Isatin inhibits the invasion of human neuroblastoma SH-SY5Y cells, based on microarray analysis

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Abstract. Neuroblastoma is the fourth most common type of extracranial malignant solid tumor in children. Isatin had been demonstrated to have inhibitory effects on neuroblastoma tumors *in vivo* and *in vitro*. The aim of the present study was to investigate the molecular mechanism related to the anti-invasion effect of isatin on SH-SY5Y cells using microarray analysis. The microarray data identified a number of genes to be differentially upregulated or downregulated between isatin-treated cells and untreated controls. A large number of these genes were associated with the mTOR signaling pathway. The differentially expressed genes involved in the mTOR signaling pathway were verified further, as well as their downstream genes associated with autophagy. The results of the present study provided an insight into the potential inhibitory mechanism of isatin on neuroblastoma metastasis.

Introduction

Neuroblastoma (NB) is the most common extracranial solid cancer in childhood and infancy, with a mortality rate of 15%, and an incidence between 5.9 and 10.5 per million children under 15 years of age (1). The majority of NB cases are metastatic, and thus are associated with a poor prognosis and a high mortality rate (2). A total of 20-50% of high-risk cases do not respond adequately to high-dose chemotherapy and are progressive or refractory (3). Novel treatments using new agents and combinations against NB are available in phase I or II clinical trials, but the outcomes remain poor (4). Single targeted agents are unlikely to be sufficient for long-term treatment for high-risk NB. New therapeutic approaches need to be developed (2).

Clinical trial results have demonstrated that multi-target inhibitor drugs are more effective compared with single-target drugs during cancer treatment (5). 1H-indole-2, 3-dione (isatin) is a derivative of the anti-cancer drug indirubin, which exhibits beneficial biological activities, including antibacterial, antifungal and antitumor properties (6-8). Isatin was initially considered to be an inhibitor of monoamine oxidase-b and has been used to treat Parkinson's disease (9,10). Recent studies have reported that isatin inhibits cell proliferation and invasion in human neuroblastoma SH-SY5Y cells (11,12).

mTOR has been identified as a key molecule in tumorigenesis and cancer progression (13). Increasing evidence has identified the mTOR pathway as a relevant target for the suppression of tumorigenesis; thus, inhibition of mTOR may be a promising method for targeting human malignancies (14). The aim of the present study was to investigate the underlying molecular mechanisms of the inhibitory effect of isatin on migration and invasion in SH-SY5Y cells, which are associated with the mTOR pathway.

Materials and methods

Cell culture. Human neuroblastoma SH-SY5Y cells (human origin) were purchased from Peking Union Medical College. The cell line had been STR authenticated (ATCC no. CRL-2266). Cells were cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. Isatin was dissolved in 0.1% dimethyl sulfoxide and added to 6-well plates at concentrations of 50, 100, 200 and 300 μM, which were determined based on our previous study (11), when the cells had reached ~70% confluence. The cells (~2x10^6/well) were harvested following isatin treatment at 37˚C for 48 h for further analysis.

Cell migration and invasion assay. Cells (1x10^6) were seeded in 6-well culture plates and exposed to isatin (200 μM) with or without rapamycin (10 μM) at 37˚C for 48 h. The concentration of isatin was determined according to previous results, which demonstrated that 200 μM isatin did not induce necrosis of SH-SY5Y cells (11). A wound healing assay was performed to monitor cell invasion, as previously described (11). For the

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Transwell invasion assay, isatin-treated cells were seeded in Boyden chambers (EMD Millipore) with and without Matrigel, and the assay was performed as previously described (11). Cells were counted in at least five fields under an inverted light microscope (magnification, x10/x20). All experiments were performed in triplicate.

