Silencing long non-coding RNA SNHG6 restrains proliferation, migration and invasion of Wilms’ tumour cell lines by regulating miR-15a

Limin Su, Aiyiing Wu, Weitong Zhang and Xianchun Kong

Department of Pediatric Medicine, Juancheng People’s Hospital, Juancheng, China; Department of Pediatric Surgery, Heze Municipal Hospital, Heze, China

ABSTRACT

Wilms’ tumour (WT) is a frequent primary malignant tumour of urinary system in children. LncRNAs small nucleolar RNA host gene 6 (SNHG6) modulates kinds of biological procedures of cancer cells. Our research is to explore the effect and associated regulatory mechanism of SNHG6 in WT. CCK8 assays and bromodeoxyuridine were used to determine cell viability and cell proliferation, respectively. Flow cytometric analysis was performed to measure cell apoptosis rate. Cell mobility was tested through transwell and migration assays. Western blotting was employed to test the expression of proteins related to cell proliferation, cell apoptosis and signal pathways. In the results, overexpression of SNHG6 was found in WT tissues. The knockdown of SNHG6 suppressed cell proliferation, migration and invasion, but promoted apoptosis. Further study found that the knockdown of SNHG6 elevated the expression of miR-15a. Then, the combination of miR-15a inhibitor abolished the inhibiting effect of si-SNHG6 on WT progression. We also found that the TAK1/JNK and Wnt/β-catenin signal pathways were inactivated by the knockdown of SNHG6 through elevating the expression of miR-15a. In summary, SNHG6 is an oncogene of WT development by targeting miR-15a.

Introduction

Wilms’ tumour (WT, nephroblastoma) is a frequent primary malignant tumour of urinary system in children. It accounts for 6–7% of malignant tumours in children under 15 years old and about 75% of them are distributed in children under 5 years old. The age of onset of WT accompanied by congenital malformation is usually before 1 year old and the incidence rate is about 1/10,000 [1,2]. The recrudescence ratio of nephroblastoma is nearly 15%, and the long-term survival rate of patients with recurrence is only 50% [3]. In recent years, with the development of comprehensive treatment based on the combination of surgery, chemotherapy and radiotherapy, almost 85% of children with WT can be cured, but there are still a few children died due to relapse, metastasis and insensitivity to chemotherapeutic drugs and other factors. With the deepening of research, although people have a better understanding of the pathogenesis of WT, there are still many problems worth exploring.

Long non-coding RNAs (LncRNAs) are functional RNAs that contain at least 200 nucleotides and lack the ability to encode proteins. But, they can provide multiple protein binding sites, and can also be paired with DNA and RNA by base pairing principle for biological interaction [4]. IncRNAs can regulate cell cycle, DNA methylation and histone modification through epigenetics, transcriptional regulation and post-transcriptional regulation to achieve gene activation or silencing, thus participating in physiological and pathological processes of the body extensively [5]. For example, Zhu et al. observed that IncRNA LINCO0473 was overexpressed in WT and LINCO0473 knockdown restrained cell activity, caused Bcl-2-rely apoptosis and G1/S arrest through CDK2 and cyclin D1 by targeting miR-195 [6]. LncRNA small nucleolar RNA host gene 6 (SNHG6) locates on chromosome 8q13 and has been regarded as a potential oncogene in kinds of cancers, such as colorectal cancer (CRC), gastric cancer and so on [7,8]. However, the role of SNHG6 in the progression of WT has not been explored before.

