Helichrysetin and TNF-α synergistically promote apoptosis by inhibiting overactivation of the NF-κB and EGFR signaling pathways in HeLa and T98G cells

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Abstract. Tumor necrosis factor-α (TNF-α) has different effects on apoptosis depending on activation or inactivation of the nuclear factor-κB (NF-κB) and epidermal growth factor receptor (EGFR) signaling pathways. Helichrysetin, a natural chalcone, inhibits NF-κB nuclear translocation in mouse pancreatic β cells. The present study aimed to identify the effect of helichrysetin on activation of the NF-κB and EGFR signaling pathways induced by TNF-α, and the synergistic effect of helichrysetin and TNF-α on apoptosis of HeLa and T98G cells. Cell proliferation was measured by Cell Counting Kit-8 assay, while apoptosis was measured by Hoechst 33258 and Annexin V/PI staining. NF-κB activity was detected by luciferase assay, protein expression was measured by western blotting and mRNA expression was detected by quantitative PCR assay. The results revealed that in HeLa and T98G cells helichrysetin blocked the increased phosphorylation of NF-κB p65 induced by TNF-α. Although helichrysetin alone decreased cell viability, helichrysetin and TNF-α synergistically decreased cell viability. Helichrysetin, not TNF-α, promoted apoptosis, while the combination of helichrysetin and TNF-α synergistically increased apoptosis. In addition, helichrysetin and TNF-α synergistically enhanced the activation of caspase-3 and poly-(ADP-ribose)-polymerase compared with helichrysetin alone. Helichrysetin inhibited the phosphorylation of transforming growth factor-β activated kinase (TAK1), IκB kinase-α/β (IKK-α/β), NF-κB p65 and EGFR induced by TNF-α. Consistent with the inhibition of NF-κB activation, the increased TNF-α-induced mRNA expression levels of NF-κB, IL-1β, CCL2, CCL5 and CXCL10 were significantly downregulated by helichrysetin. Therefore, helichrysetin and TNF-α synergistically promoted apoptosis by inhibiting TAK1/IKK/NF-κB and TAK1/EGFR signaling pathways in HeLa and T98G cells, indicating a potential therapeutic strategy for cancer.

Introduction

Inflammatory cytokine-induced chronic inflammation is a high risk factor for the development of numerous malignancies (1,2). One of the most important inflammatory cytokines is tumor necrosis factor-α (TNF-α). TNF-α can induce cancer malignancy as well as induce apoptosis of cancer cells (3). To promote apoptosis, TNF-α receptor 1 (TNFR1) serves a crucial role (4). TNFR1 contains a death domain in the membrane region and once TNF-α binds to TNFR1, the Fas-associated death domain adaptor protein and caspase-8 associate with trimerized TNFR1 to direct activation of caspase-3 (5). Subsequent cleavage of poly-(ADP-ribose)-polymerase (PARP) finally results in apoptosis (6).

TNF-α induction of cancer malignancy often depends on nuclear factor-κB (NF-κB) (7). Transforming growth factor-β activated kinase (TAK1) phosphorylates downstream IκB kinase-α/β (IKK-α/β) (8). Subsequently, IKKs phosphorylate IκBα at Ser-32/36 to promote IκBα degradation by the ubiquitin-proteasome pathway to release NF-κB from IκBα (9). At the same time, they activate NF-κB p65 by phosphorylation at Ser-536 to induce NF-κB transcriptional activity (10). NF-κB p65 regulates inflammatory factors as well as anti-apoptotic genes (7,11,12). Therefore, the suppression of NF-κB activation may be an effective strategy to inhibit cancer malignancy.

Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is also involved in TNF-α-induced anti-apoptotic signaling (13). TAK1 induces p38 MAPK activation, leading to EGFR phosphorylation at Ser-1046/7 to block apoptosis (14). Therefore, TNF-α acts as a double-edged sword in the tumor microenvironment (15).

