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Hesperetin Activates the Notch1 Signaling Cascade, Causes Apoptosis, and Induces Cellular Differentiation in Anaplastic Thyroid Cancer

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Abstract

Background—Anaplastic thyroid cancer (ATC) is characterized by very aggressive growth with undifferentiated features. Recently, it has been reported that the Notch1 signaling pathway, which affects thyrocyte proliferation and differentiation, is inactivated in ATC. However, it remains largely unknown whether Notch1 activating compounds can be an effective therapeutic strategy in ATC. Therefore, in this study, we aimed to evaluate the drug effects of a potential Notch activator Hesperetin on ATC cell.

Methods—A unique ATC cell line HTh7 was used to evaluate the drug effects of Hesperetin. The Notch1 activating function and cell proliferation were evaluated. The mechanism of growth regulation was investigated by the detection of apoptotic markers. The expression levels of thyrocyte-specific genes were quantified for ATC re-differentiation.

Results—Up-regulated expression of Notch1 and its down-stream effectors Hes-1 and Hey-1 was observed in Hesperetin treated ATC cells. The enhanced luciferase signal also confirmed the functional activity of Hesperetin-induced Notch1 signaling. Hesperetin led to a time- and dose-dependent decrease in ATC cell proliferation. The cell growth inhibition was mainly caused by apoptosis as evidenced by increased levels of cleaved poly ADP ribose polymerase (PARP) and cleaved Caspase-3 as well as decreased survivin. Additionally, Hesperetin induced the expression levels of thyrocyte-specific genes including thyroid transcription factor 1 (TTF1), TTF2, paired box gene 8 (PAX8), thyroid stimulating hormone receptor (TSHR), and sodium/iodide symporter (NIS).

Conclusion—Hesperetin activates the Notch1 signaling cascade and suppresses ATC cell proliferation mainly via apoptosis. Hesperetin also induces cell re-differentiation of ATC, which could be useful clinically.

Keywords

thyroid cancer; Hesperetin; anaplastic; differentiation; Notch1 signaling

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Introduction

Thyroid cancer makes up 2.5% of all malignancies in the United States and the incidence continues to rise.¹ Anaplastic thyroid carcinoma (ATC) accounts for less than 5% of all thyroid cancer cases, but it represents over half of thyroid cancer-related deaths.² The prognosis of ATC is extremely poor, with a median overall survival between 3–5 months.^{3,4} ATC is characterized with very aggressive growth, extensive local invasion, and distant metastasis. Additionally, ATC has an undifferentiated feature. As such, the American Joint Committee on Cancer usually classifies ATC as TNM stage IV regardless of the overall tumor burden.^{3,5} The employment of surgery, radiotherapy, and chemotherapy are usually palliative but not curative in nature.^{2,6,7} Therefore, new treatment modalities and novel approaches are desperately needed for patients with ATC.

The Notch1 signaling pathway, first found in *Drosophila*, controls cell fate by regulating cellular differentiation and proliferation.⁸ The aberrant regulation of the Notch pathway has been implicated in the oncogenesis of variant types of cancers. Most of the endocrine-related cancers such as epithelial thyroid cancers, medullary thyroid cancer and gastrointestinal neuroendocrine cancers demonstrate down-regulated Notch1 signaling activity.^{9,10} Regarding the role of Notch1 in ATC, a recent study has reported much lower expression levels of Notch1 in human ATC tissue compared with normal thyroid tissue. Furthermore, overexpression of Notch1 results in cell growth suppression and restores the differentiated phenotype of thyroid cancer cells.¹¹ Thus, activation of Notch1 could yield therapeutic effects in the context of ATC.

Previous studies using a novel high-throughput screening method discovered 27 potential Notch activating compounds out of over 7,000 tested.¹² Hesperetin, one of the potential Notch activators, is a naturally occurring flavanone found in citrus fruits. The antitumor effect of Hesperetin has been observed in breast cancer, colon cancer, melanoma and pancreatic cancer.^{13–16} Most of these studies have found that Hesperetin suppresses cancer growth by inhibition of cell proliferation. However, the drug effect of Hesperetin on ATC cells remains largely underexplored.

In the present study, we aimed to evaluate whether Hesperetin treatment can activate the Notch1 signaling cascade and suppress cell proliferation in ATC cells. We also explored the underlying mechanisms for cell growth inhibition in ATC. We further assessed the therapeutic effect of Hesperetin on ATC cell re-differentiation.

