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Aminoacylase 1 (ACY-1) Mediates the Proliferation and Migration of Neuroblastoma Cells in Humans Through the ERK/Transforming Growth Factor β (TGF-β) Signaling Pathways

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Aminoacylase 1 (ACY-1) is a cytosolic enzyme that catalyzes amino acid deacylation and has been reported to participate in various human diseases. However, the role and mechanism of ACY-1 in neuroblastoma (NB) are not completely understood. The aim of this study was to elucidate the role of ACY-1 in NB.

Material/Methods: Overexpression and knockdown of ACY-1 in human NB cells were performed, and the transfection efficiency was assessed through fluorescence microscopy, real-time PCR, and western blotting. The effect of ACY-1 on tumorigenesis and metastasis was determined by cell counting, colony formation, wound healing, flow cytometry, and transwell invasion assays *in vitro*, and the signaling pathway was examined using western blotting.

Results: ACY-1 overexpression inhibited proliferation and induced apoptosis in human NB cells. ACY-1 inhibited the colony formation ability, migration, and invasion of SH-SY5Y cell lines. Moreover, the ERK1/2 and TGF-β1 signaling pathways were more active when ACY-1 was overexpressed in NB cells. However, the knockdown of ACY-1 in SH-SY5Y cell lines showed the opposite effects.

Conclusions: ACY-1 regulates the proliferation, migration, and invasion of human NB cells through the ERK1/2 and TGF-β1 signaling pathways, implying that ACY-1 may serve as a therapeutic target for patients with NB.

MeSH Keywords: Calcium Signaling • Canavan Disease • Neuroblastoma

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Background

Neuroblastoma (NB) is one of the most common extracranial solid tumors in children, accounting for 7% of malignancies and 15% of mortality in childhood cancer worldwide [1,2]. Many investigations have shown that over 50% of NBs occur under the age of 2 years [3,4]. In recent years, the incidence of NB has increased quickly, and the 2-year survival of children after diagnosis is only 38% [5]. The tumor mainly manifests as masses and appears in the sympathetic nervous system tissues [6]. NB is classified based on differentiation degree as undifferentiated, poorly differentiated, and differentiated subtypes. Few patients with NB develop benign tumors, with minimal or no systemic treatment [7]. NB has a wide range of biological and clinical characteristics, including heterogeneity, high malignancy, poor prognosis, and easy metastasis, which are key indicators for predicting prognosis. Presently, the main clinical treatments for NB are surgery, radiotherapy, chemotherapy, and immunotherapy [8,9]. However, NB treatment remains unsatisfactory and the prognosis is poor. Hence, it is crucial to understand the genetic and pathogenic mechanisms of NB, which will greatly improve the response of the disease to therapy.

Aminoacylase 1 (ACY-1) is a cytosolic enzyme that catalyzes amino acid deacylation during the degradation of proteins within cells [10]. In eukaryotic cells, most cellular proteins are acylated at the N-terminus, which might affect the function and stability of the protein through cooperative or post-translational modification [11]. The main functions of ACY-1 are to accelerate the hydrolysis of N-acetylated peptides, especially N-acetylated neutral aliphatic amino acids, and participate in protein synthesis through the release of free amino acids. ACY-1 is expressed mostly in the kidney, followed by the liver, brain, skeletal muscle, pancreas, and other organs at lower levels [12, 13]. Studies have shown that ACY-1 plays a crucial role in the pathogenesis of numerous tumor diseases, such as colorectal, liver, and small cell lung cancers and renal cell carcinoma [14,15]. In liver cirrhosis, ACY-1 is associated with protein metabolism, participates in protein degradation, and serves as a valuable candidate biomarker for diagnosing the disease [16]. ACY-1 is involved in the disease development and prognosis of colorectal cancer and it is related to lymph node metastasis, vascular invasion, and shorter disease-free survival [17]. However, current research on the specific function and mechanism of ACY-1 in tumor proliferation and migration is still limited. One study indicated that ERK1/2 activation and TGF-β expression may be involved in this process [18].

In the present study, we systematically explored the effects of ACY-1 on the proliferation, migration, and invasion of human NB cells. We further investigated the signaling pathways of ACY-1 in NB cells involved in tumorigenesis.

