ALDOB Promotes Neuroblastoma Metastasis by Affecting EMT Pathway and is a Potential Prognostic Factor

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Research Article

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Abstract

**Background** Aldolase B (ALDOB) is a member of the aldolase family, which is the fourth enzyme in glycolysis process. In recent years, the non-enzymatic effects of some glycolytic enzymes have been reported to promote the formation of several human tumors, but the non-enzymatic action of ALDOB in neuroblastoma (NB) remains unclear. This study aims to explore the non-enzymatic effect of ALDOB in neuroblastoma.

**Methods** We used immunohistochemistry to examine 63 patients tissue microarray samples and 3 pairs of lymph node metastases and the primary tissue samples, and evaluated the relationship between ALDOB expression level and clinical characteristics. We then analyzed the public datasets of NB based on microarray to verify the immunohistochemistry results. In addition, we conducted *in vitro* experiments on SK-N-BE(2) and SH-SY5Y cell lines to explore the molecular mechanism.

**Results** Immunohistochemistry indicated ALDOB is significantly associated with INSS stage and tumor metastasis in NB, public dataset analysis showed ALDOB is related to NB patient survival remarkably. *In vitro* experiments displayed silencing ALDOB may inhibit the cell migration by epithelial-mesenchymal transition (EMT) pathway.

**Conclusions** Our finding demonstrated that ALDOB can affect the metastasis of NB by EMT pathway and may be a potential target for neuroblastoma therapy in the future.

**Background**

Neuroblastoma (NB) is the most frequently seen childhood malignancy, with 25–50 cases per million population [1], accounting for more than 7% of childhood malignancies and a mortality rate of 15% [2]. Neuroblastoma is a neuroendocrine tumor, may originate from any neural crest part of the sympathetic nervous system, the commonest location is the adrenal glands, but they can also occur in the neck, chest, abdomen and pelvic nerve tissue[3]. Many patients have tumor metastasis at the time of diagnosis[4], about half of the children who had metastasized developed distal metastases to bone marrow, cortical bone, non-adjacent lymph nodes, and liver[5], mortality in patients with bone metastases occurred in more than 93% of patients [6]. For more than a decade, the clinical staging and risk classification for neuroblastoma has dramatically improved[7], which is beneficial for guiding treatment. However, the prognosis with metastasis is poor and the chance of recurrence with neuroblastoma is still high[8]. Therefore, understanding the molecular mechanism of NB invasion and metastasis and finding biomarkers that lead to NB metastasis and relapse are of great significance for monitoring NB progression and guiding diagnosis and therapy.

ALDOB is a member of the aldolase family, which is the fourth enzyme in glycolysis process, including ALDOA, ALDOB and ALDOC three subtypes. The association between aerobic glycolysis (oxyglycolysis) and tumorigenesis has been known for decades after the "Warburg hypothesis" (also termed the "Warburg effect") was proposed by Otto Warburg, a German scientist[9]. However, in recent years, lots of studies
have suggested that some glycolytic enzymes not only function in the glycolytic pathway[10], but also have extra non-glycolytic enzyme functions in many ways such as; the aldolase family has been reported to activate WNT signaling in a GSK-3-dependent mechanism that influences the course of cancer development [11]. The non-enzymatic action of ALDOA, one of the isoenzymes of ALDOB, has been demonstrated in a variety of tumorigenesis[12–14]. ALDOA has been suggested to promote lung cancer metastasis through prolyl hydroxylases (PHD)-mediated stabilization of HIF-1 and subsequent activation of MMP9[15]. It has also been reported that the expression of ALDOA can be used to predict poor prognosis of pancreatic cancer, partly due to its role in regulating E-cadherin expression[12]. ALDOB is mainly overexpressed in liver [16] and large intestine [17] tissue samples. ALDOB has been shown to promote tumor cell proliferation in liver metastasis of colon cancer [16] and it is a key molecule that promotes the metastasis of colorectal cancer (CRC) by regulating epithelial-mesenchymal transition (EMT) pathway[17]. However, the non-enzymatic role of ALDOB in neuroblastoma has not been studied. Therefore, a series of molecular biological studies were conducted on the relationship between ALDOB expression and the development of neuroblastoma. We predict based on tissue microarray immunohistochemistry and public databases that ALDOB will promote the metastasis of neuroblastoma, leading to poor prognosis, and through in vitro experiments such as wound healing assay, transwell experiment, western blot, etc., it was verified that silencing ALDOB can inhibit the proliferation and metastasis of NB cells by inhibiting EMT pathway.

