

MiR-34-a acts as a suppressor in neuroblastoma progression by targeting CD44

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Abstract

Objective: To verify whether micro ribonucleic acid 34-a can exert its negative effects in human neuroblastoma cells.

Methods: The study was conducted at The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China during 15 months (from March 2015 to about June 2016). Quantitative reverse transcription polymerase chain reaction was used to find the differences of micro ribonucleic acid 34-a between metastatic neuroblastoma and primary tumours. We transfected micro ribonucleic acid 34-a mimics and antisense oligonucleotides into neuroblastoma cell line to explore its function in vitro through the variations. Additionally, fluorescent reporter assay was used to clear the targeting site of micro ribonucleic acid 34-a and CD44. Furthermore, protein levels of CD44, the putative target gene of micro ribonucleic acid 34-a, was assessed after transfection by Western blot.

Results: Compared to the primary neuroblastoma tumours, micro ribonucleic acid 34-a was lower in metastatic neuroblastoma using quantitative reverse transcription polymerase chain reaction ($p < 0.05$). Transfection of micro ribonucleic acid 34-a mimics and antisense oligonucleotides into a neuroblastoma cell line significantly affected cellular activity, migration and invasion ($p < 0.05$). Fluorescent reporter assays proved that CD44 acts as the target spot of micro ribonucleic acid 34-a for repression in post-transcription level. Micro ribonucleic acid 34-a inhibited the expression of CD44, and increased concentration of micro ribonucleic acid 34-a mimics resulted in a greater decrease in the expression of CD44.

Conclusion: Micro ribonucleic acid 34-a might suppress the progression of neuroblastoma through inhibiting the expression of the potential target gene CD44.

Keywords: CD44, In vitro, HA receptor, MiR-34-a, Neuroblastoma, Tumourigenesis, Tumour therapy. (JPMA 67: 1524; 2017)

Introduction

Metastasis is the distinctive biological characteristic that differentiates malignant tumours from benign, and is a major cause of cancer mortality.¹ Neuroblastoma (NB) is an embryonic tumour of the sympathetic nervous system, occurring primarily in children under the age of 5 years, and is characterised by early metastasis. Over 70% of NB children have metastatic lesions at diagnosis in addition to the primary tumour, primarily observed in the lung, brain, and bone marrow.²⁻⁴ Despite great strides in NB treatment, the overall five-year survival rate is still quite poor (25% - 40%).⁵ Poor prognosis makes it urgent to explore the mechanism underlying NB metastasis.

In cancer research, much interest has focussed on a category of genes which transcribes small non-coding ribonucleic acid (RNAs), contains 20-22 nucleotide molecules, called microRNAs (miRNAs). The regions of the genome-producing miRNA are located mainly in the 3'-

untranslated region (3'-UTR) of target mRNAs.⁶ By binding to miRNAs of protein-coding genes to act at post-transcriptional level, miRNAs have been found to be involved in tumourigenesis and tumour aggression. Increasing numbers of studies have demonstrated that the garbled expression of microRNAs triggers tumour cell proliferation, apoptosis and differentiation by mediating expression of genes such as P53, MYCN, and CCND1. Several miRNAs have been found linked to MYCN, the best characterised genetic marker for NB. Zhang et al. identified miR-375 as a negative regulator of MYCN. MYCN down-regulated by miR-375 led to inhibition of tumour growth and tumourigenicity.⁷ Furthermore, several miRNAs have been shown to function as mediators of metastasis in NB. MiR-659-3p has been reported to serve as a metastasis promoter by down-regulating a focal adhesion pathway through CNOT1 in bone marrow-infiltrating cells.⁸ Cheung et al. found that miR-29a was a biomarker for NB central nervous system (CNS) metastasis, and a lower level of miR-29 was significantly correlated with CNS progression.⁹

MiR-34-a has been reported to exhibit a potential negative regulatory role in tumour invasion, in bladder cancer,¹⁰ colorectal cancer¹¹ and breast cancer.¹² Sachin et al. found miR-34a suppressed cell proliferation and

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tumour growth of glioma stem cells by targeting Akt and Wnt signalling.¹³ An in vivo research about miRNA-34-a in NB found that over-expression of miR-34-a in nude mice resulted in significant reduction in tumour volume up to 21 days post-injection relative to pre-miR-negative control-treated groups.¹⁴

