Long non-coding RNA HAND2-AS1 targets glucose metabolism and inhibits cancer cell proliferation in osteosarcoma

SHUNGUANG CHEN*, XIAOMING XU*, SHENGJUN LU and BIAO HU

Department of Orthopedics, Jingzhou Central Hospital, Jingzhou, Hubei 434020, P.R. China

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Abstract. Long non-coding RNA heart and neural crest derivatives expressed 2-antisense RNA 1 (lncRNA HAND2-AS1) is a known tumor suppressor gene in endometrioid endometrial carcinoma; however, its function in osteosarcoma is currently unknown. In the present study, HAND2-AS1 expression in the tumor tissues and adjacent healthy tissues of patients with osteosarcoma, and in the serum of patients and healthy controls was detected by reverse transcription-quantitative polymerase chain reaction. lncRNA HAND2-AS1 small interfering RNA was transfected into osteosarcoma cells, and cell proliferation, glucose transporter 1 (GLUT1) expression and glucose uptake were detected using the Cell Counting Kit-8, western blotting and glucose uptake assays, respectively. The results revealed that the expression levels of HAND2-AS1 were reduced in cancer tissues compared with those in healthy tissues. Levels of HAND2-AS1 were also reduced in the serum of patients with osteosarcoma compared with those of the control subjects. A significant association was observed between serum levels of HAND2-AS1 and tumor size, but not tumor metastasis. HAND2-AS1-knockdown promoted osteosarcoma cell proliferation, increased glucose uptake and upregulated GLUT1 expression. It was therefore concluded that lncRNA HAND2-AS1 may inhibit the proliferation of osteosarcoma cells by targeting glucose metabolism.

Introduction

As a malignancy that develops in the bone, osteosarcoma primarily affects children, adolescents and young adults (1). Although the incidence is low, osteosarcoma is one of the leading causes of cancer-associated mortality among teenagers (13-19 years old) and young adults (20-30 years old) worldwide (2). With the development and application of systemic chemotherapy, the survival rate of patients with osteosarcoma has improved significantly (3). However, this is challenged by the high prevalence of tumor metastasis by the time of diagnosis, and despite appropriate treatment, the 5-year survival rate of patients with distant tumor metastasis remains poor (≥20%) (4). Therefore, early diagnosis and treatment are prominent factors in the survival of patients with osteosarcoma. Unclear pathogenesis is a principal cause of treatment failure in osteosarcoma (3), and in-depth investigation of the mechanisms of development and progression of osteosarcoma may improve its diagnosis and treatment.

It has been demonstrated that the development and progression of osteosarcoma is frequently associated with the altered expression of specific long non-coding RNAs (lncRNAs) (5), indicating the involvment of lncRNA in disease pathogenesis. Glucose metabolism serves a pivotal role in cancer growth by providing energy for survival and cellular proliferation (6). lncRNA heart and neural crest derivatives expressed 2-antisense RNA 1 (HAND2-AS1) is a recently identified lncRNA that serves as a tumor suppressor gene in endometrioid endometrial carcinoma by inhibiting the invasion and migration of cancer cells (7). However, its involvement in other cancer types is unknown. It has been reported that certain lncRNAs may interfere with glucose metabolism in cancer cells, promoting or inhibiting growth, development and progression (8). In the present study, HAND2-AS1 was downregulated in osteosarcoma, where its influence on glucose metabolism may regulate tumor growth. This revealed a novel function for HAND2-AS1 and provides a potential therapeutic target for osteosarcoma.

Materials and methods

Subjects. A total of 48 patients with osteosarcoma were recruited. Patients were diagnosed by pathological examination and treated in Jingzhou Central Hospital (Jingzhou, Hubei, China) between January 2015 and January 2017. Inclusion criteria were as follows: i) Osteosarcoma confirmed by pathological examinations; ii) patients who were willing to participate; and iii) patients who received surgical resection. Exclusion criteria were as follows: i) Patients suffering from other types of malignancies; ii) patients with other types of severe disease, such as metabolic diseases and severe infections; and iii) patients who were treated in other hospitals prior...
to the study. The cohort included 28 males and 18 females (age range, 14-67 years; mean age, 32±8.5 years), and 44 healthy volunteers were recruited as controls. The control group included 26 males and 18 females (age range, 14-66 years; mean age, 34±7.9 years). No significant differences in age and sex were present between the two groups. All patients provided written informed consent.

