

Long Noncoding RNA LINC01410 Suppresses Tumorigenesis and Enhances Radiosensitivity in Neuroblastoma Cells Through Regulating miR-545-3p/HK2 Axis

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Background: Abnormal expression of long noncoding RNAs (lncRNAs) was often involved in tumorigenesis and radiosensitivity of various cancers. The aim of this study was to explore the biological function and regulatory mechanism of lncRNA long intergenic non-protein coding RNA 1410 (LINC01410) in tumorigenesis and radiosensitivity of neuroblastoma (NB).

Methods: The expression of LINC01410, microRNA-329-3p (miR-545-3p) and hexokinase 2 (HK2) was detected by quantitative real-time polymerase chain reaction (qRT-PCR). Methylthiazolyl-diphenyl 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 interaction between miR-545-3p and LINC01410 or HK2 was predicted by starBase v2.0 and verified by dual-luciferase reporter, RNA Immunoprecipitation (RIP) and RNA pull-down assays. Western blot was used to measure the protein expression of HK2. The mice xenograft model was established to investigate the role of LINC01410 in vivo.

Results: LINC01410 and HK2 were highly expressed while miR-545-3p was lowly expressed in NB tissues and cells. LINC01410 knockdown inhibited tumorigenesis by repressing cell proliferation and invasion, and increased the radiosensitivity via inhibiting colony formation rates and glycolysis. LINC01410 knockdown also suppressed tumor growth in vivo. Moreover, miR-545-3p could bind to LINC01410 and its downregulation reversed the effects of LINC01410 knockdown on tumorigenesis and radiosensitivity. Additionally, HK2 was a direct target of miR-545-3p and its overexpression attenuated the effects of miR-545-3p restoration on suppression of tumorigenesis and promotion of radiosensitivity. Besides, LINC01410 functioned as a molecular sponge of miR-545-3p to regulate HK2 expression.

Conclusion: LINC01410 interference inhibited tumorigenesis and increased radiosensitivity via regulating miR-545-3p/HK2 axis, providing a novel therapeutic strategy for NB.

Keywords: NB, LINC01410, miR-545-3p, HK2, tumorigenesis, radiosensitivity

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Introduction

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} In spite of advances in treatment have improved the

survival times of NB patients, children with regional or distant metastatic disease still have a poor prognosis.³ Radiotherapy is the main treatment for tumors, but the efficacy of radiotherapy in treating NB is limited due to obtaining radioresistance in the treatment of this disease.⁴ Thus, it is necessary to explain the molecular mechanisms of NB and identify more effective therapeutic targets to increase the radiosensitivity.

Long non-coding RNAs (lncRNAs), highly conserved transcripts (>200 nucleotides), lack protein-coding capacity and play regulatory roles in various physiopathology processes.⁵ Currently, numerous studies have demonstrated that lncRNAs are abnormally expressed in a variety of cancers, including NB.⁶ For instance, lncRNA NBAT-1 down-regulation promoted aggressive NB through enhancing proliferation and attenuating differentiation of neuronal precursors.⁷ Moreover, lncRNA RMRP was overexpressed in NB tissues and its knockdown suppressed the progression of NB.⁸ Importantly, recent researches have proven that lncRNA long intergenic non-protein coding RNA 1410 (LINC01410) expression is tightly linked to the progression of multiple cancers, such as thyroid cancer,⁹ cervical cancer,¹⁰ and gastric cancer.¹¹ Besides, previous study uncovered that LINC01410 was highly expressed in NB tissues and its expression was related to NB development.¹² However, the underlying mechanisms of LINC01410 involved in regulating the progression of NB and radiosensitivity remain largely unknown.

Increasing evidence reveals that lncRNAs serve as competing endogenous RNAs (ceRNAs) to modulate gene expression through sponging microRNA (miRNA).¹³ 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.^{14,15} Currently, accumulating evidence demonstrated that aberrant expression miRNA was closely related to the development and progression of cancers, and could affect the cells response to radiation.^{16,17} MiR-545-3p has been suggested to function as a tumor suppressor or tumor promoter in different cancers.^{18,19} Moreover, miR-545 was also reported to be lowly expressed in NB.²⁰ Nevertheless, the functions of miR-545-3p on the tumorigenesis and radiosensitivity of NB are poorly understood.

Several miRNAs target hexokinase 2 (HK2; a metabolism-related factor) to influence the progression of diverse types of cancer.^{21,22} HK2 has been reported to be upregulated in NB and acts as a target of miR-143-3p in NB.²³ However, there is no report on the interaction

between miR-545-3p and HK2, and exact roles of HK2 in NB progression and radiosensitivity should be explored.

In the present study, the expression of LINC01410, miR-545-3p and HK2 was analyzed in NB tissues and cells. In addition, we explored their effects on proliferation, invasion, glycolysis, and radiosensitivity, and investigated the regulatory network of LINC01410/miR-545-3p/HK2 in NB cells, aiming to offer a new biological target for treatment 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. This research was permitted by Research Ethics Committee of People's Hospital of Rizhao and performed in accordance with the Declaration of Helsinki. 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 bought 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 cultivated in Dulbecco's modified eagle medium (DMEM; Invitrogen, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. The cells were grown in an incubator of CO_2 (5%) at 37°C .

For this study, small interfering RNA against LINC01410 (si-LINC01410) and the corresponding control (si-NC), miR-545-3p mimic (miR-545-3p) and the corresponding control (miR-NC), miR-545-3p inhibitor (anti-miR-545-3p) and the corresponding control (anti-miR-NC), LINC01410 or HK2 overexpression plasmid (LINC01410 or HK2) and the corresponding control (pcDNA) were bought from GenePharma (Shanghai, China). Lentivirus-mediated shRNA interference targeting LINC01410 (sh-LINC01410) and the corresponding control (sh-NC) were constructed by Genechem (Shanghai, China). GI-LI-N and SK-N-BE(2) cells 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). In this study, the primers were as follows: LINC01410 (Forward, 5'-GTGACAAG AATGGCCCAAGC-3'; Reverse, 5'-ACTGTGCACCTG TTACACCA-3'); miR-545-3p (Forward, 5'-GCCGAG TCAGCAAACATTTAT-3'; Reverse, 5'-CAGTGCCT GTCGTGGAGT-3'); HK2 (Forward, 5'-ACAGCCT GGACGAGAGCATC-3'; Reverse, 5'-AGGTCAAACCTCC TCTCGCCG-3'); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Forward, 5'-CGCTCTCTGCTCCT CCTGTTTC-3'; Reverse, 5'-ATCCGTTGACTC CGACCTTCAC-3'); U6 snRNA (Forward, 5'-CTCGC TTCGGCAGCACATATACT-3'; Reverse, 5'-ACGCTT CACGAATTTGCGTGTC-3'). The LINC01410, HK2 or miR-545-3p expression was evaluated with the $2^{-\Delta\Delta C_t}$ method, followed by normalizing to GAPDH or U6 snRNA, respectively.

