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MiR-424 suppressed viability and invasion by targeting to the DCLK1 in neuroblastoma

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Abstract. – OBJECTIVE: Neuroblastoma is the most frequent tumor of sympathetic nervous system in infants. MiRNAs acted as oncogenes or tumor suppressors in the process of tumor development. We aim at exploring the functions of miRNA in neuroblastoma.

PATIENTS AND METHODS: Cell viability and invasion were evaluated by Cell Counting Kit-8 (CCK-8) and transwell assays. Western blot was utilized to assess the protein expression associated with epithelial-mesenchymal transition (EMT) markers. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to calculate the mRNA levels of miRNA and gene.

RESULTS: MiR-424 was downregulated while doublecortin like kinase 1 (DCLK1) was upregulated in neuroblastoma tissues and cells compared to adjacent non-tumor and normal spongiocyte cells. MiR-424 suppressed cell viability, invasion, and EMT by targeting DCLK1. MiR-424 regulated the expression of DCLK1 by directly binding to the 3'-untranslated region (UTR) of DCLK1 mRNA in SK-N-SH and Be2C cells. DCLK1 reversed partial functions of miR-424 in neuroblastoma.

CONCLUSIONS: MiR-424 suppressed cell viability, invasion, and EMT by directly targeting the 3'-UTR of DCLK1 mRNA. The newly identified miR-424/DCLK1 axis provides novel insights into the pathogenesis of neuroblastoma.

Key Words:
MiR-424, DCLK1, Viability, EMT, Neuroblastoma.

Introduction

Neuroblastoma (NB) is the most frequent tumor in the sympathetic nervous system of infants, with three subtypes: schwann/substrate-adherent S-type cells, neuroblastic N-type cells, and intermediate I-type cells[1,2]. Intermediate I type cells are intermediate of those exhibited by N-type and S-type NBs, and their gene expression patterns are similar to those of cancer stem cells[3,4]. Therefore, targeting type I cells has become a therapeutic strategy for the treatment of neuroblastoma. Thus, it is urgent to identify novel targets for the early diagnosis of neuroblastoma patients.

MiRNAs were involved in various biological processes that included cell proliferation, metastasis and angiogenesis[5]. MiR-424 was usually downregulated in a variety of tumors that included glioma, osteosarcoma and endometrial cancer[6-8]. MiR-424 emerged as a tumor suppressor *via* inhibiting tumor growth *in vitro* and *in vivo* in colorectal cancer and in breast cancer[9,10]. MiR-424 suppressed cell viability and increased cell apoptosis in cervical cancer[11]. MiR-424 was sensitive to cis-platinum in ovarian cancer cells via reducing cell proliferation and increasing cell apoptosis[12]. Similarly, miR-424 attenuated hepatocellular carcinoma cell proliferation and metastasis, and improved cell apoptosis[13,14]. However, miR-424 was overexpressed in esophageal squamous cell carcinoma and non-small cell lung cancer, and promoted cell viability, cell cycle and metastasis[15,16]. Therefore, we mainly explore the roles of miR-424 on the viability and invasion of neuroblastoma cells.

Doublecortin like kinase 1 (DCLK1) contains two N-terminal double cortisol domains that mediates a variety of protein-protein interactions[17]. DCLK1 is involved in several different cellular processes, such as DNA damage response, retrograde transport, angiogenesis, and neurogenesis[18,19]. DCLK1 was able to increase

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tumor stem cells, and leaded to predict the regulation of tumor cell pluripotency[20]. Besides, DCLK1 was overexpressed in colorectal cancer and promoted cell metastasis and induced cell cycle arrest[21,22]. DCLK1 improved cell viability *in vitro* and *in vivo*, and promoted cell metastasis in pancreatic cancer[23]. In addition, DCLK1 improved cell metastasis and EMT ability of colorectal cancer *via* the PI3K/AKT/NF-κB pathway[24].

Patients and Methods

Patients and Samples

Patients undergoing neuroblastoma were treated at Qingdao Women and Children's Hospital were collected from January 2015 and December 2018. 49 pairs of neuroblastoma and adjacent non-tumor tissue samples were enrolled from patients who underwent surgery. The tissues were instantly frozen in liquid nitrogen and stored at -80°C refrigerator until use. None of the patients received local or systemic treatment before surgery. All the patients provided the written informed consent, and the study was approved by the Ethical Committee of Qingdao Women and Children's Hospital.