**Methods**

**Clinical specimens**

The clinical samples used in this research were primary pediatric NB patients histologically diagnosed in Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine from September 2012 to February 2015. Tumor samples from all the patients in the study were surgically removed. All specimens were first quick-frozen in liquid nitrogen and then stored at -80°C for tissue microarray analysis. The research and consent procedure have been validated by the Clinical Committee of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine.

**Tissue microarray (TMA) preparation and immunohistochemistry (IHC)**

Separate a small part of the tissue specimen and shape it in a customized mold for chip production. Then fix overnight in 4% paraformaldehyde, after that tissue blocks were embedded in paraffin in a prepared array. Then the sample was sliced (5 μm) and adhered to a poly-L-lysine coated glass slide for immunohistochemical staining, which was performed as previously described [18, 19], using specific antibody against ALDOB (1:100 dilutions, Cat.18065-1-AP, Proteintech Group, Chicago, USA). Without knowing the clinicopathological characteristics of the tumor, the immunoreactivity in tissue sections was observed under three random microscopes, and then evaluated by three pathologists. Differences in scoring were discussed until consensus was reached. The tissue sections were scored under an optical microscope according to the degree of staining (0~3 points were negative staining, light yellow, light
brown, dark brown) and the positive range (1~4 points were 0~25%, 26~50%, 51~75%, 76~100%). The two scores are then multiplied to get the final score for comparison. Finally, the intensity of staining was divided into four levels according to the final score: "0" means negative expression (the final score was 0), "1" means weak positive expression (the final scores were 1~4 points), "2" means moderate expression (the final scores were 5~8 points), and "3" means strong expression (the final scores were 6~12 points).

**Public database analysis**

Four publicly available data sets were selected from the R2: Microarray analysis and Visualization platform (http://r2.amc.NL): Oberthuer-251-custom-ampexp255, Maris-101-custom-u95a, NRC-283-rma_sketch(bc)-huex10t and Asgharzadeh-249-Custom-huex10Tx, which contained information on the clinical characteristics and prognosis of patients with neuroblastoma, the target genes were obtained and selected for analysis. All Kaplan-Meier analyses were performed online, and the optimal $P$ value and corresponding cutoff value for separating the high-expression group from the low-expression group were selected by the median.

**Cell Culture**

Human neuroblastoma cell lines SK-N-BE (2) and SH-SY5Y were acquired from American Type Culture Collection (Manassas, USA) and maintained in a mixture of Eagle basic medium and Ham nutrient mixture F12 1:1, 10% fetal bovine serum (FBS), both from Gibco, USA, and cultivated in a 5% CO2 moist incubator at 37°C.

**Production and infection of lentivirus**

Lentivirus particles expressing shRNA sequence targeting human ALDOB gene (5'‐CCTATTGTGAACGAGGTATA‐3') were designed and constructed, and shRNA particles expressing nonsense sequences were used as negative control (shNC). Lentivirus particles expressing shALDOB or shNC were added to 6-well plates with SH-SY5Y and SK-N-BE(2) growing to 30%-60% confluency, and the infection complex (MOI) was 20. After 48 hours of transfection, fluorescent microscope (Olympus) was used to detect the transfection efficiency of stably expressed green fluorescent protein (GFP) cells, and 1 g/ml puromycin (Cat. #ST551, Beyotime, China) was used to screen the transfected cells, and stable transfected cell lines were obtained after 48 hours of continuous treatment. After screening, some cell RNA was collected to confirm the ALDOB mRNA level by qPCR, and the extracted protein was detected by Western blot.