Cell Viability Assay

Methylthiazolyl-diphenyl tetrazolium bromide (MTT) was utilized for detecting cell viability. In brief, GI-LI-N and SK-N-BE(2) cell suspension (100 μ L, 2×10^3 cells) were seeded in 96-well plates and then transfected with si-LINC01410, si-LINC01410 + anti-miR-545-3p, miR-545-3p, miR-545-3p + HK2, or matched controls. MTT reagent (5mg/mL, 10 μ L, Beyotime, Shanghai, China) was added to each well after transfection. After incubation for 4 h, the cultured medium from per well would be carefully discarded and dimethyl sulfoxide (150 μ L) was added. 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 were placed 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 paraformaldehyde (4%) for 30 min at 4°C. After that, GI-LI-N and SK-N-BE(2) cells were washed by PBS and then stained by crystal violet (0.1%, Sigma-Aldrich, St. Louis, MO, USA). Microscope (Olympus, Tokyo, Japan) was applied to count colonies containing more than 50 cells. The survival fraction was calculated following the equation: the number of colonies/(number of plated cells \times plating efficiency).²⁴

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 placed 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. After 24 h of incubation, non-invaded cells were carefully removed with cotton bud, and invaded cells were fixed using ethanol (95%) and then stained using crystal violet (0.1%). Finally, a microscope (Olympus, Tokyo, Japan) was applied to observe and count invaded cells in five random fields.

Measurement of Glucose Consumption and Lactate Production

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. The consumption glucose and lactate production were analyzed by detecting the absorbance of each tube against the reagent blank at 540 nm and 570 nm, respectively.

Dual-Luciferase Reporter Assay

The possible binding sites of miR-545-3p and LINC01410 or HK2 were predicted by starBase v2.0. The LINC01410 and HK2 3'UTR fragments that contained wild-type (WT) or mutant (MUT) binding sites of miR-545-3p were amplified and inserted into pmirGLO luciferase reporter vector

(Promega, Madison, WI, USA) to create the WT plasmids (WT-LINC01410, HK2 3'UTR-WT) or MUT plasmids (MUT-LINC01410, HK2 3'UTR-MUT). Afterward, GI-LI-N and SK-N-BE(2) cells were co-transfected with reporter plasmids and miR-545-3p or miR-NC. After incubating the cells for 48 h, the luciferase activity was estimated via Dual-Luciferase Reporter assay system (Promega).

RNA Immunoprecipitation (RIP) Assay

EZ-Magna RIP Kit (Millipore, Billerica, MA, USA) was used to validate the interaction between miR-545-3p and LINC01410 or HK2. In brief, cells were lysed by complete RIP lysis buffer. After that, whole cell extract (100 μ L) was incubated by RIP buffer containing magnetic beads coated with human anti-argonaute2 (Ago2) or anti-immunoglobulin G (IgG) (as control). Next, samples were digested using the proteinase K. Finally, the RNAs in the immunoprecipitates were isolated with Trizol reagent, and the expression levels of LINC01410, miR-545-3p and HK2 were examined by qRT-PCR.

RNA Pull-Down Assay

RNA pull-down assay was performed to verify the endogenous interaction between miR-545-3p and LINC01410 or HK2 in NB cells. Biotinylated miRNAs (bio-miR-NC and bio-miR-545-3p) were obtained from Sangon Biotech (Shanghai, China). In brief, cells transfected with bio-miR-NC or bio-miR-545-3p were lysed by lysis buffer. The cell lysates were incubated with Dynabeads M-280 Streptavidin (Thermo Fisher Scientific). Next, the beads were washed, the RNA complexes bound to the beads were extracted with Trizol reagent. Finally, the enrichment of LINC01410 and HK2 was detected by qRT-PCR.

Western Blot Assay

Transfected cells were lysed using RIPA lysis buffer (Sigma-Aldrich) supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich) to extract the total protein. After quantification by using bicinchoninic acid protein assay kit (Tanon, Shanghai, China), 30 μ g of protein in each group was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, polyvinylidene fluoride (Beyotime) membranes were employed to transfer the protein. 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. Afterward, membranes

were maintained in horseradish peroxidase-conjugated anti-rabbit IgG (1:4000, D110058, Sangon Biotech) after washing with PBST. Immunoreactive bands were visualized through 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 Vital River Laboratory Animal Technology Co., Ltd, (Beijing, China). The sh-LINC01410 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: length \times width²/2. After injection for 10 days, sh-LINC01410 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 LINC01410, miR-545-3p and HK2 expression levels. The animal experiments were approved by the Committee on the Ethics of Animal Experiments of People's Hospital of Rizhao. All animal procedures were conducted following the Guidelines for Care and Use of Laboratory Animals of "National Institutes of Health".

Immunohistochemistry (IHC) Analysis

Tumor tissues from transplanted nude mice were fixed in paraformaldehyde (4%, Beyotime) and then were dehydrated, embedded in paraffin, and cut. After that, consecutive tumor sections (4 μ m in thickness) were incubated by antibodies against HK2 (1:200, ab227198, Abcam) overnight at 4°C, followed by incubation with secondary antibody (1:5000, ab205718, Abcam) for 1 h. The sections were stained by diaminobenzidine (DAB), and cell nucleus was counterstained with haematoxylin. At last, stained sections were photographed using a fluorescence microscope (Olympus).

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-545-3p and LINC01410 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

LINC01410 Was Upregulated and miR-545-3p Was Downregulated in NB Tissues and Cells

Firstly, we explored the expression of LINC01410 and miR-545-3p in NB tissues and cells by qRT-PCR. As presented in Figure 1A and B, LINC01410 was overexpressed in NB tissues and cells in contrast to their matched controls. Moreover, we found that the expression of miR-545-3p was decreased in NB tissues and cells compared to their corresponding controls (Figure 1C and D). Besides, the correlation between of LINC01410 and miR-545-3p was analyzed in NB tissues. As depicted in Figure 1E, LINC01410 expression was negatively correlated with miR-545-3p in NB tissues ($r = -0.5852$, $p < 0.001$). These data indicated that LINC01410 and miR-545-3p might be involved in the pathogenesis of NB.

