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Original Article

Chrysin inhibits growth and induces apoptosis of anaplastic thyroid cancer cells via Notch-1/Slug/PUMA signals

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Abstract: Aims: Anaplastic thyroid carcinoma (ATC) is one of the most aggressive malignancies in humans. Although it is rare (<2%), accounts for 14-39% deaths for thyroid cancer. There is no effective treatment for ATC so far. Targeting therapy for ATC might represent a feasible therapeutic step. Chrysin, a flavone subgroup of flavonoids, has been reported as a potential anti-proliferative agent in ATC cells. However, the mechanisms of how Chrysin functions remains unclear. In the present study, we observed the anti tumor effect of Chrysin on ATC cells in vitro, and explored its mechanisms. Methods: SW1736 and 8505C cells were transfected with plasmid SLUG cDNA or small interfering RNA (siRNA) targeting Notch-1 or PUMA for 24 hs, then treated with 50 μM Chrysin for 72 hs. Cell survival and apoptosis was detected by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Annexin V-FITC staining for flow cytometric analysis. The signaling pathway protein, such as Notch-1, SLUG, PUMA, and other pro- or anti-apoptotic proteins such as Bad, Bid, Bim, Noxa, Mcl-1 and Bcl-2 was detected by Western blot assay. Results: We showed that Chrysin treatment (50 Noxasignificantly suppressed growth and induced apoptosis of ATC cells. Notch-1 and PUMA was activated, and Slug was inactivated with Chrysin treatment. Knockdown of Notch-1 or PUMA by siRNA, or Slug overexpression by Slug cDNA transfection totally abrogated Chrysin-induced PUMA activation and cell apoptosis as well cell survival inhibition. Conclusion: Chrysin could be an effective therapeutic agent to inhibit growth and induce apoptosis of ATC cells. The effect of which depends on the Notch-1 activation and SLUG inactivation, and eventually makes PUMA free from Slug. Our study suggests that Chrysin could be a effect agents for the treatment of ATC cells. Thus, further clinical investigations are warranted.

Keywords: Anaplastic thyroid carcinoma, Chrysin, apoptosis, Notch-1, slug, PUMA

Introduction

Anaplastic thyroid carcinoma (ATC) is one of the most lethal of all human malignancies. Although less than 2% of all thyroid cancer patients occurs, it contributes to (14-50)% of the deaths for thyroid cancer [1]. Because of its highly malignant, all the ATC patients are classified as having stage IV disease by the American Joint Committee on Cancer [2]. Rare morbidity and short survival time (median survival of 3 to 5 months) made it difficult for the scientists to find an effect and widely accepted methods for the treatment of ATC [3].

Not expressing thyroid-specific genes, so treatment with radioiodine is no use for ATC. Eradication by complete surgical resection with total thyroidectomy was only applied for the patients of early stage of pathological changes [3, 4]. Furthermore, these patients must accepts radiation or chemotherapy or in combination treatment. Even so, the long-term survival was not significantly enhanced for such patients [4, 5]. As to the patients of late stage of pathological changes, radically surgical organ resection could only improve temporary local symptoms, but increased the morbidity [6]. Traditional chemotherapy has little effect on metastatic ATC cases. Aggressive radiotherapy can only reduce locoregional recurrences, but does not improve the median overall survival rate in over 50 years [7]. Although some new agents has been used

for targeting treatment of this disease, unexpected good results occurs [8]. Therefore, there is a critical for us to develop new agents for the treatment of patients of ATC.

Notch signaling is a highly conserved pathway, which is activated by its ligands [9]. When the Notch receptor is activated, the receptor-ligand then triggers a second Notch extracellular domain cleavage by a metalloproteinase ADAM, which in turn downregulates ligand activity [10]. Up to date, only four Notch genes have been identified in mammals (Notch-1 to 4) [9]. All the Notch receptors are very similar in structures, although there are some subtle differences in their cytoplasmic domains and extracellular [11].