Materials and Methods

Cell Culture and Reagents

HTh7 cell line (kindly provided by Dr. Rebecca Schweppe, University of Colorado Denver, CO) is a human derived ATC cell line authenticated by DNA profiling.¹⁷ Cells were maintained in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) in a humidified atmosphere of 5% CO₂ in air at 37°C. Hesperetin (MP Biomedicals, Solon, OH) was

dissolved in dimethyl sulfoxide (DMSO) to prepare a 200 mM stock solution and stored at -20°C . Fresh dilutions in cell culture medium were made for each experiment.

Cellular Proliferation Assay

ATC cell proliferation was measured by viable cell counts. HTh7 cells were first plated at the density of 10,000 cells per well. On the other day, cells were treated with 25–200 μM Hesperetin or DMSO vehicle control followed with incubation for up to 72 hours. Viable cell number was counted for each dose on the day of treatment and every 24 hours post-treatment. On the day of measurement, cells were washed and trypsinized. After the centrifuging, the cell pellet was re-suspended in equal volumes of medium and Trypan blue (0.05% solution, Bio-Rad Laboratories, Hercules, CA). Trypan blue dye exclusion was used to assess cell viability. The viable cell number was counted using a TC10 Automated Cell Counter (Bio-Rad Laboratories). Each experiment was repeated at least for three times.

Western Blot Analysis

The whole cell lysates were harvested as previously described¹⁸ and cellular protein extracts were quantified using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) following the manufacturer's instructions. Protein extracts (20 μg for each sample) were denatured, resolved by 8–12% SDS-PAGE, and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). After blocking, membranes were incubated with primary antibody overnight at 4°C . The following primary antibody dilutions in blocking solution were used: Notch1 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), cleaved poly-ADP ribose polymerase (PARP) (1:3,000), cleaved caspase-3 (1:1,000), β -actin (1:2,000), survivin (1:1,000) and Bcl-2-associated death promoter (BAD) (1:1,000) (Cell Signaling Technology, Beverly, MA). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, Cell Signaling Technology) the other day. The immunoreactive protein bands were visualized by the detection systems of Immunarstar (Bio-Rad Laboratories) or SuperSignal West Femto (Pierce Biotechnology, Rockford, IL). Immunoblot analyses were repeated at least twice and the expression levels of β -actin were used as the loading control.

Luciferase Assay

To measure the functional Notch activity, HTh7 cells were transiently transfected with both wild type CBF-1 luciferase reporter plasmid (kindly provide by Dr. Diane Hayward, Johns Hopkins University, Baltimore, MD) and cytomegalovirus β -galactosidase (CMV- β -gal; 0.5 μg) using Lipofectamine transfection reagent (Invitrogen Life Technologies). After the transfection overnight, cells were treated with 25–200 μM Hesperetin or DMSO and incubated for an additional 48 hours. Luciferase activity (luminescence) was measured using a luciferase assay system (Promega, Madison, WI) by a moonlight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) according to manufacturer's directions. The activity of β -galactosidase in transfected cells was assessed using a β -galactosidase enzyme assay system (Promega) and measured at 420 nm by a spectrophotometer (μQuant ; Bio-Tek Instruments). Luciferase activity was determined relative to β -galactosidase activity and plotted as relative induction compared to DMSO control (mean $\pm$ SEM).

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from the cells treated with Hesperetin or DMSO control for 48 hours using an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Total RNA concentrations were determined using a NanoVue Plus spectrophotometer (General Electric Healthcare, Madison, WI). Complementary DNA was synthesized from 2µg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Sequences for the PCR primers were listed in Supplementary Table S1.

The quantitative real-time PCR were performed in triplicate on MyiQ Thermal Cycler (Bio-Rad Laboratories) and the results of each gene of interest were normalized to GAPDH mRNA expression. The comparative cycle threshold (Ct) method was used to calculate relative expression levels of target genes. Normalized expression for each treatment was plotted as relative induction fold compared to DMSO control (mean ± SEM).

Statistical Analysis

Statistical Analyses were performed utilizing the Statistical Package for the Social Sciences (SPSS, version 17, IBM SPSS). Unless specifically noted, all data are represented as mean ± SEM. One-way ANOVA or two-tailed Student's t test was used to determine statistical significance. A *P* value <0.05 was considered significant.