To better define the molecular mechanism of miR-34-a in tumorigenesis, we initiated a search for its targets with the help of biological information websites (TargetScan and miRanda),⁵ where CD44 was identified as a predicted target for miR-34-a. CD44 is an adhesion molecule expressed on various cell types, and is an important participant in a number of signalling pathways.¹⁵ Data from descriptive studies showed CD44 expression in glial and neuronal cells can play a crucial role in nervous system pathology, such as response to injury and tumour invasion.¹⁶ Recently, two urinary tumour studies pointed to miR-34-a function as an anti-metastatic microRNA by directly targeting CD44.^{10,17} Another predicted target gene identified was MYCN, the most relevant gene for NB prognosis, progression and treatment, which was reported to change in parallel with miR-34-a.¹⁸

The current study was planned to verify whether miR-34-a can exert its negative effects in human NB cells, and what the effect of miR-34-a expression is on its hypothetical target genes CD44.

Materials and Methods

The study was conducted at the Affiliated Hospital of Qingdao University, Qingdao, Shandong, China, from March 2015 to about June 2016. In this study, we collected 18 matched NB tissue sections which contained the primary NB tissues and their metastatic lesions. All the samples were immediately snap frozen and stored in liquid nitrogen (-180°C) until RNA extraction. All the clinical data and detailed pathological information were gathered, including primary sites with the corresponding metastatic organs data and Edmondson tumour grade. Two expert pathologists again verified the diagnoses of all samples for this study. Approval was obtained from the ethics committee of Qingdao University.

Cell-line culture and transfections of miRNA mimics and antisense oligonucleotides (ASO) were arranged.

Two human NB cell lines (SH-SY5Y, IMR-32) obtained from American Type Culture Collection (ATCC) and MiR-34-a mimics and miR-34-a ASO provided from Shanghai Gene Pharma (Shanghai, China) were used. All cell lines placed in a humidified incubator with 5% carbon dioxide (CO₂) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Multicell; Wisent, Saint-Bruno, Quebec, Canada)

with 10% foetal bovine serum (FBS), 50 U/mL penicillin, and 50 mg/mL streptomycin (Gibco, Carlsbad, California (CA), Unites States) at 37°C. Using Lipofectamine-2000 (Invitrogen, Carlsbad, CA), transfections of miR-34-a mimics and ASO were operated according to the manufacturer's directions.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of mRNA and miRNA expression was done.

The mirVana miRNA isolation kit (Ambion, USA) was used in RNA extraction from cells and tissue samples. According to the manufacturer's instructions, fractions of the RNA smaller than 200 nt were gained through separation and purification. Then, using 1 µg of isolated RNA and murine leukaemia virus (M-MLV) (Promega, USA), complementary deoxyribonucleic acid (cDNA) was obtained. Quantitative polymerase chain reaction (qPCR) was performed using SYBR Premix Ex Taq™ (Perfect Real Time) kits (TaKaRa, Japan) for detecting the relative level of miR-34-a to endogenous control U6 according to the manufacturer's directions (cycling conditions: 95°C for 3 minutes, and further 40 cycles of amplification: at 94°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds).

Detection of cell proliferation capacity was done next. To assess the result that miR-34-a on NB cell proliferation, we performed a colony formation assay after transfection. Transfected SH-SY5Y and IMR-32 cells were seeded at a density of 200 cells/well into 12 well plates. The cell lines were washed and stained after 10 days' culture. Then the viable cell colonies were counted and the colony formation ratio: the number of colonies/the number of seeded cells x 100%.

Cell activity evaluation was measured by MTT assay. Briefly, cells were plated at density of 1×10^3 cells per well into 96 well plates (BD Biosciences, Bedford, Massachusetts [MA], USA). Cell growth was analysed over a period of 6 days. At every 24-hour interval, 20µL of MTT (5 mg/ml) was added to each well and the plate was incubated for 4 hours at 37°C in a CO₂ incubator. The formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and absorbance was measured at 570 nm in a Bio-Rad microplate reader 680 (BioRad, Hercules, CA, USA).

Transwell assays were performed to estimate the invasion capacity of transfected NB cell lines. We seeded a density about 5×10^4 SH-SY5Y and IMR-32 cells in 0.2 ml Roswell Park Memorial Institute (RPMI) 1640 into the upper part of Transwells chamber (Corning, USA) coated with Matrigel matrix (BD Science, Sparks, Maryland (MD), USA). Medium

with 10% FBS was deposited in the lower chamber. After 20 hours, we fixed the cells that had translocated across the membranes with 4% neutral formalin. Observed under microscope after stained with crystal violet, cells with invasion capacity were counted. Aforementioned assays were performed independently three times in triplicate according to the manufacturer's directions.