More and more evidences show that Notch signaling plays an important role in cell apoptosis and proliferation, the roles of which are involved in the development of organ's function [12-14]. Notch-1 is the most studied gene among Notch1 to Notch-4. Many studies have demonstrated that the Notch-1 gene is abnormally activated in many human malignancies [15-20]. Notch-1activation might predict poorer survival and more aggressive behavior in patients with malignancies [15, 21-24].

Although Notch-1 activation is generally held to promote cancer development, however, Notch-1 was inactivated in thyroid cancer tissue and cell lines [25, 26]. Further research proved that Notch-1overexpression could inhibit cell proliferation and induce apoptosis in thyroid cancer cells [27], suggesting that activation of Notch-1 signaling may be have an effectively therapeutic role for thyroid cancer.

Chrysin, a natural flavonoid, was widely found in fruit- and vegetable derived foods [28]. Recently, it has reported that Chrysin has pro-apoptosis and anti-proliferative activities against various cancers [29-32]. In ATC *in vitro* and *in vivo*, Chrysin was also showed obvious antitumor effect, by which Notch-1 signal was activated [33-34]. However, the detailed mechanisms or signaling pathway of Notch-1 functions is not very clear.

PUMA (p53 upregulated modulator of apoptosis), a BH3-only Bcl-2 family protein, plays an essential role in cancer cell or normal cell apoptosis induced by various stimuli [35, 36]. Slug, a zinc finger transcriptional repressor, a pro-metastasis and antiapoptotic factor, could inhibit activation of PUMA, protecting cancer cells or normal cells from apoptosis [37-39]. Further study showed that Slug was the strong inhibitor of PUMA [37].

Chang et al. has found that Slug inhibition by Notch-1 activation played an important role in LPS-induced endothelial cells death, suggesting that Slug was required for survival in Notch-activated endothelial cells [40]. Considering that Slug was overexpressed in ATC [41], and Notch-1 was negative in ATC cell lines [25, 26], we supposed that Notch-1 and SLUG has negatively relation in ATC tissue and cell line.

In the present study, we first investigated the role of chrysin treatment on cell growth and apoptosis of ATC cells. We then observed the effect of chrysin on Notch-1 signaling pathway. Our study suggests that Chrysin could be an effective agent for the treatment of ATC cells. Thus, further clinical investigation are warranted.

Materials and methods

Cell culture

The human anaplastic thyroid cancer (ATC) SW1736 and 8505C cell lines was purchased from DSMZ (Beijing, China). The cells were cultured as the DSMZ's instruction. Briefly, SW-1736 and 8505C cells were grown in RPMI1640 (Hangzhou, China) medium, supplemented with 10% fetal bovine serum (FBS), penicillin (100 uM/l), non-essential amino acids and sodium pyruvate. The adherent monolayer cultures were maintained on plastic and incubated at 37°C in 95% air and 5% carbon dioxide. The cultures were free of Mycoplasma species. In all of the assays, a monolayer of cells that was 50-70% confluent was used.

Plasmid and agents

siRNA targeting Notch-1 and its scratching siRNA were purchased from Santa Cruz Biotechnology (Shanghai, China). PUMA siRNA and SLUG cDNA plasmid was preserved in our laboratory. Anti-Notch-1, PUMA, SLUG, SNAIL, Mcl-1, TWIST, bcl-2, Bak, Bid, Noxa, Bim and β-actin antibody were purchased from Santa Cruz Biotechnology (Shanghai, China. Chrysin was purchased from ToYongBio (Shanghai, China).

