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Original Article

Isatin inhibits SH-SY5Y neuroblastoma cell invasion and metastasis through PTEN signaling

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Abstract: Objective: Isatin has gained attention in recent years owing to its anticancer properties and is thought to offer medical benefits. Isatin is an endogenous oxidized indole with various behavioral and metabolic properties and is commonly found in mammalian tissues and fluids. It has several plausible applications in biomedical research and has also been investigated as a potential anticancer agent. However, its effects on neuroblastoma (NB) cells are unclear. Here, we evaluate the effects of isatin on neuroblastoma cell metastasis and invasion and reveal the underlying mechanism. Methods: NB cell viability was evaluated with the cell counting kit (CCK)-8 assay. NB cell invasion and migration abilities were tested with transwell and wound healing experiments. The relative mRNA expression of associated molecules was detected with real-time polymerase chain reaction (RT-PCR) and quantitative PCR. The expression level of related proteins was detected with western blotting. Results: Isatin inhibited the proliferation, invasion, and migration of neuroblastoma cells in a dose-dependent manner. Isatin increased the expression level of H3K4m1 and phosphatase and tensin homolog (PTEN) and decreased the phosphorylation level of PTEN downstream proteins phosphoinositide 3-kinase, protein kinase B, mammalian target of rapamycin, focal adhesion kinase, and SHC. Together, these results support the potential anti-metastatic effects of isatin on NB cells.

Keywords: Isatin, neuroblastoma, PTEN, LSD1, invasion

Introduction

Neuroblastoma (NB), one of the most common solid extracranial neoplasms in children, accounts for more than 7% of malignancies in patients younger than 15 years old and is responsible for around 15% of all pediatric cancer-related deaths [1, 2]. Although substantial improvement has been observed in the outcomes of a few well-defined subsets of patients during the past few decades, not much improvement has been reported for those with a high-risk clinical phenotype [3, 8]. NB is a disease of the sympatico-adrenal lineage of the neural crest, wherein the tumors may develop anywhere in the sympathetic nervous system. About half of all cases are classified under a high risk for disease relapse, and the overall survival rate is less than 40% despite intensive multimodal therapy [4, 9]. The leading cause of death in patients with NB is metastasis, which in most cases is observed to inexplicably migrate to the bone marrow. Therefore, it is important to understand the specific mechanism underlying NB invasion and metastasis and [5, 10] to discover safer and better compounds to inhibit NB [4, 6] invasion and metastasis.

Monoamine oxidase (MAO) is localized in the outer membrane of the mitochondria and catalyzes the oxidative deamination of neuroactive cells. An increase in MAO activity was shown to be accompanied by apoptotic cell death of various neuronal cells following growth factor deprivation [7, 11]. Monoamine oxidase A (MAOA) was reported to induce the epithelial to mesenchymal transition (EMT) and consequently mediate the growth, invasiveness, and metastasis of tumor cells.