Cells and Cell Culture

Normal human neuroblastoma cell lines (SK-N-SH and Be2C) and a normal spongiocyte cell lines HEB were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) contained with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific) at 37°C incubator with 5% CO_2.

RNA extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The Mir-XTM miRNA qPCR SYBR Kit (TaKaRa, Otsu, Shiga, China) and SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, China) were used for the qRT-PCR on a QuantStudio 6 Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The primers were listed in Table I. GAPDH and U6 acted as the standardization of mRNA and miRNA, respectively. The relative expression was analyzed using the $2^{-\Delta\Delta Cq}$ method[25].

Western Blot

Total proteins were lysed from tissues and cells by cold radioimmunoprecipitation assay buffer (RIPA, Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was calculated by using the Bicinchoninic Acid Protein Assay Kit (PCA, Pierce; Thermo Fisher Scientific, Waltham, MA, USA). Equal proteins were separated using a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and followed transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked by 5% non-fat milk, and followed it was incubated using primary antibodies at 4 °C overnight. The primary antibodies were against E-cadherin, N-cadherin and GAPDH. Next, the membrane was incubated by secondary antibody (Abcam, Cambridge, CA, USA) for 2h at room temperature, and measured the immunoreactive protein bands using enhanced chemiluminescence (ECL) System.

Cell Viability Assay

SK-N-SH and Be2C cells were seeded at a density of 1,000 cells per well in 96-well plates containing 0.1 ml DMEM. Cell Counting Kit-8

Table I. Primer sequences for RT-qPCR.

Gene		Primer sequences
miR-424	Forward	5'-ATGGTTCAAAACGTGAGGCGT-3'
	Reverse	5'- ACCTTCTACCTTCCCCACGA-3'
U6	Forward	5'-AACGCTTCACGAATTTGCGT-3'
	Reverse	5'-CGCTTCACGAATTTGCGTGTCAT-3'
DCLK1	Forward	5'-CGGTCCACATGCAATAAAAA-3'
	Reverse	5'-GATATCACCGATGCCATCAAG-3'
GAPDH	Forward	5'-GATATCACCGATGCCATCAAG-3'
	Reverse	5'-TGGACTCCACGACGTACTCA-3'

(CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was employed to calculate cell viability after 24, 48, 72, and 96 h of incubation. After incubating CCK-8 reagent for over 3 hours, the absorbance of each well was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Transwell Assay

Cell invasive ability was evaluated using transwell insert (Corning, Beijing, China) covered with Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA). SK-N-SH and Be2C cells were trypsinized and suspended in serum-free RPMI-1640 medium. The transwell insert was placed in a 24-well plate, and then, the suspended cells were seeded in the upper chamber. In the meantime, the lower chamber was filled with 600 ml of RPMI-1640 medium containing 20% FBS. After 24 h, the non-invaded SK-N-SH and Be2C cells were removed by a cotton swab. Meanwhile, the invaded SK-N-SH and Be2C cells was fixed for 30 min with 100% methanol, and then, were stained with 0.1% crystal violet for 20 min. Finally, the images of SK-N-SH and Be2C cells were captured under a phase-contrast microscope.

Cell Transfection

SK-N-SH and Be2C cells were seeded in 24-well plates and incubated overnight. Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect the miR-424 mimic, the miR-424 inhibitor, or inactive control vectors (Life Technologies, Carlsbad, CA, USA) into SK-N-SH and Be2C cells.

Luciferase Assay

To verify whether miR-424 targeted DCLK1, the predicted miR-424 binding sequences on 3'UTR of DCLK1 mRNA was mutated, and both the wild type and the mutant sequences were inserted into the pGL3 vectors. SK-N-SH and Be2C cells were co-transfected with the miR-424 mimic or NC mimic and wild type pGL3-DCLK1 or mutant pGL3-DCLK1 plasmid using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). After 24 h of cell culture, the firefly Luciferase activity was measured using a Dual-Luciferase assay (Promega, Madison, WI, USA), and Renilla Luciferase activity was utilized as the internal control.

Statistical Analysis

Statistical Product and Service Solutions (SPSS; IBM Corp., Armonk, NY, USA) software was conducted to perform the statistical analysis, and the data was expressed as means ± standard deviation (SD). The differences between the two groups were compared by *t*-test, while the the differences between three or more groups were analyzed through One-way analysis of variance. Pearson's correlation coefficient was employed to estimate the relationship between the expression of miR-424 and DCLK1 in neuroblastoma tissues. Statistical analysis was significant when $p < 0.05$.

