Original Article

miR-143-3p functions as a tumor suppressor by targeting HK2 in neuroblastomas

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Abstract: Hexokinase 2 (HK2) is strongly expressed in multiple human cancers. Dysregulation of HK2 has been associated with malignant progression of tumor cells, including neuroblastomas (NB). Accumulating evidence has verified the obvious roles of miR-143-3p in anti-tumor growth. Thus, it was hypothesized that miR-143-3p may function as a tumor suppressor in NB via modulation of HK2. The current study detected the abundance of miR-143-3p and HK2 mRNA in NB tissues and cell lines using qRT-PCR. Interactions between miR-143-3p and HK2 were predicted using an online database, further confirmed by Dual-Luciferase reporter and Western blot assays. Glucose consumption, cell proliferation, and apoptosis were determined by colorimetry, MTT, and flow cytometry assays, respectively. Cell migration and invasion abilities were evaluated using Transwell assays. Protein levels of HK2, cleaved caspase-3, and Ki-67 were measured with Western blotting assays. Finally, the roles of miR-143-3p and HK2 on tumor growth were demonstrated in vivo. Results showed that miR-143-3p was significantly downregulated and HK2 was upregulated in tumor samples and cancer cells. HK2 was, therefore, identified as a potential target of miR-143-3p. The addition of miR-143-3p attenuated glucose consumption, proliferation, migration, and invasion. It also stimulated apoptosis of NB cells, which was reversed by HK2 restoration. Furthermore, miR-143-3p suppressed tumor growth in vivo by downregulating HK2. Present findings indicate that miR-143-3p suppresses NB progression through direct targeting of HK2, providing a novel therapeutic target for NB patients.

Keywords: Neuroblastoma, miR-143-3p, HK2, epigenetics

Introduction

Neuroblastomas (NB) are the third most common solid tumors in children, originating from primitive neural crest cells of the sympathetic nervous system. An estimated 710 new cases of NB have been diagnosed among children younger than 14 years old, accounting for nearly 7% of all types of childhood malignancies [1]. Although development of novel therapeutic strategies, such as surgery and/or chemotherapy and radiotherapy, have improved survival times of patients with NB, prognosis for children with regional or distant-metastatic disease remains unsatisfactory [2, 3]. Further clarification of the molecular mechanisms will potentially aid in identifying suitable agents for therapy.

Previous studies have identified a series of biomarkers implicated in occurrence and development of NB. MYCN is a widely known proto-oncogene universally expressed in high-stage NB [4]. In contrast, neurotrophic receptor tyrosine kinase 1 (NTRK1) is essential for the development and survival of nerve cells. High levels of NTRK1 occur in low-stage NB and have been associated with good prognosis for NB patients [5]. Aerobic glycolysis is a well-recognized hallmark of multiple cancers. Increased glycolysis can provide enough energy to support cell growth, leading to the malignant progression of a wide range of tumors [6, 7]. Hexokinase 2 (HK2), a major glycolytic enzyme for the Warburg effect located on chromosome 2, has the potential to stimulate aerobic glycolysis through catalyzing the phosphorylation of glucose to glucose-6-phosphate (G6P) [8]. Previous studies have reported that amplification of HK2 functions as a pivotal oncogene in various malignancies, including melanoma, colorectal can-
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In NB, HK2 stimulates cell proliferation and metastasis, conferring high resistance of NB cells to chemotherapy [11]. However, the regulatory mechanisms of HK2 have not been elucidated in NB.

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNA molecules 19-24 nucleotides in length. MicroRNAs can negatively regulate gene expression post-transcriptionally through binding to 3’-untranslated regions (3’-UTR) of target mRNAs, in a sequence-specific manner, by base-pairing. This leads to mRNA degradation or translational repression [12, 13]. Recent studies have verified abnormal expression patterns of miRNAs in human cancers, implicated in momentous pathways linked to cell proliferation, apoptosis, and metastasis [14]. Several miRNAs target HK2, a metabolism-related factor, to modulate the progression of various types of tumors [15, 16]. miR-143-3p has been reported as a tumor suppressor in multiple cancers, including breast cancer [17], cervical cancer [18], and esophageal squamous cell carcinoma (ESCC) [19]. Yang et al. also revealed that RBM3 protected NB cells from apoptosis via abolishing the induction of miR-143 by NO, indicating the functional implication of miR-143 in NB [20]. The current study sought to address the interaction of miR-143-3p and HK2 in the regulation of NB tumor biology, aiming to provide a novel biological target for NB treatment.

