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Resveratrol inhibit the proliferation and migration of HepG2 cells through SIRT1-MAPK signaling pathway

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Author contributions

Chenchen Feng and Yijiao Xu contributed to formal analysis, investigation, data curation, writing original draft preparation, visualization and should be considered co-first authors. Yu Chen and Xiao Wei contributed to data curation. Chenchen Feng, Wanwei Yang and Rendong Zheng contributed to partial funding acquisition and data curation. Xiao Wei and Rendong Zheng contributed to conceptualization, validation, writing-review and editing, project administration, and supervision.

Competing interests

The authors declare no conflicts of interest.

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Abstract

Background: Resveratrol is a widely recognized anti-inflammatory and antioxidant agent, and it has been suggested to possess anti-tumor effects. But the effect of resveratrol on hepatocellular carcinoma and its molecular mechanisms are unknown. This study confirmed the effects of resveratrol on HepG2 cell proliferation and migration, and the underlying mechanism. **Methods:** Viability of resveratrol (0-200 μmol/L)-treated HepG2 cells was detected by CCK-8. Wound healing assay was employed to evaluate cell migration. The expression levels of proteins including Bcl-2, Bax, Caspase3, SIRT1, and components of the MAPK pathway were analyzed via Western blot. **Results:** Resveratrol significantly inhibited the migration and proliferation of HepG2 cells at concentrations above 100 μmol/L ($P<0.01$). The expression of Bax, cleaved Caspase3 and SIRT1 was up-regulate ($P<0.05$) and Bcl-2, p-JNK、p-p38 MAPK was down-regulate ($P<0.05$) by resveratrol. **Conclusion:** Resveratrol suppresses the proliferation and migration of HepG2 cells by activating the SIRT1 signaling pathway and inhibiting the JNK and p38 MAPK pathways.

Keywords: Resveratrol, Hepatocellular carcinoma, HepG2 cells, SIRT1, MAPK

Introduction

Liver cancer is the 6th most prevalent malignancy and ranks 3rd in cancer-related deaths globally [1]. More than 90% of all primary liver cancers are hepatocellular carcinoma, referred to HCC [2]. The initiation and development of HCC are closely related to chronic liver diseases, including infection of hepatitis virus, and NAFLD or NASH [3]. The malignancy of HCC is due to its ability to proliferate and metastasize. The stronger the ability of proliferation and metastasis, the higher the malignancy of HCC and the greater the harm caused. Drug therapy is an important way of early intervention for HCC patients, but drug resistance and escape reactions often occur, which brings heavy economic burden to patients' families [4].

Many medicines based on natural products are isolated from nature because they are low in toxicity, readily available, inexpensive and targeted. Therefore, some researchers have focused on natural plant polyphenols in food, such as resveratrol and curcumin, which possess attractive features in regulating multiple processes of tumor occurrence or development [5]. Resveratrol is widely known as an antioxidant, which is rich in grape, polygonum cuspina and mulberry, has various effects for example regulation of metabolism, anti-inflammation and anti-aging [6]. Additionally, clinical and basic research has found that it has therapeutic effects on a wide range of tumors, including liver, pancreatic, and lung cancers [7–10], which is related to the activation of silent information regulator 2 homologue 1 (sirtuin1, SIRT1) signaling pathway, but the specific mechanism is still unclear [11]. SIRT1 is a NAD^+-dependent protein deacetylase. Resveratrol-mediated activation of SIRT1 is considered to be one of the main targets of cancer therapeutic strategies [5–7]. Even in HepG2 cancer cells, resveratrol reduces proliferation and migration via activating SIRT1[12]. Nevertheless, the molecular mechanisms of SIRT1 in cancer cells is controversial.