LINC01410 Knockdown Inhibited Cell Proliferation and Invasion and Enhanced Radiosensitivity by Inhibiting Glycolysis in NB Cells

Next, the effects of LINC01410 on cell proliferation, invasion, glycolysis, and radiosensitivity were investigated using loss-function experiments. The transfection efficiency of si-LINC01410 was determined by qRT-PCR. The results showed that the expression of LINC01410 was decreased in GI-LI-N and SK-N-BE(2) cells transfected with si-LINC01410 compared to those cells transfected with si-NC or control cells (Figure 2A), suggesting the transfection of si-LINC01410 was successful. MTT assay revealed that LINC01410 interference decreased GI-LI-N and SK-N-BE(2) cell viability (Figure 2B). Moreover, colony formation assay indicated that LINC01410 deficiency decreased the number of colonies in GI-LI-N and SK-N-BE(2) cells (Figure 2C). Furthermore, silencing LINC01410 repressed GI-LI-N and SK-N-BE(2) cell invasion ability (Figure 2D). GI-LI-N and SK-N-BE(2) cells were transfected with si-LINC01410 or si-NC and then irradiated with 0 Gy to 8 Gy to explore the effect of LINC01410 on radiosensitivity. The data showed that LINC01410 downregulation strikingly decreased the survival fraction of GI-LI-N

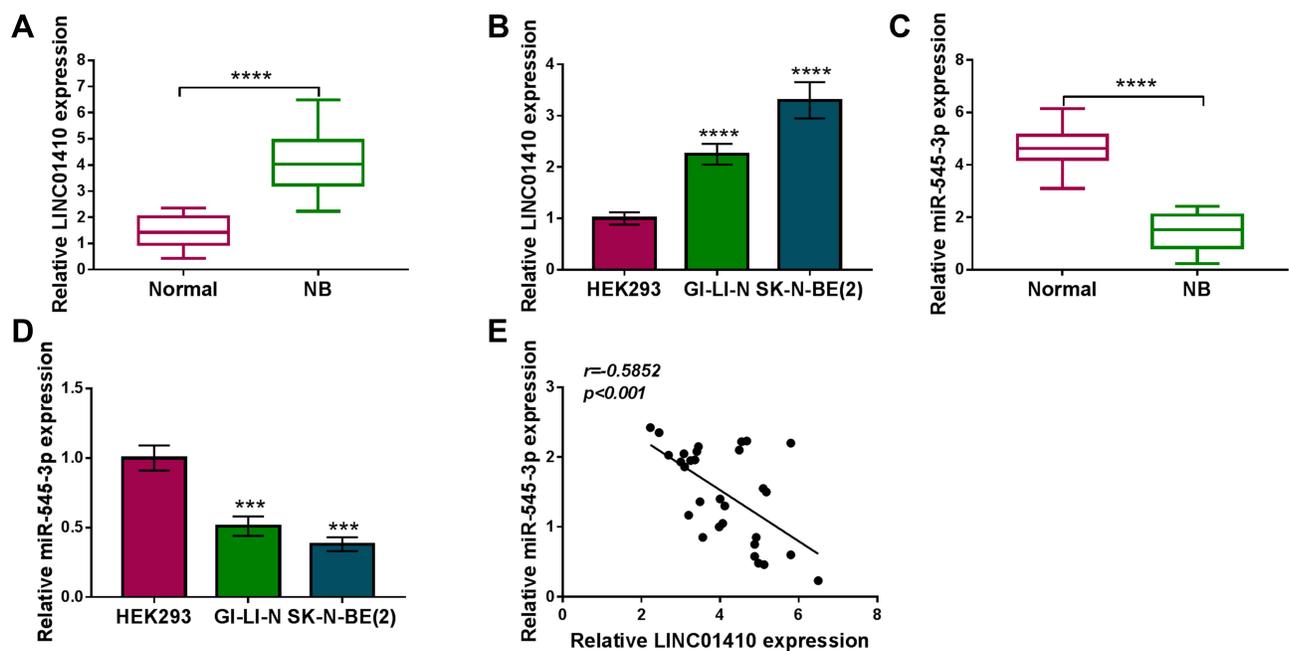


Figure 1 LINC01410 was upregulated while miR-545-3p was downregulated in NB tissues and cell lines. (A and B) Relative LINC01410 expression was detected by qRT-PCR in NB tissues, normal tissues, NB cells (GI-LI-N and SK-N-BE(2)) and HEK293 cells. (C and D) Relative miR-545-3p expression was measured by qRT-PCR in NB tissues, normal tissues, NB cells (GI-LI-N and SK-N-BE(2)) and HEK293 cells. (E) Correlation between LINC01410 and miR-545-3p expression was verified by Spearman rank correlation. *** $P < 0.001$, **** $P < 0.0001$.