MicroRNAs (miRNAs), non-coding little RNAs with 20–24 nucleotides in length, regulate protein coding through 3′-untranslated region (3′-UTR) of messenger RNA (mRNA). They widely exist in animals and plants and are involved in post-transcriptional gene expression and regulation, such as cell proliferation, apoptosis and cell cycle regulation [9]. At present, only a few of the biological functions of miRNAs have been elucidated and proved to be extensively related to kinds of physiological and pathological procedures. Besides that, it has been confirmed that some miRNAs expression in various cancerous tissues was notably different with that in adjacent regular tissues. These malignant tumour-related miRNAs in cancer is similar to tumour suppressor genes or oncogenes, suggesting that miRNAs may be
involved in the pathogenesis of malignant tumors [10,11]. For example, miR-15a was markedly negatively regulated in sphere culture hepatocarcinoma (HCC) cells and miR-15a mimic could suppress HCC progression through the repression of cMyb [12]. Besides that, inhibition of IncRNA LINC00473 in vivo could overcome the Taxol resistance of CRC cells through positively regulating tumour suppressor miR-15a [13]. However, whether there is a regulatory relationship between SNHG6 and miR-15a is still unknown. So, we explored the biological roles of SNHG6 and its regulation mechanism to provide molecular target for WT.

Materials and methods

Clinical specimens
Twenty couples of clinical human nephroblastoma tissues and adjacent regular tissues were got from sufferers who suffered surgical resection in Heze Municipal Hospital. Specimens were stored in liquid nitrogen after removal and kept at −80°C until RNA extraction. Our research was done following the Helsinki declaration and was approved by the Medical Ethics Committee of the Heze Municipal Hospital.

Cell
Human kidney (Wilms’ tumour) cell line G401 and SK-NEP-1 were got from the American Type Culture Collection (ATCC) and were kept in McCoy’s medium (Life Technologies Inc., Gaithersburg, MD) including 15% FBS (HyClone, South Logan, UT), 1 mg/mL penicillin/streptomycin and 2 mmol/L L-glutamine (ThermoFisher Scientific, Waltham, MA) in a wet 37°C incubator with 5% CO2.

Transfection
Si-SNHG6, si-NC, miR-15a inhibitor and the NC inhibitor were designed and compounded through GenePharma Co. (Shanghai, China). Cell transfections were conducted through Lipofectamine 3000 reagent (Invitrogen, Karlsruhe, Germany) according to producer’s direction.

qRT-PCR
Experimental RNA in tissues or tumour cells was got from transfected cells through TRIzol reagent (Invitrogen, Karlsruhe, Germany) following producer’s instructions and was handled with DNasel (Promega, Madison, WI). qRT-PCR was performed through PrimeScript RT Reagent Kit and SYBR Premix Ex Taq (Takara, Dalian, China) following producer’s directions. GAPDH and U6 were worked as control of SNHG6 and miR-15a, respectively. Fold change of SNHG6 and miR-15a was computed through the equation $2^{ΔΔCt}$.

CCK-8 experiment
Cell viability was tested through cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). First, G401 and SK-NEP-1 cells were seeded in 96-well plate with 5000 cells/well and were handled as indicated, CCK-8 solution was added to the medium, and the cultures were incubated for 2 h at 37°C in wet 95% air and 5% CO2. Four hundred and fifty nanometres was used to test absorbance through multifunctional microplate reader spectraMaxM5 (Molecular Devices, San Jose, CA) at indicated time points.

Proliferation experiment
1 mg/mL bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) was added to the cultured cells for 3 h prior to analysis to label proliferating cells. At least, there were 1000 cells under every condition.

Apoptosis assay
Cell apoptosis was valued through Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). Cells were seeded in six well plate (100,000 cells/well) and were treated as indicated. Treated cells were cleaned twice with cold PBS and re-suspended in buffer. Then, the cells double-labelled with Annexin V-FITC and PI apoptosis detection kits (Annexin V-FITC Apoptosis Detection Kit, eBioscience, San Diego, CA) following the producer’s directions and were tested through flow cytometer (Beckman Coulter, Brea, CA) to separate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

Western blot
Total protein was gathered through RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) and the proteins were quantified through BCA™ Protein Assay Kit (Pierce, Appleton, WI). Then, 20 μg of isolated protein was isolated by SDS-PAGE and was then transferred onto a PVDF membrane (Millipore, Billerica, MA). After blocked, the membrane was incubated with corresponding primary antibodies overnight at 4°C, followed with washing and incubating with secondary antibody signed with horseradish peroxidase at room temperature for 1 h. After rinsing, bands were detected and visualized through a chemiDoc xrs imaging system and analysis software (Bio-Rad, San Francisco, CA).