The MAPK pathway is integral to various intracellular responses, including cell growth, differentiation, inflammation, and oxidative stress. Classical MAPK signaling pathways include c-Jun N-terminal kinase (JNK), p38 MAPK, extracellular regulated protein kinase (ERK) 1/2 and ERK5, which can be activated through a cascade of sequential phosphorylation events [13]. Substantial evidence indicates that JNK, p38 MAPK, and ERK1/2 play roles in the migration and proliferation of different tumors [14]. The process of HCC is also accompanied by the abnormal activation of MAPK pathways [15]. Therefore, this study aims to investigate the impact of resveratrol on the growth and migration of HepG2 cells and to explore the molecular mechanisms of SIRT1 and MAPK signaling pathways in the apoptosis and migration of HCC cells. This research provides a theoretical foundation for the clinical application of resveratrol in the treatment of HCC.

Materials and methods

Materials

Resveratrol (34092, Sigma) was from Sigma (St. Louis, MO, USA). CCK-8 (CK04, Dojindo, Kumamoto, Japan) was from Dojindo. Primary antibodies used included Caspase3 (9662, CST), Bcl-2 (3498, CST), Bax (2772, CST), SIRT1 (9475, CST), ERK (4695, CST), phospho-ERK (4370, CST), JNK (9252, CST), phospho-JNK (4668, CST), p38 MAPK (8690, CST), phospho-p38 MAPK (4511, CST), β-actin (3700, CST) antibodies, and secondary antibodies all from CST (Beverly, MA, USA).

Cell and Cell Culture

The HepG2 cell line was purchased from the Cell Bank of Chinese Academy of Sciences (SCSP-510, China), cultured in DMEM high glucose medium (11995065, Gibco) containing 10% FBS (C0227, Beyotime) at 37 ℃ in a 5% CO2 incubator. Resveratrol was dissolved in DMSO to formulate a stock solution (200 mmol/L), aliquoted, and stored at -20℃. The drug containing medium, concentrations 0-200 μmol/L, was prepared with whole culture medium. All culture solutions were purchased from Sigma.

Analysis of Cell Viability

Viability was assessed by the CCK-8 assay. HepG2 cells were seeded in 96-well plates (5,000 cells per well). After overnight adherence, cells were treated with varying concentrations of resveratrol (0, 25, 50, 100, and 200 μmol/L) for 24 hours. Six replicates were conducted for each concentration. Following treatment, 10 μL (g/L) CCK-8 was added to each well and incubated for 2 hours. Finally, absorbance was measured at 450 nm on a multifunctional plate reader to evaluate the cell viability of HepG2.

Analysis of Cell Migration

Migration was assessed by wound-healing assay. HepG2 cells were seeded in 24-well plates (100,000 cells per well) and cultured to 100% confluence. Then, scratch wounding was performed using a 200 μL pipette tip. The suspended cells were washed out with PBS without Ca^{2+} & Mg^{2+}. After that, cells were treated with resveratrol (0, 50, 100, and 200 μmol/L) for 12 and 24 hours with three replicates of each treatment, and were placed in the 37℃, 5% CO2 incubator. The wounded areas were observed and imaged with a inverted phase-contrastmicroscope (×200). Image contrast was enhanced using Adobe Photoshop 2023 to better show the edges of the scratches. The migration distances were imaged at 0, 12 and 24 h after scratching.

Western Blot Analysis

HepG2 cells were seeded into 6-well plates 500,000 cells/well. After treatment with 0, 50, 100, and 200 μmol/L resveratrol for 24 hours, cells were collected and lysed using protein extraction buffer. The lysates underwent three freeze-thaw cycles and were centrifuged at 14,000 rpm for 30 minutes to obtain supernatants. Protein concentrations were measured at 562 nm using the BCA Protein Assay Kit (P0010S, Beyotime). Protein samples were separated by SDS-PAGE (P0012A, Beyotime), then transferred to PVDF membranes (ISEQ00010, Millipore). Incubate the membranes overnight at 4℃ with different primary antibodies and then incubate for 2 hourwith the appropriate secondary antibody at room temperature . The bands were finally detected by using an Enhanced ECL Chemiluminescence Detection kit (E411-05, Vazyme). Densitometry analysis of protein band was calculated by Quantity One v4.6.6.

Statistical Analysis

Statistical analyses were carried out using SPSS software version 22.0. Data are presented as means ± SEM. ANOVA was used to assess differences among groups, followed by unpaired Student's t-tests for comparisons between two groups. $P<0.05$ was considered statistically significant.