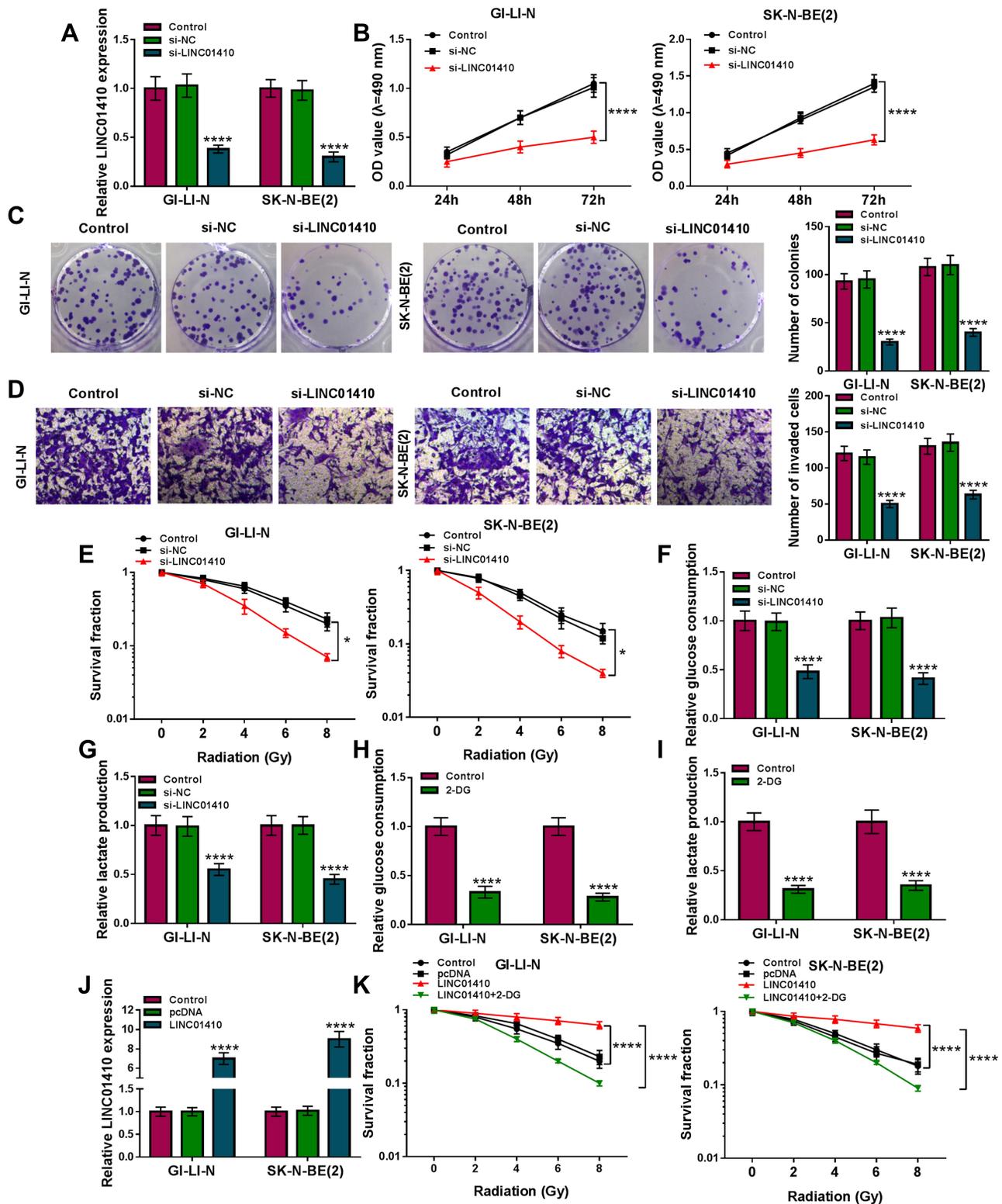


Figure 2 LINC01410 knockdown suppressed cell proliferation and invasion, and promoted radiosensitivity through inhibiting glycolysis in NB cells. (A–D) GI-LI-N and SK-N-BE(2) cells were divided into 3 groups: Control, si-NC, and si-LINC01410. (A) The expression of LINC01410 was examined using qRT-PCR analysis. (B) MTT assay was employed to analyze cell viability. (C) Colony formation assay was utilized to determine the number of colonies (incubation for 14 days). (D) Cell invasion was detected by transwell assay (incubation for 24 h). (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-LINC01410 and exposed to radiation (incubation for 14 days). (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-LINC01410 as well as the cells treated with 2-DG by glucose assay kit and lactate assay kit, respectively. (J) The expression of LINC01410 was analyzed in GI-LI-N and SK-N-BE(2) cells transfected with pcDNA or LINC01410 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-LINC01410 or si-LINC01410 + 2-DG under radiation condition. *P<0.05, ****P<0.0001.

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.²⁵ This aerobic glycolysis will result in increased glucose consumption and lactate production. We observed that knockdown of LINC01410 inhibited glucose consumption and lactate production in GI-LI-N and SK-N-BE(2) cells (Figure 2F and G). 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 I). QRT-PCR analysis showed that the expression of LINC01410 was enhanced in cells transfected with LINC01410 compared to pcDNA group (Figure 2J). Besides, the promotive effect of LINC01410 on cell survival fraction was overturned by treatment with 2-DG (Figure 2K), suggesting that LINC01410 knockdown enhanced the radiosensitivity through inhibiting glycolysis. Taken together, these results demonstrated that LINC01410 interference inhibited the tumorigenesis and increased the radiosensitivity in NB cells.

LINC01410 Directly Interacted with miR-545-3p in NB Cells

In view of the alteration of LINC01410 and miR-545-3p in NB tissues and cell lines and the negative correlation between LINC01410 and miR-545-3p expression, we wondered whether the function of LINC01410 was mediated by miR-545-3p through complementary binding sites. StarBase v2.0 predicted that LINC01410 contained putative binding sites of miR-545-3p (Figure 3A). Transfection of miR-545-3p increased the expression of miR-545-3p expression, while transfection of anti-miR-545-3p decreased miR-545-3p expression in GI-LI-N and SK-N-BE(2) cells (Figure 3B). To verify whether miR-545-3p was a direct target of LINC01410, the dual-luciferase reporter assay, RIP assay and RNA pull-down assay were performed. As presented in Figure 3C, overexpression of miR-545-3p led to a significant decrease in the luciferase activity of WT-LINC01410, while luciferase activity of MUT-LINC01410 was not evidently affected after transfection with miR-545-3p in GI-LI-N and SK-N-BE(2) cells. RIP assay revealed that both miR-545-3p and LINC01410 were immunoprecipitated in argonaute2 (Ago2) group instead of immunoglobulin G (IgG) group (Figure 3D). RNA pull-down assay showed that the expression of LINC01410 was significantly elevated in biotinylated miR-545-3p (bio-miR-545-3p) group rather than biotinylated negative (bio-miR-NC) group (Figure 3E), suggesting that

miR-545-3p could pull down LINC01410. Moreover, the expression of LINC01410 was reduced in GI-LI-N and SK-N-BE(2) cells transfected with si-LINC01410, while its expression was elevated in cells transfected with LINC01410 (Figure 3F). Next, we explored the effect of LINC01410 on expression of miR-545-3p. As expected, knockdown of LINC01410 increased the expression of miR-545-3p, whereas overexpression of LINC01410 presented an opposite effect (Figure 3G), suggesting that miR-545-3p was negatively regulated by LINC01410. Meanwhile, we observed that co-transfection of anti-miR-545-3p and si-LINC01410 abated the effect of si-LINC01410 on promotion of miR-545-3p expression (Figure 3H). Additionally, inhibition of miR-545-3p reversed the inhibitory effects of LINC01410 knockdown on viability, colony formation and invasion of GI-LI-N and SK-N-BE(2) cells (Figure 3I–K). Moreover, the suppressive effect of LINC01410 interference on the survival fraction was abolished by downregulation of miR-545-3p in GI-LI-N and SK-N-BE(2) cells exposed to IR (Figure 3L). Furthermore, miR-545-3p silencing abated the inhibitory effect of LINC01410 knockdown on glucose consumption and lactate production (Figure 3M and N). Our findings suggested that miR-545-3p could bind to LINC01410 and its knockdown reversed the effects of LINC01410 interference on tumorigenesis and radiosensitivity in NB cells.

HK2 Was a Direct Target of miR-545-3p in NB Cells

To explore the underlying mechanism of miR-545-3p in progression of NB, the function targets of miR-545-3p were searched by starBase v2.0. As presented in Figure 4A, miR-545-3p and HK2 3'UTR had complementary binding sequence, indicating that HK2 might be a target of miR-545-3p. Dual-luciferase reporter assay displayed that the luciferase activity of HK2 3'UTR-WT was obviously inhibited by transfection of miR-545-3p, whereas no clear change of the luciferase activity of HK2 3'UTR-MUT was found (Figure 4B). RIP assay demonstrated that miR-545-3p and HK2 were obviously enriched in the Ago2 immunoprecipitation compared with the IgG immunoprecipitation (Figure 4C). Moreover, HK2 was strikingly enriched in GI-LI-N and SK-N-BE(2) cells transfected with bio-miR-545-3p (Figure 4D). 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 4E and F). Additionally, HK2 expression was negatively correlated with miR-545-3p level in NB