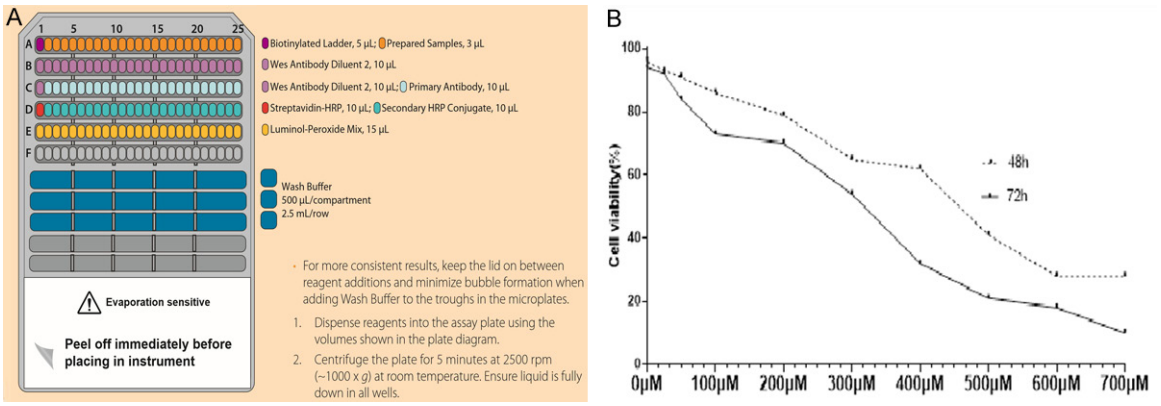

Figure 1. Isatin inhibits SH-SY5Y cell growth. All western reagents were added to the wells of the plate, as shown in (A). The effect of isatin on the proliferation of neuroblastoma cells. The cells were incubated with 0, 25, 50, 100, 200, 300, 400, 500, 600, and 700 µmol/L of isatin for 48 or 72 h in a CCK-8 assay (B). *P < 0.05.

As a member of the MAO family [8, 12], lysine-specific demethylase 1 (LSD1) which specifically removes the dimethyl and monomethyl modifications of H3K4 in the presence of flavine adenine dinucleotide (FAD) [9, 13] is strongly associated with the development, invasiveness, and metastasis of tumor cells [10, 14]. For instance, LSD1 could be recruited by the SNAG domain of Snail to the E-cadherin promoter for transcription suppression and EMT [11, 15]. An epigenetic marker, LSD1 overexpression is one of the characteristics of malignant tumors [12, 16] and has been correlated with the malignant progression of multiple cancers, including primary neuroblastic tumors, estrogen receptor-negative breast cancer [13, 17], and poorly differentiated NBs [14, 18]. LSD1 may also form a co-inhibitory complex with the SNAG domain of Snail and allow the recruitment of LSD1 to the phosphatase and tensin homolog (PTEN) promoter for H3K4 demethylation [15, 19] and PTEN transcription repression [16, 20].

PTEN exerts its role as a tumor suppressor through the downregulation of the phosphoinositide 3-kinase (PI3K)/protein kinase (AKT) signaling pathway, which is highly related to the invasion and metastasis of cancer cells [17, 18, 21, 22]. For instance, PTEN inhibits the migration and invasion of HepG2 cells by decreasing the expression of matrix metalloproteinase (MMP) via the PI3K/AKT pathway [19, 23]. PTEN also interacts with focal adhesion kinase (FAK) by reducing its tyrosine phosphorylation and negatively regulates the interaction between the cell and the extracellular matrix [20, 24] to inhibit cell migration, cell spreading, and focal adhesion formation [21, 25].

Previous studies have shown that isatin is an endogenous indole that inhibits MAOB and may induce SH-SY5Y cell death in a dose- and time-dependent manner [22, 26]. Isatin was recently shown to inhibit SH-SY5Y cell migration and invasion through various pathways, especially through the downregulation of MMP-2/MMP-9 expression [23, 27]. However, the precise mechanism involved in the anti-metastasis activity of isatin is incompletely understood.

We thought that the inhibition of LSD1 activity may cause H3K4 demethylation and isatin may upregulate the expression of PTEN, which would negatively regulate SH-SY5Y cell invasion and metastasis through the PI3K/AKT and PTEN/p-SHC/p-FAK signaling pathways. To test our assumption, we applied real-time polymerase chain reaction (RT-PCR), western blotting, and transwell assays and determined the molecules involved in this process. The results obtained confirm our hypothesis.

