mTOR inhibitor PP242 increases antitumor activity of sulforaphane by blocking Akt/mTOR pathway in esophageal squamous cell carcinoma

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Abstract

Background Sulforaphane (SFN) is a kind of isothiocyanate from cruciferous vegetables with extensive anti-tumor activity. Esophageal squamous cell carcinoma (ESCC) is a popular malignancy in East Asia, East and South Africa, while the more efficient medicines and therapeutic strategies are still lack. This study aims to explore the anti-tumor activity of SFN alone and combined with Akt/mTOR pathway inhibitors as well as the potential molecular mechanism in ESCC.

Methods and results Cell proliferation, migration, cell cycle phase, apoptosis and protein expression were detected with MTT assay, clone formation experiment, wound healing assays, flow cytometry and Western blot, respectively, after ESCC cells ECa109 and EC9706 treated with SFN alone or combined with Akt/mTOR inhibitors. Xenograft models were used to evaluate the efficiency and mechanism of SFN combined with PP242 in vivo. The results showed that SFN significantly inhibited the viability and induced apoptosis of ECa109 and EC9706 cells by increasing expression of Cleaved-caspase 9. SFN combined with PP242, but not MK2206 and RAD001, synergetic inhibited proliferation of ESCC cells. Moreover, compared to SFN alone, combination of SFN and PP242 had stronger inhibiting efficiency on clone formation, cell migratory, cell cycle phase and growth of xenografts, as well as the more powerful apoptosis-inducing effects on ESCC. The mechanism was that PP242 abrogated the promoting effects of SFN on p-p70S6K (Thr389) and p-Akt (Ser473) in ESCC.

Conclusions Our findings demonstrate that PP242 enhances the anti-tumor activity of SFN by blocking SFN-induced activation of Akt/mTOR pathway in ESCC, which provides a rationale for treating ESCC using SFN combined with Akt/mTOR pathway inhibitors.

Keyword Akt · Esophageal squamous cell carcinoma · PP242 · p70S6K · Sulforaphane

Introduction

Esophageal cancer (EC), a common gastrointestinal tumor, includes two main subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) [1]. ESCC is a popular subtype in East Asia, East and South Africa [2]. Surgical resection is currently the main treatment measure for early-stage ESCC, while patients with advanced ESCC have to suffer conservative treatment, such as radiotherapy and chemotherapy [3]. However, conventional chemotherapy drugs such as cisplatin exhibited highly toxicity and widespreadly adverse effects on patients [4].
Although targeted drugs have favourable therapeutic efficiency to ESCC, resistance and poor prognosis of patients are still not neglectful [5]. Thus, developing new drugs and exploring combined strategies of drugs are desired for ESCC therapy.

Due to the low toxicity and side effects of plant-based drugs, researchers have been keen to develop natural small molecules with antitumor effects from plants, such as curcumin, a polyphenolic compound isolated from spice turmeric [6] and dihydroartemisinin, a new anti-malarial drug isolated from Artemisia annua [7]. Recent studies have shown that cruciferous vegetables are rich in many phytochemicals with anticancer properties, such as Indole-3-carbinol (I3C) and SFN [8]. SFN, a kind of glucose isothiocyanate extracted from cruciferous vegetables such as cauliflower and broccoli, has been demonstrated to have excellent efficacy against cardiovascular diseases [9], diabetes [10] as well as cancers such as breast cancer, prostate cancer and lung cancer, etc. [11–13]. There are extensive molecular mechanisms about the anti-tumor effects of SFN [14]. For instance, SFN could inhibit STAT3 pathway by inducing ROS production [15], activating Keap1/Nrf2 [16] and ERK1/2 [17] pathway and so on. In our previous study, we have demonstrated SFN induces cell autophagy and inhibiting autophagy enhances sulforaphane-induced apoptosis via targeting Nrf2 in ESCC [18]. Akt/mTOR pathway has the critical regulating function to cell autophagy [19] and we have demonstrated the inhibition effects of Akt/mTOR pathway inhibitors on ESCC [20, 21], while how these inhibitors affecting anti-ESCC efficiency of SFN is still obscure. In this study, we therefore explored effects and the potential mechanism of SFN alone or combined with Akt/mTOR pathway inhibitors on ESCC.