Material and Methods

Cell line culture

We purchased the human NB cell line, SH-SY5Y cells, from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were cultured in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). The SH-SY5Y cells were incubated at 37°C with 5% CO_2 in a humidified incubator.

Overexpression and knockdown of ACY-1

siRNA interference was used to knock down the expression of ACY-1 in the SH-SY5Y cells; the negative control siRNA-scramble was synthesized by Invitrogen. Briefly, SH-SY5Y cells were transfected with siRNA-ACY-1 or siRNA-scramble in transfection reagent for 48 h, according to the manufacturer's protocol. We used recombinant adenovirus to overexpress ACY-1; human ACY-1 complementary deoxyribonucleic acid was subcloned and validated by sequencing, and the empty vector (mock group) or ACY-1 construct was transfected into SH-SY5Y cells using the transfection reagent. After 48 h, the expression of ACY-1 in SH-SY5Y cells was determined by fluorescence microscopy, real-time PCR, and western blotting to assess the transfection efficiency.

Real-time PCR

Total RNA was extracted from the SH-SY5Y cells treated as mentioned above, following which cDNA was synthesized via reverse transcription according to the manufacturer's protocol (Takara, Japan). The cDNA was used to conduct real-time PCR, and the following primers were used:
ACY-1 forward: 5'-GGCTGCATGAGGCTGTGTT-3',
reverse: 5'-CTTGGCACTGGTTGGGATG-3';
and Actin forward: 5'-TGGCACCCAGCACAATGAA-3',
reverse: 5'-CTAAGTCATAG TCCGCCTAGAAGCA-3'.
The mRNA expression of ACY-1 was calculated with the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation and colony formation assay

We used a cell counting kit-8 to determine cell proliferation. Briefly, the SH-SY5Y cells were treated as mentioned above and cultured in a 96-well plate. The optical density was determined 24 h later using a microplate reader. For the colony formation assay, the cells were seeded into 6-well plates and incubated for 1 week. Subsequently, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. Next, 0.1% crystal violet was added to the plates, which were further incubated at room

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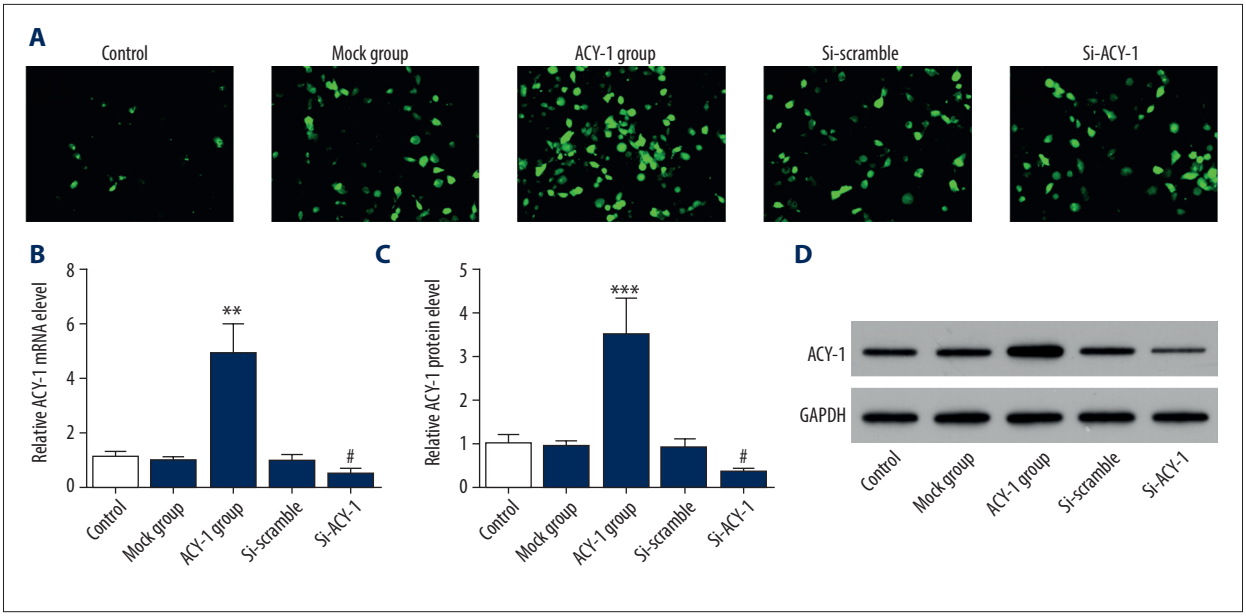