Microarray analysis. Microarray expression analysis was performed using total RNA from SH-SY5Y cells that were incubated at 37˚C with or without 200 μM isatin for 48 h. Total RNA was extracted using TRIzol® (Beyotime Institute of Biotechnology). An Affymetrix Microarray kit (Thermo Fisher Scientific, Inc.) was used for the gene expression analysis. Raw data intensities were quantile-normalized, and genes exhibiting significantly higher intensities compared with the background and a fold-change (FC)>1.5 following isatin treatment, compared with the control, were selected. This selection uncovered a total of 429 genes. Expression values of the genes were rescaled to mean 0 and SD 1, and hierarchical clustering was performed using Ward's algorithm (15). The number of clusters was determined using the Akaike Information Criterion (16). A Mann-Whitney test was performed to discriminate clusters of genes with differential expression levels between the two groups. Differentially expressed genes were selected based on FC in average gene expression with $P<0.05$, as determined by Student's t-test. Gene Ontology (GO) enrichment ('biological process', 'cellular component' and 'molecular function') (17,18) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (19-21) of differentially expressed genes were analyzed using the Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources, version 6.7 (http://david.abcc.ncifcrf.gov). Subsequent selection of enriched GO terms was performed based on the calculated q-values using the threshold $q<0.05$.

Reverse transcription-qPCR (RT-qPCR). Total RNA of SH-SY5Y cells was extracted using TRIzol and was reverse transcribed to cDNA using TransScript™ Reverse Transcriptase (Beijing Transgen Biotech Co., Ltd.) at 42˚C for 15 min and 85˚C for 5 sec. Then, qPCR was performed independently three times using SYBR Green mix (Takara Bio, Inc.). Gene expression was analyzed using the following primers: β-actin forward, 5'-GCGTGACATTAAGGAGAAGC-3' and reverse, 5'-CCACGTCACACTTCATGATGG-3'; mTOR forward, 5'-CTGGGACTCAAATGTGTGGAGTTC-3' and reverse, 5'-GAACAATAGGGTGAATGATCCGGG-3'; ribosomal protein S6 kinase B1 (RPS6KB1) forward, 5'-GGTGGAGTTTGGGAGCATTA-3' and reverse, 5'-TGTGAGGTAGGGAGGCAAAT-3'; eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1) forward, 5'-GTCGGAACTCACCTGTGACC-3' and reverse, 5'-CCGCTTATCTTCTGGGCTATT-3'; Ras homolog mTORC1-binding (RHEB) forward, 5'-AGCTTTGGCAGAATCTTGGA-3' and reverse, 5'-GCATGAAGACTTGCCTTGTG-3'; DNA damage-inducible transcript 4 (DDIT4) forward, 5'-AGACACGGCTTACCTGGATG-3' and reverse, 5'-CAGTAGTTCTTTGCCCACCTG-3'. The thermocycling conditions were 94˚C for 5 min, then 40 cycles of 93˚C for 30 sec, 55˚C for 30 sec and 72˚C for 60 sec. Data are expressed as fold change compared with control. The $2^{-\Delta\Delta Cq}$ method was employed to analyze the relative expression of genes (22).

Protein extraction and western blot analysis. Total protein was extracted from SH-SY5Y cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) following isatin treatment for 48 h, and the protein concentration was quantified using a bicinchoninic acid protein assay. Proteins (20-40 μg/lane) were separated on SDS-polyacrylamide gels (8-12%) and transferred onto PVDF membranes. Membranes were blocked with 5% bovine serum albumin for 2 h at room temperature, and then incubated with the following primary antibodies diluted in 5% BSA: β-actin (1:3,000; cat. no. ab8227; Abcam), AMPK (1:500; cat. no. ab131512; Abcam), phosphorylated (p)-AMPK (1:500; cat. no. ab131357; Abcam), mTOR (1:300; cat. no. ab32028; Abcam), p-mTOR (1:5,000; cat. no. ab109268; Abcam), microtubule associated protein 1 light chain 3α (LC3; 1:1,000; cat. no. 3868; Cell Signaling Technology), Beclin-1 (1:1,000; cat. no. 3738; Cell Signaling Technology) and p62 (1:1,000; cat. no. 5114; Cell Signaling Technology) at 4˚C overnight, washed with TBS with Tween-20, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibodies (1:1,000; cat. no. HS101-01; Beijing Transgen Biotech Co., Ltd.) for 1 h at room temperature. Protein expression levels were detected using the Enhanced Chemiluminescence Plus kit (Wuhan Boster Biological Technology, Ltd.) and densitometric analysis was performed using Quantity One analysis software (version 4.52; Bio-Rad Laboratories, Inc.).