**Specimen collection and processing.** Tumor tissues and adjacent healthy tissues (within a 2-cm area around the tumor) were collected during surgical resection, and confirmed by pathological examination. On the day of admission, blood was extracted from the elbow vein of each of the patients and healthy controls. The blood was stored at room temperature for 2 h, followed by centrifugation at 1,250 x g at room temperature for 20 min for serum. All specimens were stored in liquid nitrogen for long-term use.

**Cell lines and transfection.** The human osteosarcoma MG-63 and SAOS-2 cell lines, and the normal bone hFOB cell line were obtained from the American Type Culture Collection (ATCC). The MG-63 and SAOS-2 cells were cultured in Eagle’s minimum essential medium (cat. no. 30-2003; ATCC) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). hFOB cells were cultured in McCoy’s 5a modified medium (cat. no. 30-2007; ATCC) containing 10% FBS. All cells were cultured at 37˚C with 5% CO₂ and 0.2% SDS]. Radioactivity was measured at 37˚C. Cells were washed twice with ice-cold KRH buffer 1 µCi [3H]-2-deoxyglucose (PerkinElmer, Inc.) for 25 min followed by centrifugation at 1,250 x g at room temperature for further 4 h, and OD values were measured at 450 nm using the Fisherbrand™ accuSkan™ GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Inc.). The experiment was performed in triplicate.

**Glucose uptake assay.** A total of 5x10⁵ cells were cultured in each well of a 6-well plate. Cells were incubated for 24 h and washed twice with PBS. Glucose uptake was initiated by incubating cells with Krebs-Ringer-HEPES (KRH) buffer [120 mM NaCl, 25 mM HEPES (pH 7.4), 1.2 mM MgSO₄, 1.3 mM CaCl₂, 5 mM KCl and 1.3 mM KH₂PO₄] containing 1 µCi [3H]-2-deoxyglucose (PerkinElmer, Inc.) for 25 min at 37˚C. Cells were washed twice with ice-cold KRH buffer to halt glucose uptake, and lysed using lysis buffer [10 mM Tris- HCl (pH 8.0) and 0.2% SDS]. Radioactivity was measured using liquid scintillation spectrometry, and glucose uptake was presented as disintegrations per min. The experiment was performed in triplicate.

**RT-qPCR.** TRizol® reagent was used to extract the total RNA from tumor tissues, adjacent healthy tissues and the 3 cell lines, MG-63, SAOS-2 and hFOB. The RNA concentration was measured using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.), and samples with an A260/A280 ratio between 1.8 and 2.0 were reverse transcribed. The PrimeScript RT reagent kit (Takara Bio, Inc.) was used to perform reverse transcription reactions with poly(T) as the primer. Reaction conditions were: 25˚C for 6 min, 54˚C for 20 min and 80˚C for 5 min. The SYBR® Green Real-Time PCR Master mix (Thermo Fisher Scientific, Inc.) was used to prepare the PCR. The sequences of primers were as follows: HAND2-AS1 forward, 5'-GGGTGTTTACGT AGACCAGAAC-3' and reverse, 5'-CTTCCAAAGGCTCTC TGCCCTTAG-3'; and β-actin forward, 5'-GACCTCTATGCG AACACAGT-3' and reverse, 5'-AGTACTTGGCTCAGGAG GA-3'. PCR was conducted using the CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories Inc.). PCR conditions were as follows: 95˚C for 40 sec, followed by 40 cycles at 95˚C for 15 sec and 55˚C for 45 sec. HAND2-AS1 expression was normalized to β-actin using the 2-ΔΔCq method (9). The experiment was performed in triplicate.