Materials and methods

Cell culture

SH-SY5Y cells (Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium (DMEM)/high glucose (HyClone, USA) supplemented with 10% fetal bovine serum (FBS; BI, California, USA) at 37°C with 5% CO_2

Table 1. Primer sequences for *LSD1*, *PTEN*, and *GAPDH*

Gene	Forward	Reverse
LSD1	5'-TGGTGGTAACAGGTCTTGGAGG-3'	5'-GGCTTCATAAAGTGGGCATTTTTG-3'
PTEN	5'-AGTTCCCTCAGCCGTTACCT-3'	5'-ATTTGACGGCTCCTCAACTG-3'
GAPDH	5'-GGAGCCAAAAGGGTCATCATCT-3'	5'-AGGGGCCATCCACAGTCTTCT-3'

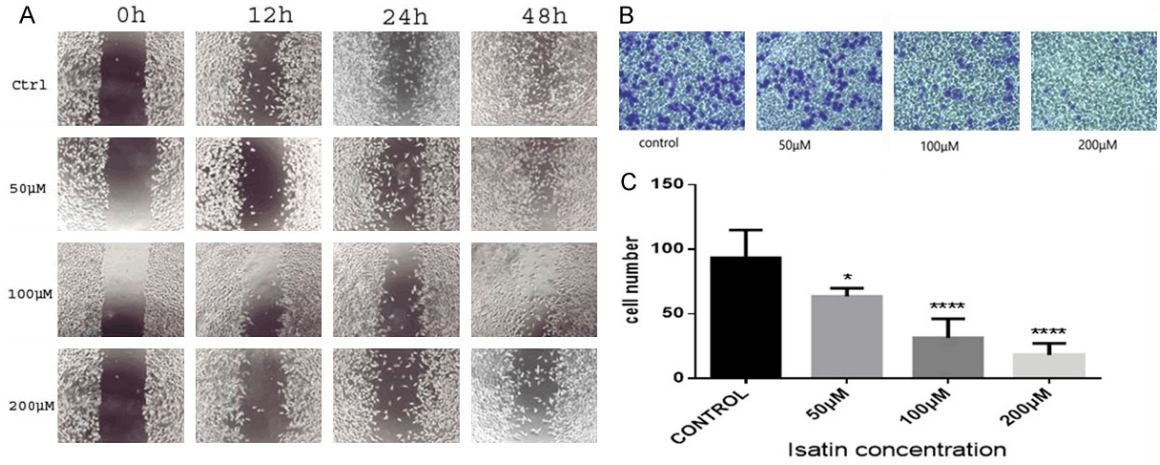

Figure 2. Isatin inhibits SH-SY5Y cell invasion and migration. Isatin inhibits SH-SY5Y cell migration and invasion (A). The effect of isatin on neuroblastoma cell migration at × 100 magnification under an inverted microscope (B). Statistical analysis of SH-SY5Y cell Matrigel invasion counted in five random fields (C). *P < 0.05, **P < 0.01 as compared with the control.

and 98% relative humidity in a culture incubator. The cells were incubated with different concentrations (0, 50, 100, and 200 μmol/L) of isatin (99.0%; Sigma, California, USA) for 24, 48, or 72 h after reaching 80% confluency. The cells (about 9 × 10^5 cells/well) were harvested and used for proliferation, migration, and protein analyses.

Cell viability assay

The viability of the cells was measured with a cell counting kit-8 (CCK-8) assay. The cells were seeded in a 96-well plate in a final volume of 100 μL of complete culture medium containing 1 × 10^4 cells/well and exposed to different concentrations (0, 25, 50, 100, 200, 300, 400, 500, 600, and 700 μmol/L) of isatin (six wells for each concentration) for 48/72 h at 37°C. After treatment, 10 μL of CCK-8 solution was added to each well and the cells were incubated at 37°C for 3 h. The absorbance of the samples was measured at a wavelength of 450 nm with a microplate reader (Synergy H1; BioTek, Vermont, USA). Each experiment was performed thrice.