Cell migration and invasion experiments
Cell migration and invasion were detected through Transwell and Matrigel Transwell (Corning Costar, Corning, NY) membrane filter inserts into 24-well tissue culture plates, respectively. Cells suspended in serum-free DMEM were added to the top chamber of transwell filters and allowed to migrate through the filter. The lower chamber was full of DMEM containing 20% FBS and then incubated for 24 h. The cells were removed from the top of the filter, and the cells across the membrane were fixed with 4% formaldehyde, stained with Crystal Violet.
Statistics analysis

Results are revealed as mean ± SD of at least three independent assays in triplicate. Statistical analyses were done through SPSS 19.0 statistical software (SPSS, Chicago, IL). The p values were computed through a one-way analysis of variance (ANOVA) or t-test. p Value < .05 was statistically significant. Each experiment was duplicated for three times.

Results

LncRNA SNHG6 is highly expressed in nephroblastoma

The level of LncRNA SNHG6 in clinical human nephroblastoma tissues was first detected through qRT-PCR. SNHG6 was highly expressed in nephroblastoma compared with the corresponding normal tissues (Figure 1) in our results.

Si-SNHG6 restrained cell proliferation and caused apoptosis

To further explore the consequent of SNHG6 in nephroblastoma, specific si-SNHG6 was transfected into G401 and SK-NEP-1 cells. The expression of SNHG6 was effectively suppressed contrasted with NC (Figure 2(A)). Then, we found that si-SNHG6 effectively restrained cell proliferation and activity (Figure 2(B,C)). Besides that, the results of western blot in Figure 2(D–G) showed that cyclinD1 and p53 in both cells were restrained through si-SNHG6 and was then upgraded through the adding of miR-15a inhibitor in si-SNHG6 + miR-15a inhibitor group. Moreover, the promoting consequent of si-SNHG6 on cell apoptosis was also offset through the combination of miR-15a inhibitor which was detected through flow cytometry assay (Figure 5(H)). At the same time, Figure 5(I–L) reveals that the inhibiting effect of si-SNHG6 on the levels of cleaved-caspase-3 and cleaved-caspase-9 in both cells was neutralized through the adding of miR-15a inhibitor. Thus, we conclude that si-SNHG6 suppressed cell proliferation and caused apoptosis through positively regulating miR-15a.

Si-SNHG6 suppressed cell migration and incursion through up-regulating miR-15a

Then, the function of miR-15a in the inhibiting effect of si-SNHG6 was further investigated. Figure 6(A,B) reveals that the inhibiting effect of si-SNHG6 on cell migration and invasion was both counteracted by the adding of miR-15a inhibitor.

Si-SNHG6 suppressed TAK1/JNK and Wnt/β-catenin signal pathways through positively regulating miR-15a

The associated signal pathways were further investigated. Figure 7(A–D) reveals that the rate of p-TAK1/TAK1 and p-JNK/JNK in G401 and SK-NEP-1 cells was restrained through si-SNHG6 and was then upgraded through the adding of miR-55a inhibitor, identifying that the JNK signal pathway was suppressed by si-SNHG6 through up-regulating miR-15a. Besides that, the expression of wnt3α and β-catenin was
Figure 2. Si-SNHG6 suppressed cell proliferation and induced cell apoptosis. G401 and SK-NEP-1 cells were transfected with si-NC or si-SNHG6 or left untreated as the control, respectively. (A) SNHG6 expression was tested through qRT-PCR. (B) Cell proliferation was detected using bromodeoxyuridine as described. (C) Cell viability was valued through CCK8 assay. (D, E) Levels of cell proliferation related proteins in G401 cells were tested through western blot. (F, G) Levels of cell proliferation related proteins in SK-NEP-1 cells were tested via western blot. (H, I) Levels of apoptosis related proteins in G401 cells were tested via western blot. (J, K) Levels of apoptosis related proteins in SK-NEP-1 cells were tested via western blot. (L) Apoptosis was tested via flow cytometry. *p < .05, **p < .01, ***p < .001 contrasted with CTRL set.

