Long noncoding RNA XIST suppresses tumorigenesis and enhances radiosensitivity in neuroblastoma cells through regulating miR-653-5p/HK2 axis

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Abstract

Background: Abnormal expression of long noncoding RNAs (lncRNAs) was usually involved in tumorigenesis and radiosensitivity of various cancers. The aim of this study was to explore the biological function and regulatory mechanism of lncRNA X-inactive specific transcript (XIST) in tumorigenesis and radiosensitivity of neuroblastoma (NB).

Methods: The expression levels of XIST, microRNA-653-5p (miR-653-5p) and hexokinase 2 (HK2) were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Methylthiazolyldiphenyl tetrazolium bromide (MTT) assay, colony formation assay and transwell assay were utilized to detect cell viability, colony formation and cell invasion abilities. Glucose consumption or lactate production was measured by glucose assay kit or lactate assay kit, respectively. The mice xenograft model was established to investigate the role of XIST in vivo. The interaction between miR-653-5p and XIST or HK2 was predicted by starBase v2.0 and verified by dual-luciferase reporter assay. Western blot was used to measure the protein expression of HK2.

Results: XIST and HK2 were highly expressed whilst miR-653-5p was lowly expressed in NB tissues and cells. XIST knockdown inhibited tumorigenesis by repressing NB cell proliferation and invasion. Meanwhile, XIST downregulation increased the radiosensitivity via inhibiting colony formation rates and glycolysis. Moreover, miR-653-5p could bind to XIST and its downregulation reversed the effects of XIST knockdown on tumorigenesis and radiosensitivity. Additionally, HK2 was a direct target of miR-653-5p and its overexpression attenuated the effects of miR-653-5p restoration on suppression of tumorigenesis and promotion of radiosensitivity. Besides, XIST functioned as a molecular sponge of miR-653-5p to regulate HK2 expression. Furthermore, XIST knockdown also suppressed tumor growth by upregulating miR-653-5p and downregulating HK2 in vivo.

Conclusion: XIST interference inhibited tumorigenesis and increased radiosensitivity in NB by regulating miR-653-5p/HK2 axis, providing a novel therapeutic strategy for NB.

Background

Neuroblastoma (NB) is one of the most frequent extracranial childhood tumors and originates from primitive neural crest cells of the sympathetic nervous system, accounting for 7% of malignant tumors in children and approximately 15% of all childhood cancer deaths [1, 2]. NB is notable for its phenotypic diversity, from undifferentiated tumors to tumors containing a neural crest-derived differentiated cell range [3]. Although advances in treatment have improved the survival times of NB patients, children with regional or distant metastatic disease still have a poor prognosis [4]. Radiotherapy is the main treatment for tumors, but the efficacy of radiotherapy is limited due to obtaining radioresistance in the treatment of NB [5]. Thus, it is necessary to explain the molecular mechanisms of NB and identify more effective therapeutic targets to increase the radiosensitivity.

Long noncoding RNAs (lncRNAs) are highly conserved transcripts (>200 nucleotides) with limited or without protein-coding ability and play regulatory roles in various physiopathology processes [6]. Currently, numerous studies have demonstrated that lncRNAs are abnormally expressed in a variety of cancers, including NB [7]. For instance, lncRNA NBAT-1 downregulation promoted aggressive NB through enhancing proliferation and attenuating differentiation of neuronal precursors [8]. Moreover, lncRNA RMRP was overexpressed in NB tissues and its knockdown suppressed the progression of NB [9]. X inactive-specific transcript (XIST) belongs to a class of RNA molecules known as non-coding transcripts. XIST gene is located in the X inactivation center, and its product is transcribed from the inactive X chromosome [10]. Besides, XIST plays critical roles in the differentiation, proliferation, and genome maintenance of human cells. Specifically, it has been suggested that dysregulation of XIST is tightly associated with the progression of multiple cancers, such as lung cancer [11], hepatocellular carcinoma [12] and gastric cancer [13]. Besides, previous study uncovered that XIST was highly expressed in NB tissues and its expression was related to NB development [14]. However, the underlying mechanisms by which XIST is involved in regulating NB progression and radiosensitivity remain largely unknown.