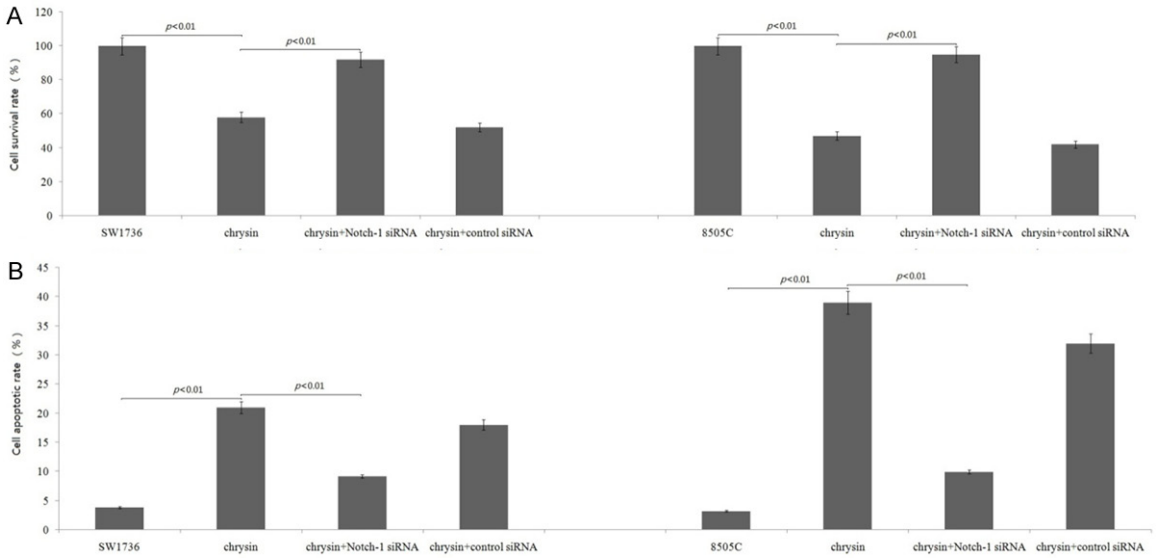

Figure 1. Effect of Chrysin on proliferation and apoptosis of SW1736 and 8505C cells. SW1736 and 8505C cells were treated with 50 μM chrysin for 72 hs or transfected with Notch-1 siRNA/control siRNA for 24 hs then treated with 50 μM chrysin for 72 hs. A. Cell survival rate was detected by MTT assay; B. Cell apoptosis was analyzed by annexin V/PI staining followed by flow cytometry. It was showed that chrysin treatment inhibits proliferation and induces apoptosis in the cells. Knockdown of Notch-1 by siRNA inhibited chrysin-induced the cell apoptosis and the proliferation.

Transient transfection

SW1736 and 8505C (1×10^5) cells were transfected with either 2 uM of Notch-1 siRNA or PUMA siRNA (siGENOME SMARTpool) or scrambled siRNA or SLUG cDNA plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The effect of protein knockdown was achieved by western blot assay after 24 h transfection.

Chrysin treatment

SW1736 and 8505C cells were exposure to 50 μM Chrysin for 72 h. Cell viability and cell apoptosis was detected. To determine the effect of Notch-1 or SLUG or PUMA on Chrysin-induced apoptosis and cell viability, SW1736 and 8505C cells were transfected with Notch-1 siRNA or SLUG cDNA or PUMA siRNA or their control for 24 hs, then exposured the cells to 50 μM Chrysin for 72 h.

Cell viability assay

SW1736 and 8505C cells in different treatment groups were seeded in 96-well plates at a density of 5000 cells/well in 100 mL of medium, and incubated for 24 hs. After changing the medium as usually, the cells above were treated with 50 μM Chrysin for 72 hs. DMSO was as negative control. Then 100 ml medium containing 10 ml MTT solution (5 mg/ml stock in PBS) per well was added to the wells, and incubated 4 h at 37°C with 5% CO_2. Dissolved the crystals formed in each well in 100 mL DMSO and mix them on Shaker for 1 min. The absorbance of each well at 595 nm was measured on a Multilabel counter. The relative cell viability was calculated as the formular. Relative cell viability =[A^{595} (treated)-A^{595} (blank)/(A^{595} (control)-A^{595} (blank)]×%. Each experiment was repeated at least 4 times, and Each treatment was performed in 3 times.

Cell apoptosis assay

Apoptotic cells were detected by Annexin V-FITC (AV-FITC) Apoptosis Detection Kit according to the manufacturer's instructions. Briefly, SW-1736 and 8505C cells (1×10^6) in different treatment groups were stained with AV-FITC for 30 min at 4°C in the dark, and stained with propidium iodide (PI) for 10 min. Then flow cytometric analysis was performed according to the manufacturer's instructions.