Materials and methods

Patients and tissue specimens

Thirty-two NB tumor tissues and paired normal tissues were collected from patients undergoing surgical resections at Fenghua Hospital, between March 2014 and February 2017. None of the patients received preoperative treatment. According to the International Neuroblastoma Staging System (INSS), 10 samples were defined as INSS I and II tumor tissues (non-metastases), 9 were defined as INSS III tumor tissues (regional metastases), and 13 were defined as INSS IV tumor tissues (distant metastases). Excised specimens were quick-frozen in liquid nitrogen and stored at -80°C. Written informed consent was obtained from all participants. This study was approved by the Research Ethics Committee of Fenghua Hospital.

Cell culture and transfection

Two human NB cell lines, SK-N-SH and SH-SY5Y, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), with human neuroblast CHP-126 as the normal control. All cells were grown in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 µg/mL streptomycin (Sigma-Aldrich). The medium was maintained in an incubator of 5% CO₂ at 37°C.

For this study, miR-143-3p mimics (miR-143-3p) and matched negative controls (NC) were obtained from GenePharma (Shanghai, China). HK2-overexpressed plasmid (HK2) was generated by inserting full-length HK2 sequences into pcDNA3.1 plasmid. MicroRNAs or plasmids were transiently transfected into SK-N-SH and SH-SY5Y cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions.

RNA preparation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from NB tissues and cell lines using TRIZol Reagent (Invitrogen), according manufacturer protocol. This was followed by detection of RNA purities using a spectrophotometer (Mapada, Shanghai, China). Next, 1 µg RNA was reversely transcribed into first-strand cDNA using TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher, Waltham, MA, USA) or High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Reaction conditions of reverse transcription included annealing at 16°C for 30 minutes, cDNA synthesis at 42°C for 30 minutes, and enzyme denaturation at 85°C for 5 minutes.

Expression patterns of miR-143-3p and HK2 were determined using Universal SYBR Green PCR Kit (Takara, Dalian, China) and performed on an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), using the procedures of 15 seconds at 95°C and 1 minute at 60°C, for a total of 40 cycles. All operations were repeated three times with U6 or GAPDH as an endogenous control for miR-143-3p or HK2, respectively. Moreover, qPT-PCR primers for HK2 were: 5’-AACAGCCTGGAGCAGGATC-3’ (forward), 5’-
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AGGTCAAACTCTCCTCGC CG-3’ (reverse); GAPDH: 5’-TATGGATATCAAGGGGTAGT-3’ (forward), 5’-TGTATGACACTTGCTAC-3’ (reverse). Primers for miR-143-3p and U6 were obtained from GenePharma.

Dual-Luciferase reporter assay

Partial sequences of the HK2 3’ untranslated region (3’-UTR) containing putative miR-143-3p binding sites were inserted into psiCHECK-2 vectors (Promega, Madison, WI, USA) to generate wild-type HK2 luciferase reporter (HK1-wt). Next, mutant HK2 luciferase reporter (HK2-mut) containing mutant miR-143-3p binding sites was constructed using TaKaRaMutanBEST kit (Takara), according to manufacturer instructions. SH-SY5Y and SK-N-SH cells (5×10^3) were seeded into 96-well microplates and hatched for 24 hours. Afterward, NC or miR-143-3p was transfected into cells, along with HK1-wt or HK2-mut reporter. About 48 hours after transfection, luciferase activity was determined using the Dual-Luciferase Reporter System (Promega).