Results

Resveratrol Suppresses Proliferation of HepG2 Human Hepatocellular Carcinoma Cells

Resveratrol, a natural compound renowned for its anti-cancer properties, has demonstrated cytotoxic effects on various human cancers, including hepatocellular carcinoma (HCC). To assess its impact on HCC cell proliferation, HepG2 cells were treated with 0 to 200 μmol/L resveratrol for 24 hours, and then subjected to CCK-8 assay. The results indicated a concentration-dependent decrease in cell proliferation upon resveratrol treatment (Figure 1). Notably, at 50 μmol/L, resveratrol reduced cell viability by approximately 50% *vs.* the DMSO control group.

Resveratrol Inhibits Migration of HepG2 Cells

Following the observation that resveratrol hampers HepG2 cell proliferation, we investigated its effect on cell migration. Wound healing test were conducted on HepG2 cells treated with 100 μmol/L or 200 μmol/L resveratrol for 24 hours (Figure 2A). The migration of HepG2 cells was significantly suppressed by resveratrol in a dose dependent manner (Figure 2B), and the inhibition rate gradually increased in a concentration-dependent manner. When the drug

concentration reached 100 μmol/L, statistically significant differences in migration rates.

Resveratrol Induces Apoptosis in HepG2 Cells

The imbalance between cell apoptosis and proliferation is common in cancer development. To test whether these changes were caused by resveratrol-induced apoptosis, Western blot was used to detect the effects of different resveratrol on the expression of apoptosis-related proteins in HepG2 cells. Bcl-2, Bax and Caspase3 are important markers of apoptosis. As shown in Figure 3A, the expression of Bcl-2 (anti-apoptotic) was down regulated. While Bax (pro-apoptotic protein) was up regulated. Meanwhile, the expression of the cytosolic level of cleaved Caspase3 was increased dose dependently (Figure 3B), and the quantitative analysis showed that the Bcl-2/Bax ratio was decreased (Figure 3C). These data suggested that resveratrol significantly induces concentration-dependent apoptosis in HepG2 cells. We also observed that at a concentration greater than 100 μmol/L, resveratrol significantly promoted the apoptosis in HepG2.

Resveratrol Activates SIRT1 and Inhibits JNK and p38 Pathways in HepG2 cells

Previous studies have confirmed that the activation of SIRT1 and MAPK pathway are involved in the growth of HCC. Learn more about that resveratrol induces apoptosis in HepG2 cells, we identified the expression of proteins in SIRT1 and MAPK pathway via resveratrol treatment. Our western blot results clearly showed that resveratrol-treated resulted in activation of SIRT1 and inhibition of JNK and p38 MAPK phosphorylation in a does-dependent manner in HepG2 cells (Figure 4A). Here we found a dramatic increase in the expression of SIRT1 after 24 hours of exposure to 100 μmol/L resveratrol (Figure 4B). At the same time, resveratrol did not significantly change the phosphorylation level of ERK in HepG2 cell cytoplasm (Figure 4C), but inhibited the levels of and p-JNK and p-p38 MAPK (Figure 4D and 4E), suggesting that it could inhibit the JNK and p38 MAPK pathways. Furthermore, resveratrol-mediated dramatic activation of SIRT1 signaling pathway and inhibition of JNK and p38 MAPK signaling pathways were consistent with decreased cell proliferation and migration (Figure 1 and 2) and increased apoptosis (Figure 3) in HepG2 cells, revealing that resveratrol-induced HepG2 cell apoptosis might be associated with its activation of SIRT1 and inhibition of p38 MAPK and JNK signaling pathways.

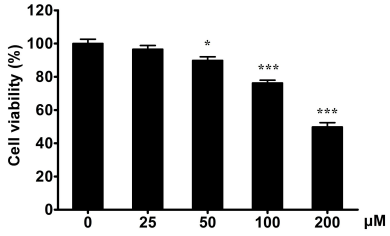

Figure 1 Effects of resveratrol on the viability of HepG2 cells for 24 hours. *vs.* control group,* $P<0.05$, ***$P<0.001$.