**Cell proliferation assay.** A 96-well plate was seeded at 4x10³ cells/well and cultured at 37˚C (5% CO₂). At the 24-, 48-, 72- and 96-h time points, 10 µl Cell Counting Kit-8 (CCK-8) solution was added to each well; cells were cultured for a further 4 h, and OD values were measured at 450 nm using the Fisherbrand™ accuSkän™ GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Inc.). The experiment was performed in triplicate.

**Western blot analysis.** Radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) was used to extract the total protein from cells, and the bicinechonic acid assay method was used to measure protein concentration. Proteins were separated using SDS-PAGE with a 10% gel (30 µg protein/lane), and transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk in PBS at room temperature for 1 h, followed by incubation with the following primary antibodies overnight at 4˚C: Rabbit anti-glucose transporter 1 (GLUT1; 1:2,000; cat. no. ab15309) and anti-GAPDH (1:1,000; ab8245; all Abcam, Cambridge, UK). The following day, the membranes were
incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. MBS435036; MyBioSource, Inc.) for 1 h at room temperature. Protein bands were visualized using an Enhanced Chemiluminescence detection reagent (Sigma-Aldrich; Merck KGaA), and Image J software, v1.8.0 (National Institutes of Health) was used to normalize the expression levels of GLUT1 to the endogenous β-actin control. The experiment was performed in triplicate.

Statistical analysis. SPSS software version 19.0 (IBM Corp.) was used for all statistical analyses. Data are expressed as the mean ± standard deviation. Comparisons between two groups and among multiple groups were performed using the Student’s t-test and one-way analysis of variance, followed by the Tukey’s test, respectively. Count data were analyzed using the χ² test. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic value of HAND2-AS1 serum levels in osteosarcoma, with patients as true-positive samples and healthy controls as true-negative samples. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of HAND2-AS1 in the tumor tissues and adjacent healthy tissues of 48 patients with osteosarcoma. Expression levels of HAND2-AS1 in the osteosarcoma tissues and healthy tissues of 48 patients were measured using RT-qPCR. As illustrated in Fig. 1, expression levels of HAND2-AS1 were significantly lower in osteosarcoma tissues compared with those in the adjacent tissues in 41 of the 48 patients (P<0.05). Only 3 patients displayed significantly lower expression levels of HAND2-AS1 in adjacent tissues compared with those in osteosarcoma tissues (P<0.05). No significant differences were observed in the remaining 4 patients.

Comparison and diagnostic values of HAND2-AS1 serum levels between patients with osteosarcoma and healthy controls. Serum levels of HAND2-AS1 were determined using RT-qPCR. Serum levels of HAND2-AS1 were significantly higher in control subjects compared with those in osteosarcoma patients (P<0.05; Fig. 2A). ROC curve analysis was performed with osteosarcoma patients as true-positive and healthy controls as true-negative samples. The area under the curve (AUC) was 0.8685, with a 95% confidence interval of 0.7989-0.9382 (P<0.0001; Fig. 2B). Therefore, serum HAND2-AS1 level may hold potential diagnostic value in osteosarcoma.

Correlation between serum levels of HAND2-AS1 and clinicopathological features. Patients were divided into high and low expression groups according to the median serum level of HAND2-AS1. The χ² test was performed to analyze correlations between serum levels of HAND2-AS1 and clinicopathological patient data. As displayed in Table I, there were no significant correlations between the serum levels of HAND2-AS1 and patients’ age, sex and distant tumor metastasis. However, a significant correlation was observed between serum levels of HAND2-AS1 and tumor size.

Effects of HAND2-AS1 siRNA silencing on glucose uptake. The data in Table I indicate that HAND2-AS1 is associated with tumor growth in osteosarcoma. Glucose metabolism is critical for the survival and proliferation of cancer cells (10). In the present study, the effects of HAND2-AS1 siRNA silencing on glucose uptake were investigated in osteosarcoma cells. HAND2-AS1 siRNA silencing significantly promoted glucose uptake in the human osteosarcoma MG-63 and SAOS-2 cell lines (P<0.05; Fig. 3), but not in the normal bone hFOB cell line.