Glucose consumption

Glucose consumption was detected using Screen Quest Colorimetric Glucose Uptake Assay Kit (AAT Bioquest, Sunnyvale, CA, USA). Briefly, transfected cells (5×10^4) supplemented with serum-free medium were starved in 96-well plates overnight at 37°C. After washing twice with KRPH buffer, the cells were resuspended in 90 μL Glucose Uptake Buffer for 1 hour, stimulated with 10 μL insulin for 20 minutes, and treated with 10 μL 2-deoxyglucose (2-DG) for 40 minutes. They were then washed with KRPH buffer and lysed with Acidic Lysis Buffer. This was followed by glucose consumption detection at an indicated absorbance of 570 nm using a microplate reader (Thermo Fisher).

Western blot assay

Proteins from cells or tumors were extracted using RIPA lysis buffer (ThermoFisher), supplemented with proteinase inhibitors and quantified using BCA Protein Assay Kit (Thermo Fisher Scientific), according to manufacturer protocol. Extracted proteins denatured at 98°C for 5 minutes were separated by SDS-PAGE gel and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk powder at 37°C for 2 hours, the membranes were hatched with rabbit anti-HK2 (1:1000), anti-active caspase-3 (1:1000), anti-Ki-67 (1:1000), and anti-beta actin (1:5000) (Abcam, Cambridge, MA, USA) overnight at 4°C. Subsequently, they were further incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:10000) (Abcam) for another 1.5 hours. Proteins were detected using ECL reagents (Pierce, Rockford, IL, USA) on the Bio-Rad ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

Cell proliferation assay

Cell proliferation was measured using Cell Counting Kit-8 (CCK-8 kit) (Dojindo, Tokyo, Japan), referring to manufacturer instructions. NB cells transfected with miR-143-3p or HK2 were plated in 96-well microplates at a density of 5×10^4 cells/well. This was followed by the addition of 10 μl CCK-8 reagent at 0, 24, 48, and 72 hours after transfection. After incubating for another 2 hours, absorbance at 450 nm was measured using a microplate reader.

Cell apoptosis assay

Cell apoptosis was measured using Annexin V-FITC/PI Apoptosis Detection Kit (BestBio, Shanghai, China), in accordance with manufacturer instructions. NB cells were treated with miR-143-3p or HK2 for 48 hours. The cells were then digested with trypsin free from EDTA, washed twice with PBS, and adjusted to a concentration of 5×10^5 cells/mL with the binding buffer. Afterward, 200 μl suspensions were added to each labeled tube. This was followed by the introduction of 5 μl Annexin V-FITC and 10 μl propidium iodide (PI). After incubation in a dark room for 10 minutes, apoptotic cells were observed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Cell migration and invasion assays

Cell migration and invasion abilities were evaluated using uncoated Matrigel (migration) or coated Matrigel (invasion) Transwell chambers with 8 μm aperture (BD Biosciences) in 24-well plates. Briefly, SK-N-SH and SH-SY5Y cells treated with miR-143-3p or HK-2 were harvested and supplemented with serum-free medium. Cell suspension was then added in the upper chamber and complete medium containing
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10% FBS was added in the bottom chamber. After incubation for 16 hours, cells migrating or invading the basal side of the membrane were fixed with methanol and stained with hematoxylin (Sigma-Aldrich). Finally, cells in random three visual fields were counted using a microscope (Olympus, Tokyo, Japan).

Tumor xenograft

Female BALB/c nude mice (4-6 weeks old) were purchased from Huafukang (Beijing, China) and raised under indicated conditions with a 12-hour light/dark cycle for one week. Next, $1 \times 10^7$ NB cells transfected with miR-143-3p or HK2 were subcutaneously inoculated into the backs of the mice after acclimatization. Tumors were measured using Vernier calipers every three days after injection. Volume was calculated by $(\text{length} \times \text{width}^2)/2$. Reaching end points, the mice were sacrificed. Tumors were collected to determine protein expression levels of HK2, Ki-67, and cleaved caspase-3. All experiments were performed in accordance with the Guiding Principles in the Use of Laboratory Animals and approved by the Committee of Animal Research of Fenghua Hospital.

Statistical analysis

Data are displayed as mean ± standard deviation (SD). Statistical differences between two or more groups were assessed by Student’s t-test or ANOVA using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ indicates statistical significance.