The protocol for in vitro migration assessment was a wound healing assay. In brief, 5×10⁴ SH-SY5Y and IMR-32 cells seeded into plates were cultured 24 hours before scratch-wound. Subsequently, wounds were created in the confluent cell layers using a 200 µl pipette tip. Debris was removed by washing several times with serum-free medium. Cells that migrated into the gap or protruded from the margin of the wound were photographed under an inverted microscope after 0 h and 48 h. Each experiment was independently performed at least three times.

To verify whether miR-34a directly targets CD44 mRNA, GenPharm (Shanghai, China) synthesised us the human CD44 3'UTR containing miR-34a potential target-binding sequences according to microRNA databases. NB cells with miR-34a mimics or ASO and controls were co-transfected with the reporter vector CD44 3'UTR or CD44 3'UTRmut in 48-well plates. After 48 hours post-transfection and lysed with Radio Immuno Precipitation Assay (RIPA) sufficiently, harvested protein fluorescence of NB cells were detected on Fluorescence Spectrophotometer (Hitachi) according to the internal reference.

The detection of CD44 protein expression was measured by Western blotting. Using Trizol reagent (Invitrogen, USA) total cellular protein was extracted after 48-hour post-transfection according to the manufacturer's instructions. Then the proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to membranes. The blots were probed with primary antibodies to CD44 (Abcam). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Saierbio) was chosen as the endogenous control. Finally, proteins were visualised using the ECL plus detection system (Pierce).

The target genes of miR-34a were predicted by TargetScan (<http://www.targetscan.org/>) and miRanda (<http://www.microrna.org/>) as shown below:

Data from all experiments was presented as mean ± standard deviation (SD). Student's t-test was used for analysis. $P < 0.05$ was considered significant.

Results

Detecting the differences of miR-34a between metastatic

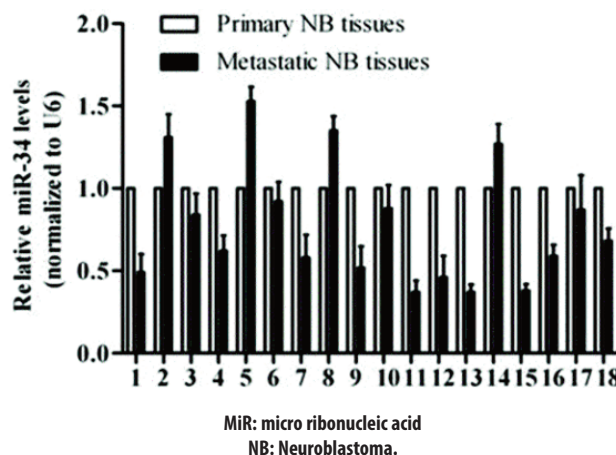


Figure-1: Identification of the differential expression of miR-34-a in NB tissues. The expression level of miR-34-a in eighteen pairs of tissues from metastasis sites and primary sites were detected.

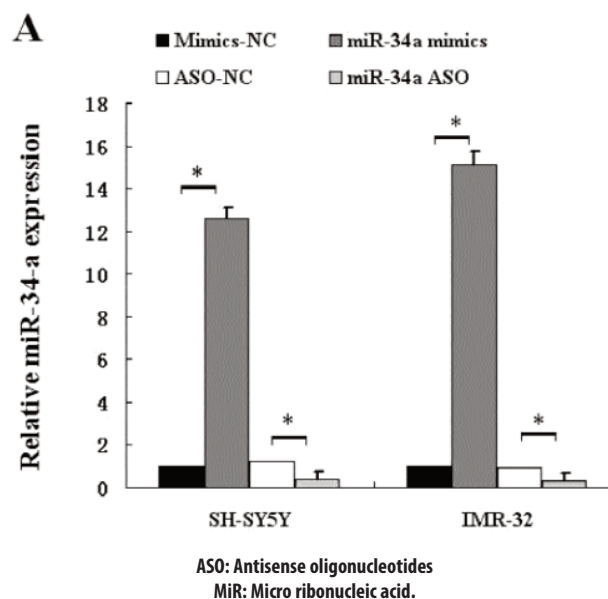


Figure-2A: The relative expression of miR-34-a and the effect of it on SH-SY5Y and IMR-32 cell proliferation after transfection. (A) SH-SY5Y and IMR-32 cells were transfected with miR-34-a mimics or ASO, or negative control (NC) mimics or ASO.