Figure 1. Transfection efficiency of Aminoacylase 1 (ACY-1) in SH-SY5Y cells. (**A**) ACY-1 expression was determined in SH-SY5Y cells through fluorescence microscopy. (**B**) ACY-1 mRNA expression was examined through real-time PCR. (**C, D**) ACY-1 protein expression was determined through western blotting. * Compared to mock group; # compared to siRNA-scramble group.

temperature for 20 min. Finally, the cells were washed with PBS, dried, and imaged.

Wound healing assay

The SH-SY5Y cells were scraped off the 6-well plates using 200-µL pipette tips. Next, the cells were washed with PBS and incubated for 12 h and 24 h. The migration distance of the cells was then observed under a phase-contrast microscope at 0, 12, and 24 h and measured using Image J software.

Migration and invasion assays

The transwell assay was used to detect cell migration and invasion ability. The SH-SY5Y cells were seeded onto the upper chamber coated with or without Matrigel (BD Biosciences, San Jose, CA, USA) and cultured in serum-free RPMI 1640 medium. The RPMI 1640 medium supplemented with 10% FBS was added as a chemoattractant to the lower chamber. After incubation for 24 h, the cells on the upper surface of the membrane were removed and those that migrated to the underside of the membrane were fixed with 4% paraformaldehyde for 30 min. The cells were stained with 0.1% crystal violet for 10 min and enumerated under a microscope.

Flow cytometry

Flow cytometry was used to determine the effect of ACY-1 on apoptosis using an apoptosis staining kit (BD Biosciences). We seeded the cells onto 6-well plates and cultured them for 24 h, followed by washing with PBS and incubation with Annexin V for 10 min in the dark. The cells were washed again, immediately stained with propidium iodide, and detected using a flow cytometer (Accuri C6, BD Biosciences).

Western blotting

The SH-SY5Y cells were treated as mentioned above, and proteins were extracted with RIPA lysis buffer (Beyotime, Shanghai, China). The following antibodies were used: anti-GAPDH, anti-Bax, anti-ACY-1, anti-phospho-ERK1/2, anti-ERK1/2, anti-TGF-β, anti-Bcl-2, and anti-Bad (Cell Signaling Technology, Danvers, MA, USA). The samples were visualized using an enhanced chemiluminescence system, and the bands were analyzed with the Image J software.

Statistical analysis

Data are presented as the mean±SD for 5 independent experiments. The statistical differences between different groups were analyzed through one-way analysis using SPSS version 17.0. A value of $P<0.05$ was considered statistically significant.

Results

Transfection efficiency of ACY-1 in human NB cells

To explore the influence and understand the function of ACY-1 in human NB, we used the SH-SY5Y cell line. We performed

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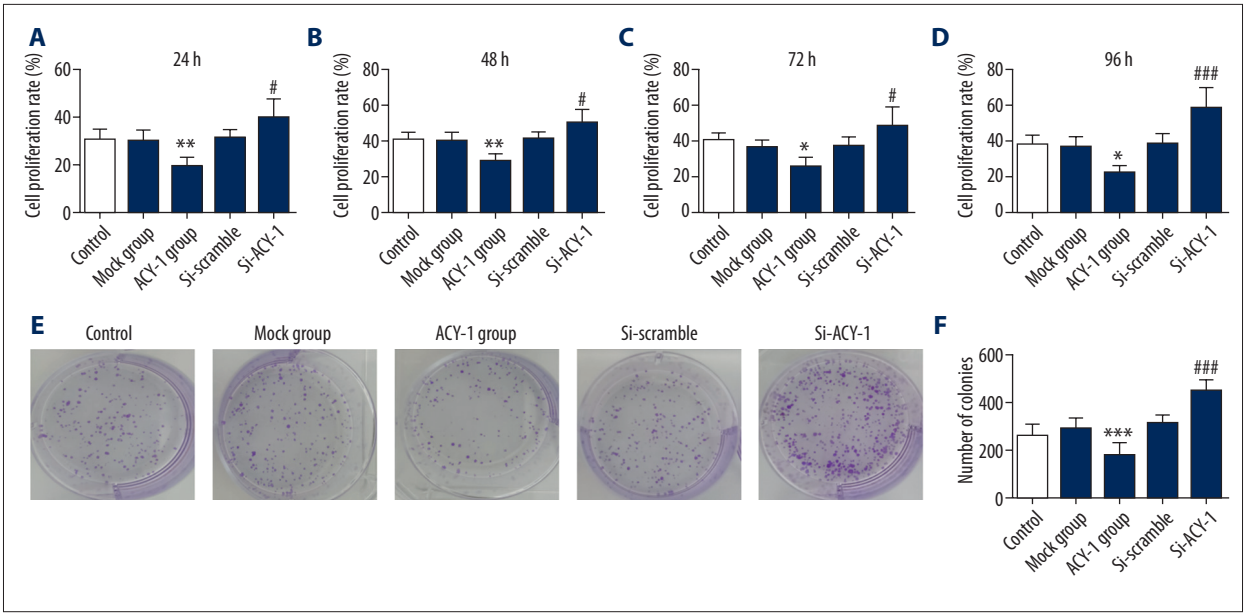