Results

The Connection Between the Expression of MiR-424 and Prognosis in Neuroblastoma

To explore the great functions of miR-424 in neuroblastoma, qRT-PCR was used to calculate the expression of miR-424 in neuroblastoma tissues and non-tumor tissue samples. The expression of miR-424 was lower in neuroblastoma tissues than that in non-cancer tissue samples ($p<0.05$) (Figure 1A). Similar results were found in cells, and miR-424 was downregulated in neuroblastoma cells SK-N-SH ($p<0.01$) and Be2C ($p<0.05$) *vs.* normal HEB cells (Figure 1B).

QRT-PCR assay was conducted to assess the expression of DCLK1 in tissues and cell lines. Contrary to the results of miR-424, DCLK1 was overexpressed in neuroblastoma tissues versus normal tissues ($p<0.05$) (Figure 1C). Moreover, the expression of DCLK1 was higher in SK-N-SH ($p<0.05$) and Be2C cells ($p<0.01$) than that of HEB cells (Figure 1D).

MiR-424 Suppressed the Viability in Neuroblastoma Cells

To explore the functions of miR-424, the miR-424 mimic was used to upregulate the expression of miR-424 in SK-N-SH ($p<0.01$) and Be2C cells ($p<0.05$), while the miR-424 inhibitor was utilized to downregulate the expression of miR-424 ($p<0.05$) (Figure 2A and 2C). CCK-8 assay was carried out to evaluate the roles of miR-424 on cell viability in neuroblastoma cells. Cell viability was decreased by the miR-424 mimic in SK-N-SH ($p<0.05$) and Be2C cells ($p<0.05$) (Figure 2B). On the contrary, cell viability was improved by the miR-424 inhibitor in SK-N-SH ($p<0.05$) and Be2C cells ($p<0.05$) (Figure 2D), which elucidated that miR-424 inhibited cell viability in SK-N-SH and Be2C cells ($p<0.05$).

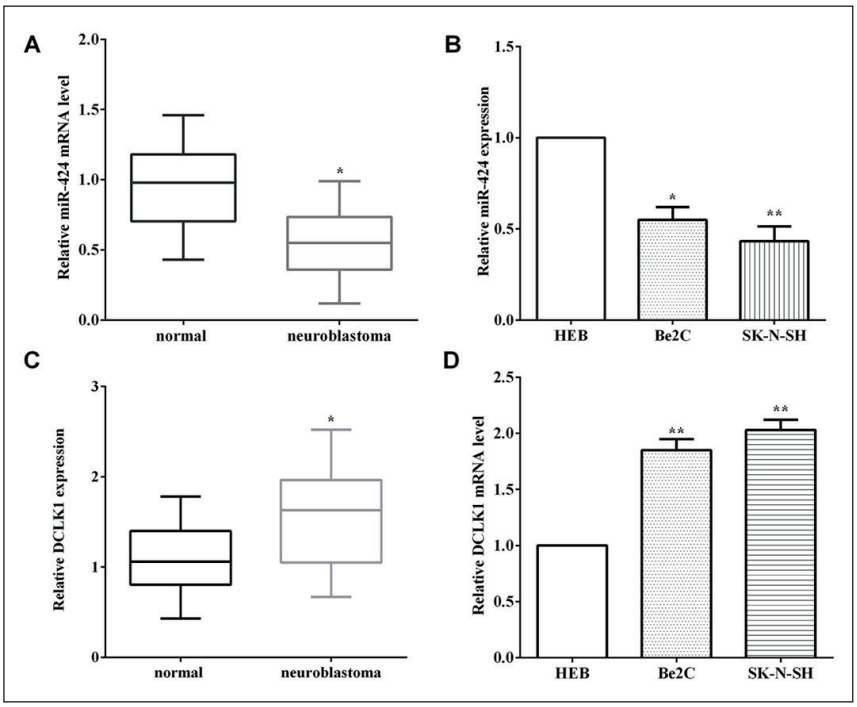

Figure 1. The connection between the expression of miR-424 and prognosis of neuroblastoma. **A,** The expression of miR-424 was lower in neuroblastoma tissues than that of non-coding tissue samples. **B,** MiR-424 was downregulated in neuroblastoma cells SK-N-SH and Be2C versus normal HEB cells. **C,** DCLK1 was overexpressed in neuroblastoma tissues versus normal tissues. **D,** The expression of DCLK1 was higher in SK-N-SH and Be2C cells than that of HEB cells.