Statistical analysis. Each experiment was performed at least three times. Data are expressed as the mean ± SD. Multiple comparisons were performed by one-way ANOVA followed by a least significant difference or Tamhane's T2 post hoc test in SPSS 22.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Isatin treatment affects gene expression in SH-SY5Y cells. To investigate the underlying molecular events of the anti-invasive activity of isatin in SH-SY5Y cells, RNA was extracted from SH-SY5Y cells (untreated or treated with 200 μM isatin for 48 h) for Affymetrix cDNA microarray analysis. The GeneChip results revealed 284 differentially upregulated and 145 downregulated genes between cells treated with isatin and controls (FC>1.5; Fig. 1A). GO term analysis demonstrated that the differentially expressed genes were involved in redox activity, binding and transcription function, cell metabolism and transport (data not shown). The results of GO term analysis indicated that isatin is involved in cell proliferation and the cell cycle, and functions in cell translation, biosynthesis and metabolism. According to the results of KEGG analysis, GeneChip predicted that isatin-modulated gene pathways were likely to be associated with chemokine signaling pathways, ribosome pathways and mTOR signaling pathways (Fig. 1B). The DDIT4, RHEB, EIF4EBP1 and RPS6KB1 genes, which are associated with mTOR activation, were selected for further analysis.

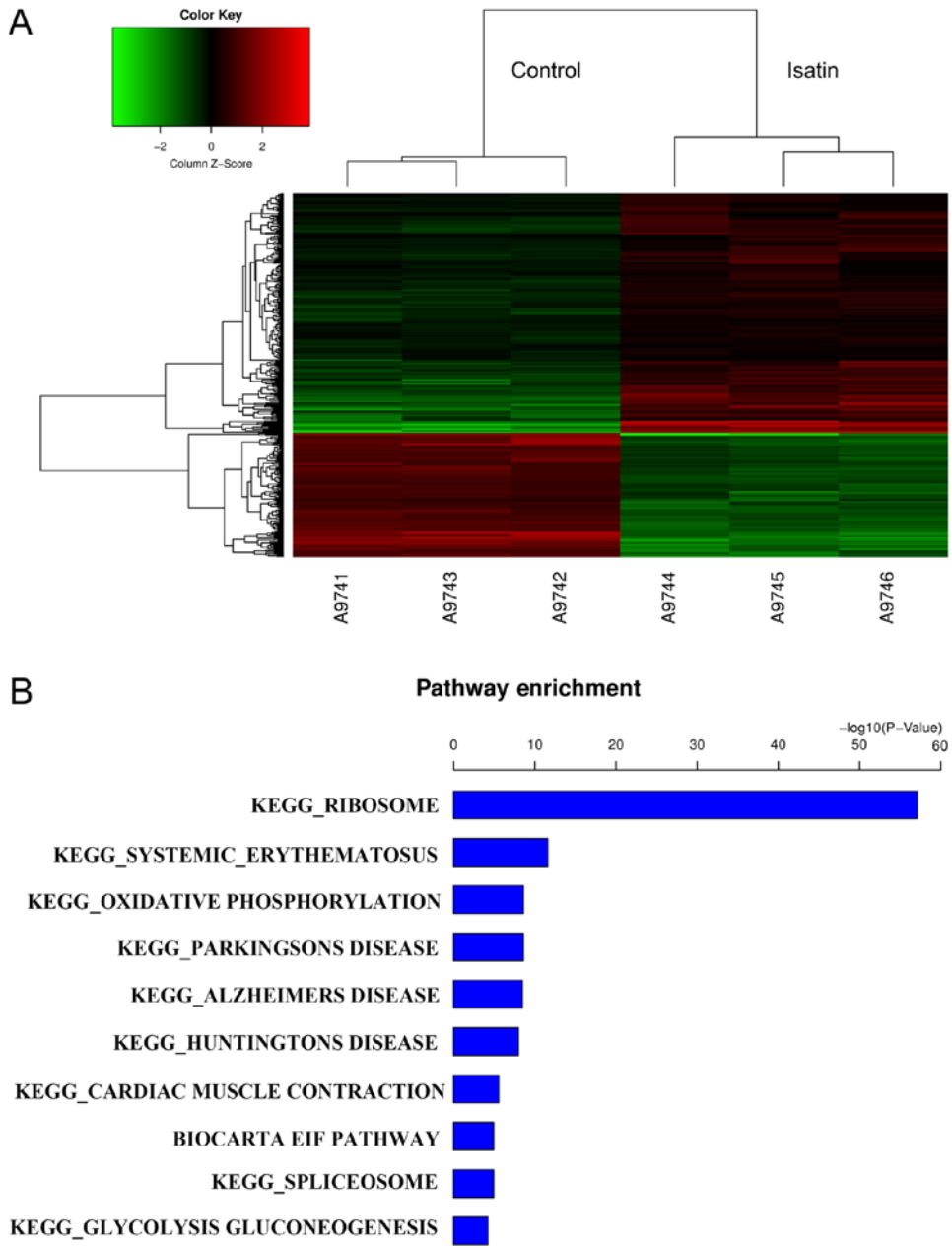

Figure 1. Differentially expressed genes in SH-SY5Y cells following isatin treatment. (A) Heat map of differentially expressed genes. (B) Pathway enrichment results of KEGG analysis. KEGG, Kyoto Encyclopedia of Genes and Genomes.