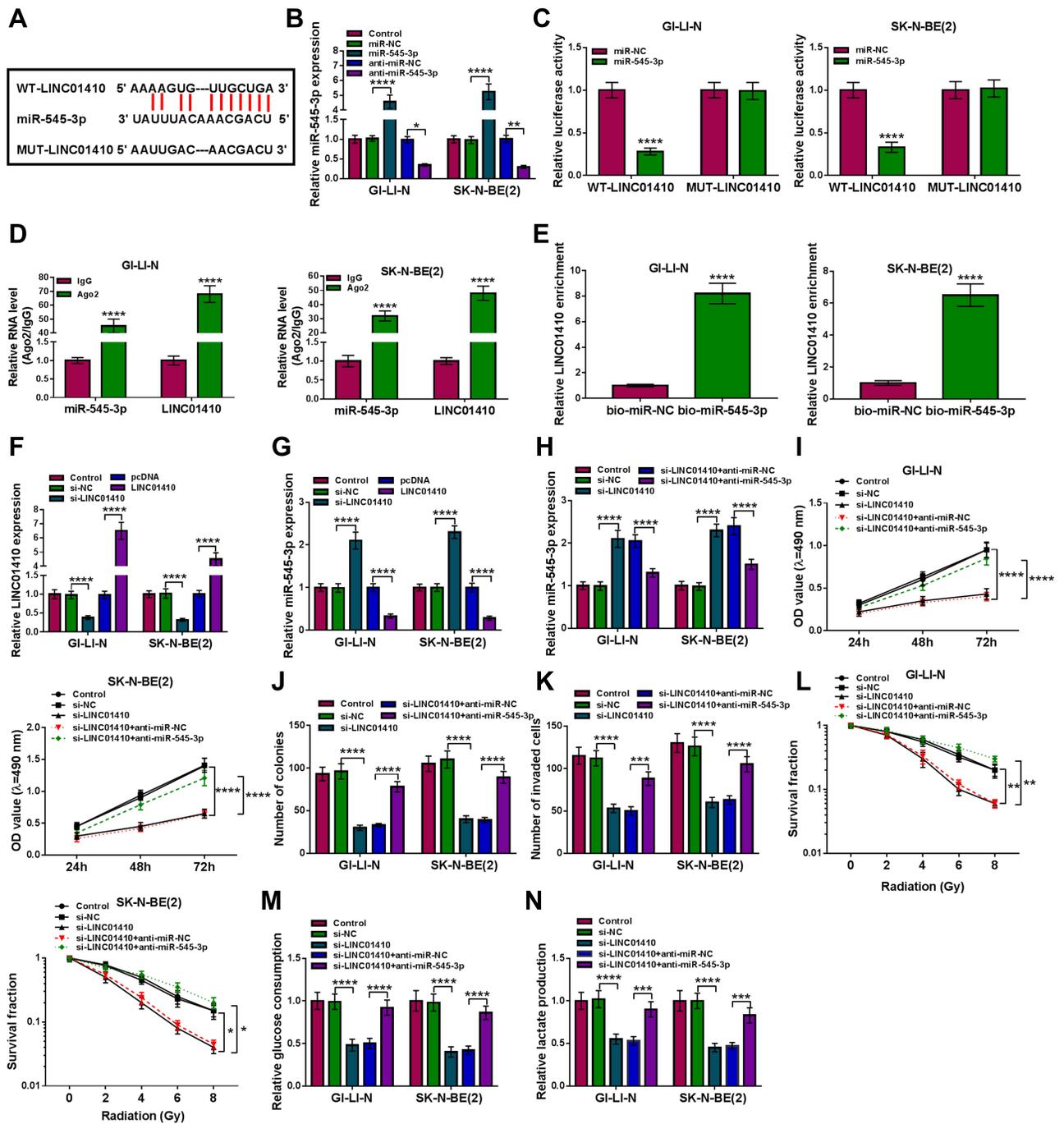


Figure 3 LINC01410 knockdown inhibited tumorigenesis and promoted radiosensitivity in NB cells by sponging miR-545-3p. (A) Predicted binding sites between miR-545-3p and LINC01410 were shown. (B) The expression of miR-545-3p was examined by qRT-PCR in GI-LI-N and SK-N-BE(2) cells transfected with miR-NC, miR-545-3p, anti-miR-NC, or anti-miR-545-3p. (C) Dual-luciferase reporter assay was conducted to determine luciferase activity in GI-LI-N and SK-N-BE(2) cells co-transfected with miR-545-3p or miR-NC and WT-LINC01410 or MUT-LINC01410. (D) The levels of miR-545-3p and LINC01410 were measured by RIP assay in GI-LI-N and SK-N-BE(2) cells incubated with Ago2 or IgG. (E) The level of LINC01410 was examined in GI-LI-N and SK-N-BE(2) cells transfected with bio-miR-545-3p or bio-miR-NC by RNA pull-down assay. (F and G) LINC01410 and miR-545-3p expression were detected using qRT-PCR analysis in GI-LI-N and SK-N-BE(2) cells transfected with si-NC, si-LINC01410, pcDNA, or LINC01410. (H–L) GI-LI-N and SK-N-BE(2) cells were transfected with si-NC, si-LINC01410, si-LINC01410 + anti-miR-NC, or si-LINC01410 + anti-miR-545-3p. (H) The expression of miR-545-3p was measured by qRT-PCR. (I) MTT assay was performed to examine cell viability. (J) The number of colonies was determined by colony formation assay (incubation for 14 days). (K) Transwell assay was carried out to detect the number of invaded cells (incubation for 24 h). (L) Cell survival fraction was determined by colony formation assay under radiation condition (incubation for 14 days). (M and N) Glucose consumption or lactate production was measured by glucose assay kit or lactate assay kit, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.00001$.