MiR-424 Suppressed Invasion and EMT in Neuroblastoma Cells

Transwell assay was used to calculate invasive capacity of SK-N-SH and Be2C cells. Similar findings were obtained with cell viability; the miR-424 mimic inhibited cell invasion in SK-N-SH ($p<0.05$) and Be2C ($p<0.05$) cells (Figure 3A). On the contrary, miR-424 inhibitor promoted the invasive ability in SK-N-SH ($p<0.05$) and Be2C ($p<0.05$) cells (Figure 3B).

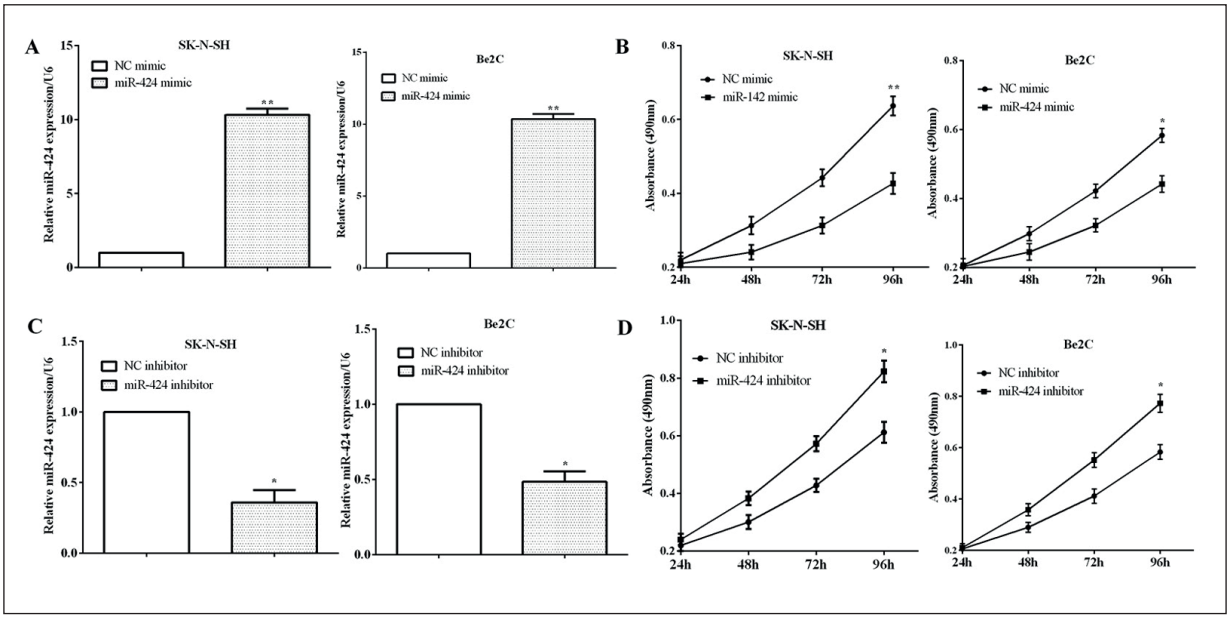

Figure 2. MiR-424 suppressed the viability of neuroblastoma cells. **A,** The miR-424 mimic was up-regulate the expression of miR-424 in SK-N-SH and Be2C cells. **B,** The miR-424 mimic decreased the viability in SK-N-SH and Be2C cells. **C,** The miR-424 inhibitor utilized to downregulate the expression of miR-424. **D,** Cell viability was improved by the miR-424 inhibitor in SK-N-SH and Be2C cells.