Increasing evidence has shown that lncRNAs serve as competing endogenous RNAs (ceRNAs) to modulate gene expression through sponging microRNA (miRNA) [15]. MiRNAs are usually small RNAs (18-25 nucleotides) that lack protein-coding potential and negatively gene expression via combining with complementary sequences on target mRNA [16, 17]. Currently, accumulating evidence demonstrated that aberrant expression miRNA was closely related to the development and progression of cancers, and could affect the cell's response to radiation [18, 19]. MiR-653-5p targets 615 transcripts with conserved sites, containing a total of 662 conserved sites and 487 poorly conserved sites, and miR-653-5p can exert regulatory functions in mammalian evolution [20]. MiR-653-5p has been suggested to function as a tumor suppressor or tumor promoter in different cancers [21, 22]. Moreover, miR-653-5p was also reported to be lowly expressed in NB [23]. Nevertheless, the biological functions of miR-653-5p in the tumorigenesis and radiosensitivity of NB are poorly understood.

Several miRNAs target hexokinase 2 (HK2; a metabolism-related factor) to influence the progression of multiple types of cancer [24, 25]. HK2, a member of the human glandular kallikrein family, is a serine protease expressed by the prostate gland with 80% identity in primary structure to prostate-specific antigen (PSA) [26]. HK2 has been shown to act as a critical regulator in cell growth, invasion and glycolysis [27]. Moreover, HK2 was reported to be upregulated in NB and can act as a target of miR-143-3p in NB [28]. However, there is no report on the interaction between miR-653-5p and HK2, and the exact roles of HK2 in NB progression and radiosensitivity should be explored. Interestingly, starBase v2.0 software online predicted that XIST and HK2 shared the complementary binding sites of miR-653-5p. Hence, we hypothesized XIST might regulate the tumorigenesis and radiosensitivity of NB by acting as a sponge of miR-653-5p to modulate HK2 expression.

In the present study, the expression levels of XIST, miR-653-5p and HK2 were analyzed in NB tissues and cells. In addition, we explored their effects on proliferation, invasion, glycolysis, and radiosensitivity, and investigated the regulatory network of XIST/miR-653-5p/HK2 in NB cells. The aim of this study was to

identify promising therapeutic targets for treatment of NB and explore a novel mechanism for better understanding the pathogenesis of NB.

Materials And Methods

Clinical tissue samples

In this study, thirty NB tissues and paired normal tissues were obtained from patients undergoing surgery at People's Hospital of Rizhao. The patients did not receive any treatment before surgery. Each patient has signed written informed consent. Excised tissues were collected and promptly frozen in liquid nitrogen, and then cryopreserved at -80°C for subsequent study.

Cell culture and transfection

Human embryonic kidney (HEK293) and NB cell line (SK-N-BE(2)) were purchased from BeNa Culture Collection (Beijing, China). The NB cell line (GI-LI-N) was obtained from Conservation Genetics CAS Kunming Cell Bank (Yunnan, China). These cells were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin in an incubator with 5% CO₂ at 37°C.

For this study, small interfering RNA against XIST (si-XIST) and the corresponding control (si-NC), miR-653-5p mimic (miR-653-5p) and the corresponding control (miR-NC), miR-653-5p inhibitor (anti-miR-653-5p) and the corresponding control (anti-miR-NC), XIST or HK2 overexpression plasmid (XIST or HK2) and the corresponding control (pcDNA) were purchased from GenePharma (Shanghai, China). Lentivirus-mediated shRNA interference targeting XIST (sh-XIST) and the corresponding control (sh-NC) were constructed by Genechem (Shanghai, China). GI-LI-N and SK-N-BE(2) cells with 50–60% confluence were transfected with oligonucleotide (50 nM) or plasmid (2 µg) using Lipofectamine 3000 (Invitrogen).

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen) was applied to isolate total RNA from tissues (NB tissues and normal tissues) and cells (HEK293, GI-LI-N and SK-N-BE(2)). Next, the first strand of complementary DNA (cDNA) was synthesized with a High-Capacity cDNA Reverse Transcription Kit and TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR was carried out using the SYBR Green PCR kit (Thermo Fisher Scientific) on the ABI 7300 system (Thermo Fisher Scientific). Primers used for qRT-PCR were exhibited as below: XIST (Forward, 5'-CCTCTCCACATACCTCAGT-3'; Reverse, 5'-ACATAATCACACGCATACCA-3'); miR-653-5p (Forward, 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCAGTAGAG-3'; Reverse, 5'-ACACTCCAGCTGGGGTGTGAAACAATCT-3'); HK2 (Forward, 5'-ACAGCCTGGACGAGAGCATC-3'; Reverse, 5'-AGGTCAAACCTCTCGCCG-3'); 18S RNA (Forward, 5'-CGAACGTCTGCCCTATCAACTT-3'; Reverse, 5'-ACCGTGGTCACCATGGTA-3'), U6 snRNA (Forward, 5'-CTCGCTTCGGCAGCACATATACT-3'; Reverse, 5'-

ACGCTTCACGAATTTGCGTGTC-3'). The expression levels of XIST, HK2 and miR-653-5p were calculated with the $2^{-\Delta\Delta C_t}$ method, followed by normalizing to GAPDH or U6 snRNA, respectively.