**RNA extraction, reverse transcription and real-time PCR**

Total RNA was extracted from cells by Trizol Reagent (Thermo Fisher Scientific, USA). Reverse transcription was executed using PrimeScript RT kit (TaKaRa Biotech, Shanghai, China), bio-RAD Labs (Hercules, CA) perform the Real-time PCR with SYBR Green PCR Master Mix (TaKaRa Biotech), following the manufacturer's protocol. The expression of ALDOB mRNA was normalized to GAPDH and used the 2-
ΔΔCt method for relative quantitative. We repeated three independent porous experiments for statistical analysis. Primer sequences used in this research are

ALDOB forward: 5′-AGCTATGGCCACCGTAACAG-3′,
ALDOB reverse: 5′-GGGCTTTTGGTAGAGGGCAA-3′
GAPDH forward: 5′-TGTGGGCATCAATGGATTTGG-3′
GAPDH reverse: 5′-ACACCATGTATTCCGGGTCAT-3′

Cell viability assay

Stably transfected SH-SY5Y and SK-N-BE(2) cells were inoculated in 96-well plates at a density of 5×10^3/ well and cultured in 100 microliters of MEM/ F12 containing 10% FBS for 4 days. Cell viability was detected by cell counting kit (CCK-8) (Dojindo Molecular Technologies Inc., Shanghai, China) every 24 hours. The specific procedure was to add 10 microliters of CCK-8 solution to each well, and then to measure the absorbance value (OD) of each well at 450nm and 630nm after incubation at 37°C for 1 hour.

Colony formation test

The stably transfected SH-SY5Y and SK-N-BE(2) cells were inoculated into a 6-well plate at a density of 1000 cells per well. The cells were cultured in a cell incubator for 14 days, and the liquid was changed every three days, the cell state and the size of the clones was observed under the microscope. When each clone was larger than 20 cells, the culture medium was removed, paraformaldehyde fixed the cell, then crystal violet was used to stain cells. Next the cells were washed with PBS, and photographs were taken.

Determination of wound healing

SH-SY5Y and SK-N-BE(2) cells were cultured in a six-well plate and grown to confluence 90%. Artificial wound marks were created by scraping a single layer of converging cells with a pipette suction head and incubating the cells with a sera free medium for 48 hours. After PBS rinsing, photographed transplanted cells at 0 and 48 hours, and calculated the migration distance during wound healing was.

Cell invasion

Cell invasion assay was performed in transwell chamber using Matrigel (BD, Biosciences, CA). 6×10^3 cells in the matrix medium with no fetal bovine serum were incubated in Transwell upper chamber with matrix gel. In the lower chamber, there was the cell culture medium with 10% FBS. Then cultivated at 37°C for 48 hours. After 48 hours, 4% paraformaldehyde fixed the cells on the submembrane surface of the compartment and then stained with crystal violet.

Western blot analysis
Lyse cells on ice with RIPA buffer which contains PMSF (Cat. # ST506, Beyotime, China) (Cat. # P0013B, Beyotime, China) for 30 minutes, centrifugation at 20,000 g for 15 minutes, collected the supernatant, and used the BCA kit (Thermo Scientific, Rockford) to test the protein concentration. Gel electrophoresis used 10% SDS-PAGE gel and contains the same amount of protein per lane. Protein transfer used PVDF membranes (Millipore, Sigma Aldrich, USA). Then took the 5% BSA TBST (Tris buffered saline, pH 7.6, 0.1% Tween®20) to seal the PVDF membranes at room temperature for 1 hour, followed by primary incubation at 4°C overnight. TBST washed the membrane 3 times for 5 minutes each time, then incubated with TBST and an appropriate secondary antibody at room temperature for 30 minutes. Next, TBST wash the membrane 3 times for 5 minutes each time. Proteins are visualized by electrochemical luminescence (ECL). The primary antibodies of ALDOB (catalog number AB75751) and β-actin (catalog number AB8227) were purchased from Abcam. E-cadherin, Vimentin, N-cadherin, Claudin-1, Slug, and ZEB1 were purchased from Cell Singling Technology (CST). Image J (X64, v. 2.1.4) software for quantitative analysis of imprinting.

