Chronic Diseases Prevention Review 6 (2018) 6-9

Available at http:// www.cancercellresearch.org ISSN 2158-0820

Isatin inhibits FAK signaling pathway in neuroblastoma

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Abstract: **Isatin has been shown to initiate apoptotic processes in neuroblastoma SH-SY5Y cells. Immunohistochemical (IHC) results indicated that 73% samples of neuroblastoma have detected FAK, and the phosphorylation of p125FAK increased obviously in stage IV tumors. Therefore, this study aimed to investigate whether isatin inhibited FAK signaling pathway in SH-SY5Y cells. According to the results, isatin reduced the levels of phosphorylated FAK following treatment with isatin for 48 h. In addition, isatin significantly inhibited MMP9 expression. These effects may be exerted by isatin via down-regulating the expression level of p-FAK.**

Keywords: SH-SY5Y; Neuroblastoma; Isatin; FAK

Received 26 December 2017, Revised 2 March 2018, Accepted 25 March 2018

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1. Introduction

Isatin, with simply chemical structure, is easily susceptible substitution and addition reactions, which is the monomer of anti-tumor drug indirubin. Previous research has shown that isatin has an inhibitory effect for a variety of tumors. With the same principle of various anti-cancer drugs, isatin is capable of altering the proliferation of human neuroblastoma cells (SH-SY5Y) by blocking cell cycle[1]. Neuroblastoma is the most common childhood extracranial solid tumor, which originates from the neural crest of sympathetic ganglia. In the proportion of child cancer, neuroblastoma is as high as 7%-10% and the long-term survival rate in patients remains less than 55%[2]. Neuroblastoma cells have invasive characteristics and are less sensitive to chemotherapy. Main patients are infants and young children who must be careful medication, which greatly increased the difficulty of treatment [3,4].

The new treatments gradually focus on metastasis of neuroblastoma. Fas, a large protein complexes, through which cytoskeleton can connect with extracellular matrix. Through the complex adhesion, SH-SY5Y transmits signals and regulates cellular behavior. The most important part of FAs system is focal adhesion kinases (FAK). In a study[5], immunohistochemical (IHC) results showed that 73% samples of neuroblastoma have detected FAK and the expression of FAK in ganglion type tumor cells is associated with rich mRNA expression in advanced cancer. In stage IV tumors, the phosphorylation of p125FAK increases obviously. Bozzo[6] found that FAK phosphorylated in SH-SY5Y cell lines after adhesion to laminin and collagen Ⅳ. FAK can accept extracellular signals to mediate cell proliferation, invasion and migration. Paxillin was phosphorylated directly by FAK, enhancing cell motility, which is critical for the rearrangement of the cytoskeleton. Additionally, isatin was used to investigate the role of FAK signaling pathways in SH-SY5Y cell.

2. Materials and methods

Cells and cell culture. The SH-SY5Y human neuroblastoma cell line was purchased from Conservation Genetics CAS Kunming Cell Bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Besides, the cells were cultured at 37℃ in an humidified atmosphere of 5% CO_2.

2.1. Cell survival assay

Cells (10^3 cells/well) were seeded into 96-well plates and isatin was added to a final concentration of 25μmol/L, 50μmol/L, 100μmol/L, 200μmol/L, 300μmol/L. Control cells treated without isatin only add 10% DMEM/F12. Following incubation for 48h, the cells were incubated with MTT (1mg/mL) for 3h at 37℃, after which formazan crystals were dissolved in 100 μL DMSO. The absorbance was measured at 490nm using a microplate reade.

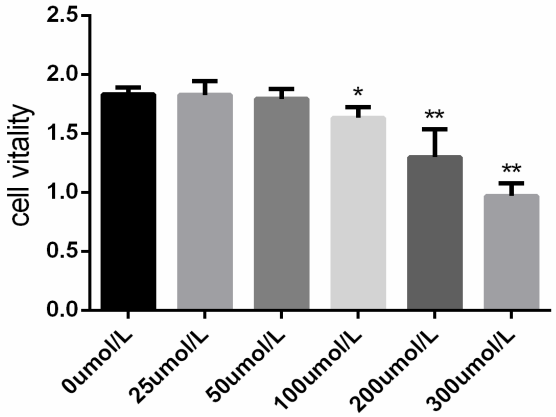

Figure 1. Inhibitory of isatin on SH-SY5Y cell vitality.