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Helichrysetin, 2',4',4'-trihydroxy-6'-methoxy chalcone, is commonly found in the *Alpinia* species (16). The structure of helichrysetin is shown in Fig. 1A. Helichrysetin has anti-inflammatory (17), apoptosis-inducing (18,19), anti-platelet aggregation (20) and antioxidant (21) effects. Regarding inflammatory signaling, helichrysetin decreases the transcriptional activity of NF-κB by inhibiting NF-κB nuclear translocation in mouse pancreatic β MIN-6 cells (17). Additionally, helichrysetin inhibits the cell viability in pancreatic cancer, fibrosarcoma (22), cervical adenocarcinoma (19,21), liver cancer, breast cancer (16), colon cancer (23) and lung cancer (18) cell lines. To the best of our knowledge, no molecular studies of helichrysetin have been performed. Therefore, the present study aimed to identify the effect of helichrysetin on activation of the NF-κB and EGFR signaling pathways induced by TNF-α, and the synergistic effect of helichrysetin and TNF-α on the apoptosis of HeLa and T98G cells.

**Materials and methods**

**Reagents.** Helichrysetin was supplied by Professor Jingshan Shen (Shanghai Institute of Material Medica, Chinese Academy of Sciences, Shanghai, China), and high-performance liquid chromatography analysis was performed as previously described (16) to confirm that it had a purity of >95%. Recombinant human TNF-α (cat. no. 210-TA) was purchased from R&D Systems, Inc. Primary antibodies against caspase-3 (cat. no. 9665s), PARP (cat. no. 9532s), TAK1 (cat. no. 5206s), pTAK1 (cat. no. 4536s), TAK1 binding protein 1 (TABI) (cat. no. 3226s), TAB2 (cat. no. 3745s), IKKβ (cat. no. 8943s), phosphorylated (p)IKKα/β (cat. no. 2697s), EGFR (cat. no. 2467s), pEGFR-SI046/7 (cat. no. 2238s), pNF-κB p65-SS36 (cat. no. 3033s) and β-actin (cat. no. 4970s) were purchased from Cell Signaling Technology, Inc. Primary antibodies against IKKα (cat. no. c0514) and NF-κB p65 (cat. no. k0515) were obtained from Santa Cruz Biotechnology, Inc. Cell Counting Kit-8 (CCK-8) and Annexin V-FITC Apoptosis Detection kit (cat. no. AD10) were purchased from Dojindo Molecular Technologies, Inc. The Hoechst 33258 staining kit (cat. no. MA0160) was purchased from Beyotime Institute of Biotechnology.

**Cell culture.** Human cervical cancer (HeLa) and human glioma (T98G) cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and cultured in DMEM (high glucose) containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc.), in a humidified 5% CO₂ atmosphere at 37°C. Luciferase reporter plasmid p65 NF-κB under the control of 4x κB sites; the luciferase reporter plasmid was provided by Professor Hiroaki Sakurai (University of Toyama, Toyama, Japan) and also contained a neo resistance gene. A stable clone (HeLa-κB) was isolated in medium containing 500 µg/ml G418. Cells (1.0x10⁴) were seeded in a 96-well plate at 37°C for 48 h. After pretreatment with 50 µM helichrysetin at 37°C for 30 min, cells were stimulated with TNF-α (20 ng/ml) at 37°C for another 6 h. Luciferase activity was detected using the ONE-Glo™ Luciferase Assay System (Promega Corporation) and measured using a microplate reader.

**CCK-8 assay.** HeLa cells were seeded at 1.6x10⁴ cells/well, and T98G cells were seeded at 2x10⁵ cells/well in 96-well plates and cultured overnight to adhere at 37°C. After pretreatment with helichrysetin at 37°C for 30 min, cells were stimulated with TNF-α (20 ng/ml) at 37°C for another 24 h. Subsequently, cells were incubated with CCK-8 solution (10 μl CCK-8 added to 90 μl medium per well) at 37°C for 1 h. Absorbance was detected at 450 nm on a Varioskan Flash microplate reader (Thermo Fisher Scientific, Inc.). The cell viability rate (%) was calculated as follows: (absorbance of drug-treated sample-blank)/absorbance of control sample-blank) x100.

