Berberine Combined with Formononetin Induces Autophagy Through Downregulation of the MAPK/ERK Signaling Pathway and Inhibits the Proliferation of Nasopharyngeal Carcinoma Cells

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Primary research

Keywords: Berberine, Formononetin, MAPK/ERK signaling pathway, Autophagy, Nasopharyngeal carcinoma, proliferation

Posted Date: September 9th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-829474/v1>

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Abstract

To investigate whether the combination of berberine and formononetin can inhibit the proliferation of nasopharyngeal carcinoma cells by regulating the MAPK/ERK signaling pathway and affecting cell autophagy.

Methods: RTCA and Cytation™ 5 cell imaging systems were used to detect cell proliferation. MDC staining and transmission electron microscopy were used to detect cell autophagy. Western blot was used to detect the expression levels of autophagy and proliferation-related proteins and the expression levels of key proteins in the MAPK/ERK signaling pathway.

Results: The combination of berberine and formononetin can inhibit the proliferation of nasopharyngeal carcinoma cells ($P < 0.05$) and induce autophagy ($P < 0.05$) and. After berberine combined with formononetin for 24 h, the expression of LC3 II/I and Beclin1 increased, whereas the expression of P62 and PCNA decreased. The expression of key proteins p-c-Raf, p-MEK, and p-ERK1/2 in the MAPK/ERK signaling pathway was downregulated ($P < 0.05$). Inhibiting autophagy or activating the MAPK/ERK signaling pathway significantly reduced the effect of berberine combined with formononetin on upregulating the expression of LC3 II/I and Beclin1 and downregulating the expression of P62 and PCNA. At the same time, it significantly reduced the effects of berberine combined with formononetin in inducing autophagy and inhibiting cell proliferation ($P < 0.05$).

Conclusion: Berberine combined with formononetin inhibits the proliferation of nasopharyngeal carcinoma cells by downregulating the activity of the MAPK/ERK signaling pathway and then upregulating LC3 II/I and Beclin1, and downregulating the expression of P62 and PCNA.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a malignant epithelial tumor of the nasopharyngeal mucosa, which occurs in Southeast Asia[1,2].The etiology of NPC is mainly related to epstein-barr (EB) virus infection, environm[3,4]ental factors, genetic characteristics, and eating habits. Chemoradiation is the main treatment for NPC, but it has considerable toxicity and poor tolerance. Therefore, there is an urgent need to find new adjuvant treatment methods to provide patients with more effective and personalized treatment.

Combination therapy can be more effective for inhibiting cancer and improving the survival rate of cancer patients. Previous studies [5]showed that berberine (Ber) inhibited the proliferation of NPC cells and induced apoptosis in a dose- and time-dependent manner. Formononetin (Form) [6] can inhibit the proliferation of NPC cells, induce apoptosis, and inhibit cell migration. Autophagy is a highly conserved eukaryotic cell recycling process [7]. Many research groups [8,9] have identified autophagy as a potential therapeutic target for cancer. At the same time, the literature [10] has reported that the excessive activation of the MAPK/ERK signaling pathway is closely related to the occurrence and development of a variety of tumors. Therefore, this study aimed to investigate whether the combination of berberine and

formononetin can inhibit the proliferation of NPC cells by downregulating autophagy mediated by the MAPK/ERK signaling pathway.

2. Materials And Methods

2.1. Drugs

Berberine(Ber) and formononetin were purchased from Shanghai Jinsui Biotechnology Co., Ltd. (Shanghai, China), and the purity was > 99 %. The powder was dissolved in dimethyl sulfoxide (DMSO) to prepare a 200 mM stock solution, which was separated and stored at -20 °C in the dark. Roswell Park Memorial Institute (RPMI) 1640 medium was used to dilute the stock solution to prepare working concentrations. EGF(Meilun Bio) was dissolved in 20 µg/ml DMSO, and 1640 medium was used to dilute the working solution to 50 ng/ml. PD98059 (MCE) was dissolved in 50 mM DMSO, and 1640 medium was used to dilute the working solution to 50 µM. 3-Methyladenine(3-MA, MCE, Monmouth Junction, NJ, USA) was dissolved in 200 mM DMSO, and 1640 medium was used to dilute the working solution to 2 mM. Rapamycin (RAPA, MCE) was dissolved in 25 µM DMSO, and 1640 medium was used to dilute the working solution to 50 nM.