Effects of HAND2-AS1 siRNA silencing on GLUT1 expression. Compared with that in normal bone cells (hFOB), HAND2-AS1 expression was significantly downregulated (Fig. 4A), while GLUT1 protein was significantly upregulated (Fig. 4B), in the osteosarcoma MG-63 and SAOS-2 cell lines. HAND2-AS1 siRNA silencing led to significantly upregulated expression of GLUT1 in MG-63 and SAOS-2 cells (P<0.05), but not in hFOB cells (Fig. 4C).
Table 1. Correlation between serum levels of heart and neural crest derivatives expressed 2-antisense RNA 1 and the clinicopathological data of the patients.

| Variable                        | Group | Cases, n | High expression, n | Low expression, n | $\chi^2$ value | P-value |
|---------------------------------|-------|----------|--------------------|-------------------|---------------|---------|
| Sex                             | Male  | 28       | 12                 | 16                | 1.37          | 0.24    |
|                                 | Female| 20       | 12                 | 8                 |               |         |
| Age, years                      | >30   | 26       | 11                 | 15                | 1.34          | 0.25    |
|                                 | <30   | 22       | 13                 | 9                 |               |         |
| Primary tumor diameter, cm      | >5    | 23       | 17                 | 6                 | 10.1          | 0.001   |
|                                 | ≥5    | 25       | 7                  | 18                |               |         |
| Tumor distant metastasis        | Yes   | 19       | 11                 | 8                 | 0.78          | 0.38    |
|                                 | No    | 29       | 13                 | 16                |               |         |

Figure 3. Effects of HAND2-AS1 siRNA silencing on glucose uptake. The effects of HAND2-AS1 siRNA silencing on glucose uptake in osteosarcoma cells were investigated by glucose uptake assay. (A) HAND2-AS1 siRNA silencing significantly reduced the levels of HAND2-AS1 in MG-63, SAOS-2 and hFOB cell lines, and (B) promoted glucose uptake in MG-63 and SAOS-2, but not hFOB cells. *P<0.05. n=3. HAND2-AS1, heart and neural crest derivatives expressed 2-antisense RNA 1; siRNA, small interfering RNA; C, control; NC, negative control.
Figure 4. Effects of HAND2-AS1 siRNA silencing on GLUT1 expression. The effects of HAND2-AS1 siRNA silencing on GLUT1 expression were investigated by western blotting. Compared with normal bone hFOB cells, in MG-63 and SAOS-2 cells, (A) The non-coding RNA, HAND2-AS1 was significantly downregulated, while (B) GLUT1 expression was significantly upregulated. (C) HAND2-AS1 siRNA silencing led to significantly upregulated expression of GLUT1 in MG-63 and SAOS-2 cells, but not in hFOB cells. *P>0.05. n=3. HAND2-AS1, heart and neural crest derivatives expressed 2-antisense RNA 1; GLUT1, glucose transporter 1; siRNA, small interfering RNA; C, control; NC, negative control.

Figure 5. Effects of HAND2-AS1 siRNA silencing on cell proliferation. The effects of HAND2-AS1 siRNA silencing on cell proliferation were investigated using the CCK-8 assay. HAND2-AS1 siRNA silencing significantly promoted the proliferation of human osteosarcoma MG-63 and SAOS-2 cell lines, but not cells of the normal bone hFOB cell line. *P>0.05. n=3. HAND2-AS1, heart and neural crest derivatives expressed 2-antisense RNA 1; siRNA, small interfering RNA; C, control; NC, negative control.
Effects of HAND2-AS1 siRNA silencing on cell proliferation. The proliferation rates of the 3 cell lines were detected using the CCK-8 assay. HAND2-AS1 siRNA silencing significantly promoted the proliferation of the human osteosarcoma MG-63 and SAOS-2 cell lines (P<0.05; Fig. 5), but not the cells of the normal bone cell line, hFOB.

