LncRNA STK4 antisense RNA 1(STK4-AS1) affects the osteosarcoma cell cycle by regulating p53 protein

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Research Article

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Abstract

Background: LncRNA STK4-AS1 has been identified as a potential biomarker associated with multiple cancers. We proposed that STK4-AS1 plays a role in the proliferation of osteosarcoma by regulating its cell cycle.

Methods: We compared the expression of STK4-AS1 in osteosarcoma vs normal samples both in clinical tissues and cell lines. We overexpressed and knockdown STK4-AS1 in p53 expressing osteosarcoma cells U2OS and p53 muted expressing osteosarcoma cells MG63 and analyzed cell viability and cell cycle. We overexpressed p53 in STK4-AS1 knockdown cells to explore the association of STK4-AS1 and p53 in the cell cycle.

Results: The STK4-AS1 expression was higher in osteosarcoma tissue than adjacent normal bone tissues and was higher in osteosarcoma cell lines (U2OS, MG63, and SAOS-2) than in osteoblast cell lines (hFOB and HOB). Knockdown of STK4-AS1 in U2OS decreased the cell viability, increased cells in the G0/G1 phase, decreased cells in the S and G2/M phase, decreased expression of cyclin A and B, increased p53 and p21, and had no effect on cyclin D and cyclin E, while overexpression did the opposites. MG63 cell viability was not affected by altered STK4-AS1 levels. P53 overexpression in STK4-AS1 knockdown cells recovered cell viability, p21, cyclin A, and cyclin B expression.

Conclusion: LncRNA STK4-AS1 affected p53 expressing osteosarcoma cells U2OS cell viability through regulating cell cycle, which is mediated by p53/p21 pathway.

1. Introduction

Osteosarcoma is the most common primary bone malignancy [1]. The incidence of osteosarcoma is 5.4 cases per year per million in males and 4 cases per year per million in females [2]. Although the death rates for osteosarcoma have steadily declined by approximately 1.3% per year, the 5-year overall survival rate is only about 68% [2]. Studies have revealed multiple regulation pathways of bone metabolism [3-7], and many effective therapeutic approaches to bone disease have been developed [8, 9]. Yet, to date, the understanding of the pathology of osteosarcoma is still insufficient [10]. Hence, the study of osteosarcoma is necessary.

Long non-coding RNAs (lncRNAs) are RNAs that transcripts with lengths over 200 bp but are not translated into protein [11]. LncRNAs function as transcriptional regulators for the expression of many genes [12]. Recently, more and more ncRNAs were found to be involved in bone cell regulations [13-16]. Many IncRNAs were identified to be associated with human diseases including cancers [17-19]. Studies revealed that the IncRNAs expressed in bone plays a critical role in regulating bone formation [20] and differentiation [21-23]. Osteosarcoma derives from primitive bone-forming mesenchymal cells [24] and it is also suggested to be regulated by IncRNAs [25]. An increasing number of IncRNAs were identified as the prognostic biomarkers or potential therapeutic targets of cancer by bioinformatics study [26, 27] and
the role of many of them in osteosarcoma has been validated in vivo or in vitro[28-31]. As the number of lncRNAs is large and the regulation network of lncRNAs is complex, more works are required in this field.

A lncRNA, STK4-AS1, has previously been identified as a cancer biomarker associated with the progression of breast cancer[32], lung cancer [27], and colon cancer [26]. STK4-AS1 orientates from the minus strand and is located at 20q13.12 in the human genome with a size of 2,665 bases [33]. It is one of the antisense RNA of the STK4 gene. STK4 protein was reported to regulate many types of cancers, including liver cancer [34], prostate cancer [35], and thyroid carcinoma [36]. However, the effect of its antisense RNA was still unclear. Some lncRNAs can regulate cell growth by regulating the cell cycle [37]. We proposed that STK4-AS1 plays a role in the proliferation of osteosarcoma by regulating its cell cycle. In this study, we investigated the expression of lncRNA STK4-AS1 in osteosarcoma, both in clinical samples and cell models, and study its effect on the osteosarcoma cell cycle. Our study aims to deepen the understanding of the role of STK4-AS1 in osteosarcoma and develop it as a potential novel target for the treatment of osteosarcoma.