Western blot analysis

SW1736 and 8505C cells in different treatment groups were trypsinized and washed with cold PBS. Total cell protein was extracted with

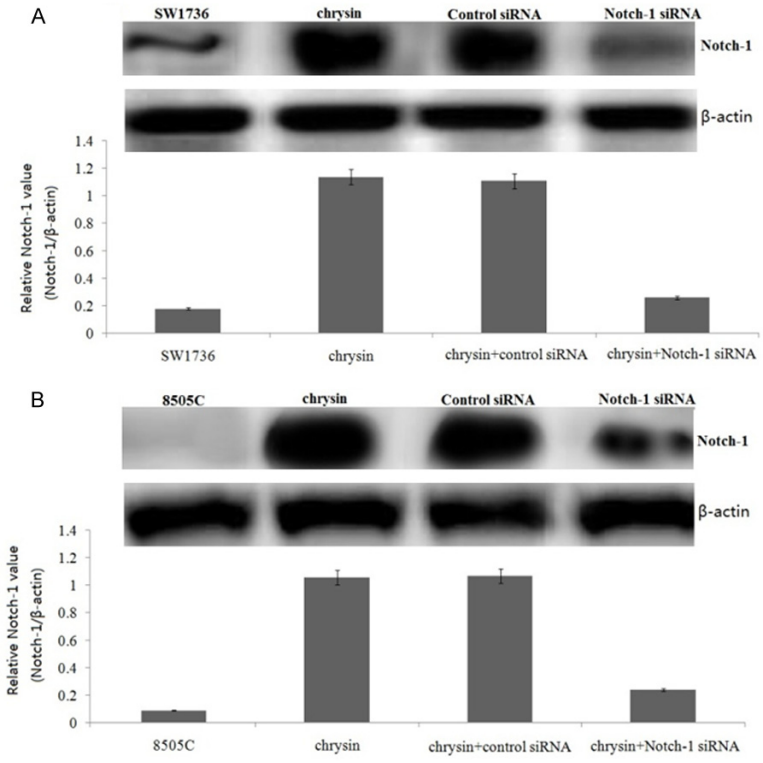

Figure 2. Effect of Chrysin on expression of Notch-1 in SW1736 and 8505C cells. SW1736 and 8505C cells were treated with 50 μM chrysin for 72 hs or transfected with Notch-1 siRNA/control siRNA for 24 hs then treated with 50 μM chrysin for 72 hs. Notch-1 expression was detected by western blot assay in SW1736 (A) and 8505C cells (B). It was showed that chrysin treatment induced Notch-1 expression in both of the cells. Knockdown of Notch-1 by siRNA inhibited chrysin-induced Notch-1 upregulation.

ABC protein extraction solution. Protein concentration of the cell extraction was determined using BAC protein assay kit according to the manufacturer's instructions. Protein samples were separated by 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. After the membranes were blocked at room temperature, protein blots were probed with specific antibodies (Notch-1, PUMA, SLUG, SNAIL, TWIST, bcl-2, Mcl-1, Bak, Bid, Bim and Noxa) overnight at 4°C followed by incubation with corresponding secondary antibody. β-actin was used as loading control.

Statistical analysis

The data were expressed as means ± (standard error) SE. Statistical differences between each group were determined by one-way ANOVA followed by *t*-test. All calculations and statistical analysis were performed using SPSS. 22 soft (SPSS Inc., Chicago, IL, USA). Statistical results were regarded as statistically significant at $P<0.5$.

Results

Chrysin inhibits proliferation and induces apoptosis of ATC cells

Previously study has found that chrysin could statistically inhibit proliferation of ATC cells in a dose- and time-dependent manner [34]. In the present study, we exposured the SW1736 and 8505C cells to 50 μM Chrysin for 72 h and explored its effect on cell growth and cell apoptosis. Our results showed that 50 μM Chrysin could resulted in 42% and 53% cell growth inhibition in SW1736 and 8505C cells, respectively ($P<0.01$) (Figure 1A).