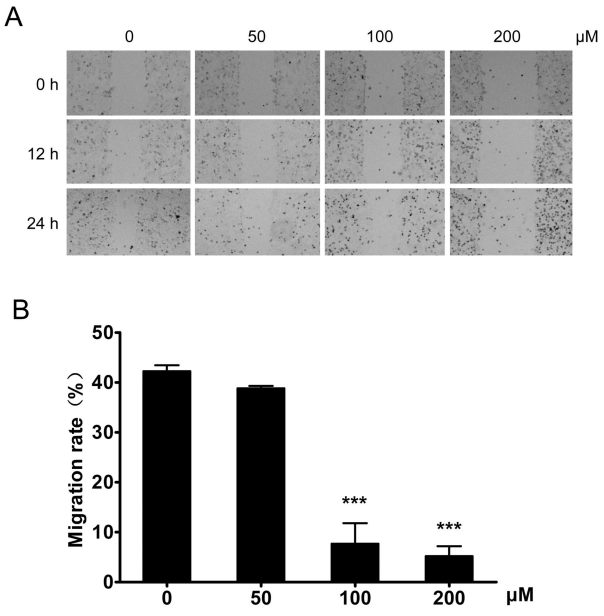

Figure 2 Effect of resveratrol on the migration rate of HepG2 cells at 24 hours. (A) Scratch assay of HepG2 cells treated with 0-200 μmol/L resveratrol for 12 and 24 hours (×200); (B) Bar chart of cell mobility for 24 hours in wound healing test. *vs.* control group,*** $P<0.001$.

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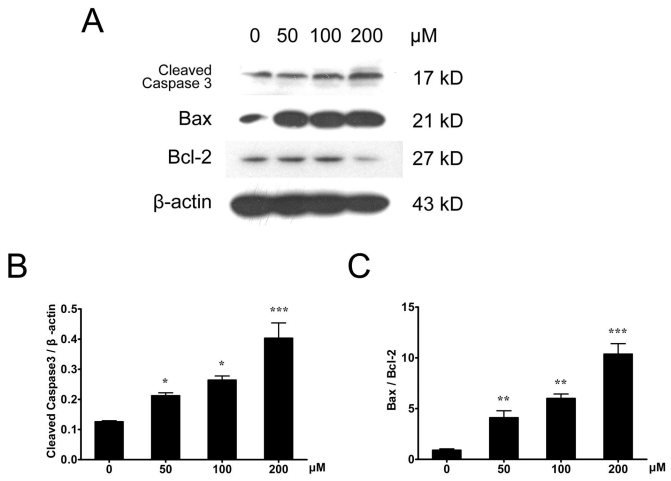

Figure 3 Effect of resveratrol on the expression of Bcl-2, Bax and Caspase3 spliceosomes in HepG2 cells. (A) Western blot treated with 0, 50, 100, and 200 μmol/L resveratrol in HepG2 cells ; (B-C) Bar chart of protein expression in western blot experiment. *vs*. control group,* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

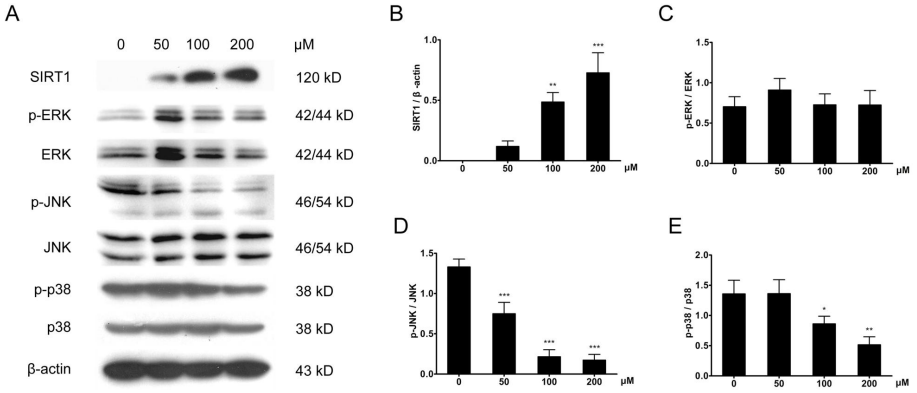

Figure 4 Effects of resveratrol on SIRT1 and MAPK signaling pathway proteins in HepG2 cells. (A) Western blot of HepG2 cells treated with 0, 50, 100, and 200 μmol/L resveratrol; (B-D) Bar chart of protein expression in western blot experiment. *vs*. control group,* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