Figure 2. Aminoacylase 1 (ACY-1) affected the proliferation and colony formation of human neuroblastoma cells. (**A–D**) Cell proliferation was determined in SH-SY5Y cells. (**E, F**) The colony formation capacity was determined in SH-SY5Y cells. * Compared to mock group; # compared to siRNA-scramble group.

ACY-1 overexpression and knockdown in the SH-SY5Y cells. The transfection of ACY-1 into SH-SY5Y cells increased ACY-1 levels, whereas the transfection of siRNA-ACY-1 reduced ACY-1 expression in the cells (Figure 1A). We further confirmed the results with real-time PCR and western blotting, and the results exhibited similar trends (Figure 1B–1D). Collectively, our results showed that ACY-1 overexpression and knockdown were successfully performed in human NB cells.

ACY-1 affected the proliferation, colony formation, and apoptosis of human NB cells

Next, we performed a cell proliferation assay to determine the influence of ACY-1 on cell proliferation *in vitro*. ACY-1 overexpression in the SH-SY5Y cells inhibited proliferation at 24, 48, 72, and 96 h, compared to the mock group (Figure 2A–2D). In contrast, the knockdown of ACY-1 in SH-SY5Y cells promoted cell proliferation. Moreover, the colony formation assay showed that ACY-1 markedly inhibited the colony formation ability of SH-SY5Y cells when ACY-1 was overexpressed, whereas ACY-1 knockdown significantly increased the number of colonies in the siRNA-scramble group (Figure 2E, 2F).

We further analyzed the effect of ACY-1 on apoptosis through flow cytometry. ACY-1 overexpression significantly promoted the generation of apoptotic cells, compared to the mock group (Figure 3A, 3B). However, apoptosis was greater in the down-regulation of ACY-1 group compared to the siRNA-scramble group. Furthermore, we explored the apoptosis-related proteins in the SH-SY5Y cells through western blot analysis to confirm our results. The expression of Bax and Bad proteins increased in the ACY-1 group and decreased in the siRNA-ACY-1 group. In contrast, the level of Bcl-2 decreased in the ACY-1 group and increased in the siRNA-ACY-1 group, compared with that of the siRNA-scramble group (Figure 3E, 3F). Collectively, the results demonstrated that ACY-1 promoted proliferation and inhibited apoptosis in NB cells.

ACY-1 regulated the migration and invasion ability of human NB cells

We subsequently evaluated the potential role of ACY-1 on cell migration and invasion through wound healing and transwell assays. ACY-1 overexpression inhibited the migration ability, while ACY-1 knockdown exhibited an opposite trend in the SH-SY5Y cells (Figure 4). Furthermore, the transwell assay showed that ACY-1 overexpression in SH-SY5Y cells inhibited invasion ability, while this ability was promoted in the siRNA-ACY-1 group, compared with the controls (Figure 5A–5D). These findings showed that ACY-1 regulated the migration and invasion of human NB cells.