Results

Hesperetin Inhibited ATC Cell Proliferation Mainly by Apoptosis

Hesperetin treatment for up to 72 hours resulted in a dose- and time-dependent reduction in ATC cell proliferation (Fig. 1). By 72 hours, 50µM and 100µM Hesperetin treatment resulted in a 27% and 47% reduction respectively in viable cell number compared with control.

Next, we explored the mechanism by which Hesperetin inhibited growth of ATC cells. As shown in Figure 2, the protein expression of cleaved PARP and cleaved caspase-3, both of which are pro-apoptotic markers in the execution phase of cell apoptosis, were increased in a dose-dependent manner with Hesperetin treatment. Additionally, BAD, a member of the Bcl-2 family and also a pro-apoptotic protein,¹⁹ was found to be up-regulated. In contrast, the levels of Survivin, a member of the inhibitor of apoptosis protein (IAP) family, were down-regulated with increasing concentrations of Hesperetin treatment (Fig 2). The observed changes in expression levels of apoptotic mediators suggested that the Hesperetin induced ATC cell growth inhibition mainly by apoptosis.

Hesperetin Activated the Notch1 Signal Transduction Pathway in ATC cells

To determine whether Hesperetin acts as a functional Notch1 activating compound in ATC, a luciferase reporter assay using the CBF-1 binding site was carried out. Hesperetin, including the lowest treatment concentration (25µM), yielded a significant induction of luciferase activity compared with DMSO control (Fig. 3A). Treatment of 100µM and 200µM Hesperetin caused a 4- and 7-fold increase in luciferase units respectively, indicating that the Notch pathway may be activated by Hesperetin. Since the cleavage of Notch reflects

activation of the signaling pathway,⁸ we further evaluated the protein levels of Notch1 intracellular domain (NICD) in control and treated ATC cells. Hesperetin induced protein expression of NICD in a dose-dependent manner (Fig. 3B). Notch1 mRNA levels were also measured in ATC cells upon 48 hours of Hesperetin treatment. As shown in Figure 3C, Hesperetin up-regulated Notch1 mRNA with increasing concentration of treatment, mirroring the increase observed in NICD protein expression. Significant induction of Notch1 mRNA levels was seen in the cells treated with 100 μ M and 200 μ M Hesperetin compared with 50 μ M treatment ($P<0.01$ for both), which was consistent with the observations on luciferase activity (Fig. 3A).

Next, we analyzed the expression of Notch1 response genes, hairy and enhancer of split 1 (Hes1) and hairy and enhancer of split 1 related with YRPW motif (Hey1). Hes1 and Hey1 are responsible for the transduction of ligand-stimulated notch activation into phenotypic effects.²⁰ Hesperetin treatment for 48 hours caused an up-regulation of Hes1 (Fig. 4A) and Hey1 (Fig. 4B) in a dose-dependent fashion in ATC cells. Treatment of 100 μ M and 200 μ M Hesperetin caused a 2.3- and 2.6-fold induction respectively in Hes1 mRNA expression relative to DMSO control. The increase of Hey1 mRNA expression was more substantial: 100 μ M and 200 μ M Hesperetin resulted in a 2.0- and 5.4-fold increase respectively in mRNA expression relative to DMSO control. Additionally, both Hes1 and Hey1 mRNA levels were significantly increased with the treatment of 100 μ M and 200 μ M compared with 50 μ M treatment (Fig. 4, $P<0.01$).

Hesperetin Up-regulated the Expression of Thyroid-specific Differentiation Markers in ATC Cells

Since the up-regulation of Notch1 signaling has previously been shown to be involved in the expression of thyrocyte differentiation markers,^{11,21} we further evaluated the drug efficacy of Hesperetin on ATC cell re-differentiation by the measurement of mRNA levels of thyroid-specific genes. ATC cells treated with Hesperetin (0–200 μ M) for 48 hours showed a dose-dependent increase in thyroid-specific transcription factors: TTF1, TTF2 and PAX8 (Fig. 5A-C). Similarly, mRNA expression of thyrocyte markers TSHR and NIS was also up-regulated in ATC cells treated with Hesperetin (Fig. 5D-E). TSHR levels increased 7.1 and 13.6 times upon treatment with 100 μ M and 200 μ M Hesperetin respectively. Similarly, NIS levels showed significant increases of 3.3 and 6.4 folds upon treatment with 100 μ M and 200 μ M Hesperetin respectively. Furthermore, 200 μ M treatment of Hesperetin resulted in a profound induction of NIS mRNA level compared with 50 μ M treatment (Fig. 5E, $P<0.01$).