2. Materials And Methods

2.1. Clinical tissue samples

Clinical osteosarcoma and adjacent normal bone tissues were collected from osteosarcoma patients who underwent surgery from 2016 to 2019 in the Affiliated Tumor Hospital of Zhengzhou University (Hunan, China). This work was approved by the Ethical Committee of Zhengzhou University and all of the patients consented to participate in this study. Samples were immediately snap-frozen in liquid nitrogen for further study.

2.2. Cell lines and cell culture

Human osteosarcoma cell lines MG-63 (ATCC® CRL-1427™), Saos-2 (ATCC® HTB-85™), U-2 OS (ATCC® HTB-96™), and human osteoblast cell line HOS (ATCC® CRL-1543™) was obtained from ATCC (Washington, USA). Human primary osteoblast cell line HOB was obtained from PromoCell (Heidelberg, Germany). Cell lines were cultured in Osteoblast Growth Medium C-27001 (Heidelberg, Germany) in a humidified atmosphere of 5% CO2 at 37°C.

2.3. MTT assay

The MTT assay was first reported by Mossmann in 1983 [38]. It is commonly used for both cancer cell and non-cancer cell viability determination [39, 40]. Briefly, cells were grown in 96 well plates and at the endpoint of the culture time, the culture medium was removed and 0.8 mg/ml of MTT working solution (dissolved in serum-free medium) was added to the wells. After 4 hours, DMSO was added to the wells, and absorbance was recorded at 560 nm using the microplate spectrophotometer system (Spectra max190-Molecular Devices).

2.4. RNA extraction and QPCR
The expression of STK4-AS1 was determined by QPCR assay which was described previously [41]. Total RNA from tissue or cell samples was extracted by using Trizol reagent (Invitrogen, Calsbad, CA, USA). Then the total RNA was reversely transcribed into cDNA using a reverse transcription kit (Thermo, USA). Quantitative real time-PCR was done with PowerUp™ SYBR™ Green Master Mix (Thermo, USA) using the ViiA 7 Real-Time PCR System (Applied Biosystems). The protocol for PCR: 95°C for 3 min, 40 cycles of 95°C for 30 s, 58°C for 15 s, and 72°C for 30 s. Gene expression was quantified using the 2-△△CT method. The primers used for RT-PCR were designed by Eurofine Genomic primer design online tools and synthesized by Sigma-Aldrich, Inc. (St. Louis, MO, USA). The sequences are as follows: GAPDH forward: 5'-AATGGGCAGCCGTTAGGAAA-3' and reverse: 5'-TGAAGGGTTCATTGATGGCA-3'. STK4-AS1 forward: 5'-CGGAGCGCACAAAATACTCG-3' and reverse: 5'-CCACAAATACCTCCAGCGT-3'.

2.5. Cell transfection

The STK4-AS1 knockdown and overexpression cells and p53 rescue cells were conducted by transfection of siRNA or expression vector. The cell lines were transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The expression levels of STK4-AS1 were validated by qPCR. The expression of p53 was validated by immunofluorescence imaging and western blotting. The cell transfection method was described previously [42].

The experimental details are as follows: (1) STK4-AS1 knockdown: STK4-AS1 small interfering (si)RNA and negative control siRNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sequences were as follows: STK4-AS1 siRNA, 5'-ACCTCGGCCAGAGAGGTGAAGTATCTTCAAGAGAGATACTTCACCTCTCTGGCCTT-3'. A scramble siRNA (siN05815122147) was used as the NC siRNA. The transfection concentration of siRNA was 60 nM. (2) STK4-AS1 overexpression: The entire sequence of human STK4-AS1 was amplified from hFOB cell lines using PCR and cloned into the pcDNA3.1 vector. The negative control empty vector, which was purchased from Sigma-Aldrich (St. Louis, MO, USA). The transfection concentration of the plasmid was 2 µg/ml. (3) P53 rescue: p38 expressing vectors, TR200pa-pGP-p53-mCMV-EF1α-Puro (plasmid), and negative vectors were purchased from System Biosciences (Palo Alto, CA, USA). The transfection concentration of the plasmid was 2 µg/ml.

The control of cell transfection was set as follows: Ctrl1: cells without treatment; Ctrl2: cells with NC siRNA transfection; Ctrl3: cells with empty pcDNA3.1 vector transfection; Ctrl4: cells with NC siRNA and p53 negative vector transfection.