Cell migration and invasion assay

SH-SY5Y cells were seeded in a serum-free medium in a six-well plate and exposed to isatin (0, 50, 100, and 200 μmol/L) for 48 h. A micropipette tip was used to scratch and create a wound each well. The cells were monitored during regrowth, and images were captured at different time points (0, 12, 24, and 48 h). The transwell invasion assay was performed in Boyden chambers (Millipore, California, USA). About 2 × 10^5 cells/well seeded in 200 μL of serum-free medium and pretreated with isatin for 24 h were added to the upper chamber coated with Matrigel, while the medium supplemented with 10% FBS was added to the lower chamber. At the end of the incubation, the cells from the upper surface were completely removed, and the filter was fixed in methanol and stained with crystal violet. Cells invading the Matrigel were counted under an inverted microscope. Data were expressed as the aver-

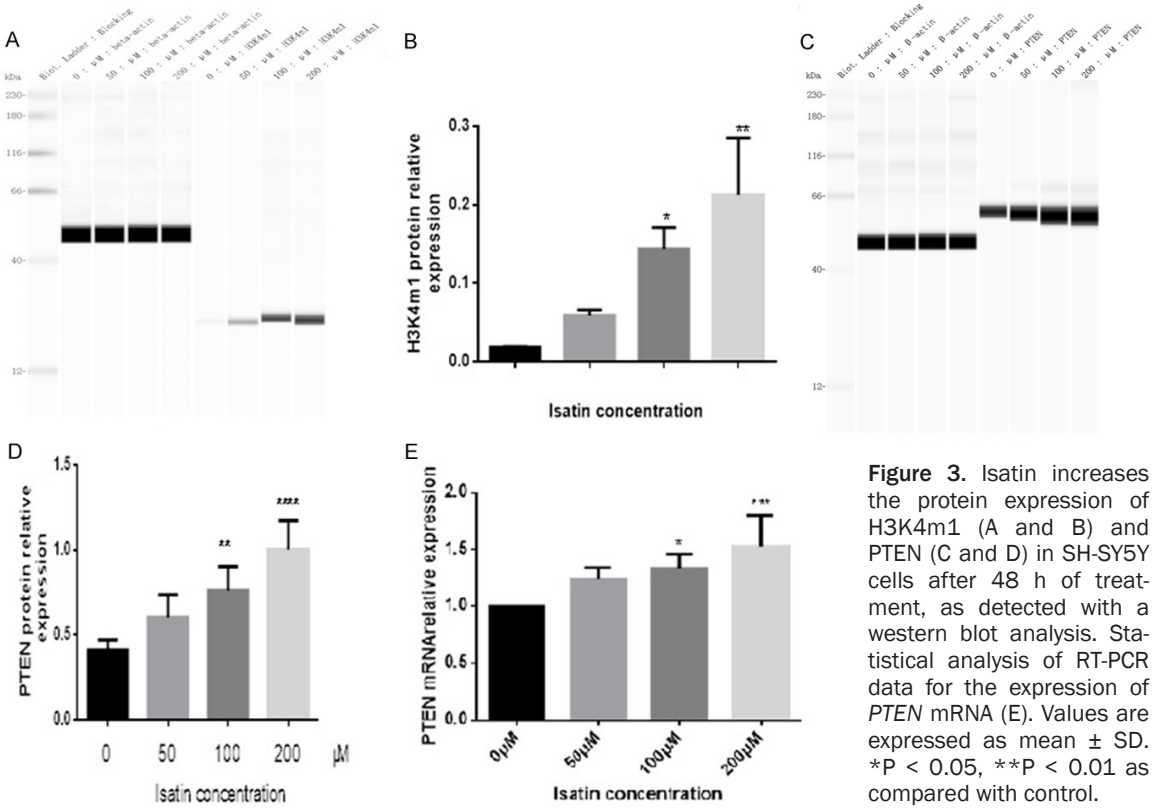

Figure 3. Isatin increases the protein expression of H3K4m1 (A and B) and PTEN (C and D) in SH-SY5Y cells after 48 h of treatment, as detected with a western blot analysis. Statistical analysis of RT-PCR data for the expression of *PTEN* mRNA (E). Values are expressed as mean ± SD. *P < 0.05, **P < 0.01 as compared with control.

age cell number in five fields and the experiment was repeated thrice.