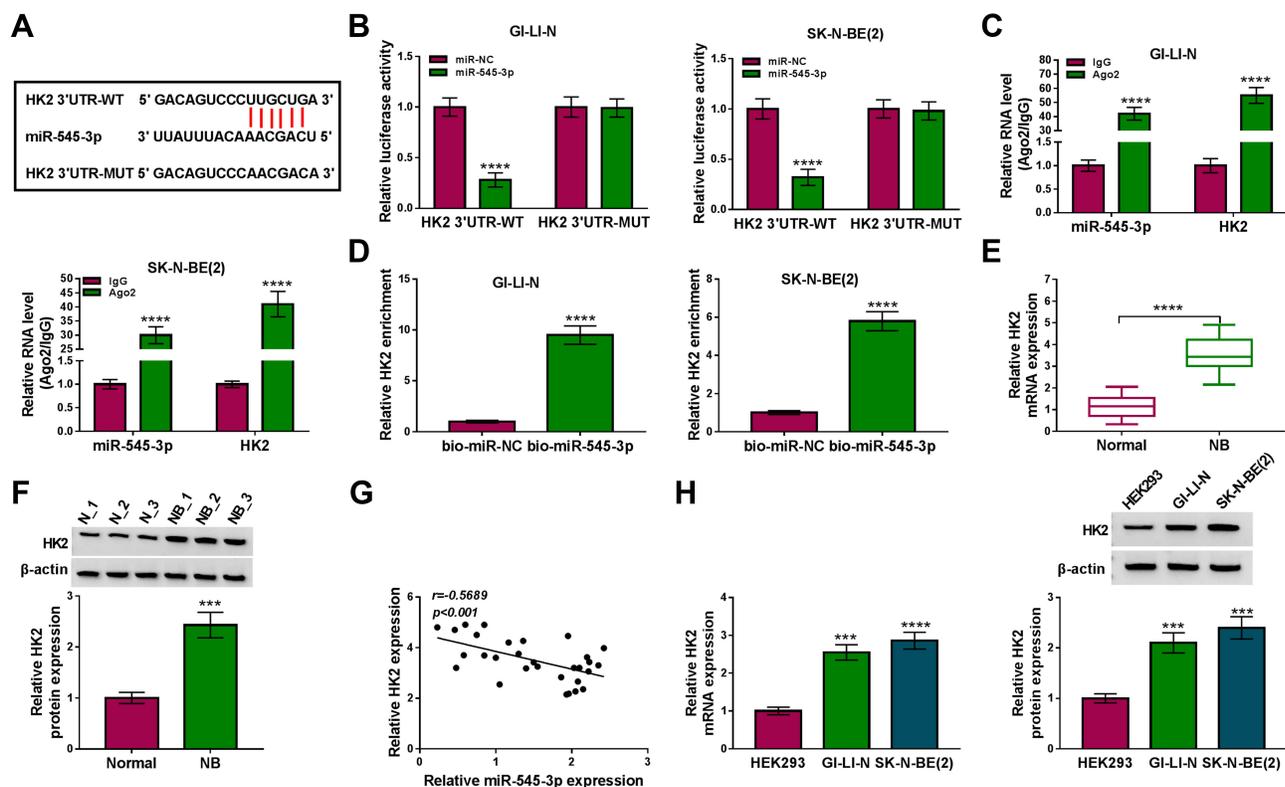


Figure 4 HK2 was a downstream target of miR-545-3p in NB cells. (A) The putative binding sites between miR-545-3p and HK2 were predicted by starBase v2.0. (B) Relative luciferase activity was determined in GI-LI-N and SK-N-BE(2) cells co-transfected with HK2 3'UTR-WT or HK2 3'UTR-MUT and miR-545-3p or miR-NC. (C) RIP assay was used to explore the interaction between HK2 and miR-545-3p in GI-LI-N and SK-N-BE(2) cells. (D) RNA pull-down assay was performed to confirm the targeted relationship between miR-545-3p and HK2 in GI-LI-N and SK-N-BE(2) cells. (E and F) The mRNA and protein expression of HK2 were evaluated in NB tissues and normal tissues by miR-545-3p and Western blot analysis, respectively. (G) Correlation between HK2 and miR-545-3p expression was determined by Spearman rank correlation. (H) 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. *** $P < 0.001$, **** $P < 0.0001$.

tissues ($r = -0.5689$, $p < 0.001$) (Figure 4G). 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 4H). These data illustrated that miR-545-3p directly interacted with HK2.

Overexpression of HK2 Reversed the Effects of miR-545-3p on Inhibition of Tumorigenesis and Promotion of Radiosensitivity in NB Cells

To determine whether the effects of miR-545-3p were regulated by HK2 expression, GI-LI-N and SK-N-BE(2) cells were transfected with miR-NC, miR-545-3p, miR-545-3p + pcDNA, or miR-545-3p + HK2. Western blot showed that HK2 protein expression was decreased in cells transfected with miR-545-3p, while the effect was abated by co-transfection of HK2 (Figure 5A). Additionally, overexpression of miR-545-3p 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–D). Moreover, the inhibitory effects of miR-545-3p upregulation on survival fraction and glycolysis were also overturned by overexpression of HK2 (Figure 5E–H). Collectively, these findings demonstrated that miR-545-3p exerted its biological functions through regulating HK2 expression.

HK2 Was Regulated by LINC01410 and miR-545-3p in NB Cells

Next, knockdown efficiency of miR-545-3p was tested by qRT-PCR. As shown in Figure 6A, the expression of miR-545-3p was significantly reduced in GI-LI-N and SK-N-BE(2) cells transfected with anti-miR-545-3p compared to those cells transfected with anti-miR-NC or control group. To explore whether LINC01410 served as a ceRNA of miR-545-3p to modulate HK2 expression, GI-LI-N and SK-N-BE(2) cells were transfected with si-NC, si-LINC01410, si-LINC01410 + anti-miR-NC, or si-LINC01410 + anti-miR-545-3p. Western blot assay demonstrated that the protein level of HK2 was decreased after transfection with si-LINC01410, while the effect was reversed by