2.2. Cell culture

The human NPC cell line CNE1 , CNE2 were purchased from BeNa Culture Collection (Beijing, China). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin. Cells were cultured at 37 °C with 5% CO₂.

2.3. Real-time monitoring of cell proliferation by real-time cellular analysis (RTCA)

The RTCA monitoring system uses cell index (CI) to reflect cell proliferation. The logarithmic growth phase cells were taken, digested, and resuspended into a single cell suspension, and 5×10^3 cells/well were seeded on the RTCA cell proliferation monitoring culture plate, and each group was set with three multiple wells.

2.4. CytationTM5 cell imaging multifunctional detection system

The cells are routinely digested and the cell suspension prepared. 5×10^3 cells/well were seeded in a 96-well plate, and the drug was added to the CytationTM5 cell imaging multifunctional detection instrument.

Pictures were taken at an interval of 6 h to calculate the number of cells and the relative cell proliferation rate.

Relative cell proliferation rate = cell proliferation number in the drug group/ cell proliferation number in the control group × 100%

2.5. MDC staining

Take the logarithmic growth phase cells, make a single cell suspension and inoculate it overnight in a 6-well culture plate. After 24 h of drug intervention, the culture was terminated, the supernatant was discarded and washed with phosphate buffer solution(PBS), 1 mL of 50 μ M MDC solution was added to each well, incubated for about 40 min, washed with PBS three times, and photographed under a CKX31 inverted fluorescence microscope. Image-Pro Plus 6.0 software was used to calculate the fluorescence intensity.

2.6. Transmission Electron Microscope

The cells were inoculated in culture flasks at 5×10^5 and cultured overnight. After drug intervention for 24 h, the cells were collected into Ep pendorf (EP) tubes, and centrifuged at 1000g for 5 min, the supernatant were discarded, and the cells were resuspended by adding 1 mL PBS. The centrifugation was repeated twice, the supernatant discarded, 1 mL 2.5% glutaraldehyde (prepared with PBS) was added to each tube, before placing in a refrigerator at 4 °C for more than 4 h, and then fixed with 1% osmium acid fixative for 2 h. After dehydration with gradient acetone, soaking, embedding, curing, and ultrathin sectioning, it was stained with 3% uranyl acetate and lead nitrate, and the film was observed and photographed with a Hitachi H7700 transmission electron microscope.

2.7. Western Blot analysis

After 24 h of drug intervention, the Petri dish was placed on ice to extract the total protein. Protein extracts were quantified using a BCA protein assay kit (Lianke, Hangzhou, China). Equal amounts of proteins were loaded on 12% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% fat-free milk in TBST at room temperature for 1 h, then incubated with primary antibodies anti- β -actin (1:1000, 3700S), anti-LC3 a/b (1:1000, 12741P), anti-Beclin1 (1:1000, 3495P), anti-P62 (1:1000, 5114S), anti-proliferating nuclear antigen (PCNA, 1:1000, 13110) , anti-p-c-Raf (1:1000, 9427), anti-p-MEK (1:1000, 9154), and anti-p-ERK1/2 (1:1000, 4370S) at 4 °C overnight (all from Cell Signaling Technology, Beverly, MA, USA). After primary antibody incubation, membranes were washed three times in TBST then incubated with secondary antibody (1:5000, LI-COR, Lincoln, NE, USA) for 1 h at room temperature. After washing,

signals were visualised by an Odyssey Infrared Imaging System (Lincoln, NE, USA). Endogenous beta-actin was used for normalisation.