Western blot analysis

SH-SY5Y cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China) supplemented with a protease inhibitor cocktail (Sigma, Germany) on ice for 1 h and centrifuged for 20 min at 4000 × g. The supernatant was collected and the protein concentration was measured with bicinchoninic acid (BCA) assay (Beyotime, Jiangsu, China). DTT from the kit was mixed with 40 μL of deionized water to obtain a 400 mM solution. The fluorescent 5 × master mix was mixed with 20 μL of 10 × sample buffer and 20 μL of the prepared DTT solution. The biotinylated ladder provided with the kit was mixed with 16 μL of deionized water, 2 μL of 10 × sample buffer, and 2 μL of the prepared DTT solution and gently mixed. The entire volume of the ladder was transferred to a 0.6 mL tube (blue), and the 10 × sample buffer was diluted 1:100 with water to obtain 0.1 × sample buffer. The protein lysate was diluted with 0.1 × sample buffer and the final concentration of the protein sample was adjusted to 0.2 mg/mL. The samples and biotinylated ladder were denatured at 95°C for 5 min and stored on ice. The primary antibody was diluted with antibody diluent 2 (1:50). The secondary horseradish peroxidase (HRP) conjugate was provided in the detection module and was ready to use. About 200 μL Lumino-S and 200 μL peroxide supplied in the detection module were mixed in a microcentrifuge tube and gently pipetted and stored on ice. All the reagents were added to the wells of the plate (**Figure 1A** lower panel) for the immunoassay. The assay was loaded in Compass software and a capillary cartridge was inserted into the cartridge holder. After the change in the interior light from orange to blue, the assay plate lid was removed, and the plate was held firmly on bench and its evaporation seal was carefully peeled-off. Any bubbles observed in the separation matrix wells were removed with a pipette tip. The assay plate was placed on the plate holder, the door was closed, and the run was started. After run completion, the plate and cartridge were discarded. Data were collected and analyzed by Compass software.