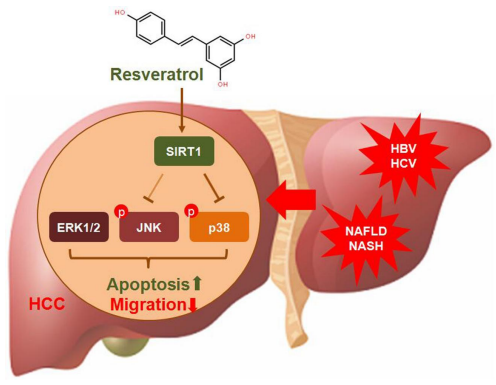

Figure 5 Resveratrol inhibits proliferation and migration in HepG2 cells via activating SIRT1 pathway and reducing the phosphorylation levels of JNK and p38 MAPK.

Discussion

HCC is a malignant cancer with high mortality and morbidity. Its development is a complex process influenced by multiple factors. However, the key driving factors for HCC development are still unclear. HepG2, the human liver tumor cell line used in this study, is often used as a model for HCC [16]. Therefore, the HepG2 cell line was selected for studying the anti-cancer activity of natural compound further in detail.

Among many promising anticancer drugs from food, resveratrol has been widely used in anti-HCC research due to its good anticancer effect and limited toxicity [17–22]. Usually, the so-called “resveratrol” in the literature refers to trans-resveratrol [23]. Its antioxidant and biological activities are mediated by hydroxyl groups at the molecular level [24]. Resveratrol is reported to act as an activator of SIRT1 both in vitro and in vivo [25]. However, whether resveratrol is a direct activator of SIRT1 is still controversial [26, 27]. Studies have proved that resveratrol can indeed increase the level of SIRT1 [28], and even binds to the N-terminal region of the SIRT1 protein, thereby ectopically activating SIRT1[29]. In our study, exposure to varying concentrations of resveratrol (0-200 μmol/L) significantly increased SIRT1 expression (Figure 4). However, we haven’t measured the change of SIRT1 activity, which should be further detected.

SIRT1 has been shown to inhibit cell apoptosis by suppressing p38 MAPK phosphorylation and caspase-3 activation [30]. In addition, during oxidative stress, JNK interacts with SIRT1 to phosphorylate Thr530, Ser47 and Ser27 on SIRT1, thus enhancing nuclear localization and enzyme activity [31]. These suggested that MAPK signaling pathway may also interact with SIRT1 in some biological events. However, the interaction between MAPK pathway and SIRT1 in the development of tumors remains unclear. It was found that resveratrol could promote the cell apoptosis of HepG2 cells and inhibit migration via SIRT1. The results suggest that there may be signal crosstalk between MAPK and SIRT1 signaling pathways. The effect of resveratrol on HepG2 cells may be dependent on the level of p-JNK and p-p38 MAPK, but not dependent on p-ERK. It can promote the apoptosis and inhibit the migration of HepG2 cells.

The CCK-8 assay results showed that resveratrol concentrations above 50 μmol/L significantly inhibited HepG2 cell viability (Figure 1). Concurrently, Western blot analysis revealed that concentrations exceeding 100 μmol/L increased the Bax/Bcl-2 ratio (Figure 3). Bax promotes cytochrome C release from mitochondria and activates Caspase3 cleavage [32, 33]. Bcl-2 inhibits Bax, blocking apoptosis and promoting survival [34]. An increased Bax/Bcl-2 ratio directly initiates apoptosis [35]. Resveratrol modulates this ratio and elevates cleaved Caspase3 levels, consistent with other studies demonstrating its anti-tumor properties, suggesting that 50 μmol/L resveratrol can induce apoptosis in HepG2.