NB and primary NB may help us find its potential role in the process of human NB metastasis. The expression levels of miR-34-a were measured by qPCR in eighteen pairs of human NB samples. MiR-34a levels in 14(77.8%) of the 18 metastatic NB samples were significantly lower than in the primary NB samples. Compared to primary NB, the distinct down-regulation of miR-34-a in metastatic NB implied its potential critical role in NB metastasis (Figure-1).

Growth is an earlier step before metastasis. We

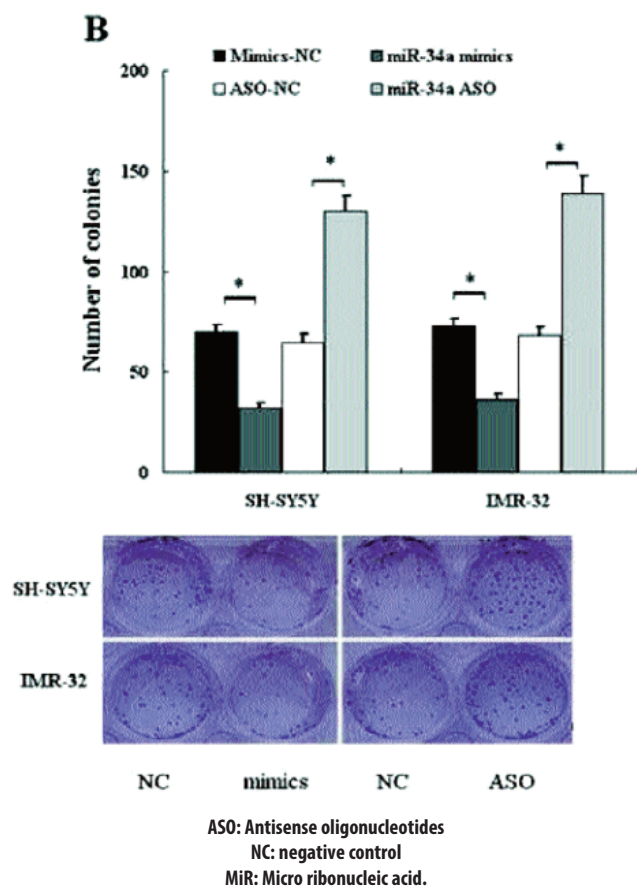


Figure-2B: The relative expression of miR-34-a and the effect of it on SH-SY5Y and IMR-32 cell proliferation after transfection. (B) Colony formation assay of SH-SY5Y and IMR-32 cells. Transfecting with miR-34-a mimics reduced colony formation.

transfected mimics and ASO into NB-derived cell lines in order to observe its effect on NB cell growth. Transfection efficiency was assessed by qRT-PCR (Figure-2A) where we can see that miR-34-a mimics and ASO were altered significantly compared to the controls in accordance with the expectations. The effect of ectopic expression of miR-34-a on cell proliferation for NB cell-lines SH-SY5Y and IMR-32 was determined by colony formation assay. Over-expression of miR-34-a inhibited cell proliferation and low expression of miR-34-a had the reverse effect on both SH-SY5Y and IMR-32 cells, significantly increasing the number of colonies compared to relative control cells (Figure-2B). These results indicate that miR-34-a expression suppressed NB cell proliferation in vitro.

MTT assays were used to further analyse the effect of miR-34a on the growth of NB cells. During six days of observation, it was found that transfection with miR-34-a mimics resulted in a decline of cell activity, while transfection with miR-34-a ASO had the opposite effect