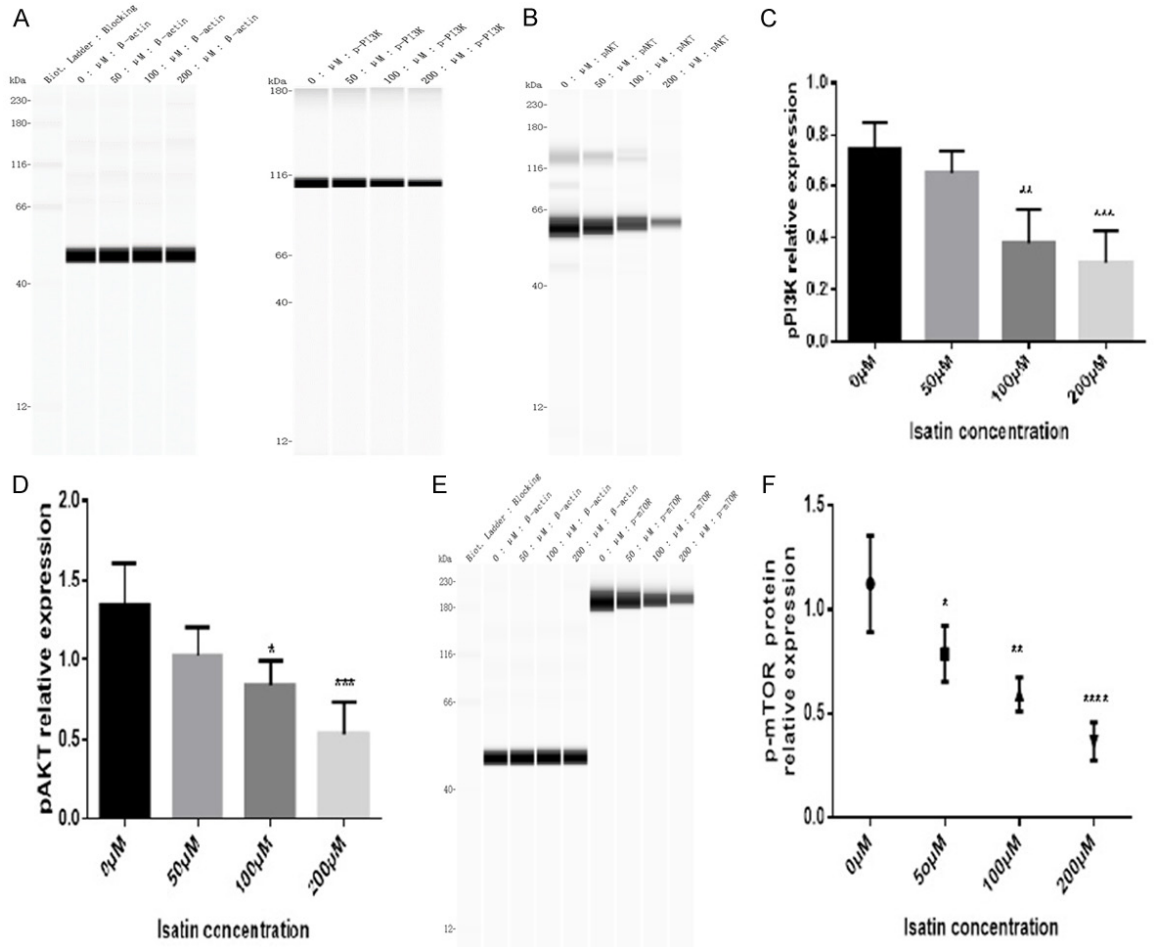

Figure 4. The phosphorylation level of PI3K (A and C), AKT (B and D), and mTOR (E and F) in SH-SY5Y cells decreased after 48 h of treatment with isatin, as detected with western blot analysis. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01 as compared with control.

Quantitative RT-PCR assay

Total RNA was extracted from the cultured SH-SY5Y cells with Trizol reagent (Solarbio, Beijing, China). A reverse transcription kit (Transgene, Beijing, China) was used to construct the template cDNA for real-time PCR with Trans Start Probe RT-PCR Super Mix (Transgene, Beijing, China). The data were obtained on Bio-Rad (California, USA) One-Step Plus system. The primer sequences used are shown in **Table 1**.

Statistical analysis

The data were obtained from three independent experiments and represented as the mean ± standard deviation (SD). The corresponding data were compared with a one-way analysis of variance (ANOVA) using GraphPad Prism 6 statistical software (GraphPad, La Jolla, CA, USA), and significance was set at $P < 0.05$.

Results

Detection of cell viability with the CCK-8 assay

Isatin inhibited the invasion and metastasis of NB cells in vitro. The growth inhibitory effects of isatin on SH-SY5Y cells were evaluated with a CCK-8 assay. As shown in **Figure 1B**, isatin at various concentrations influenced the survival rate of SH-SY5Y cells after treatment for 48/72 h. Isatin was nontoxic at concentrations below 200 μmol/L, as evident from the survival of 80% of the cells. So the concentrations of 0 μmol/L, 50 μmol/L, 100 μmol/L, 200 μmol/L were used in subsequent experiments.