Materials and methods

Reagent & antibodies

Sulforaphane, PP242, MK2206, RAD001 were purchased from Med Chem Express (Monmouth Junction, NJ, USA). Primary antibodies p-Rictor (Thr1135), Rictor, p-Akt (Ser473), Akt (pan), PRAS40, p-PRAS40 (Thr246), p-p70S6K (Thr389), p70S6K, Bax, Bcl-2, were purchased from Cell Signaling Technology (Danvers, MA, USA), and Cleaved-caspase 9 were acquired from Abcam (Cambridge, UK). The second antibody was obtained from Zhongshan Golden Bridge Biotechnology (Beijing, China).

Cell lines and animals

Human ESCC cell lines ECa109 and EC9706 purchased from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) were cultured as described before [20]. 4- to 6-week-old athymic male BALB/c nude mice were purchased from Silikejingda Experimental Animal Ltd (Changsha, China) and fed in an individual ventilated caging (IVC) system with alternating 12 h dark and 12 h light. The operation of animals was agreed upon by the Animal Ethics Committee, Zhengzhou University and in accordance with the ARRIVE guidelines.

MTT assay

ECa109 and EC9706 cells (5 × 10^3 cells/well) seeded triply in 96-well plates were treated with different measures, and cell proliferation was detected by MTT assay as before [18]. When ECa109 and EC9706 cells were treated with SFN combined with MK2206, PP242 and RAD001, respectively, combined Index (CI) was calculated according to the proliferation inhibition rate of cells by CompuSyn software. CI < 1 indicates the combination had synergistic effects, and the reverse indicated antagonism [22].

Colony formation assay

ECa109 or EC9706 cells seeded in 6-well plates at a density of 1 × 10^3 cells/well were treated with drugs and cultured for 10 days, cell colonies were fixed with 4% paraformaldehyde for 30 min, followed by being rinsed with PBS for 2–3 times and stained with 1% crystal violet (Beyotime, China), the number of colonies was subsequently counted by ImageJ software (NIH, Bethesda, MA, USA).

Wound healing assay

After ECa109 and EC9706 cells (3 × 10^5 cells/well) were seeded in a 6-well plate were scratched a line with a sterile tip, cells were treated with 20 μM of SFN and 4 μM of PP242 alone or combined for 48 h, the migration distance was measured by NIS-Elements BR software under the microscope. The relative migration rate (%) = migration distance of cells in experiment group/migration distance of cells in control group × 100%.

Cell cycle assay

After ECa109 and EC9706 cells were treated with 20 μM of SFN and 4 μM of PP242 alone or combined for 48 h, the proportion of cells in different cell cycle phases were investigated by flow cytometry as before [20].

Cell apoptosis assay

After ECa109 and EC9706 cells (3 × 10^5 cells/well) were treated with different measures, cells were collected,
washed, and resuspended in the binding buffer and stained with Annex-V and PI, cell apoptosis was investigated by a flow cytometer as our previous study [23].

Mitochondrial membrane potential (ΔΨm) assay

After ECa109 and EC9706 cells (3 × 10^5 cells/well) seeded in 6-well plates were treated with 20 μM of SFN and 4 μM of PP242 alone or combined for 48 h, cells were collected and incubated with JC-1 working solution (Beyotime Biotech, Shanghai, China) at 37 °C for 20 min in the dark. Cells were washed with PBS and re-suspended with JC-1 staining buffer, and ΔΨm of cells was investigated by a Flow cytometer [24].

Western blot

30 μg of total protein abstracted from cells or xenograft tissues was separated using SDS-PAGE and transferred onto PVDF membrane, expression of proteins was detected by Western blot as before [20]. GAPDH was used as an internal control, the density of bands was analyzed by ImageJ software.

Animal experiment

After 20 nude mice were adaptively fed for one week, ECa109 cells (1 × 10^7 cells in 0.2 mL PBS) were inoculated hypodermically into the right forelimbs of the mice. When the average tumor volume reached 100–120 mm^3, the mice were divided randomly into four groups (5 mice/group), and the experimental group received an intraperitoneal injection of SFN (5 mg/kg) and PP242 (4 mg/kg) alone or combined every other day for 14 days. At the end of treatment, the mice were sacrificed and the tumor tissue was separated and weighed. Measure of tumor size and evaluation of the efficacy of the drugs were as described previously [18].

H&E staining and in situ TUNEL assay

Tissues from mice fixed in a 4% paraformaldehyde solution and embedded in paraffin were sliced into 4–6 μm slides and subjected to hematoxylin and eosin (H&E) staining. Cell apoptosis in tumor tissues was detected by Transferrase-mediated dUTP nick end labeling (TUNEL) kit as described before [25].