**Hoechst 33258 staining.** HeLa or T98G cells were seeded in 96-well culture plates and cultured overnight to adhere at 37°C. After pretreatment with helichrysetin at 37°C for 30 min, cells were stimulated with TNF-α (20 ng/ml) for 24 h at 37°C. Subsequently, cells were fixed with 4% paraformaldehyde at room temperature for 10 min and washed with PBS. Cells were incubated with 50 µl Hoechst 33258 staining solution at room temperature for 5 min and then washed twice with PBS. Cell morphology was observed and captured under a fluorescence microscope (magnification, x200).

**Annexin V/PI staining.** An Annexin V-FITC Apoptosis Detection kit was used for apoptosis assays. HeLa cells were plated at 3.2x10⁵ cells/well and T98G cells were plated at 4x10⁵ cells/well in 35-mm cell culture dishes. Cells were pretreated with helichrysetin at 37°C for 30 min, followed by stimulation with TNF-α at 37°C for 24 h. Subsequently, the cells were harvested and washed with PBS, and the cell number was adjusted to 1x10⁶ cells/well. After collection by centrifugation at 300 x g at 4°C for 5 min, the cells were resuspended in 1x Binding Buffer, and stained with Annexin V for 15 min and PI for 5 min at room temperature in the dark. Apoptosis was analyzed on a CytoFlex S flow cytometer (Beckman Coulter, Inc.) using the CytExpert v2.3 software (Beckman Coulter, Inc.).

**Western blot analysis.** HeLa and T98G cells were cultured in 35-mm dishes at 37°C overnight, then incubated with fresh culture medium containing 0.5% FBS at 37°C for another 24 h. Cells were then pretreated with helichrysetin at 37°C for 30 min, followed by stimulation with TNF-α at 37°C for 5 min, 10 min, 6 or 12 h. Cells were harvested and lysed in CellLytic™ MT Cell Lysis Reagent (cat. no. C3228; Sigma-Aldrich; Merck KGaA) containing protease and phosphatase inhibitors (cat. nos. 04693116001 and 04906837001; Roche Diagnostics). The protein concentration was determined by BCA assay. A total of 20 µg protein from each sample was separated by standard SDS-PAGE (7.5, 10 or 12.5%) and transferred to Immobilon-P membranes (EMD Millipore) by semi-dry transfer. The membranes were incubated with SuperBlock™ (PBS) Blocking Buffer (Thermo Fisher Scientific, Inc.) at room temperature for 2 h, and then rinsed twice with 1x PBS-Tween (PBST) containing 1% Tween-20. Membranes were incubated with primary antibodies (1:1,000) against...
caspase-3, PARP, TAK1, pTAK1, TAB1, TAB2, pIKKα/β, EGFR, pEGFR-s1046/7, pNF-κB p65-s536, β-actin, IKKα, IKKβ and NF-κB p65 overnight at 4˚C. After washing twice with PBS, membranes were incubated with HRP-conjugated anti-rabbit secondary antibody (1:5,000; cat. no. 122107; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. The target protein bands were visualized with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) using a Tanon-5200 chemiluminescent imaging system (cat. no. 20182351; Tanon Science and Technology Co., Ltd.). For quantification, target proteins were normalized to β-actin within the same sample using ImageJ v1.52a (National Institutes of Health).