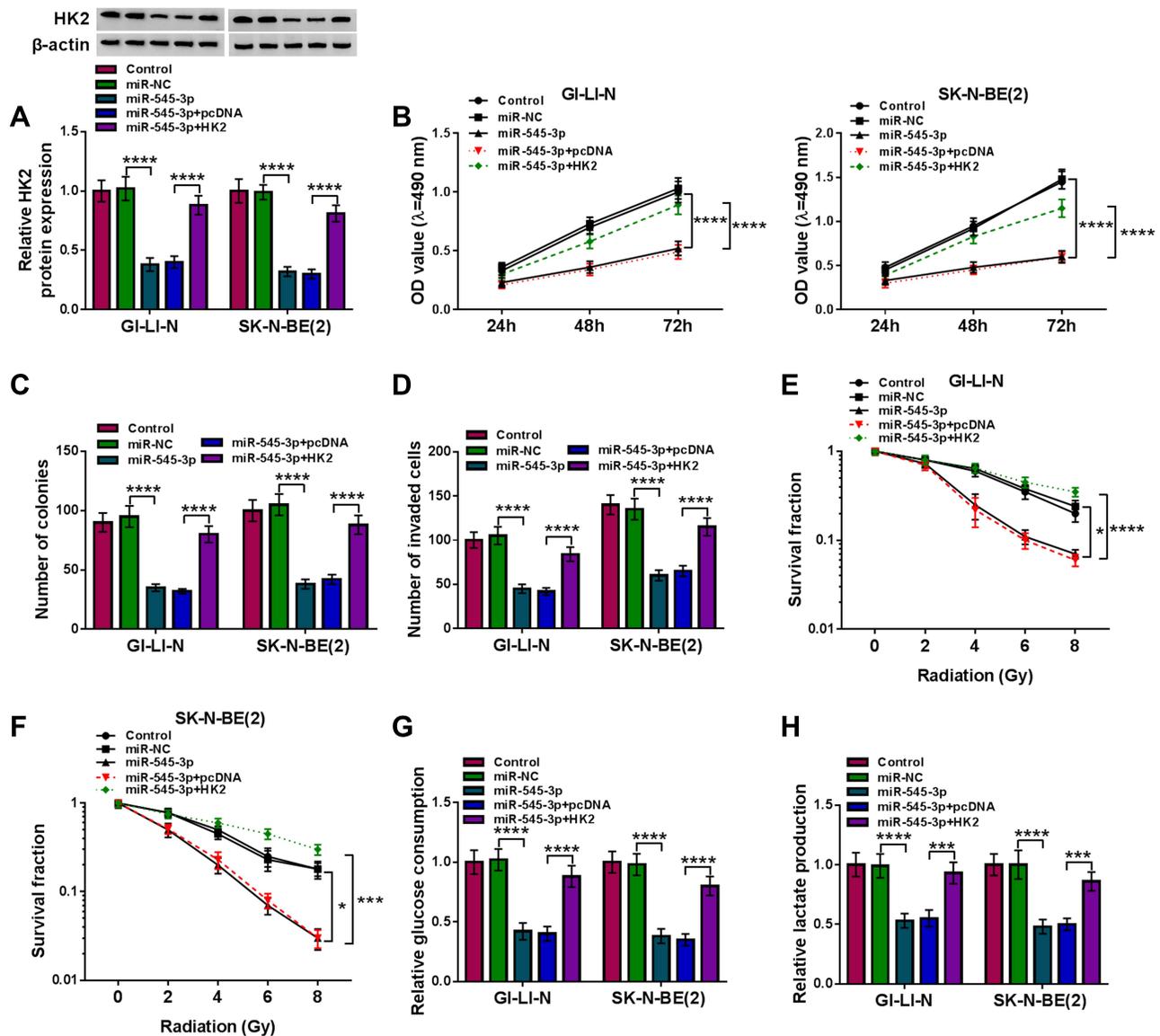


Figure 5 Overexpression of miR-545-3p suppressed tumorigenesis and increased radiosensitivity in NB cells by downregulating HK2. GI-LI-N and SK-N-BE(2) cells were divided into 5 groups: Control, miR-NC, miR-545-3p, miR-545-3p + pcDNA, and miR-545-3p + HK2. (A) The protein abundance of HK2 was detected by Western blot assay. (B) Cell viability was evaluated by MTT assay. (C) The number of colonies was determined using colony formation assay (incubation for 14 days). (D) Transwell assay was performed to assess cell invasion ability (incubation for 24 h). (E and F) Cell survival fraction was measured by colony formation assay under radiation condition (incubation for 14 days). (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.

downregulating miR-545-3p (Figure 6B). In a word, our results proved that LINC01410 regulated HK2 expression by sponging miR-545-3p in NB cells.

Downregulation of LINC01410 Inhibited Tumor Growth and Increased Radiosensitivity by Regulating miR-545-3p and HK2 in vivo

To explore the effect of LINC01410 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-LINC01410 or sh-NC were subcutaneously injected into BALB/c nude mice and irradiated with 6Gy X-ray once a week or did not irradiate. In line with in vitro results, LINC01410 knockdown or IR treatment suppressed tumor volume and weight, and combination of LINC01410 interference and IR treatment significantly inhibited tumor volume and weight in xenograft model compared with only sh-LINC01410 group or IR group (Figure 7A and B). In addition, knockdown of LINC01410 decreased the expression of LINC01410 and HK2

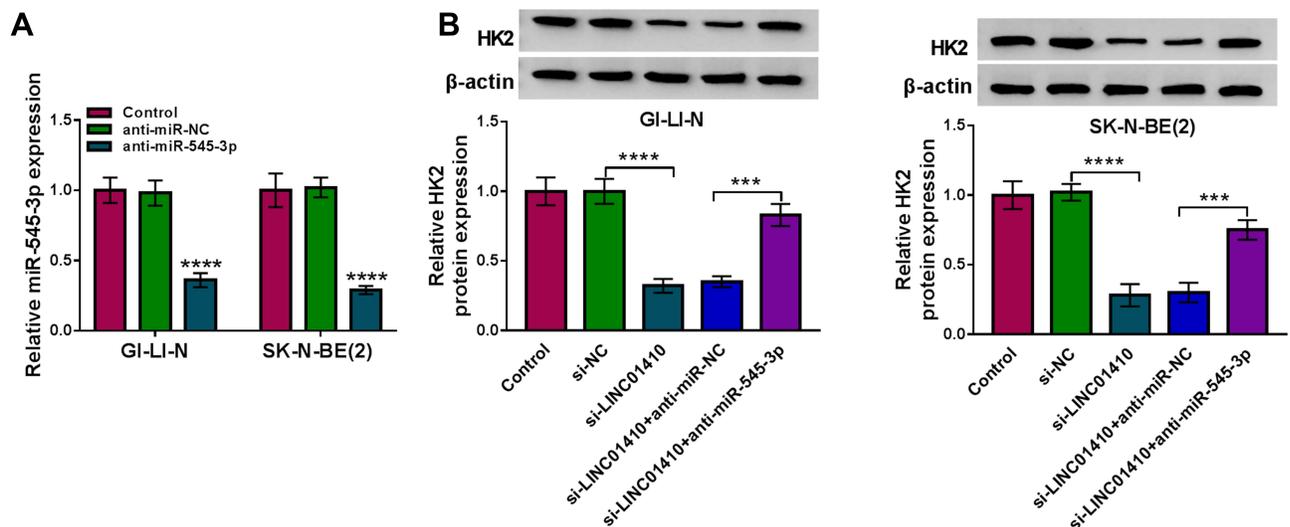


Figure 6 LINC01410 positively regulated HK2 expression via sponging miR-545-3p. (A) Knockdown efficiency of miR-545-3p was determined by qRT-PCR. (B) Western blot assay was conducted to measure the protein expression of HK2 in GI-LI-N and SK-N-BE(2) cells transfected with si-NC, si-LINC01410, si-LINC01410 + anti-miR-NC, or si-LINC01410 + anti-miR-545-3p. *** $P < 0.001$, **** $P < 0.0001$.

while increased the expression of miR-545-3p in excised tumor masses (Figure 7C and D). IHC analysis showed that LINC01410 deficiency reduced HK2-positive cells (Figure 7E), suggesting that LINC01410 knockdown decreased the expression HK2 in tumor tissues. These above findings indicated that downregulation of LINC01410 could enhance the radiosensitivity to inhibit tumor growth in vivo.

Discussion

NB is considered to be a common and aggressive malignancy in children. Recently, it has been increasingly recognized that dysregulation of lncRNAs is involved in the progression of NB.^{23,26} Radiotherapy has become an effective strategy for NB treatment, however, the influence of lncRNAs on radiosensitivity of NB cells is not well elucidated. The purpose of this research was to explore the effects of LINC01410 on NB progression and radiosensitivity.

Up to now, a growing number of researches have shown that LINC01410 serves as an oncogene and plays essential roles in cellular behaviors, such as cell growth, cell cycle, metastasis and apoptosis.²⁷ Besides, Lu et al pointed out that LINC01410 was overexpressed in endometrial cancer tissues, and accelerated endometrial cancer cell progression through miR-23c/CHD7 axis.²⁸ Moreover, Jiang et al uncovered that LINC01410 knockdown inhibited cholangiocarcinoma cell proliferation and metastasis via sponging miR-124-3p to decrease SMAD5 expression.²⁹ In our research, it was found that LINC01410 abundance was elevated in NB tissues and

cells. Additionally, interference of LINC01410 suppressed cell proliferation and invasion, and elevated radiosensitivity via suppressing glycolysis in NB cells as well as restrained tumor growth through enhancing radiosensitivity in vivo. In agreement with our findings, Mi et al disclosed that LINC01410 expression was markedly enhanced in NB tissues and its knockdown inhibited the cell growth and tumor growth and facilitated the apoptosis in NB cells by regulating miR-506-3p/WEE1 axis.¹² These results revealed that LINC01410 was involved in the tumorigenesis of NB and radiosensitivity.