2.8. Statistical analysis

SPSS 23.0 software (IBM Corp., Armonk, NY, USA) was used to analyze the data results. All experiments were performed at least three times. Results are presented as the mean $\pm$ standard deviation (SD). The single-factor design and the measurement data comparison between multiple groups use single-factor analysis of variance. Multiple comparisons that meet the homogeneity of variance use the LSD test. The Dunnett T3 test was used for multiple comparisons that met the homogeneity of variance, the rank sum test was used for those that did not meet the normal distribution. If $P < 0.05$, the difference was considered to be statistically significant. All analysis diagrams were made using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. The effect of berberine combined with formononetin on the proliferation of nasopharyngeal carcinoma cells

The effect of berberine combined with formononetin on the proliferation of NPC cells was dynamically monitored using RTCA. The results showed that compared with the control group, different concentrations of berberine combined with formononetin had different levels of inhibitory effects on NPC cells, which was concentration-dependent. Among them, the concentration of Ber10 μ M + Form10 μ M had the most obvious inhibitory effect. (Fig. 1A). CompuSyn software calculated CI values for the two drugs under different concentration combinations, and the results showed that Ber10 μ M + Form10 μ M had a greater synergistic effect ($CI < 1$, Table 1,2). Therefore, this concentration combination was selected for subsequent experiments. Western blot results showed that compared with the control group, the expression of PCNA, a proliferation-related protein, was significantly downregulated, and the difference was statistically significant (Fig. 1B). Overall, these results indicate that both the single and combined use of Ber and Form could inhibit cell proliferation, and the combined use results in a synergistic effect.

Table 1 Inhibitory effect of berberine combined with formononetin on proliferation of CNE1 cells by CI.

Dose Ber (μ M)	DoseForm (μ M)	Combination Drug index(CI)		
		24h	36h	48h
5	5	1.19	0.82	0.78
5	10	1.35	0.9	0.73
10	5	0.87	1.06	0.56
10	10	0.86	0.76	0.61
10	20	0.34	0.33	0.34
20	10	0.43	0.25	0.31
20	20	0.37	0.28	0.27

Table 2 Inhibitory effect of berberine combined with formononetin on proliferation of CNE2 cells by CI.

Dose Ber (μ M)	Dose Form (μ M)	Combination Drug index(CI)		
		24h	36h	48h
5	5	2.27	0.45	0.31
5	10	1.66	0.6	0.3
10	5	0.62	0.78	0.36
10	10	0.65	0.73	0.48

3.2. The effect of berberine combined with formononetin on autophagy of nasopharyngeal carcinoma cells

The results of MDC staining showed that the fluorescence intensity of NPC cells after treatment with different concentrations of berberine combined with formononetin for 24 h was increased compared with the control group. Among them, the concentration of Ber10 μ M + Form10 μ M compatible fluorescence intensity is the largest (Fig. 2A). Transmission electron microscopy showed that compared with the control group, after berberine combined with formononetin treatment of NPC cells for 24 h, a large number of autophagosomes (indicated by the red arrow) appeared in the cells, the nucleus was pyknotic, and the chromatin was marginalized. There was an increase in vacuoles in the cytoplasm (in the red box) (Fig. 2B). Western blot results showed that compared with the control group, berberine combined with

formononetin upregulated the expression of LC3 II/I and Beclin1 and down-regulated the expression of P62 (Fig. 2C).

Ber combined with Form on expression of autophagy-related proteins in CNE1–CNE2 cells. Expression of LC3 II/I, Beclin1, and P62 in CNE1–CNE2 cells examined by western blotting after treatment with Ber (10 μ M), Form (10 μ M) and Ber combined with Form (10 μ M+10 μ M) for 24 h. (* p < 0.05, ** p < 0.01 compared to the Control group, n = 3).

3.3. The relationship between berberine combined with formononetin inducing autophagy and inhibiting cell proliferation

RTCA dynamic monitoring with the CytationTM5 cell imaging multifunctional detection system found that compared with the berberine combined with formononetin group, the inhibitory effect of berberine combined with formononetin with 3-MA pretreatment of NPC cells decreased (Fig. 3A), and the expression of PCNA, a proliferation-related protein, was relatively upregulated (Fig. 3B). Compared with the result of **Section 3.2**, it shows that 3-MA reversed the inhibitory effect of berberine combined with formononetin on NPC cells.