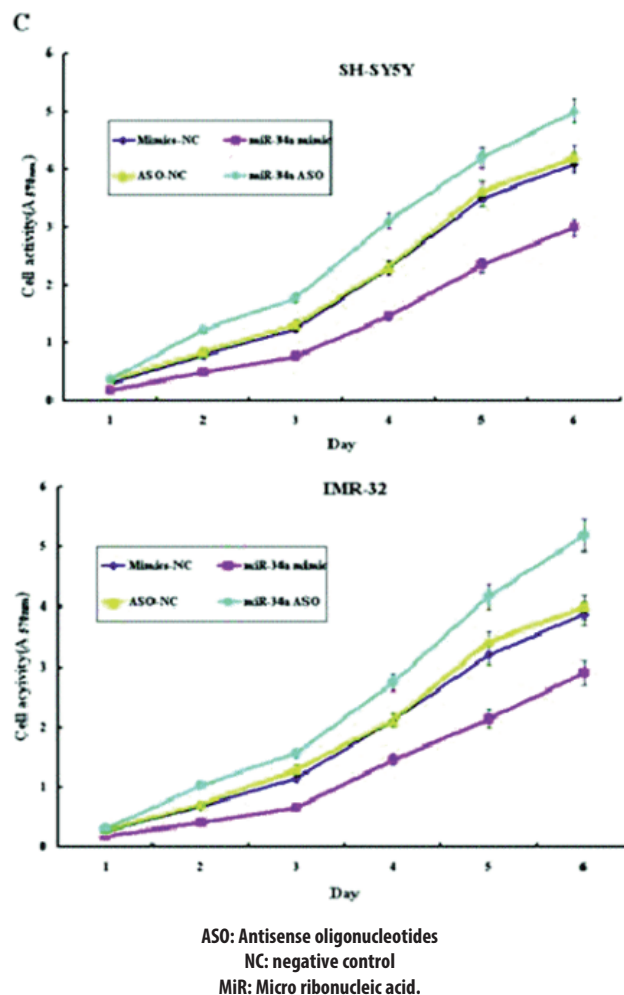


Figure-2C: The relative expression of miR-34-a and the effect of it on SH-SY5Y and IMR-32 cell proliferation after transfection. (C) MTT assay was used to assess the effect of miR-34-a on cell activity.

compared to the controls. The significant activity curves further confirmed that miR-34-a served as a tumour suppressor in NB cells in vitro (Figure-2C).

Metastasizing to a number of organs and leading to a poor prognosis is an important character of NB tumours. Research on the movement of NB, for example the invasion ability and its migration ability may therefore be of benefit to understand the mechanism of NB metastasis. A Transwell assay was utilised to evaluate whether miR-34-a can affect the invasion ability of NB cell lines. Over-expression of miR-34-a reduced 43% invasion ability of SH-SY5Y cells and 47% of IMR-32 cells compared to the control cells. On the contrary, low-expression of miR-34-a increased 1.85-fold invasion ability of SH-SY5Y cells and 2.25-fold of IMR-32 cells compared to ASO-negative control (NC) (Figure-3A).

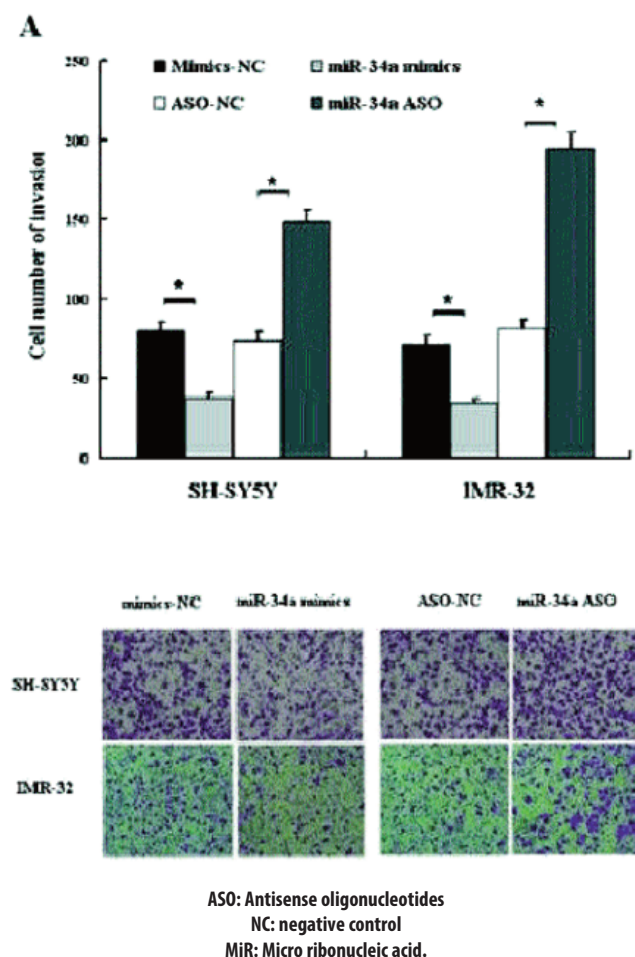


Figure-3A: Influences of miR-34-a on migration and invasion ability in NB cells.

Wound healing assays were performed to evaluate the effect that miR-34-a acts on migration of SH-SY5Y and IMR-32 cells. After 48 hours, the wound gap of miR-34-a mimic-transfected cells was significantly wider than that of control in both cell lines (Figure-3B). This demonstrated that miR-34-a suppressed NB cell migration in vitro. These two experiments further suggest that miR-34-a plays a role in inhibiting NB invasion.