Next, we examined the effect of chrysin on cell apoptosis of SW1736 and 8505C cells. Flow cytometry analysis showed that treatment of SW1736 and 8505C cells with 50 μM chrysin for 72 h resulted in 21% and 39% cell apoptosis, respectively ($P<0.01$) (**Figure 1B**). These results here showed that chrysin could induce apoptosis in ATC cells, which might be related with the cell growth inhibition.

Chrysin induces ATC cell apoptosis by Notch-1-dependent signaling

Western blot assay showed that rich baseline expression of Notch-1 was detected in SW1736 cells (**Figure 2A**), and poor baseline expression of Notch-1 in 8505C cells (**Figure 2B**). Chrysin (50 μM) treatment for 72 h induced Notch-1 protein expression in both SW1736 and 8505C cells (**Figure 2A**, **2B**).

We transiently transfected Notch-1 siRNA or scramble siRNA into SW1736 and 8505C cells for 24 h before chrysin treatment, then the siRNA transfected cells were exposure to chrysin (50 μM) for 72h. We found that knockdown

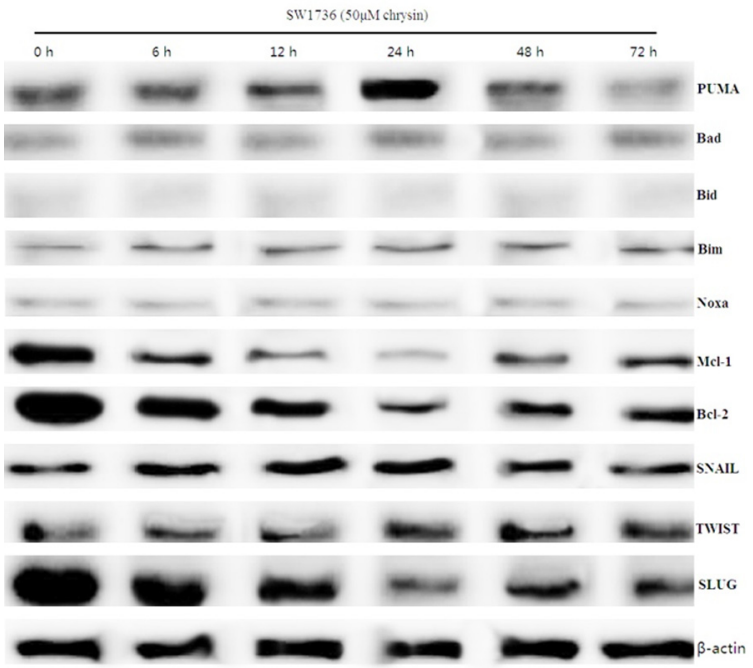

Figure 3. Effect of Chrysin on SNAIL family and BH3-only proteins expression in SW1736 cells. SW1736 cells were treated with 50μM chrysin for 6-72 hs. BH3-only proteins Bad, Bid, Bim, Noxa, Mcl-1 and Bcl-2 as well as SNAIL family SLUG,SNAIL and TWIST protein was detected by western blot assay. The results showed that SLUG expression was decreased and PUMA expression was increased after Chrysin treatment.

of Notch-1 by Notch-1 siRNA transfection inhibited chrysin-induced Notch-1 activation in the two cells (**Figure 2A**, **2B**). Further, knockdown of Notch-1 blocked chrysin-induced cell apoptossis (**Figure 1B**) and growth inhibition (**Figure 1A**). Scramble siRNA has no affect on chrysin-induced Notch-1 upregulation, apoptosis and growth of the two cells (data not shown). These results here showed that chrysin induces ATC cell apoptosis and growth inhibition by activation of Notch-1 signaling.

Chrysin selectively induces PUMA in ATC cells

PUMA protein expression was detected by western blot after SW1736 and 8505C cells were exposured to chrysin (50 μM) for 72 hs. The results showed that PUMA protein was strongly induced in a time-dependant manner in SW1736 cells (**Figure 3**). Furthermore, PUMA protein reached the peak value at 24 h after chrysin exposure, then decreased gradually (**Figure 3**).