RT-qPCR verification of mTOR-associated differentially expressed genes. The expression levels of mTOR signaling pathway-associated genes DDIT4, RHEB, EIF4EBP1 and RPS6KB1, which exhibited differential expression in the microarray datasets, were verified by RT-qPCR. The results demonstrated that the differences in the expression of the four genes were consistent with the GeneChip data (Fig. 2A). In addition, the mTOR mRNA expression level was also downregulated in SH-SY5Y cells following isatin treatment compared with untreated controls (Fig. 2B).

Rapamycin partially reverses the effects of isatin. To validate the role of mTOR in the anti-invasive effects of isatin on SH-SY5Y cells, migration and invasion assays were performed in the presence and absence of rapamycin. The rate of wound healing of SH-SY5Y cells co-treated with isatin and rapamycin was lower compared with that of isatin-treated cells (Fig. 3A and B). In addition, the invasion analysis showed that 200 μM isatin decreased the invasive ability of SH-SY5Y cells compared with untreated controls, which was partly restored by co-treatment with rapamycin (Fig. 3C and D), and the mean numbers of invasive cells were 64 and 115, respectively.

Isatin treatment affects the expression of proteins associated with the mTOR pathway. SH-SY5Y cells were treated with a range of isatin concentrations (0-300 μM) for 48 h and total or phosphorylated cell proteins were extracted for western blotting. The mTOR phosphorylation level was inhibited by isatin in a concentration-dependent manner (Fig. 4A and B). By contrast, the phosphorylation of AMPK, which is an inhibitor kinase of mTOR, was increased by isatin compared

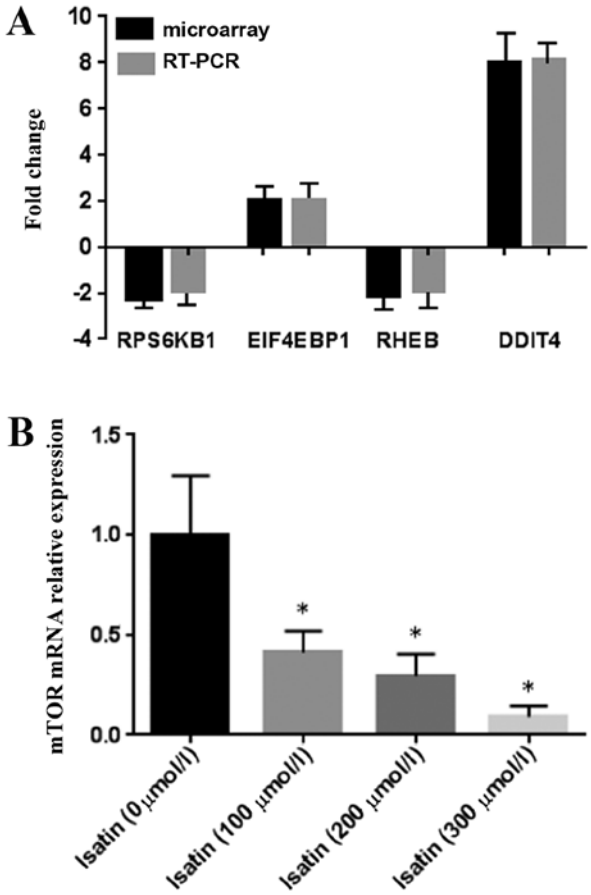

Figure 2. mTOR and mTOR-related gene expression is affected by isatin treatment. (A) Comparison of RT-qPCR and GeneChip results. (B) Relative mRNA expression of mTOR normalized to GAPDH following a 48-h isatin treatment. *P<0.05 vs. Isatin (0 μmol/l). RT-qPCR, reverse transcription-quantitative PCR.