2.6. Cell cycle analysis

The cell cycle was analyzed using flow cytometry with propidium iodide (PI) staining, which was described previously [43]. Briefly, cells were washed with PBS and resuspended at a concentration of 1 × 106/mL. Cells were fixed with 100% ethanol for three hours at 4 °C. Then suspended cells were washed with PBS two times and incubated with propidium iodide staining solution (0.1% Triton X-100, 0.2 mg/mL
DNAse-free RNAse A, 0.02 mg/mL in cold PBS) at 37 °C for 15 min. BD FACSCalibur was used to acquire cell cycle data. FlowJo Version 10 was used to analyze the data.

2.7. Western blotting

The western blotting method was described previously [44]. Briefly, proteins were extracted from cells using RIPA buffer with protease inhibitor (Sigma-Aldrich, USA). Total protein concentrations were determined using a BCA protein assay kit to control the loading amount (25 μg). SDS gel electrophoresis was performed to separate proteins. Afterward, the proteins were transferred to 0.2-μm polyvinylidene difluoride membranes which were subsequently blocked with 5% skim milk in Tris-buffered saline with 0.5% Tween-20 (TBST). Membranes were then reacted with primary (1:1000 dilution of 5% skim milk in TBST) and secondary antibodies (1:3000 dilution of 5% skim milk in TBST) subsequently. The band intensities were photographed using a super-sensitive gel imaging system after reacted with ECL reagents (Bio-Rad, Hercules, CA, USA).

The primary antibodies used in the experiment are as follow: Anti-cyclin A antibody (sc-271682), Anti-Cyclin B antibody (ab72), Anti-p53 antibody (ab26), Anti-p21 antibody (ab109520), Cyclin D1 Antibody (#2922), and Anti-Cyclin E1 antibody (ab33911). The secondary antibodies are all purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.8. Immunofluorescence imaging

Immunofluorescence imaging was used to measure the expression of p53. The method was described previously [45]. Cell cultures were fixed in 3.7% formaldehyde and permeabilized with 0.25% Triton X-100. Nuclei were stained with 40, 6diamidino-2-phenylindole (DAPI). Cells were immunolabeled using an anti-p53 antibody (ab32389) and a Goat Anti-Rabbit IgG H&L (HRP) (ab6721) secondary antibody. Fluorescence imaging was performed using an inverted fluorescence microscope (Leica, Germany) and Imaris 4.0 software.

2.9. Statistics

T-test or one-way ANOVA and Dunnett’s post hoc tests were used to compare the significant difference between the control and experimental group (p < 0.01). GraphPad Prism (version 6) was used to plot the figures and calculate statistical significance.

3. Results

3.1. STK4-AS1 was overexpressed in osteosarcoma tissue and cells

This study started with clinical osteosarcoma samples. We collected clinical osteosarcoma and adjacent normal tissues from patients who underwent surgery and compared the STK4-AS1 level in these samples. Results showed that STK4-AS1 expression in osteosarcoma samples was significantly higher than in the adjacent normal tissues (Fig.1 A), suggesting that STK4-AS1 might be a cancer biomarker associating
with the development of osteosarcoma. We also compared the expression of STK4-AS1 in osteosarcoma cells vs normal osteoblast cells. Results showed that the expression of STK4-AS1 in osteosarcoma cell lines we tested was much higher than in normal osteoblast cell lines (Fig. 2A).

As we are interested in the role of STK4-AS1 in the p53 pathway, we also determined p53 levels in clinical samples and these cell lines. Results showed that p53 was expressed higher in normal tissue than in osteosarcoma (Fig. 1BC). Results also revealed that p53 was expressed lower or not expressed in osteosarcoma cells compared to normal osteoblast cells (Fig. 2BC). Therefore, we proposed that a higher expression of STK4-AS1 might associate with the lower level of p53 in osteosarcoma.