The possible binding site in the 3'-UTR of CD44 mRNA was identified and its mutated version was synthesised. The miR-34-a mimics or ASO was transfected into SH-SY5Y cells together with a CD44 3'UTR luciferase reporter construct. The direct interaction between miR-34-a and CD44 3'UTR was evaluated in 2 large groups and 8 squads. It was observed that a 60% reduction of luciferase activity in miR-34-a mimics - CD44 3'UTR group and 1.7-fold increase of it in miR-34-a ASO - CD44 3'UTR group compared to the controls. On the contrary, no significant change in luciferase intensity was detected in the mutated CD44

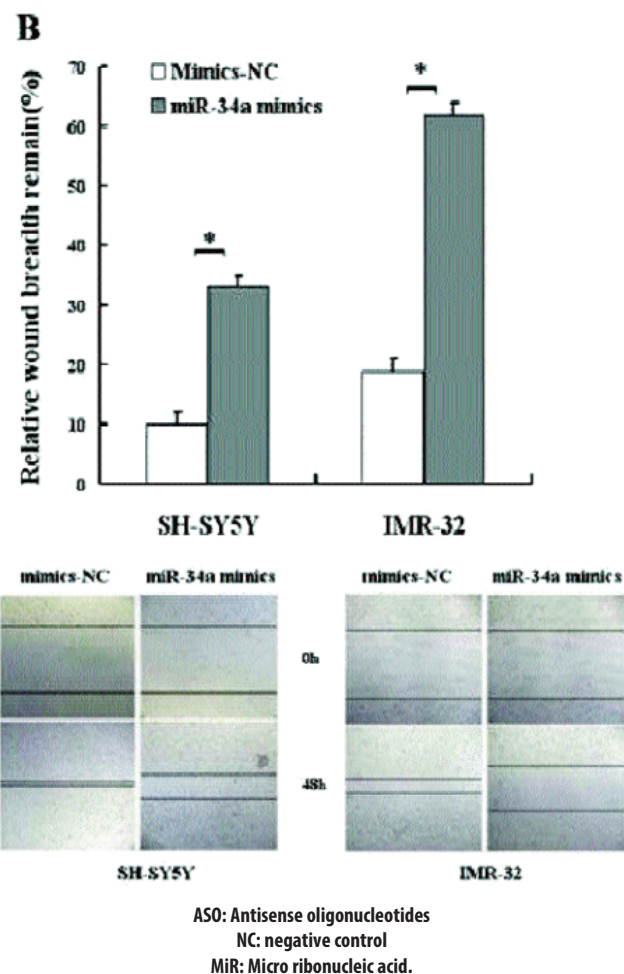


Figure-3B: Influences of miR-34-a on migration and invasion ability in NB cells.

3'UTR reporter construct group (Figure-4). The results demonstrated that the binding site of miR-34-a and CD44 was specific and it can be destroyed when mutation altered the CD44 3'UTR sequence. In conclusion, these data indicated that MiR-34-a can directly target and negatively regulate CD44 via miR-34-a 3'-UTR binding site.

To further assess whether miR-34-a can mediate the endogenous CD44 expression and how it regulates, miR-34-a mimics or miR-34-a ASO were both transfected into SH-SY5Y and IMR-32 cells (Figure-5). Over-expression of miR-34-a caused by mimics led to a 55% degree repression of CD44 protein levels in SH-SY5Y cells while a corresponding 40% degree repression in IMR-32 cells (Figure-5A). In the meantime, inhibition of miR-34-a caused by ASO resulted in a significant increase of CD44 protein expression level in SH-SY5Y cells (1.8-fold) and IMR-32 cells (1.6-fold). Taken together, the data suggested that miR-34-a can inhibit the expression of CD44.

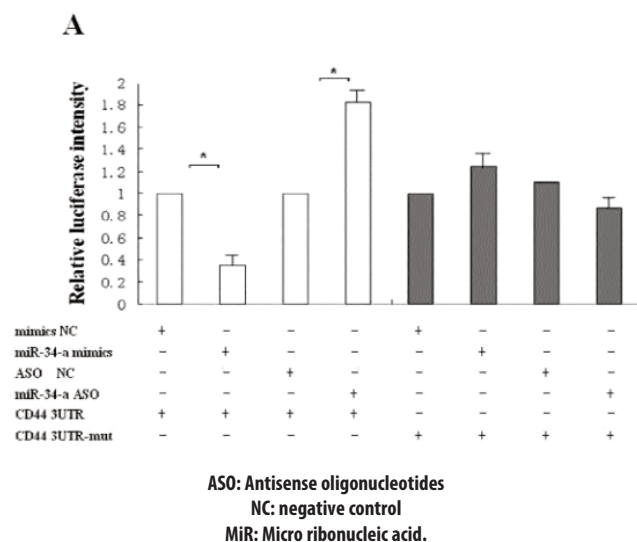


Figure-4: MiR-34-a directly targets CD44. Effect of miR-34-a on the luciferase activity of CD44 3'UTR and CD44 3'UTR-mut. For 3'UTR reporter assays, cells were co-transfected with miR-34-a mimics.