Cell viability assay

Methylthiazolyldiphenyl tetrazolium bromide (MTT) was utilized for detecting cell viability. In brief, GI-LI-N and SK-N-BE(2) cells (100 μ L) were seeded in 96-well plates at a density of 1×10^4 /mL and then transfected with si-XIST, si-XIST + anti-miR-653-5p, miR-653-5p, miR-653-5p + HK2, or matched controls. MTT reagent (5mg/mL, 10 μ L, Beyotime, Shanghai, China) was added to each well after transfection for 24, 48 h, or 72 h. After incubation for 4 h, the cultured medium was carefully discarded and dimethyl sulfoxide (DMSO; 150 μ L) was added to per well. Lastly, the absorbance was evaluated at 490 nm under a microplate reader (Bio-Rad, Hercules, CA, USA).

Irradiation (IR) and colony formation assay

After transfection for 48 h, GI-LI-N and SK-N-BE(2) cells (150 cells per well) were seeded into six-well plates and the medium was updated every three days. For treatment of IR, cells were exposed to different doses of radiation by a linear accelerator (Varian, Palo Alto, CA, USA) at a dose rate of 3.5 Gy/min. After incubation for 2 weeks, GI-LI-N and SK-N-BE(2) cells were carefully washed with cold phosphate-buffered saline (PBS; pH=7.2) and subsequently fixed with 4% paraformaldehyde for 30 min at 4°C. After that, GI-LI-N and SK-N-BE(2) cells were washed by PBS and then stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Colonies containing more than 50 cells were counted by a microscope (Olympus, Tokyo, Japan). The survival fraction was calculated as previously described [29].

Transwell assay

Transwell assay was performed using a 24-well plate inserts with 8 μ m pores (Corning Incorporated, Corning, NY, USA) to evaluate cell invasion capacity. GI-LI-N and SK-N-BE(2) cells (2×10^4 cells/well) re-suspended in DMEM medium alone (100 μ L) were seeded into the top chamber pre-coated with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). To induce cells invading through the membrane, the bottom chamber was filled with DMEM containing 10% FBS (600 μ L). After incubation for 24 h, non-invaded cells were carefully removed with a cotton bud, and invaded cells were fixed with 95% ethanol and then stained with 0.1% crystal violet. Finally, invaded cells from five random fields were photographed and counted by a microscope.

Measurement of glucose consumption and lactate production

GI-LI-N and SK-N-BE(2) cells (5×10^4 cells/well) were seeded into six-well plates and transfected with oligonucleotide or/and plasmid. Cell culture media were collected 48 h after the transfection. In accordance with the manufacturer's instructions, the glucose consumption and lactate production were detected with the glucose assay kit (Sigma-Aldrich) and lactate assay kit (BioVision, Mountain View, CA,

USA), respectively. A standard calibration curve was used for determining the data, followed by normalizing to the amount of total protein.

Dual-luciferase reporter assay

The possible binding sites of miR-653-5p and XIST or HK2 were predicted by starBase v2.0. The XIST and HK2 3'UTR fragments containing wild-type (WT) or mutant (MUT) binding sites of miR-653-5p were amplified and inserted into pmirGLO luciferase reporter vector (Promega, Madison, WI, USA) to create the WT plasmids (WT-XIST, HK2 3'UTR-WT) or MUT plasmids (MUT-XIST, HK2 3'UTR-MUT). Afterward, GI-LI-N and SK-N-BE(2) cells were seeded in 24-well plates (2×10^4 cells/well) and were co-transfected with reporter plasmids and miR-653-5p or miR-NC. After incubating the cells for 48 h, the luciferase activity was estimated via Dual-Luciferase Reporter assay system (Promega).

Western blot assay

GI-LI-N and SK-N-BE(2) cells (1×10^5 cells/well) were seeded into six-well plates and transfected with oligonucleotide or/and plasmid. After transfection for 48h, cells were lysed using RIPA lysis buffer (Sigma-Aldrich) supplemented with 1mM phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich) to extract the total protein, followed by being centrifuged at $12\,000 \times g$ at 4°C for 15 min. After quantification by using bicinchoninic acid protein assay kit (Tanon, Shanghai, China), 30 μg of protein in each group was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene fluoride membranes (Beyotime). After blocking with 5% non-fat milk in PBS containing 0.05% Tween 20 (PBST), the membranes were immunoblotted by primary antibody against HK2 (1:5000, ab227198, Abcam, Cambridge, UK) or GAPDH (1:2500, ab9485, Abcam) for 12-16 h at 4°C . After washing with PBST, membranes were incubated in horseradish peroxidase-conjugated anti-rabbit IgG (1:4000, D110058, Sangon Biotech, Shanghai, China) for 2 h at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL; Tanon) reagent following the manufacturer's protocol. ImageJ software was utilized to quantify the protein expression. GAPDH was used as an internal control.