Precious research has shown that miR-545-3p has pivotal roles in the regulating cellular behaviors, such as proliferation, differentiation and migration.^{30,31} It has recently been identified as a cancer-related miRNA for several cancers. For example, Changjun et al declared that miR-545-3p was highly expressed in hepatocellular carcinoma tissues and cells, and miR-545-3p inhibition limited hepatocellular carcinoma cell proliferation and metastasis via regulating MTIM.¹⁸ In contrast, Shi et al proved that miR-545-3p acted as a tumor-suppressive miRNA through targeting HDAC4 to suppress epithelial ovarian cancer proliferation, migration and invasion.³² These findings suggested that miR-545-3p could exert a tumor-suppressive or tumor-promotive function depending on the type of cancer. More importantly, Chen et al showed that miR-545 was a downregulated miRNA in NB,²⁰ suggesting that miR-545-3p might act as a tumor suppressor in NB. Here, we found that miR-545-3p abundance was declined in NB tissues and cells, and was inversely correlated with LINC01410. Previous studies have

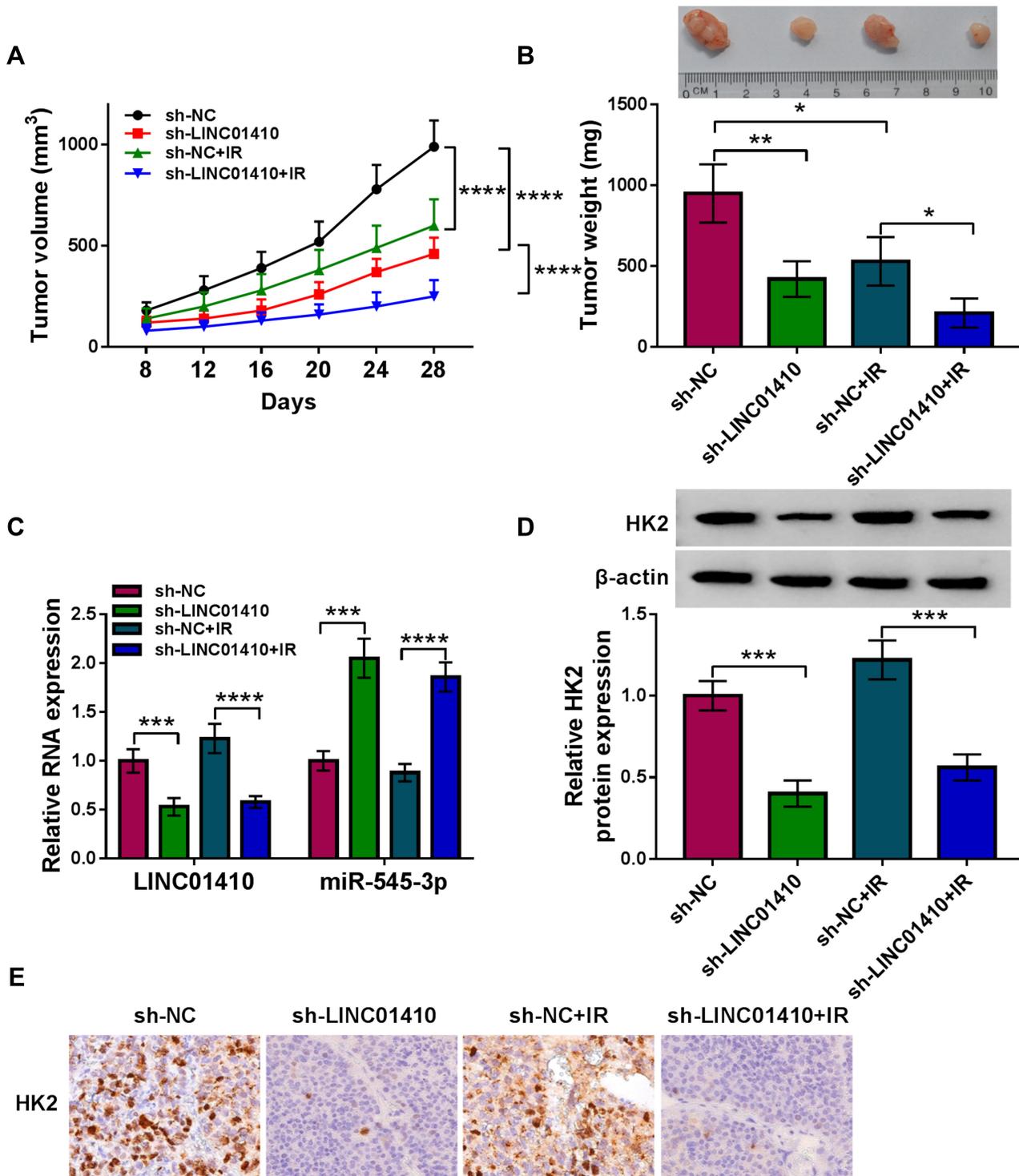


Figure 7 Interference of LINC01410 treatment of IR inhibited tumor growth by upregulating miR-545-3p and downregulating HK2 in vivo. SK-N-BE(2) cells transfected with sh-NC or sh-LINC01410 were injected subcutaneously into nude mice, and the mice were treated with IR or without IR. (A and B) Tumor volume and weight were detected. (C) The expression levels of LINC01410 and miR-545-3p 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. (E) The expression level of HK2 in resected tumor tissues was detected by IHC analysis * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

demonstrated that lncRNAs can execute their functions through binding with their downstream miRNAs.³³ To further explore the relationship between LINC01410 and miR-545-

3p, starBase v2.0 was used to predict the targeting relationship. Interestingly, starBase v2.0 showed that miR-545-3p contained predicted binding sites with LINC01410. Next, the

prediction was validated through dual-luciferase reporter, RIP and RNA pull-down assays. In addition, rescue experiments demonstrated that miR-545-3p knockdown could reverse the impact of LINC01410 interference on cell proliferation, invasion, radiosensitivity, and glycolysis in NB cells. Collectively, our data indicated that LINC01410 exerted its function through regulating miR-545-3p expression.

It is well known that the biological functions of miRNAs are realized via modulating mRNA expression, so the potential target genes for miR-545-3p would be 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-545-3p due to its tumor-promoting effect. Further, we proved that miR-545-3p 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.^{34,35} In addition, Botzer et al presented that high expression of HK2 promoted NB metastasis.³⁶ 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 restoration on progression of NB.²³ In line with previous findings, we also demonstrated that HK2 was highly expressed in NB tissues and cells. Moreover, HK2 upregulation could weaken the influence of miR-545-3p restoration on inhibition of tumorigenesis and promotion of radiosensitivity in NB cells. Furthermore, we uncovered that LINC01410 acted as a molecular sponge of miR-545-3p to modulate HK2 expression. In a word, these findings disclosed that LINC01410 regulated the progression and radiosensitivity of NB cells by sponging miR-545-3p to modulate HK2 expression.

In conclusion, LINC01410 and HK2 were upregulated while miR-545-3p was downregulated in NB tissues and cells. Knockdown of LINC01410 inhibited the tumorigenesis and enhanced the radiosensitivity in NB cells by regulating miR-545-3p and HK2 expression. These findings might provide a potential therapeutic strategy for NB.

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Disclosure

The authors declare that they have no competing interests.

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