Discussion

In the present study, a novel IncRNA with characterized functionality in endometrioid endometrial carcinoma, and the growth of osteosarcoma tumors, was reported. It was also observed that the action of this IncRNA in osteosarcoma is likely achieved by inhibiting osteosarcoma cell proliferation through disruption of glucose metabolism. This highlighted a potential therapeutic target for osteosarcoma.

The pathogenesis of osteosarcoma is influenced by numerous IncRNAs and their varied roles in the onset, development and progression of cancer. Upregulation of IncRNA-highly upregulated in liver cancer (HULC) was previously observed in osteosarcoma tissues compared with that in adjacent healthy tissues, and reducing the expression level of IncRNA HULC was suggested to be a potential treatment for osteosarcoma (11). Overexpression of nuclear paraspeckle assembly transcript 1 is involved in the development of drug resistance in osteosarcoma cells. Therefore, knockdown of this oncogenic IncRNA may improve the outcome of drug treatment in osteosarcoma (12). By contrast, growth arrest specific 5 is considered to be a tumor suppressor IncRNA and displays a downregulated expression pattern in osteosarcoma (13). IncRNA HAND2-AS1 is a recently identified IncRNA with decreased expression levels in endometrioid endometrial carcinoma tissues (7), indicating its potential role as a tumor suppressor in this disease. In the present study, significantly lower expression levels of IncRNA HAND2-AS1 in tumor tissues (compared with adjacent healthy tissues) was observed in the majority of patients with osteosarcoma, indicating its potential role as a tumor suppressor.

The survival rate of osteosarcoma patients with distant tumor metastasis is low, and an increase in the early diagnostic rate is required to improve treatment outcomes. The blood not only transports nutrients around the body, but also delivers signaling molecules, and the development of human diseases is usually accompanied by alterations in certain blood constituents (14,15). Detecting these alterations in the blood may provide guidance for the treatment of human diseases (16,17). In the present study it was observed that serum levels of IncRNA HAND2-AS1 were significantly lower in osteosarcoma patients compared with those in healthy controls. ROC curve analysis also revealed that serum HAND2-AS1 may be used to effectively distinguish osteosarcoma patients from healthy individuals. These data suggest that serum HAND2-AS1 may serve as a potential diagnostic marker for osteosarcoma. It is worth noting that HAND2-AS1 is a recently identified IncRNA with an unknown expression pattern in the majority of human diseases. Therefore, multiple markers may be used to improve diagnostic accuracy.

The present study revealed an association between HAND2-AS1 and tumor growth, but not metastasis. Glucose metabolism provides energy for all cell types, although abnormal glucose metabolism is a unique marker of cancerous cells (6). HAND2-AS1 siRNA silencing significantly promoted glucose uptake in 2 osteosarcoma cell lines, indicating that HAND2-AS1 may be an inhibitor of glucose uptake in osteosarcoma. As a key component of glucose metabolism, GLUT1 is ordinarily upregulated in cancer cells (18). HAND2-AS1 siRNA silencing significantly promoted the expression of GLUT1, and also promoted proliferation in 2 osteosarcoma cell lines. The data suggest that HAND2-AS1 may inhibit the proliferation of osteosarcoma cells by targeting glucose uptake through the downregulation of GLUT1.

Additionally, HAND2-AS1 siRNA silencing did not have significant effects on cells of the normal bone hFOB cell line. Therefore, HAND2-AS1 may serve as a potential therapeutic target for osteosarcoma specifically.

In conclusion, HAND2-AS1 is downregulated in osteosarcoma and may inhibit the growth of osteosarcoma tumors through its interaction with glucose metabolism.

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Availability of data and materials

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

Author's contributions

SC, XX, SL and BH were responsible for the conception and design of the study. SC and XX performed the experiments. SC, XX, SL and BH analyzed and interpreted the data. SC and XX drafted the article. SC, XX, SL and BH were responsible for the revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocols of the present study were approved by the Ethics Review Committee of Jingzhou Central Hospital (Jingzhou, China). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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