Analysis of other Bcl-2 family members showed that chrysin treatment did not upregulate pro-apoptotic protein, such as Bim, Noxa, Bad and Bid, but downregulate antiapoptotic protein Mcl-1 and Bcl-2 (**Figure 3**). Chrysin has the same effect on 8505C cells (data not shown).Our results showed that chrysin could selectively induce PUMA and mediate its proapoptotic effects.

PUMA is required for chrysin-induced apoptosis in ATC cells

We transiently transfected PUMA siRNA or scramble siRNA into SW1736 and 8505C cells for 24 h, then the siRNA transfected cells were exposure to chrysin (50 μM) for 72 h. We found that knockdown of PUMA by PUMA siRNA transfection inhibited chrysin-induced PUMA activation in the two cells (**Figure 4A**, **4B**). Furthermore, knockdown of PUMA blocked chrysin-induced cell apoptossis (**Figure 4C**) and growth inhibition (**Figure 4D**).

These results here showed that chrysin induces ATC cell apoptosis and growth inhibition by activation of PUMA signaling.

Chrysin selectively inhibited Notch-1 mediated Slug expression in ATC cells

Considering the potential relation between Notch-1 and Slug, we assessed the effects of chrysin on SLUG protein and RNA expression in SW1736 and 8505C cells. We observed that treatment with 50 μM chrysin for 72 h significantly inhibited Slug protein expression in Slug-rich SW1736 cells (**Figure 3**). However, Slug protein was increased gradually 24 h after chrysin exposure (**Figure 3**). Chrysin has the same effect on 8505C cells (data not shown).

The Snail family members mainly includes SLUG, SNAIL and TWIST, we next studied whether chrysin treatment have effect on SNAIL and TWIST expression. The results showed that chrysin treatment only slightly increased SNAIL protein at 24 hours following chrysin treatment, but did not induce the TWIST expression (**Figure 3**). These results indicated that chrysin only selectively inhibited Slug signaling.