Compared with the berberine combined with formononetin group, the fluorescence intensity was weakened by adding 3-MA pretreated berberine combined with formononetin MDC staining (Fig. 3C). Transmission electron microscopy showed that the number of autophagosomes in the cell decreased (Fig. 3D), Western blot results showed that the expression of autophagy-related proteins LC3 II/I and Beclin1 was downregulated, and the expression of P62 protein was upregulated (Fig. 3E). Compared with the results in **Section 3.1**, it is indicated that 3-MA reversed the effect of berberine combined with formononetin in inducing autophagy.

3.4. Berberine combined with formononetin induces autophagy and inhibits cell proliferation through the MAPK/ERK signaling pathway

Western blot was used to detect the expression of key proteins in the MAPK/ERK signaling pathway. The results showed that compared with the control group, the berberine combined with formononetin group MAPK/ERK signaling pathway key proteins p-c-Raf, p-MEK, and p-ERK1/2. The expression was significantly downregulated, indicating that berberine combined with formononetin reduced the expression of key proteins in the MAPK/ERK signaling pathway (Fig.4).

The **Section 3.3** results have confirmed that berberine combined with formononetin can induce autophagy and inhibit the proliferation of NPC cells. To explore whether berberine combined with

formononetin can exert the above effects through the MAPK/ERK signaling pathway, the MAPK/ERK signaling pathway activator EGF was used as a single treatment in the EGF group or combined with a drug group to clarify further that berberine combined with formononetin can induce autophagy and inhibit NPC cells by inhibiting the MAPK/ERK signaling pathway proliferation. Western blot results showed that compared with the berberine combined with formononetin group, the expression level of p-ERK1/2 decreased after EGF activated the MAPK/ERK signaling pathway (Fig.5), that is, EGF could significantly downregulate the combination of berberine and formononetin expression of key signal proteins for MAPK/ERK.

Compared with the berberine combined with formononetin group, after activation of the MAPK/ERK signaling pathway, CytationTM5 cell imaging multifunctional monitoring results showed that the inhibitory effect of berberine combined with formononetin on NPC cells was weakened (Fig.6A). Western blotting showed that the expression of proliferation protein PCNA is upregulated. Compared with the control group, the expression of PCNA in the EGF group was upregulated, whereas the expression of PCNA in the berberine combined with formononetin group was significantly downregulated (Fig.6B). That is, EGF reversed the effect of berberine combined with formononetin on the proliferation of NPC cells, suggesting that the inhibition of berberine combined with formononetin on the proliferation of NPC cells is related to its downregulation of the MAPK/ERK signaling pathway.

After activation of the MAPK/ERK signaling pathway, MDC staining revealed that the fluorescence intensity of berberine combined with formononetin was reduced (Fig. 6C), while downregulating the expression of LC3 II/I and Beclin1, and upregulating the expression of P62 (Fig. 6D). It is suggested that the combination of berberine and formononetin inducing autophagy in NPC cells is related to the downregulation of the MAPK/ERK signaling pathway.

4. Discussion

Nasopharyngeal cancer is likely to occur in hidden areas, and treatment is difficult. In recent years, many traditional Chinese medicines and their monomers have been confirmed to have obvious antitumor effects, such as curcumin [11], berberine [12,13], artemisinin [14] and formononetin [15]. Among them, Wang et al.[16] found that berberine can inhibit the proliferation of NPC cells, induce cell cycle arrest and apoptosis, and at the same time, it is effective against gastric cancer [17], colon cancer [18] breast cancer [19], etc., having a significant inhibitory effect. Formononetin [20] has antitumor properties in vivo and in vitro, and induces apoptosis and cycle arrest through a variety of signal pathways, inhibits cell proliferation and cell invasion, and is effective against cervical cancer [21], prostate cancer [22], breast cancer [23], etc. with obvious anticancer effects. Based on this, in this study, we mainly used berberine combined with formononetin to play an antinasopharyngeal cancer effect.