Reverse transcription-quantitative PCR assay. HeLa and T98G cells cultured in 24-well plates were pretreated with helichrysetin at 37˚C for 30 min and then stimulated with TNF-α at 37˚C for 4 h. Total RNA was isolated from the harvested cells using an RNA Faster 2000 kit (cat. no. 220011; Fastagen) according to the manufacturer’s protocol. cDNA was reverse transcribed from RNA (1 µg) using PrimeScript™ RT Master Mix (Perfect Real Time) (cat. no. RRd36A; Takara Bio, Inc.) according to the manufacturer’s protocol. Quantitative PCR was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus), ROX plus (Takara Bio, Inc.) on a Quant Studio 6 Flex System (Thermo Fisher Scientific, Inc.) under the following conditions: 95˚C for 30 sec; 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec; 95˚C for 15 sec; 60˚C for 1 min; and 95˚C for 15 sec. Quantification of target genes was determined using the 2−ΔΔCq method (24). The relative expression of individual target genes was normalized to that of GAPDH in the same sample. The sequences of the primers (Generay Biotech co., Ltd.) used are listed in Table I.

Thermal shift assay. HeLa and T98G cells were cultured in 100-mm dishes at 37˚C overnight and then incubated with fresh culture medium containing 0.5% FBS at 37˚C for another 24 h. After treatment with helichrysetin at 37˚C for 30 min, cells were collected and washed with PBS, then resuspended in PBS with protease inhibitors. The cell suspension was evenly distributed into PCR tubes and heated at 4, 40, 43, 46, 49 and 52˚C for 3 min, and then cooled for another 3 min at room temperature. Subsequently, cells were lysed by rapid freeze-thawing. The lysates were centrifuged at 20,000 x g for 20 min at 4˚C. Soluble fractions were transferred to new tubes and samples were prepared for western blot analysis as aforementioned.

Statistical analysis. All data are presented as the mean ± SD. Differences between 2 groups were analyzed via Student’s unpaired t-test, and differences among ≥3 groups were analyzed via one-way ANOVA with Tukey’s post-hoc test using GraphPad 7.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Table I. Quantitative PCR primers.

| Gene        | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|-------------|------------------------|------------------------|
| GAPDH       | GGGAAAGTGAAGTTCGGAGT   | GGGGTCAATGATGGCAACA    |
| TNF-α       | GCTGCATTTGAGGTATCG     | CTTGTACTCGGGGTTCCGAG   |
| IL-1β       | GGAGCTATGCTGTGGTACAA   | ACAGGTCTGTGGAGGATGGT   |
| CCL2        | AATCAATGCCCCAGTACCT    | GGCTGACAGAGTCCCTTT     |
| CCL5        | TGTGTGCCAAAACCAGAGAG   | GAAGCCTCCAAAGCTAGGAC   |
| CXCL10      | GCTGCTTATCCTTGT        | CTCTTCTACCTTCTT        |

Figure 1. Effect of helichrysetin on NF-κB activation. (A) Structure of helichrysetin. (B) HeLa-κB cells were pretreated with 50 µM helichrysetin for 30 min and then stimulated with TNF-α (20 ng/ml) for 6 h. The transcriptional activity of NF-κB was then measured. (C) HeLa and (D) T98G cells were pre-treated with 50 µM helichrysetin for 30 min and then stimulated with TNF-α (20 ng/ml) for 5 min. Whole cell extracts were prepared, and the protein expression levels of phosphorlated NF-κB p65, NF-κB p65 and β-actin were analyzed by western blotting. Data are shown as the mean ± SD (n=3). **P<0.01; ***P<0.001. Heli, helichrysetin; p, phosphorylated.
Results