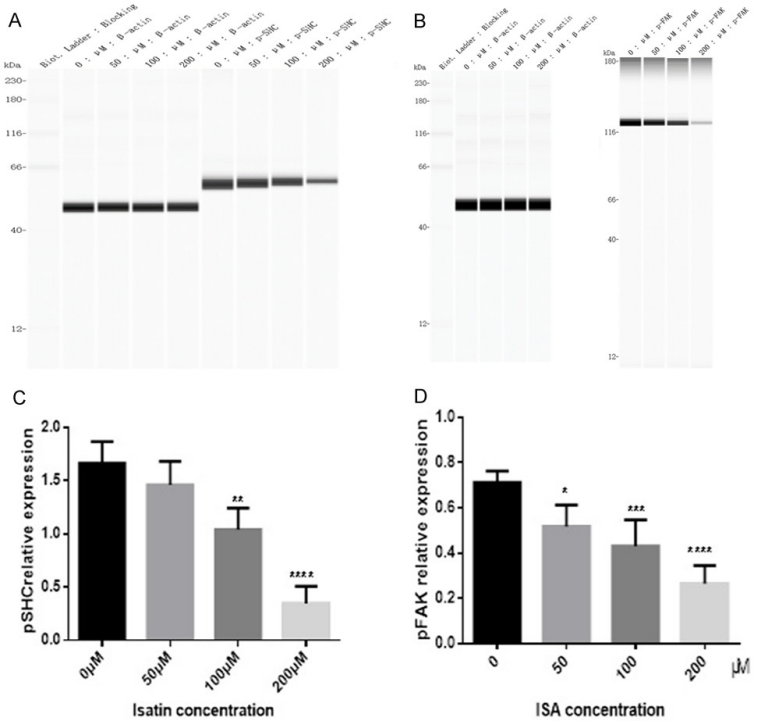

Figure 5. The phosphorylation level of p-SHC (A) and p-FAK (B) in SH-SY5Y cells decreased after 48 h of treatment with isatin, as detected with a western blot analysis. Statistical analysis of the expression of p-SHC (C) and p-FAK protein (D). Values are expressed as the mean ± SD. *P < 0.05, **P < 0.01 as compared with control.

Decrease in the invasion and migration ability of isatin-treated cells as compared with control cells

The wound healing rate was significantly lower in isatin-treated SH-SY5Y cells than in the untreated cells. At 48 h, the wound closure was almost complete in the control cells, but the isatin-treated cells showed a noticeable wound gap (**Figure 2A**). In addition, isatin at 200 μmol/L concentration decreased the invasion of cells to 20% as compared with the control cells (**Figure 2B** and **2C**). Isatin also exerted an anti-proliferation effect on the SH-SY5Y cells (**Figure 1B**). All these results suggest that isatin exhibits a potentially inhibitory effect on NB cell metastasis.

Increase in the expression of H3K4m1 and PTEN

As reported in previous studies, isatin inhibits MAOA activity in SH-SY5Y cells [24, 28]. Here, we investigated whether isatin modulates LSD1 activity by evaluating the expression of its target protein H3K4m1 [9, 13]. The expression of the H3K4m1 protein significantly increased in the cells incubated with 200 μmol/L of isatin as compared with the control cells (**Figure 3A** and **3B**), indicating that isatin may influence SH-SY5Y cell invasion and metastasis through the inhibition of LSD1 activity. LSD1 is known to maintain the proliferation of carcinoma cells through the PI3K/AKT pathway [25, 29]. Whether LSD1 decreases the expression of PTEN, a typical inhibitor of PI3K/AKT pathway [26, 27, 30, 31], remains unclear in NB cells. In this direction, we tested the expression of related molecules through western blot analysis and RT-PCR. As a result, we found that the protein (**Figure 3C** and **3D**) and mRNA (**Figure 3E**) expression levels of PTEN in SH-SY5Y cells exposed to isatin increased in a dose-dependent manner (**Figure 3E**).

Decrease in the phosphorylation of PI3K, AKT, mTOR, FAK, and SHC

We observed a decrease in the phosphorylation levels of PTEN downstream molecules, such as PI3K (**Figure 4A** and **4C**), AKT (**Figures 4B** and **5D**), mTOR (**Figure 4E** and **4F**), FAK (**Figure 5A** and **5C**), and SHC (**Figure 5B** and **5D**), as confirmed with western blotting.

No change in the expression of LSD1

We failed to observe any change in the expression of LSD1 at the protein (**Figure 6A** and **6B**) and mRNA (**Figure 6C**) levels, suggesting that isatin may inhibit cell invasion not by decreasing the expression of LSD1 but through the inhibition of LSD1 activity.

All these results suggest that isatin may inhibit LSD1 activity and increase PTEN expression, leading to the inhibition of SH-SY5Y cell invasion and metastasis through the PI3K/AKT and PTEN/p-SHC/p-FAK signaling pathways.