Statistical analysis

The experimental data were showed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism8 software (GraphPad Software, Inc. La Jolla, USA). Between the two groups, we used Student's T-test to estimate the differences, and between the three groups, we used and one-way ANOVA. Kaplan-Meier analysis was used for survival assessment, and survival differences was analyzed by log-rank test. $P<0.05$ was considered as a statistically significant difference. Significance was displayed as: * $P<0.05$, ** $P<0.01$, and *** $P<0.001$

Results

**ALDOB is significantly associated with INSS stage and tumor metastasis in NB**

Firstly, we stained the tissue chips from patients with 63 primary neuroblastomas using immunohistochemical (IHC) staining. We observed that ALDOB was predominantly located in the cytoskeleton of neuroblastoma cells, with no staining in adjacent non-tumor and normal tissues (Fig. 1a, b). We next analyzed the expression of ALDOB and found that ALDOB was expressed in 56 out of 63 (88.9%) primary neuroblastomas. Among them, 24 patients (38.1%) showed weak expression of ALDOB, 27 patients (42.9%) showed moderate expression, and 5 patients (7.9%) showed strong expression (Fig. 1c). Subsequently, we analyzed whether the expression level of ALDOB in tissue samples was significantly correlated with clinical characteristics, and one-way ANOVA showed that the expression level of ALDOB was significantly correlated with tumor stage ($P=0.0223$). ALDOB expressed in International Neuroblastoma Staging System(INSS) stage 3, 4 neuroblastoma tissues were significantly higher than those in the INSS stage 1, 2 neuroblastoma tissues (Fig. 1d). The expression level in patients with both lymph node metastasis and bone marrow metastasis was obviously higher than that of patients without
metastasis \( (P = 0.0202) \) (Fig. 1e), but it was not related to gender, histopathology diagnosis or differentiation degree (Table 1).

We then stained paired samples of both the lymph node tissue and primary tumor tissue of three patients with lymph node metastasis (Fig. 1f). We found that ALDOB expression in lymph node tissues was higher than that in primary tumor tissues significantly \( (P = 0.0232) \) (Fig. 1g). Together, the above mentioned results indicate that overexpression of ALDOB in neuroblastoma patients may be associated with the metastasis of neuroblastoma.

**ALDOB is significantly related to NB patient survival and is a potential prognostic factor for NB**

To further verify the role of ALDOB in neuroblastoma, we analyzed the expression of ALDOB in four different public datasets of tumor neuroblastoma (Oberthuer-251-custom-AMPexp255, Maris-101-custom-U95a, CNC-283-RMA-Sketch(BC)-Huex10T and Asgharzadeh-249-custom-huex10Tx) online on R2 platform. The KM curves reflecting the relationship between ALDOB expression and overall survival rate (OS) were drawn. Patients with high ALDOB mRNA level showed poor OS with \( p \) values of 4.2E-05, 0.034, 0.029 and 0.014 (Fig. 2a, b, c, d), respectively. From the above, our data suggested that a high level of ALDOB expression correlates with a poor prognosis in neuroblastoma.

**Aldob Knockdown Reduces Invasion And Migration Of Nb Cells**

SH-SY5Y cells with high ALDOB expression (Fig. S1 shows this in more detail, see additional file 1) were selected for transfection with shALDOB and non-silenced shNC lentivirus to clarify the biological function of ALDOB in NB; SK-N-BE(2) cells with stable knockdown were also constructed for verification. Through fluorescence microscope observation, it was found that more than 80% of the cells expressed GFP, showing a high efficiency of infection (Fig. 3a). As shown in Fig. 3b and Fig. 3c, mRNA and protein expression levels of ALDOB in SH-SY5Y cells infected with shALDOB were significantly lower than those of cells infected with shNC. Cell viability of SH-SY5Y and SK-N-BE (2) were detected by CCK-8 and colony formation tests. The results showed that cell proliferation of shALDOB group was lower than that of shNC group (Fig. 3d, e), which proved the effect of ALDOB knockdown on cell proliferation *in vitro*. Then, in order to explore the effect of ALDOB knockdown on migration and invasion of SH-SY5Y and SK-N-BE (2) cells, wound healing test and Transwell Matrigel assay were performed. The results were shown in Fig. 4a and Fig. 4b. The scratch closure rate of SH-SY5Y cells transfected with shALDOB was lower than that of the control group and the decrease of ALDOB expression significantly inhibited the invasion of SH-SY5Y cells \( (P < 0.001) \). These findings suggested that ALDOB may promote NB cells migration and invasion.