Figure 3. Si-SNHG6 suppressed cell migration and invasion. G401 and SK-NEP-1 cells were transfected with si-NC or si-SNHG6 or left untreated as the control, respectively. (A) The effect of SNHG6 knockdown on cell migration was tested via wound-healing experiment. (B) Invasive number of SNHG6-downregulated both cells was detected via transwell invasion assays. **p < .01 contrasted with CTRL set.
down-regulated by si-SNHG6 and was then up-regulated by the adding of miR-55a inhibitor (Figure 7(E-H)), identifying that the Wnt/β-catenin signal pathway was suppressed by si-SNHG6 through up-regulating miR-15a. Thus, we concluded that si-SNHG6 suppressed TAK1/JNK and Wnt/β-catenin signal pathways through positively regulating miR-15a.

Discussion

The occurrence of tumours is a multifactorial process, and associated causes may be involved in numerous molecular pathways. The same tumour is not only regulated by a variety of miRNAs and lncRNAs, respectively, but also affected by the interaction network of miRNAs and lncRNAs, which is important in the occurrence, growth, invasion and metastasis of malignant tumours. Thus, it is necessary to study its mechanism of action in malignant tumours to provide potential therapeutic targets at the molecular level. Wilms’ tumour accounts for about 90% of all paediatric tumours and the whole alive ratio of WT has been ameliorated to over 90% in sufferers with good histology. However, new molecules and curing methods for more effective curing strength are still needed to identify high-risk WT. LncRNA SNHG6 has been

Figure 4. Si-SNHG6 up-regulates the level of miR-15a. G401 and SK-NEP-1 cells were transfected with si-NC or si-SNHG6 or left untreated as the control, respectively. The level of miR-15a was tested through qRT-PCR. **p < .01 contrasted with CTRL set.

Figure 5. Si-SNHG6 suppressed cell proliferation and caused apoptosis via positively regulating miR-15a. G401 and SK-NEP-1 cells were transfected with si-NC + NC inhibitor or si-SNHG6 + NC inhibitor or si-SNHG6 + miR-15a inhibitor or left untreated as the control, respectively. (A) miR-15a expression was tested through qRT-PCR. (B) Cell proliferation was detected using bromodeoxyuridine as described. (C) Cell viability was valued through CCK8 assay. (D, E) Levels of cell proliferation related proteins in G401 cells were tested via western blot. (F, G) Levels of cell proliferation related proteins in SK-NEP-1 cells were tested via western blot. (H) Apoptosis was tested via flow cytometry. (I, J) Levels of apoptosis related proteins in G401 cells were tested via western blot. (K, L) Levels of apoptosis related proteins in SK-NEP-1 cells were tested via western blot. **p < .01, ***p < .001 contrasted with CTRL set. #p < .05 contrasted with si-SNHG6 + NC inhibitor group.
proved to play promotion roles in cell proliferation, invasion and migration [14,15]. So, SNHG6 may be a potential molecular target for WT. Our study first proved this guess that si-SNHG6 inhibited cell proliferation, migration, invasion and TAK1/JNK and Wnt/β-catenin signal pathways through up-regulating miR-15a, suggesting the promotion biological functions of SNHG6 in the development of WT.

Up to now, numerous studies have been conducted to prove that lncRNA exerts tumour-suppressive or -promoting (oncogenic) functions to influence cell growth, pressure response, apoptosis and chemical resistance in cancer [16]. LncRNA SNHG6, a recently identified cancer-related lncRNA [17], has been reported that it was up-regulated in CRC and high expression level of SNHG6 elevated proliferation...
through an immediate inhibition of p21 in CRC [18]. In addition, SNHG6 was overexpressed in breast cancer tissues and SNHG6 knockdown restrained cell proliferation, migration and incursion [17]. However, the effect of lncRNAs including SNHG6 in WT has not been widely explored. Here, we first observed that SNHG6 was strongly positively regulated in WT and the knockdown of SNHG6 effectively suppressed cell proliferation, caused obvious apoptosis, restrained cell migration and incursion in G401and SK-NEP-1 cell lines. Our data were identical with the past research that we cleared the function of SNHG6 in promoting tumour development. And these results indicated that SNHG6 may be considered as a marker predicting the occurrence of WT.