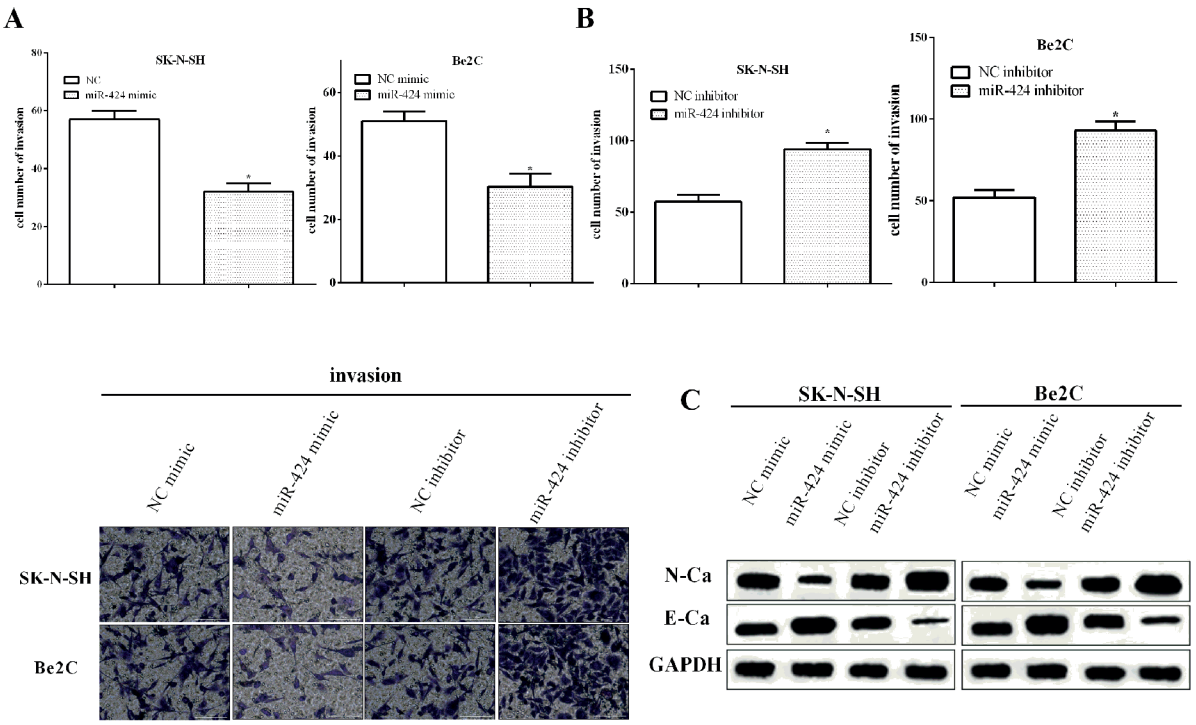

Figure 3. MiR-424 suppressed invasion and EMT of neuroblastoma cells. **A,** The invasion was reduced by the miR-424 mimic in SK-N-SH and Be2C cells. **B,** The miR-424 inhibitor promoted the invasive ability in SK-N-SH and Be2C cells (200×). **C,** MiR-424 suppressed the EMT ability in SK-N-SH and Be2C cells.

Besides, Western blot was used to calculate the expression of EMT-related proteins, such as N-cadherin and N-cadherin. As expected, the miR-424 mimic decreased the expression of N-cadherin, while increased the expression of E-cadherin in SK-N-SH and Be2C cells. By contrast, the expression of N-cadherin was enhanced, whereas E-cadherin was reduced by the miR-424 inhibitor in SK-N-SH and Be2C cells (Figure 3C). All the results revealed that miR-424 suppressed the invasive and EMT abilities of neuroblastoma cells.

DCLK1 Is a Target Gene of MiR-424 in Neuroblastoma Cells

Potential targets of miR-424 were predicted by a public database TargetScan, and DCLK1 was predicted as a target of miR-424 (Figure 4A). To verify that miR-424 directly targeted to DCLK1, we performed the Lluciferase reporter assay. Compared with mimic NC, the miR-424 mimic reduced the Luciferase activity of wild type DCLK1 3'-UTR in both SK-N-SH and Be2C cells ($p<0.05$). However, the miR-424 mimic did not alter the Luciferase activity of mutant 3'-UTR of DCLK1 mRNA in SK-N-SH and Be2C cells ($p>0.05$) (Figure 4B). QRT-PCR was used to evaluate the expression of DCLK1 after overexpression or knockdown of miR-424 in SK-N-SH and Be2C cells. As expected, the expression of DCLK1 was reduced by the miR-424 mimic in Be2C and SK-N-SH cells ($p<0.05$). On the contrary, the miR-424 inhibitor increased the expression of DCLK1 in SK-N-SH and Be2C cells ($p<0.05$) (Figure 4C). All findings elucidated that miR-424 directly targeted DCLK1 *via* binding to the 3'-UTR of its mRNA.