Autophagy is involved in the occurrence and development of cancer and has become a research hotspot in cancer treatment [24]. However, there are few studies on autophagy for NPC. Therefore, this study used experimental methods such as RTCA real-time monitoring confirmed that berberine combined with

formononetin can significantly inhibit the proliferation of NPC cells, and the inhibitory effect increases with the increase of drug concentration, and downregulates the expression of proliferation-related protein PCNA, suggesting that berberine combined with formononetin can inhibit the proliferation of NPC cells. MDC staining and transmission electron microscopy to explore the mechanism of autophagy in the treatment of NPC. Our results show that after berberine combined with formononetin intervenes in NPC cells, a large number of autophagosomes and even cell death appear. At the same time, berberine combined with formononetin upregulates LC3 II/I and Beclin1 expression, with downregulation of P62 expression. All of the above suggest that berberine combined with formononetin can induce autophagy in NPC cells. To understand further whether the combination of berberine and formononetin can inhibit the proliferation of NPC cells by inducing autophagy, we added the autophagy inhibitor 3-MA for pretreatment. We found that compared with the berberine combined with formononetin group alone, 3-MA reduced the expression of berberine combined with formononetin on LC3 II/I and Beclin1 expression, upregulated the expression of P62, and also reduced the expression of the proliferation-related protein PCNA. That is to say, 3-MA can reverse the proliferation and autophagy effects of berberine combined with formononetin on NPC cells, which indicates that berberine combined with formononetin can inhibit the proliferation of NPC cells by inducing autophagy. Monomers of traditional Chinese medicine have an antitumor effect by inducing autophagy, which has become a common model. Previous studies have found that ginsenosides [25], curcumin [26], and Cepharanthine [27] can inhibit tumor cell proliferation by inducing autophagy.

The occurrence and development of tumors are closely related to the MAPK/ERK signaling pathway[28]. Triptolide[29], catechin flavonoids[30], parthenolide[31], etc. all exert antitumor effects by regulating the MAPK/ERK signaling pathway. To explore further the molecular mechanism of berberine combined with formononetin to induce autophagy in tumors, we detected the expression levels of the key proteins p-c-Raf, p-MEK, and p-ERK1/2 in the MAPK/ERK signaling pathway, and found that berberine combined with formononetin significantly inhibited the activation of the MAPK/ERK signaling pathway. After activation of the MAPK/ERK signaling pathway, it reduced the effect of berberine combined with formononetin on proliferation and autophagy of NPC cells. This indicates that berberine combined with formononetin may inhibit the proliferation of NPC cells by downregulating the MAPK/ERK signaling pathway to induce autophagy.

In summary, this experiment confirmed that berberine combined with formononetin can inhibit cell proliferation by inducing autophagy. This effect may be through downregulation of key proteins p-c-Raf, p-MEK, and p-ERK1/2 in the MAPK/ERK signaling pathway.

Declarations

Author contributions

RU preformed the main experiments, JL wrote the manuscript, JYF and TL performed the statistical analysis, FLZ helped with literature search, FQT collected data, XWW, DFT and YCH revised and proofed

of the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Rui Su: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization. **Jie Liu:** Methodology, Software, Validation, Investigation. **Jingying Fan:** Resources. **Ting Lin:** Resources. **Fangliang Zhou:** Data curation, Investigation. **Faqing Tang:** Formal analysis. **Xianwen Wang:** Methodology. **Daofa Tian:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition. **Yingchun He:** Conceptualization, Writing - review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81874408), the Natural Science Foundation of Hunan (No. 2019JJ40216), and the Postgraduate Research and Innovation Project of Hunan (No. CX20200794)

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Figures

Figure 1

Effects of berberine and/or formononetin on the proliferation of CNE1–CNE2 cells. (A) RTCA assays showing inhibition of the proliferation of CNE1–CNE2 cells by Ber and/or Form. (B) Effect of Ber combined with Form on expression of proliferation-related proteins in CNE1–CNE2 cells. Expression of PCNA in CNE1–CNE2 cells examined by western blotting after treatment with Ber (10 μ M), Form (10 μ M) and Ber combined with Form (10 μ M+10 μ M) for 24 h. (* p < 0.05, ** p < 0.01 compared to the Control group, n =3).