***In vivo* tumor growth assay**

BALB/c nude mice (male, 5-week-old) were purchased from Huafukang (Beijing, China). The sh-XIST or sh-NC was transfected into SK-N-BE(2) cells. Subsequently, stably transfected cells (3×10^6) were subcutaneously injected in BALB/c nude mice. From the 8th day, tumor volume was examined using a caliper every 4 days and calculated with the formula: $\text{length} \times \text{width}^2/2$. After injection for 10 days, sh-XIST group or sh-NC group were randomly divided into two groups. One group was irradiated with 6Gy X-ray once a week, and the other group served as control. All mice were sacrificed after injection for 4 weeks, and tumor samples were weighted and collected for detection of XIST, miR-653-5p and HK2 expression levels.

Statistical analysis

All data in this study were displayed as mean $\pm$ standard deviation (SD) and all experiments were repeated at least 3 times. Correlation between miR-653-5p and XIST or HK2 was detected by Spearman rank correlation. The results from different groups were assessed using the two-tailed Student's *t*-test (for two groups) and a one-way analysis of variance (ANOVA; for more than two groups). Statistical analyses were performed using Graphpad Prism version 6.0 software (GraphPad Software, San Diego California, USA). $P < 0.05$ indicates a statistically significant result. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Results

XIST was upregulated and miR-653-5p was downregulated in NB tissues and cells

Firstly, we explored the expression of XIST and miR-653-5p in NB tissues and cells by qRT-PCR. As presented in Figure 1A and 1B, XIST was overexpressed in NB tissues and cells in contrast to their matched controls. Moreover, we found that the abundance of miR-653-5p was decreased in NB tissues and cells with respect to their corresponding controls (Figure 1C and 1D). Besides, the correlation between XIST and miR-653-5p was analyzed in NB tissues. As depicted in Figure 1E, XIST expression was negatively correlated with miR-653-5p expression in NB tissues ($r = -0.8114$, $p < 0.001$). These data indicated that XIST and miR-653-5p might be involved in the pathogenesis of NB.

XIST knockdown inhibited cell proliferation and invasion, and enhanced radiosensitivity by inhibiting glycolysis in NB cells

Next, the effects of XIST on cell proliferation, invasion, glycolysis, and radiosensitivity were investigated using loss-function experiments. The transfection efficiency of si-XIST was determined by qRT-PCR. The results showed that the expression of XIST was decreased in GI-LI-N, SK-N-BE(2) and HEK293 cells transfected with si-XIST relative to those cells transfected with si-NC or control cells (Figure 2A, Supplementary Figure 1A), suggesting that si-XIST was successfully transfected into GI-LI-N, SK-N-BE(2) and HEK293 cells. MTT analysis revealed that XIST interference inhibited the viability of GI-LI-N and SK-N-BE(2) cells (Figure 2B). However, downregulation of XIST did not significantly inhibit the viability of HEK293 cells (Supplementary Figure 1B), indicating that XIST knockdown had little effect on HEK293 cells. Moreover, colony formation assay indicated that XIST deficiency decreased the number of colonies in GI-LI-N and SK-N-BE(2) cells (Figure 2C). Furthermore, GI-LI-N and SK-N-BE(2) cell invasion were inhibited by silencing XIST (Figure 2D). GI-LI-N and SK-N-BE(2) cells were transfected with si-XIST or si-NC and then irradiated with 0 Gy to 8 Gy to explore the effect of XIST on radiosensitivity. The data showed that XIST downregulation strikingly decreased the survival fraction of GI-LI-N and SK-N-BE(2) cells exposed to radiation compared to si-NC or control group (Figure 2E). Most cancer cells mainly rely on aerobic glycolysis to produce the energy needed for cellular processes [30]. This aerobic glycolysis will result in increased glucose consumption and lactate production. We observed that knockdown of XIST inhibited glucose consumption and lactate production in GI-LI-N and SK-N-BE(2) cells (Figure 2F and 2G). Moreover, we found that glucose consumption and lactate production were decreased in GI-LI-N and SK-N-BE(2) cells treated with 2-deoxyglucose (2-DG; glycolytic inhibitor) (Figure 2H and 2I). QRT-PCR analysis

showed that the expression of XIST was enhanced in cells transfected with XIST compared to pcDNA group (Figure 2J). Besides, the promoting effect of XIST on cell survival fraction was overturned by treatment with 2-DG (Figure 2K), suggesting that XIST knockdown enhanced the radiosensitivity through inhibition of glycolysis. Taken together, these results demonstrated that XIST interference inhibited the tumorigenesis and increased the radiosensitivity in NB cells.