Statistical analysis

All experiments were repeated independently at least three times. The results were analyzed statistically by one-way analysis of variance (ANOVA) using GraphPad Prism 5 software. The data were expressed as mean ± standard deviation and P < 0.05 was considered as statistically significant.

Results

SFN inhibited ESCC but activated Akt/mTOR pathway

For investigate the inhibition effects of SFN on ESCC cells, cell proliferation, clone formation and cell apoptosis were detected after cells were treated with SFN. As shown in Fig. 1a, SFN inhibited the proliferation of ECa109 and EC9706 cells in a dose- and time-dependent manner, the IC_{50} values were 31.58 ± 1.50 and 14.95 ± 1.72 μM in ECa109 cells and 38.78 ± 1.59, 22.79 ± 1.36 μM in EC9706 cells at 48 and 72 h, respectively. Moreover, SFN inhibited cell colony formation, the clone formation rate decreased 43.1% and 69.8% for ECa109 and 26.4% and 52.3% for EC9706 at 5 and 10 μM, respectively) compared to the control group (176 ± 32 for ECa109 and 202 ± 23 for EC9706) (P < 0.05, Fig. 1b). And the cell apoptosis rates assayed by a flow cytometer in Fig. 1c were 6.93 ± 0.37%, 26.60 ± 0.73% and 36.63 ± 1.02% in ECa109 cells, and 5.00 ± 0.73%, 19.20 ± 0.57% and 29.37 ± 1.23% in EC9706 cells treated with 0, 10, and 20 μM of SFN, respectively, for 48 h, which had a statistical difference between control group and experimental group (P < 0.01), indicating that SFN induced apoptosis of ESCC cells. In addition, expression of proteins in apoptotic and Akt/mTOR pathway was detected by Western blot after ECa109 and EC9706 cells were treated with 20 μM of SFN for 0, 3, 6, 12 and 24 h, respectively, and the results showed that SFN promoted the expression of Cleaved-caspase 9, but not Bcl-2 and Bax (Fig. 1d). However, SFN promoted phosphorylation of Akt (Ser473) and p70S6K (Thr389) (Fig. 1e). The results suggested that SFN inhibited ESCC through activating apoptotic pathways, while SFN also activated Akt/mTOR pathway.

PP242 enhanced inhibitory effects of SFN on ESCC by blocking activation Akt/mTOR pathway by SFN

Considering the activation of SFN on Akt/mTOR pathway might impair anti-tumor efficiency of SFN, we speculated the combination of Akt/mTOR pathway inhibitors and SFN might have better anti-tumor effects. Therefore, cell proliferation was investigated after ESCC cells were treated with SFN (0, 10, 20 μM) combined with Akt/mTOR inhibitors MK2206 (0, 5, 10, 15 and 20 μM), PP242 (0, 0.5, 1, 5 and 10 μM) and RAD001(0, 5, 10, 20 and 30 μM) for 48 h, respectively, and the combination efficiency was judged according to the CI value. As shown in Fig. 2a–c and Table 1, for SFN + RAD001, CI > 1 in both ECa109 and
EC9706 cells; For SFN + MK2206, CI > 1 in ECa109 cells and CI < 1 in EC9706 cells; while for SFN + PP242, CI < 1 in the two cell lines, indicating that SFN combined with PP242, but not MK2206 or RAD001, had synergistic inhibiting efficiency on ESCC (P < 0.001 or P < 0.01).

To further demonstrate the combined efficiency of SFN and PP242, colony formation, migration, cell cycle and apoptosis of ECa109 and EC9706 cells treated with SFN and PP242 alone or combined were subsequently investigated. Compared to control group, SFN and PP242 alone
or combined inhibited the formation of colonies and cell migration as well as retarded cells in G2 phase \((P < 0.05)\), especially when they were combined, which had obvious statistic difference compared with that of the single-drug group \((P < 0.05, \text{Fig. 2d–f})\). Moreover, PP242 had no obvious effects on apoptosis and JC-1 of ESCC cells at the current concentration, while it enhanced the apoptosis-inducing and JC-1 monomer-promoting effects of SFN on ESCC cells \((P < 0.05, \text{Fig. 2g, h})\). The results above indicated that PP242 promoted inhibition efficiency of SFN on ESCC.