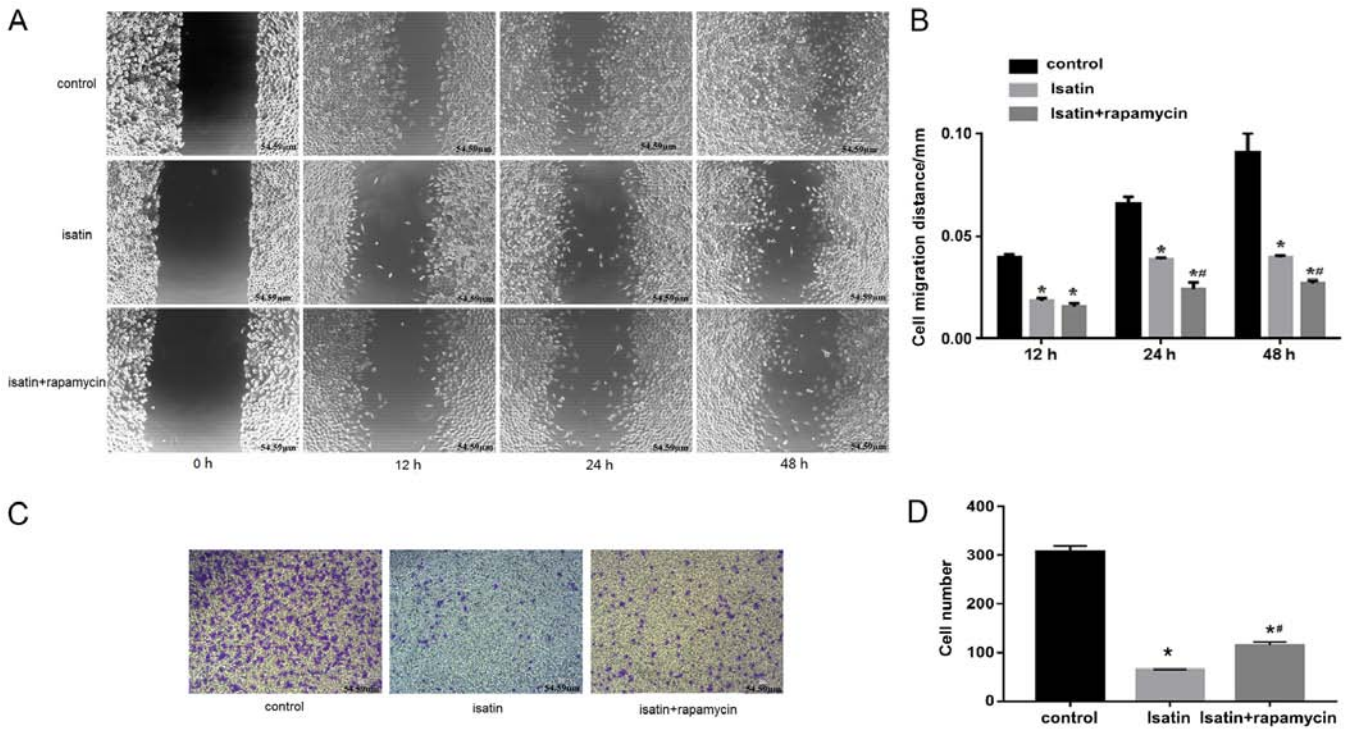

Figure 3. Isatin inhibit migration and invasion of SH-SY5Y cells via mTOR. (A) Effects of isatin (200 μM) and rapamycin (10 μM) on neuroblastoma cell migration. Scale bar=54.59 μm. (B) Statistical analysis of SH-SY5Y cell migration distance at 12, 24 and 48 h. (C) Effects of isatin (200 μM) and rapamycin (10 μM) on neuroblastoma cell invasion. (D) Statistical analysis of the invasion of SH-SY5Y cells, counted in three random fields of view. The statistical analyses were performed by one-way analysis of variance followed by least significant difference or Tamhane's T2 post hoc test. *P<0.05 vs. respective control. #P<0.05 co-treatment of isatin and rapamycin vs. isatin treatment.