3.2. Effects of STK4-AS1 on osteosarcoma cell viability

Although all three osteosarcoma cell lines tested expressed STK4-AS1, only U2OS expressed p53 while MG63 and SAOS-2 expressed almost no detectable level of p53. According to previous studies, MG63 and SAOS-2 cells have inactivated p53 while U2OS have wild type p53 [46, 47]. Therefore, we compared p53 expressing cell line U2OS and p53 muted cell line MG63. Cell transfection was used to knock down or overexpress STK4-AS1 in U2OS and MG63, which were validated by QPCR assay (Fig. 2DF). We determined the cell viability using MTT assay. Results showed that knockdown of STK4-AS1 in osteosarcoma cells decreased cell proliferation while overexpression of STK4-AS1 in U2OS cells increased cell proliferation (Fig. 2E). However, artificially altered levels of STK4-AS1 failed to affect the cell viability of p53 muted cell line MG63 (Fig. 2G). This indicated that p53 might mediate the effects of STK4-AS1 on the viability of osteosarcoma cells.

3.3. Effect of STK4-AS1 on the cell cycle

To further explore the exact effect of STK4-AS1 on cell growth, we measured its effect on the U2OS cell cycle. Results showed that, compared with the control, the knockdown of STK4-AS1 increased cells in G0/G1 phase and caused a significant decrease in the S phase and G2/M phase cells. On the other hand, the overexpression of STK4-AS1 decreased cells in G0/G1 phase cells but caused an increase in S phase cells and G2/M phase cells (Fig. 3AB). This indicated that the expression of STK4-AS1 might facilitate cell division. Hence, we suggested that STK4-AS1 promotes osteosarcoma by regulating the cell cycle.

3.4. Effect of STK4-AS1 on the expression of cyclin proteins

This suggestion was confirmed by the determination of key regulators of the cell cycle, cyclin proteins. Western blotting experiments revealed that the knockdown of STK4-AS1 decreased the expression of cyclin A and cyclin B while the overexpression of STK4-AS1 did the opposite (Fig. 3CD). Cyclin A is a critical factor to initiate and complete DNA replication [48, 49] and Cyclin B regulates the G2 checkpoint [50]. The alteration of these two critical factors further confirmed that STK4-AS1 promotes osteosarcoma by regulating the cell cycle. These results could account for the changes in cell numbers in the S and G2 phases. However, although cell in G1 phase was affected, G1 regulators cyclin D and cyclin E [51] were not affected (Fig. 3CD).
3.5. P53 mediated the regulation of STK4-AS1 on cell cycle

Cancer suppressor p53 has long been reported to function as a cell cycle controlling protein in osteosarcomas [52]. It has been proved to regulate the G2 phase by affecting cyclin B [53]. Here we tested it as a potential target underlying the effect of STK4-AS1 on osteosarcomas cells. Results showed that the knockdown of STK4-AS1 decreased the expressions of both p53 and p21 while the overexpression increased the expression of p53 and p21 (Fig. 4AB). Previously, the p53/p21 pathway was reported to regulate G2/M cell cycle genes [54]. Our results provided evidence that p53 and p21 are potential downstream targets of STK4-AS1.

3.6. Rescue experiments of p53 protein

A rescue experiment of p38 in STK4-AS1 knockdown U2OS cells was conducted to confirm the involvement of p53 and p21 in the effect of STK4-AS1 on cells. Both immunofluorescence and western blotting validated that most of the p53 expression was rescued in STK4-AS1 knockdown U2OS cells (Fig.4CDFG). The recovery of p53 expressions also recovered the viability of STK4-AS1 knockdown U2OS cells (Fig.4E), confirming that p53/p21 was involved in U2OS cell viability regulations. Western blotting results showed that the rescue of p53 also upregulated the expressions of p21, cyclin A, and cyclin B, indicating that these three proteins might be potential downstream targets of the STK4-AS1/p53 pathway.

4. Discussion

This study tried to reveal the role of a lncRNA STK4-AS1 in the proliferation of osteosarcoma. From clinical samples, we found an association between the expression of STK4-AS1 and osteosarcoma: the cancer bone tissue expressed a higher level of STK4-AS1 over noncancer bone tissues. We also found that the expression of STK4-AS1 in osteosarcoma is much higher than in representative osteosarcoma tissue mixture which we used as a control. The difference in tissue extraction and cell extraction might account for part of these results, but we suggested that STK4-AS1 might have different expression profiles in cells in different subsets. For example, cancer stem cells [55] might express a different level of STK4-AS1 from other cancer cells. Cell lines were optimized for growth and might have a higher proportion of cancer stem cells [56]. The cancer tissue might include cancer cells with fewer cancer stem cells which resulted in a lower average STK4-AS1 expression.