DCLK1 Partially Reversed Functions of MiR-424 on Cell Viability and Invasion

qRT-PCR was conducted to assess the transfection efficiency of transfecting DCLK1 overexpression plasmid or control in miR-424 mimic-transfected SK-N-SH ($p<0.05$) and Be2C ($p<0.05$) cells ($p<0.05$) (Figure 5A). Cell viability and invasive abilities were calculated by CCK-8 and transwell assays in SK-N-SH and Be2C cells. The overexpression of DCLK1 enhanced cell viability compared with transfected negative control in miR-424 mimic-transfected SK-N-SH ($p<0.05$) and Be2C cells ($p<0.05$) (Figure 5B). In addition, invasive ability was improved by trans-

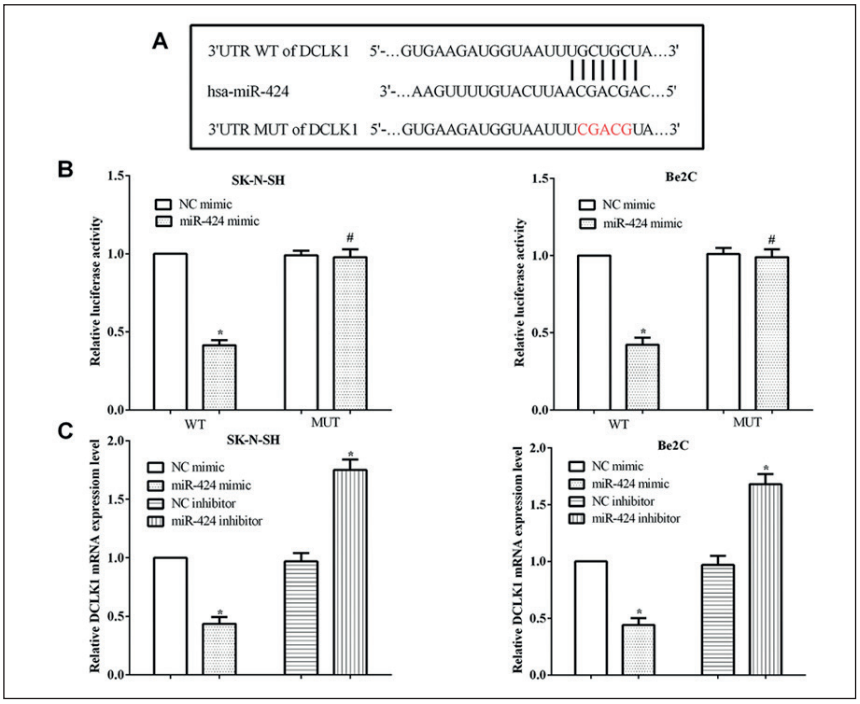

Figure 4. DCLK1 is a target gene of miR-424 in neuroblastoma cells. **A,** DCLK1 was predicted as a target of miR-424. **B,** The miR-424 mimic reduced luciferase activity of wild type DCLK1 3'-UTR in SK-N-SH and Be2C cells. **C,** MiR-424 regulated the expression of DCLK1 in SK-N-SH and Be2C cells.

fecting DCLK1 overexpression plasmid in SK-N-SH ($p<0.05$) and Be2C cells ($p<0.05$) (Figure 5C). Rescue experiments elucidated that miR-424 regulates cell viability and invasion by directly binding to the 3'-UTR of DCLK1 mRNA, and DCLK1 partially reversed the roles of miR-424.

Discussion

MicroRNAs mediate gene expression *via* base pairing with the 3'-UTR of target gene[26]. MiR-424 functioned as a suppressive miRNA by impairing glioma cell invasion and migration, and promoted