Results

miR-143-3p was significantly downregulated while HK2 was upregulated in NB tissues and cells

To explore the roles of miR-143-3p and HK2 in NB, expression levels were determined using the samples from 32 NB patients with different INSS stages via qRT-PCR. miR-143-3p was consistently downregulated in NB tissues relative to adjacent normal tissues. This was especially true in tumor tissues obtained from patients with advanced INSS stage (INSSIII and INSSIV), compared to early stages (INSSI and INSSII) (Figure 1A). In contrast, HK2 exhibited elevated levels in NB tissues and expression levels of HK2 were higher in INSSIII or INSSIV stage groups than in early stage groups (Figure 1B). Correlation analysis further verified a negative correlation between miR-143-3p and HK2 expression (Figure 1C). Furthermore, miR-143-3p expression was significantly diminished (Figure 1D), while HK2 was increased in NB cell lines SK-N-SH and SH-SY5Y, compared with that in normal CHP-128 cells (Figure 1E).
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Present findings indicate that miR-143-3p and HK2 might be involved in the pathogenesis of NB.

HK2 directly targeted by miR-143-3p

MiRNAs can function as an oncogene or anti-tumor factor in multiple cancers via targeting cancer-related molecules [21]. In view of the alteration of miR-143-3p and HK2 in NB tissues and cells, as well as the negative correlation between expression levels of the two genes, present researchers asked whether HK2 was mediated by miR-143-3p through complementary binding sites. Bioinformatics analysis predicted the putative binding sites of miR-143-3p within the 3'-UTR of HK2 using TargetScan online website (Figure 2A). To further confirm the true interaction between miR-143-3p and HK2, HK2-wt or HK2-mut reporter containing wild-type or mutant miR-143-3p binding sites was transfected into NB cells, along with miR-NC or miR-143-3p. Dual-Luciferase analysis disclosed that an elevated abundance of miR-143-3p suppressed the luciferase activity of HK2-wt. It showed no effects on the luciferase activity of HK2-mut reporter both in SH-SY5Y (Figure 2B) and SK-N-SH cells (Figure 2E). Moreover, Western blotting revealed that the addition of miR-143-3p notably suppressed HK2 protein expression, while restoration of HK2 weakened the inhibitory effects of miR-143-3p on HK2 expression in SH-SY5Y (Figure 2C and 2D) and SK-N-SH cells (Figure 2F and 2G). In summary, data suggests HK2 as a potential target of miR-143-3p.
miR-143-3p inhibited glycolysis and proliferation while inducing apoptosis in NB cells by targeting HK2

Since glycolysis, proliferation, and apoptosis are required for tumor progression, the current study probed the function and mechanisms of miR-143-3p in NB. Addition of miR-143-3p suppressed glucose consumption (Figure 3A) and cell proliferation (Figure 3B) in SH-SY5Y and SK-N-SH cells. This was overturned by HK2 restoration (Figure 3A and 3B). In contrast, apoptosis of SH-SY5Y and SK-N-SH cells was significantly induced in the presence of miR-143-3p, while HK2-overexpression abolished miR-143-3p-induced cell apoptosis (Figure 3C). Caspase-3 plays a key role in the regulation of cell apoptosis and Ki-67 has been considered a biomarker of cell proliferation. Protein levels of cleaved caspase-3 were strikingly upregulated, while Ki-67 was downregulated in miR-143-3p-transfected SH-SY5Y and SK-N-SH cells (Figure 3D and 3E). Introduction of HK2 abrogated stimulatory effects of miR-143-3p on cleaved caspase-3 expression, as well as inhibitory effects on Ki-67 expression (Figure 3D and 3E). Present findings indicate that miR-143-3p attenuated glycolysis and proliferation, while stimulating apoptosis in NB cells by directly targeting HK2.

miR-143-3p inhibited cell migration and invasion by directly interacting with HK2 in NB cells

Metastasis is a major threat to NB-related death. NB patients with distant-metastasis often develop malignant lesions. The current study verified the regulatory effects of miR-143-3p on cell migration and invasion in NB cells. Results revealed that migration and invasion abilities of SH-SY5Y and SK-N-SH cells were significantly suppressed following the transfection of miR-143-3p, compared with the NC group (Figure 4A and 4B). However, migration and invasion abilities inhibited by miR-143-3p were induced with the restoration of HK2 in SH-SY5Y and SK-N-SH cells (Figure 4A and 4B). Results suggest that miR-143-3p inhibits cell migration and invasion via directly interacting with HK2.