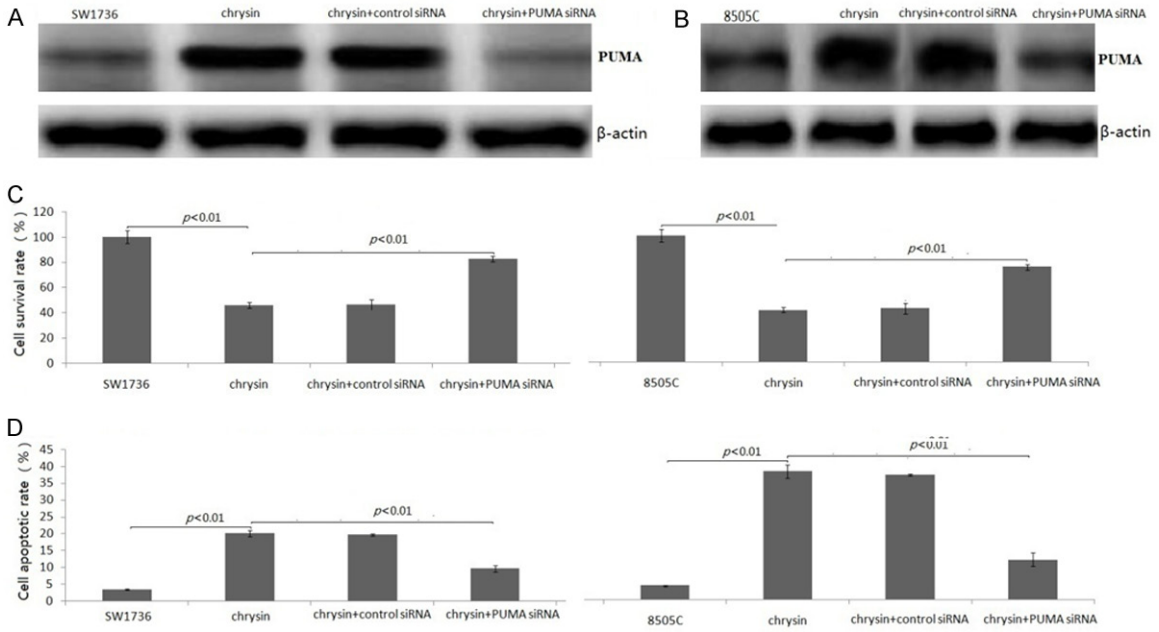

Figure 4. Chrysin -induced apoptosis and growth inhibition in SW1736 and 8505C cells via PUMA upregulation. SW1736 and 8505C cells were transfected with PUMA siRNA or control siRNA for 24 h, then treated the transfected cells with 50 μM chrysin. PUMA protein expression was detected by western blot assay in SW1736 (A) and 8505C cells (B). Cell survival rate was detected by MTT assay in SW1736 and 8505C cells (C). Cell apoptosis was analyzed by annexin V/PI staining followed by flow cytometry in SW1736 and 8505C cells (D). These results showed that knockdown of PUMA by siRNA inhibited chrysin-induced apoptosis in ATC cells.

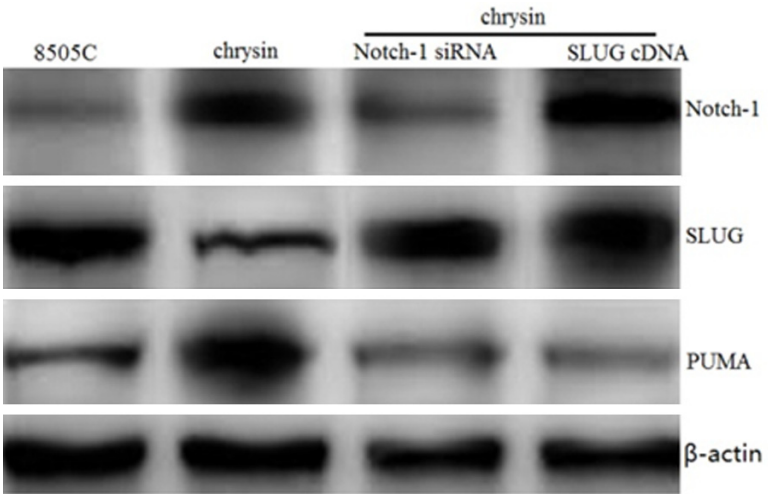

Figure 5. PUMA activation by chrysin is mediated by Notch-1/SLUG pathway. 8505C cells were transfected with Notch-1 siRNA, SLUG cDNA or PUMA siRNA, then treated with 50 μM chrysin for 72 hs. Notch-1, SLUG and PUMA protein expression was detected by Western blot assay.

PUMA activation by chrysin is mediated by Notch-1/SLUG pathway

We next investigated the relation between Notch-1, SLUG and PUMA. In 8505C cells, treatment with 50 μM chrysin induced Notch-1 and PUMA protein expression, but decreased the Slug protein expression (**Figure 5A**). We transfected Notch-1 siRNA into 8505C cells, then treated the transfected cells with 50 μM chrysin for 72 h, only to find that Notch-1 and PUMA protein expression was blocked, but Slug protein expression was increased (**Figure 5A**). We transfected Slug cDNA into 8505C cells , then treated the transfected cells with 50 μM chrysin, only to find that Notch-1 and Slug protein expression was increased with chrysin treatment, but PUMA expression was blocked (**Figure 5A**). Chrysin has the same effect on SW-1736 cells (Data not shown).

Discussion

As highly conserved Notch signaling pathway, Notch activation could, on the one hand, promote cancer development [18-20, 41, 42], on the other hand, inhibit cancer development [25, 26, 43], which might depend on its cellular con-

text. Many studies have reported that targeting Notch-1 in Notch-1-rich expressed cancer cells suppressed cancer cell growth and metastasis [44]. However, Notch-1 was inactivated in thyroid cancer or cell lines [25, 26], and overexpression of Notch-1 play an effective anti tumor effect in thyroid cancer cells [26]. So we can imagine that activation of Notch-1signaling in ATC cells could produce antitumor effect. However, except for gene therapy, there is no method to deliver activated *Notch-1* to tumor cells up to date. Therefore, identification of an activitor to activate Notch-1 signal would likely provide a therapeutic benefit for ATC.