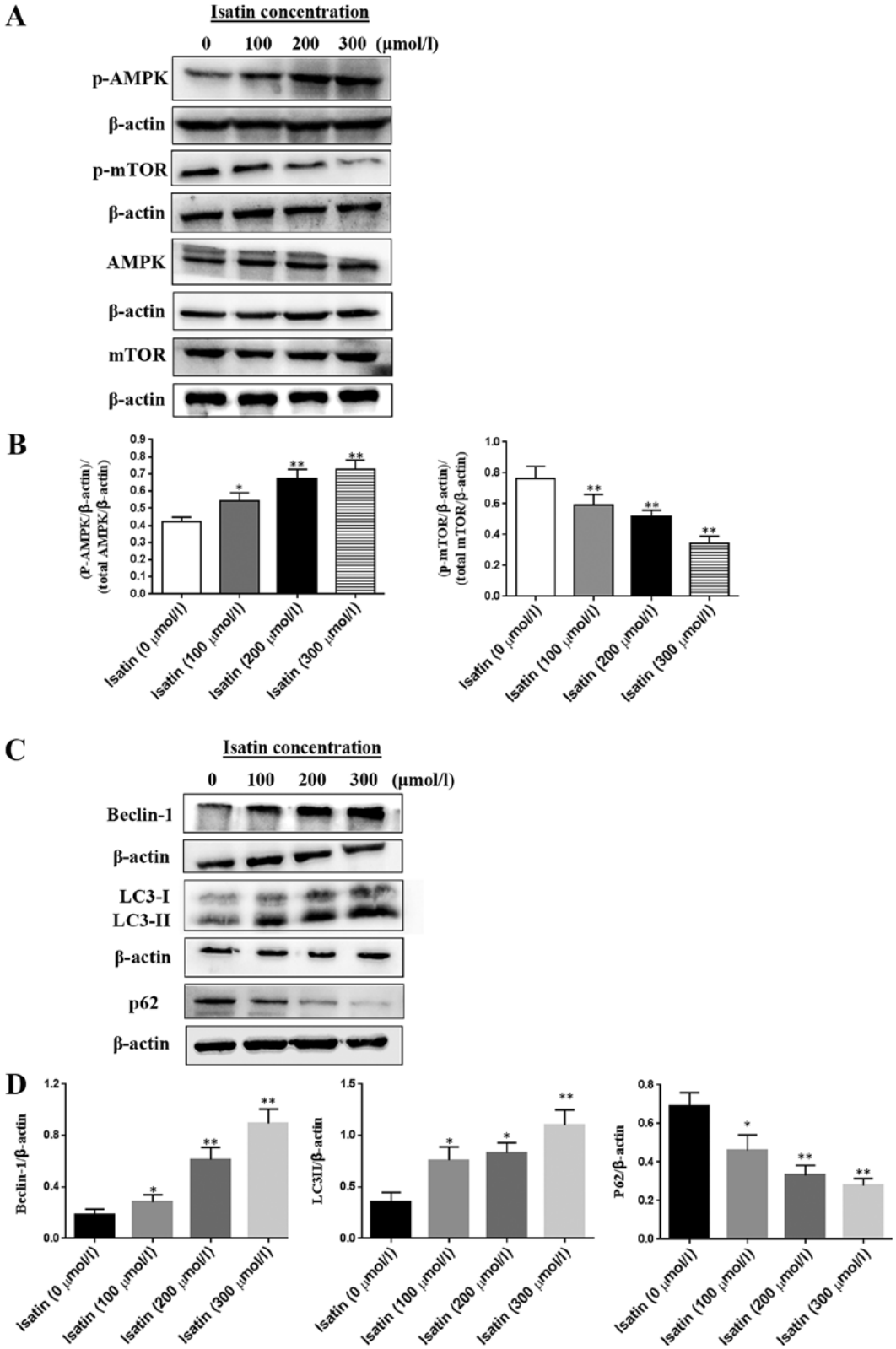

Figure 4. Isatin affects the expression of mTOR-associated proteins in SHSY-5Y cells. (A) Western blot analysis of the expression levels of mTOR, p-mTOR, p-AMPK and AMPK in SH-SY5Y cells following a 48-h isatin treatment. (B) Statistical analysis of the expression levels of p-mTOR and p-AMPK protein relative to β-actin. (C) Expression of LC3, p62 and beclin-1 in SH-SY5Y cells following a 48-h treatment with isatin. (D) Statistical analysis of the expression of LC3, p62 and beclin-1 protein relative to β-actin. Values are expressed as the mean ± SD and were compared by ANOVA with least significant difference or Tamhane's T2 post hoc tests. *P<0.05 and **P<0.01 vs. 0 μmol/l isatin. p, phosphorylated; LC3, microtubule associated protein 1 light chain 3α.

with that in untreated cells (Fig. 4A). LC3-II expression was upregulated and p62 was downregulated in SH-SY5Y cells treated with 200 or 300 μM isatin compared with the control group (P<0.01; Fig. 4C and D). In addition, tumor suppressor Beclin-1 was also activated following isatin treatment (Fig. 4C and D).

Discussion

Tumor cell migration and invasion are the principal steps in tumor metastasis (23,24). The majority of neuroblastoma-related mortalities are due to the infiltration of tumor cells to lymph nodes, bones and bone marrow (25). Controlling tumor metastasis is a promising approach for neuroblastoma treatment. In the present study, microarray analysis was conducted to investigate the underlying mechanism of the anti-metastatic effects of isatin on neuroblastoma.

The mTOR gene is a key regulator of cell growth, proliferation, differentiation and survival (26,27). The activated mTOR signaling pathway accelerates tumor progression and downregulates genes such as RHEB, DDIT4, EIF4EBP and RPS6KB1, all of which are positive regulators of mTOR (28,29). The results of the microarray analysis in the present study demonstrated that the expression levels of these genes were among the most significantly altered by isatin treatment, which was further verified by RT-qPCR. These findings suggested that the mTOR signaling pathway may be involved in tumor progression following isatin treatment.

The results of the present study also revealed that the anti-metastatic effect of isatin was weakened by the inhibition of mTOR expression by rapamycin. AMPK is a serine/threonine protein kinase that has inhibitory effects in certain types of tumor, including prostate, pancreatic and thyroid cancer (30). Activated AMPK activates the tuberous sclerosis complex, which leads to inhibition of mTOR activity, subsequently inhibiting tumor angiogenesis (31). In the present study, activated phosphorylation of AMPK was observed, which subsequently inhibited the phosphorylation level of mTOR.