Western Blotting detected protein of phosphorylation and relative expression. SH-SY5Y cells (10^7) were cultured in the presence or absence

of isatin for 48 h. Before centrifugation at 10,000g for 20 min, the cells were scraped and lysed in buffer for 20min on ice. The protein concentration of the supernatants was determined using Bradford protein assay reagent, separated in 10% SDS-PAGE and blotted onto PVDF membrane. The blots were blocked in BSA in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 2h at room temperature, before incubation with the monoclonal primary antibodies overnight at 4℃. The blots were washed for 3x5 min in TBS-T and then incubated in peroxidase-conjugated donkey anti-rabbit secondary antibodies for 1h at room temperature. The blots were again washed for 3x5 min in TBS-T and proteins were detected using an enhanced chemiluminescence plus kit. Densitometric analysis was performed based on Quantity One software.

2.2. Statistical analysis

Each experiment was performed at least for three times. Values are expressed as the mean ± standard deviation. Statistical analysis included one-way analysis of variance, which was performed using SPSS software, version 22. In addition, P<0.05 was considered to indicate as statistically significant difference.

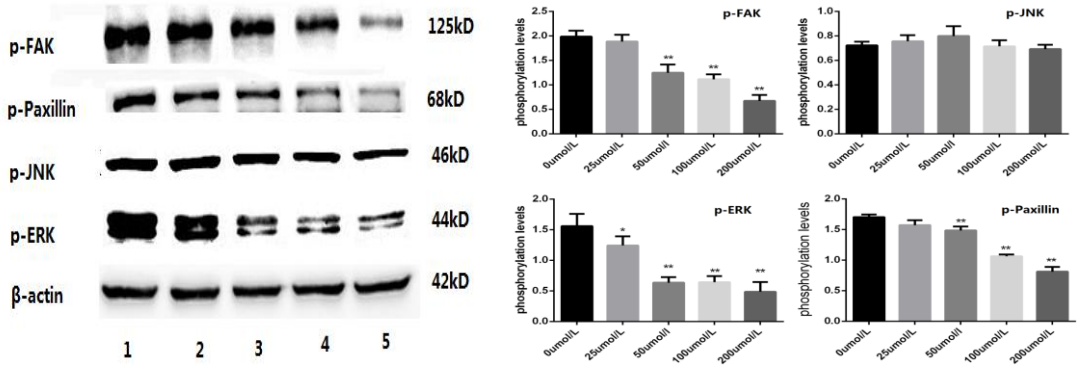

Figure 2. Isatin inhibition of phosphorylation of related proteins in SH-SY5Y cells，lanes1-5:SH-SY5Y cells were treated with 0, 25, 50, 100, 200μmol/L isatin. Levels of phosphorylated FAK, Paxillin, ERK and SAPK/JNK were determined by densitometric analysis with normalization to β-actin.

3. Results

Cell survival assay. After being treated with isatin for 48h, the relative number of cells with different concentration in each group was shown in Figure 1. The data displayed that in the lower concentration, the relative number of cells did not significantly change, P>0.05. When the drug concentration was 100μmol/L, the inhibitory rate was 18%, P<0.05. With the concentration of 200μmol/L and 300μmol/L, the inhibitory rate was 28%, 49%, respectively, P<0.01.

Western Blotting detect protein of phosphorylation and relative expression. After being treated with isatin for 48 h, FAK (Tyr397), Paxillin (Tyr118) and ERK (Thr202/Tyr204) phosphorylation levels decreased with the increasing concentration of isatin P<0.05. However, when isatin concentration was 25μmol/L, the change of FAK (Tyr397) and Paxillin (Tyr118) had no significant difference P>0.05. Besides, SAPK/JNK (Thr183/Tyr185) phosphorylation levels change had no significant difference with the increasing concentration of isatin, P>0.05 (Figure 2).

Western Blotting detect protein of MMP9. After being treated with isatin for 48 h, MMP9 decreased with the increasing concentration of isatin P<0.05 as described in Figure 3. However, when isatin concentration was 25μmol/L, the expression of MMP9 had no significant difference P>0.05. With the concentration of 50, 100 and 200μmol/L, the expression of MMP9s were 41%, 34% and 31%, respectively, P<0.01.

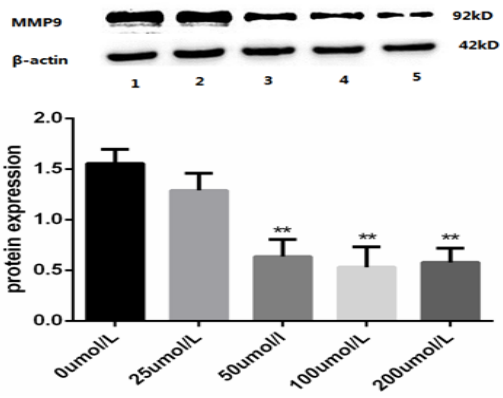

Figure 3. Isatin inhibition of MMP9 protein expression in SH-SY5Y cells， lanes1-5： SH-SY5Y cells were treated with 0, 25, 50, 100, 200μmol/L isatin.