Discussion

The Notch1 signaling pathway has been established to be essential for cell proliferation and differentiation in many cellular contexts.⁸ Notch1 has also been reported to be a tumor suppressor, with minimal to no signaling activity in small cell lung cancer,²² medullary thyroid cancer,²³ gastrointestinal neuroendocrine tumors,²⁴ myeloid leukemia,²⁵ neuroblastoma tumors,²⁶ and other cell types. Similarly, in ATC, Notch1 acts as a tumor suppressor. The unregulated growth and lack of differentiation markers in ATC can be partly attributed to the aberrantly inactivated Notch1 signaling. Overexpression of Notch1 in

ATC cells via cell transfection of NICD reduces cellular proliferation and restores differentiation.¹¹ Thus, restoration of functional Notch1 signaling in ATC may serve as a promising therapeutic strategy.

In the current study, we evaluated the drug efficacy of Hesperetin, a potent Notch1 inducer found in citrus fruits. Hesperetin was able to restore functional Notch1 signaling in the context of ATC. Additionally, Hesperetin treatment resulted in a dose- and time-dependent decrease in ATC cell proliferation. More importantly, Hesperetin was found, for the first time, to be capable of inducing the expression of thyroid specific differentiation markers, including TTF1, PAX8 and NIS.

The growth inhibition observed with Hesperetin treatment was mainly mediated by apoptotic proteins. Caspase-3, an important effector kinase of both the intrinsic and extrinsic apoptotic pathways,²⁷ and PARP, a regulator of genomic integrity,²⁸ both were seen to be cleaved and activated with treatment. BAD – a pro-apoptotic marker – was also increased with treatment, while expression of Survivin – an inhibitor of apoptosis – was decreased. Survivin binds specifically to effector apoptotic proteases such as caspase-3, and thus its decrease was consistent with the increase observed with cleaved caspase-3 protein.²⁹ These observations suggested apoptosis as the major mechanism of ATC cell death with Hesperetin treatment.

The activation of Notch1 cascade could be in-part responsible for the reduced cell proliferation observed in the current study, since Hesperetin has been reported to be involved in the regulation of multiple signaling pathways including TGF- β and NF κ B signaling.^{30,31} Up-regulation of Notch1 signaling either by transient transfection of NICD or through the use of drug-inducible NICD cell lines has been found to inhibit the proliferation of many different cell types, including medullary thyroid cancer and ATC.^{11,23} Similar effects also have been reported in carcinoid tumors.³² Furthermore, it has been shown that Hes1, a Notch1 effector gene, inhibits the growth of various kinds of cells including carcinoid tumor cells, breast cancer cells, PC12 cells, and 3T3 cells.^{33–35} In our experiment, we demonstrated an increase in Hes1 mRNA expression concomitant with the Hesperetin-induced increase in Notch1 signaling, making it a possible mediator of cell growth inhibition.

The induction of cell re-differentiation has obvious therapeutic implications in the context of ATC. The undifferentiated features of ATC, including the inability to concentrate iodine, lead to the subsequent lack of treatment options such as the use of radioactive iodine therapy. In our study, we demonstrated that Hesperetin induced the mRNA expression of TTF1, TTF2, PAX8, TSHR and NIS. Hesperetin shares a similar chemical structure with resveratrol, while it is more potent than resveratrol to induce the expression of TSHR. This may indicate a possible combination with TSH to enhance the expression of NIS. Other strategies and compounds have also previously been used in an attempt to enhance thyrocyte re-differentiation, especially for the expression of NIS.³⁶ Retinoic acid, via action on Retinoic acid nuclear receptors, induces NIS expression in de-differentiated follicular thyroid carcinoma and papillary thyroid carcinoma.^{36–39} Histone deacetylase inhibitors, such as depsipeptide, have been shown to induce NIS mRNA expression in ATC cells via

the up-regulation of TTF1, but not via Pax8.⁴⁰ Interestingly, all these compounds have been reported as Notch1 signaling inducers.^{10,41–44}