The inhibitory effect of resveratrol on HepG2 is not only reflected in the increase of apoptosis level, but also the inhibition of migration ability. In this study, the effect of resveratrol on the migration ability of HepG2 cells were detected by cell wound healingtest. More than 100 μmol/L resveratrol treatment group basically did not move the cell boundary, and the scratch spacing was not significantly shortened (Figure 2). Tumor cell migration is involved in the formation of epithelial-mesenchymal transition (EMT) and metastasis, which includes cell dedifferentiation and loss of cell connection. EMT of tumor cells will give them the ability to invade the blood circulation and other tissues and organs [36, 37]. Resveratrol can effectively prevent this process from occurring.

The above results confirmed that resveratrol could regulate the apoptosis and migration of HepG2 cells, and further explore the related molecular mechanisms behind this phenomenon. Previous studies have found that resveratrol is a natural activator of SIRT1 pathway and can regulate the SIRT1 in cells. Present study, when resveratrol concentration was greater than 100 μmol/L, it showed a significant effect on up-regulating SIRT1 protein expression, suggesting that SIRT1 signaling pathway was activated (Figure 3). SIRT1 can deacetylate substrate proteins in the nucleus and cytoplasm, and is an important "Eraser" involved in epigenetic modification [38]. It also known as senescence protein, has the effect of prolonging life span, which is closely related to its functions of regulating metabolism, activating autophagy and inhibiting tumor [39]. Resveratrol promotes apoptosis and inhibits migration of HepG2 cells, which are related to the activation or inhibition of SIRT1 pathway. Activation of SIRT1 protein can inhibit the expression of c-Myc, a gene highly expressed in cancer cells, which can increase the proliferation ability of cancer cells [40]. Meanwhile, SIRT1 can promote the expression of p53 protein, which is one of the most common anti-cancer genes. Additionally, SIRT1 inhibits matrix metalloproteinases (MMPs), reducing tumor migration [41], and may regulate tumor development via intracellular miRNAs [42]. Thus, resveratrol's anti-tumor effects may depend on SIRT1 activation.

In HCC, resveratrol's anti-tumor actions are not only mediated through SIRT1's regulation of downstream proteins but also involve interactions with other signaling pathways. The MAPK pathway is a key intracellular regulatory route. Our study showed that resveratrol reduced p-JNK and p-p38 MAPK, but not p-ERK1/2 (Figure 4). ERK1/2, regulated by the Ras-Raf pathway, induces cyclin expression, but its abnormal activation is rare in HCC (about 5%), making Ras pathway inhibitors less effective [15]. In this study, resveratrol intervention did not down-regulate the phosphorylation level of ERK1/2, which may be related to the absence of abnormal activation of ERK1/2 in HepG2 cells.

Furthermore, resveratrol decreased the activation of p-JNK and p-p38 MAPK. JNK pathway is involved in the process of inflammation and tumorigenesis of HCC. The phosphorylation of JNK can induce the expression of proto-oncogenes c-Jun and c-Myc, and promote tumor proliferation and migration [43]. Resveratrol's inhibition of these pathways likely underlies its suppressive effects on HepG2 cells. The p38 pathway is involved in stress responses related to inflammation, metabolism, growth, and differentiation. Chronic inflammation can induce the longterm activation of p38, so p38 could be an important intervention target for the transformation of chronic liver inflammation into HCC [44]. It has also been found that inhibition of p38 MAPK can inhibit the activity of myosin light chain kinase, thereby reducing the migration and proliferation of tumor cells after tumorigenesis [45]. Thus, the inhibition of JNK and p38 MAPK pathways may be part of the molecular mechanism by which resveratrol affects HepG2 cells.

In conclusion, resveratrol can induce apoptosis and inhibit the migration of HCC HepG2 cells, which may be achieved by activating SIRT1 signaling pathway and reducing JNK and p38 MAPK phosphorylation levels (Figure 5). However, no counter evidence experiments have been conducted to confirm the upstream and downstream relationship of the related signaling pathways. In the future studies, more evidence is needed to support the following views.

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