR results showed that NEAT1 was enriched in anti-AGO2 antibody compared with in controls. Similar results were yielded in miR-34a-5p (Figure 3e). The above results imply that miR-34a-5p can bind to NEAT1 in vitro.

### 3.5 NEAT1 regulates HOXA13, the target gene of miR-34a-5p

To investigate the potential role of miR-34a-5p in the development of osteosarcoma, we screened out the target genes of miR-34a-5p by bioinformatics prediction (TargetScan, Starbase, RegRNA). Finally, HOXA13 was selected for further analyses. After construction of luciferase plasmids pGL3- HOXA13-WT and pGL3- HOXA13-MUT, they were cotransfected with miR-34a-5p mimics or NC in HOS and Saos2 cells, respectively (Figure 4a). Luciferase activity of the WT reporter was inhibited, while MUT reporter group did not change (Figure 4b). The above results indicate that HOXA13 is a potential target gene of miR-34a-5p. Subsequently, HOXA13 expression in osteosarcoma tissues and paired paracancerous tissues was determined by qRT-PCR. The mRNA levels of HOXA13 were remarkably elevated in osteosarcoma tissues compared to paracancerous tissues (Figure 4c). Western blot analysis revealed the same result at the protein level (Figure 4d).

To elucidate whether NEAT1 regulated HOXA13 expression via targeting miR-34a-5p, we detected expression levels of HOXA13 in osteosarcoma cells after altering NEAT1 or miR-34a-5p expressions. Transfection with miR-34a-5p inhibitor in HOS cells upregulated HOXA13 expression, which was reversed by cotransfection with miR-34a-5p inhibitor and NEAT1 siRNA (Figure 5a and b). Furthermore, transfection with miR-34a-5p mimics in Saos2 cells inhibited HOXA13 expression, which was reversed by cotransfection with miR-34a-5p mimics and NEAT1 overexpression plasmid (Figure 5c and d). Subsequently, Saos2 cells were transfected with NEAT1 overexpression plasmid and its mutant
overexpression plasmid, followed by the determination of HOXA13 expression. Both qRT-PCR and Western blot results showed that overexpression of wild-type NEAT1 upregulated HOXA13 expression in osteosarcoma cells, whereas mutant-type NEAT1 did not disrupt base pairing between NEAT1 and miR-34a-5p (Figure 5e and f). To sum up, our findings confirm that NEAT1 positively regulates the expression of HOXA13 by directly binding to miR-34a-5p.

3.6 | NEAT1/miR-34a-5p axis regulates behaviors of osteosarcoma cells

We next explored whether miR-34a-5p could affect proliferative and apoptosis potentials of HOS and Saos2 cells. Downregulation of miR-34a-5p in HOS cells markedly promoted proliferative and inhibited apoptosis potentials compared to controls, which were partially reversed by cotransfection with miR-34a-5p inhibitor and NEAT1 siRNA (Figure 6a and c). In addition, overexpression of miR-34a-5p inhibited proliferative and promoted apoptosis potentials of Saos2 cells, and were partially reversed by NEAT1 overexpression (Figure 6b and d). Based on the above results, NEAT1/miR-34a-5p axis exerts great effects on regulating behaviors of osteosarcoma cells.

4 | DISCUSSION

Osteosarcoma is ranked among the leading causes of cancer-related death in the pediatric age group. The cancer's low
prevalence and its large tumor heterogeneity make it difficult to obtain meaningful progress in patient’s survival (Botter, Neri, & Fuchs, 2014). LncRNA has been shown to be a regulator of various cellular processes. Dysregulated LncRNAs have been identified to be related to the disease development (Yu, Chuang, & Kuo, 2018). NEAT1 has been confirmed to participate in cell proliferation (Wang, Wang, Zhang, Deng, & Long, 2017; Zhu et al., 2019), so we hypothesized that NEAT1 may be involved in the pathogenesis of osteosarcoma.

Our study showed a higher expression of NEAT1 in osteosarcoma cells relative to normal osteoblast cells. In addition, downregulation of NEAT1 expression remarkably reduced proliferative and induced apoptosis capacities, suggesting that NEAT1 was an important regulator in the growth of osteosarcoma cells as an oncogene. Therefore, explorations on the effect of NEAT1 on accelerating growth of osteosarcoma cells are of great significance for in-depth studies of the occurrence and development of osteosarcoma.

Through separation of cytoplasm and nucleus, we confirmed that NEAT1 was mainly distributed in cell cytoplasm, indicating that NEAT1 may serve as a ceRNA. Subsequently, RIP and dual-luciferase reporter gene assay clarified that NEAT1 could bind to miR-34a-5p. So far, miR-34a-5p has been proved to be lowly expressed in ovarian cancer and glioma (Ding, Wu, Tao, & Peng, 2017; Xu et al., 2018). Our study demonstrated that miR-34a-5p was downregulated in osteosarcoma cells, and transfection with miR-34a-5p mimics promoted apoptosis and suppressed proliferative capacities of osteosarcoma cells, which could be reversed by NEAT1 overexpression. We believed that both NEAT1 and miR-34a-5p may participate in the development and progression of osteosarcoma.

HOX genes play a fundamental role in the development of the vertebrate central nervous system, axial skeleton, limbs, gut, urogenital tract and external genitalia, but mutations in two of the 39 human HOX genes (HOXD13 mutated in synpolydactyly, and HOXA13 mutated in hand-foot-genital syndrome) have been shown to cause congenital malformations (Goodman & Scambler, 2001; Grier et al., 2005). Studies have shown that HOXA13 exerts an important regulatory effect on organ development, cell differentiation, and tumorigenesis (Luo, Rhie, Lay, & Farnham, 2017). Quagliata et al. (2018) declared that high expression of HOXA13 is correlated with poorly differentiated hepatocellular carcinomas and modulates sorafenib response in in vitro models. Hu, Chen, Cheng, Li, & Zhang (2017) suggested that dysregulated expression of homebox gene HOXA13 is related to the poor prognosis in bladder cancer. Accordingly, our study verified that upregulated NEAT1 increased expression of HOXA13, the target gene of miR-34a-5p, further leading to abnormal proliferation and apoptosis of osteosarcoma cells.

To sum up, NEAT1 functioned as a competitive endogenous RNA to regulate HOXA13 expression by sponging miR-34a-5p, thus regulating the development of osteosarcoma.

**CONFLICT OF INTEREST**

None.

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