XIST directly interacted with miR-653-5p in NB cells

In view of the alteration of XIST and miR-653-5p in NB tissues and cell lines, as well as the negative correlation between XIST and miR-653-5p expression, we wondered whether the function of XIST was mediated by miR-653-5p through complementary binding sites. StarBase v2.0 predicted that XIST contained putative binding sites of miR-653-5p (Figure 3A). To verify whether miR-653-5p was a direct target of XIST, the dual-luciferase reporter assay was performed. As presented in Figure 3B, overexpression of miR-653-5p led to a significant decrease in the luciferase activity of WT-XIST, while luciferase activity of MUT-XIST was not evidently affected after transfection with miR-653-5p in GI-LI-N and SK-N-BE(2) cells. Moreover, the expression of XIST was reduced in GI-LI-N and SK-N-BE(2) cells transfected with si-XIST, while its expression was elevated in cells transfected with XIST (Figure 3C). Next, we explored the effect of XIST on expression of miR-653-5p. As expected, knockdown of XIST increased the expression of miR-653-5p, whereas overexpression of XIST presented an opposite effect (Figure 3D), suggesting that miR-653-5p was negatively regulated by XIST. At the same time, we observed that co-transfection of anti-miR-653-5p and si-XIST abated the effect of si-XIST on promotion of miR-653-5p expression (Figure 3E). Additionally, deficiency of miR-653-5p reversed the inhibitory effects of XIST knockdown on viability, colony formation and invasion of GI-LI-N and SK-N-BE(2) cells (Figure 3F-3H). Moreover, the suppressive effect of XIST interference on the survival fraction was abolished by downregulation of miR-653-5p in GI-LI-N and SK-N-BE(2) cells exposed to IR (Figure 3I). Furthermore, miR-653-5p silence abated the inhibitory effect of XIST knockdown on glucose consumption and lactate production (Figure 3J and 3K). Our findings suggested that miR-653-5p could bind to XIST and its knockdown reversed the effects of XIST interference on tumorigenesis and radiosensitivity in NB cells.

HK2 was a direct target of miR-653-5p in NB cells

To explore the underlying mechanism of miR-653-5p in progression of NB, the function targets of miR-653-5p were searched by starBase v2.0. As presented in Figure 4A, miR-653-5p and HK2 3'UTR had complementary binding sequence, indicating that HK2 might be a target of miR-653-5p. Dual-luciferase reporter assay displayed that the luciferase activity of HK2 3'UTR-WT was obviously inhibited by transfection of miR-653-5p, whereas no clear change of the luciferase activity of HK2 3'UTR-MUT was found (Figure 4B). Next, we detected the expression of HK2 in NB tissues and cells. The results suggested that the mRNA expression and protein expression of HK2 were increased in NB tissues in comparison with normal tissues (Figure 4C and 4D). Additionally, HK2 expression was negatively correlated with miR-653-5p level in NB tissues ($r=-0.5985$, $p<0.001$) (Figure 4E). Likewise, the mRNA expression and protein

expression of HK2 were also enhanced in NB cells (GI-LI-N and SK-N-BE(2)) compared to HEK293 cells (Figure 4F). These data illustrated that miR-653-5p directly interacted with HK2.

Overexpression of HK2 reversed the effects of miR-653-5p on inhibition of tumorigenesis and promotion of radiosensitivity in NB cells

To determine whether the effects of miR-653-5p were regulated by HK2 expression, GI-LI-N and SK-N-BE(2) cells were transfected with miR-NC, miR-653-5p, miR-653-5p + pcDNA, or miR-653-5p + HK2. Western blot showed that HK2 protein expression was decreased in cells transfected with miR-653-5p, while the effect was abated by co-transfection of HK2 (Figure 5A). Additionally, overexpression of miR-653-5p reduced the cell viability, colony formation and invasion of GI-LI-N and SK-N-BE(2) cells, which was reversed by upregulating HK2 (Figure 5B-5D). Moreover, the inhibitory effects of miR-653-5p upregulation on survival fraction and glycolysis were also overturned by overexpression of HK2 (Figure 5E-5H). Collectively, these findings demonstrated that miR-653-5p exerted its biological functions through regulating HK2 expression.