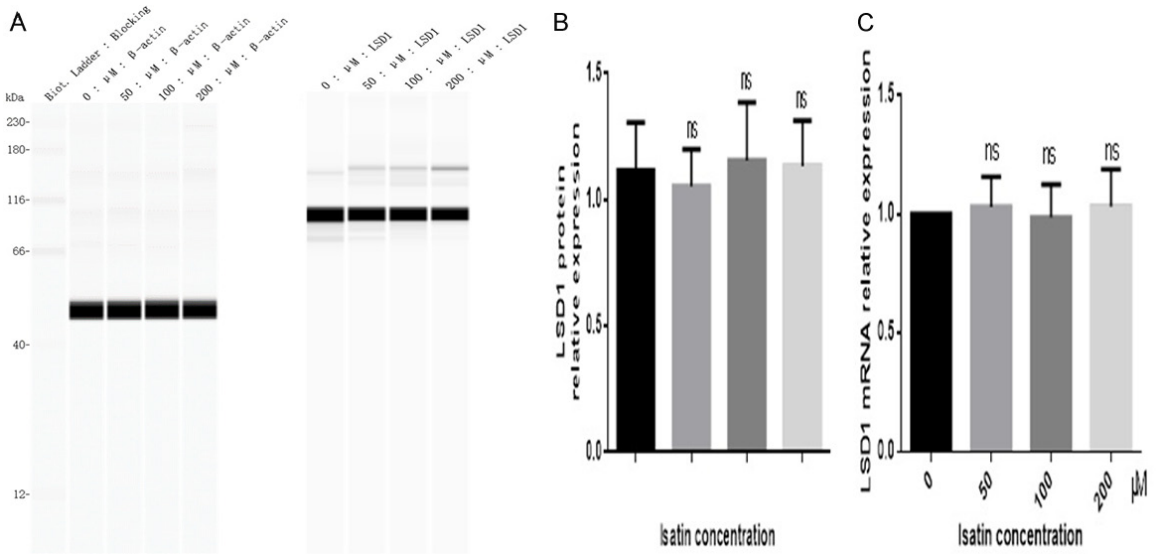

Figure 6. The protein (A and B) and mRNA (C) expressions levels of LSD1 in SH-SY5Y cells showed no change after 48 h of treatment with isatin, as detected with a western blot analysis. Values are expressed as the mean ± SD. *P < 0.05, **P < 0.01 as compared with control.

Discussion

Prior work has revealed the effectiveness of isatin in the prevention of cancer cell proliferation and progression. A study by Havrylyuk [28, 32] showed that isatin exhibits a remarkable antiproliferative effect on cancer. However, these studies have either focused only on the anti-proliferation property of isatin or have not clarified the anti-invasive mechanism isatin's underlying effects in NB cells. In the present study, we investigated the anti-invasive effects of isatin on NB cells and revealed the underlying mechanism using western blotting and RT-PCR. As a result, we found that isatin treatment increased the expression of H3K4m1 in SH-SY5Y cells, wherein H3K4m1 acts as a substrate of LSD1. The expression of LSD1, however, showed no change, indicating that isatin is likely to downregulate H3K4m1 expression by inhibiting the activity of LSD1 and not by decreasing LSD1 expression. The upregulation in H3K4m1 expression resulted in an increase in the level of PTEN. We also observed a decrease in the expression of the downstream molecules involved in PTEN signaling, including p-SHC, p-FAK, p-PI3K, p-AKT, and p-mTOR. These findings indicate that isatin exerts its anti-invasion and anti-metastasis effects on SH-SY5Y cells by increasing the expression level of H3K4m1, which then activates PTEN signaling through the inhibition of LSD1 activity. To our knowledge, this is the first study to systematically investigate the influence of isatin on PTEN signaling-related molecules in NB cells. However, our study has a few limitations. Although our hypotheses were statistically supported by the results of biochemical experiments in vitro, whether the mechanism is reproducible in animals or humans is questionable. Further studies are warranted to evaluate the effects of isatin on tumors in animal models.

Acknowledgements

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Disclosure of conflict of interest

None.

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