Introduction of miR-143-3p inhibited tumor growth via regulation of HK2 in vivo

In present study, effects of miR-143-3p on tumor growth in vivo were evaluated. SH-SY5Y cells transfected with miR-143-3p or miR-143-3p+HK2 were subcutaneously injected into BALB/c nude mice. Volumes of NB tumors in different groups continually increased during the whole tumor growth period (Figure 5A). Presence of miR-143-3p lowered tumor volumes. They were inverted by the restoration of HK2 (Figure 5A). The abundance of HK2 and Ki-67 prominently decreased, while cleaved caspase-3 was increased in miR-143-3p-overexpressed NB tumors. HK2 introduction overturned inhibitory effects of miR-143-3p on HK2 and Ki-67 expression, as well as stimulatory effects on cleaved caspase-3 expression (Figure 5B and 5C). Present data suggests that miR-143-3p suppresses NB growth in vivo via negative modulation of HK2.

Discussion

Increasing evidence has suggested the functional implication of miRNAs in various cancers by the modulation of cellular proliferation, differentiation, apoptosis, migration, and invasion. Ectopic expression of miRNAs can serve as oncogenes or tumor-suppressors in NB. Buechner et al. suggested that let-7 and miR-101 attenuate cell proliferation and colony formation of MYCN-amplified NB cells via targeting MYCN, a prime oncogenic transcription factor [22]. Lynch et al. reported that an elevated abundance of miR-335 distinctly weakens the migratory and invasive capacity of NB cells through inactivation of non-canonical TGF-β pathways by negatively regulating ROCK1, MAPK1, and LRG1 [23]. Moreover, Althoff et al. also confirmed that low expression of miR-137 is associated with poor prognosis of primary NB patients. Restoration of miR-137 activates cell phenotypes consistent with tumor suppression by directly targeting KDM1A [24]. These findings have demonstrated the carcinogenic or anti-tumor effects of miRNAs in NB pathology. The current study observed a significant reduction of miR-143-3p and a striking elevation of HK2 in NB. Changes in miR-143-3p and HK2 levels were even more pronounced in tissues from patients with advanced INSS stage. Dysregulation of miR-143-3p and HK2 was associated with functional analyses, revealing that miR-143-3p weakened glycolysis, cell proliferation, migration, and invasion, while inducing cell apoptosis via binding to the 3'-UTR of HK2.
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A

SK-N-SH

SH-SY5Y

Glucose consumption (μmol)

NC
miR-143-3p
miR-143-3p+HK2

Glucose consumption (μmol)

NC
miR-143-3p
miR-143-3p+HK2

B

SK-N-SH

SH-SY5Y

Relative absorbance

NC
miR-143-3p
miR-143-3p+HK2

NC
miR-143-3p
miR-143-3p+HK2

Time (h)

Time (h)

C

SK-N-SH

SH-SY5Y

Annexin V-FITC

Annexin V-FITC

Apoptotic cells (%)