ACY-1 mediated the ERK1/2 and TGF-β signaling pathways in human NB cells

The ERK1/2 and TGF-β1 signaling pathways are part of many cellular processes, such as cell proliferation, differentiation, and apoptosis [19]. To explore the signaling pathway involved in ACY-1 in NB cells, the levels of ERK1/2, p-ERK1/2, and TGF-β1 were determined in the SH-SY5Y cells. TGF-β1 and ERK 1/2

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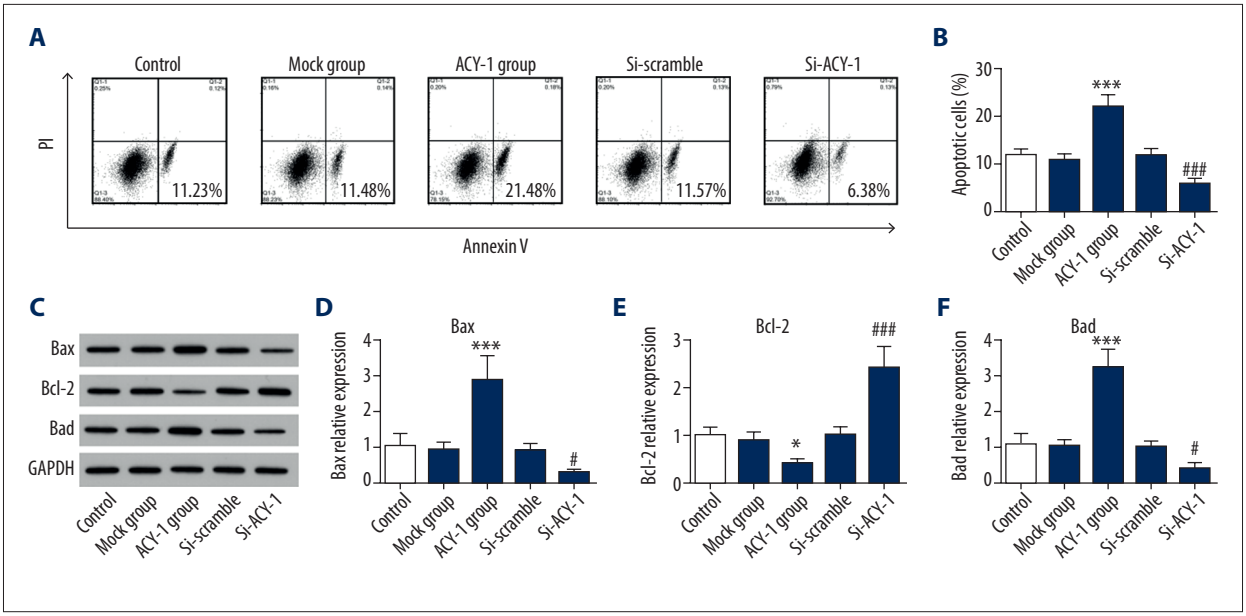

Figure 3. Effect of Aminoacylase 1 (ACY-1) on human neuroblastoma cell apoptosis. (**A, B**) Flow cytometry was performed to test apoptosis in SH-SY5Y cells. (**C–F**) The expression of Bax, Bcl-2, and Bad in SH-SY5Y cells were detected using western blotting. * Compared to mock group; # compared to siRNA-scramble group.