**Aldob Knockdown Inhibits Emt Pathway**
To further understand the molecular mechanism of ALDOB knockdown on NB cell migration and invasion, we performed western blot experiments on SH-SY5Y cells. As the Fig. 5 shows, down-regulation of ALDOB enhances the expression of E-cadherin, claudin-1, while the expression of Vimentin, N-cadherin, Slug, and ZEB1 decreased. These findings suggest that ALDOB may promote the metastasis of neuroblastoma by regulating the EMT pathway and affect the prognosis.

Discussion

NB is one of the most common pediatric neuroectodermal solid tumors in infancy and childhood. It can occur in the tissues of the sympathetic nervous system, usually in the paravertebral ganglia or adrenal medulla, and detectable masses can occur in the chest, neck, pelvis, and abdomen[1]. NB accounts for 7% of all pediatric cancers, and the 5-year survival rate of low-risk people is > 95%, while the 5-year survival rate of high-risk patients is only 40%[1]. About 65% of cases have metastasized at the time of diagnosis[20]. Surgery alone or in combination with minimal treatment regimens can improve survival in low-risk patients, but in most cases, surgery is not an option because the disease is clearly metastatic at the time of diagnosis. In many stage III and IV NB patients, recurrence occurs immediately after chemotherapy, and resistance to chemotherapy is also associated with organ metastasis. In neuroblastoma, the main cause of death is associated with metastasis. Preventing recurrence and reducing metastasis remains a major clinical challenge in treating the NB.

The role of glycolysis in tumorigenesis and development has long been recognized, glycolytic enzymes control the progress of glycolysis, and these metabolic changes regulate the biosynthesis and energy production of glucose, amino acids, and fatty acid-dependent metabolites[21, 22]. However, in recent years, studies have found that some glycolytic enzymes have extra non-enzymatic action in tumorigenesis and development[10], which brings us new research directions. To name only a few, pyruvate kinase M2 (PKM2) promotes the migration and drug resistance of colon cancer cell by regulating STAT3 signaling[23, 24]. In non-small cell lung cancer, enolase (ENO1) promotes tumor growth, invasion and migration through the pseudo-drug-mediated PI3K/AKT pathway[25]. In particular, aldolase interacts with a variety of proteins independent of glycolytic enzymes, including F-actin[26], ARNO[27] and Tubulin[28] and is essential for cancer cells to promote their proliferation [29]and metastasis [30] through non-enzymatic pathways. In this research, we have 63 patients of primary neuroblastoma tissue microarray and 3 pairs of samples of lymph node metastases and the primary tissue samples from different patients with lymph node metastasis with immunohistochemical staining were analyzed, we found that the expression level of glycolytic enzyme ALDOB in cancerous tissue is associated with tumor metastasis and stages. Further a public database through the analysis of the R2 platform for verification, found that glycolytic enzyme ALDOB will affect the survival of patients with neuroblastoma and the higher its expression, the more unfavorable to the survival of patients. Therefore, we suspect ALDOB can be used as a prognostic marker of NB. Our series of \textit{in vitro} experiments also demonstrated that silencing ALDOB expression can inhibit the metastasis and invasion of neuroblastoma cells.
The EMT pathway is a key event that promotes the dissociation, invasion, and metastasis of cancer cells[31]. Loss of E-cadherin is a marker of EMT and is usually associated with high tumor stage and metastasis. Several transcription factors, such as Slug and ZEB1, have been involved in transcriptional inhibition of E-cadherin and induction of EMT[32]. Since ALDOB knockdown suppressed the NB cell proliferation, metastasis and invasion, EMT was one of the critical processes to enhance the invasion and metastasis of tumor, we speculated whether ALDOB played a role on neuroblastoma by inducing EMT. As we expected, the down-regulation of ALDOB expression resulted in an increase in epithelial marker proteins (E-cadherin, Claudin-1) and a decrease in mesenchymal marker proteins (N-cadherin, Vimentin, Slug, and ZEB1), displaying that silencing of ALDOB may cause the reversal of EMT progress in neuroblastoma. However, the non-enzymatic mechanism of glycolytic enzyme ALDOB in neuroblastoma has not been discovered and proven so far.