4. Discussion

FAK degrades the extracellular matrix, which is mainly by regulating MMPs. Phenotype of Invasive is related to v-Src-FAK signaling complex gathering in the invasion pseudopodia. Cell migration contributes to the increase of integrins and Metalloproteinases (MMPs). Overexpression of FAK in Squamous Cell Carcinoma has been demonstrated that it can promote invasion of lung cancer cells[7,8]. MMPs can degrade type IV collagen and matrix proteins around the cell, hydrolysis laminin, type V collagen and finally undermine the integrity of basement membranes, providing the basis of tumor cells out of place[9,10]. MMP9 (also called gelatinase b) is an important member of MMP family, as it overexpresses in numerous tumor and associates with poor prognosis in oral cancer and gastric cancer, attracting more attention[11-13]. After integrins activated FAK signaling pathway, actively FAK polymerized actin filaments to the plasma membrane and formed bumps in the forefrontby raising effect[14,15]. Through the formation of FAK/Src complexes and kinase activity, subsequent phosphorylation of p130Cas promoted the formation of Cas/Crk complexes and then affected the cell migration[16,17]. In addition to the formation of focal adhesion, FAK signals regulated by integrins ensure membrane constantly highlight the dynamic and complex process in migration through controlling focal adhesion turnover. For example, by forming FAK/Src complexes in focal adhesion, increasing ERK2 activity leads to the activation of calpain 2, which allows calpain 2 opening molecules such as FAK and Talin in adhesion, and facilitates the focal adhesion turnover in motor cell[15,18,19]. In addition, FAK may also affect the actin cytoskeleton rearrangement and the adhesion stability. Myosin light chain kinase (MLCK) regulates dismantling of adhesion and phosphorylation of paxillin promotes cytoskeletal rearrangements. Paxillin is a typical joint protein with more than one available binding domains to other proteins, which is not only involved in the formation of focal adhesion, but also has many functions of signal transduction. The research conducted by Petit[20] has shown that, Tyrosine phosphorylation of paxillin has an important role in cell movement. By mutating two main sites of paxillin (Tyr118 and Tyr31), cell movements decreased significantly. In the process, paxillin served as a signal and hindered forming complexes with Crk due to the reduction of its phosphorylation, which then affected the cell motility. The cytoskeleton rearrangement regulated by FAK cellular signals cause good mobility in cell, which form the basis of distal migration of tumor cells together with the degradation of the extracellular matrix.

In any case, numerous studies have indicated that FAK-Src-Paxillin system is overexpressed in the invasiveness neuroblastoma. The result shows that the FAK-Src-Paxillin over expression in invasive tumors in recent clinical oncology and pathology. Multiple studies have shown proved that the migration of tumors involves many factors which combines with inhibition of more than one target in tumor with a good effect on cancer treatment.

In this experiment, we analyze several critical proteins in FAK signaling pathways using Western Blotting technology. Results showed that after 48 h treatment of isatin, phosphorylation of FAK activation (Tyr397) decreased, phosphorylation of FAK were affected, and the phosphorylation of ERK, which is the FAK downstream protein, was reduced. Eventually, the expression of MMP9 was decreased. However, as the concentration of the drug increases, phosphorylation of JNK levels, FAK downstream protein, did not change significantly, which may be caused by interaction of cross signaling pathway. As downstream of the FAK protein, paxillin is important for cell motility. Through reducing the phosphorylation of FAK, isatin reduced phosphorylation of paxillin levels, thus affecting the SH-SY5Y rearrangement of the cytoskeleton and cell motility. In SH-SY5Y cells, isatin reduced the level of phosphorylation of FAK and paxillin and then affected the rearrangement of the cytoskeleton and cell motility. Thus, the decrease of FAK phosphorylation levels led to ERK phosphorylation levels decrease, thus affecting the expression of MMP9. Isatin may inhibit neuroblastoma tumor invasion though inhibiting FAK signaling pathway. As a result, we predict that FAK is a potential target for treating neuroblastoma, and isatin serves as a potential drugs for treating neuroblastoma.

5. Conclusion

In summary, MMP9 protein expression level is inhibited in SH-SY5Y cells after being treated with Isatin. The mechanism may involve the down-regulating of p-FAK. Though we have study little work, further study is required. Nevertheless, isatin might be a possible treatment for human neuroblastoma.

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