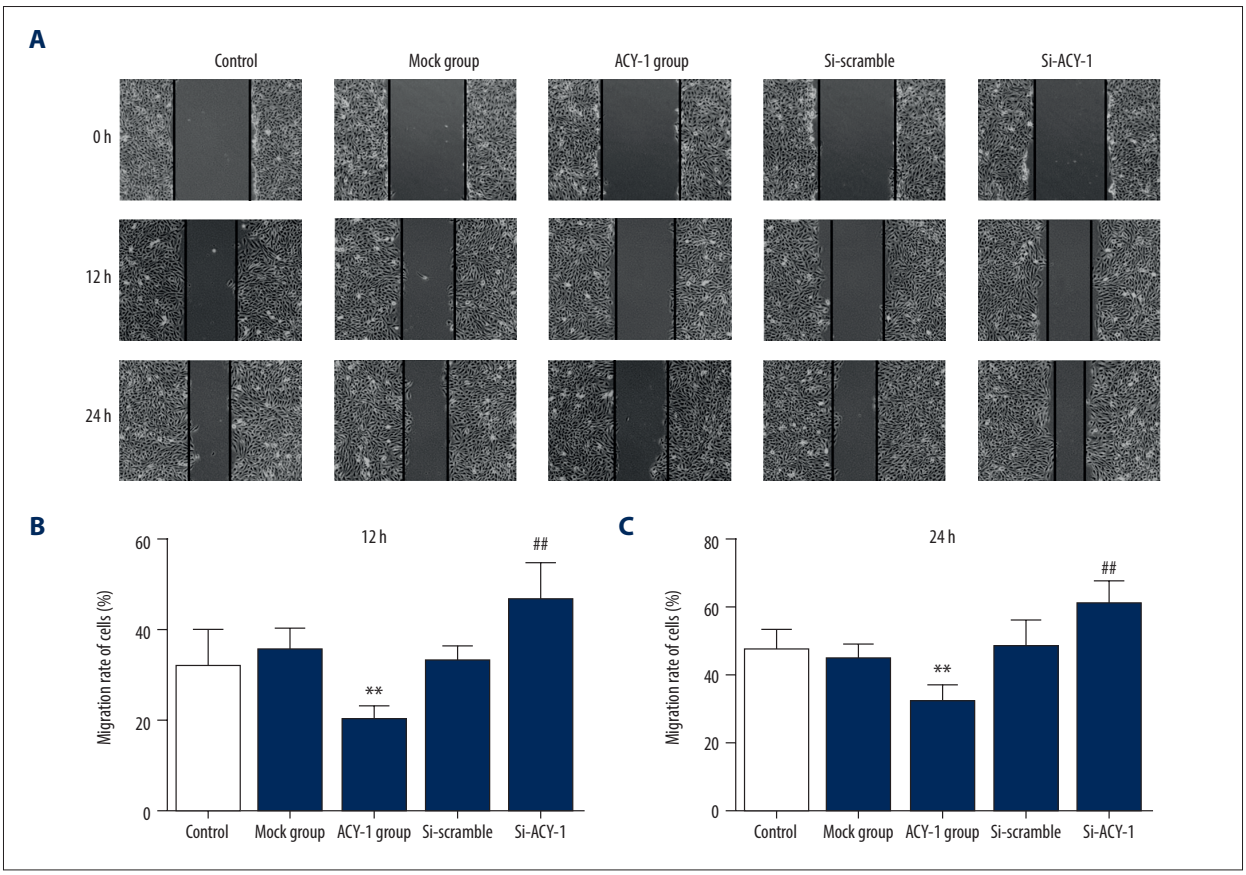

Figure 4. Influence of Aminoacylase 1 (ACY-1) on the migration of SH-SY5Y cells determined by wound healing assay. (**A–C**) SH-SY5Y cells were scraped off with pipette tips and cultured for 0, 12, and 24 h. Cell migration was determined under a microscope before and after injury. * Compared to mock group; # compared to siRNA-scramble group.

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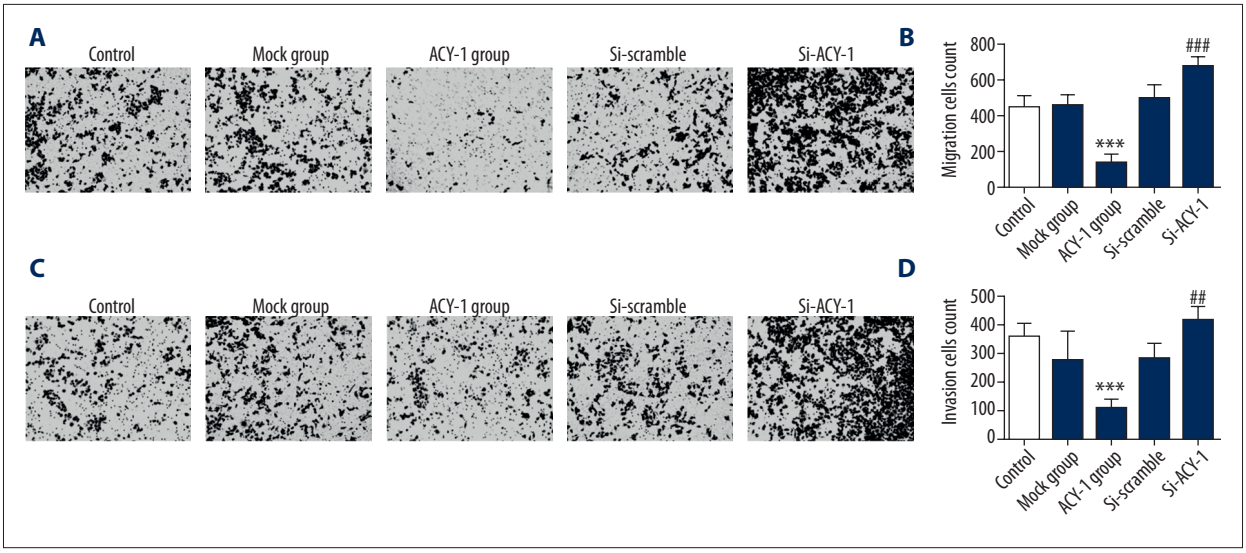