Among the three osteosarcoma cell lines tested in this study, MG63 expressed most STK4-AS1. However, MG63 had no p53 expression, thus, it is a good negative control cell line for us to explore the role of p53 in STK4-AS1 regulation. As for clinical value, many preclinical studies have contributed to clinical cancer treatment [57-60]. On one hand, this study revealed that STK4-AS1 can be a potential pharmacological target for osteosarcoma treatment. On the other, osteoblast cell lines hFOB and HOB expressed a very low level of STK4-AS1 compared with cancer cells, therefore, STK4-AS1 can also be developed as a potential clinical prognosis marker for osteosarcoma.
As STK4-AS1 was found to be a cancer “booster” in breast cancer [32], lung cancer[27], and colon cancer[26], we hypothesized that it also promoted the progression of osteosarcoma by p53/p21 pathway. MTT assay with transfected cells revealed that the expression of STK4-AS1 level was associated with cell viability, which was consistent with our hypothesis. The cell viability of cancer has been studied wildly to develop pharmacological targets [61, 62]. We analyzed the effect of STK4-AS1 on the cell cycle of the p53 expressing osteosarcoma cell line U2OS and found that STK4-AS1 affected cell viability by regulating the cell cycle. Although some studies showed that the arresting of the cell cycle at the S or G2 phase can also lead to suppression of proliferation [63, 64], based on our viability results, we suggested that the increased cells in S and G2 phase resulted in more proliferation of U2OS cells. On the other hand, in the p53 negative control, MG63 cells, STK-AS1 failed to affect cell viability, which was consistent with the hypothesis.

To further confirm the hypothesis, we observed the effect of STK4-AS1 on cyclin A, a regulator of the S phase regulation [65], cyclin B, a regulator of the G2 phase [50], and cyclin D/cyclin E, regulators of the G1 phase [51]. Furthermore, we also determined p53/p21 pathway. P53 has long been reported to regulate the cell cycle in osteosarcomas[52]. Here we tested it as a potential target underlying the effect of STK4-AS1 on osteosarcomas cells. The p53/p21 pathway is responsible for the negative regulation of cyclin B and cdk1, regulating activities of the G2 phase in cancer cells[50]. The accumulation of p53 can increase the expression of its transcriptional target gene p21 which can potentially inactivate the cyclin B/cdk1 complex [66]. Our results showed that the knockdown or overexpression of STK4-AS1 affects the expression of p53, and the p53 subsequently affected p21 and negatively regulates activities in the G2 phase. P53 rescue experiment confirmed the hypothesis that the cell viability, p21 expression, cyclin A expression, and cyclin B expression were largely recovered to the same level of wild type cells. Besides, p21 can also potentially binds and inactivate the cyclin E/CDK2 complex. Interestingly, although cyclin D and cyclin E have been found regulated by the p53/p21 pathway [67, 68], in this study the cyclin D and cyclin E levels were not affected even with p53/p21 level altered. We suggested that STK4-AS1 might have other impacts on cyclin D and cyclin E besides the p53/p21 pathway that subset the effect of the p53/p21 pathway (Fig.5). We supposed that this unidentified regulation might involve other cancer-related mechanisms such as ion channel regulations[69, 70].

5. Conclusion

This study demonstrated that LncRNA STK4-AS1 plays a role in the proliferation of p53 expressing osteosarcoma by affecting its cell cycle. This effect is mediated by p53/p21 pathway. We suggested that STK4-AS1 is a potential upstream target of the p53/p21 pathway. Given the potential value of STK4-AS1 for clinical osteosarcoma therapy, our study is conducive to the development of STK4-AS1 as a novel treatment target for osteosarcoma.

Abbreviations

STK4-AS1: LncRNA STK4 antisense RNA 1
Declarations

Ethics approval and consent to participate

This work was approved by the Ethical Committee of Affiliated Tumor Hospital of Zhengzhou University.

Consent for publication

All the author consent for this publication.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

There is no potential conflict of interest in this paper.

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Authors’ contributions

Weitao Yao performed experiments and data analysis. Jingyu Hou, Guoqing Liu, Fangxing Wu, and Qiang Yan contributed to the design of the experiment. Weitao Yao and Guoqing Liu wrote the manuscript. Liangyu Guo and Chuchu Wang reviewed and edited the manuscript. Chuchu Wang supervised and directed the project.

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