HK2 was regulated by XIST and miR-653-5p in NB cells

Next, knockdown efficiency of miR-653-5p was tested by qRT-PCR. As shown in Figure 6A, the expression of miR-653-5p was significantly reduced in GI-LI-N and SK-N-BE(2) cells transfected with anti-miR-653-5p compared to those cells transfected with anti-miR-NC or control group. To explore whether XIST served as a ceRNA of miR-653-5p to modulate HK2 expression, GI-LI-N and SK-N-BE(2) cells were transfected with si-NC, si-XIST, si-XIST + anti-miR-NC, or si-XIST + anti-miR-653-5p. Western blot assay demonstrated that the protein level of HK2 was decreased after transfection with si-XIST, while the effect was reversed by downregulating miR-653-5p (Figure 6B). In a word, our results proved that XIST regulated HK2 expression by sponging miR-653-5p in NB cells.

Downregulation of XIST inhibited tumor growth and increased radiosensitivity by regulating miR-653-5p and HK2 *in vivo*

To explore the impact of XIST on tumor growth and radiation response *in vivo*, we established a xenograft model in which the SK-N-BE(2) cells stably transfected with sh-XIST or sh-NC were subcutaneously injected into BALB/c nude mice and irradiated with 6Gy X-ray once a week or didn't irradiate. In line with *in vitro* results, XIST knockdown or IR treatment suppressed tumor volume and weight, and combination of XIST interference and IR treatment significantly inhibited tumor volume and weight in xenograft model compared with only sh-XIST group or IR group (Figure 7A and 7B). In addition, knockdown of XIST decreased the expression of XIST and HK2 while increased the expression of miR-653-5p in excised tumor masses (Figure 7C and 7D). These above findings indicated that downregulation of XIST could enhance the radiosensitivity to inhibit tumor growth *in vivo*.

Discussion

NB is a common and aggressive malignancy in children. Recently, it has been increasingly recognized that dysregulation of lncRNAs is involved in the progression of NB [31]. Radiotherapy has become an effective strategy for treatment of NB; however, the influence of lncRNAs on radiosensitivity of NB cells is not well elucidated. The purpose of this study was to explore the effects of XIST on NB progression and radiosensitivity.

Up to now, a growing number of studies have shown that XIST serves as an oncogene and plays essential roles in cellular behaviors, such as cell growth, cell cycle, metastasis and apoptosis [32, 33]. Besides, Song *et al.* pointed out that XIST was overexpressed in nasopharyngeal carcinoma tissues, and accelerated nasopharyngeal carcinoma cell growth through targeting miR-34a-5p [34]. Moreover, Chen *et al.* uncovered that XIST knockdown inhibited colorectal cancer cell proliferation and metastasis via sponging miR-200b-3p to decrease ZEB1 expression [35]. In our research, it was found that XIST abundance was elevated in NB tissues and cells. Additionally, interference of XIST suppressed cell proliferation and invasion, and elevated radiosensitivity via suppressing glycolysis in NB cells. In agreement with our findings, Zhang *et al.* disclosed that XIST expression was markedly enhanced in NB tissues and its knockdown inhibited cell proliferation and metastasis in NB cells by regulating H3 histone methylation of DKK1 [14]. These results revealed that XIST was involved in the tumorigenesis and radiosensitivity of NB.

Previous studies showed that miR-653-5p played pivotal roles in the regulating cellular behaviors, such as proliferation, cell cycle and apoptosis [36]. It has recently been identified as a cancer-related miRNA for several cancers. For example, Fu *et al.* declared that miR-653-5p was highly expressed in prostate cancer tissues, and miR-653-5p knockdown limited prostate cancer cell proliferation and metastasis via inhibiting Wnt/ β -catenin signaling [37]. Han, *et al.* proved that miR-653-5p acted as a tumor-suppressive miRNA through targeting TIAM1 to suppress lung cancer cell proliferation and invasion [38]. These findings suggested that miR-653-5p could exert a tumor-suppressive or tumor-promotive function depending on the type of cancer. More importantly, Chi *et al.* showed that miR-653-5p expression was reduced in NB tissues, and miR-653-5p knockdown reversed the inhibitory abilities of cell proliferation, migration, and invasion induced by downregulating SNHG7 in NB cells, suggesting that miR-653-5p might act as a tumor suppressor in NB [23]. Here, we found that miR-653-5p abundance was declined in NB tissues and cells, and miR-653-5p expression was inversely correlated with XIST. Previous studies have demonstrated that lncRNAs can execute their functions through binding with their downstream miRNAs [39]. To further explore the relationship between XIST and miR-653-5p, starBase v2.0 was used to predict the targeting relationship. Interestingly, starBase v2.0 showed that miR-653-5p contained predicted binding sites with XIST. Next, the prediction was validated through dual-luciferase reporter assay. In addition, rescue experiments demonstrated that miR-653-5p knockdown could reverse the impact of XIST interference on cell proliferation, invasion, radiosensitivity, and glycolysis in NB cells. Collectively, our data indicated that XIST exerted its function through regulating miR-653-5p expression.