NC
miR-143-3p
miR-143-3p+HK2

NC
miR-143-3p
miR-143-3p+HK2

D

NC
miR-143-3p
miR-143-3p+HK2

Cleaved caspase3

Ki-67

β-actin

SK-N-SH

Relative caspase 3 protein expression

Relative Ki-67 protein expression

NC
miR-143-3p
miR-143-3p+HK2

NC
miR-143-3p
miR-143-3p+HK2


8456
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Figure 3. miR-143-3p inhibited cell glycolysis and proliferation, while inducing apoptosis by targeting HK2. SH-SY5Y and SK-N-SH cells were transfected with NC, miR-143-3p, or miR-143-3p+HK2. (A) About 48 hours after transfection, glucose consumption was detected by colorimetry. Cell proliferation (B), apoptosis (C), and protein levels of cleaved caspase-3 and Ki-67 (D and E) in SH-SY5Y and SK-N-SH cells were measured by MTT, flow cytometry, or Western blot assay, respectively. *P<0.05.
**Figure 4.** miR-143-3p attenuated cell migration and invasion by interacting with HK2. A and B. Migration and invasion abilities of SH-SY5Y and SK-N-SH cells were determined by Transwell assay after transfection of NC, miR-143-3p, or miR-143-3p+HK2. *P<0.05.

**Figure 5.** Presence of miR-143-3p repressed tumor growth in vivo via negatively regulating HK2. BABL/c nude mice were injected subcutaneously with NC, miR-143-3p, or miR-143-3p+HK2-transfected SH-SY5Y cells. A. Tumors were measured using Vernier calipers every three days after injection and volume was calculated as the formula of (length × width²)/2. B and C. Abundances of HK2, Ki-67, and cleaved caspase-3 proteins were measured by Western blot assay. *P<0.05.
Previous studies have indicated that the aggressive potential of cancer cells was inhibited after miR-143-3p overexpression. Moreover, overexpressed miR-143-3p inhibited proliferation and motility, while inducing cell cycle arrest of triple-negative breast cancer (TNBC) cells via negative modulation of LIM domain kinase 1 (LIMK1) at mRNA and protein levels [17]. In addition, miR-143-3p was notably downregulated in esophageal squamous cell carcinoma (ESCC) and has been correlated with poor prognosis of patients. Functionally, upregulation of miR-143-3p recedes cell proliferation and migration through directly binding to QKI-5 [19]. The addition of miR-143 significantly suppresses the malignant progression of epithelial ovarian carcinoma (EOC) by targeting connective tissue growth factor (CTGF). This was reflected by the decreased proliferation, migration, and invasion of EOC cells [25]. The current study provides the first evidence that miR-143-3p plays a prime role in the modulation of NB cell glycolysis. Moreover, miR-143-3p could retard the progression of NB, as demonstrated by decreased cell proliferation, migration, and invasion, as well as induced cell apoptosis. These findings support the view that miR-143-3p could function as a vital tumor-suppressor in NB. This view is in agreement with previous studies, suggesting that miR-143-3p suppresses tumor progression in various cancers.

HK2, a major type of hexokinase family, is mostly overexpressed in several types of malignancies [26, 27]. Ectopic HK2 expression has been implicated with the occurrence and development of human cancers through catalyzing the first step of glycolysis. The current study confirmed HK2 as a functional target of miR-143-3p. Re-overexpression of HK2 abrogated miR-143-3p-suppressed cell glycolysis, proliferation, migration, and invasion, as well as miR-143-3p-induced apoptosis in NB cells. In vivo experiments further illuminated the stimulatory effects of HK2 on miR-143-3p-inhibited tumor growth. In line with present findings, Patra et al. revealed HK2-induced tumor initiation and progression in KRAS or ErbB2-driven mouse models of lung cancer [28]. Zhu et al. suggested the important role of HK2 in miR-98-mediated suppression of glucose uptake, lactate production, and proliferation of colon cancer cells through acting as a molecule target of miR-98 [29]. Likewise, reduction of HK2 by miR-125a lowered lactate production and glucose consumption, as well as ATP and reactive oxygen species (ROS) expression, in HCC cells [30]. These findings, together with present results, verify the crucial roles of HK2 in miRNA-mediated cancer progression.

In summary, the current study indicates that miR-143-3p suppressed glycolysis, proliferation, migration, and invasion, while stimulating apoptosis in NB cells and weakening tumor growth in vivo. Mechanically, inhibitory effects of miR-143-3p on the malignant progression of NB cells were overturned by its target HK2. Present findings not only support the concept that aerobic glycolysis is required for tumor growth, but also provide a novel therapeutic avenue for NB patients, especially for those with advanced INSS stage.

**Disclosure of conflict of interest**

None.

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