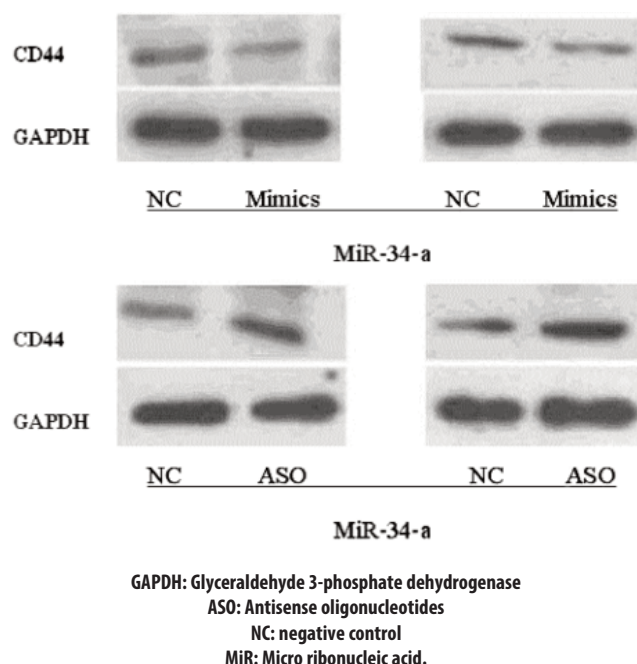


Figure-5: MiR-34-a inhibit the expression of CD44 in protein level. (A) Western blot was used to detect the CD44 protein level.).

Moreover, SH-SY5Y and IMR-32 cells were transfected with different concentrations of miR-34-a mimic. Protein levels of CD44 were also detected by Western blot. Comparing with the control group, the expression of CD44 protein in miR-34-a mimic-transfected cells

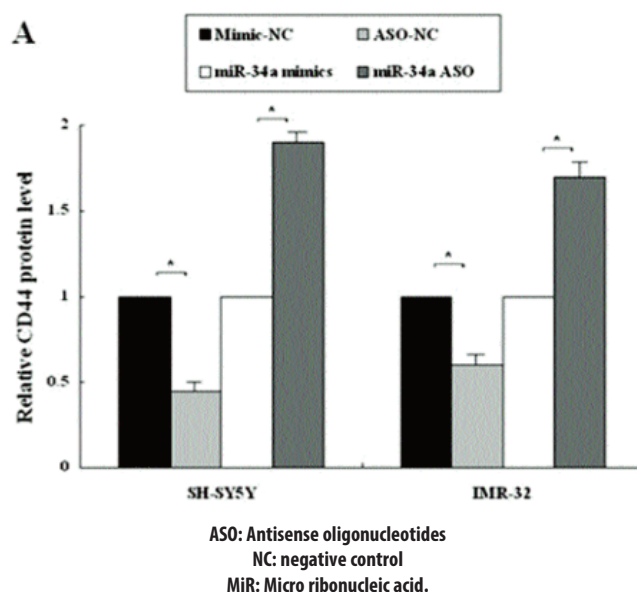


Figure-5A: MiR-34-a inhibit the expression of CD44 protein. (A) Western blot was used to detect the CD44 protein level.)

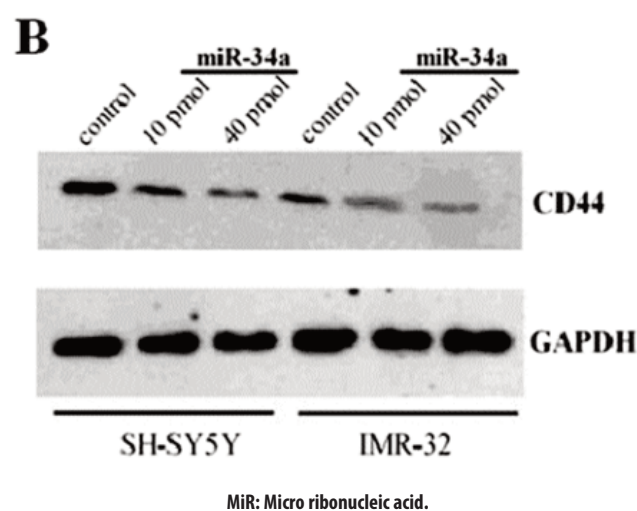


Figure-5B: MiR-34-a inhibit the expression of CD44 in protein level. (B) Expression of CD44 after transfection with different concentrations of miR-34-a mimics.

decreased, and the decrease degree in CD44 expression was correlated with the miR-34-a mimic concentration closely (Figure-5B). This result further verified that miR-34-a down-regulated the expression of CD44 and CD44 is the target gene of miR-34-a in NB.