Figure 2

Effects of berberine and/or formononetin on the autophagy of CNE1 and CNE2 cells. (A) CNE1–CNE2 cells were treated with different doses of Ber and/or Form for 24 h, after which the fluorescence intensity was measured by MDC staining. (bar = 100 μm, 20×) (**p < 0.01 compared to the Control group at the same time point, n = 3). (B) Berberine combined with formononetin induced autophagosome formation in CNE2 cells.(bar=5μm, ×5000). (C) Effect of Ber combined with Form on expression of autophagy-related

proteins in CNE1–CNE2 cells. Expression of LC3 II/I, Beclin1, and P62 in CNE1–CNE2 cells examined by western blotting after treatment with Ber (10 μ M), Form (10 μ M) and Ber combined with Form (10 μ M+10 μ M) for 24 h. (*p < 0.05, **p < 0.01 compared to the Control group, n = 3).

Figure 3

CNE1–CNE2 cells were treated with Ber 10 μ M + Form 10 μ M, 3-MA (inhibitors of autophagy), and 3-MA + Ber 10 μ M + Form 10 μ M + RAPA (activators of autophagy). (A) Effect of autophagy activators and

inhibitors on inhibition of cell proliferation by Ber combined with Form analysed by RTCA and CytationTM5 experiments. (B) Expression of proliferation-related proteins examined by western blotting. (C) The fluorescence intensity was measured by MDC staining. (D) Observation of autophagosomes using a transmission electron microscope. (E) Expression of LC3 II/I, Beclin1 and P62 in CNE1–CNE2 cells examined by western blotting (*p < 0.05, **p < 0.01 compared to the Control group, #p < 0.05, ###p < 0.01 compared to the Ber 10 µM + Form 10 µM group, n = 3).

Figure 4

Effect of Ber combined with Form on the expression of key proteins in the MAPK/ERK pathway in CNE1–CNE2 cells. Expression of p-c-Raf, p-MEKand p-ERK in CNE1–CNE2 cells examined by western blotting after treatment with Ber (10 µM), Form (10 µM) and Ber combined with Form (10 µM+10 µM) for 24 h. (*p < 0.05, **p < 0.01 compared to the Control group, n = 3).

Figure 5

Effect of activating MAPK/ERK signaling pathway on the down-regulation of p-ERK in CNE1–CNE2 cells by berberine combined with formononetin. CNE1–CNE2 cells were treated with 10 µM Ber + 10 µM Form, EGF (activators of MAPK/ERK), and EGF + Ber 10 µM + Form 10 µM + PD98059 (inhibitors of MAPK/ERK). Expression of p-ERK examined by western blotting. (*p < 0.05, **p < 0.01 compared to the Control group, #p < 0.05, ##p < 0.01 compared to the Ber 10 µM + Form 10 µM group, n = 3).

Figure 6

CNE1/CNE2 cells were treated with Ber 10 μM + Form 10 μM, EGF (activators of MAPK/ERK), and EGF + Ber 10 μM + Form 10 μM + PD98059 (inhibitors of MAPK/ERK). (A) Effect of MAPK/ERK signalling pathway activators and inhibitors on inhibition of cell proliferation by Ber combined with Form analysed by CytationTM5 experiments. (B) Expression of proliferation-related proteins examined by western blotting. (C) The fluorescence intensity was measured by MDC staining. (D) Expression of LC3 II/I, Beclin1

and P62 in CNE1–CNE2 cells examined by western blotting (*p < 0.05, **p < 0.01 compared to the Control group, #p < 0.05, ##p < 0.01 compared to the Ber10 μ M + Form10 μ M group, n = 3).