Due to its biological anticancer properties, Chrysin, a natural flavonoid, has got a wide range of concerns in the world. At present, Chrysin has found to induce apoptosis in many cancer cells, such as human leukemia U937 cells [45, 46], esophageal squamous cell carcinoma cell [47] and colon carcinoma cell lines [48]. In ATC cells, chrysin has been reported to inhibit survival and induce apoptosis of culture cells in vitro and xenograft in vivo of ATC cells [43]. In our results, chrysin also showed a significant antiproliferation and proapoptotic effect on ATC cells in vitro, a finding similar to those observed before [43]. However, the exact mechanism of how Chrysin induces apoptosis remains unclear.

In our study, treatment with Chrysin activated the Notch-1 signaling, followed by increased cell apoptosis and cell growth inhibition in ATC cells. However, knockdown of Notch-1 could block Chrysin-induced apoptosis in ATC cells, suggesting that Chrysin-induced apoptosis was achieved by activation of Notch-1 signaling.

Monasterio et al. have found Chrysin induced apoptosis in human leukemia U937 cells via activation of caspase-3 dependent pathway [45, 46]. Zhang et al. have found that Chrysin induced cell apoptosis was mitochondria-mediated through an up-regulation of cleavage of caspase-9 and caspase-3 [47]. Yu et al. have found that Chrysin induced apoptosis in ATC cells by activation of cleaved-PARP [43]. From the studies above, we could conclud that Chrysin might function by mitochondrial pathway.

Proapoptotic protein PUMA mediates death signals primarily through the mitochondria. Through interaction with Bcl-2 family members Bax and/or Bak, PUMA induces mitochondrial dysfunction and caspase-activation, and inducing cell apoptosis [48]. To explore the effect of PUMA on Chrysin-induced apoptosis in ATC cells, PUMA siRNA was transfected into the ATC cells, then treated with Chrysin for 72 h. The results showed that PUMA is required for chrysin-induced apoptosis in ATC cells. PUMA upregulation, followed by decreased bcl-2, suggested that Chrysin function by mitochondrial pathway. In addition, Chrysin treatment did not induce other BH3-only proteins Bad, Bid, Bim, and Noxa, suggesting that chrysin selectively activate PUMA and mediate its anticancer effects. In our study, activity of Notch-1 and PUMA were all necessary for Chrysin-induced apoptosis of ATC cells. Furthermore, knockdown of Notch-1 blocked Chrysin-induced PUMA upregulation. We therefore suggested that Notch-1/PUMA pathway was necessary for Chrysin-induced apoptosis and growth inhibition in ATC cells.

Slug was the antiapoptotic protein, which was found to protect cells from apoptosis induced by various stimuli [49]. Numerous studies have reported that the protective effect of Slug was achieved by downregulation of PUMA [49, 50]. In addition, the activated Notch pathway could up-regulated Slug, but not Snail, and launch epithelial-mesenchymal transition [51-53]. Therefore, close connection exists among Notch-1, Slug and PUMA.

We showed here that SLUG targeted *PUMA*, and no other pro-apoptotic gene, including the Noxa and Bim to suppress apoptosis. This was in line with the previous study [51]. In addition, Slug, but not Snail and Twist, was negatively regulated by Notch-1. It was in line with the previous study [52, 53]. In addition, knockdown of PUMA and Notch-1 or Slug overexpression blocked Chrysin-induced cell apoptosis. The results showed that chrysin induced apoptosis of ATC cells by activation of Notch-1/Slug/PUMA axis.

Conclusion

Our results showed for the first time that Chrysin activated Notch-1, which resulted in the slug inactivation, leading to PUMA activation and onset of mitochondria-mediated apoptosis in ATC cells. We believe that Chrysin could

be an effective therapeutic agent for the treatment of ATC. Thus, further clinical investigations are warranted.

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Disclosure of conflict of interest

None.

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