To explore the molecular mechanism, expression of proteins in the Akt/mTOR pathway was detected after ECa109 and EC9706 cells were treated with 20 \(\mu\)M of SFN and 4 \(\mu\)M of PP242 alone or in combination for 24 h. As shown in Fig. 2i, compared to the corresponding control group, SFN promoted but PP242 inhibited the expression of p-p70S6K (Thr389), p-Akt (Ser473) and p-PRAS40, but their expression was still inhibited in ESCC cells treated with SFN combined with PP242, indicated that PP242 blocked the activation of SFN on Akt/mTOR pathway in ESCC \((P < 0.05)\).
**SFN combined with PP242 had stronger inhibiting effects on xenografts of ESCC**

Effects and mechanism of SFN combined with PP242 were further explored in vivo using xenograft models of ESCC. As shown in Fig. 3a, b, along with the prolonging of treatment time, tumor volume increased in every group, while tumor growth was obvious slower in experimental groups than that in control group, especially in the combined group. Moreover, apoptosis-related features such as cell shrinkage and nuclear merging, as well as more brown positive cells were observed in experimental groups, especially in SFN + PP242 group (Fig. 3c). The tumor inhibition rate in SFN + PP242 group is 56.51%, which was obviously higher than SFN (20.55%) or PP242 (39.97%) group, and the relative tumor growth rate (T/C%) in SFN + PP242 group was <40% (Table 2). These results above indicated SFN and PP242 alone could inhibit the growth of xenografts of ESCC, while the combination of them had more significant efficiency. In addition, SFN stimulated expression of p-Akt (Ser473), p-p70S6K (Thr389) and p-PRAS40 (Thr246), and inhibited expression of p-Rictor (Thr1135), while PP242 weakened the activation of SFN on them, which were consistent with that in vitro (Fig. 3d). The toxicity of drugs was primarily evaluated according to the body weight, H&E staining of liver and kidney, blood routine, indicators of liver and kidney function, as well as organ coefficients of nude mice, and the data above had no obvious difference in each group (Fig. S1, Table S1-3), indicating that SFN and PP242 had no obvious toxicity at the current dose.

**Discussion**

Akt/mTOR pathway, as a crucial pathway in tumorigenesis and development, participates in various biological processes such as cell migration, apoptosis, and autophagy [26]. mTOR is a core regulator in the pathway and involved in two multisubunit complexes, mTORC1 and mTORC2.
mTORC1 could be activated by growth factor, chemokines and nutrients (glucose, amino acids) through PI3K/Akt pathway, thus regulating protein synthesis and cell growth by phosphorylating p70S6K, while mTORC2 mainly regulated actin cytoskeleton network through phosphorylating Akt at Ser473 site via Rictor [20, 27]. Many Akt/mTOR pathway inhibitors such as RAD001, MK2206 and PP242 have been developed to treat cancers [28]. RAD001 is an inhibitor of mTORC1 [29], MK2206 is an allosteric Akt inhibito[30], and PP242 is an ATP-competitive mTORC1 and mTORC2 inhibitor with more powerful anti-proliferative and pro-apoptotic effects than mTORC1 inhibitor [31]. All of them have obvious inhibition efficiency on ESCC according to our previous study [20, 32].

![Figure 2 (continued)](image-url)
Many studies have demonstrated the regulation effects of SFN on PI3K/Akt/mTOR pathway, but the results are not consistent and even opposite. Some researchers have shown that SFN inhibits tumorigenesis by suppressing this pathway [17, 33], while others demonstrate the activation of SFN on it [34, 35]. In this study we demonstrated firstly that SFN inhibited ESCC through activating caspase 9. However, we also found SFN activated Akt/mTOR pathway in ESCC (Fig. 1), which may impair the anti-tumor effect of SFN, we therefore speculated that Akt/mTOR pathway inhibitor combined with SFN might have stronger efficiency on ESCC. Thus, we explored subsequently the efficiency of SFN combined with RAD001, MK2206 and PP242, respectively, on proliferation of ESCC cells. Based on the CI value, we found that SFN combined with PP242 had better proliferation-inhibiting effects on ESCC cells than MK2206 and RAD001 (Fig. 2a-c). The excellent combined efficiency of PP242 and SFN on migration, cell cycle phase and apoptosis of ESCC cells was also confirmed (Fig. 2d-h). Mechanically, PP242 suppressed activation of p-Akt (Ser473) and p-p70S6K (Thr389) and inhibition of Rictor (Thr1135) by SFN (Fig. 2i).