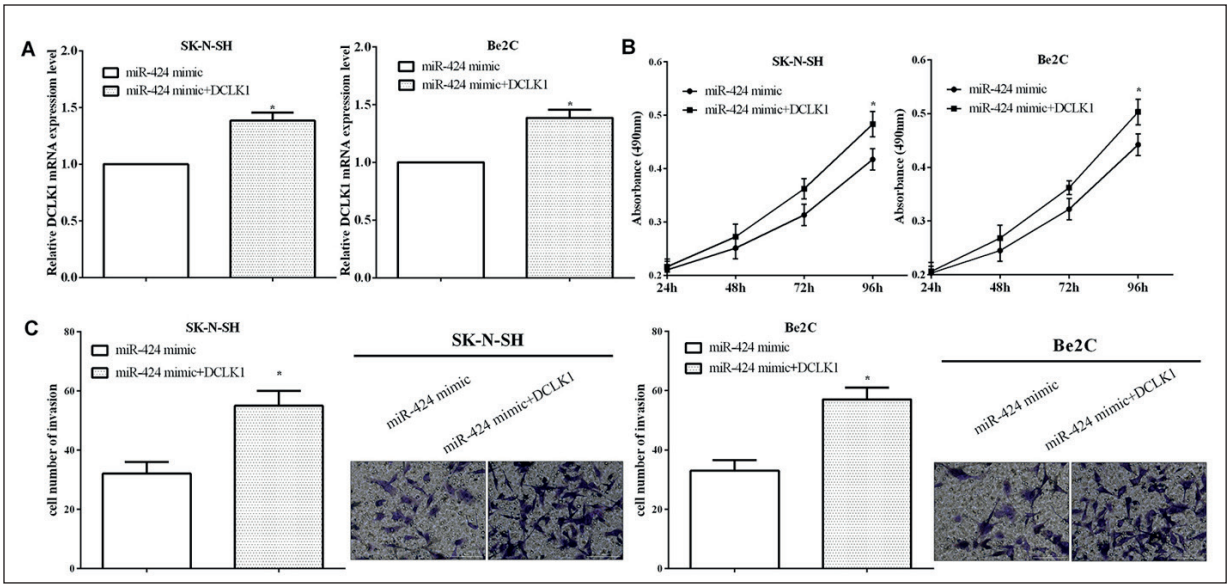

Figure 5. DCLK1 partially reversed the functions of miR-424 on cell viability and invasion**. A,** RT-qPCR was performed to assess the transfection efficiency of transfected DCLK1 overexpression plasmid or control in cells transfected with miR-424 mimic. **B,** DCLK1 overexpression plasmid enhanced the viability in miR-424 mimic-transfected SK-N-SH and Be2C cells. **C,** Invasive ability was improved by transfecting DCLK1 overexpression plasmid in SK-N-SH and Be2C cells (200×).

cell apoptosis[27,28]. Consistent with all the findings, we discovered that miR-424 was lowly expressed in neuroblastoma tissues and cell lines in comparison with the corresponding normal tissues and normal cell line. It was discovered that miR-424 inhibited cell viability in endometrial cancer[29]. Similarly, miR-424 suppressed cell proliferation and metastasis of hepatocellular carcinoma[30]. We also discovered that the upregulation of miR-424 inhibited cell viability and invasion, while the downregulation of miR-424 improved cell viability and invasion in neuroblastoma. Our results elucidated that miR-424 suppressed the EMT ability of neuroblastoma, which was consistent with the findings of endometrial carcinoma[31].

DCLK1 acted as a recurrence predictor and was overexpressed in salivary gland malignancies[32]. The overexpression of DCLK1 was related to poor prognosis of several malignant tumors, such as hepatocellular carcinoma[33,34]. DCLK1 emerged as a CSC marker in bladder carcinomas and was associated with tumor aggressiveness[35]. In addition, DCLK1 promoted the EMT ability of colorectal cancer *via* PI3K/Akt/NF-κB pathway [24]. Consistent with all the findings, we found that the expression of DCLK1 was higher in neuroblastoma tissues and cells than non-tumor tissue samples and normal cell. Moreover, DCLK1 was mediated by miR-424, which can regulate cell viability, migration and invasion in basal-like breast cancer[36]. Similarly, miR-424 suppressed cell metastasis and EMT abilities *via* directly targeting DCLK1 in ovarian clear cell carcinoma[37]. Our data were consistent with the above results, miR-424 regulated the expression of DCLK1 by directly binding to the 3'-UTR of DCLK1 mRNA in SK-N-SH and Be2C cells. Moreover, the overexpression of DCLK1 improved the viability and invasion in miR-424 mimic-transfected cells, which validated that DCLK1 partially reversed the functions of miR-424 in neuroblastoma.

Conclusions

All together these results revealed that the differential expression of miR-424 between the tumor and surrounding tissues may be related to the metastasis of neuroblastoma patients. MiR-424 suppressed cell viability, invasion, and EMT *via* targeting the 3'-UTR of DCLK1 mRNA in SK-N-SH and Be2C cells. These data provide further insight into the study of neuroblastoma. MiR-424 might be a useful marker and potential therapeutic target for neuroblastoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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