Studies have identified that there exist close interrelationships between miRNAs and IncRNAs as they could play an important biological function by regulating each other [19–21]. Moreover, lncRNAs are reported to regulate miRNAs by acting as endogenous “miRNA sponge” and inhibiting the expression of miRNA or IncRNA competes with miRNAs for 3’-UTR of the same target gene to inhibit the regulation of miRNAs on target genes [17,22]. Studying the specificity, diversity and multiplicity of biological effects between miRNAs and IncRNAs is becoming the hotspot in current molecular biology research. Lv et al. found that SNHG6 promoted breast cancer cells proliferation, migration and invasion via down-regulating miR-26a-5p [17]. Zhu et al. found that SNHG6 in CRC cells promoted tumour growth and liver metastasis through directly sequestered miR-760 [23]. These findings indicated that there was a certain relationship between SNHG6 and miRNAs. Besides, miR-15a functions as tumour suppressor in most of tumours, such as pituitary tumour [24] and colorectal carcinomas [25]. Feng et al. has reported that miR-15a was down-regulated by IncRNA HULC in promoting pancreatic cancer development [26]. But whether there is a certain regulatory relationship between SNHG6 and miR-15a is still remain unclear. This research first observed that SNHG6 knockdown elevated miR-15a expression. The combination of miR-15a inhibitor abolished the inhibiting effect on cell proliferation and cell mobility in G401and SK-NEP-1 cell lines, suggesting that SNHG6 acts as an oncogene in WT by suppressing the expression of tumour suppressor miR-15a.

In our further study, associated signal pathways were investigated. TAK1 is proved to participate in pathophysiological processes by regulating downstream signalling pathways such as NF-kappa B and MAPK (extracellular signal-regulated kinases ERK, JNK and p38) [27]. TAK1-mediated signal transduction pathway is significant in cell cycle regulation and cell differentiation, and can participate in pathophysiological processes such as inflammation, immune response and apoptosis [28]. Previous studies showed that crosstalk between JNK and other signal pathways elevates cancer cell existence [29]. For example, the TAK1-JNK pathway causes the phosphorylation and latter stable of the anti-apoptotic factor Mcl1 in activated T cells [30]. β-Catenin is evolutionarily conserved and acts as an effector of Wnt family to regulate developmental and biological processes [31]. It has been reported that Wnt ligands were activated after irradiation and elevated survival in head and neck cancers [32]. Besides, Han et al. reported that IncRNA CRNDE modulated the development of CRC through regulating miR-181a-5p and Wnt/β-catenin signalling [33]. All the above findings indicated that TAK1/JNK and Wnt/β-catenin signal pathways played a positive effect on tumour development. Here, we innovatively found the regulatory mechanism among SNHG6, miR-15a and these pathways that the knockdown of SNHG6 inactivated the TAK1/JNK and Wnt/β-catenin signal pathways through positively regulating miR-15a in G401and SK-NEP-1 cell lines. Our findings provided a theoretical basis for SNHG6 to predict WT.

Taken together, we found that the SNHG6 level was elevated in WT cells. Our findings supported that SNHG6 can be regarded as an oncogene to promote WT development. We found that SNHG6 knockdown notably restrained cell proliferation, migration, incursion and TAK1/JNK and Wnt/β-catenin signal pathways, while increased cell apoptosis. Additionally, the negative relationship between SNHG6 and miR-15a was first found in our study, suggesting that this was the first report of a possible mechanism of SNHG6 in promoting WT development through down-regulating miR-15a and promoting TAK1/JNK and Wnt/β-catenin pathways. Our research provided a novel potential target for WT therapy.

Acknowledgements

Ethical standards: All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Disclosure statement

The authors declare that there is no conflict of interest.

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