Although this study brings us initial revelation about the role and clinical significance of ALDOB in NB, there are still lots of limitations to be further discussed. First of all, the number of patients included in this research is not large enough, especially the lack of samples of stage 4S patients. Second, we'd better do an overexpression experiment to rule out the off-target effect. Thirdly, in vivo experiments are necessary to strengthen the evidence that ALDOB promotes the metastasis of neuroblastoma.

To sum up, this study confirmed that the high expression of ALDOB is an independent poor prognostic factor of OS in NB. It plays an important part in the progression and metastasis of neuroblastoma by inducing the EMT pathway. ALDOB is expected to become a new predictor for NB to guide novel clinical therapy. It may help clinicians to identify patients with a high risk of early recurrence. These patients should be recommended for close follow-up and appropriate adjuvant therapy to prolong survival.

**Conclusions**

There is still a need to find potential biological targets for NB. Our study found for the first time that high expression of ALDOB will promote the metastasis of NB by regulating the EMT pathway and affect the prognosis, indicating a critical tumor promoting role in NB progression.

**Abbreviations**
| Abbreviation | Description |
|--------------|-------------|
| ALDOB        | aldolase B   |
| ALDOA        | aldolase A   |
| ALDOC        | aldolase C   |
| NB           | neuroblastoma|
| EMT          | epithelial-mesenchymal transition |
| WNT          | wg (wingless) and int |
| GSK          | glycogen synthase kinase |
| PHD          | prolyl hydroxylases |
| HIF          | hypoxia-inducible factors |
| MMP          | matrix metallopeptidase |
| CRC          | colorectal cancer |
| ATCC         | American Type Culture Collection |
| TMA          | tissue microarray |
| IHC          | immunohistochemistry |
| R2           | Genomics Analysis and Visualization Platform |
| MOI          | multiplicity of infection |
| GFP          | green fluorescent protein |
| GAPDH        | glyceraldehyde-3-phosphate dehydrogenase |
| MEM          | modifiedagle medium |
| FBS          | fetal bovine serum |
| CCK-8        | cell counting kit 8 |
| PBS          | phosphate buffered saline |
| PMSF         | phenylmethanesulfonylfluoride |
| BCA          | bicinechonic acid |
| SDS-PAGE     | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| PVDF         | polyvinylidene difluoride |
| BSA          | bovine serum albumin |
| TBST         | tris-buffered saline and tween20 |
| ECL          | electrochemical luminescence |
| Acronym | Description |
|---------|-------------|
| ZEB1    | zinc finger E-box-binding homeobox 1 |
| RT-PCR  | reverse transcription polymerase chain reaction |
| ANOVA   | analysis of variance |
| INSS    | International Neuroblastoma Staging System |
| PKM2    | pyruvate kinase M2 |
| STAT3   | signal transducer and activator of transcription 3 |
| ENO1    | enolase 1 |
| P13K/AKT| phosphatidylinositol 3-kinase /protein kinase B |
| ARNO    | cytohesin-2 |
| GNB     | ganglioneuroblastoma |

### Declarations

**Ethics approval and consent to participate**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Clinical Committee of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine (Approval No. XHEC-D-2016-037).

**Consent for publication**

Not applicable

**Availability of data and materials**

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

**Competing interests**

The authors declare that they have no conflict of interest.