It is well known that the miRNAs exerted their biological functions via modulating the expression of target mRNAs, so the potential target genes for miR-653-5p were analyzed in further analysis. Despite the fact

that numerous tumor-associated genes were predicted using starBase v2.0, HK2 was selected as the candidate target gene of miR-653-5p due to its tumor-promoting effect. Further, dual-luciferase reporter assay proved that miR-653-5p directly targeted HK2. HK2 (a major type of hexokinase family) was reported to be overexpressed and facilitated rates of glucose metabolism necessary for tumor growth in multiple cancers [40, 41]. In addition, Botzer *et al.* reported that high expression of HK2 promoted NB cell metastasis [42]. Besides, Cen *et al.* revealed that HK2 was upregulated in NB tissues and cells, and HK2 accumulation weakened the repressive effects of miR-143-3p on progression of NB [28]. In line with previous findings, we also demonstrated that HK2 expression was increased in NB tissues and cells. Moreover, HK2 upregulation reversed the effect of miR-653-5p overexpression on inhibition of tumorigenesis and promotion of radiosensitivity in NB cells. Furthermore, we uncovered that XIST acted as a molecular sponge of miR-653-5p to modulate HK2 expression. Besides, knockdown of XIST restrained tumor growth and enhanced radiosensitivity by upregulating miR-653-5p and downregulating HK2 *in vivo*. In a word, these findings disclosed that XIST regulated the progression and radiosensitivity of NB cells by sponging miR-653-5p to modulate HK2 expression.

Conclusion

XIST and HK2 were upregulated and miR-653-5p was downregulated in NB tissues and cells. Knockdown of XIST inhibited the tumorigenesis and enhanced the radiosensitivity in NB cells by regulating miR-653-5p and HK2 expression. These findings might provide a potential therapeutic strategy for NB.

Abbreviations

lncRNAs: long noncoding RNAs; XIST: X-inactive specific transcript; miR-653-5p: microRNA-653-5p; HK2: hexokinase 2; qRT-PCR: quantitative real-time polymerase chain reaction; MTT: Methylthiazolyldiphenyl tetrazolium bromide; ceRNAs: competing endogenous RNAs; miRNA: microRNA; PBS: phosphate-buffered saline; WT: wild-type; MUT: mutant

Declarations

Ethics approval and consent to participate

All the cervical cancer tissues samples were collected with written informed consent in accordance with the Declaration of Helsinki and with the approval of the Ethics Committee of People's Hospital of Rizhao (No. SU493565, Date: 2019/01/22, Shandong, China).

Animal studies were performed in compliance with the ARRIVE guidelines and the Basel Declaration. Experimental procedures were approved by the Institutional and Local Committee on the Care and Use of Animals of People's Hospital of Rizhao (ACU146379) on January 25, 2019. All animals received humane care according to the National Institutes of Health (USA) guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

None.

Authors' contributions

Liping Mou participated in the conception and design of the study. Lili Wang performed the analysis and interpretation of data. Shaoming Zhang contributed to drafting the manuscript. Qinghua Wang reviewed and approved the final submitted manuscript.

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Figures

Figure 1

XIST was upregulated while miR-653-5p was downregulated in NB tissues and cell lines. (A and B) Relative XIST expression was detected by qRT-PCR in NB tissues, normal tissues, NB cells (GI-LI-N and SK-N-BE(2)) and HEK293 cells. (C and D) Relative miR-653-5p expression was measured by qRT-PCR in NB

tissues, normal tissues, NB cells (GI-LI-N and SK-N-BE(2)) and HEK293 cells. (E) Correlation between XIST and miR-653-5p expression was verified by Spearman rank correlation. ***P<0.001, ****P<0.0001.