In cancer cells, autophagy is triggered in response to cellular stress, such as nutrient or growth factor starvation and hypoxia (32). The occurrence of autophagy can inhibit tumor cell development in a number of tumors, such as osteosarcoma and glioblastoma (33,34). Further studies have demonstrated that autophagy is also closely related to tumor invasion and metastasis (35,36). The mTOR signaling pathway serves a crucial role in autophagy (37,38). Among proteins related to autophagy, LC3 is an essential marker; the level of LC3-II expression in the membrane of autophagosomes reflects the level of autophagy (39,40). p62 ubiquitin-like binding protein is also a marker protein for autophagy detection; the expression level of p62 is negatively correlated with autophagic activity (41). The tumor suppressor function of autophagy is induced by certain ATG-proteins, such as Beclin-1, which exhibit anti-oncogenic functions (42). Inactivation of autophagy-related genes, including Beclin-1, leads to increased tumorigenesis, whereas overexpression of these genes inhibits the formation of human breast, ovarian and prostate tumors in mouse models (43). The results of the present study revealed high levels of autophagy-related protein expression in isatin-treated cells, which further explained the mechanism of action of isatin in neuroblastoma metastasis.

In conclusion, isatin is an effective inhibitor of neuroblastoma cell invasion and migration; the mechanism of the inhibition may be associated with the mTOR signaling pathway. Further studies are necessary to confirm whether isatin is a possible anti-metastasis drug for human neuroblastoma.

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Availability of data and materials

All data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LZ, LH and WS performed statistical analyses, and contributed towards the conception and design of the present study. LH supervised the study. All authors including CJ, YC and XW were involved in the acquisition and interpretation of data. LZ, WS and LH drafted the manuscript. All authors contributed to revision of the manuscript for important intellectual content. All authors approved the final version of the manuscript to be submitted.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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