Notch1 is implicated in differentiation of many cell types. In ATC cells, it has been shown that up-regulation of Notch1 and Hes1 causes increased NIS expression. Two potential Hes1 responsive sites exist in the human NIS promoter and the NIS gene is a direct target of Notch1 signaling via transcriptional activation of Hes1.¹¹ In our study, the increased levels of Hes1 along with NIS mRNA levels were supported by this finding. In addition to the direct Notch1-mediated activation of NIS, cell re-differentiation including NIS expression may also be induced via direct effects on the NIS promoter by TTF1 and PAX8,^{36,45} both of which were demonstrated to be up-regulated by Hesperetin in our study. In this study, TSHR was seen to be elevated with Hesperetin treatment. The expression of this differentiation marker, in vivo and in the presence of TSH (thyroid stimulating hormone), could supplement and potentiate the direct Notch1 effects on NIS expression since TSH stimulation also causes up-regulation in Notch1 activity, Hes1, and NIS.¹¹ TSH leads to direct increases in NIS expression via the NIS TSH-responsive factor-1 (NTF-1) – another thyroid specific transcription factor.⁴⁶

In summary, Hesperetin activates functional Notch1 signaling in human ATC cells in vitro. It inhibits ATC cell growth via apoptosis and induces the expression of thyroid-specific differentiation markers. Hesperetin could be an attractive source of potential cancer therapeutics for patients with ATC, either as single agent or as part of the combination therapy with other chemotherapeutic agents. Therefore, this novel Notch1 activating compound could be useful clinically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SYNOPSIS

Anaplastic thyroid carcinoma (ATC) is an extremely aggressive malignancy with undifferentiated features. In the current study, we found that a novel Notch1 inducer Hesperetin inhibited ATC cell proliferation and induced the expression of thyroid-specific genes including TTF1, NIS and Pax8.

Figure 1. Effect of Hesperetin on ATC cell viability

Hesperetin treatment resulted in dose- and time-dependent reduction of growth in ATC cells. HTh7 cells were treated with Hesperetin (0-400 μ M) for up to 72 hours. Cell viability was measured by viable cell counts every 24 hours after treatment and expressed as mean $\pm$ SEM (representative of four experiments).

Figure 2. Apoptosis in ATC induced by Hesperetin treatment

Detection of apoptotic markers including cleaved-PARP, cleaved caspase-3, BAD and Survivin by Western blot in HTh7 cells treated with Hesperetin or vehicle control (DMSO) for 48 hours. Equal loading was confirmed with β -actin.

Figure 3. Hesperetin activated Notch1 Signaling in ATC cells

A, HTh7 cells transiently transfected with a CBF1 luciferase plasmid showed increased luciferase activity with Hesperetin treatment. Luciferase activity was first normalized to β -galactosidase unit, and data were expressed relative to control cells without Hesperetin treatment. B, Notch1 intracellular domain expression levels detected by western blot in HTh7 cells treated with different concentrations of Hesperetin (0-200µM). Equal loading was confirmed with β -actin. C, Notch1 mRNA expression levels in HTh7 cells treated with Hesperetin were assessed by real-time RT-PCR. Data were plotted relative to control cells

without Hesperetin treatment. All values were expressed as mean $\pm$ SEM (n=3, *P<0.05 and **P<0.01 compared with control).

Figure 4. Hesperetin induced expression of Notch1 response genes

The mRNA levels of Notch1 response genes Hes1 (A) and Hey1 (B) in HTh7 cells treated with Hesperetin were assessed by real-time RT-PCR. Data were plotted relative to control cells without Hesperetin treatment. All values were expressed as mean $\pm$ SEM (n=3, **P<0.01 compared with control).

Figure 5. Hesperetin induced the expression of differentiation markers in ATC cells

The mRNA levels of differentiation markers including TTF1 (A), TTF2 (B), Pax8 (C), TSHR (D) and NIS (E) were quantified by real-time RT-PCR in HTh7 cells treated with Hesperetin or vehicle control. All data were plotted relative to cells treated with vehicle control. All values were expressed as mean $\pm$ SEM (n=3, *P<0.05 and **P<0.01 compared with control).