Figure 5. Aminoacylase 1 (ACY-1) regulates the migration and invasion of human neuroblastoma cells. (**A, B**) The transwell assay was used to assess the influence of ACY-1 on SH-SY5Y cell migration. (**C, D**) The influence of ACY-1 on the invasion of SH-SY5Y cells was determined by transwell assay. * Compared to mock group; # compared to siRNA-scramble group.

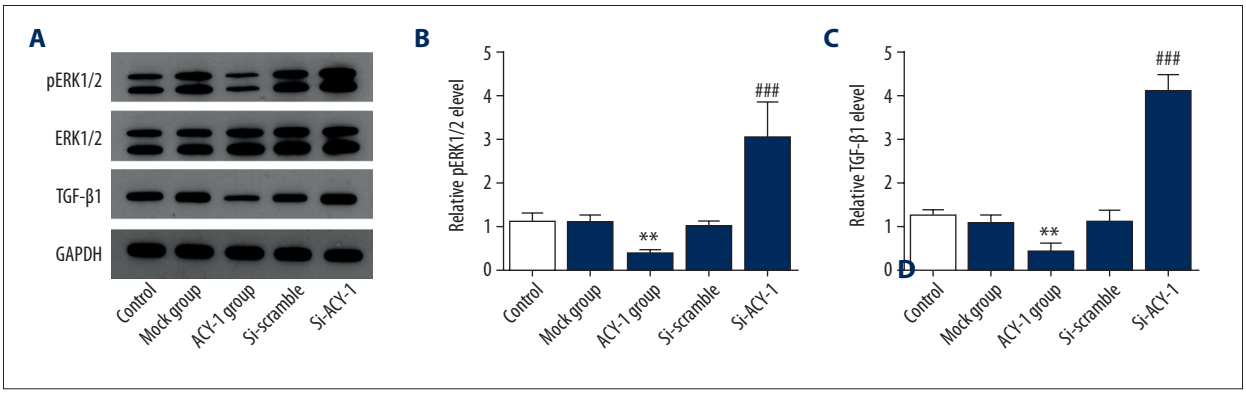

Figure 6. Aminoacylase 1 (ACY-1) mediates the ERK1/2 and TGF-β1 signaling pathways in SH-SY5Y cells. (**A–C**) The expression of ERK1/2, p-ERK1/2, and TGF-β1 were determined in the SH-SY5Y cells by western blotting. * Compared to mock group; # compared to siRNA-scramble group.

phosphorylation levels were reduced following the overexpression of ACY-1 and were increased when the expression of ACY-1 was downregulated, compared with the controls (Figure 6A–6C). These results indicated that ACY-1 might affect tumorigenesis in NB cells through the ERK1/2 and TGF-β1 signaling pathways.

Discussion

ACY-1 has been most intensively studied for its catalytic activity in the hydrolysis of N-terminally acylated amino acids into an acyl group and a corresponding free amino acid during intracellular protein catabolism [20]. In addition, ACY-1 has functions in biological use of N-acylated food proteins in normal mammalian intestine [21]. Many studies showed that ACY-1 plays a pivotal role in the process of tumor development [14,15]. However, the mechanism of ACY-1 in cancer cells, especially in NB, remains unclear. In this study, the SH-SY5Y cell line was used to investigate the effect of ACY-1 on NB *in vitro*. Our results indicated that ACY-1 overexpression might inhibit the proliferation, migration, and invasion ability of SH-SY5Y cells, whereas ACY-1 knockdown exhibited completely opposite effects, indicating that ACY-1 plays a crucial role in human NB cells.