Discussion

Since early metastasis leads to poor prognosis in NB patients, efforts to stop metastasis are the key focus in NB study all the time. In recent years, advanced bioinformatics

techniques have helped researchers identified numerous miRNAs that are thought to be related to NB invasion and progression.¹⁹ Garbled miRNAs and their target genes form an enormous and complicated network. For example, miR-29-a/b has been reported over-expressed in metastatic NB in vivo. The network established by miR-29-a/b and its predicted targets CASP8 and integrins were identified to involve in NB metastasis.²⁰ Over-expression of miR-558 in NB cell lines promoted invasion, metastasis and angiogenesis in vitro and in vivo via heparanase (HPSE); in contrast, knockdown of endogenous miR-558 had the opposite effect.²¹ Other relevant miRNAs which related to NB metastasis such as miR-181,²² let-7 miRNA family²³ and miR-542-3p,²⁴ etc. also attracted the attention of researchers.

MiR-34-a, which maps to the chromosome 1p36 region, was reported as an important tumour suppressor gene by its targeting of numerous genes associated with cell proliferation and invasion.¹⁰⁻¹³ In the current study, compared with primary tumour tissues, we found that miR-34-a expression was reduced 77.8% in metastatic tumour tissues. In addition, we found that increasing levels of miR-34-a could suppress NB cells growth, invasion and migration. Decreased levels of miR-34-a in metastatic NB tissues, and the in vitro experimental data support a role for miR-34-a in tumour progression, and identify it as a tumour suppressor.

Although several targets of miR-34-a have been identified in some other tumours, including CCND1, BCL-2, AKT and the MMP (matrix metalloproteinases) family,^{13,25} the role of miR-34-a in NB has not been investigated so far. These studies all highlight a key role for miR-34-a in regulating cell cycle, differentiation and invasion. CD44 was previously identified as a direct target of miR-34-a in bladder cancer and prostate cancer.^{10,17} CD44 is the major surface hyaluronan (HA) receptor implicated in intercellular and cell matrix adhesion, cell migration and signalling.²⁶ In this study, increased levels of miR-34-a mimic were correlated with a decrease in the expression of CD44 as analysed by Western blot.

Since the discovery that CD44 is a stem cell marker, targeting of CD44 for anticancer therapy has been attempted using CD44 vaccines, anti-CD44 monoclonal antibodies, and nanoparticle-mediated delivery of CD44 small interfering RNA (siRNA).²⁷ A recent study delivered foreign antigens to target CD44hi tumour cells using a polymeric ovalbumin (foreign antigen) and HA delivery system. Tumour cells whose surface class-I major histocompatibility complex (MHC) antigens displayed an ovalbumin peptide were injected in mice immunised with ovalbumin, and tumour growth was reduced due to

ovalbumin peptide-specific cytotoxic T-lymphocytes.²⁸ HA-coated nanoparticles have been used to deliver multi-drug resistance 1 (MDR1) siRNA in xenograft models. Following the down-regulation of P-glycoprotein, these xenografts become sensitive to paclitaxel treatment.²⁹ Anti-CD44 antibodies have also been evaluated as an anticancer therapeutic agent in many tumours, for example in chronic lymphocytic leukaemia cells.³⁰ In summary, targeting of CD44 for therapy is considered a bright prospect in cancer.

In conclusion, miR-34-a was first identified down-regulated in NB metastatic tumour tissues and acts as a tumour suppressor in our study. We found that increased levels of miR-34-a inhibit SH-SY5Y and IMR-32 cell proliferation, activity, migration and invasion. After verifying the direct binding site of miR-34-a and CD44, we observed that miR-34-a can inhibit the expression of its potential target gene CD44 and CD44 expression level changes parallel to the level of over-expressed miR-34-a further suggested a specific targeting of CD44 by miR-34-a.

Conclusion

MiR-34-a might have suppressed NB progression by targeting CD44 in NB. Based on the research of CD44-mediated tumour therapy, our result may reveal a new chapter for NB prognosis and therapy.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: None.

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