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**Authors’ contributions**
Conception and design: Z-RL, Z-XW and Y-MW. Implementation of the experiment: X-XH, Y-YG, K-C, H-PD and J-H. Analysis and interpretation of the data: X-XH and Y-YG. Drafting of the article: X-XH. Critical revision of the article for important intellectual content: Z-RL, Z-XW and Y-MW. Final approval of the article: Z-XW. The authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures
Figure 1

The ALDOB expression level in NB clinical samples. a General observation of ALDOB expression in TMA of 63 neuroblastoma samples. b Four grades of IHC staining expressed by ALDOB in NB samples (0 for negative, 1 for weak, 2 for intermediate, and 3 for strong). c The ratio of 4 grades in 63 NB samples. d Clinical staging in TMA of 63 neuroblast tumor samples based on ALDOB expression, and P values were obtained using one-way ANOVA. e Tumor metastasis in TMA of 63 neuroblast tumor samples based on
ALDOB expression, and P values were obtained by t test. f The expression levels of ALDOB in three pairs of lymph node metastases. Tissue staining under 40x microscope showed lymph node tissue on the left and primary tumor tissue on the right, with three different patients from top to bottom (sample1, sample2, sample3). g Expression of ALDOB in lymph node tissue and primary tissue of patients with lymph node metastasis, and P value was shown by Student's T test. The values were mean ±SEM, and the statistical significance was *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2
The prognostic value of ALDOB in four NB databases. a Kaplan-Meier analysis of OS of the Oberthuer database based on ALDOB expression, and the log-rank test P value was shown (n = 251). b Kaplan-Meier analysis of OS of the Maris Database based on ALDOB expression, which shows the log-rank test P value (n = 97). c Kaplan-Meier analysis of OS of the NRC database based on ALDOB expression, and P value of log-rank test (n = 283) was indicated. d Kaplan-Meier analysis of OS of the Asgharzadeh database based on ALDOB expression, and the log-rank test P value (n = 247) was displayed. e The ALDOB expression levels in stage I, III and IV tumors of Oberthuer database. The values were mean ±SEM, and the statistical significance was *P< 0.05, **P<0.01, *** P< 0.001
Figure 3

The silencing efficiency of ALDOB and the effects of ALDOB knockdown on NB cell proliferation. a The silencing efficiency of ALDOB using shRNA lentivirus infection in the SH-SY5Y cell line. Bright field and fluorescence micrographs of SH-SY5Y cells were taken 48 h after lentivirus infection. b ALDOB expression in SH-SY5Y cells infected with shALDOB or negative control (NC) shRNA lentivirus was determined by real-time PCR c The ALDOB expression in SH-SY5Y cells infected with shALDOB or
negative control (NC) shRNA lentivirus was determined by Western blot. d An analysis of cell viability of SH-SY5Y and SK-N-BE(2) cells transfected with shALDOB based on CCK-8 assay. e Micrographs and statistical analysis of SH-SY5Y and SK-N-BE(2) cell colonies after transfection with shALDOB or shNC. The values were mean ±SEM, and the statistical significance was *P< 0.05, **P<0.01, *** P< 0.001

**Figure 4**

The effects of ALDOB knockdown on NB cell migration and invasion in vitro. a Wound healing test of SH-SY5Y cells after transfection with shALDOB or shNC was performed in vitro to investigate the migration ability. b SH-SY5Y and SK-N-BE(2) were transfected with shALDOB or shNC to conduct Transwell test to assess the invasion ability, and the invasion was quantified by counting the cells in 3 random microscope fields. The values were mean±SEM, and the statistical significance was *P< 0.05, **P<0.01, *** P< 0.001
Figure 5

The effect of ALDOB knockdown on the regulation of migration and invasion in SH-SY5Y cells, as confirmed using western blot analysis. β-actin was used as the internal control. The values were mean ±SEM, and the statistical significance was *P< 0.05, **P<0.01, *** P< 0.001

Supplementary Files

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- table1.pdf
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