Cell proliferation refers to an increase in the number of cells due to cell growth and division, which is necessary for growth of normal tissue and tumorigenesis [22]. From embryological development through senescence, cell proliferation is maintained by a strict coordination of cellular signals, and deregulation of cell proliferation is the defining feature of all tumors [23].

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Moreover, manipulation of cell proliferation pathways may decrease the malignant potential of tumors, including NB, making these pathways an attractive target for novel therapeutics. In addition to cell proliferation, cell apoptosis plays an important role in the development of tumors.

Cell apoptosis is mediated by caspase-dependent and caspase-independent pathways. Caspase 3 has a key role in cell apoptosis. Once caspase 3 is activated, cytochrome C release from mitochondria increases by cleaving Bcl-2 expression and converting it from an anti-apoptotic to a pro-apoptotic protein [24]. The mitochondria-dependent pathway is the classical apoptotic pathway. Bax and Bcl-2 are the predominant regulators in controlling the release of cytochrome C in mitochondria-dependent apoptosis [25]. When Bax inserts into the outer membrane of mitochondria, cytochrome C is released from the organelle. However, when Bcl-2 instead of Bax binds to the outer mitochondrial membrane, release of cytochrome C is blocked. Many anti-cancer agents can induce the release of cytochrome C through either upregulating Bax expression and/or downregulating Bcl-2 expression. Our results showed that ACY-1 overexpression remarkably increased the apoptotic cells and protein expressions of Bax and Bad, but inhibited the expression of Bcl-2, suggesting the activation of the mitochondria-mediated apoptosis pathway. Furthermore, ACY-1 knockdown dramatically decreased the level of apoptosis, indicating that ACY-1 has a pro-apoptotic function in NB.

The mitogen-activated protein kinase (MAPK) signaling pathway consists mainly of 3 subfamilies, including ERK 1/2, p38, and JNK, which play significant roles in cellular events, such as cell growth, proliferation, differentiation, and apoptosis [26,27]. In the MAPK subfamilies, ERK1/2 serves as the most important component, contributing to cell proliferation and metastasis during tumorigenesis [28]. Many studies have shown increased p-ERK1/2 levels in many types of cancer, including pancreatic and lung cancers, and NB [29,30]. In pancreatic cancer tissue specimens, activated p-ERK was observed to be associated with poor survival in patients with pancreatic adenocarcinoma [31].

TGF-β is another important factor that participates in tumor progression and may promote the proliferation of tumor cells and attenuate the immune system directly [32]. In tumors, cell invasion caused by TGF-β is accompanied by the activation of the ERK signaling pathway. Furthermore, TGF-β-induced proliferation, migration, and invasion are blocked when ERK1/2 signaling is inhibited in a pancreatic cancer cell line and non-small cell lung cancer cells [33,34]. Previous studies have confirmed that ACY-1 participates in cell proliferation through the ERK1/2 and TGF-β pathways, showing that these pathways may function downstream of ACY-1 [18]. Another study showed that silenced ACY-1 may promote the activation of the ERK pathway [35]. These studies revealed that the ERK pathway plays an important role in regulating ACY-1 functions. Hence, we hypothesized that ACY-1 might modulate ERK1/2 and TGF-β signaling and be involved in the pathogenesis of NB. As expected, our results showed that the phosphorylation of ERK1/2 and activation of TGF-β1 were inhibited in the ACY-1-overexpressed SH-SY5Y cells. Moreover, when we inhibited the expression of ACY-1 in SH-SY5Y cells, the phosphorylation of ERK1/2 and TGF-β1 signaling was more active. These results suggest that ACY-1 regulated the ERK1/2 and TGF-β1 signaling pathways participating in the development of NB. However, the exact mechanism by which ACY-1 silencing regulates these genes requires further validation.

Conclusions

In summary, our study demonstrated that ACY-1 can regulate the proliferation, migration, and invasion of human NB cells, and the ERK1/2 and TGF-β1 signaling pathways play a crucial role in this process. Considering the importance of ACY-1 in human NB cells, ACY-1 may serve as a potential therapeutic target for the treatment of human NB.

Conflict of interest

None.

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