### Table 1

| CI of SFN combined with AKT/mTOR inhibitor on ESCC cells |
|---------------------------------|
| SFN (μmol/L) | CI EC80 | CI EC90 |
|----------------|-----------|
| PP242 (μmol/L) | 0.5 | 0.83 ± 0.09 | 0.69 ± 0.26 |
| | 1 | 0.72 ± 0.53 | 0.63 ± 0.25 |
| | 5 | 0.56 ± 0.14 | 0.40 ± 0.02 |
| | 10 | 0.45 ± 0.33 | 0.31 ± 0.09 |
| MK2206 (μmol/L) | 5 | 1.39 ± 0.21 | 1.92 ± 0.23 |
| | 10 | 1.06 ± 0.48 | 0.93 ± 0.47 |
| | 15 | 1.03 ± 0.38 | 0.89 ± 0.27 |
| | 20 | 0.83 ± 0.36 | 0.72 ± 0.30 |
| RAD001 (μmol/L) | 5 | 1.80 ± 0.95 | 0.96 ± 0.29 |
| | 10 | 1.27 ± 0.24 | 0.69 ± 0.30 |
| | 20 | 1.12 ± 0.87 | 0.61 ± 0.46 |
| | 30 | 0.58 ± 0.46 | 0.48 ± 0.09 |

![Fig. 3](image-url) Effects of SFN and PP242 alone or combined on xenografts of ESCC cells (n = 5). a Tumor growth curve. b Xenograft from in nude mice at the end of treatment. c H&E staining and TUNEL assay of tumor tissue. d Expression of proteins in Akt/mTOR pathway in tumor tissues from mice. *p < 0.05, **p < 0.01, ***p < 0.001, versus control group; #p < 0.05, ##p < 0.01, ###p < 0.001, versus single treatment drug.
Because bioavailability and metabolism of medicines in vivo, results in vitro might not reflect exact efficiency of drugs, which must need further verification in vivo [36]. We, therefore, established xenograft models of ESCC and investigated the inhibitory efficiency of SFN combined with PP242 on ESCC in vivo. The data showed that the small dose of SFN (5 mg/kg) and PP242 (4 mg/kg) alone had slight inhibiting effects on tumor growth, while SFN combined with PP242 could significantly suppress the growth of xenografts and induce cell apoptosis (Fig. 3a-c), and the molecular mechanism (Fig. 3d) was consistent with that in vitro.

### Table 2: Efficiency of drugs on nude mice

| Group  | RTV   | T/C (%) | Tumor weight (g) | Tumor inhibitory rate (%) |
|--------|-------|---------|-------------------|--------------------------|
| Control| 12.61 | –       | 1.42 ± 0.13       | –                        |
| SFN    | 7.84  | 62.19   | 1.13 ± 0.11       | 20.55                    |
| PP242  | 7.03  | 55.78   | 0.85 ± 0.05       | 39.97                    |
| S + P  | 4.10  | 32.55*  | 0.62 ± 0.04       | 56.51*                   |

Relative tumor volume (RTV) = \( V_t / V_0 \), in them \( V_0 \) was tumor volume at the beginning of treatment, and \( V_t \) was tumor volume at every treatment. \( \text{T/C}\% = \text{TRTV}/\text{CRTV} \times 100\% \), in them TRTV was the mean of RTV in experiment group during the treatment, and CRTV was the mean of RTV in the control group during the treatment. \( \text{T/C}\% \leq 40\% \) and \( P < 0.05 \) were considered as effective treatment, \( \text{T/C}\% > 40\% \) was considered as invalid treatment.
Conclusion

Our current study demonstrated that SFN could inhibit ESCC by activating caspase 9, and PP242 could enhance anti-tumor efficiency of SFN on ESCC by blocking the activation of SFN to Akt/mTOR pathway (Fig. 4), which provides theoretical and experimental basis for combination of SFN and Akt/mTOR inhibitors in ESCC treatment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.107/s11333-021-06895-9

Author contributions Guiqin Hou and Zhaoming Lu conceived the study. Zhaoming Lu and Yalin Zhang wrote the manuscript. Yalin Zhang, Yujia Xu, Huiyun Wei, Yan Li conducted the experiments and analyzed the data. Guiqin Hou, Wen Zhao, and Pengju Wang secured the fundings. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Declarations

Conflict of interest The authors declare there is no conflict of interest in this study.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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