Figure 2

XIST knockdown suppressed cell proliferation and invasion, and promoted radiosensitivity through inhibiting glycolysis in NB cells. (A-D) GI-LI-N and SK-N-BE(2) cells were divided into 3 groups: Control, si-NC, and si-XIST. (A) The expression of XIST was examined using qRT-PCR analysis. (B) MTT assay was

employed to analyze cell viability. (C) Colony formation assay was utilized to determine the number of colonies. (D) Cell invasion was detected by transwell assay. (E) Cell survival fraction was detected by colony formation assay in GI-LI-N and SK-N-BE(2) cells transfected with si-NC or si-XIST and exposed to radiation. (F-I) Glucose consumption and lactate production were measured in GI-LI-N and SK-N-BE(2) cells transfected with si-NC or si-XIST as well as the cells treated with 2-DG by glucose assay kit and lactate assay kit, respectively. (J) The expression of XIST was analyzed in GI-LI-N and SK-N-BE(2) cells transfected with pcDNA or XIST using qRT-PCR analysis. (K) Colony formation assay was applied to determine cell survival fraction in LI-N and SK-N-BE(2) cells transfected with si-NC or si-XIST or si-XIST + 2-DG under radiation condition. * $P < 0.05$, **** $P < 0.0001$.

Figure 3

XIST knockdown inhibited tumorigenesis and promoted radiosensitivity in NB cells by sponging miR-653-5p. (A) Predicted binding sites between miR-653-5p and XIST were shown. (B) Dual-luciferase reporter

assay was conducted to determine luciferase activity in GI-LI-N and SK-N-BE(2) cells co-transfected with miR-653-5p or miR-NC and WT-XIST or MUT-XIST. (C and D) XIST and miR-653-5p expression were detected using qRT-PCR analysis in GI-LI-N and SK-N-BE(2) cells transfected with si-NC, si-XIST, pcDNA, or XIST. (E-K) GI-LI-N and SK-N-BE(2) cells were transfected with si-NC, si-XIST, si-XIST + anti-miR-NC, or si-XIST + anti-miR-653-5p. (E) The abundance of miR-653-5p was measured by qRT-PCR. (F) MTT assay was performed to examine cell viability. (G) The number of colonies was determined by colony formation assay. (H) Transwell assay was carried out to detect the number of invaded cells. (I) Cell survival fraction was determined by colony formation assay under radiation condition. (J and K) Glucose consumption or lactate production was measured by glucose assay kit or lactate assay kit, respectively. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.00001$.

Figure 4

HK2 was a downstream target of miR-653-5p in NB cells. (A) The putative binding sites between miR-653-5p and HK2 were predicted by starBase v2.0. (B) Relative luciferase activity was determined in GI-LI-N and SK-N-BE(2) cells co-transfected with HK2 3'UTR-WT or HK2 3'UTR-MUT and miR-653-5p or miR-NC. (C and D) The mRNA and protein expression of HK2 were evaluated in NB tissues and normal tissues by miR-653-5p and western blot analysis, respectively. (E) Correlation between HK2 and miR-653-5p expression was determined by Spearman rank correlation. (F) The mRNA and protein expression of HK2 were assessed in NB cells (GI-LI-N and SK-N-BE(2)) and HEK293 cells by qRT-PCR and western blot, respectively. *** $P<0.001$, **** $P<0.00001$.

Figure 5

Overexpression of miR-653-5p suppressed tumorigenesis and increased radiosensitivity in NB cells by downregulating HK2. GI-LI-N and SK-N-BE(2) cells were divided into 5 groups: Control, miR-NC, miR-653-5p, miR-653-5p + pcDNA, and miR-653-5p + HK2. (A) The protein abundance of HK2 was detected by western blot assay. (B) Cell viability was evaluated by MTT assay. (C) The number of colonies was determined using colony formation assay. (D) Transwell assay was performed to assess cell invasion ability. (E and F) Cell survival fraction was measured by colony formation assay under radiation condition. (G and H) Glucose consumption and lactate production were measured using glucose assay kit and lactate assay kit, respectively. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 6

XIST positively regulated HK2 expression via sponging miR-653-5p. (A) Knockdown efficiency of miR-653-5p was determined by qRT-PCR. (B) Western blot assay was conducted to measure the protein abundance of HK2 in GI-LI-N and SK-N-BE(2) cells transfected with si-NC, si-XIST, si-XIST + anti-miR-NC, or si-XIST + anti-miR-653-5p. *** $P < 0.001$, **** $P < 0.0001$.

Figure 7

Interference of XIST treatment of IR inhibited tumor growth by upregulating miR-653-5p and downregulating HK2 in vivo. SK-N-BE(2) cells transfected with sh-NC or sh-XIST were injected subcutaneously into nude mice, and the mice were treated with IR or without IR. (A and B) Tumor volume and weight were detected. (C) The expression levels of XIST and miR-653-5p were analyzed by qRT-PCR in resected tumor tissues. (D) Western blot assay was used to measure the protein expression of HK2 in resected tumor tissues. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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