Helichrysetin inhibits the activation of NF-κB. Helichrysetin inhibits NF-κB nuclear translocation in mouse pancreatic β cells (17). However, the effect of helichrysetin on NF-κB activation in cancer cells has not been clarified. Therefore, the present study measured the effect of helichrysetin on the transcriptional activity of NF-κB and the phosphorylation of NF-κB in human cancer cells. HeLa-κB cells were firstly used to measure the inhibitory effect of helichrysetin on the transcriptional activity of NF-κB induced by TNF-α. HeLa-κB cells were pretreated with 50 µM helichrysetin for 30 min and then stimulated with TNF-α for 6 h. Helichrysetin significantly inhibited the transcriptional activity of NF-κB induced by TNF-α (Fig. 1B). Phosphorylation of NF-κB at Ser-536 is crucial for its transcriptional activity. Thus, the phosphorylation of NF-κB p65 at Ser-536 was analyzed. In order to detect the effect of helichrysetin on the NF-κB and EGFR signaling pathways, T98G and HeLa cells were used for further experiments, since in the present study, both HeLa and T98G cells had a good response upon TNF-α stimulation, which strongly induces NF-κB activation, and both of them express wild-type EGFR. As expected, although helichrysetin exhibited no effect on the protein levels of p65 NF-κB, it inhibited the phosphorylation of p65 NF-κB induced by TNF-α stimulation in both HeLa and T98G cells (Fig. 1C and D). Overall, helichrysetin inhibited NF-κB activation in HeLa and T98G cells.

Helichrysetin and TNF-α synergistically promote apoptosis of HeLa and T98G cells. To mimic the tumor microenvironment, HeLa and T98G cells were treated with TNF-α and the synergistic effect of helichrysetin and TNF-α on the apoptosis of cancer cells was measured. Cells were pretreated with 50 µM helichrysetin for 30 min and then stimulated with TNF-α for 24 h. The results revealed that helichrysetin, but not TNF-α, had an inhibitory effect on cell viability in both cell lines; additionally, the combination of helichrysetin and TNF-α synergistically decreased cell viability (Fig. 2A and B).

Consistent with the CCK-8 assay results, Hoechst 33258 staining demonstrated that the combination of helichrysetin and TNF-α synergistically increased the number of T98G and HeLa cells with dense stained nuclei, compared with cells treated with helichrysetin alone (Fig. 2C and D).

For apoptosis analysis, cells were pretreated with 50 µM helichrysetin for 30 min and then stimulated with TNF-α for 8 h (for HeLa cells) or 24 h (for T98G cells). Annexin V/PI staining detected by flow cytometry demonstrated that helichrysetin, but not TNF-α, significantly enhanced apoptosis, and that the combination of helichrysetin and TNF-α synergistically increased the ratio of apoptotic cells in both cell lines (Fig. 2E and F).

Helichrysetin and TNF-α synergistically enhance the activity of apoptosis-associated proteins in HeLa and T98G cells. Next, the activity of apoptosis-associated proteins was determined. As demonstrated in Fig. 3, HeLa and T98G cells were pretreated with 50 µM helichrysetin for 30 min and then stimulated with TNF-α for 6 or 12 h. After stimulation with TNF-α for 6 h, compared with the control group, helichrysetin, but not TNF-α, significantly increased the protein expression levels of cleaved PARP in both cell lines. The combination of helichrysetin and TNF-α synergistically enhanced this increase. Additionally, helichrysetin significantly increased the protein expression of cleaved caspase-3 in HeLa but not T98G cells. After stimulation with TNF-α for 12 h, helichrysetin, but not TNF-α, increased the protein expression levels of cleaved PARP, and the combination of helichrysetin and TNF-α synergistically enhanced the protein levels of cleaved PARP and cleaved caspase-3 in both cell lines (Fig. 3). Overall, these results demonstrated that the combination of helichrysetin and TNF-α had a synergistic promoting effect on apoptosis.

Helichrysetin inhibits TAK1/IKK/NF-κB signaling induced by TNF-α in HeLa and T98G cells. To elucidate the detailed molecular mechanisms for the observed effects, the phosphorylation of TAK1 and IKKs was analyzed. As shown in Fig. 4, TNF-α stimulation significantly upregulated the phosphorylation of TAK1 and IKKα/β in both cell lines. Although helichrysetin alone had no effect on the phosphorylation of these molecules, it significantly counteracted the phosphorylation induced by TNF-α in both cell lines (Fig. 4).

Activation of NF-κB promotes the expression levels of many proinflammatory factors, such as TNF-α, IL1β, CCL2, CCL5 and CXCL10 (25-27). To further confirm the inhibitory effect of helichrysetin on NF-κB activation induced by TNF-α, HeLa and T98G cells were pretreated with 50 µM helichrysetin for 30 min and then stimulated with TNF-α for 4 h. After TNF-α stimulation, the mRNA expression levels of TNF-α, IL1β, CCL2, CCL5 and CXCL10 were significantly increased in both cell lines; this was completely reversed by helichrysetin treatment (Fig. 5). Overall, these results indicated that helichrysetin blocked TAK1/IKK/NF-κB signaling pathway.

Helichrysetin inhibits the TNF-α-induced phosphorylation of EGFR at Ser-1046/7. Finally, whether helichrysetin affected EGFR phosphorylation at Ser-1046/7 was analyzed. As shown in Fig. 6, helichrysetin alone had no effect on the phosphorylation of EGFR Ser-1046/7, but it significantly inhibited TNF-α-induced EGFR phosphorylation.

Helichrysetin does not directly bind to the TAK1/TAB1/TAB2 complex. Whether helichrysetin could directly bind to the TAK1/TAB1/TAB2 complex to inhibit TAK1 activity was further analyzed using a thermal shift assay. When compound-protein interactions exist, the stability of the complex will be increased compared with that of a single protein at certain temperatures. In other words, the expression of the complex will be higher than that of a single protein in a thermal shift assay. As shown in Fig. 7, TAK1, TAB1 and TAB2 expression was not increased in helichrysetin-treated cells compared with that in helichrysetin-untreated cells. Therefore, it was demonstrated that helichrysetin did not directly bind to the complex.

Discussion

Pro-inflammatory factors serve an important role in cancer (28). TNF-α is a double-edged sword for apoptosis; TNF-α has an anti-apoptotic effect that depends on NF-κB activation and a pro-apoptotic effect when the NF-κB signaling pathway is inhibited (29). The imbalance between...
proliferation and apoptosis results in cancer growth (30–32). Helichrysetin inhibits NF-κB activation in mouse pancreatic β cells (17). However, the effect of helichrysetin on NF-κB activity in cancer cells has not been previously investigated.

Figure 2. Effect of helichrysetin and TNF-α on apoptosis. HeLa and T98G cells were pre-treated with 50 μM helichrysetin for 30 min and then stimulated with TNF-α (20 ng/ml) for 24 h. Viability of (A) HeLa and (B) T98G cells was determined by Cell Counting Kit-8 assay. Apoptosis in (C) HeLa and (D) T98G cells was detected by Hoechst 33258 staining and measured under a fluorescence microscope (scale bar, 100 μm). Apoptosis of (E) HeLa or (F) T98G cells was analyzed by Annexin V/PI staining and detected using flow cytometry. Data are shown as the mean ± SD (n≥3). *P<0.05; **P<0.01; ***P<0.001. Heli, helichrysetin.
Chemotherapeutic drugs fight cancer by enhancing the apoptosis of cancer cells (33) and some flavonoids induce apoptosis (34,35). Although helichrysetin has an antitumor activity in several types of human cancer cells, including pancreatic cancer, fibrosarcoma (22), cervical adenocarcinoma (19,21), liver cancer, breast cancer (16), colon cancer (23) and lung cancer (18) cell lines, the detailed molecular mechanisms for these effects are unclear. Therefore, the present study aimed to elucidate the molecular targets of helichrysetin. It was revealed that helichrysetin and TNF-α synergistically enhanced the apoptosis of cancer cells by inhibiting TAK1 activation. PARP is the main substrate of cleaved caspase-3, which is a key executor of apoptosis, and cleaved PARP is an important indicator of apoptosis (36-38). In the present study, the combination of helichrysetin and TNF-α synergistically enhanced the cleavage of caspase-3 and PARP, indicating that helichrysetin and TNF-α synergistically promoted the apoptosis of cancer cells in a caspase-3-dependent manner.
To elucidate the detailed molecular mechanism of the synergistic effect of helichrysetin and TNF-α on the apoptosis of cancer cells, NF-κB and EGFR phosphorylation was analyzed. TNF-α induced NF-κB activation by phosphorylating NF-κB p65 at Ser-536 mediated by TAK1. TAK1 also activates the phosphorylation of EGFR to promote TNF-α-induced anti-apoptotic signaling (14,39). The present findings revealed that helichrysetin inhibited TNF-α-promoted NF-κB activation by blocking the phosphorylation of TAK1, IKKα/β and NF-κB p65, resulting in attenuated expression levels of NF-κB targeted genes. This indicated that helichrysetin and TNF-α synergistically enhanced apoptosis by repressing TAK1-mediated NF-κB activation. Furthermore, the present results

Figure 4. Effect of helichrysetin on the TNF-α-induced activation of NF-κB signaling. (A) HeLa or (B) T98G cells were pretreated with 50 µM helichrysetin for 30 min followed by TNF-α (20 ng/ml) stimulation for 5 min. Whole cell extracts were prepared, and the phosphorylation of TAK1 and IKKα/β and the protein expression levels of TAK1, IKKα, IKKβ and β-actin were analyzed by western blotting. Quantification of pTAK1/TAK1 and pIKKα/β/IKKα in (C) HeLa and (D) T98G cells. Data are shown as the mean ± SD (n≥3). *P<0.05; **P<0.01; ***P<0.001. Heli, helichrysetin; p, phosphorylated; TAK1, transforming growth factor-β activated kinase; IKKα/β, IκB kinase-α/β.
demonstrated that helichrysetin did not directly bind to the TAK1/TAB1/TAB2 complex. Therefore, helichrysetin may affect other molecules that lead to TAK1 inactivation. When TNF-α binds to TNFR1, numerous adaptor molecules associate with TNFR1 to activate downstream molecules, including TAK1 (1,40). The hypothesis of the present study is that helichrysetin may bind to TNFR1 itself or its adaptor molecules to inhibit TAK1 activation. The detailed mechanism by which helichrysetin blocks the activation of TAK1 requires further study.

Depending on its tyrosine phosphorylation, EGFR participates in the regulation of genes that regulate cell proliferation, survival, differentiation, autophagy and metabolism (41-43). Additionally, TNF-α controls TAK1-dependent phosphorylation of EGFR at Ser-1046/7, which blocks TNF-α-induced apoptosis (14). The current study demonstrated that
helichrysetin inhibited the phosphorylation of EGFR Ser-1046/7 in HeLa and T98G cells. Hence, helichrysetin and TNF-α may synergistically promote apoptosis by blocking the phosphorylation of EGFR. Similar results were obtained in both HeLa and T98G cells. Therefore, the present findings may be adapted to the cancer cell lines that express wild-type EGFR and have a good response upon TNF-α stimulation.

Overall, as shown in Fig. 8, helichrysetin and TNF-α may synergistically promote the apoptosis of cancer cells by inhibiting TNF-α-induced TAK1/IKK/NF-κB and TAK1/EGFR signaling pathways in HeLa and T98G cells. This may indicate a potential therapeutic strategy for human cervical cancer and glioblastoma.
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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZhiW and XL performed most of the experiments, and wrote the original draft. WL and LD performed flow cytometry and analyzed the data. AX and LY contributed to data analysis and interpreted the results. XW and ZheW contributed to the conception and design of the study. YZ and HS designed the experiments, wrote and revised the manuscript. ZhiW, HS and YZ confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

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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